Sujatha Mulpuri · Nicolas Carels
Bir Bahadur *Editors*

Jatropha, Challenges for a New Energy Crop

Volume 3: A Sustainable Multipurpose Crop

Springer

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Foreword – I

Despite its proven potential as a cost-efficient bioenergy feedstock, *Jatropha curcas* L. (Jatropha) remains to be a domesticated plant in terms of various traits. These include processes such as its reproductive biology, which is critical in gaining an insight into breeding for synchronous flowering. That insight would accrue through a molecular understanding of the process, for which genomic information on the plant is required.

Nearly more than a decade and a half of systematic, international, collaborative studies has been conducted on understanding the morphological and genetic diversity of Jatropha in order to guide parent selection for cross-pollination. Numerous genetic markers have been developed for the purposes of uncovering genetic diversity to assist in the breeding efforts. Hence, the first wave of Jatropha improvement is coming from traditional breeding approaches that utilize natural variations within *J. curcas* and in other genetically compatible species. There have also been efforts to introduce more variation through mutagenesis. Commercially relevant traits of shorter stature, higher number of female flowers, better self-branching, increased seed oil content, and decreased input requirements are being targeted. While efforts through traditional breeding are beginning to show initial successes in these target traits, there are many problem areas where traditional breeding-based solutions have limitations. These include Jatropha plant diseases, oilseed toxicity, and its less than ideal fatty acid profile.

Modern plant science-based approaches provide a valuable avenue in further improvement of Jatropha, similar to crops such as cotton, canola, soybean, and maize. Such approaches include the biotechnology-mediated possibility of introducing some well-characterized genes from other organisms to test for effects in Jatropha. Fine-tuning the seed's fatty acid profile and its biosynthesis and that of other metabolites such as the seed oil toxins is a primary target. A precondition to Jatropha biotechnology is the availability of efficient and robust regeneration systems and transformation systems, preferably through *Agrobacterium*. There have been several reports on regeneration and genetic transformation of Jatropha, and it remains to be tested if there is an efficient and robust protocol across laboratories.

With good transformation system, it is also important to have promoters that can drive spatiotemporal-specific gene expression to suit various objectives. The primary source of such promoters would be known from promoters of other plants, but a systematic understanding based identification of endogenous promoters is also required. Similarly, a good structural, functional, and comparative understanding of the Jatropha genes, genomes, and genotypes will help in achieving the goal of manipulating its secondary metabolism. A recent report of seed-specific enhancement of oleic acid in Jatropha illustrates the beginning of utilizing biotechnology in Jatropha improvement. Also noteworthy are the use of one endogenous promoter and the deletion of antibiotic selection marker gene in the final transgenic Jatropha. The use of CRISPR technologies, absence of foreign genes, and the fact that humans will not be directly exposed to the transgenic Jatropha products will all help in regulatory approvals for commercialization. With such possibilities in understanding and manipulating the most desirable traits of better biotic and abiotic stress tolerance, and quality and quantity improvement in seed oil, the doors will open to addressing traits that impact a whole range of products, including oils and bio-plastics, along value addition, in establishing Jatropha plant as a natural bioreactor. Thus the "explosion" of scientific activity on Jatropha since 2008 leading to the availability of a genome sequence and a lot of upstream research within 4 years since, coming up to successes with regeneration and transformation, is already establishing Jatropha as a model non-edible oilseed crop, including as a first crop to be purposely domesticated in modern times. Availability of records on such a process would be very useful in comparative evolution.

Publication of this book intends to produce a synthesis of what has been achieved and to help scientists move forward in understanding and utilizing Jatropha. It serves as a milestone but also showcases how quickly we can achieve a critical understanding on a novel bioenergy plant with the help of accumulated knowledge on model food crops and with the advanced scientific and research tools and technologies. The editors of the book, Drs. Mulpuri, Carels, and Bahadur, are the doyens in Jatropha research and development and have made significant contributions in our understanding of the potential and uses of this plant. It is most appreciated that they work closely and tirelessly to bring together information to update and guide the Jatropha research into the future through the present volume on *Jatropha, Challenges for a New Energy Crop*: *Volume 3 – A Sustainable Multipurpose Crop*. Their efforts and results, and those of all the scientists, presented in this volume and as a body of research outputs, will hopefully contribute to an increased funding from public and private sector to further support Jatropha research and development, which will further expedite the process of developing Jatropha as a new energy crop.

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Foreword – II

During his tenure as Director General, Indian Council of Agricultural Research, Dr. R.S. Paroda made several efforts to significantly boost oil seed productivity in the country.

I am indeed happy that the learned Editors have decided to bring out the present volume on *Jatropha curcas*, considered as a potential bioenergy feedstock plant. This is the third volume which speaks of their dedication, diligence, and sheer determination to popularize this oil seed plant.

The present volume covers several areas like *physiology and plant production, selective breeding and genetic diversity, feeding use, coproducts, processing, and socioeconomic sustainability*.

Undeniably, the plant has been around for the past several decades, though it is only recently that attempts are being made to understand its reproductive features and genome information. Initially, improvement was based on traditional breeding that utilizes natural variants existing within *J. curcas* and in other genetically compatible species. With a view to raising desired hybrids, suitable parents with desired traits, based on morphological and genetic diversity, need to be identified for crossing. Consequently several genetic markers have been developed with a view to unravel the genetic diversity. With a focus on several desirable traits, e.g., increasing oil productivity and decreasing input, better self-branching, shorter stature, higher number of female flowers, and higher oil content are being developed, and time-tested techniques like mutagenesis and transgenesis have been exploited.

Unarguably, traditional breeding poses several "roadblocks," for instance, *Jatropha* pathogens, toxicity of oil seeds, and its less than ideal desired fatty acid profile. In this context, biotechnological techniques provide valuable alternative to traditional methods as demonstrated in several cash crops including oilseeds. I am happy to state that the umpteen impressive publications on Jatropha of the editors of the present volume, in high-impact journals, need special mention.

In my view, the present volume provides a deep glimpse of our revered experts in their respective fields of specialization and explores all conceivable horizons in the field.

I am of the considered opinion that it offers a broad perspective on the current status on the economic and sustainable aspects of this important bioenergy plant.

I congratulate the eminent editors for timely bringing out this publication which will enthuse the growers to domesticate this important oilseed species.

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Preface

We initiated the Jatropha books project about 9 years back, and the first volume, entitled *Jatropha, Challenges for a New Energy Crop: Farming, Economics and Biofuel* (30 chapters), was published in 2012 followed by the second volume *Genetic Improvement and Biotechnology* (31 chapters) in 2013 both published by Springer New York. At that time, physic nut (*Jatropha curcas* L.) was emerging as an oilseed option to expand biodiesel production especially in marginal land poorly suitable for the cultivation of crops producing edible oil. These books gave a comprehensive account of the research going on internationally with the purpose of stimulating future development in this area. Five years went by since the publication of the first two volumes, and an update is now proposed with a third volume. Considering its potential as an alternative to fossil fuel, there have been considerable works on various technological aspects of physic nut, but it must still be considered as a semi-wild species. Further efforts considering selective breeding are necessary to increase yield and improve agricultural features to bring physic nut to the status of an industrial crop, which is needed for a commodity such as oil for biodiesel, given that selling prices can only be low. Volume 3 (25 chapters) intends to give a positive global picture on physic nut, a crop that has suffered from the fact that it is not yet fully domesticated despite its promising agronomical and economical features. Physic nut has the benefit of a high potential productivity larger than 7 tons of seeds per hectare (but actually commonly less than 2 tons per hectare) associated to proper oil composition for biodiesel conversion. In addition, physic nut is easy to subculture in vitro and to manipulate in laboratory as well as to transform in vitro genetically. Furthermore, it has a significant genetic diversity in its center of origin (Central America) and is relatively easy to cross-hybridize within the various species of the genus. Because of these promising features, physic nut is definitively on the rise as a crop, and some companies have already successfully understood how to handle it up. Thus, it is our concerted duty to sustain the efforts at domestication of this crop in order to provide additional solutions to the still too few industrial oilseed crops available for biofuel production.

The Editors have the expertise in agronomy, botany, selective breeding, biotechnology, molecular biology, genomics, and bioinformatics, which enabled them to gather worthy and sounding contributions. Actually, at this stage of physic nut journey as an industrial crop, the understanding of its physiology and its selective breeding remain the main bottlenecks to improve its economic status. In addition, we dedicated some chapters to the discussion on (i) how its return can be improved by the exploration of by-products such as animal feed, biomass, and chemicals for health and medicinal aspects, (ii) how its oil can be better processed into biofuel, and (iii) what are the objectives to be reached to warrant its sustainability in the future. Thus, Volume 3 should interest biologists, biotechnologists, agronomists, breeders, decision-makers, and investors of the biodiesel chain.

By publishing this book, we aimed at supporting the people in developing a crop that should help populations from marginal areas to gain access to a biofuel that may boost their economy. In that respect, we believe that the book will be seen as helpful by the interested communities as has already been proven by the success of the two previous volumes.

We wish to express our gratitude to all the contributing authors from all over the world for readily accepting our invitation not only for sharing their knowledge but also for admirably integrating their expertise to the vast information from diverse sources and enduring editorial suggestions to finally produce this venture. We also acknowledge the huge support received from many colleagues in the preparation of the manuscripts as well as to our family members and relatives for bearing with our commitment to the book. We wish to express our appreciation for the help given by Dr. Mamta Kapila (Senior Editor, Springer Nature India, New Delhi), Mr. Daniel Ignatius Jagadisan, Project Coordinator (Books) for Springer Nature, and their team for the excellent cooperation being extended and many valuable suggestions. We wish to thank Dr. Kenneth Teng from Springer New York, USA, where the book proposal for Volume 3 was submitted and approved prior to be subsequently transferred to Springer India.

We wish a pleasant reading of Volume 3 to scientists and students around the world who are interested in the subject of physic nut as a multipurpose crop.

Finally, we would like to apologize for any omissions or mistakes, or failures that may subsist in the book.

Hyderabad, India — Sujatha Mulpuri
Rio de Janeiro, Brazil — Nicolas Carels
Warangal, India — Bir Bahadur

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Editors and Contributors

About the Editors

Sujatha Mulpuri graduated in Plant Sciences from the University of Hyderabad (UoH), India. She has a Ph.D. in Genetics from Osmania University (OU), Hyderabad, and has worked on intergeneric and interspecific affinities between Ricinus and Jatropha. Dr. Sujatha is a versatile researcher, adopting conventional and modern tools for the improvement of oilseed crops encompassing the areas of genetics, tissue culture, and biotechnology. Her achievements include the development of stable male sterile lines in safflower, sunflower, and niger, optimization of tissue culture and genetic transformation protocols, development of transgenic events in castor for foliage feeders and sunflower for resistance to necrosis disease, and use of molecular markers in diversity analysis and tagging of desirable traits in sunflower (downy mildew, fertility restoration) and Jatropha (non-toxicity). Dr. Sujatha has also carried out pioneering work on Jatropha with regard to tissue culture, genetic diversity analysis of native and world collections, and interspecific hybridization, which have provided valuable leads for genetic enhancement of *J. curcas*.

Nicolas Carels graduated in Agronomy in Belgium and completed a Ph.D. in Plant Pathology (FUSAGx, Gembloux) prior to working as a scientist on the elaboration of the first genetic map of sugar beet at the end of the 1980s (ICIseed-SES, Belgium). He then moved to Paris (IJM, CNRS, France) where he completed a second Ph.D. on the genome organization in plants. He continued his work on genomics in Italy (SZN, Naples) and Spain (INTA-CAB, Madrid, Torrejon de Ardoz) before moving to Brazil (Bahia, Ilhéus, UESC), where he contributed to the application of bioinformatics and genomics to the improvement of cocoa and rubber tree for resistance to fungal diseases. His initial investigations on Jatropha covered the measurement of

the genome size by flow cytometry and the application of reverse genetics to detect QTLs for oil production with the purpose of breeding Jatropha for this trait. He is now a Federal Officer of Fiocruz (Rio de Janeiro, Brazil) and is interested in the exploration of genomics, system modeling, bioinformatics, computational biology, and natural products for the benefit of human health, with a particular focus on therapeutics for cancer.

Bir Bahadur former Professor, Chairman and Head of the Department, and Dean of the Faculty of Science at Kakatiya University, Warangal, India, has also taught at Osmania University, Hyderabad, India. He obtained his Ph.D. in Plant Genetics from Osmania University and was closely associated with Prof. J.B.S. Haldane, F.R.S, a renowned British geneticist. He made significant contributions in several areas of plant biology, especially heteromorphic incompatibility, genetics, mutagenesis, plant tissue culture morphogenesis, biotechnology, plant asymmetry and handedness, ethnobotany, application of SEM pollen and seeds in relation to systematics, medicinal plants, and Jatropha and Castor. He has mentored thousands of graduates and postgraduate students and taught genetics, biotechnology, plant molecular biology, plant reproduction, and related subjects for over 45 years and has accumulated 50 years of research experience in these areas. He has been the recipient of numerous awards, fellowships, and honors, including the Prof. Vishwambhar Puri Gold Medal, Bharath Jyoti Award, and Royal Society Bursary & Honorary Fellow of Birmingham University (UK).

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Part I
Selective Breeding and Genetic Diversity

Chapter 1
Genetic Improvement of Edible and Non-edible Jatropha for Marginal Environments in Sub-Saharan Africa

Matthias Martin, Brigitte Bohlinger, Elisa Senger, Euloge Dongmeza, Zafitsara Tantely Andrianirina, and Juan M. Montes

Abstract Jatropha has been grown in the past mainly for producing oil for biofuels preferably in marginal environments in sub-Saharan Africa, yet many projects collapsed due to overestimated yields and underestimated costs. The cultivation of jatropha genotypes that produce seeds lacking toxic phorbol esters ("non-toxic jatropha," "edible jatropha") currently receives increasing attention for animal feeding and food production. We give an overview of the challenges of jatropha cultivation in sub-Saharan Africa, discuss results from field trials in marginal environments from that region, and propose strategies for jatropha genetic improvement. Average seed yields obtained from selected hybrids at marginal places in Cameroon and Madagascar over 4 years demonstrated superiority of hybrids (2.2–8.3 t/ha) over wild germplasm, considerable extent of midparent heterosis (~400%), and potential to select for stably performing hybrids exhibiting less genotype-by-environment interaction. Cultivation of edible and non-edible jatropha hybrids had positive contribution margins per hectare and year (124–665 €/ha) in contrast to negative contribution margins of wild germplasm. The main breeding objective for edible and non-edible jatropha is to increase seed yield and stability across years and environments. Breeding objectives for seed quality parameters differ depending on the market segment. New hybrid varieties adapted to different climates have now become available. Jatropha companies and institutions providing

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solutions for superior genetics and technical guidance will lead to a new start in jatropha cultivation to turn future projects into success stories.

Keywords Breeding · Edible jatropha · Genetic improvement · Marginal environments · Non-toxic jatropha · Sub-Saharan Africa

1.1 Introduction

Jatropha curcas L. (jatropha) is considered a promising crop for the production of feedstock for biofuel production, preferably on wastelands and in areas where food production is hardly economical (marginal environments). Due to enduring low petrol prices however, the production of jatropha oil for biofuels has become less attractive, and alternative uses of jatropha are being investigated. Jatropha has traditionally been used as a medicinal plant in Africa (Sabandar et al. 2013), and nearly all plant parts (leaves, fruits, seeds, stem bark, latex, and roots) have been attributed effects. For instance, dried latex is used to stimulate wound healing, and recently, research activities have focused on the antitumor effects of curcin, a Type I ribosome inactivating protein in jatropha seeds (Luo et al. 2007; Prasad et al. 2012). Currently, the cultivation of jatropha genotypes that produce seeds lacking toxic phorbol esters ("non-toxic jatropha," "edible jatropha") receives increasing attention for animal feeding or food production purposes (Makkar and Becker 2009; Senger et al. 2017; Martinez Herrera et al. 2017). Prior to being used as food, a heat treatment of press cake, kernels, or kernel fragments for reduction of antinutrients is necessary. Additionally, the growth of resilient jatropha varieties for beneficial environmental effects, such as prevention of desertification, carbon capture, and afforestation of degraded land, has been proposed by various authors (Becker et al. 2013; Diédhiou et al. 2017; Noulèkoun et al. 2017).

Jatropha oil production has high potentials in semiarid and arid regions in sub-Saharan Africa (Wicke et al. 2011). Benefits of jatropha cultivation include its potential as a driver of rural economic and social development, potential developmental benefits (local production and supply of energy, creating additional markets for agricultural products, income generation for rural populations), as well as potential environmental benefits (improving soil conditions, increasing soil carbon storage, reducing soil erosion, and increasing agricultural productivity). Most of the jatropha projects that were established during the last 15 years, however, collapsed due to overestimations of yields coupled with underestimations of costs (von Maltitz et al. 2014).

Sub-Saharan Africa, i.e., that area of the African continent south of the Sahara, is one of the major jatropha-producing zones in the world (Jingura 2012). The regional agroecological zones in this vast area have been further classified by FAO (1994) as warm arid and semiarid tropics, warm subhumid tropics, warm humid tropics, and cool tropics, and many sub-Saharan African countries share mixes of these zones.

The lack of genetically improved jatropha varieties specifically selected for marginal environments in sub-Saharan Africa has been a major constraint for the economic success of jatropha projects in this region. In this chapter, our objectives were (1) to give an overview of the challenges of jatropha cultivation in sub-Saharan African countries, (2) discuss results from jatropha field trials in marginal environments from that region, and (3) propose strategies for genetic improvement of jatropha.

1.2 Challenges of Jatropha Cultivation in Sub-Saharan African Countries

During the global jatropha hype in the first decade of the twenty-first century, many jatropha projects were initiated especially in sub-Saharan Africa. The majority of these projects however failed to demonstrate viability due to a number of challenges. Gasparatos et al. (2015) identified energy security, poverty alleviation, and economic development as major policy imperatives that were the rationale behind the promotion of large-scale cultivation of jatropha as a bioenergy crop. The various projects had significant and country-specific environmental and socioeconomic impacts (positive and negative), namely, on greenhouse gas emissions, water availability and pollution, deforestation, biodiversity loss, poverty alleviation, energy security, loss of access to land, and food security. However, agronomic, institutional, and market failure were the main barriers for viable and sustainable biofuel investments at that time. Currently, the development of biofuels is hampered by absence of a clear vision of stakeholders and a lack of coordination between public actors in many African countries. Public actors have to establish institutional frameworks to facilitate sustainable jatropha production (Gatete and Dabat 2017). Policies promoting the cultivation of jatropha in sub-Saharan Africa need to be properly informed by empirical evidence, as pointed out by Jingura and Kamusoko (2018). These authors stressed the need to support the development of elite planting germplasm of jatropha to realize optimal seed yields, thereby providing opportunities for rural people to monetize the substantial labor inputs needed in jatropha cultivation and post-harvest processing. They concluded that only best practices will lead to achieving the acclaimed attributes of jatropha (reclaiming marginal soils and wastelands, drought tolerance, water and nutrient use efficiency, resistance to pests and diseases, low labor requirements, noncompetition with food production), which needs proper support by suitable policies.

In the past, jatropha cultivation attracted a large number of investors and farmers, who were promised high yields despite claims of low nutrient requirements and little need for care in combination with resilience of the plant to arid climates. This raised high expectations regarding its revenue potential for straight vegetable oil (van Eijck et al. 2014). Once it has been purified (filtering, neutralization, degumming, bleaching), straight vegetable oil can be used to fuel diesel, flex-fuel, or plant oil

engines, as well as combined heat and power plants. The oil productivity of jatropha plantations in the past, however, has often been very limited due to cultivation of undomesticated wild germplasm (Achten et al. 2008). The seed yield in mature stands was often not viable, and knowledge of fertilization requirements and plant management was scarce (GTZ 2009). Most jatropha projects collapsed because of numerous reasons ranging from overestimated business plans, to low yield, underestimated costs, lack of market, and lack of policy framework to regulate the biofuel sector (von Maltitz et al. 2014; Gasparatos et al. 2015). One recurring and perhaps the most important reason for failure, however, is that jatropha plantations were unable to realize viable seed yields. In fact, in several instances in the past, seed yields had been far overestimated or could not be realized (Terren et al. 2012; Almeida et al. 2014; Muys et al. 2014; Romijn et al. 2014; Slingerland and Schut 2014; von Maltitz et al. 2014; Ahmed et al. 2017). Numerous examples from different sub-Saharan countries demonstrated the dependency of economic viability of jatropha plantations on seed yields.

Terren et al. (2012), for instance, reported a huge gap between expected and realized seed yields (5.25 t/ha vs. 0.5 t/ha in the 4th year) in a pilot plantation on marginal land in Senegal, although the best-known production techniques had been applied. The authors identified susceptibility to soilborne pathogens and a short vegetation period of the used plant material as main reasons for the low seed yields.

In a techno-economic assessment of the jatropha value chain for production of straight vegetable oil from jatropha to feed fuel generators for local electrification in Mali, Bouffaron et al. (2012) found that electrification projects had a high sensitivity especially to seed yields, petrol prices, characteristics of geographic locations, and labor costs. They reported that the highest financial risk was carried by the farming sector, and the most competitive jatropha projects are characterized by higher seed yields and low to medium investment costs.

Similarly, Somorin et al. (2017) reported that, based on a life cycle assessment of self-generated energy in Nigeria, replacing diesel fuel by jatropha biodiesel in electricity generators can reduce greenhouse gas emissions by 76%. The benefits however would depend on seed yields, material inputs, and environmental status of fossil diesel.

Ghana has seen a multifaceted development of jatropha-based biofuel projects (Nygaard and Bolwig 2017). High capital investments and low oil production volumes combined with market risks contributed to the collapse of the jatropha sector. Considering the technical and management perspective, discontinuation of jatropha projects was caused by a reduced access to information due to low levels of learning and knowledge-sharing and weak public research and development support. Osei et al. (2016) developed techno-economic models for optimized jatropha utilization under an outgrower scheme for Ghana. They found variability in the profitability of the models studied for farmers and processors. Key parameters influencing net present value and internal rate of return were (i) variation in prices of jatropha seeds as well as of oil and by-products, (ii) the assumed discount rate, and (iii) variation in jatropha seed yields. Acheampong and Campion (2014) reported that contrary to the expectation of growing jatropha on marginal lands, biofuel

companies in Ghana had been given fertile land by local chiefs to grow jatropha. As a consequence, local farmers were forced to move to less productive land – a policy often leading to violent confrontation between parties.

On the other hand, Bosch and Zeller (2013) reported a wide acceptance and appreciation of a jatropha plantation established on marginal land in central Madagascar. They found significant positive effects on incomes and food security for the local population due to additional income generated by the labor-intensive jatropha production on the plantation.

Interviews with stakeholders involved in jatropha cultivation made it clear why many projects failed to meet initial expectations in Kenya (Hunsberger 2016). Commonly mentioned reasons, why jatropha activities and optimism had diminished, were lack of performance, i.e., low seed yields, susceptibility to pests and diseases, as well as high labor intensity.

Jingura (2011) emphasized the need to supply elite planting materials for optimization of seed yield and quality in Zimbabwe and considered the use of improved jatropha germplasm over wild germplasm, an objective for successful plantation establishment. Furthermore, the author suggested increasing the efficiency of jatropha cultivation through the development of improved plant varieties with a better response to fertilization.

In Tanzania, first commercial jatropha projects started in 2005. Van Eijck et al. (2014) compared two jatropha production systems: a smallholder system with a central oil processing facility (Diligent Tanzania Ltd.) and a plantation system (BioShape Tanzania Ltd.). Both models only showed marginal profitability at the time due to low yields and revenues, preventing sustained positive societal impacts. However, the authors expected that jatropha breeding would lead to more reliable varieties with higher yields consequently increasing financial feasibility. A profitability boost was expected by improved valorization of jatropha by-products, in particular by the use of the press cake for animal feeding.

Romijn et al. (2014) studied jatropha projects in Mali, Mozambique, and Tanzania. They found weak business cases and economically inviability of plantations due to a combination of reasons (investment costs, low yields caused by slow and unreliable crop maturation, inefficient oil pressing, inadequate utilization of by-products, and competitive prices of fossil diesel and palm oil). For smallholders on the other hand, jatropha only had limited value as a hedge crop in disadvantaged areas. Western support organizations phasing out their support for jatropha, based on the somewhat premature conclusion of general inviability of jatropha cultivation, further aggravated the situation. The authors concluded that the prospects would improve with more reliable and better yielding jatropha varieties due to plant breeding efforts.

To fully exploit the potential of jatropha as an energy source for the rural areas of Sudan, Abdelraheem et al. (2013) considered genetically improved cultivars, adequate agronomy and environments, as well as presence of insect pollinators as necessary conditions. Furthermore, the authors identified the complete utilization of by-products (e.g., press cake) as a key aspect for optimizing the economics of this industry.

Reasons for rise and fall of so many jatropha biofuel projects in Southern Africa were manifold. As outstanding positive examples on the other hand, von Maltitz et al. (2014) compared a smallholder project in Malawi (BERL) and a large-scale plantation in Mozambique (Niqel), which had in common to have based their operations on relatively modest seed yield assumptions ($\leq$3 t/ha). Despite promising frame conditions (climatic suitability, low yield expectations, sound economic planning, good management, sensitivity to local issues) shown by these projects, von Maltitz et al. (2014) stressed that viability at the time was still not guaranteed and jatropha needed to be considered as a high-risk crop until proven otherwise. Furthermore, the authors assumed that jatropha would remain a low-value crop due to high labor requirements and in particular due to the low value of the press cake of toxic jatropha varieties, which reduces overall profitability by limiting its utilization as valuable by-product. They concluded that plant breeding may partly resolve the problem of low seed yields by developing high-yielding varieties.

In the past, jatropha projects in sub-Saharan Africa often have been risky undertakings due to socioeconomic, market, cost, and agronomic uncertainties. From an agronomic point of view, realized seed yield is the most crucial factor for economic viability. Seed yield is determined by a number of factors, such as edaphic and climatic suitability of the cultivation site, crop management practices, and choice of variety, just to mention a few of them. Many of the aforementioned studies demonstrated that selective breeding and development of adapted varieties of jatropha is a crucial element for its successful cultivation in sub-Saharan Africa. Therefore, new jatropha varieties will be characterized by high seed yield potential and stability in the edapho-climatic conditions of this region. In addition, new varieties will contribute to the reduction of management costs (resource use efficiencies, mechanization potential) and to the increase of return opportunities based on a larger exploitation of seeds (oil content, non-toxicity) and their potential by-products (press cake, animal feeding, biomass). In the next section, we will present results from our breeding program focusing on jatropha performance in marginal areas of sub-Saharan Africa.

1.3 Performance of Jatropha in Marginal Environments of Cameroon and Madagascar

Perennial and multi-site field testing is necessary for selection of superior jatropha genotypes. After a 3-year screening of a global jatropha germplasm collection in multi-site field trials (Martin and Montes 2015), a set of superior and promising edible and non-edible jatropha accessions was available. Using molecular marker information, Montes et al. (2013) had shown that these accessions had a high degree of homozygosity and formed distinct clusters. Subsequently, we selected crossing

partners from both clusters based on complementary characteristics as well as based on genetic distance estimates with the goal of exploiting heterosis, taking advantage of its increase with genetic distance (Reif et al. 2005). The selected accessions were further self-fertilized, and finally controlled crossings were conducted to produce testcross hybrid seeds.

Within the framework of our global field trial network, our goal was to evaluate the performance of edible and non-edible jatropha hybrids and conduct quality analyses on seeds at the end of the harvest season. Seeds of 44 non-edible and 11 edible testcross genotypes as well as seeds of 2 non-edible check lines and 1 edible check line were sown in nurseries at two sites in Cameroon (Batchenga, Garoua) and at one site in Madagascar (Ihosy). Seedlings were raised for an average period of 3.8 months and then transplanted to the field in 4 m by 2 m spacing. At all locations, the genotypes were evaluated in plots comprising four plants in an alpha lattice field trial design with three replications. Agronomic management followed standard practices.

The two locations in Cameroon have been described in detail by Senger et al. (2016). Cameroon has been termed *Afrique en miniature* (which means *Africa in miniature*) due to its wide range of climatic conditions from the humid south to the arid north of the country. The same is true for soil quality, as one can find fertile and marginal land in this country. The growing number of regions characterized by marginal land and the expanding desertification are major threats for agriculture in sub-Saharan Africa. Here, we refer to *marginal environments* as lower quality agricultural land, where food production is less productive (Shortall 2013) and/or unfavorable tropical climates particularly characterized by limited water availability. The trial in the south of Cameroon (Batchenga) is located in a humid environment with high temperatures throughout the year and a growing season of 9.5 months with high amounts of rainfall (1342 mm/year, on average over the trial period). Based on the regional agroecological zones (AEZ) framework of the FAO (1994), it falls into AEZ4 (warm humid tropics). Its sandy loam soil has low pH values and low amounts of available phosphorus. The trial site in the north of Cameroon (Garoua) is located in an arid environment with a short growing season of 5 months, moderate amounts of rainfall (716 mm/year, on average over the trial period), and high temperatures throughout the year. It falls into AEZ1 (warm arid and semiarid tropics). Its loamy sand soil is characterized by low to medium pH values, limited availability of phosphorus, low content of organic matter, and limited water holding capacity. Agricultural production in Madagascar, Earth's fourth largest island, frequently has to cope with extreme weather events, particularly with cyclones leading to temporary flooding of arable land, as well as with droughts. The trial site in central Madagascar (Ihosy) is characterized by a short growing season of 5 months and a moderate amount of rainfall (770 mm/year, on average over the trial period). It falls into AEZ1 (warm arid and semiarid tropics). The soil quality of the loamy sand at this location is characterized by acidity, limited availability of phosphorus, and very low cation exchange capacity.

1.3.1 Performance of Edible and Non-edible Jatropha in Perennial Field Experiments

Average seed yields at Batchenga, Garoua, and Ihosy increased with each growing season (Fig. 1.1). In the past, non-edible jatropha hybrids and lines have shown a better and more stable performance than edible jatropha genotypes. Correspondingly, average seed yields in the non-edible genotypes were superior to those of the edible genotypes. At the time of finalizing this manuscript, the harvest in 4th year was just completed at Garoua and Batchenga, where the best performing jatropha hybrids yielded, on average, 8334 kg/ha and 2153 kg/ha, respectively. At Ihosy, the first 2 years of growth were characterized by negligible seed yields. The best performing non-edible hybrid at Ihosy yielded on average 2161 kg/ha in the 3rd year. Results from the 4th year are not presented, because harvest was still ongoing at the time of finalizing this manuscript. Edible jatropha genotypes are known to be more susceptible to environmental constraints such as drought, frost, or excess of water. Nevertheless, it was possible to identify certain edible hybrids that significantly outperformed less performing non-edible genotypes at all three testing locations. Despite very high temperatures and low amounts of rainfall at Garoua, edible jatropha performed unprecedentedly well. The best performing edible jatropha hybrid yielded on average 4935 kg/ha at Garoua in the 4th year. On the other hand, the performance of the edible genotypes at Batchenga stagnated in the 3rd and 4th year on a lower level compared to non-edible hybrids. The difference between edible and non-edible jatropha in terms of seed yield was less pronounced at Ihosy. In the 3rd year, the best performing edible jatropha hybrid yielded on average 2020 kg/ha.

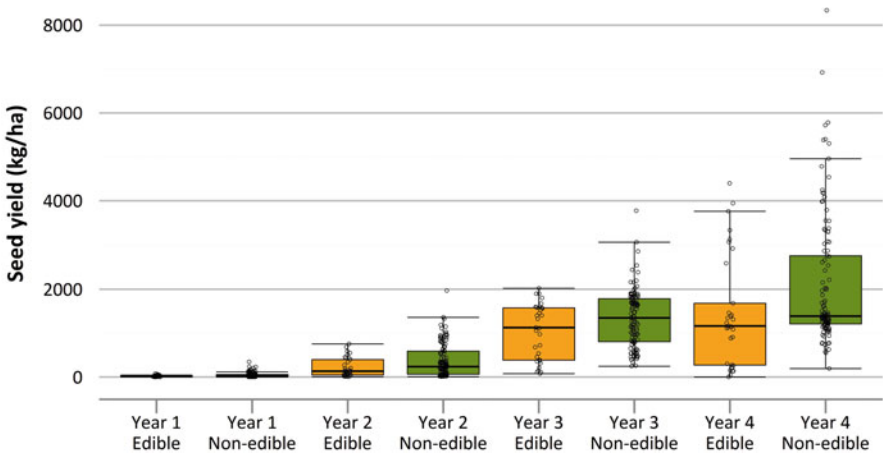

Fig. 1.1 Boxplots displaying the distribution of the population-wise mean seed yields of edible and non-edible jatropha lines and hybrids across two locations in Cameroon and one in Madagascar. Genotype means (circles) are based on three replications

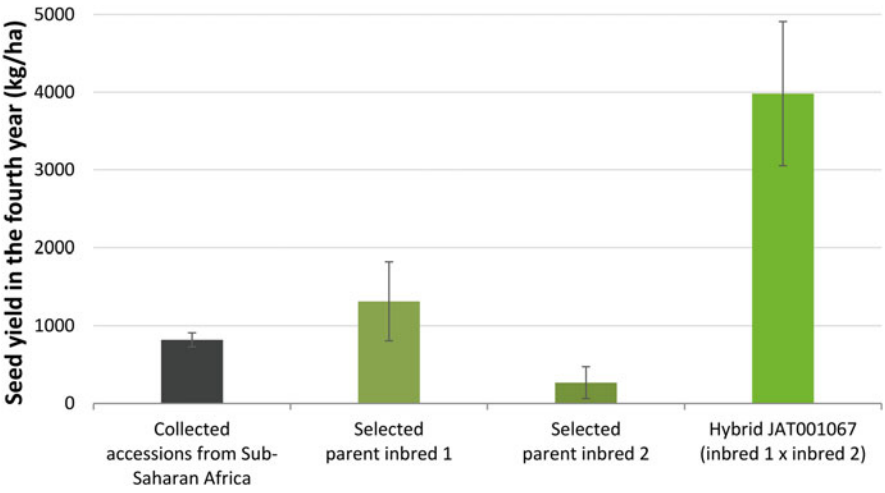

Fig. 1.2 Comparison of mean seed yields (kg/ha) in the fourth growing year at the semiarid location in Cameroon (Garoua) for 35 accessions collected in Cameroon, Chad, Gambia, Madagascar, and Tanzania, as well as selected inbred parents and their corresponding hybrid progeny

Many jatropha projects in sub-Saharan Africa in the past failed due to the cultivation of unselected jatropha germplasm, which was locally available at the time of project launch. Usually, such material had not undergone scientific evaluation in multi-year or multi-site field experiments and has a limited seed yield potential. Figure 1.2 shows the realized mean seed yield of 35 accessions from 5 different sub-Saharan African countries compared to that of a selected non-edible hybrid and its corresponding inbred parents randomly grown at the same location (Garoua). The hybrid genotype showed a significantly better performance than the wild-type germplasm. Interestingly, compared to the wild-type population, the inbred parent lines had only slightly higher or even less seed yields, respectively, which might be due to inbreeding depression. The hybrid progeny on the other hand was superior to both the wild-type population and its parents, exhibiting considerable midparent heterosis (~400%). Such an extent of heterosis ("hybrid vigor") as shown by this example is comparable to what is known from corn and shows that the prospects of hybrid breeding for jatropha are very promising.

Jatropha seeds are a rich source of oil. The average oil content in the seeds measured across 3 years was significantly higher at Ihosy (40.5%) than at Batchenga (36.2%) and Garoua (34.9%). While there was no significant difference at Ihosy between edible and non-edible jatropha, the edible hybrids had significantly higher oil contents than non-edible hybrids, namely, on average more than 1% at Batchenga and more than 2% at Garoua (Fig. 1.3a). Additionally, seeds of edible jatropha represent a valuable source of high-quality protein for animal feeding or food purposes (Senger et al. 2017). The protein content was measured in the 3rd year in the kernels of edible jatropha, i.e., after removing the seed shell, which has no nutritional value. The average protein contents in the kernels from Batchenga

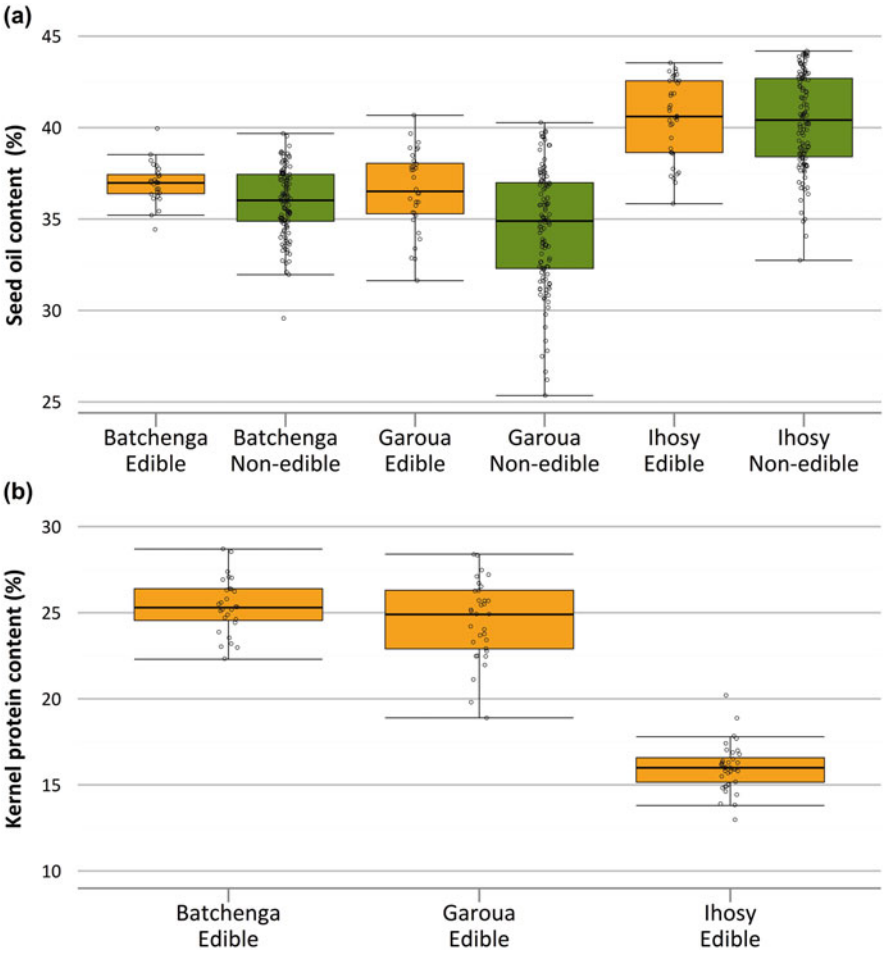

Fig. 1.3 Boxplots displaying the distribution of population-wise oil content of seeds harvested from edible and non-edible jatropha hybrids and lines (**a**), and kernel protein content harvested from edible jatropha hybrids and lines (**b**) grown at two locations in Cameroon (Batchenga, Garoua) and one location in Madagascar (Ihosy)

(25.4%) and Garoua (24.5%) were significantly higher than that of kernels harvested from edible jatropha seeds in Ihosy (16.0%) (Fig. 1.3b). These results corroborate the findings of Montes et al. (2013), who reported a negative correlation between oil and protein content in jatropha seeds.

The boxplots in Fig. 1.3 clearly show the variation for the two parameters; it is important to note that there was significant genotypic variation for oil and protein content. Thus, there are strong effects on these quality parameters that are due to the genetic set up of jatropha plants. Hence, the prospects are good to select parental

components and develop jatropha varieties with particular seed quality parameters, as desired by the corresponding markets.

1.3.2 Genotype-by-Environment Interaction

Genotype-by-environment interaction describes the phenomenon that certain genotypes perform differently in varying environments. We compared the seed yields of the best performing edible and non-edible jatropha hybrids at all three locations in the 3rd year, and we found strong indications for genotype-by-environment interaction (Fig. 1.4). Correspondingly, we estimated the relevant variance components, and the estimated genotype-by-environment interaction variance component was highly significant.

For instance, the best performing non-edible hybrid in the 3rd year at Batchenga and Ihosy (JAT001011) had only average performance at Garoua. Interestingly, however, it exhibited very stable yields across all three locations (between 1817 and 2023 kg/ha). On the other hand, the best yielding hybrid at Garoua in the 3rd year (JAT001026) was not significantly different from the highest yielding hybrids neither at Batchenga nor at Ihosy (Fig. 1.4a).

Among edible hybrids, there was no change in ranking at Batchenga and Garoua; however, the best performing hybrid (JAT001036) was ranked second at Ihosy, though not significantly different from the hybrid with the highest seed yield (JAT001037) (Fig. 1.4b).

Plant breeders usually pursue the objective of selecting varieties that exhibit a superior and stable performance for the most relevant characteristics across environments. If this objective cannot be reached, the varieties available to the breeder have to be grouped and assigned to target environments, which might be characterized by climatic or agronomic parameters. For the non-edible jatropha segment, it was possible to select superior and stable hybrids (JAT001026, JAT001067). Similarly, for the edible jatropha segment, it was possible to select a superior and stable hybrid (JAT001036), which had a convincing performance across locations and years. The analysis of results across years from the locations in Cameroon and Madagascar clearly demonstrated their superiority over other hybrids and over wild jatropha germplasm.

1.3.3 Economic Considerations of Growing Improved Jatropha Varieties

Based on the results shown, we compared the economics of four different cultivation scenarios at the semiarid site of Garoua (Cameroon). The scenarios varied with

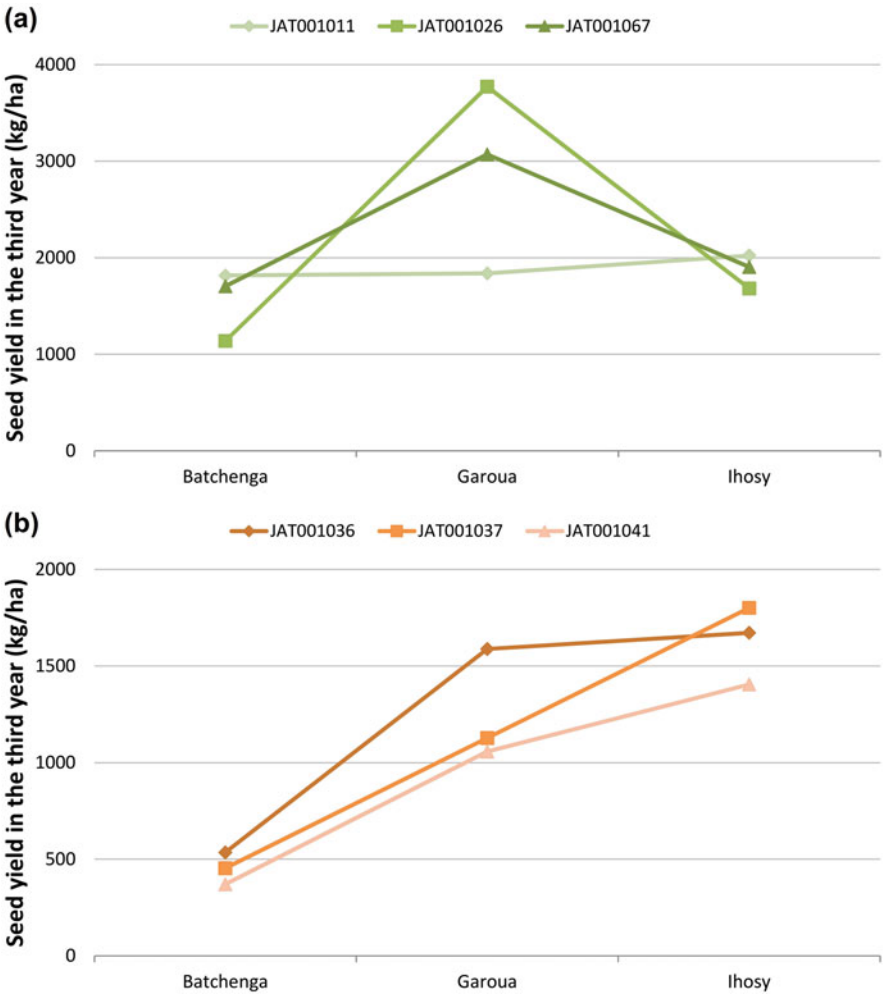

Fig. 1.4 Mean seed yields of the best performing three non-edible (**a**) and edible (**b**) jatropha hybrids across two locations in Cameroon (Batchenga, Garoua) and one location in Madagascar (Ihosy) in the third growing year

regard to the genetic material used and their seed yield and market potentials (wild type, non-edible hybrid with moderate seed yield, non-edible hybrid with high seed yield, and edible hybrid). All costs were adapted to the local situation (e.g., fertilizer, fuel, or labor costs). To calculate the contribution margin, a standard calculation model developed by JatroSolutions was used.

The assumptions of the different scenarios are summarized in Table 1.1. An average selling price for seeds harvested from wild-type jatropha was taken from the available literature data (100–150 €/t). The price of seeds harvested from non-edible hybrid varieties was adapted to 175 €/t because of their higher seed oil content

Table 1.1 Production factors of jatropha in different scenarios

Factors	Units	Wild type	Non-edible hybrid (moderate yield)	Non-edible hybrid (high yield)	Edible hybrid (feed/food)
Yield from 4th year onward	t/ha	0.8	4.0	8.3	4.3
Average annual yield during 20 years	t/ha	0.7	3.6	7.4	3.8
Seed selling price	€/t	125	175	175	350
Seed oil content	%	31	39	38	39
Oil yield after extraction[a]	t/ha	0.2	1.3	2.7	1.4
Seeds needed to produce 1 m^3 of JSVO	t/m^3	3.4	2.8	2.8	2.8
Cultivation area needed to produce 1 m^3 of JSVO[b]	ha/m^3	4.2	0.69	0.34	0.64

[a]Efficiency of oil mill 85%
[b]*JSVO* jatropha straight vegetable oil

compared to wild-type jatropha seeds, which reduces oil production costs in different areas (logistics, energy, labor, mill capacity utilization, maintenance). The annual production of an oil mill is nearly 30% higher when processing seeds harvested from improved hybrid varieties with 39% oil content rather than wild-type seeds with only 31% oil content. The market price of edible jatropha seeds was assumed to be comparable to the average price of groundnuts (with shell) and soybean in Cameroon for the period 2012–2016 (350 €/t) (FAOSTAT 2018).

Assuming the production factors in Table 1.1, wild-type jatropha shows a negative annual contribution margin (sales per hectare minus variable costs per hectare) of −209 €/ha (Fig. 1.5). The moderate seed yield scenario using non-edible jatropha hybrid gives an annual positive contribution margin of 124 €/ha. The high yield scenario using the best performing jatropha hybrid with 8.3 t/ha in the 4th year increases the yearly margin to 590 €/ha. In the edible hybrid scenario with use of jatropha for animal feeding or food, the yearly margin rises to 665 €/ha.

The cultivation of new jatropha hybrid varieties has the potential to significantly improve the incomes and livelihoods of small-scale farmers. However, one should also take into consideration the costs, which will probably arise compared to wild-type jatropha (higher costs for planting material in the 1st year, increased management costs, higher inputs). The proportion of the establishment costs may be considerable in the 1st year (costs for seed, seedlings and nursery costs). In the hybrid scenarios, seedling costs may rise up to 50% in the year of establishment, which is due to higher costs for elite varieties compared to undomesticated accessions. However, over the assumed cultivation period of 20 years, the costs are less than 3% of total costs (Fig. 1.5). Therefore, farmers are advised to invest in higher yielding varieties in order to secure higher rates of return in the long term.

The costs in the present scenarios are dominated by fertilizer and manual harvesting costs, which varied between 52–62% and 19–25%, respectively, of the

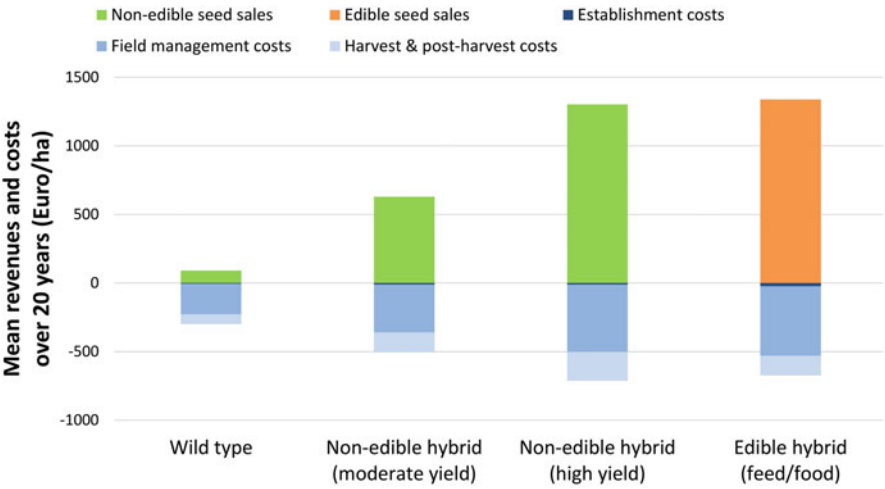

Fig. 1.5 Mean revenues and costs over 20 years for different scenarios based on data from Garoua, Cameroon, and a standard model calculation (JatroSolutions)

total costs (Fig. 1.5). The press cake remaining after oil extraction is a valuable by-product that can further be commercialized by oil mills. For instance, Ravaoarinirina et al. (2016) recommended the use of press cake from non-edible jatropha as manure due to its fertilizing and potential pesticidal properties. In our calculation model for non-edible jatropha, the press cake was returned completely as organic fertilizer to the field. In contrast, for edible jatropha, we assumed higher returns from seed sales, and fertilization was based on mineral and other organic fertilizers. Using the press cake as additional income only made sense if it was priced at a rate larger than 100 €/t.

Irrigation, fertilization, and plant protection may constitute an important proportion of the total production costs. Further studies will therefore be conducted to validate the increased efficiency of water and nutrient use as well as pest and disease resistance of the new improved varieties.

Production of jatropha straight vegetable oil is a valuable solution for rural areas to produce electricity in off-grid systems. The production of biodiesel on the other hand depends on a well-adapted logistical system for material supply. To keep the transport costs of seeds at a reasonable level, the maximal distance for seed collection to the transformation units should not exceed 100–150 km (Chapuis et al. 2013). The increase in jatropha yield will lead to a significant diminution of the radius of seed collection area to the oil pressing factories and consequently lead to a diminution of logistics costs.

The availability of improved jatropha varieties along with validated agronomic know-how (Senger et al. 2016) and specialized markets will certainly lead to a "new start" in the jatropha sector especially in rural African areas. Growing improved jatropha varieties will allow for more reliable and more accurate business plans than those from past years. The prospects for profitability will certainly lead to a new flux

of funding (for small- and large-scale projects) in jatropha production. Therefore, the advent of improved jatropha varieties will restore the hope and probably boost the economy in the Sahelian rural areas.

1.4 Genetic Improvement of Jatropha

1.4.1 Breeding Objectives

The breeding objectives are determined by the traits that define economic success of growers. The overall goal of breeders is to develop varieties that maximize revenues and minimize costs of jatropha production. Seed yield is a key factor for economic viability, and many jatropha projects in the past failed due to low yield levels. The main breeding objective in jatropha is therefore to increase seed yield and seed yield stability across years and environments.

Improved varieties will contribute to cost efficiency and optimization of agronomic practices. Therefore, fast and early development of juvenile plants, high efficiency of water and nutrient use, and tolerance and resistance to abiotic and biotic stresses represent important breeding objectives. Furthermore, the plant architecture is crucial for optimization of agronomic management practices (harvest, spacing, and pruning). Especially for development of a mechanized harvest technique, dwarfs or varieties with reduced plant height and canopy spread are needed.

The non-edible germplasm pool of jatropha is used for sustainable production of vegetable oil for biofuel purposes. Straight oil can either be used directly in diesel engines or it may be transesterified to obtain biodiesel. Biodiesel has better properties, if the source oil has higher contents of mono-unsaturated fatty acids (Ramos et al. 2009). An important breeding objective is therefore to further improve the proportion of fatty acids in jatropha oil. Utilization of the oil as raw material for soap production can be a very attractive alternative for growers. The potential of jatropha to grow in harsh environments with minimal irrigation (waste water) in hot deserts makes it an ideal candidate for carbon capture to mitigate climate change and curb desertification (Becker et al. 2013). The advancing desertification in the Sudano-Sahelian zone is a huge menace for agriculture and biodiversity in that region. Resilient jatropha varieties that better tolerate prolonged periods of drought and increased levels of salinity will therefore be needed. In the future, different products or product combinations will be obtained from jatropha plantations (Fig. 1.6). Therefore, breeding objectives will vary depending on the pursued production path.

The edible germplasm pool has a great potential for combined use as biofuel feedstock and for animal feed or food production (Fig. 1.7). The seeds produced in these jatropha genotypes lack toxic phorbol esters. Quality traits of the kernels are therefore a high priority among breeding objectives. The goal is to further reduce anti-nutritive substances such as curcin, trypsin inhibitors, and similar in the seeds. Currently, technical elimination or neutralization (heat treatment) of these substances is still needed, similar to the situation in soybean and peanut production.

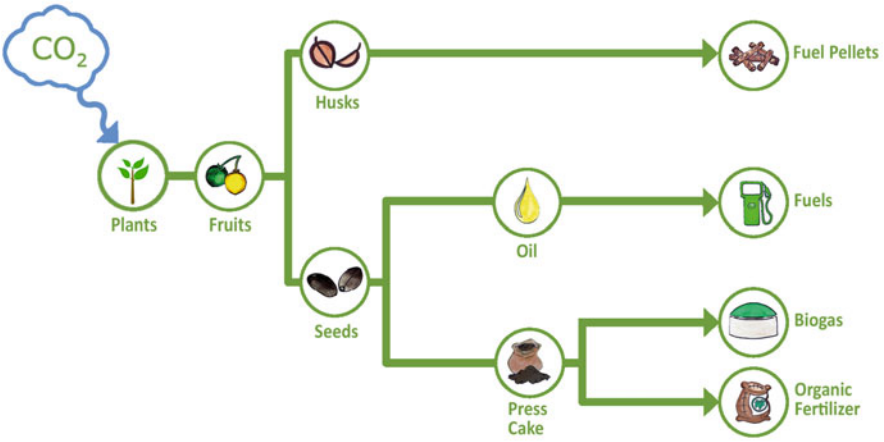

Fig. 1.6 Product paths for non-edible jatropha. (JatroSolutions 2018)

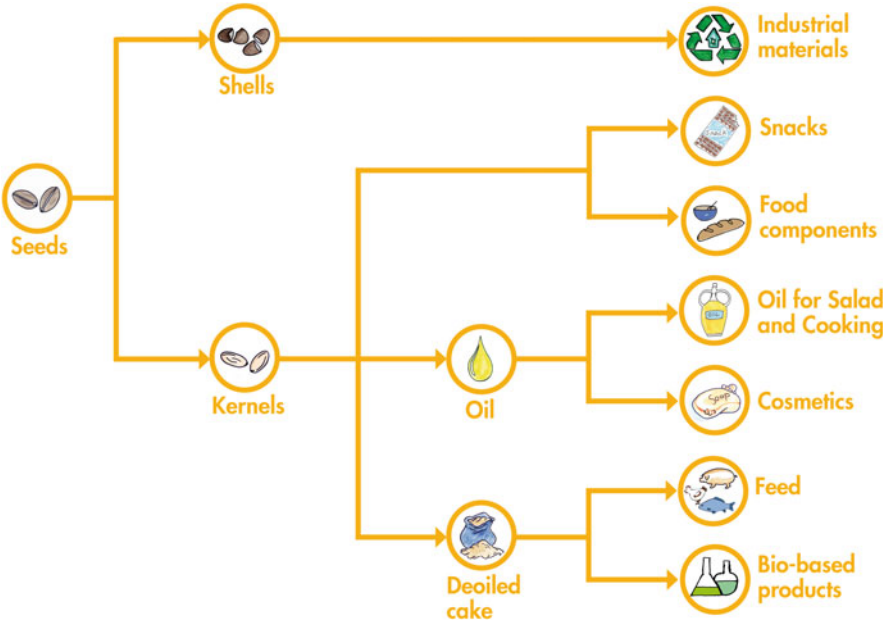

Fig. 1.7 Product paths for edible jatropha. (JatroSolutions 2018)

The contents either of oil or of protein in the seeds are to be increased, depending on the targeted product. Furthermore, the fatty acid profile of the oil and the amino acid profile of the protein fraction are being addressed by breeders. Adaptation of plant characteristics of edible jatropha to fit into modern agronomic practices will be similar to the non-edible jatropha segment described above.

In the short term, feed production in particular for fish and chicken is most probable to be realized because edible jatropha is a rich source of high-quality protein. We expect a rapid increase of the cropping area for food production, once feed production from edible jatropha has widely been established and novel food registrations are completed. To obtain feed or food, shelling of seeds, i.e., separation of kernel and seed shell, is required. Afterward, a heat treatment is necessary similar to soybean or peanut processing. Kernels or kernel fragments can be roasted entirely and used as snack (which is one of the traditional uses in Mexico) or as ingredient in bakery products or energy bars. Additionally, they can be processed to oil-rich kernel meal for bakeries or used as basic material for meat substitutes. If edible oil is produced, the remaining press cake of kernels can be used as source of plant protein for animal feeding or might be further channeled into production of bio-based products (bioenergy, organic fertilizer, bioplastics) (Fig. 1.7). Food production from edible jatropha is currently intensively investigated and needs further improvement mainly in the areas of post-harvest treatment and processing (Senger et al. 2017).

1.4.2 Genetic Diversity

Genetic diversity forms the basis for selection and is crucial for a successful breeding program. In jatropha, Montes et al. (2014), who analyzed 70 jatropha accessions from a global jatropha collection with SSR and SNP markers, found higher genetic diversity in the germplasm pool that lacks phorbol esters in seeds ("edible jatropha," "non-toxic jatropha") than in the germplasm pool that produces these toxins. Recent studies confirmed high genetic diversity, especially among accessions from Mexico and Central America, which is assumed to be the center of origin of *J. curcas* (Montes Osorio et al. 2014; Outtara et al. 2014; Santos et al. 2016). Large genetic variation was found for plant morphology and yield components (Martin and Montes 2015; Aguilera-Cauich et al. 2015), as well as for product quality traits such as phorbol ester content (He et al. 2011; Montes et al. 2013; Senger et al. 2017).

Genetic diversity in jatropha is not necessarily related to geographic distance. Ouattara et al. (2014), for instance, studied the genetic diversity among 103 jatropha accessions collected mainly from African sites (82 from different agroecological zones in Senegal, 10 from Burkina Faso, 4 from Benin, as well as 1 each from Mali, DR Congo, Mozambique, Tanzania, Cambodia, India, and Guatemala). Despite the relatively large geographic distances among the accessions, no differences on the DNA level were detected except for two accessions from Burkina Faso. The narrow genetic base is most likely due to introduction of a limited number of jatropha plants in the past by Portuguese seafarers and the subsequent clonal propagation of these jatropha accessions. In contrast to such recent introduction on a broad geographic scale, the diversification of jatropha in its center of origin occurred over a period of millions of years, which explains the high genetic diversity found in Central America

today. Consequently, exploring the genetic diversity in the countries of origin for crop improvement to develop high yielding jatropha genotypes is required.

Similarly, Sanou et al. (2015) found only very low genetic diversity in landraces from several East- and West-African countries and from India in contrast to the genetic diversity observed in Mexican jatropha populations. There was a strong differentiation, however, between Mexican and non-Mexican origins. The authors expected heterosis (hybrid vigor) to occur because of the high level of homozygosity and probable inbreeding depression in non-Mexican accessions.

In a field trial in Mozambique, Rodrigues et al. (2013) compared quantitative seed parameters originating from 12 different accessions from a commercial breeding program as well as from collections from Ghana, Mozambique, and Brazil. They found significant differences among accessions for seed oil content as well as for content and compositions of free fatty acids, sterol composition, and gamma-tocopherol content in the oil itself, which illustrates the potential for genetic improvement of seed quality traits.

On the other hand, little genetic diversity was found by King et al. (2009), who analyzed genetic diversity using AFLP markers as well as fatty acid composition of seeds collected from 23 different sites from Madagascar alone. They concluded that the observed phenotypic differences in fatty acid composition are largely due to environmental effects. The authors suggested the development of high seed yielding varieties, which lack phorbol esters. Similarly, He et al. (2011) found low genetic diversity at DNA level in accessions from Madagascar, yet high genetic diversity in those from Mexico. Phorbol ester content in seed kernels from non-Mexican accessions varied within the range of 120–515 μg/g, while accessions lacking phorbol esters were only found in Mexico. The authors suggested therefore to analyze genetic diversity before starting any jatropha selective breeding program and to focus on germplasm evaluation and conservation efforts on the basis of plant materials from Mesoamerica.

In summary, low genetic diversity among jatropha accessions is generally observed in sub-Saharan Africa, which is most likely due to the introduction history of jatropha from the Caribbean via the Cape Verde islands to Africa by Portuguese seafarers (Heller 1996). This is a good example for a genetic founder effect caused by introduction of a limited number of plants due to a species going through a genetic bottleneck leading to subsequent genetic drift. It is reasonable to assume that this situation had caused the failure of jatropha breeding in Africa and Asia in the past (Li et al. 2017). Low genetic variation drastically limits the chances for successful selection. In order to improve jatropha for sub-Saharan conditions, it is therefore imperative to search diversity within a genetic pool constituted by accessions from other parts of the world and especially from Central America. Genetically diverse germplasm collections need to be assessed in the target regions to select promising parental candidates for further recombination.

1.4.3 Selection of Jatropha for Sub-Saharan African Environments

Efforts to conduct genetic improvement of jatropha for sub-Saharan environments have been proposed by many authors. However, only a limited number of researchers reported successful selection of genotypes particularly for sub-Saharan African environments (Diédhiou et al. 2012; Ngugi et al. 2012; Martin and Montes 2015; Tiendrebeogo et al. 2016; Senger et al. 2016).

Diédhiou et al. (2012), for example, compared growth and yield parameters of different accessions from Senegal and found significant variation for certain seed yield parameters, although the observed seed yields were generally on a low level. The authors concluded that breeding for high yielding varieties for large-scale production is a priority.

Senger et al. (2016) studied the genetic variation and genotype-by-environment interactions for important agronomic and quality traits (seed and oil yield, harvest and plant architectural parameters) in a set of 227 jatropha genotypes in three climatically different locations in Cameroon. They estimated genetic variation and heritability and concluded that prospects for selection and breeding of improved cultivars were excellent. Furthermore, the authors stressed the need of testing in multiple environments, because jatropha showed significant genotype-by-environment interaction effects.

Similarly, Ngugi et al. (2012) studied the performance of 49 accessions originating mostly from East Africa in 2 contrasting environments in Kenya and found significant genotypic, environmental, as well as genotype-by-environment interaction effects for seed yield and oil content. They identified promising accessions that might show stable performance across locations yet stressing the need for replicated testing of more genotypes in more locations and seasons.

Jatropha breeding studies in the past have shown that the prospects for genetic improvement were promising. However, the aspect of genotype-by-environment interaction needs to be taken into account for selection of stable candidates. Testing jatropha genotypes in the target environments of sub-Saharan Africa is a prerequisite for the goal of selecting varieties that exhibit high and stable seed yield performance in that region. Testing in many environments also increases the accuracy for selection and helps separating environmental (nonheritable) variation from the genotypic (heritable) variation.

1.4.4 Breeding Methodology and Variety Types

The main breeding objectives in jatropha, improvement of seed yield and seed yield stability, have a quantitative nature. In other words, these traits are influenced by

genotypic and by environmental effects and interaction among these. Selection of superior genotypes and separation of genotypic from environmental effects requires testing across environments and years. Therefore, multi-locational perennial field trials to evaluate the crop performance in sub-Saharan Africa were set up in the past (Martin and Montes 2015; Senger et al. 2016).

Sub-Saharan Africa comprises a huge region with different agroecological zones. Selection efficiency and selection gain can be increased by a large number of testing environments in the target regions. The goal is to develop stable jatropha varieties with broad adaptation. However, it might be necessary to develop varieties with specific adaptation to particular zones (e.g., genotypes specifically adapted to hot and dry regions for afforestation purposes). The optimal allocation of resources (number of testing sites and duration in years, number of test candidates, etc.), however, will in the end depend on economic considerations as well as on availability of skilled personnel and necessary infrastructure.

Major limitations for successful jatropha breeding in the past were lack of expertise in agronomic management and breeding methodology (Martin and Montes 2015). In the last couple of years, however, substantial progress has been made in both areas. New superior jatropha hybrid varieties, which are adapted to various climates and agro-technologies, have become available. Current jatropha companies provide superior genetic material as well as technical guidance. These will be the ingredients for a new start in jatropha cultivation to turn future projects into success stories.

The increasing availability of affordable molecular tools will help to develop varieties for sub-Saharan Africa faster. Future jatropha breeding strategies will therefore rely on marker-assisted selection and genomic selection more intensively than today. The application of marker-assisted selection is particularly interesting for monogenic traits, for example, phorbol ester absence (King et al. 2013). Genomic selection strategies will considerably increase the efficiency of selection by shortening the length of breeding cycles (Alves et al. 2015). These techniques have good prospects to efficiently improve complex traits like seed yield, which is particularly interesting for perennial tree crops like jatropha. In the near future, genome editing approaches will play an increasingly important role for tailoring monogenic and oligogenic traits. However, the corresponding technologies must not be in conflict with national legislations.

The pursued breeding strategy depends to a great extent on the breeding category, which is determined by the final propagation method of the commercial variety. Jatropha shows a mixed mating system with a substantial amount of autogamy (Bressan et al. 2013) and can be propagated via seeds, stem cuttings, and micropropagation. Therefore, different methods for plant genetic improvement and all four breeding categories are possible for jatropha (line, population, clone, and hybrid breeding) (Montes and Melchinger 2016). Other factors to be considered are the genetic variation available to reach the breeding goals, the target markets, the level of plant variety protection required, and the available budget. The target markets determine the quality standards and agro-environmental conditions, for which new varieties are developed. The level of plant variety protection is

country-specific, determines the security of the return on investment in breeding, and affects the commercialization strategy. The available budget has great influence in setting the boundaries of the breeding program (optimal allocation of resources).

The magnitude of heterosis and the availability of an efficient hybridization system are key factors in the choice of the optimal breeding method. Thanks to the high degree of heterosis (Fig. 1.2), the most promising breeding methods for edible and non-edible jatropha are hybrid and clone breeding. The cultivation of jatropha varieties for afforestation purposes to prevent desertification and soil erosion might, however, require a different breeding category. Biomass accumulating varieties with resilience to drought and hot temperatures might only be viable in a low-price seed market. Therefore, open pollinated varieties might fit better in this market segment, because they have better flexibility to changing environmental conditions and the propagation method is cheaper than that of hybrids and clones. Like in other crops, combination of different breeding strategies may be the best way forward, for example, open pollinated varieties in low-price seed markets and hybrid and clone varieties in high-price seed markets.

The past years have shown that the huge genetic variation present in jatropha germplasm allows to rapidly derive parental inbred lines for a series of phenotypic characters and to develop hybrid test crosses. Promising experimental hybrids have been selected recently after undergoing intensive performance tests and are now being propagated and commercialized. In parallel, intensive research is being conducted on optimal propagation methods for hybrids. The optimal strategy for jatropha breeding for different markets will depend on the level of heterosis, the final propagation method, and the costs of breeding and variety production.

1.4.5 Seed Market and Variety Protection

Based on the results presented in the previous sections, the logical objective is a reliable system to provide farmers with high-quality seeds or planting material for growing purposes. From a farmer's point of view, it will be necessary that the varieties are superior to local landraces especially in terms of seed yield and yield stability. In addition, the seeds of jatropha varieties for growing purposes must conform to clearly defined criteria, such as minimum seed germination capacity, genetic, and physical purity. An established seed production and seed trading system will be needed to provide higher security to the farmers to be supplied with high-quality seeds. Furthermore, having a transparent seed pricing scheme will allow farmers to estimate the chances of economic success of growing jatropha for biofuel or for animal feeding and food purposes. The same is true for organizations that want to grow jatropha to curb desertification, for soil conservation, carbon capture, and climate change mitigation.

To our knowledge, there are no regulations for the market of jatropha seeds for growing purposes, and the situation is complicated in the majority of sub-Saharan African countries. From a plant breeder's point of view, a variety registration and

variety protection system will be necessary to warrant intellectual property rights to plant breeders and, consequently, ensure sustainable investments in jatropha seed commercialization as well as in research and development. So far, jatropha varieties have officially been registered in Mexico (Zamarripa-Colmenero and Pecina-Quintero 2017). It will be necessary to adapt the registration procedures for jatropha varieties to sub-Saharan countries, i.e., to realize a system of plant variety protection and registration with clearly defined procedures for the characterization of jatropha varieties in terms of distinctiveness, uniformity, and stability (DUS criteria).

Acknowledgments This chapter is dedicated to the late Klaus Tropf, co-founder of JatroSolutions GmbH and jatropha pioneer.

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Chapter 2
Genetic Resources and Advances in the Development of New Varieties of *Jatropha curcas* L. in México

José Luis Solís Bonilla, Biaani Beeu Martínez Valencia, Guillermo López-Guillén, and Alfredo Zamarripa Colmenero

Abstract The objective of this chapter is to present the studies carried out in Mexico on *Jatropha curcas* L. for the generation of new varieties to satisfy the demand of the industries. The importance of genetic diversity to develop new improved varieties is discussed. Varietal trials were established in four tropical environments of Mexico based on genotypes selected according to their promising agronomical and industrial attributes. The main selection criteria addressed were grain yield, oil content, growth habit, and the presence of female flowers. *J. curcas* presents a large variation in yield over the years with several types of behavior. The best genotypes of the clonal trials were two clones with 100% female flowers and one clone with a predominance of male flowers, but also with the presence of female flowers. The oil content, fatty acid composition, and physicochemical characteristics of 13 selected elite genotypes were evaluated based on their yield, resulting in an oil content between 48.3% and 56.8%. The oil of *J. curcas* is considered unsaturated with the major components, in the genotypes evaluated, being oleic acid (21.5–39.7%) and linoleic acid (29.2–46.7%). Two female varieties with 100% female flowers were registered with the names "Gran Victoria" and "Doña Aurelia," while a variety with the highest proportion of male flowers was used as a pollinator for the two female varieties and registered as "Don Rafael."

Keywords Mexican jatropha · breeding · biodiesel

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2.1 Introduction

The growing need to reduce greenhouse gas (GHG) emissions has promoted the interest in renewable energy sources, in general, and in the production of liquid bioenergy from oilseeds, in particular; biodiesel is considered as a renewable source of non-toxic fuel that can be produced from a wide range of nonedible oleaginous raw materials.

Jatropha curcas L. has become a crop of industrial interest since the oil produced by this crop can easily be transformed into liquid biofuel that meets American and European quality standards by transesterification (Azam et al. 2005; Tiwari et al. 2007). In addition, the oil of *J. curcas* can also be used for alternative products, such as soap, candles, varnish, lubricant, hydraulic oil, and biocides (insecticide, molluscicide, fungicide, and nematicide) (Adebowale and Adedire 2006; Shanker and Dhyani 2006).

With the purpose of domesticating *J. curcas*, several Asian countries, Indonesia and India in particular, have investigated its genetic diversity; their findings indicate that it was low among the material collected in different geographical regions of India (Pandey et al. 2012). In Latin America, there has been a great interest in working with *J. curcas* to produce biodiesel as well, and plantations of *J. curcas* were undertaken on a large scale. Unfortunately, in Mexico and other countries of America, such as Nicaragua and Brazil, crop productivity was very low, and profitability was zero or even negative, which promoted programs of selective breeding for improved varieties (Zamarripa and Solís 2013a; Zamarripa and Pecina 2017).

Plant breeding uses principles from a variety of sciences to improve the genetic potential of plants; it is an efficient and sustainable economic strategy to meet agronomical challenges, such as low production and biotic or abiotic limitations. The process involves the combination of elite parental plants to obtain the next generation with improved characteristics. Breeders improve plants by selecting individuals with the greatest potential to transmit their valuable features to their progenies. Plants are improved for food, feed, fiber, fuel, and a variety of other features useful to human activities.

Considerable genetic variations can be expected in the growth, chemical composition, and characteristics of seeds according to their provenance, variety, or progeny, particularly in cross-species, such as *Jatropha*, *Acacia*, and *Prosopis*, which are widely used in agroforestry systems across landscapes throughout the world (Kaushik et al. 2007). Variation would be useful as a source of future genetic selection provided that the types desired for agroforestry systems are clearly defined (Cannel 1982; Burley et al. 1984; Von Carlowitz 1986).

According to Zamarripa and Pecina (2017), genetic improvement can be successful if it starts with a selection process that contains high genetic variability that enables to identify genotypes and genes that regulate the agronomical and industrial traits of interest. In this context, this chapter will discuss advances in the development of new varieties of *J. curcas* obtained from the great diversity of genetic resources existing in the southern part of Mexico.

2.2 Genetic Resources

According to the International Plant Genetic Resources Institute (IPGRI), the Plant Genetic Resources (PGRs) are living beings that carry valuable traits and have been used in the development of improved crops since the beginning of agriculture. The importance of PGRs has increased in contemporary times, to face current and future challenges such as the adaptation of crops for changing climatic conditions and to tolerate biotic and abiotic stresses.

Germplasm banks have assumed a paramount importance for the safe ex situ conservation of genetic resources. Germplasm banks have the main responsibility of collecting, regenerating, conserving, characterizing, evaluating, documenting, and distributing their stored germplasm and warrant its secure conservation by maintaining duplicates of unique and important genetic resources (Tyagi and Agrawal 2015).

The value of a germplasm bank of *J. curcas* lies in the use of its genetic resources for the domestication of the species and the obtention of new varieties. The characterization of a plant is defined as the description of the variation that exists in a collection of germplasm in terms of morphological, biochemical, agronomical, and molecular characteristics and allows its relative according to the accessions established in germplasm banks as well as the genetic diversity of the whole (Hidalgo 2003), which is the first step that determines to a large extent the success of future commercial crops.

In 2008, Mexico voted the Law of Promotion and Development of Bioenergetics, which incepted the diversification of energy resources through the promotion of the agro-industry of oilseeds to produce biodiesel (Zamarripa and Solís 2013b). The Federal Government of Mexico, since that date, has fostered the research and development of bioenergetics, which is why the Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias (INIFAP) in the Rosario Izapa Experimental Station has germplasm banks of species such as jatropha (*Jatropha curcas* L.), castor bean (*Ricinus communis* L.), moringa (*Moringa oleifera* Lam.), coyol (*Acrocomia mexicana* Karw. ex Mart.), jícaro (*Crescentia* sp.), tevetia (*Thevetia peruviana* Pers.), napahuite (*Trichilia hirta* L.), totoposte (*Licania arborea* Seem.), corozo (*Scheelea lundellii* Bartlett), and forage species.

INIFAP through its research program on bioenergy realizes the conservation of national germplasm of *J. curcas*. In the Rosario Izapa Experimental Station, there are about four hectares of plantations that make up the National Germplasm Bank of *J. curcas* with 422 accessions from Chiapas, Oaxaca, Yucatan, Tamaulipas, Veracruz, Puebla, Guerrero, Morelos, San Luis Potosí, Michoacán, and Jalisco. There is also an exclusive germplasm bank with non-toxic *Jatropha* with a total of 25 accessions that were collected in the state of Puebla, Oaxaca, and Veracruz. The collections were made in the years 2007–2009 under different conditions of soil, climate, and altitudes that varied from 0 to 1950 m above sea level. The national

collection is in the municipality of Tuxtla Chico, Chiapas, an area of humid tropical forest (Zamarripa 2011; Zamarripa and Solis 2013a).

2.3 Selection Criteria

The genetic improvement of plants has occurred empirically since the beginning of agriculture with the domestication of plant species of anthropocentric interest. As a result of this selection process that holds for some thousands of years, farmers have selected plants with outstanding shape, color, and taste, among other characteristics. Following the emergence of modern genetics with the publication of the Principles of Inheritance of Gregor Mendel in the nineteenth century, genetic improvement began to lay its scientific basis into what we know today.

The improvement of plants is the continuous search for a genetic gain. Breeders use preexisting genetic material that constitutes its raw material. According to Demarly (1977), selective breeding is the search to produce a genetic structure adapted to the criteria and needs of humans, from an imperfect preexisting construction. Progresses in selective breeding occur only if the available plant materials have significant genetic variability. The variation observed in plants depends on the interaction between genetic factors and the environment. The genetic constitution determines a variation that is intrinsic to each individual and depends on its origin. The variation due to the medium is independent of the origin of the individual and is not heritable.

The genetic improvement of plants is an economic, efficient, and sustainable strategy in the solution of agronomical, as well as biotic and abiotic, constraints, which enables to increase yields and product quality to warrant a better competitiveness. For the genetic improvement process to be successful, large genetic variability is necessary for the identification of genotypes and genes that regulate the agronomical and industrial traits of desirable interest.

In 2007, INIFAP began the collection, conservation, and characterization of genetic resources of *J. curcas* in various states of the Republic of Mexico. From a collection of 422 accessions of diverse geographic origins (Zamarripa 2011), the characterization of morphological (Avendaño and Zamarripa 2012) and biochemical (Zamarripa et al. 2012b) traits as well as the genetic variability within the germplasm through molecular biology confirmed the existence of a large genetic base (Pecina et al. 2011). Pecina et al. (2011) showed that the diversity index of *Jatropha* germplasm in Chiapas was as high as 60%. Another study where a representative set of 175 *Jatropha* accessions from nine states of central and southeastern Mexico was used for the analysis of diversity by means of amplified fragment length polymorphism (AFLP) suggested that Chiapas could be the center of origin of *J. curcas* and that domestication was carried out in the states that border the Gulf of Mexico (Pecina et al. 2014). Thus, breeding materials for selection programs have been collected according to the agronomical features of different genotypes.

2.4 Agronomical Assessment and Selection for Crop Varieties

The agronomical characterization of varieties can be defined as the description of the behavior of a genotype or a population in a given environment. For the agronomic evaluation, we worked with a population of 288 individuals during the fourth year of production and recorded the number of inflorescences, number of male and female flowers, number of fruits, weight of fruit, and yield of fruit and seed (Table 2.1).

The number of fruits varied from 0 to 1018 fruits per plant, with a variation in weight of 4.9–30 g per fruit. The yield varied depending on the accession between 10 g and 4.9 kg of fruit per plant. Zamarripa and Pecina (2017) mentioned that the number of inflorescences in the plant and the number of female flowers are good predictors of yield because they were found to be positively correlated with the yield (0.83 and 0.78, respectively). In addition, the total number of fruits, the number of fruits harvested, the average weight of fruits, the total number of seeds, and the average weight of seeds showed high positive correlations with seed yield. For the variability of toxicity, we found plants without phorbol esters whose material is native to Puebla and plants with a high phorbol ester content of up to 3.56 mg/g.

The number of inflorescences varied from 1 to 230 per plant. The maximum value of male flowers was 17,883 flowers per plant. The maximum value of female flowers was 1064 flowers per plant. The ratio of male to female flowers was 12:1; however, trees with complete female flowers were detected. *J. curcas* is a species which is able to produce fruits by self-pollination and cross-pollination. According to Qing et al. (2007), *J. curcas* is self-compatible, but its cross-pollination achieves 86.6% when performed artificially and 76.4% occurring naturally, which allows a large degree of genetic variation and a broad set of possibilities to select plants with desired traits.

Obtaining varieties with high energy efficiency and good agro-industrial yield will grant security and profitability to Mexican and international producers to face competition in the market of agroenergetic supplies.

Given the great heterogeneity of environments present in Mexico, it was essential to perform experiments in a wide range of environmental conditions to examine both the adaptation and the adaptability of *J. curcas* varieties. The varietal assessment allowed selecting five best genotypes for evaluation in different environments.

Table 2.1 Variation of flower, fruit, and seed characteristics per plant in *J. curcas* accessions from the germplasm bank of INIFAP (Zamarripa and Solis 2013a)

Characteristics	Range
No of inflorescences	1–230
No of male flowers	6–17,883
No of female flowers	1–1064
No of fruits	0–1018
Average seed weight (g)	0.6–1.3
Seed length (cm)	1.1–2.1
Seed thickness (cm)	0.7–1.7
Seed weight per plant (kg)	0.01–4.9
Toxicity (phorbol esters) (mg/g)	Absent–3.56

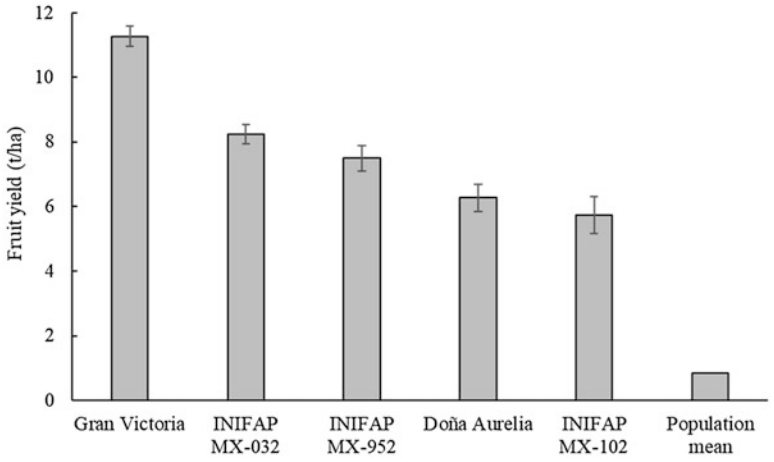

Fig. 2.1 Fruit yield of selected elite genotypes in the germplasm bank of *J. curcas*, compared to the average of 288 genotypes

Figure 2.1 shows the fruit yield of elite selected genotypes of *J. curcas* compared to the population average of 288 varieties that reaches an average yield of 0.839 t/ha of fruits. In contrast, the best five accessions reached between 5.7 and 11.2 t/ha which means that one can obtain genotypes that have 7–13 times higher yield just by individual selection.

Figure 2.2 shows the grain yield of selected elite genotypes of *J. curcas*; the best five accessions reached yields of 1.07–1.98 t/ha, which means that individual selection enables to obtain genotypes that present up to 12 times higher yield.

Considering that *J. curcas* is a perennial crop, the yields of the first three harvests are insufficient to determine the best genotype of the group under study, which makes necessary to assess the yield of at least five harvests (Zamarripa 2011). Consequently, the suitable productive cycle at which a representative selection based on seed yield can be performed was assessed over a period of time, and the seed yield of the genotypes under investigation was recorded during 6 years, as shown in Fig. 2.3. These genotypes were cultivated with the same agronomic management and under the same environmental conditions. It has been observed that yield of these genotypes continuously increased until the fourth year and decreased in the fifth year, as shown in Fig. 2.3. For example, INIFAP-MX 03 produced a yield of 1979 kg/ha in the fourth year; thereafter, the yield fell to 1334 kg/ha in the fifth year and continued to decrease to 826 kg/ha per plant in the sixth year. In general, the yield of these genotypes remained low in the following years. *J. curcas* thus exhibits a great variation in performance over the years and shows varied behavior in terms of yield. However, genotypes with early and sustained production were found, and these genotypes were good candidates for multilocation trials.

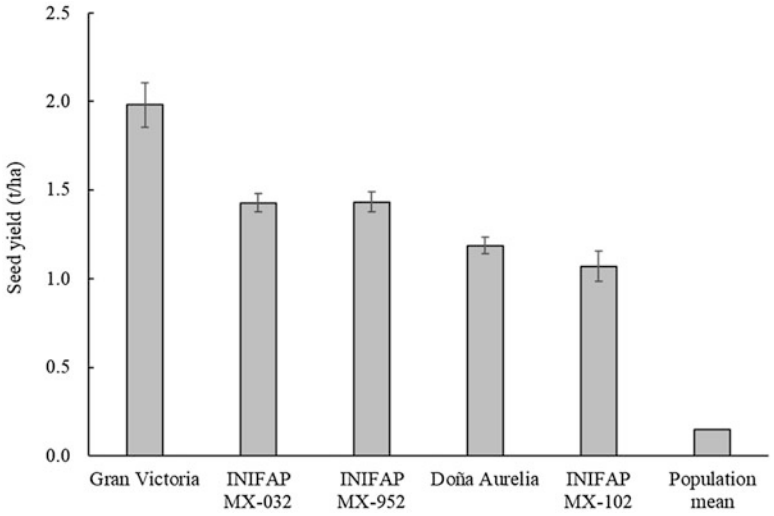

Fig. 2.2 Yield of selected elite genotypes in the germplasm bank of *J. curcas*, compared with the average of 288 genotypes

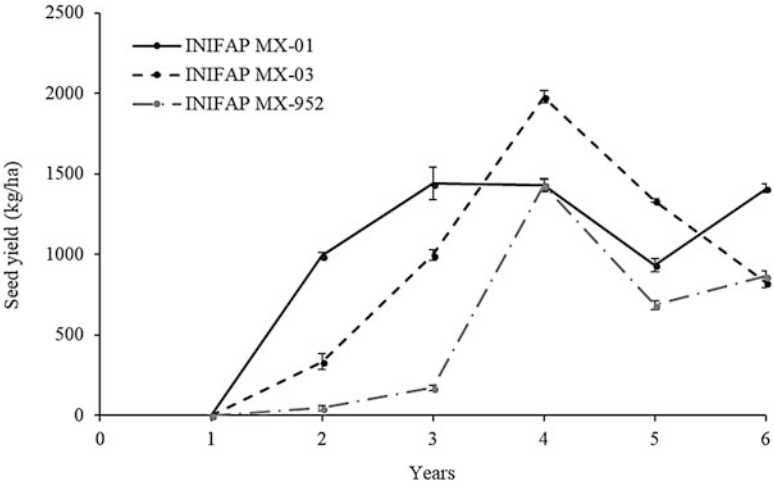

Fig. 2.3 Performance of dry seed yield (kg/ha) during six production cycles in three genotypes of *J. curcas*, in the humid tropics of Mexico

2.5 Physicochemical Characterization of Seeds and Oil of *J. curcas*

Zamarripa et al. (2012a) reported a large variation in oil content, protein content, and fatty acid composition in the germplasm collection of INIFAP. The quality of the biodiesel depends on the fatty acid composition of the vegetable oil from which it is

derived, that is to say, the degree of unsaturation and branching of oil influence fuel parameters such as cetane number, point of fusion, and oxidative stability, among others (Knothe 2005; Steen et al. 2010). Samples of 13 elite genotypes selected for their yield characteristics were assessed for their physicochemical properties and fatty acid composition.

In Table 2.2, the results of the physical characterization of the seeds are presented; these were classified in internal (kernel) and external (shell). For the variable of seed weight and humidity, significant differences were detected ($P \leq 0.05$), among the elite genotypes evaluated. The seed weight varied from 0.69 to 1.05 g; the genotypes INIFAP-MX 351, INIFAP-MX 721, and INIFAP-MX 254 showed the highest weight of 0.88, 0.90, and 1.05 g, respectively. The average of the 13 individuals evaluated was 0.82 g. The seed moisture ranged from 3.91% to 5.35%. The water content in the seed is a very important factor during the extraction of the oil regardless of the method chosen. Bernardini (1981) mentions that each seed must have an optimum humidity of less than 9%; a humidity above this value represents a decrease in the effectiveness of transformation, generating emulsions between water and oil, which occurs in the surface of the particles, which prevents the solvent from penetrating the kernel fibers and solubilizing the oil.

In Table 2.3, the oil content, fatty acid composition, and some physicochemical characteristics of *J. curcas* oil are given. The oil content varied from 48.3% to 56.8%. The results of the seed oil content are similar to those obtained by Achten et al. (2008) reporting an oil content of 54.6% and Foidl et al. (1996) reporting 57.4%. It should be noted that ten genotypes had values greater than 50% oil. Two of them stand out for having oil contents between 55% and 60% and were selected as parents in genetic improvement programs. The quality of biodiesel also depends on

Table 2.2 Average weights of *J. curcas* seeds and shells

Genotype	Seed weight (g)	Kernel weight (g)	Shell weight (g)	Humidity (%)
INIFAP-MX 821	0.77bc	0.47b	0.30bcdef	4.66c
INIFAP-MX 254	1.05a	0.67a	0.38a	5.36a
INIFAP-MX 331	0.88ab	0.55ab	0.34abcd	5.26a
"Don Rafael"	0.82bc	0.54ab	0.29cdef	4.94b
INIFAP-MX 721	0.90ab	0.55ab	0.35ab	4.87b
"Gran Victoria"	0.75bc	0.49b	0.26ef	4.50c
INIFAP-MX 952	0.69bc	0.45b	0.24f	3.93d
INIFAP-MX 32	0.80bc	0.51b	0.29cdef	3.80d
INIFAP-MX 102	0.71bc	0.45b	0.25ef	4.49c
INIFAP-MX 341	0.86bc	0.54ab	0.32bcd	4.86b
INIFAP-MX 244	0.86bc	0.51b	0.34abc	4.58c
"Doña Aurelia"	0.81bc	0.51b	0.30bcde	3.84d
INIFAP-MX 362	0.74bc	0.46b	0.28def	3.92d
Average	0.82	0.52	0.30	4.54
CV%	14.10	15.18	14.92	11.47

Averages with the same letters are not statistically different (Tukey = 0.05)

Table 2.3 Chemical characteristics of oil from 13 genotypes of *J. curcas*

Genotype	Oil content (%)	Fatty acids (%)			Iodine (mg iodine/g)	Density at 15 °C (g/cm^3)	Viscosity at 40 °C (mm^2/s)
		Saturated	Monounsaturated	Polyunsaturated			
INIFAP-MX 821	54.6ab	34.60ab	29.72def	35.67cdef	106.183cd	0.920e	35.04d
INIFAP-MX 254	51.0cd	33.27abc	32.86cd	33.87def	96.762g	0.920de	34.01f
INIFAP-MX 331	50.8cd	31.5abcd	39.12ab	29.34f	106.013cd	0.921bc	33.60h
"Don Rafael"	51.1cd	35.9a	29.10def	34.95cdef	105.409d	0.920bc	33.92g
INIFAP-MX 721	48.3cd	23.45fg	41.39a	35.15cdef	106.013cd	0.921bc	33.22i
"Gran Victoria"	53.4bc	20.9g	34.95bc	43.94ab	109.169bc	0.921bc	32.79j
INIFAP-MX 952	55.1ab	24.23efg	35.54bc	40.23abcd	106.323cd	0.920cde	33.94g
INIFAP-MX 32	56.8a	27.77cdef	31.63cde	40.58abc	101.616ef	0.920e	33.22i
INIFAP-MX 102	49.7d	29.69bcde	26.68ef	43.62ab	111.198b	0.922ab	35.56b
INIFAP-MX 341	49.5d	26.38defg	26.90ef	46.72a	115.319a	0.922ab	37.39a
INIFAP-MX 244	50.5cd	35.23ab	32.62cd	32.15ef	94.642g	0.922ab	35.24c
"Doña Aurelia"	53.4bc	35.36ab	24.89f	39.75bcd	98.571fg	0.921ab	34.89e
INIFAP-MX 362	54.8ab	36.43a	25.34f	38.22bcde	103.995de	0.901f	19.12k
CV%	5.12	17.59	16.32	13.41	4.25	0.638	13.018

Averages with the same letters are not statistically different (Tukey = 0.05)

the physicochemical properties of the oil. The excess of free fatty acids determines the necessity of an additional prestep of esterification in order to avoid saponification during the transesterification process.

Significant differences were found ($P \leq 0.05$) for fatty acids (saturated, monounsaturated, and polyunsaturated), iodine, density, and viscosity. For saturated fatty acids (palmitic, stearic, arachidic), the proportion interval ranged from 20.9% to 36.4%, while monounsaturated (mainly oleic) varied between 24.9% and 39.1%, and polyunsaturated (linoleic, linolenic, and icosenoic) varied from 29.3% to 46.7%. The unsaturated fatty acids (monounsaturated and polyunsaturated) ranged from 64.7% to 78.9% of the total fatty acid content in the oil of *J. curcas*. Saturated fatty acid content increases the point of cloud or fog (solidification of the oil) and the cetane number (quality of combustion) and improves the oxidation stability of biodiesel. The genotypes with the highest monounsaturated fatty acid content were INIFAP-MX 721 and INIFAP-MX 331 with 41.4% and 39.1%, respectively. Ideal oils should have low content of saturated fatty acids and high content of monounsaturated fatty acids, especially oleic acid.

The iodine index varied between 94.64 and 115.64 mg iodine/g. According to the European standard EN-14214, the maximum permissible value of iodine index of biodiesel is 120 g I_2/100 g. Very low values of this index indicate a higher risk of solidification at cold temperatures. ASTM 6751 establishes permissible ranges of 1.9–6 mm^2/s for fuel viscosity. The oil viscosity of elite genotypes was lower than the value of 42.88 mm^2/s indicated by Akbar et al. (2009) and ranged from 19.12 to 37.39 mm^2/s. It is worth mentioning that the viscosity of *J. curcas* oil decreases by more than 85% when it is transformed into biodiesel and falls within the range of 2.59–5.08 mm^2/s, which is in agreement with international standards.

The oil density of the evaluated elite materials ranged from 0.901 to 0.922 g/cm^3. The European standard states that oils destined for the production of biodiesel should not have densities lower than 0.860 g/cm^3. When transformed into biodiesel, the oil density decreases by 4% and was found in the evaluated genotypes to be in the range of 0.866–0.885 g/cm^3. This parameter determines the maximum proportion of biodiesel that can be used in mixture with fossil diesel (B5, B10, B15, etc.). It may be the case that the mixtures do not comply with the official standard; this may occur with mixtures containing a high amount of biodiesel, or in those in which the density of the mixture of diesel and biodiesel is close to the upper limit allowed, which is 0.900 g/cm^3.

As discussed above, fatty acid profile and quality of oil and biodiesel differ largely between elite genotypes (data not shown), so that genetic improvement through intraspecific hybridization and/or individual selection is a high priority for the future success of *J. curcas* as a main supplier of oil for biofuel production in Mexico. It is clear that the supply of quality oil is key to the biodiesel industry. Therefore, the quality of the biofuels generated is directly dependent on the type of raw material used.

The research and selective breeding programs are key elements in the development of a sustainable and efficient biofuel industry. The species *J. curcas* presents physicochemical characteristics suitable to meet the supply of a sustainable source of raw material to produce biodiesel.

2.6 Variety Selection

According to the productivity of genotype selected individually on the basis of their agronomical performances over several years and in various environments, three clonal varieties were retained for their outstanding seed yield, oil content, growth habit, and/or female flower-only features. "Doña Aurelia "and "Gran Victoria" are female only, while "Don Rafael" serves as a good pollinator since it presents a larger proportion of male flowers. To initiate the commercial sowing and to take care of the high demand of plants and oil to feed the national energy industry, INIFAP registered officially in September 2014 the three clonal varieties just outlined in the "National Catalog of Plant Varieties" (CNVV) of the Inspection and Certification of Seeds of National Service (SNICS) belonging to the Secretariat of Agriculture, Livestock, Rural Development, Fisheries, and Food. The elaboration of the varietal description of the new varieties was based on the "Technical guide for the description of the variety of *Jatropha*" published by SNICS (2014). The breeding methodology used to obtain the varieties was the clonal selection (Zamarripa and Pecina 2017). The clonal varieties are a set of plants that are derived from the same parent plant by vegetative propagation. These collections allow us the first evaluation of a clone for interesting features such as yield, vigor, growth habits, disease resistance, and oil quality. Elite plants are multiplied as rooted cuttings and evaluated in cloning trials along with standard varieties. The best selected clones in a country of origin are not necessarily suitable for other countries due to the environmental interaction. Given the ease of asexual propagation of *J. curcas*, this method is interesting to be considered in genetic improvement programs (Zamarripa and Pecina 2017). Next, we present the characteristics of the elite three varieties of *J. curcas* registered by INIFAP.

2.6.1 Variety "Doña Aurelia"

The tree has an intermediate shape, open canopy, and drip area of 4.0 m^2. The stem is green with a diameter of 4.3 cm, branched from the base with 3 axes and 61 branches on average, which have apical leaves. The leaves are small in web form with five lobes. The shoots are green with tan tones (Fig. 2.4).

This variety is female only with 100% female flowers and has a yield of 211 bunches, with 909 female flowers. It presents yields of 820 seeds with an

Fig. 2.4 Tree characteristics of the variety "Doña Aurelia." (**a**) Branching from the base and structure, (**b**) canopy coverage, and (**c**) height

average weight of 0.83 g. The seeds have a length of 1.99 cm, 1.09 cm in width, and 0.89 cm in thickness (Zamarripa and Pecina 2017). This variety identified for the conditions of Chiapas, Mexico, presents yields at the fourth year of 1,182 kg/ha of seed with oil content of 53.4%.

2.6.2 Variety "Gran Victoria"

This variety is of intermediate size with open cup and drip area of 4.01 m^2. The stem is green with a diameter of 5.5 cm, branched from the base with 4 axes and 95 branches on average, which have apical leaves. The leaves are large heart-shaped with three lobes. The shoots are tanned (Fig. 2.5).

The variety presents 100% female flowers with large sepals, with 94 clusters on average, which produce 408 pistillate flowers (Fig. 2.5). The seed is black, elliptical in shape. This variety presents a production of 799 seeds with an average weight of 0.77 g. The seeds have a length of 1.74 cm, 1.15 cm in width, and 0.93 cm in thickness (Zamarripa and Pecina 2017). For the region of Chiapas, Mexico, this variety presents yields of 1, 979 kg/ha of seeds at the fourth year with oil content of 53.4%.

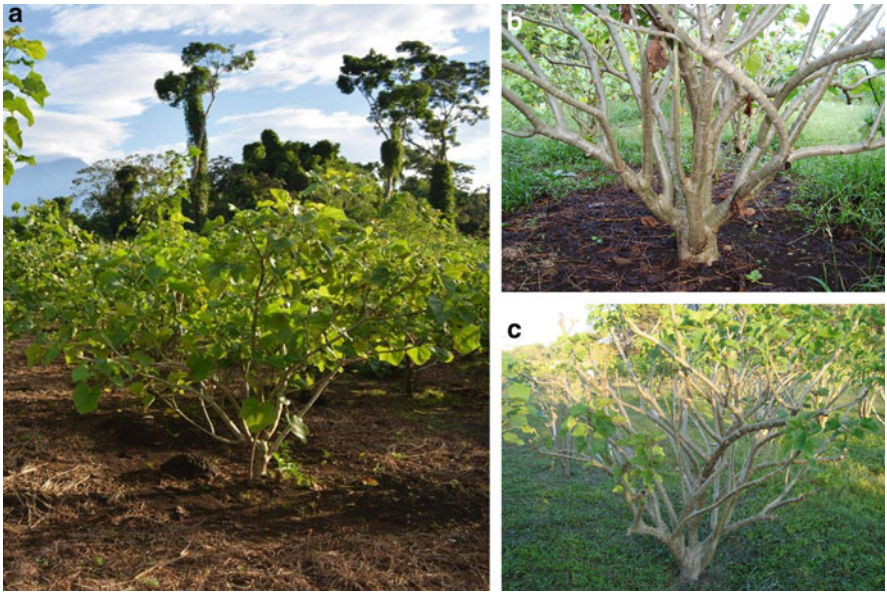

Fig. 2.5 Tree characteristics of the variety "Gran Victoria." (**a**) Canopy coverage and height, (**b**) branching from the base, and (**c**) structure

2.6.3 Variety "Don Rafael"

The trees of this variety are of intermediate size, erect, and with intermediate branching; they have an average of 20 male flowers for each female flower, which is why it is considered as a pollinating variety. The oil content in the grain is 49.8%. The seed yield at the fourth year of planting is 900 kg/ha (Fig. 2.6).

2.7 Conclusions and Future Perspective

To preserve the varietal identity, these three important varieties must be propagated asexually by stem cuttings and established in clonal gardens. The cuttings should be a minimum of 40 cm long and 3 cm thick for greater field strength. The clonal varieties "Don Rafael," "Gran Victoria," and "Doña Aurelia" are found in clonal gardens of 0.5 ha in four experimental stations of the INIFAP: in the Rosario Izapa Experimental Station, in the municipality of Tuxtla Chico, Chiapas; C.E. Valley of Apatzingán, in Michoacán; C.E. Las Huastecas, in the municipality of Altamira, Tamaulipas; and C.E. Mocochá, in the municipality of Uxmal, Yucatán. The use of these three clonal varieties of *J. curcas* will allow to meet

Fig. 2.6 Tree characteristics of the variety "Don Rafael": canopy coverage, height, and structure

the short- and medium-term demand of both the plants and oil for the energy industry. Farmers in Mexico may have other alternatives for profitable and competitive production in terms of production of biofuels and generation of employment through crop production and biofuel processing industry.

The use of clonal varieties of the INIFAP will enable to increase the production of raw material to warrant a sustainable production of biofuels. In Mexico, according to the maps of productive potential generated by the INIFAP, more than 1 million hectares under dryland conditions have been identified with high potential for *J. curcas* cultivation with an altitude of 0–900 m, temperature range of 18 and 28 °C, and rainfall between 900 and 1500 mm per year, excluding the areas currently occupied by natural forests and jungles, in which these varieties could be cultivated not only to increase the current yields but also to address the ecological concerns and issues regarding climate change. The development of *J. curcas* as a crop through the use of the three varieties of INIFAP for the production of biofuels will have a favorable impact on the environment since they yield biodegradable compounds, with positive energy balances of 1:5.1 ratio, which reduce the emission of polluting gases by more than 70% with respect to the fossil diesel reference that equals 83.8 kg CO_2eq GJ. Further, it reduces the greenhouse effect which directly contributes to the reduction of the problems caused by climate change and its impact on human health as well as on the ecological environment (Zamarripa et al. 2012a; Zamarripa and Solis 2013a).

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Chapter 3
Strategies in the Genetic Breeding of *Jatropha curcas* for Biofuel Production in Brazil

Bruno Galvêas Laviola, Erina Vitório Rodrigues, Larissa Pereira Ribeiro, Lidiane Aparecida Silva, Leonardo de Azevedo Peixoto, and Leonardo Lopes Bhering

Abstract The global challenge is to increase food production in a sustainable way, given that most of the energy used comes from fossil fuels, which causes unsustainable damage to the environment, such as the greenhouse gas emissions. Aiming at diversifying the Brazilian energy matrix, the use of biofuels emerged as a promising alternative. In this context, it is important to emphasize that soybean sustains most of the biodiesel and biokerosene markets (79.1%), so it is highly dependent on this crop, which constitutes a threat concerning economical security issues. In this way, it is the need of the hour to invest in diversification of potential raw materials for biofuel production, such as *Jatropha*, which has been identified to present a high content of quality oil suitable for biofuels. However, the seed and oil yields per hectare of *Jatropha* are still too low to be economically sustainable for farmers. This situation requires the development of improved cultivars. Several research efforts with this crop have already been initiated in Brazil. However, there is still much to be done in order to bring *Jatropha* to the level of a commercial crop able to deliver a suitable return on farming. Considering that it presents long breeding cycles, it is important to adopt strategies for increasing the selection efficiency and genetic gain, as well as for decreasing the cultivar generation time.

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In view of the considerations given above, the purpose of this chapter is to integrate the information available in the literature and to report on the most promising approaches of genetics and biotechnology for the selective breeding of improved *Jatropha* cultivars in Brazil.

Keywords Breeding populations · Genome-wide selection · Recurrent selection · Renewable energy

3.1 Introduction

The necessity to reduce greenhouse gas emissions has promoted a sustained search for alternative resources of renewable energies in many countries around the world. The environmental impacts caused by the atmosphere warming and the depletion of easily extractable oil reserves have stimulated the use of renewable inputs that can replace fossil fuels like petroleum, coal, and natural gas. Biofuels are one of the alternatives that may contribute to the mitigation of climate changes (Cremonez et al. 2015; Kumar et al. 2016).

Currently, Brazil stands out among other countries according to the process of renewable energy production, mainly because it has extensive growing area and great hydroelectric potential. In this scenario, biodiesel is receiving great attention as an alternative fuel to totally or partially replace fossil diesel, since this biofuel is characterized as non-toxic, biodegradable, and nonpolluting, has flash point, and can be mixed with fossil diesel in any proportions due to the similar properties of both fuels (Takase et al. 2015). Hence, investigations have been encouraged to set up a sustainable production chain for biodiesel based on diversified oil sources that could supply the national energy matrix as well as to contribute to the international market.

There are several promising oilseed species grown in Brazil for biodiesel production due to the wide diversity of the national ecosystem. However, among these crops, soybean contributes for most of the raw materials used in biofuel production with an average of 79.1% (ANP 2017) because of its well-controlled farming that warrants regular oil supply. In order to complement the national production system and to meet the growing global demand for biodiesel, it is necessary to invest in the improvement of oilseeds adapted to different edapho-climatic conditions so as to extend its area of production to solve the limited amount of traditional raw materials and their high prices (Dharma et al. 2016). Moreover, the inclusion of new oilseeds to the production chain is expected to bring positive social impacts by providing employment and income generation.

In this scenario of search for raw materials suitable for biodiesel production, *Jatropha* (*Jatropha curcas* L.) has been highlighted mainly because (i) its seeds have a high content of good-quality oil, (ii) it reaches maturity in a short time, (iii) it has a long productive period, (iv) it tolerates drought, and (v) its genetic variability allows selective breeding. In standard conditions, the oil yield by this

crop reaches about 1200–1500 kg/ha from the fourth year of production (Laviola et al. 2014). This oil meets the physicochemical characteristics in compliance with the American and European standards for conversion into biodiesel by transesterification (Tiwari et al. 2007).

Some countries such as India, China, and the Philippines have *Jatropha* among the main sources for biodiesel production (Zhang et al. 2011). However in Brazil, *Jatropha* is still considered as an undomesticated species concerning its potential for biodiesel production. Thus, selective breeding from a germoplasma representative of the whole species diversity is necessary to produce elite varieties (Achten et al. 2010). The lack of genetically improved varieties with homogeneous and reproducible yield under diverse edapho-climatic conditions whose fruits could be mechanically harvested has made its large-scale planting impossible (Rocha et al. 2012).

In breeding programs, information on genetic diversity within a species is essential for its rational use as a genetic resource. Given the biodiesel production chain, it is important to define the criteria for genotype selection in order to characterize the ideotypes that will best fit successful farming and genetic progress under selective breeding (Spinelli et al. 2010). Overall, perennial plant breeders seek to shorten selective cycles and minimize the time to release superior cultivars. Among the various selective strategies, there are currently precise tools available for early selection in perennial plant breeding (Resende et al. 2014). *Genome-wide selection* (GWS) is one of the most modern and promising tools for early selection that can be applied to the selective breeding of *Jatropha* (Peixoto et al. 2017).

In view of the considerations given above, the purpose of this chapter is to integrate the information available in the literature and to report on the most promising approaches of genetics and biotechnology for the selective breeding of improved *Jatropha* cultivars in Brazil.

3.2 Breeding Strategies in *Jatropha*

Jatropha can be multiplied by means of seeds or vegetative propagation depending on the breeding strategies adopted, which involve the breeding of clones, lines, hybrids, and/or populations. Seedling is the most used method of *Jatropha* propagation because it is cheap, warrants proper rooting, and enables to benefit from heterosis, which is huge in *Jatropha*. However it may entail problems of crop management and result in large variation of production and, consequently, in oil production if seeds are not produced by breeding inbred lines (Horbach et al. 2014).

Thus, vegetative propagation has been widely used, and clonal breeding has been adopted, which allows several advantages, such as (i) homogeneous and fast multiplication of inbred lines for exploitation of heterosis, (ii) cultivar homogeneity, (iii) diagnostic fingerprints for cultivar protection, and (iv) midterm timeline to first cultivar release. However, it is worth mentioning some drawbacks with this method such as (i) high propagation costs per plant, (ii) complex logistics due to phytosanitary regulations and maintenance of cuttings during transportation,

(iii) diseases that are transmitted by clonal propagation, and (iv) uncertain field performance of vegetatively propagated plants when compared with the performance of the same genotype from seeds, due to differences in root system development (Montes and Melchinger 2016).

Moreover, vegetative propagation can be associated with other techniques such as recurrent selection, marker-assisted selection, genomic selection, mutation, and genomic engineering. Thus, the breeding program of Embrapa for *Jatropha* was planned as shown in Fig. 3.1, which will be detailed below.

3.2.1 Genetic Resources

The genetic breeding adopted in each plant species depends on several factors, and among them, it is worth highlighting the genetic resources available and the reproductive system. Thus, it is essential to know the center of origin of the species because it hosts the largest genetic variability and wide genetic variability is essential for the selection of valuable genetic combinations through breeding. *Jatropha* has Mexico and Central America (Belize, Costa Rica, El Salvador, Honduras, Nicaragua, and Panama) as its center of origin (Achten et al. 2010). This species was introduced in the islands of Cape Verde and Guinea-Bissau in the middle of the sixteenth century, after the Portuguese have verified its medicinal potential. After this introduction, *Jatropha* spread to other Portuguese colonies in Africa (Mozambique, Angola) and later in Asia (India, China, and Indonesia) (Silitonga et al. 2011). It is a perennial plant that grows in tropical and subtropical regions and presents adaptability to different regions of Brazil.

Jatropha is a diploid species with small chromosomes (2n = 22) ranging from 1.71 to 1.24 μm, of which five are metacentric and six are submetacentric (Soontornchainaksaeng and Jenjittikul 2003; Paramathma and Venkatachalam 2007). According to Carvalho et al. (2008), its base composition is AT = 61.3% and GC = 38.7%.

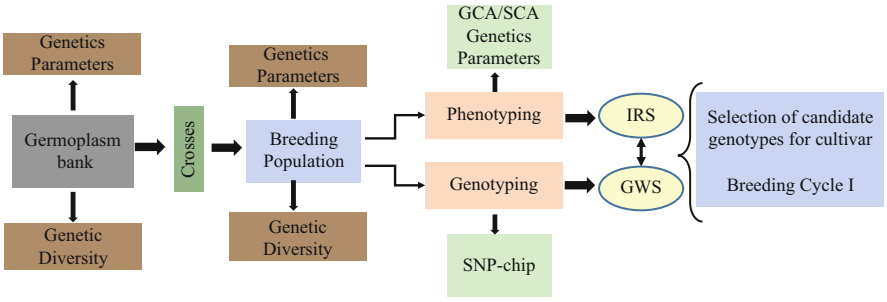

Fig. 3.1 General scheme of breeding program of Embrapa for *Jatropha*. (*IRS* intrapopulation recurrent selection, *GWS* genome-wide selection)

The reproductive system is essentially constituted of monoecious flowers, but can occasionally present hermaphrodite flowers, and is classified as allogamous being pollinated primarily by bees (Kumar and Sharma 2008; Lucena et al. 2014). The lifetime of male and female flowers is approximately 2 and 12 days, respectively, but the stigma receptivity is only high during the first 4 days and decreases after the fifth day until being null at the ninth day (Changwei et al. 2007). This information is important to determine suitable breeding strategies for *Jatropha* and how hybridizations and the number of artificial pollinations should be performed.

In general, the selective breeding of *Jatropha* follows the following steps: (i) *prebreeding*, where germplasm collections are evaluated for establishing breeding populations based on genetic information and individual performance; (ii) *breeding stage*, where the superior individuals from the breeding population are recombined to obtain promising progenies, which are evaluated in the field for the traits of interest and are subsequently selected based on their breeding value; and (iii) *release of elite varieties*, in which individuals, previously selected based on their breeding values, are vegetatively propagated for clonal testing, so that clones are selected based on their performance and commercially released as elite varieties (Bhering et al. 2011; Alves et al. 2015).

Thus, it is clear that the genetic variability available in a germplasm is the main concern to be addressed when starting a breeding program, since without sufficient genetic variability, there is no way to select new valuable genetic combinations for traits of interest. Thus, the first step to confirm is that the genetic variability available in the germplasm collection is representative of that from the species at its center of origin (Bhering et al. 2011; Alves et al. 2015). Therefore, a germplasm collection should be composed of genotypes from different regions aiming at increasing the probability to explore the largest portion of the space variability as available in the species and to maximize the chance of generating new valuable genetic combinations for the traits of interest.

In Brazil, some *active Jatropha germplasm banks* (AGB) were implemented by Embrapa Agroenergia, in partnership with Embrapa Cerrados, including genotypes from some regions of Brazil and Mexico (Laviola et al. 2010b). For the effective use of this bank, the characterization of the individuals, at both phenotypic and genotypic level, is essential. By using molecular markers, it has been possible to identify and characterize germplasm by associating a molecular phenotype to each individual, to construct genetic maps, and to estimate the genetic distance between individuals and populations. The molecular tools can contribute to optimize the estimates of genetic gain and, therefore, to minimize the time spent in evaluating the genetic potential of populations for breeding, especially in perennial crops.

Initially, Rosado et al. (2010) evaluated the Embrapa's germplasm collection by using *random amplification of polymorphic DNA* (RAPD) and *simple sequence repeats* (SSR) molecular markers. The authors observed that there was no relation among the cluster of accessions and their geographical origin, suggesting that the *Jatropha* dispersion of seeds and possibly vegetative propagules had been remixed over Brazilian regions in different times. In addition, they concluded that the genetic basis is narrow and the extent of potentially duplicate accessions probably reflects a

common ancestry, which may have occurred by intensive selection of genotypes grown since the introduction of the crop in Brazil. This result showed the need for introducing new genotypes to the Embrapa's germplasm collection for successful selective breeding of *Jatropha* cultivars adapted to the edapho-climatic conditions of Brazil. Thus, genotypes from the germplasm of other countries were introduced in order to enlarge the genetic variability of the collection.

The genetic diversity and population structure of Indonesian *Jatropha* collections using *single-nucleotide polymorphisms* (SNPs) markers were investigated (Anggraeni et al. 2018). The genetic diversity was found comparing to *Jatropha* accessions from Thailand, the Philippines, and China, reinforcing the evidence that new genotype introductions are necessary. Besides this study, several investigations have reported the low level of genetic variability in several *Jatropha* populations (Aguilera-Cauich et al. 2015; Maghuly et al. 2015; Sanou et al. 2015; Santos et al. 2016), not only in Brazil but in several regions of the world.

Trebbi et al. (2015) estimated the genetic diversity among 273 *Jatropha* genotypes from 15 countries on 3 continents based on the SSR, *expressed sequence tag*-SSR (EST-SSR), and SNP markers. They verified the formation of two groups with high genetic uniformity and high homozygosis in Africa, Asia, and South America and in some states of Mexico suggesting that only few Mexican accessions were transported abroad by Portuguese seafarers. These results were important to explore the distribution of germplasm diversity of *Jatropha* worldwide as well as to optimize the efficiency of marker-assisted selection for breeding programs. Aguilera-Cauich et al. (2015) evaluated the phenotypic diversity and contrasts in American *Jatropha* genotypes and found greater diversity among the American genotypes when compared with those from India and Malaysia. Phenotypic similarities among the accessions and their collection sites were observed in Mexico, from which it could be inferred that the effect on the genotype might be related to geographical barriers.

The next step after possessing the information about the genetic variability of the species is the formulation of the breeding population. Thus, efforts should be focused on the cross-breeding strategy in order to increase the number of favorable alleles for the traits of interest.

3.2.2 Development and Testing of the Breeding Populations

The success of a breeding program lies in the selection and evaluation of the proper parents emphasizing those with high mean for the traits under consideration and wide genetic variability, ensuring the best genetic potential of the population to produce valuable recombinations. The use of genetic designs for forming the breeding population enables to quantify the genetic variability of evaluated traits, the relative importance of additive genetic effects expressed for the effects associated with the *general combining ability* (GCA), as well as the effects due to the dominance deviations associated with the *specific combining ability* (SCA).

Diallel analysis has been routinely used in genetic breeding programs for evaluating parents, providing useful information on the different selection strategies. However, its main limitation is when a large number of parents are involved in the population, since the probability of finding those with favorable alleles for the desired trait is very low. In addition, the contribution of alleles of each parent to the population progeny would be too small that most would be lost after the first round of selection. However, if the number of parents is very low, the probability of associating the most favorable alleles for the trait under selection will also be low (Ramalho et al. 2012). Thus, to solve this challenge, circulating partial design may be employed (Bhering et al. 2011).

For forming the breeding population of the Embrapa Agroenergia, the parents were selected in the AGB based on traits that fulfilled the objectives of the *Jatropha* breeding program, so they were classified in groups according to traits such as seed yield, toxicity, and resistance to powdery mildew. In addition, a group of individuals selected according to maximum genetic divergence was also included. To obtain a consistent assessment, the genotypes were selected by their performances in four harvests. Later, they were crossed in a diallel scheme and 70 Jatropha full-sib families were obtained. Phenotypic and genotypic evaluations were performed on populations thus formed aiming at maximizing the selective accuracy.

The accurate estimation of the association between genetic and phenotypic parameters is important for the planning of efficient breeding strategies. Accurate estimates of genotypic variance and heritability are the most important parameters for quantifying the potential of a breeding population and the merit of the selection strategy to be used in order to gather the most favorable alleles for the traits under consideration. These estimates have been performed by using mixed models (REML/BLUP), since the highest priority of the breeder concerns the breeding value of an individual and not its phenotypic value because it will not be transferred to the progeny under low heritability.

The *best linear unbiased prediction* (BLUP) maximizes selective accuracy, minimizes the difference between predicted and true breeding values, maximizes the probability of selecting superior individuals, and maximizes the expected genetic gain per selection cycle (Resende 2002). Parents and/or families and/or individuals are ranked according to their respective breeding values as predicted by the BLUP for the selection of individuals for the different traits evaluated in the breeding population (Resende 2007). The *residual maximum likelihood* (REML) eliminates the bias owing to changes in allele frequencies by selection using the kinship matrix (Resende 2002). Thus, it is possible to estimate the variance components for a population and to predict the breeding values of individuals at any generation given their allele composition.

The main objectives pursued by breeders in the selective breeding of oilseeds are to obtain cultivars with homogeneous and stable seed yield with high oil content, greater tolerance to pathogens, and greater adaptability to adverse soil and climate conditions. The selection of families based on the breeding value of a cross makes it possible to select a larger number of promising individuals for the traits of interest. The efficiency of this selection is based on the fact that deviations due to

environmental effects tend to cancel out. Thus, the mean phenotypic value of a family is close to the mean of its genotype value, and the benefits obtained will be greater when the environmental effects constitute a large part of the phenotypic variance or, in other words, when the heritability is low (Falconer and Mackay 1996).

3.2.3 Selection Strategies in Jatropha

Most agronomic traits of interest, which in the case of *Jatropha* is oil yield (seed yield x oil content), are controlled by several genes, making it impossible to achieve success in a single selection cycle. Thus, one efficient strategy is the recurrent selection, which consists of a cyclical breeding process where progenies are evaluated and the best are recombined by breeding them among one another. Consequently, one expects an increase in the frequency of favorable alleles expressed by improving the trait under selection (Ramalho et al. 2012).

Despite the efficiency of recurrent selection, there are other useful strategies in shortening the selective cycles, such as marker-assisted selection and genome-wide selection, which are promising strategies for increasing the selection efficiency, lowering costs associated to the selective breeding process, and increasing the genetic gain among breeding generations. For *Jatropha* and *Acrocomia aculeata* (macauba), it is estimated that GWS could shorten the breeding cycle from 8 to 10 years to 2 years, which would cause a high impact on the release of new cultivars for planting. However, the application of these techniques is still scarce in *Jatropha* (Moniruzzaman et al. 2016). Below, we detail these strategies individually and in an integrated scheme.

3.2.3.1 Recurrent Selection

The recurrent selection was originally proposed by Hull (1945) in allogamous plants. This procedure involves the following steps: obtaining an experimental population and evaluating and recombining its progenies to form the next cycle. These steps are performed cyclically until satisfactory allele frequency levels are reached (Hallauer et al. 2010). This strategy consists in gradually increasing the frequency of favorable alleles for quantitative traits by repeated selective cycles without depleting the genetic variability of the population.

The recurrent selection strategy includes all reported steps stated above but presents some variations in the way it is conducted, depending on factors such as the species under breeding; the type of progeny evaluated, i.e., full-sib or half-sib families; the number of progenies; the number of families selected; the parental control; and the objectives of the program (Weyhrich et al. 1998). For example, if the intent is to improve the population per se focusing on additive gene to give rise to a new variety, intrapopulation methods are recommended. However, if the objective is

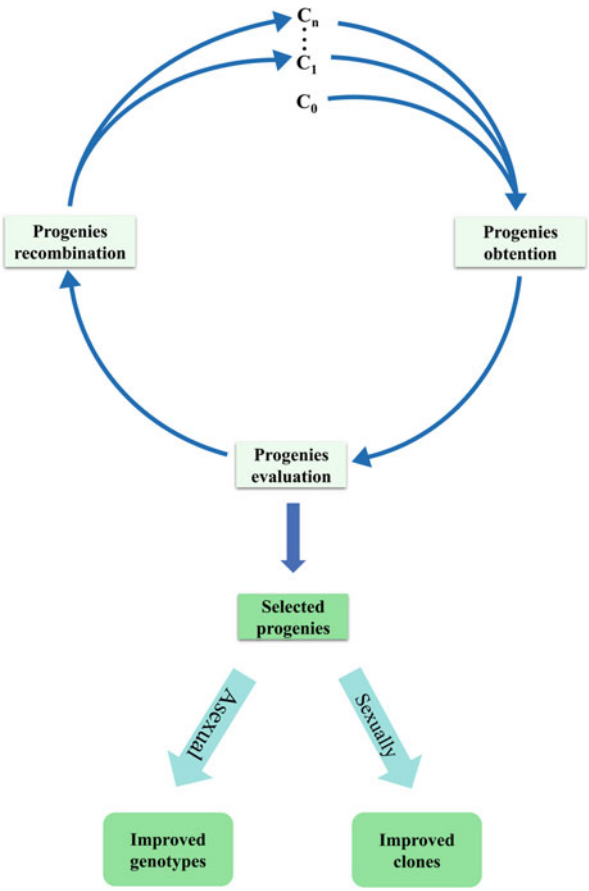

Fig. 3.2 Strategy of intrapopulation recurrent selection in *Jatropha*

to explore the heterosis obtained by crossing two populations, interpopulation methods are recommended.

Intrapopulation recurrent selection (IRS) is performed independently after each selection cycle, and in addition to the improved population, improved inbred lines can be extracted. This method has been widely used due to its simplicity and applicability for a large number of traits (Hallauer et al. 2010). Among the conduction methods, one may cite the mass selection, the selection based on individual phenotypes, and the selection using progenies. In *Jatropha* crop, this later has been shown to be more efficient, since it enables to perform tests with replicates carried out in different environmental conditions, resulting in a lower phenotypic variance and a greater genetic gain (Borém and Miranda 2013) (Fig. 3.2).

Some studies highlight the superiority of recurrent selection using *Jatropha* progenies when compared to mass selection (Divakara et al. 2010; Laviola et al. 2010a; Surwenshi et al. 2011). However, it is worth mentioning that the success of this strategy is directly related to the type of progenies evaluated during the

recombination in order to obtain higher estimates of genetic gain. The *Jatropha* breeding population implemented in Planaltina, DF (Brazil), is composed of half-sib progenies, who explore one half of the additive variance between families and one fourth of the genetic variance within families.

In the case of *Jatropha*, after the selection cycles, it is possible to follow two paths to continue the breeding program: via asexual propagation, in which the selected individuals are cloned, tested, and released as improved clones, or via sexual propagation, in which seeds are produced and improved genotypes are released. According to Punia (2007), 15 *Jatropha* open-pollinated varieties were developed by using mass selection and recurrent selection in India. After improving traits of interest, such as seed yield, oil content, and quality, as well as resistance to pests and diseases, the superior genotypes were released as varieties.

3.2.3.2 Genome-Wide Selection

Because it is a perennial crop, *Jatropha* has long breeding cycles. Thus, it is important to implement strategies that allow greater speed and efficiency in the selection process. Decreasing the breeding cycle will enable to reach a greater agility in issuing cultivars to meet the growing demand for alternative energy from biological resources. In this context, the genome-wide selection (GWS) is a promising strategy for increasing the selection efficiency, reducing the costs of cultivar release and the breeding cycle, as well as increasing the genetic gain from selective breeding (Resende et al. 2012a; Alves et al. 2015).

The methodology of selection in plant and animal breeding has markedly evolved over the last 20 years (Bernardo 2008). Plant and animal breeders have effectively used phenotypic selection to increase the mean performance in selected populations. Time-consuming and costs are evident in tree breeding due to their long life cycle. This is more evident especially in traits based on a long-time assessment of phenotypic traits using continuous variables (biomass, growth, morphology, oil content, and phorbol ester concentration) or discrete variables (resistance to pathogens, form description, etc.) expressed late in the life cycle (Peixoto et al. 2016).

GWS was proposed by Meuwissen et al. (2001) aiming at increasing the efficiency of selective breeding and accelerating the geneticist cycle. It is extremely attractive in the case of perennial crops due to the possibility of increasing the gain per unit of time and eventually to improve the selection precision for traits of low heritability. It allows the simultaneous selection for hundreds or thousands of markers, densely covering the whole genome, so that all the genes being in *linkage disequilibrium* (LD) with a *quantitative trait loci* (QTL) would be associated with at least one marker (Grattapaglia and Resende 2011; Massman et al. 2013).

Since 2007, GWS started to gain more attention when several studies described the application of the method and its accuracy in animal and plant breeding (Goddard and Hayes 2007; Meuwissen 2007; Resende 2007). This technique has been used extensively in the breeding of perennial plants, proving that it is possible to increase gains in terms of genetics and time in perennial crops such as eucalyptus

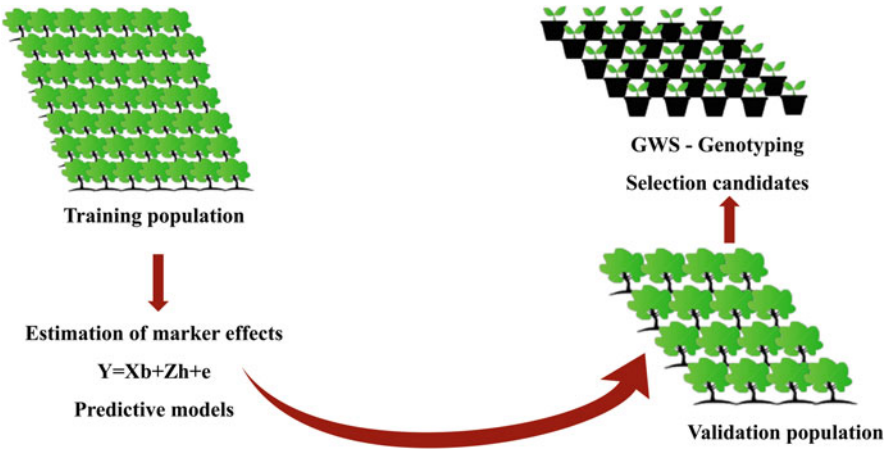

Fig. 3.3 Scheme proposed for GWS in *Jatropha*

(Resende et al. 2012a), cashew (Cavalcanti et al. 2012), apple (Kumar et al. 2012), pear (Iwata et al. 2013), *Jatropha* (Laviola et al. 2014; Peixoto et al. 2017), and grapevine (Viana et al. 2016). It is believed that GWS will positively impact methods and strategies of plant and animal genetic breeding (Viana and Resende 2014).

It has now been demonstrated that predictive models built on the basis of genome-wide marker collections allow breeders to obtain higher selection accuracy, even for traits of low heritability. Moreover, GWS may successfully reduce the time required to select elite individuals in breeding programs of perennial crops, especially when compared to traditional selection based on phenotypic data (Grattapaglia and Resende 2011; Resende et al. 2012a, b).

In the operational terms of GWS, three populations are required for (i) model training, (ii) model validation, and (iii) subpopulation selection (Fig. 3.3). The training population should be made up of a moderate number of individuals (800–1000) showing extensive phenotypic variation for the trait considered and analyzed with a large number of molecular markers (750–4500 depending on the genome size and LD structure) (Grattapaglia and Resende 2011; de Oliveira et al. 2012; Resende et al. 2012a; Massman et al. 2013).

The genotyping of a *Jatropha* population is based on a high-density chip for SNP genotyping, developed by Embrapa Agroenergia. This chip based on Axiom technology, from Affymetrix (Axiom ENERCHIP), that contemplates about 40,000 SNP selected for species with bioenergetic potential, such as *Jatropha*. The SNPs were then filtered based on multiple criteria that included (i) consensus sequence size, (ii) minimum and maximum reading depth, (iii) SNP *quality score*, (iv) *minor allele frequency* (MAF), (v) presence of other SNPs in surrounding regions, (vi) SNPs present in various populations (if they have been sampled), (vii) SNPs present in coding regions, (viii) coverage of genes of interest, and (ix) coverage at genomic level (assessed by the SNP distribution on the reference genome, when available).

To genotype the samples, the total genomic DNA is amplified and randomly fragmented into pieces of 25–125 base pairs (bp) following a modified version of the *diversity arrays technology* (DArT) (Jaccoud et al. 2001). These fragments are purified, suspended, and hybridized with Axiom® ENERCHIP. After hybridization, the binding targets are washed under stringent conditions to remove any resulting non-specific binding fragment in order to minimize background noise caused by random binding events. The DNA sample for analysis is labeled with green fluorescent dye. Fragments of the cloning vector, which are common to all elements of the array, are labeled with red fluorescent dye (control for signal intensity) and hybridized together with the green fluorescent probe (diversity representation). The ratio of signal intensity is measured at each array spot, and polymorphic spots are identified by binary distribution of signal ratios among input samples. Any new specimen can be assayed on arrays of polymorphic features to generate a genetic fingerprint.

In the case of *Jatropha* breeding program, the phenotypic and genotypic data from the breeding population can be used to elaborate the equations that predict genomic breeding values for the traits of interest, such as plant traits, seed yield, and oil content. The prediction equations can be assessed for their accuracy on independent samples within the same population. This process can be performed in the same training population in a *Jackknife* resampling scheme (taking a sample of 800 individuals for model estimation/training and 200 for model validation). Finally, the population under selection consists of independent individuals that are analyzed only through molecular marker combinations, since their phenotypes will be predicted using the validated equations. These equations are used to predict the genomic breeding value of each candidate for early selection, and the accuracy of this process is equivalent to the estimated accuracy on the validation population.

In *Jatropha*, it is estimated that GWS could shorten the breeding cycle that is at the moment between 8 and 10 years to only 2 years, which would cause a high impact on the release of new cultivars for farming (Alves et al. 2015). Laviola et al. 2014 evaluated the weight of hundred seeds in 79 *Jatropha* individuals, which were genotyped using the DArT platform. Genomic selection was modeled with the *ridge regression*-BLUP (RR-BLUP) approach (Endelman 2011) using 47 randomly selected individuals as the breeding population and the others as the cross-validation population. For comparison, the accuracy of phenotypic selection was calculated using the REML/BLUP approach. The heritability of the evaluated trait was estimated at 37%, while the accuracy of the phenotypic selection was estimated at 60%. Considering genomic selection, the accuracy scores were calculated at 63% and 71% when using SNP or DArT markers, respectively. These differences of accuracy between both technologies remained similar when analyzing other important traits. By this mean, genomic breeding values could be estimated in seedlings, reducing by at least 5 years (7 years/cycle with genomic selection vs. 12 years/cycle without genomic selection).

Although GWS has been shown to be an excellent strategy in plant breeding, it contemplates some challenges such as choosing a suitable method for predicting genomic breeding values. Thus, several methods have been proposed, which differ by the type of assumption about the genetic model associated with the quantitative

trait under selection, either with true or simulated values. Several studies have addressed these methods, either via mixed model, focusing on RR-BLUP and *genomic*-BLUP (G-BLUP) (Bernardo and Yu 2007; Endelman 2011; Cavalcanti et al. 2012), or Bayesian inference (De Los Campos et al. 2009b; Limón et al. 2012) and even studies integrating these two approaches (Habier et al. 2007; Crossa et al. 2010; Silva et al. 2013; Azevedo et al. 2015). The most efficient method of genomic prediction is one that best reflects the biological nature of the quantitative trait under analysis in terms of gene effects (Resende et al. 2008).

For example, RR-BLUP assumes that all marker effects are normally distributed and that these marker effects have identical variance (Meuwissen et al. 2001). G-BLUP assumes an equal variance for each marker and uses a genomic relationships matrix among all individuals in a reference set and a test set allowing it to compute variance components and BLUP from a mixed model (Hayes et al. 2009).

In Bayes A, markers are assumed to have different variances and are modeled as following a scaled inverse χ^2 distribution (Meuwissen et al. 2001). The prior in Bayes B assumes the variance of markers equal to zero with probability p, and the complement with probability $(1 - p)$ follows an inverse χ^2 distribution, with v degree of freedom and scale parameter S (Meuwissen et al. 2001). The definition of the probability p depends on the genetic architecture of the trait, suggesting an improvement to the Bayes B model, known as Bayes Cπ. In Bayes Cπ, the mixture probability p has a prior uniform distribution (Habier et al. 2011). In Bayesian LASSO, marker effects are assigned independent Gaussian priors with marker-specific variances ($\sigma_e^2\tau_j^2$). At the next level of the hierarchical model, the τ_j^2s are assigned exponential priors EXP $\left[\tau_j^2|\lambda^2\right]$. Considering a deeper level of the hierarchy, λ^2 is assigned a gamma prior with rate (δ) and shape (r). Finally, inverse chi-square priors are assigned to the variance parameters, and the scale and degree of freedom parameters are set to $S_u = S_e = 1$ and $d{:}f_{:e} = d{:}f_{:u} = 4$, respectively. BLASSO is described by De Los Campos et al. (2009a).

Actually, the progeny testing phase could potentially be omitted, since with GWS in hand, breeders will be able to perform early selection for yet-to-be-observed phenotypes at the seedling stage. This early selection would then allow the selected individuals to be immediately propagated for the immediate establishment of optimized clonal trials with several years of anticipation, compared to a classical breeding scheme. As the selection response is inversely proportional to the breeding cycle length, the time spent to complete a breeding generation is reduced, and consequently the selection response per time unit may be drastically increased, as has been theoretically and experimentally demonstrated (Grattapaglia and Resende 2011).

In addition, it has been demonstrated via simulation studies that for oil palm, for example, GWS can be more effective in terms of cost and time reduction than phenotype-based selection, as breeders are theoretically able to perform four breeding cycles in the same time span that usually would accommodate only two cycles when breeding is performed traditionally (Resende et al. 2012a; Wong and Bernardo 2008). Besides shortening breeding cycles, early selection may also allow breeders to increase selection intensity as the newly developed genotyping-by-sequencing

approaches allow them to have an enormous number of plants quickly and cost-effectively genotyped for thousands of markers. On the other hand, progeny trials are usually limited in size in the case of perennial crop breeding due to economics and operational aspects, which reduce the number of traits of interest that can be scored given the number of individuals. Therefore, with GWS, breeders should be able to reduce their investment in field testing, saving time and resources and also improving the selection precision for traits of low heritability.

The effectiveness of GWS depends on the correlation between the predicted breeding value and the underlying true breeding value (Goddard and Hayes 2007). This correlation of GWS, also called prediction accuracy, has been expressed as a function of the training population size (N), trait heritability on an entry-mean basis (h^2), and the effective number of QTLs or effective number of chromosome segments underlying the trait (M_e) (Daetwyler et al. 2008, 2010).

Peixoto et al. (2016) showed using REML/BLUP that the most important traits in Jatropha such as seed yield, oil content, phorbol ester concentration, and the weight of 100 seeds have different heritability and the heritability estimates were 0.32, 0.24, 0.71, and 0.85, respectively. Therefore, different strategies should be developed to use GWS in *Jatropha* and consider high accuracy for these traits. As the demand for biodiesel is constantly increasing, the development of dedicated crops has been suggested as a strategic action. On such a selective breeding basis, biodiesel production chain is expected to become feasible in the midterm, at least from the farming stand point. In that context, genomics offer innumerable technologies for collecting genetic information that could be potentially integrated into the genetic improvement programs of many crops to help the development of cultivars with outstanding performance for biodiesel production.

In the context of a long-lived perennial crop with its long breeding cycle and late-expressing traits, the achievement of such a long-term goal promises to revolutionize selective breeding (Neale and Kremer 2011). Considering that some of the most promising feedstocks for biodiesel production, such as *Jatropha*, oil palm, macaw palm (*Acrocomia aculeata*), and pongamia (*Pongamia pinnata*), are perennial crops, genomic breeding is therefore one of the most promising ways to foster the development of perennial crops dedicated to biodiesel production.

In the near future, GWS can improve the efficiency to produce oil in *Jatropha*; however, further studies are needed to prove theoretical consideration by experimental evidences because there is no research assessing the success of GWS for the selective breeding of oil traits, so far. Therefore, researches should be to evaluate how GWS method should better be implemented to capture traits related to oil production with high accuracy, to infer how many individuals and markers are needed to train the model, and to infer how the genetic vs. environment interaction can influence the prediction accuracy.

Peixoto et al. (2017) reported a new breeding strategy for *Jatropha* crop based on the use of GWS associated with intrapopulation recurrent selection, i.e., recurrent genomic selection. According to these authors, in recurrent genomic selection, segregating populations would be generated by the recombination of the best families and/or superior individuals by means of genetic design. After being

obtained, candidate elite genotypes are sown in the field, and a leaf sample is extracted from each plant at juvenile state for DNA extraction and genotyping with the markers identified in the estimation and validation process. The plants are then classified according to their estimated genomic breeding values by genomic selection methods. Elite plants are identified before flowering and gathered in subpopulation according to their similar breeding values for the trait under consideration and hybridized following the interpopulation scheme to benefit from the heterosis effect; the other individuals are eliminated.

3.3 Conclusion

Jatropha is considered as a promising crop for biodiesel production due to its high oil yield and physicochemical characteristics that enable its easy conversion into liquid biofuel meeting the American and European standards. In Brazil, genetically improved varieties that guarantee such potentiality under large-scale planting are not available. One of the main difficulties encountered by selective breeding programs conducted in Brazil is the narrow genetic variability of the Brazilian genotypes. New germplasm introductions were made to increase the likelihood of success in breeding selection.

Thus, the genetic variability of the *Jatropha* germplasm collection has been enlarged, and modern selection strategies have been adopted, always seeking to integrate classic strategies with molecular tools. With these improvements, it is believed that there will be a greater efficiency in the selection process, as well as a shorter selection cycle, since the time necessary to launch a perennial crop cultivar is huge. Hence, it is expected in the near future to launch cultivars on the market suitable for biofuel production and to contribute to the insertion of this crop in the Brazilian energy matrix, which will generate several benefits such as diversification of raw materials, reduced greenhouse gas emissions, and generation of employment and incomes.

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Chapter 4
Prebreeding and Genetic Enhancement in *Jatropha* Through Interspecific Hybridization

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Abstract Although scientists consider jatropha as a potential energy crop, not much achievement has been made on its genetic improvement mainly due to its low genetic variation. Attempts have been made to cross *J. curcas* with the other *Jatropha* species to enhance its variation. Although there are roughly 175 *Jatropha* spp. known, less than 10 species were reported to set fruits with *J. curcas*. Yet all crosses were achieved only when *J. curcas* was used as the female plant, except with *J. integerrima* (peregrina) that enabled a limited number of successful reciprocal crosses. The cross *J. curcas* × *J. integerrima* is the most promising and being studied in many aspects of genetics and breeding. Cytologically, the F_1 hybrids show disorder of chromosome segregation during meiosis, causing almost half of the microspores to contain irregular number (10 and 12) of chromosomes. Most F_2 plants have more chromosomes of *J. curcas* than *J. integerrima*, and yet interspecific translocation was frequently found. When the progenies were further intercrossed, the resulting clones exhibited many characters not found in the *J. curcas* germplasm. They were traits related to seed and oil yield, fatty acid composition, plant architecture, biomass yield and quality, and ornamental characteristics. The clones show much higher genotypic and phenotypic variation in seed yield, oil content, 100-seed weight, and canopy size as compared to *J. curcas* and thus serve as promising genetic resources for *Jatropha* improvement in the future.

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4.1 Introduction

4.1.1 Genetic Variation in Jatropha curcas *Is Low*

The center of origin of jatropha is in the Central American region around Mexico. It was introduced to Africa and Asia and grown worldwide in the waste land of the tropics and subtropics, under poor climatic conditions (Openshaw 2000). Over 10^6 ha of *Jatropha* plantations are in Asia (especially in India and China), Africa, and Latin America (Singh et al. 2014), with an expected seed yield of 4–5 t/ha/year inferred from many experimental plots. However, the actual seed yield of 0.5–2 t/ha/year was much lower than expected owing to poor crop performance under real field conditions in various countries (Edrisi et al. 2015). To produce commercial jatropha cultivars (CVs), genetic improvement for seed and oil yield is required. However, many studies showed that jatropha had insufficient genetic diversity for developing elite CVs (Basha and Sujatha 2007; Gupta et al. 2008; Ranade et al. 2008; Sun et al. 2008; Sudheer et al. 2008, 2010; Na-ek et al. 2011). Fortunately, jatropha has many related species that are intercrossable (Dehgan 1984). Thus, interspecific hybridization is one of the options to enhance genetic variability for exploitation in jatropha breeding programs. Around 175 species widely distributed in the tropics of Old and New World are known within the genus *Jatropha* (Dehgan 1984). Species and genera with desirable traits that can be used for *Jatropha* breeding include peregrina (*J. integerrima*), *J. cinerea*, bottleplant shrub (*J. podagrica*), coral plant (*J. multifida*), bellyache bush (*J. gossypifolia*), and castor bean (*Ricinus communis*) (Laosatit et al. 2014).

4.2 Enhancement of Genetic Variation in *Jatropha* Through Interspecific Hybridization

In 1968, Bijan Dehgan pollinated *J. curcas* with *J. integerrima* and obtained three viable seeds which produced two plants carrying intermediate characters between the two parental species. Although the hybrids were partially self-sterile, they could be backcrossed with their parents, and thus serving as an introgressive bridge between both species (Rupert et al. 1970). Interspecific hybridization was suggested as one of the most feasible approaches for *J. curcas* improvement. The related species are capable of gene exchange with *J. curcas* under this artificial condition resulting in a transgressive segregation of the progenies useful for further improvement. Subsequently, an intensive crossability study between *J. curcas* and other *Jatropha* species was attempted by Dehgan (1984). The author constructed a

diagram showing four possible issues in case of success, i.e., (i) no fruit or seed enlargement; (ii) fruit and seed enlarged, but without endosperm; (iii) no reciprocal crosses possible; and (iv) seeds obtained in both direct and reciprocal crosses. The only cross that belongs to the fourth group is *J. curcas* × *integerrima*. Several scientists followed Dehgan's work with the idea to enhance genetic variability of *J. curcas* through wide hybridization (Sujatha 2006; Parthiban et al. 2009; Kumar et al. 2009; Laosatit et al. 2014, 2017). All of them reported that the cross between *J. curcas* × *integerrima* produced hybrids with more seed set, while crosses of *J. curcas* with the other species either failed or were partially successful (possible to fertilize but that failed to produce seeds) due to the existence of crossability barriers (Dehgan 1984; Sujatha 2006; Parthiban et al. 2009; Kumar et al. 2009; Laosatit et al. 2014, 2017). As in other interspecific crosses, the chromosome behavior of the hybrids between *J. curcas* and *J. integerrima* was studied.

4.3 Chromosome Behavior in *J. curcas* × *J. integerrima* Hybrids

Interspecific hybridization between *J. curcas* and *J. integerrima* was successful. Most F_1 hybrid plants could produce seed and F_2 progeny. From this interspecific hybridization, improved jatropha plants with useful traits, such as seed/oil yield and biomass, have been obtained (Muakrong et al. 2013, 2014; One et al. 2014a, b). But, the rate of seed formation is low (Sujatha and Prabakaran 2003; Parthiban et al. 2009; Muakrong et al. 2014). F_1 hybrids had pollen viability lower than that of their parents (Amkul et al. 2016; Fukuhara et al. 2016). The size of pollen grains of F_1 hybrids varied, suggesting variability in their DNA content. The parent species have the same somatic chromosome number $2n = 22$ (Perry 1943; Miller and Webster 1962), and both species form 11 bivalents in regular meiosis (Soontornchainaksaeng and Jenjittikul 2003; Kikuchi et al. 2010; Sasikala and Paramathma 2010) and produce abundant pollen (Sasikala et al. 2009; Amkul et al. 2016; Fukuhara et al. 2016). Hence, genetic mismatch(es) during meiosis and/or pollen grain formation was/were thought to be the cause of pollen sterility in the F_1 hybrids. However, cytogenetic study of pollen sterility in the hybrids has not been conducted in the last 50 years from the first report by Dehgan in 1968.

Jatropha species have the same number of somatic chromosome which is small and similar in size (Dehgan and Webster 1979). Carvalho et al. (2008) measured the length of 11 *J. curcas* chromosomes showing homomorphic pairs and found that they range from 1.71 μm to 1.24 μm. Thus, it is quite difficult and laborious to study these chromosomes, and yet reliable chromosome identification is required. For species-specific chromosome painting and development of chromosomal landmarks, fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH) are two powerful cytological techniques (Schwarzacher and Heslop-Harrison 2000). In the genus *Jatropha*, FISH analysis probed with ribosomal RNA (rDNA) allowed

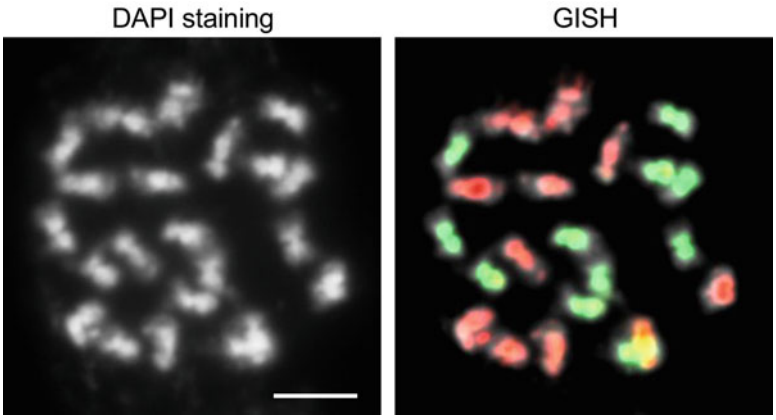

Fig. 4.1 Mitotic chromosomes in a F_1 hybrid between *J. curcas* and *J. integerrima* stained with DAPI (left) and GISH (right). GISH probes clearly show 11 chromosomes derived from *J. curcas* (red) and *J. integerrima* (green). Scale bar = 5 μm

the identification of 2 out of 11 chromosome pairs (Witkowska et al. 2009; Kikuchi et al. 2010) and the analysis of epigenetic feature (Gong et al. 2013). Telomeric repeats (Witkowska et al. 2009), subtelomeric repeats JcSat1 (Kikuchi et al. 2010), and retrotransposons (Alipour et al. 2013) can be used as FISH probes for additional chromosomal landmarks. Fukuhara et al. (2016) gave the first report describing a process of parental chromosome diagnosis by GISH analysis of interspecific *Jatropha* hybrid. The F_1 hybrids of *J. curcas* × *J. integerrima* possesses 22 chromosomes, which are constituted by the haploid number ($x = 11$) of parental chromosomes identified by differential fluorescence (Fig. 4.1). Chromocenters matched centromeric heterochromatins in *J. curcas*, *J. integerrima*, and F_1 hybrid nuclei as painted by GISH probes.

Except for GISH analysis in the F_1 hybrids of *J. curcas* × *integerrima* (Fukuhara et al. 2016), there has been no report on chromosome behavior during meiosis in *Jatropha* hybrids. In most of the cells in pachytene and diakinesis stages, chromosomes of the 2 species formed 11 bivalents by interspecies pairing (Fig. 4.2). The formation of ring-shaped bivalents with two chiasmata suggested large chromosomal affinity between the two species. Muakrong et al. (unpublished) reported the possibility of chromosomal rearrangement between *J. curcas* and *J. integerrima* owing to the different positions of rDNA in their chromosomes, but large-scale unpaired regions or loop structures are not visible on the paired pachytene/diakinesis chromosomes. Bivalents were visible in metaphase I, although several abnormalities, e.g., unsynchronized orientation of the paired chromosomes on the equatorial plate, were observed (Fig. 4.2). Fukuhara et al. (2016) found that univalents might also appear, and mean chromosome association frequency was $0.88_{I} + 10.56_{II}$. The normal dyads, tetrads, and microspores with chromocenters for the 11 chromosomes were generated at the frequencies of 37.5%, 69.2%, and 53.5%, respectively. These frequencies agree with the values of pollen viability in F_1 hybrids reported by other authors: 48.4% (Fukuhara et al. 2016), 66.2% (Rupert et al. 1970), and 72–73%

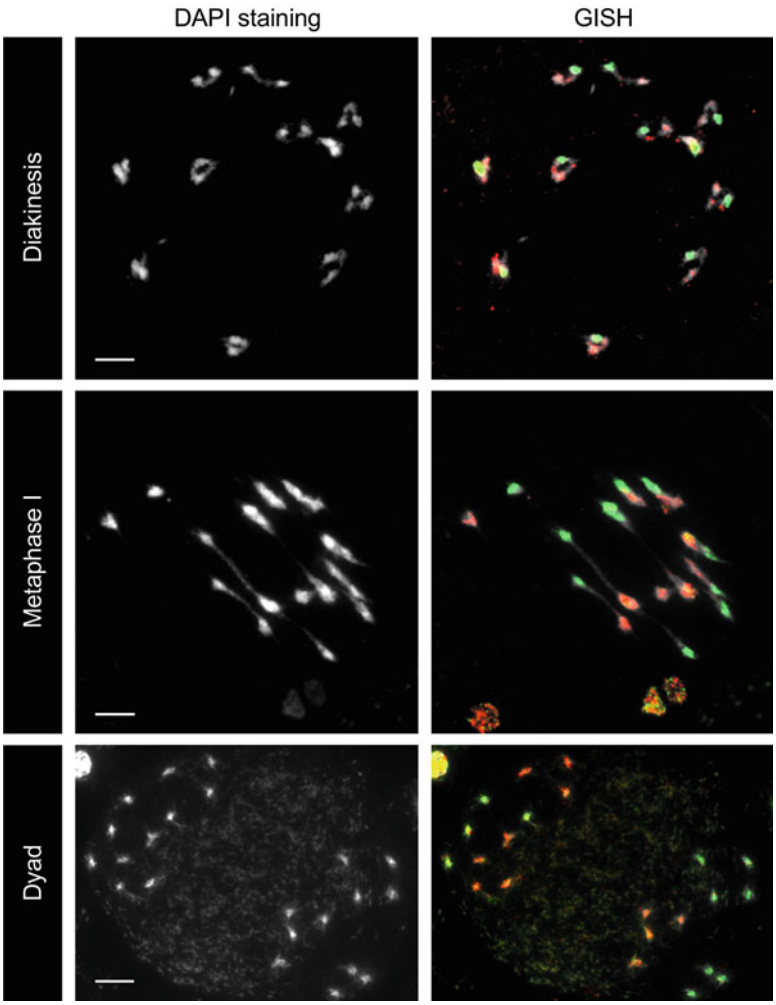

Fig. 4.2 Chromosome behavior in diakinesis, metaphase I, and dyad in a F_1 hybrid of *J. curcas* × *J. integerrima*. Red, *J. curcas* genomic DNA; Green, *J. integerrima* DNA. In diakinesis, 11 bivalents including both ring-shaped and rod-shaped chromosomes are visible. The bivalents were formed by interspecies pairing. In metaphase I, paired chromosomes are not arranged on the equatorial plate. Several chromosomes, e.g., two upper-left chromosomes, start moving to each pole earlier than the other chromosomes. In normal dyad, 11 chromosomes (fluorescence foci of centromeric heterochromatin) are observed in each daughter cell nuclei. Scale bar = 5 μm

(Amkul et al. 2016). Based on the experimental crossing during 1968–1970 by Rupert et al. (1970), self-pollination of F_1 hybrids produced no seeds, but their backcrossing with *J. curcas* or *J. integerrima* as the female parent (using the F_1 hybrid as the male parent) was successful. Most of the previous studies used

backcross progenies to develop populations for breeding and genetic analysis (Liu et al. 2011; Wang et al. 2011; Sun et al. 2012; Subashini et al. 2014; Wu et al. 2015). However, many F_2 plants were produced in some reports (Muakrong et al. 2014; One et al. 2014a, b).

F_1 hybrids showed disorder of chromosome segregation during meiosis. Half of the microspores contained irregular numbers (10 and 12) of chromosomes (Fukuhara et al. 2016). This raised the question of chromosome composition inference in progenies of these hybrids as a result of self-pollination by aneuploid gametes. In comparison to polyploids, diploid plants rarely show aneuploidy. Large populations have to be developed in order to obtain aneuploid plants. Thus, F_2 plants were with $2n = 22$ chromosomes and aneuploids could not be observed (Fukuhara et al. 2016). Actually, GISH analysis revealed that the F_2 plants of *J. curcas* × *integerrima* contained the chromosomes of the two species (Fig. 4.3). Interestingly, most F_2 plants have more chromosomes of *J. curcas* (16.04 per cell on the average) than from *J. integerrima* (5.96 per cell on the average) (Fig. 4.3) (Fukuhara et al. 2016), indicating "preferential uniparental chromosome transmission." Owing to random segregation of chromosomes from each species into the male microspores (Fukuhara et al. 2016), the non-Mendelian transmission may occur during female meiosis/gamete mitosis and pre- or postfertilization. If the preferential transmission is affected by cytoplasmic factors, it may not appear in the progenies from the reciprocal cross. Such meiotic drive for distorted segregation was found in *Mimulus* hybrids (Fishman and Willis 2005; Fishman and Saunders 2008) and mice hybrids (Chmátal et al. 2014) from polymorphism of their centromeres. Several chromosomes show obvious translocations between the two species, i.e., interspecific translocation (Fig. 4.3), supported by the evidence that the subtelomeric repeat JcSat1 are from the *J. curcas* but not from the *integerrima* genome. The irregular detection of JcSat1 at the ends of *integerrima* chromosomes (Fig. 4.3) indicates certain segment exchanges between the two species. The cause may be due to meiotic recombination (Fukuhara et al. 2016). In addition to preferential uniparental chromosome transmission, the *interspecific translocation* is useful for developing introgression lines of *J. curcas* with valuable agronomic traits of *J. integerrima*.

4.4 Useful Traits Developed from *J. curcas* × *J. integerrima* Cross

There are a number of desirable characters found in the progenies derived from the interspecific hybrids between *J. curcas* and *J. integerrima* related to seed yield, oil yield and quality, plant phenology, biomass yield and quality, and ornamental features.

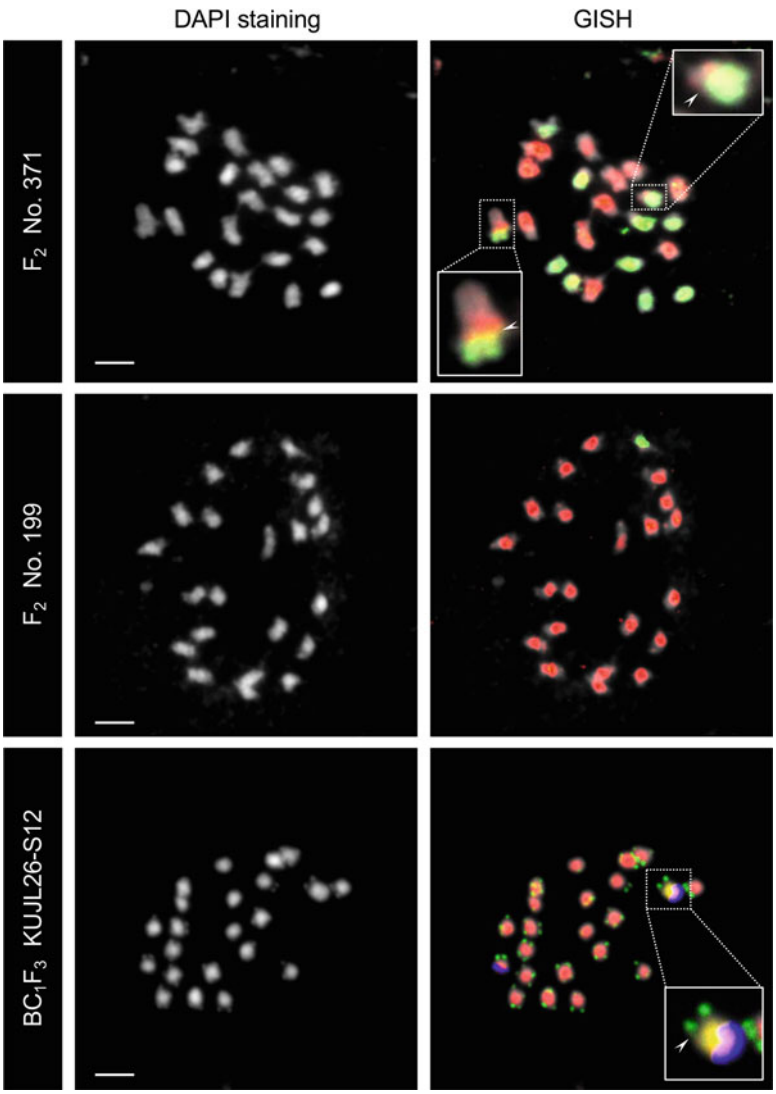

Fig. 4.3 Chromosome composition revealed by GISH and FISH analyses in F_2 plants and a BC_1F_3 plant (F_1 hybrid: *J. curcas* × *J. integerrima*). The F_2 (No. 371) plant shows roughly half mixed *J. curcas* (red) and *J. integerrima* (green) chromosomes. Several chromosomes (insets) carry irregularly adjacent GISH signals, which indicate interspecies translocations (arrow heads indicate the translocations). The F_2 (No. 19) possesses only one *J. integerrima* chromosome (green). A breeding line KUJL26-S12 (BC_1F_3) carries 21 *J. curcas* chromosomes (red) and 1 *J. integerrima* chromosome (yellow). The *J. integerrima* chromosome bears JcSat1 repeat (green) by exchange of terminal chromosome segment between the two species (arrowheads indicate the translocations). Blue, 35S rDNA. Scale bar = 5 μm

4.4.1 Seed and Oil Yield

One et al. (2014a) studied physicochemical properties of seeds and oil from an F_2 population derived from *J. curcas* × *J. integerrima* and found that the average ratio of kernel weight to seed weight ranged from 61% to 70%, which was larger than that of wild and improved *Jatropha* accessions reported by Makkar et al. (1997) and Martínez et al. (2006). The percentage of kernel weight reflects the oil yield as *Jatropha* seed contains oil mainly in the kernel. Parthiban et al. (2009) reported seed yield in interspecific BC_1F_1 clones from Indian *J. curcas*//*J. curcas*/*J. integerrima* ranging from 250 to 357 g per plant. Similarly, One et al. (2014a) reported that a clone of interspecific F_2 gave an average seed yield of 270.2 g per plant, while its *J. curcas* parent gave 66.2 g. Concerning seed oil content, Parthiban et al. (2009) found that it ranged between 37.0% and 55.3% among 27 elite clones of an interspecific backcross hybrid. One et al. (2014a) reported that the interspecific F_2 gave seed oil content of 41.5%, whereas in *J. curcas*, it varied from 17.4% in Malaysian accession (Shabanimofrad et al. 2011), 17.5% in an Indian accession (Sunil et al. 2011), 35.4–40.7% in high phorbol ester (toxic) Mexican and Indian accessions (Francis et al. 2013), and 37.7% in a Thai accession (One et al. 2014a).

4.4.2 Fatty Acid Composition

For biodiesel production, high content of monounsaturated fatty acid (especially oleic acid) and low content of polyunsaturated fatty acid (especially linoleic acid) are desirable because polyunsaturated fatty acid can negatively impact the oxidative stability of the derived fuel and cause high rate of nitrogen emission. One et al. (2014a) reported two interspecific F_2 lines showing low linoleic and high oleic acid content. F_2 line no. 198 has 46.9% oleic and 25.6% linoleic, no. 201 had 48.6% oleic and 31.1% linoleic, while the oil in the parental *J. curcas* accession contained 43.3% oleic and 32.5% linoleic acids.

4.4.3 Plant Architecture

Progeny derived from the interspecific cross of *J. curcas* × *J. integerrima* can be used to improve many agronomic characters including plant architecture. One et al. (2014b) identified a dwarf character from *J. integerrima* that is useful for introgression into *J. curcas*. They studied the inheritance of canopy height and shape from the F_2 progenies derived from the interspecific cross. *J. curcas* (female parent) had a tall canopy height (~273 cm) and erect type (canopy angle ~68°), while the dwarf *J. integerrima* was ~140 cm tall with a spreading canopy angle of ~170°. The resultant F_1 plants exhibited an intermediate canopy height (~215 cm) and angle (~115°). In the F_2 generation, nine phenotypic combinations of plant height and

growth habit were classified as depicted in their report. The tall plant showed incomplete dominance to dwarf plant, while spreading showed incomplete dominance to erectness. The dwarfness and erect growth habit were each controlled by independent genes with incomplete dominant action. Some novel growth habits, especially dwarf-erect and dwarf-upright, can have commercial value for jatropha production under high plant density, for machine harvesting and for ornamental purpose. The tall-erect and tall-upright traits have a potential in selective breeding of jatropha for high biomass.

4.4.4 Biomass Yield and Quality

Interspecific crosses have been widely used for the selective breeding of biomass of willow (Johansson and AlstrÖm 2000), poplar (Vries and Turok 2001), eucalyptus (Christine et al. 2009), and *Leucaena* (Brewbaker and Sorensson 1990). Interspecific crosses of *J. curcas* × *J. integerrima* also showed their potential as sources for biomass selection. Muakrong et al. (2013) reported that interspecific F_1 hybrids between these species were superior to both parents in fresh wood weight per plant, dry wood weight per plant, and wood density, and yet lower in moisture content. The F_1 hybrid had smaller pith than *J. curcas* and thinner bark than *J. integerrima* (Fig. 4.4). Dry wood and wood chips of the F_1 hybrid and *J. integerrima* look heavy and firm, while those of *J. curcas* look light and wrinkled (Fig. 4.5). The plant type of a F_1 hybrid was erect and V-shaped; thus it can be planted at high density with narrow spacing of 1 × 1.5 m (6667 plants/ha). In addition, most leaves of the hybrid plants dropped in the dry season, which is a trait that facilitates wood harvesting. This F_1 hybrid showed a gross heating value of 18.73 MJ/kg similar to 1-year copping willow (18.70 MJ/kg) (Peter 2002) and 3-year copping *Leucaena leucocephala* (18.94 MJ/kg) (Feria et al. 2011), but higher than *J. curcas* (17.77 MJ/kg). The hybrid has less ash (2.6%) than the parental *J. curcas* (6.93%) (Muakrong et al. 2013).

4.4.5 Ornamental Jatropha

Besides the energy purpose, interspecific hybrids from *J. curcas* × *J. integerrima* cross also showed a potential for ornamental purpose (Muakrong et al. 2014; Sujatha and Prabakaran 2003). Hybrids bearing profuse male and female flowers of different colors are attractive for ornamentation. A number of F_2 plants were released in 2012 by Kasetsart University (KU), Thailand, as "Kamphaeng Saen 1," "Kamphaeng Saen 2," and "Kamphaeng Saen 3" (Muakrong et al. 2014). Later, the same group of KU *Jatropha* breeders released more ornamental cultivars from their interspecific cross (namely, "Kamphaeng Saen 4," "Kamphaeng Saen 5," and "Kamphaeng Saen 6") (Tanya et al. 2013). These new CVs are unique in flower colors and plant type (Fig. 4.6), which are not found in the current commercial ornamental *Jatropha* spp.

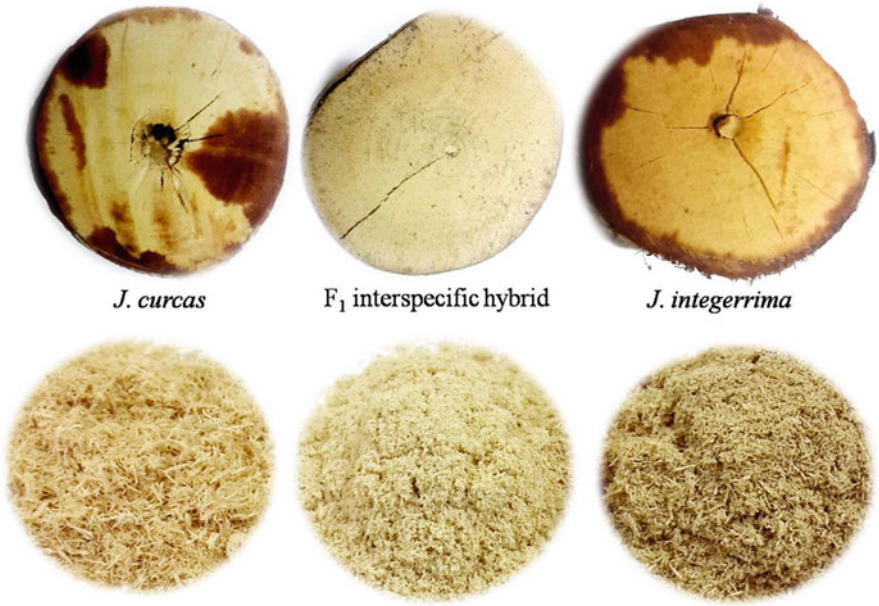

Fig. 4.4 Cross section and shredded wood of parents and a F_1 hybrid from a *J. curcas* × *J. integerrima* cross

Fig. 4.5 Timbers and chips of parents and a F_1 hybrid from a *J. curcas* × *J. integerrima* cross

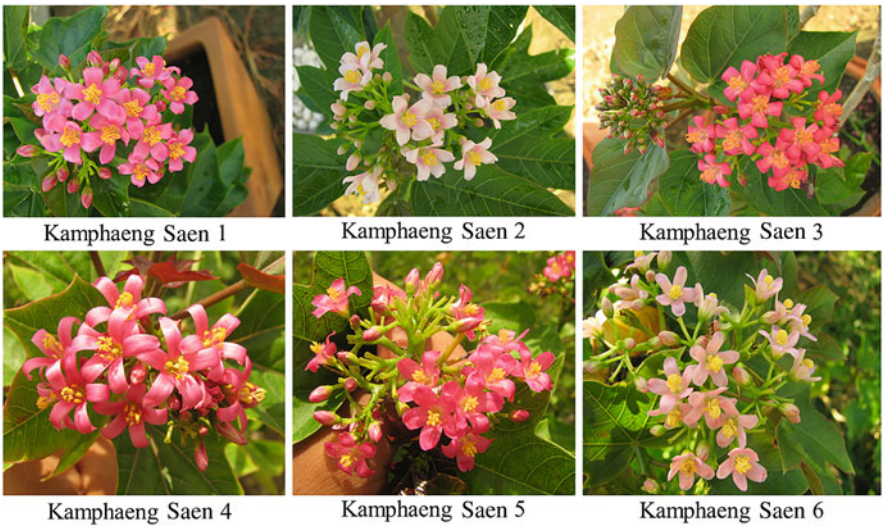

Fig. 4.6 New ornamental *Jatropha* developed from *J. curcas* × *J. integerrima* cross by Kasetsart University, Thailand

They are flowering year-round whenever there is moisture in the soil, and easy to propagate by either cutting or grafting.

4.5 Genotypic and Phenotypic Variation of Jatropha Accessions Derived from Interspecific Cross Between *J. curcas* × *J. integerrima*

Muakrong et al. (unpublished) developed new *Jatropha* accessions from polycrossing between selected clones derived from *J. curcas* × *J. integerrima* progenies. One hundred and twelve new clones/accessions were evaluated for 2 consecutive years for variability and association between 12 agronomic traits related to yield, yield components, oil content, and growth characters. The new clones showed high variation in seed yield, oil content, 100-seed weight, canopy height, and canopy width. A wide range of these traits are desirable for efficient genetic improvement through breeding. One accession (KUJL26) is already coming at its final plant height of 56 and 70 cm in the first and second year, thus promising to be a suitable germplasm for the improvement of dwarf *Jatropha* (Srinives et al. 2017, unpublished data). Some clones have high female to male flowers ratio (Fig. 4.7), which helps increase fruit (and seed) yield (Fig. 4.8), more synchronous maturity (Fig. 4.9) for easy harvesting, big fruit (Fig. 4.10) for higher seed yield, less shattering (Fig. 4.11) to extend harvesting period of the mature fruits, and short fruit bearing internodes (Fig. 4.12) for more fruits set. These novel characters

Fig. 4.7 An interspecific clone from *J. curcas* × *J. integerrima* (left) that gives a higher ratio of female to male flowers than normal jatropha (right) produces more fruits per inflorescence

Fig. 4.8 An interspecific clone from *J. curcas* × *J. integerrima* (left) shows higher number of fruits per inflorescence than normal jatropha (right)

Fig. 4.9 An interspecific clone from *J. curcas* × *J. integerrima* (two left frames) showing better synchronicity at maturity than normal jatropha (two right frames), which facilitates fruit harvesting

available in *J. curcas* will allow these interspecific clones to be utilized as a novel source of germplasm for *Jatropha* improvement, with a potential to obtain elite breeding lines for biodiesel production in the near future.

Fig. 4.10 An interspecific clone from *J. curcas* × *J. integerrima* (left) showing bigger fruits than normal jatropha (right), which is another component for higher seed and oil yield

Fig. 4.11 An interspecific clone from *J. curcas* × *J. integerrima* (left) showing less shattering than normal jatropha (right); allows mature fruits to stay on the plant for longer time

4.6 Conclusion

Genetic enhancement of *J. curcas* for traits such as seed and oil yields, fatty acid composition, plant architecture, biomass yield and quality, and ornamental characteristics is successfully demonstrated through interspecific hybridization with *J. integerrima*. These desirable characters will allow these interspecific clones to be utilized as a novel source of germplasm for jatropha improvement, with a potential to obtain elite breeding lines for biodiesel production in the near future.

Fig. 4.12 An interspecific clone from *J. curcas* × *J. integerrima* (left) showing shorter distance between the nodes that bear inflorescences than normal jatropha (right), which is a trait that increases fruit set, seed yield coupled with ease in harvesting

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Chapter 5
Genetic Transformation and Transgenics of *Jatropha curcas*, a Biofuel Plant

Qiantang Fu, Yan-Bin Tao, and Zeng-Fu Xu

Abstract *Jatropha curcas* is considered as a potential biodiesel feedstock plant. To date, however, it remains a semi-wild species. Transgenic modification is one of the most effective and rapid approaches to accelerate its breeding process. Various methods of genetic transformation, such as *Agrobacterium*- and particle bombardment-mediated transformation, have been attempted and improved over the past 10 years. This chapter presents a comprehensive account of the influence of several important factors on the genetic transformation of *Jatropha*. It also introduces studies on transgenic *Jatropha* involving functional genes for novel agronomic traits, including plant morphology, flowering time, seed development, seed oil content, oil composition and yield, as well as biotic and abiotic stress tolerance. Moreover, improvements in genetic transformation and the completion of genomic sequencing analysis give *Jatropha* the potential to become a new model species for studies on gene function and genetic improvement in woody plants.

Keywords Explants · *Jatropha curcas* · Selective agent · Transformation efficiency · Transgenic plant

5.1 Introduction

Jatropha curcas L. (hereafter referred to as *Jatropha*) is considered a potential oilseed plant for biofuel production because its seeds contain 30–40% oil (Kandpal and Madan 1995; Agarwal and Agarwal 2007; Tapanes et al. 2008), which can be easily converted to biodiesel or bio-jet fuel and used to partially or fully replace fossil fuels (Fairless 2007; Makkar and Becker 2009; Juan et al. 2011). The plant is simple to propagate, exhibits rapid growth and wide adaptability, and is ideally

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suited for growing in marginal or wastelands that are unsuitable for food production (Maghuly and Laimer 2013). However, at present, the potential of *Jatropha* is far from being realized in the biofuel industry because its seed yields are generally low in many areas (Singh et al. 2014). Investments in the large-scale planting of *Jatropha* have progressed ahead of scientific studies that aim to fully explore this plant and understand its limitations (Sanderson 2009).

Jatropha continues to remain semi-wild, and ideal commercial varieties with profitable yields are still lacking. Varieties that produce high and stable yields need to be bred to develop *Jatropha* into a successful biofuel crop (Chikara et al. 2013; Montes and Melchinger 2016). Although conventional breeding methods have been widely used to improve many plant species, this procedure is slow, especially for perennial species such as *Jatropha*. Additionally, the narrow genetic diversity among *Jatropha* germplasm (Sun et al. 2008; Rosado et al. 2010) has limited traditional cross- and selective breeding for the genetic improvement of this plant. Therefore, genetic transformation technology is needed to provide an additional tool for the genetic improvement of this crop. Compared to traditional breeding, genetic transformation techniques have many advantages, such as the directional cultivation of new breeds, reduced costs, a shorter breeding period, and the ability to introduce genes for desirable traits that may not be available within the species or that may be difficult or impossible to manage via traditional breeding methods (Li et al. 1996; Visarada et al. 2009; Herr and Carlson 2013). Moreover, genetic transformation has become an important method for basic research on gene functions and for the generation of new transgenic plants with excellent traits.

The genetic transformation of *Jatropha* has been pursued for the last 10 years, and many transformation methods have been established. However, the transformation efficiency of these methods varies greatly, ranging from only 4% to 95.4% (Li et al. 2008; Pan et al. 2010; Khemkladngoen et al. 2011; Kajikawa et al. 2012; Toppo et al. 2012; Liu et al. 2017). Several transformation techniques have been adopted for transgenesis and for the analysis of gene function (Tsuchimoto et al. 2012; Joshi et al. 2013; Li et al. 2014; Gu et al. 2015; Maravi et al. 2016; Hu et al. 2017). In this chapter, we will provide a brief overview of several common genetic transformation techniques and recent progress on gene function research in *Jatropha*.

5.2 Establishment of Transformation Methods

The efficiency of genetic transformation in plants is affected by several important factors, including the ability of explants to regenerate, the methods of gene transfer, and the selection conditions (Li et al. 2008; Kajikawa et al. 2012; Mao et al. 2013; Fu et al. 2015; Kumar et al. 2015). Table 5.1 presents a summary of various transformation studies conducted on *Jatropha*, along with various factors that may influence transformation efficiency.

Table 5.1 Genetic transformation methods for *Jatropha*

Explant tissue	Transformation method	*Agrobacterium* strain	Selection agent/concentration (mg/l)	Transformation efficiency (%)	References
Cotyledon	*Agrobacterium-mediated*	LBA4404	Phosphinothricin/1	13	Li et al. (2008)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Kanamycin/20	30.8	Pan et al. (2010)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Kanamycin/20	53	Khemkladngoen et al. (2011)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Bispyribac-sodium/4.5	4.3	Kajikawa et al. (2012)
Cotyledon	*Agrobacterium-mediated*	AGL1	Hygromycin/3–5 or glufosinate ammonium/1	Not available	Mao et al. (2013)
Cotyledon	*Agrobacterium-mediated*	EHA105	Kanamycin/40	56.0	Fu et al. (2015)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Bispyribac-sodium /4.5–11.25	23.3	Nanasato et al. (2015)
Leaf	*Agrobacterium-mediated*	LBA4404	Hygromycin/5	29	Kumar et al. (2010)
Young leaf	*Agrobacterium-mediated*	LBA4404	Kanamycin/40	23.9	Zong et al. (2010)
Leaf/ hypocotyl	*Agrobacterium-mediated*	EHA101	Kanamycin/50	5/4	Misra et al. (2012)
Leaf	*Agrobacterium-mediated*	GV3101	Mannose/20 $\times 10^3$	50	Chen et al. (2015)
Petiole	*Agrobacterium-mediated*	EHA105	Hygromycin/3	95.4	Liu et al. (2017)
Plantlet	*Agrobacterium-mediated*	EHA105	Phosphinothricin/2 $\times 10^3$	62.7	Jaganath et al. (2014)
Germinating seed	*Agrobacterium-mediated*	GV3101	Not available	15	Patade et al. (2014)
Shoot apex	Particle bombardment-mediated	Not applicable	Kanamycin/25	Not available	Purkayastha et al. (2010)
Embryo axes	Particle bombardment-mediated	Not applicable	Hygromycin/5–7	44.7	Joshi et al. (2011)

5.2.1 Explant Selection

The establishment of a highly efficient tissue culture-based regeneration system is a prerequisite for a successful transformation system. An efficient regeneration system includes highly regenerative explants and the corresponding culture medium components. Various explants from *Jatropha*, such as embryos, cotyledons, epicotyls, hypocotyls, leaves, petioles, nodal segments, axillary nodes, and shoot apices, have been successfully used to regenerate shoots (Sujatha and Mukta 1996; Khurana-Kaul et al. 2010; Kumar and Reddy 2010; Mazumdar et al. 2010; Purkayastha et al. 2010; Singh et al. 2010; Sharma et al. 2011; Toppo et al. 2012). Some explants, such as cotyledons, young leaves, and petioles, have been successfully utilized in genetic transformation protocols that employ *Agrobacterium tumefaciens* (Li et al. 2008; Kumar et al. 2010; Pan et al. 2010; Khemkladngoen et al. 2011; Misra et al. 2012; Mao et al. 2013) (Table 5.1). In addition, shoot apices and embryo axes have been utilized in particle bombardment (Purkayastha et al. 2010; Joshi et al. 2011) (Table 5.1). Among these explants, cotyledons are generally preferred for transformation because they exhibit high regeneration frequencies and produce more genetically transformed plants than other explants do. Additionally, the cotyledons of *Jatropha* are more susceptible to *A. tumefaciens* infection than are explants from other tissues, such as petioles, hypocotyls, epicotyls, or leaves (Li et al. 2006). However, cotyledons cannot maintain the genetic character of the mother plant because *Jatropha* is frequently cross-pollinated. Young leaves can maintain the good characters of the mother plant and are also available in large quantities for transformation. The methods of *Jatropha* transformation should be further developed with a focus on enhancing the transformation efficiency of leaf explants.

5.2.2 Transformation Methods

Agrobacterium- and particle bombardment-mediated genetic transformations are two popular methods used to produce stably transformed plants. Only three studies have used particle bombardment to genetically transform *Jatropha* (Purkayastha et al. 2010; Joshi et al. 2011, 2013). Purkayastha et al. (2010) established the particle bombardment-mediated transformation method by comparing the size of the gold particles, the bombardment pressure, the target distance, the travel distance of the macrocarrier, and the type and duration of osmotic pretreatment. Joshi et al. (2011) also genetically transformed *Jatropha* via particle bombardment and achieved 44.7% transformation efficiency. Using this method, salt-tolerant transgenic *Jatropha* shoots harboring *35S:SbNHX1* were created (Joshi et al. 2013). The advantages of *Agrobacterium*-mediated genetic transformation, including minimal equipment cost, are the potentially single- or low-copy transgene insertions, and preferential integration into transcriptionally active regions of chromosomes (Newell 2000), which make

this method the most widely used to generate transgenic *Jatropha* (Li et al. 2008; Pan et al. 2010; Zong et al. 2010; Kajikawa et al. 2012; Mao et al. 2013).

In the *Agrobacterium*-mediated transformation method, the *A. tumefaciens* strain is one of the most important factors in the efficiency of genetic transformation. Several *A. tumefaciens* strains have been used for the genetic transformation of *Jatropha*, including LBA4404 (Li et al. 2008; Kumar et al. 2010; Pan et al. 2010; Kajikawa et al. 2012), EHA105 (Jaganath et al. 2014; Fu et al. 2015; Liu et al. 2017), GV3101 (Patade et al. 2014; Chen et al. 2015), AGL1 (Mao et al. 2013), and EHA101 (Misra et al. 2012). However, the transformation efficiency in *Jatropha* varies greatly among these *Agrobacterium*-mediated transformation methods (Table 5.1). Different *Jatropha* explants may have different susceptibilities to different *Agrobacterium* strains (Li et al. 2008; Khemkladngoen et al. 2011; Kajikawa et al. 2012; Nanasato et al. 2015). In addition, several other conditions and factors also affect the transformation efficiency, such as the pre-culture of the explants, the density of *A. tumefaciens*, supplementation and concentration of acetosyringone, and the time course of the co-culture period (Kumar et al. 2013; Sujatha et al. 2013).

Recently, *in planta* transformation has also been successfully established in germinating *Jatropha* seeds (Patade et al. 2014) and plantlets (Jaganath et al. 2014). Both methods result in high transformation efficiency, reaching 15% and 62.66%, respectively (Table 5.1). These transformation methods are simple and easy to implement without tissue culture and can generate a relatively large number of transgenic plants in a relatively short time. We look forward to further research on gene function and transgenic breeding in *Jatropha* using this method.

5.2.3 Selection Conditions

In addition to the development of genetic transformation methods, the types of selection agents and the selection pressure are integral to developing an efficient transformation system. Kanamycin, hygromycin, and herbicides have been used widely as selection agents in genetic transformation systems. The ideal selection agent and pressure can suppress or kill untransformed cells and simultaneously allow the preferential proliferation of the transformed cells (Que et al. 2014), which may then efficiently regenerate plantlets. Li et al. (2008) first reported *Agrobacterium*-mediated transformation methods using the herbicide phosphinothricin as a selection agent. Subsequently, several groups used hygromycin (Kumar et al. 2010; Joshi et al. 2011; Mao et al. 2013), kanamycin (Pan et al. 2010; Zong et al. 2010; Khemkladngoen et al. 2011; Misra et al. 2012), bispyribac-sodium salt (Kajikawa et al. 2012), or mannose (Chen et al. 2015) as selection agents.

Many plant species, including *Jatropha*, are hypersensitive to these selection agents, and this hypersensitivity most likely causes the low transformation efficiencies observed in previous studies (Pan et al. 2010; Kajikawa et al. 2012). Alternative selection strategies, including adjusting the concentrations of the selection agents

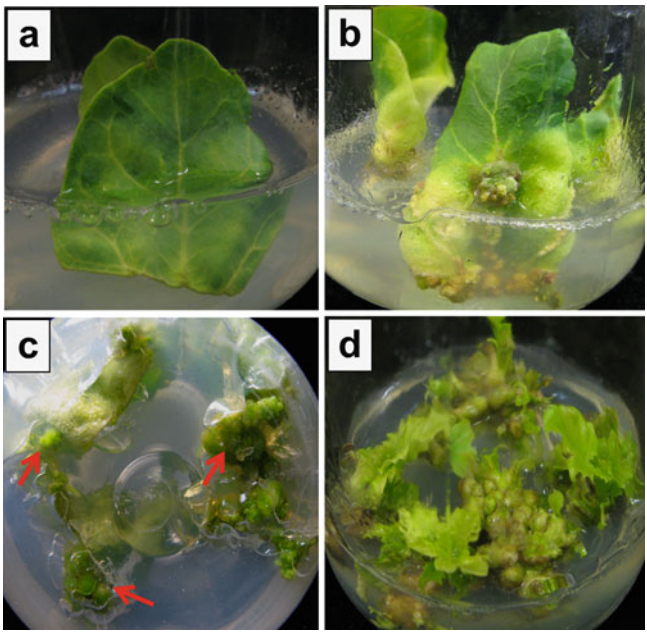

Fig. 5.1 Different stages of the *Agrobacterium*-mediated genetic transformation of *Jatropha*. (**a**) Inserting cotyledon explants into shoot-inducing medium (SIM) after cultivation on callus-inducing medium (CIM) for 7 days to induce resistant calli. (**b**) Three-week-old resistant calli in SIM after a 1-week delay in selection with 40 mg/l kanamycin. (**c**) Resistant calli (indicated by red arrows) in SIM. (**d**) Resistant shoot buds on SIM in the third cycle of selection. (Source: Fu et al. 2015)

and delaying selection, have successfully yielded transgenic apple (Yao et al. 1995) and almond plants (Miguel and Oliveira 1999; Ramesh et al. 2006). In our study, the percentage of β-glucuronidase (GUS)-positive shoots reached an average of 56.0% using 40 mg/l kanamycin with a 1-week delay in selection (Fu et al. 2015). This screening strategy also yielded transgenic *Jatropha* plants when the selection agent hygromycin (0.8–10 mg/l) or the herbicide glyphosate (1 mg/l) was used (unpublished data). In addition, the success of selection also depends on increasing the contact between explants and the selection medium through inoculation (Bhatia et al. 2005). In our method, cotyledon explants were transferred from callus-inducing medium (CIM) to shoot-inducing medium (SIM) with approximately 1 cm of their cut ends embedded in the medium (Fig. 5.1a). After 2–3 weeks, green calli and adventitious buds developed at the cut ends of the cotyledon explants in the medium (Fig. 5.1b, c), and some adventitious buds developed into transgenic shoots (Fig. 5.1d). This improved inoculation method could also lead to a reduction in escapes from kanamycin selection. The overall scheme of this transformation method is presented in Fig. 5.2.

To avoid environmental safety issues and public concerns regarding transgenic plants, an antibiotic marker-free system has been used in *Jatropha* (Qu et al. 2012;

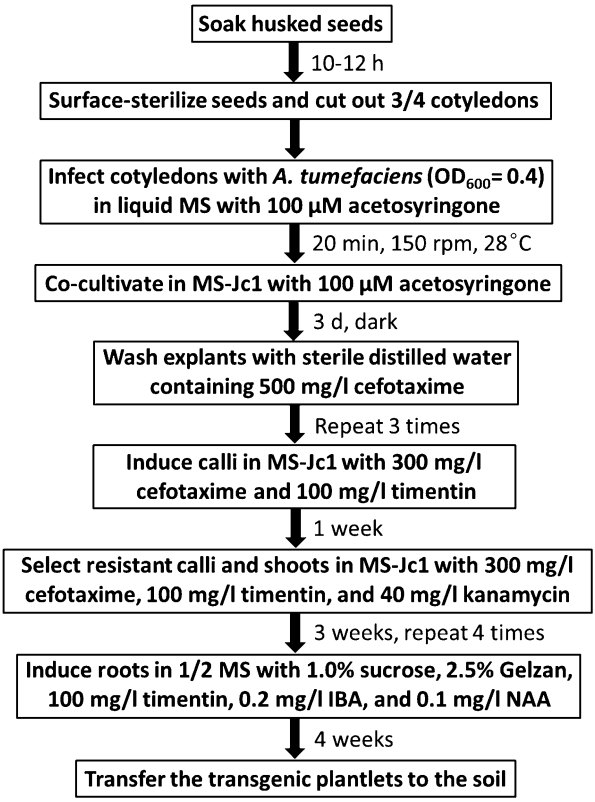

Fig. 5.2 Schematic representation of the protocol for the *Agrobacterium*-mediated transformation and regeneration of *Jatropha* using cotyledon explants. (Source: Fu et al. 2015)

Gu et al. 2014, 2015). In this technique, transgenic plants are transformed and regenerated using the conventional transformation process with a selectable marker; subsequently, the marker is removed from the host plant genome using a chemically inducible Cre-lox-mediated site-specific recombination system (Zuo et al. 2001; Guo et al. 2003). This improved technique lays the foundation for the broad planting of transgenic *Jatropha* in the field.

5.3 Progress in Transgenic *Jatropha*

In recent years, many studies on transgenic *Jatropha* have been reported by several laboratories. Transgenic *Jatropha* plants have been modified with respect to aspects such as plant architecture, flowering time, seed size, oil yield, fatty acid (FA) composition, toxic components, and biotic and abiotic stress tolerance (Table 5.2).

Table 5.2 Research on transgenic *Jatropha*

Promoter/trait genes	Promoter/ selectable marker gene	Phenotypes	References
SUC2:JcFT	*35S:NPTII*	Early flowering	Li et al. (2014)
G10-90:JcFT	*NOS:HPT*	Early flowering	Ye et al. (2014a)
35S:JcLFY	*35S:NPTII*	Moderately early flowering	Tang et al. (2016a)
35S:JcAP1	*35S:NPTII*	No effect on flowering time	Tang et al. (2016b)
35S:JcTFL1a, 35S: JcTFL1b 35S: JcTFL1c	*35S:NPTII*	Extremely late flowering	Li et al. (2017)
35S:JcTFL1b-RNAi	*35S:NPTII*	Moderately early flowering	Li et al. (2017)
35S:JcARF19	*35S:HPT*	Increase in seed size and seed yield	Sun et al. (2017)
JcUEP:JcGA2ox6	*35S:NPTII*	Dwarf phenotype with dark-green leaves and smaller reproductive organs	Hu et al. (2017)
35S:JcCYP735A-CRISPR/Cas9	*35S:NPTII*	Retardated shoot growth	Cai et al. (2018)
P_{Gm7S}*: JcFAD2-1-RNAi*	*NOS:HPT*	Increased oleic acid levels of seed oil	Qu et al. (2012)
P_{JcSDP1}*:JcSDP1-RNAi*	*NOS:HPT*	Enhanced accumulation of seed total lipid	Kim et al. (2014)
35S:AtDGAT1	*NOS:NPTII*	Dramatic increase in lipid content in leaves and seeds	Maravi et al. (2016)
$P_{JcCurcin1}$*:Curcin1*	*NOS:HPT*	Curcin-deficient in seeds	Gu et al. (2015)
P_{JcLEA1}*:dsR366*	*35S:HPT*	Reduction of phorbol ester content in seeds	Li et al. (2016)
P_{JcLEA1}*:dsSET12*			
35S:GSMT+35S: DMT	*NOS:NPTII*	Enhanced glycine betaine synthesis	Tsuchimoto et al. (2012)
35S:SbNHX1	*35S:HPT*	Enhanced salt tolerance	Joshi et al. (2013)
2X35S:ICMV-RNAi	*NOS:HPT*	Gemini viruses resistant	Ye et al. (2014b)
P_{ZmPepc}*:Cry1Ab/1Ac*	*NOS:HPT*	Strong insecticidal activity to *Archips micaceanus*	Gu et al. (2014)

5.3.1 Plant Architecture, Flowering Time, and Seed Development

Jatropha, a small tree, can reach a height of up to 5 m, which makes seed collection inconvenient. Hu et al. (2017) obtained dwarf transgenic *Jatropha* by transforming the plant with the *JcUEP:JcGA2ox6* vector, and the endogenous GA_4 content decreased. Transgenic *Jatropha* transformed with *JcUEP:JcGA2ox6* produced fewer inflorescences and flowers than those of the wild type. Using the CRISPR-Cas9 system, Cai et al. (2018) obtained transgenic *Jatropha* with the *JcCYP735A* gene knocked out; shoot growth in the *Jccyp735a* mutants was slower than in the wild type. Although *Jatropha* grows rapidly and generates abundant biomass in the growing season, excessive vegetative growth may suppress reproductive growth, resulting in an unstable flowering time and a reduction in inflorescences. FLOWERING LOCUS T (FT) plays a crucial role in the transition from vegetative to reproductive growth (Liu et al. 2013). Two laboratories have reported the function of the *Jatropha FT* ortholog (*JcFT*). The overexpression of *JcFT*, driven by the *SUC2* and *G10-90* promoters, respectively, leads to early flowering and modifies the architecture to a semi-dwarf stature of *Jatropha* (Li et al. 2014; Ye et al. 2014a). The early flowering character can therefore be used to accelerate the genetic modification of key agronomic traits. Li et al. (2017) also reported the function of the *Jatropha TFL1* (*JcTFL1*) orthologs, and all transgenic *Jatropha* that overexpressed *JcTFL1a*, *JcTFL1b*, or *JcTFL1c* showed late flowering. However, transgenic *JcTFL1b-RNAi Jatropha* consistently exhibited a moderately early flowering phenotype. JcFT and JcTFL1 may be key systemic signals regulating growth and flowering time in *Jatropha.* In addition, the overexpression of the *Jatropha LEAFY* ortholog (*JcLFY*) can cause moderately early flowering (Tang et al. 2016a). Overexpressing the *Jatropha* APETALA1 ortholog (*JcAP1*) cannot affect flowering time in *Jatropha*; however, the ectopic expression of *JcAP1* can cause early flowering in *Arabidopsis* (Tang et al. 2016b). The molecular mechanisms that control flowering may differ between herbaceous and woody plants. Sun et al. (2017) found that the overexpression of *Jatropha Auxin Response Factor 19* (*JcARF19*) significantly increased seed size and yield in transgenic *Arabidopsis* and *Jatropha*, indicating the importance of the auxin pathway in controlling seed yield in dicotyledonous plants.

5.3.2 Modifying Oil Yield, FA Composition, and Toxin Biosynthesis

The oil content of *Jatropha* seeds is an important economic trait. The oil content can be increased by manipulating the expression levels of key enzymes in the

triacylglycerol (TAG) and FA biosynthetic pathways. The silencing of *sugar-dependent 1* (*JcSDP1*), which encodes a patatin domain TAG lipase, enhances seed oil accumulation in transgenic *Jatropha* (Kim et al. 2014). Recently, the ectopic expression of *AtDGAT1*, which encodes diacylglycerol O-acyltransferase, an enzyme exclusively committed to TAG biosynthesis, was shown to enhance oil accumulation in the seeds and leaves of *Jatropha* (Maravi et al. 2016). In addition, the FA composition and content affect oil quality. A high content of monounsaturated FAs (oleate) and a low content of linoleic acid increase the quality for biodiesel production (Maghuly and Laimer 2017). Using marker-free transgenic technology together with seed-specific RNA interference (RNAi) technology to suppress the expression of 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine delta 12-desaturase (*JcFAD2-1*), Qu et al. (2012) developed transgenic *Jatropha* whose oil had a variation in FA composition; it was modified from 37% oleic acid and 41% polyunsaturated FAs to more than 78% oleic acid and less than 3% polyunsaturated FAs.

Curcin and phorbol esters are the major toxic compounds present in *Jatropha.* The reduction or loss of toxicity in the seeds can allow the use of *Jatropha* in pressed cakes for animal feed. To develop non-toxic *Jatropha* plants, RNAi technology was used to silence the curcin precursor gene in transgenic *Jatropha* (Patade et al. 2014). Further, transgenic *Jatropha* plants have been developed to produce curcin-deficient seeds through endosperm-specific RNAi-mediated gene silencing (Gu et al. 2015). *Jatropha* seeds containing a low amount of phorbol esters have also been generated by disrupting casbene biosynthesis (Li et al. 2016). The improvement of these important traits sets the stage for the development of a commercial variety of *Jatropha*.

5.3.3 Biotic and Abiotic Stress Tolerance

As cultivated areas expand, diseases and pests in *Jatropha* have become prominent problems. To improve tolerance to viruses, Ye et al. (2014b) used RNAi with a hairpin dsRNA that targeted five genes of the DNA-A genome of a geminivirus to produce virus-resistant transgenic *Jatropha*. With 94% nucleotide identity, the transgenic plant showed broad resistance to geminiviruses (Ye et al. 2014b). Gu et al. (2014) developed transgenic *Jatropha* with high insecticidal properties via the expression of the Bt-endotoxin protein Cry1Ab/1Ac.

Although *Jatropha* can be cultivated in marginal lands, thus avoiding competition with food crops for agricultural land, its tolerance to abiotic stress is limited (Cartagena 2017), and its yield is very low under these conditions. Thus, Tsuchimoto et al. (2012) overexpressed genes such as *PAT*, *NF-YB*, *GSMT*, and *DMT* to develop three types of transgenic *Jatropha* plants with improved drought tolerance characters. The glycine content is significantly increased in transgenic *Jatropha* that overexpress *GSMT* and *DMT*. In addition, the *sbNHX1* gene, which encodes an active vacuolar Na^+/H^+ antiporter isolated from the extreme halophyte *Salicornia brachiate*, has been overexpressed in *Jatropha*, resulting in transgenic plants with an

enhanced tolerance to 200 mM NaCl (Joshi et al. 2013). These desirable traits can be used to cultivate crop varieties of *Jatropha*.

5.4 Conclusions

Jatropha has a small genome, a short reproductive cycle, complete genomic information, and effective transformation systems, which are favorable features for its becoming a model woody plant. Although substantial research has been conducted on *Jatropha*, much work is still required for *Jatropha* to become a real biodiesel feedstock. The current priority is to cultivate varieties with improved seed and oil yields through the improvement of agronomic traits such as branching pattern, total flower number, the ratio of female to male flowers, and stress tolerance. We should focus on selecting candidate genes involved in regulating these agronomic traits and then modifying the expression of these genes in *Jatropha* via genetic transformation to produce varieties with the desired traits. Additionally, given the genomic information and efficient genetic transformation systems of *Jatropha*, CRISPR/Cas9 and a large-scale T-DNA insertional method will be prospective developmental tools that can be used to develop a population of *Jatropha* mutants.

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Chapter 6
Genetic Engineering for the Improvement of Oil Content and Associated Traits in *Jatropha curcas* L.

Shaik G. Mastan, Mangal Singh Rathore, Swati Kumari, Reddy P. Muppala, and Nitish Kumar

Abstract Interminably increasing petroleum rates and exhaustion of fossil reserves have ignited a global search for substitutes to renewable fuel sources. Many oil-generating plants, crops and trees have been considered for biofuel; among these *Jatropha curcas* is regarded as one of the most promising oilseed plants as its seeds contain oil content up to 35%. Because fossil oil consumption is increasing day-by-day, there is an urgent need to enhance the oil content. Transgenic technology is one of the advanced techniques that have been applied to enhance oil content and modify the composition of fatty acids in seed oils. Increasing seed oil content can be done by modifying the enzyme's level expression in the triacylglycerol biosynthetic pathway. In this chapter, an effort is made to highlight the potential of transgenic technology towards the enhancement of the oil content and in altering the candidate gene expression for biosynthesis of triacylglycerol.

Keywords Fatty acids · *Jatropha* · Kennedy pathway · Renewable biodiesel · Triacylglycerols

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6.1 Introduction

Fuel is one of the essential requirements for economic expansion of humanity, and fossil fuels fulfilled this need since the beginning of the last century. The growing concern about continuous exhaustion of fossil fuels necessitates the need to look for alternate sources of fuel. Among the alternate sources of energy to fossil fuel, biofuel, has attracted attention because of its renewable nature. Plant oils are proposed to meet the requirement for renewable energy sources that could be easily adapted to the necessity for liquid bioenergy production. Bioethanol and biodiesel are the most common forms of bioenergy that can meet such requirement. Among plant-based oils, *Jatropha curcas* L. has received attention as an energy plant for its exploitation in biodiesel production. The increasing cost of vegetable oil and the unsuitability of food crops to be used in energy production due to competition for food favoured *J. curcas* as a suitable feedstock choice for biodiesel production (Singh and Dipti 2010; Brittaine and Lutaladio 2010). Till date, *J. curcas* is considered as a wild crop due to several limitations including yield inconsistency and susceptibility to diseases and pests mainly in the mono-cropping system. Scientific and biotechnological investigations have been initiated in order to improve the agronomic attributes and seed quality traits (Puente-Rodríguez 2009; Brittaine and Lutaladio 2010; Moniruzzaman et al. 2016). Researchers and corporate companies are working to overcome these limitations, and *J. curcas* became a research topic in both developed and developing nations. !!!In the development of science in the field of classical genetics, biotechnology and biochemical engineering are the key areas in which researchers are investing their efforts. Genetic engineering can contribute to the improvement of feedstock for (i) increasing the content of oil, (ii) enhancing resistance to biotic stresses by introduction of suitable candidate gene (s), and (iii) modifying seed quality and altering fatty acid composition or modifying metabolic networks. In addition, biochemical engineering can play a critical role on downstream characteristics of biodiesel production including (i) the optimization of oil extraction, (ii) standardizing novel transesterification methods by enzymatic and chemical ways, (iii) the enhancement in the quantity and quality of biodiesel content and (iv) the standardization of chemical composition to reduce emission by engines (Ceasar and Ignacimuthu 2011). The exploitation of *J. curcas* as a plant needs a deep understanding of its physiology in order to identify the genes that need to be engineered (Fig. 6.1).

The average oil content of *Jatropha* seeds being approximately 35% with its seed endosperm rich in proteins that is suitable as animal feed after oil extraction made it a promising oilseed crop (Yang et al. 2009). Potential areas of improvement are the increase in seed and oil yields as well as reduction of seed toxicity. The major fatty acids (FAs) found to be present in plant oils include stearic (18:0), palmitic (16:0), oleic (18:1), linoleic (18:2) and linolenic acids (18:3). Stearic and palmitic acids are saturated, oleic acid is monounsaturated and linoleic and linolenic acids are polyunsaturated fatty acids (Qu et al. 2012). The quality of biodiesel to meet the distinctive

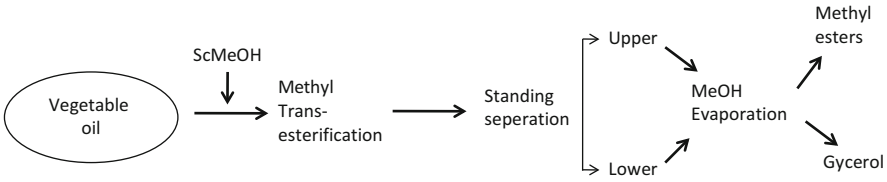

Fig. 6.1 Schematic diagram showing conversion of vegetable oil to biodiesel

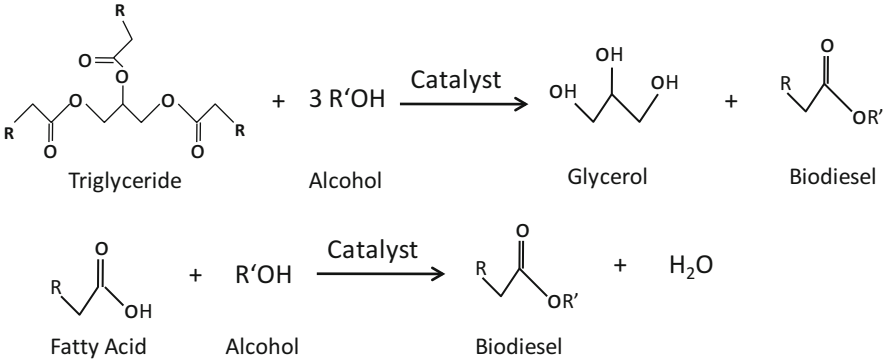

Fig. 6.2 Reactions involved in the conversion of TAGs or fatty acids into biodiesel

features is mainly regulated by the composition of FAs. FA composition of the oil influences the cetane number (CN), cloud point properties, cold flow, oxidative stability, kinematic viscosity, peroxide value, density and saponification value, which are main parameters to regulate the ignition quality of biodiesel (Knothe, 2008; Emil et al. 2010; King et al. 2011). Considering biodiesel features, vegetable oils containing more content of monounsaturated FAs are preferable to those containing polyunsaturated FAs as polyunsaturated FAs reduce biodiesel stability (Qu et al. 2012) (Figs. 6.2 and 6.3). Triacylglycerols (TAGs) are a reduced form of carbon, which constitute energy reserve in seeds and used to power consequent development of the seedling. In addition, seed lipids have nutraceutical and nutritional value, and are used as edible oils (Lung and Weselake 2006). The TAGs of seeds have similar acyl groups that are present in membrane lipids: stearic acid (18:0), oleic acid (18:1Δ^9), α-linolenic acid (18:3), palmitic acid (16:0) and linoleic acid (18:2) (Voelker and Kinney 2001). These FAs are generally referred to as common FAs that are essential for biodiesel production (Gubitz et al. 1999). In this book chapter, an effort is made to give a comprehensive account on how the transgenic technology may contribute to (i) enhance oil content, (ii) identify candidate gene(s) involved in oil synthesis and (iii) alter the candidate gene expression for biosynthesis of triacylglycerols.

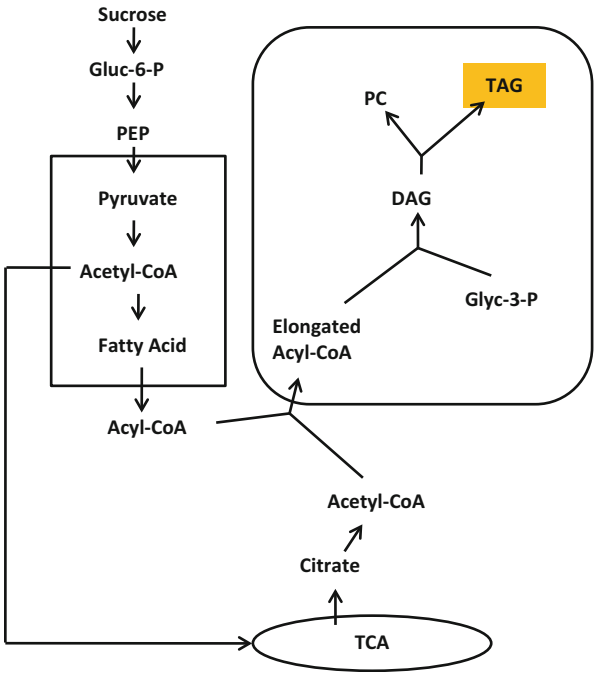

Fig. 6.3 Fatty acid synthesis cycle (Kennedy pathway) involved in synthesis of triacylglycerols (TAGs) that is essential for the production of oil efficient in conversion to biodiesel

6.2 Oil Content in *Jatropha*

The main fatty acids of *J. curcas* oil are linoleic acid (29.0–44.2%), palmitic acid (14.1–15.3%) and stearic acid (3.7–9.8%) (Table 6.1). The methyl esters of *J. curcas* seeds were studied by gas chromatography-mass spectrometry (GC-MS), and the major components were unsaturated FAs (71.93%) and saturated FAs (27.59%). According to Pramanik (2003), the *Jatropha* oil has an ideal viscosity that makes it suitable for converting to biodiesel. Banapurmath et al. (2012) recommended that 40–50% of fossil diesel can be replaced with *Jatropha* oil without any change in engine parts and preheating of the blends. Vegetable oil containing more than 1% of free fatty acid (FFA) is not considered to be efficient for conversion to biodiesel. Jatropha is reported to have 14% of FFA, and thus, attempts were made to optimize the transformation process for FFA reduction of *Jatropha* oil below 1% by esterification pretreatment, leading to a maximum yield of biodiesel in the subsequent transesterification process (Tiwari et al. 2007). They claimed that pre-esterification has enabled transesterification to give an average biodiesel yield more than 99% (Tiwari et al. 2007).

Table 6.1 Oil yield, fatty acid composition and fuel efficiency of different crops (cetane number, a quality indicating the ignition properties of diesel fuel; calorific value, quantity of heat produced by its combustion)

Crop	Oil content	Fatty acid composition	Fatty acid content	Cetane number	Calorific value of biodiesel (Mj/kg)
Castor bean	50–55%	Oleic acid	2–6%	40–42	37.1
		α-Linolenic acid	0.5–1%		
		Linoleic acid	1–5%		
		Stearic acid	0.5–1%		
		Palmitic acid	0.5–1%		
Cotton	13–15%	Oleic acid	12.4–16.5%	35–40	36.8
		α-Linolenic acid	<1%		
		Linoleic acid	61.4–68.9%		
		Stearic acid	0.9%		
		Palmitic acid	11.4–15.9%		
Jatropha	30–35%	Oleic acid	40.1%	23–41	38
		α-Linolenic acid	0.2%		
		Linoleic acid	36.9%		
		Stearic acid	6.4%		
		Palmitic acid	1.3%		
Palm oil	35%	Oleic acid	36.6%	38–40	36.9
		α-Linolenic acid	<1%		
		Linoleic acid	9.1%		
		Stearic acid	4.3%		
		Palmitic acid	43.5%		
Rapeseed	37%	Oleic acid	61%	30–36	37.4
		α-Linolenic acid	9–11%		
		Linoleic acid	21%		
		Stearic acid	2%		
		Palmitic acid	4%		
Soybean	15%	Oleic acid	23%	30–38	37.3
		α-Linolenic acid	7–10%		
		Linoleic acid	51%		
		Stearic acid	4%		
		Palmitic acid	10%		
Sunflower	32%	Oleic acid	30%	29–37	37.7
		α-Linolenic acid	1.6%		
		Linoleic acid	59%		
		Stearic acid	6%		
		Palmitic acid	5%		

6.3 Fatty Acid Cycle in Plants for Production of Oil

Glycerolipids in plants can be synthesized by means of two pathways similar to that of the phosphatidic acid (PA) in which a two-step enzymatic conversion of *sn*-glycerol-3-phosphate (G3P) is undergone (Table 6.2). The acyltransferases of G3P

Table 6.2 The key genes, their coding enzymes and their metabolic function involved in the Kennedy pathway of TAG biosynthesis in plants

S. No.	Gene	Enzyme	Function	References
1	GPAT	*sn*-glycerol-3-phosphate acyltransferase	Acylation of G3P to form lysophosphatidic acid (LPA) leading to the formation of phosphatidic acid (PA)	Voelker and Kinney (2001)
2	LPAT	Lysophosphatidic acid acyltransferase		Lung and Weselake (2006)
3	PAP	Phosphatidic acid phosphatase	Dephosphorylates PA to yield DAG	Lung and Weselake (2006) and Franca et al. (2008)
4	DGAT	Diacylglycerol acyltransferase	Transfers acyl group from acyl-CoA to sn-3 of DAG to form TAG	Durrett et al. (2008) and Kennedy (1961)
5	PDAT	Phospholipid: diacylglycerol acyltransferase	Utilize phospholipid as the acyl donor in TAG formation	Costa et al. (2010)
6	FAtA	Stearoyl-ACP thioesterase A	Produce oleic, linoleic and stearic acids	Carvalho et al. (2008)
7	FAtA and FAtB	FAtA and palmitoyl-ACP thioesterase	Produce palmitic acid	Carvalho et al. (2008)
8	FAD2	Oleate desaturase	Catalyse oleoyl-ACP (oleic) to linoleoyl-ACP (linoleic)	Carvalho et al. (2008)
9	DGAT1 and 2	Diacylglycerol acyltransferase genes	Rate-limiting enzyme in plant lipid accumulation	Xu et al. (2011)
10	KAS III	Ketoacyl synthase III	Condensation of acetyl-CoA with malonyl-ACP and formation of 3-ketobutyl-ACP	Costa et al. (2010)
11	KAS I	Ketoacyl synthase I	Condensation reactions of 4:0-ACP with malonyl-ACP giving rise to 14:0-ACP and 16:0-ACP	Costa et al. (2010)
12	KAS II	Ketoacyl synthase II	Elongation of 16:0-ACP to form 18:0-ACP	Costa et al. (2010)
13	FAH12	Oleate hydroxylase	Oleic acid undergoes a hydroxylation process yielding ricinoleic acid (C18:OH), an unusual hydroxylated (OH) fatty acid	Costa et al. (2010)

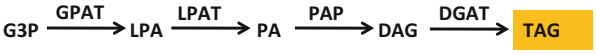

Fig. 6.4 The main steps and enzymes of the Kennedy pathway in the endoplasmic reticulum that are to be modified (overexpressed) for the enhancement of TAG production

pathway in eukaryotes use the acyl groups to produce membrane and TAGs that are used as lipid storages (Millar et al. 2000) (Fig. 6.4). The production of TAGs takes place through two metabolic pathways: one is an acyl-CoA (acyl-coenzyme A)-dependent pathway and an acyl-CoA-independent pathway. In the acyl-CoA-dependent pathway (Kennedy pathway), the substrate is acyl-CoA for successive acylation reactions of the glycerol backbone and the terminal step is the DAG acyltransferase (DGAT) that acylates *sn*-1,2-diacylglycerol (DAG) (Kennedy 1961). G3P is the source of glycerol backbone for TAG assembly, and the glycerol backbone is produced by catalytic reaction of *sn*-glycerol-3-phosphate dehydrogenase (G3PDH) from dihydroxyacetone phosphate (DHAP) which is derived from glycolysis (Weselake et al. 2009).

In Kennedy pathway, lysophosphatidic acid (LPA) is formed by the acylation of G3P through the action of *sn*-glycerol-3-phosphate acyltransferase (G3PAT). Later by second acyl-CoA-dependent acylation, phosphatidic acid (PA) is formed by the catalytic action of lysophosphatidic acid acyltransferase (LPAAT) and release of phosphate from PA to produce DAG (Lung and Weselake 2006). In the final acylation reaction, acyl-CoA as an acyl donor converts DAG to TAG through DGAT (Durrett et al. 2008). A membrane-bound glycerol-3-phosphate acyltransferase (G3PAT) transfers the fatty acids from either acyl-ACPs or acyl-CoA molecules to the *sn*-1 position of G3P, forming LPA (Voelker and Kinney 2001). Lysophosphatidic acid acyltransferase (LPAAT) catalyses the transfer of the acyl chain from the CoA ester to *sn*-2 of LPA, leading to the production of PA. The cytoplasmic enzyme PAP dephosphorylates PA to yield DAG (Franca et al. 2008). DAG formed from the PA hydrolysis is a straight precursor of TAG (Nakamura et al. 2007). Kennedy pathway's final step includes the enzyme action of DGAT that catalyses the third acyl-CoA-dependent acylation reaction that leads to the formation of TAG from DAG. Triacylglycerol is composed of three fatty acyl groups esterified to a glycerol backbone at the *sn*-1, *sn*-2 and *sn*-3 positions.

In plastids, FAs are synthesized from acetyl-coenzyme A (acetyl-CoA) in a two-step process: (i) acetyl-CoA carboxylase catalyses the formation of malonyl-CoA from acetyl-CoA by irreversible carboxylation and subsequently, malonyl-ACP is formed from acyl carrier protein by transfer of malonyl group of malonyl-CoA which acts as primary substrate of FA synthase complex (Durrett et al. 2008). This carboxylation reaction is the key step of FA synthesis (Nikolau et al. 2003); (ii) 16:0-ACP is formed by recurrent condensation of malonyl-CoA with a developing ACP-bound acyl chain by action of the fatty acid synthase complex, with the repeated accumulation of two carbon units for each elongation cycle. (Durrett et al. 2008).

For every cycle, four different reactions are required. The initial step includes the development of 3-ketobutyl-ACP by the acetyl-CoA condensation with malonyl-ACP by the action of ketoacyl synthase III (KAS III), later by reduction to 3-hydroxylacyl-ACP, dehydration to an enoyl-ACP and further reduction to form the elongated 4:0-ACP. Successive series of condensation reactions of 4:0-ACP with malonyl-ACP give rise to 14:0-ACP and 16:0-ACP by the action of KAS I enzyme (Tai and Jaworski 1993). The elongation of 16:0-ACP to form 18:0-ACP is catalysed by KAS II, and Δ^9–desaturase is the enzyme that converts 18:0-ACP into 18:1-ACP in the plastids in the first desaturation step. These three fatty acids (16:0-ACP, 18:0-ACP and 18:1-ACP) are transported into the acyl-CoA and acyl-lipid pools of the cytosol (Lung and Weselake 2006; Dyer et al. 2008). The closure of FA elongation is catalysed by enzyme acyl-ACP thioesterases (acyl-ACP hydrolases). Thioesterases catalyse the acyl-ACP hydrolysis to form free fatty acids, which have the capacity to enter the plastidial envelope to be reactivated as acyl-CoAs on the outside of the organelle (Weselake et al. 2009).

Two main types of thioesterases are described in plants and are (i) stearoyl-ACP thioesterase A (FAtA), which preferentially removes oleate from ACP, and (ii) palmitoyl-ACP thioesterase B (FAtB) that are active with saturated and unsaturated acyl-ACPs and, in some species, with shorter-chain-length acyl-ACPs (Pollard et al. 1991; Salas and Ohlrogge 2002; Mayer and Shanklin 2007). In the process of TAG production in plastids, the ratio of acyl chains is determined by the interplay between the FA synthase complex, Δ^9 desaturase and the two thioesterases (Voelker and Kinney 2001). After it has been exported from the plastids, oleic acid enters the cytosolic pool and is imported into the ER in association with CoA. Thus substrate-specific desaturases convert available oleic acid to linoleic and α-linolenic acids (Stymne 1987; Somerville et al. 2000; Durrett et al. 2008; Dyer et al. 2008). Alternatively, other FA chain modifications can occur in the ER. Finally, part of the FAs are reserved as structural components of cellular membranes (ER phospholipids and plastid galactolipids), and the rest is transferred to TAG and accumulated as an energy source (Dyer et al. 2008; Cagliari et al. 2011).

Triacylglycerol in higher plants is the principal constituent of seed oil or fruits of oleaginous plants and mostly provides as energy storage to sustain the growth of young seedlings during the early stages of germination. TAGs are also prominent components of any bioeconomy, providing a highly reduced carbon source for both food and non-food applications, such as supplying a feedstock for the production of renewable energy. Insights into the details of biosynthesis of TAG and information on the genes and enzymes linked in this work have led to experimental approaches to alter the FA components of TAG and increase the oil content in seeds.

6.4 Genes of Fatty Acid Cycle in *Jatropha* Responsible for Oil Production

Genomic and transcriptomic studies have been performed by various researchers to generate background information to accelerate the genetic improvement of many crops (Carels 2009). The data of *Jatropha* complete genome is now publicly available (Sato et al. 2011). Ruuska et al. (2002) have found 80% reduction of oil in mutant *wrinkled1* compared to that of wild-type *Arabidopsis* by cDNA microarrays. O'Hara et al. (2002) found that the majority of FA and biosynthetic genes of lipids were expressed at stable molar ratios, but altered absolute levels during embryogenesis. In another study, Dong et al. (2004) demonstrated that the number of differentially expressed genes at 15 and 25 days after fertilization was different and is reduced from 104 to 63 in *Brassica napus*. Niu et al. (2009) performed cDNA chip hybridization and found that the crucial stage for the transition of seed-to-sink tissue was 17–21 DAF, whereas FA biosynthesis-related genes were highly expressed primarily at 21 DAF.

Jatropha has gained vast attention in biological studies, especially regarding the genes that are involved in lipid and FA biosynthetic pathways (Costa et al. 2010; Purushothaman et al. 2010; Chen et al. 2011; King et al. 2011; Sato et al. 2011). Identification of processes to manipulate and alter the FA composition of candidate oil resources such as *Jatropha* would be highly desirable for the biofuel industry (Jiang et al. 2012). The saturated FA content of *J. curcas* oil includes 3.7–9.8% stearic acid, 14.1–15.3% palmitic acid, 29.0–44.2% linoleic acid and 34.3–45.8% oleic acid (Gubitz et al. 1999). Therefore, to improve *Jatropha* biodiesel qualities, it requires altering the FA composition in *Jatropha* seeds with higher oleic acid (>70%) and lower saturated FA (<10%) (Ye et al. 2013). Transcriptomic studies of genes involved in the biosynthesis of FA can provide primary molecular understanding of storage and synthesis of proteins and lipids in *Jatropha* seeds. The expression intensities of various important genes involved in biosynthesis of FA in growing seeds 14–45 days after pollination (DAP) exhibited that maximum genes were up-regulated between 29 and 41 DAP (Jiang et al. 2012). Electron microscopy studies disclosed formation of oil bodies at 28 DAP, which was actively developing by 42 DAP and gained the maximum size and number after 56 DAP (Gu et al. 2012).

Costa et al. (2010) sequenced 13,249 ESTs (Expressed Sequence Tag) from two cDNA libraries of *J. curcas* that included developing (JD) and germinating (JG) seeds and paved way for researchers to evaluate differential expression and discover maximum genes that are related to lipid metabolism. They also studied 12 ESTs coding for oleoyl-ACP desaturase (FAD2) that is involved in the catalysis of polyunsaturation of oleoyl-ACP (18:1) to linoleoyl-ACP (18:2). This enzyme has become a possible biological marker for the alteration in oil composition of *Jatropha* as oleic and linoleic acids are its major constituents. Gu et al. (2012) observed that

genes with similar role were expressed differentially during the development of endosperm, and the majority of genes related to lipid and FA biosynthesis are highly associated with the oil bodies and endosperm development in seeds of *Jatropha*. Twenty-one lipid genes linked to the TAG and FA pathways disclosed expression of 17 genes specific to growing seeds of *Jatropha* as compared to the leaves (Xu et al. 2011). King et al. (2011) observed transcripts corresponding to the plastidial glycolytic Kennedy pathway and the cytosolic glycolysis pathways related to TAG biosynthesis. The first stage to address this matter may be to explore the temporal and spatial expression of lipid and FA biosynthetic genes. Documentation of 7009 unigenes from a normalized cDNA library of *Jatropha* seed endosperm indicated 17 genes encoding enzymes for lipid and FA biosynthesis, and their expression was further characterized by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Purushothaman et al. 2010).

ESTs coding for biosynthesis of FA enzymes were observed in JD; the FA degradation pathway is enhanced in JG ESTs, which is probable considering that these stages are committed to accumulation and oil breakdown, respectively. In the FA biosynthesis pathway, Xu et al. (2011) found ESTs encoding for enzymes catalysing reactions that finally yield linoleic (PCH) and palmitic acids (FatA and FatB), oleic and stearic acids (FatA), the key components of *Jatropha* seed oil and ESTs of DGAT in both JD and JG that transfers an acyl group from acyl-CoA to *sn*-3 of DAG to form TAG. Acylglycerols are another class of lipids, which serve as energy reserves in many living organisms including plants and are one of the major constituents of seed oils. TAG is the most common acylglycerol in seed oils. In the cytoplasm, FFAs become esterified to CoA and play as substrates for biosynthesis of TAG from *sn*-glycerol-3-phosphate. After the formation of 1,2-DAG, the synthesis of TAG can take place in two ways that involve acyl group transfer from acyl-CoA to *sn*-3 of DAG by DGAT to form TAG while the second pathway involves a phospholipid:diacylglycerol acyltransferase (PDAT) that uses phospholipid as the acyl donor in the formation of TAG. The expression of β-ketoacyl-acyl carrier protein (ACP) synthase I (*KAS I*) gene is enhanced prior to that of *KAS II* and *KAS III* and catalyses the first step of fatty acid biosynthesis (Jiang et al. 2012). Expression of genes linked in biosynthesis of TAG was also discovered (Troncoso-Ponce et al. 2011; Jiang et al. 2012; Sood et al. 2015). Studies of the expression of *Jatropha* KAS III in different tissues showed maximum expression in growing seeds and roots which was enhanced with time (Li et al. 2008; Raorane et al. 2013). Overexpression of DGAT has been reported to enhance the oil content in *Arabidopsis* (Jako et al. 2001). The regulation of seed development and biosynthesis of TAG in seeds has been reported in some depth (Santos-Mendoza et al. 2008).

Annarao et al. (2008) observed that lipid biosynthesis is initiated 3 weeks after fertilization and TAGs were synthesized between IV and VII developing stages. Subsequently, only two DGAT genes (DGAT1 and 2), indicating rate-limiting enzymes in accumulation of lipid, were found to be mainly linked with the TAG biosynthesis (Xu et al. 2011). Costa et al. (2010) identified ESTs encoding enzymes that produce linoleic and palmitic (FAtA and FAtB) acids, oleic and stearic (FAtA) and oleate desaturase (FAD2), which catalyse oleoyl-ACP (oleic) to linoleoyl-ACP

(linoleic). Although the genome size of *Jatropha* is comparatively small ($C = 416$ Mb) (Carvalho et al. 2008), expression profiles of genes linked to lipid and FA biosynthesis during the development of *Jatropha* seeds have yet to be understood. Therefore, documentation of the genes linked to lipid and FA characterization and biosynthesis of their patterns are the two genetic parameters regulating lipid biosynthesis in the developing seeds of *Jatropha* (Gu et al. 2012).

DGAT is an integral ER enzyme which is also found in plastids and oil bodies (Siloto et al. 2009). Though DGAT1 and DGAT2 are the key categories of DGAT enzymes, other structurally different enzymes with DGAT activity have been reported in plants (Turkish et al. 2005; Cahoon et al. 2007). The acyl composition of the DAG pool, acyl-CoA concentration and temperature are the factors on which the substrate selectivity of DGAT depends (Lung and Weselake 2006). The involvements of DGAT1 and DGAT2 in the production of oils are seemingly species dependent. In the plant system, DGAT1 seems to be the main enzyme involved in the accumulation of seed oil; whereas, DGAT2 seems to play a vital role in the selective unusual FA accumulation, such as hydroxy and epoxy FAs, into seed oils (Shockey et al. 2006; Xu et al. 2008; Li et al. 2010). DGATs are considered as the rate-limiting enzymes in the storage of plant lipids (Jako et al. 2001; Lung and Weselake 2006; Xu et al. 2008) and seem to be critical for controlling qualitative and quantitative aspects of seed oil synthesis in transgenic plants (Cahoon et al. 2007). In this scenario, DGAT appears to be a probable target for the genetic engineering of plant lipid biosynthesis in oilseeds for commercial profit (Xu et al. 2008). Several studies proved that the accumulation of oil is regulated by the regulation of DGAT expression in *Arabidopsis* seeds (Routaboul et al. 1999; Zou et al. 1999; Jako et al. 2001). It is also reported that DGAT expression appears to be critical for the right channelling of unusual fatty acids into seed oils as well (Cahoon et al. 2007).

6.5 Genetic Engineering Strategies for Increasing the Oil Content

Many transgenic plants have reached the field for commercial cultivation (Nindita et al. 2015; Moniruzzaman et al. 2016). Till date, limited attempts are being made at the genetic transformation of *Jatropha*. Li et al. (2006) initiated a preliminary work on genetic transformation in *J. curcas*. For the first time, a comprehensive *Agrobacterium*-mediated method of transformation for *J. curcas* was developed in 2008 (Li et al. 2008). The bacterial strains EHA105 and LBA4404 were used for co-cultivation with phosphinothricin as the selective agent. Approximately 55% of phosphinothricin-resistant calli were recovered when the cotyledon explants were transformed with the two strains of bacteria and ß-glucuronidase and molecular analyses through PCR, and Southern hybridization was carried out in the primary transformants. The results showed that 13% of the total co-cultivated explants formed transgenic plants after 4 months period. Several studies were carried out

later for the development of transgenic plants in *J. curcas*. Purkayastha et al. (2010) used pBI426 in which the *npt*II gene for kanamycin resistance and the GUS reporter gene are under the control of CaMV 35S promoter and developed a direct DNA delivery system to shoot apices by particle bombardment using a biolistic PDS-1000/Helium system. They optimized several components such as bombardment pressure, target distance between stopping screen and target plate, microparticle size and osmotic pretreatment for genetic transformation, and confirmation analysis was done by PCR analysis using GUS gene primers, GUS staining and Southern blot hybridization for *npt*II gene. Mazumdar et al. (2010) utilized *Agrobacterium*-mediated transformation and established a different plant regeneration method by using explants of varying age and orientation. They used EHA105 *A. tumefaciens* strain harbouring the binary vector pCAMBIA2301 harbouring the *npt*II and GUS genes, which are regulated by CaMV 35S promoter. They proved that the activity of GUS at the cut points showed the amenability of target explants to *Agrobacterium*. More recently, successful experiments of *Agrobacterium*-mediated transformation were also described by Franco et al. (2016).

RNA interference (RNAi) technology was used to silence the expression of the *J. curcas FAD2-1* gene. Transgenic plants were generated using the traditional transformation techniques with hygromycin phosphotransferase (*hpt*) gene as the selectable marker to produce transgenic *Jatropha* plants with high oleic acid content in seeds by Qu et al. (2012). Since the *FAD2* gene plays an important role in environmental adaptation in the vegetative growth of the plant, it is essential to precisely alter the content of oleic acid in seeds only. In this study, the soybean seed storage protein 7S gene promoter which shows seed-specific expression was deployed. Further analysis done by quantitative RT-PCR proved that the engineered *FAD2-1* was gene-specific as it had no effect on the expression of *FAD2-2* (the paralog of *FAD2-1*) in the endosperm of the transgenic plant. The study also proved that the 7S promoter precisely controlled *FAD2-1* silencing only in seeds (the target organ) since there is no significant change of *FAD2-1* transcript levels in vegetative organs, such as leaves.

Following GC characterization of FA methyl esters extracted from the transformed endosperm of *Jatropha*, the *Jatropha* biodiesel CN was found to be enhanced by ~8 fold in comparison to the non-transformed control in more than 60% in transgenic plants producing oleic acid (Ye et al. 2013). Transgenic technology in *J. curcas* is exploited to overcome the problems like insect damage (Gu et al. 2014), fungal diseases (Franco et al. 2016) and alteration of FAs concentration of the oil (Kley 2000; Qu et al. 2012; Ye et al. 2013). This technique will help in the genetic enhancement of *Jatropha* for desired attributes to serve as a suitable feedstock to warrant stable biodiesel supply. Genetic modification approaches may suggest some important, interesting and new options in the genetic improvement of *Jatropha* with alteration of lipid profiles and tolerance to abiotic and biotic stresses.

Several studies reported that it is promising to enhance the seed's oil content through changes in the level of expression of key seed oil accumulation regulators. Tong et al. (2006) reported successful transfer and expression of stearoyl-acyl carrier protein desaturase in *Escherichia coli.* Lindqvist et al. (1996) reported that stearoyl-

acyl carrier protein desaturase is one of the important biosynthetic enzymes in plants that also plays a critical role in determining the ratio of unsaturated and saturated FAs. A full-length cDNA of aquaporin (JcPIP2) was isolated from *J. curcas* seedlings for understanding the mechanism at the molecular level of drought and salt resistance. Zhang et al. (2007) reported that the occurrence of JcPIP2 was enhanced by high dry condition, and it plays a critical part in fast development and growth of *Jatropha* under drought stress. A betaine aldehyde dehydrogenase gene (BADH) termed JcBD1 was cloned by RT-PCR and RACE from *J. curcas*. *JcBD1* was successfully transferred and expressed in leaves under drought (30% PEG), heat (50 °C) and salt (300 mM NaCl) stresses (Zhang et al. 2008).

6.6 Conclusions

One of the main limitations for *J. curcas* cultivation is its still inadequate oil yield. The oil content and composition of *J. curcas* can be enhanced through FA modification using genetic engineering techniques. The best pathway for TAG production is the Kennedy pathway. The genes found playing a vital role in FA biosynthesis were identified through biotechnological techniques. Some progress has been made in the genetic engineering of genes related to FA biosynthesis. The advance in understanding of *Jatropha's* biology and the integration of biotechnology and system biology to classical approaches of selective breeding is expected to expedite the domestication process.

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Chapter 7
Transcriptomic View of *Jatropha curcas* L. Inflorescence

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Abstract The inflorescence is an important component for the reproductive success in plants. At the onset of vegetative to reproductive tissue transition, a series of biological processes which affect the yield component of a plant take place within the inflorescence: shoot apical meristem to inflorescence meristem transition, floral commitment and flowering (inflorescence meristem to floral meristem), floral sex differentiation, male-to-female flower ratio, seed setting, and fruiting. *Jatropha curcas* or the physic nut is gaining recognition worldwide for its lucrative biofuel potentials; however, the present planting material offers considerable yield constraints such as poor seed yield, predominantly attributed by the unpredictable number of inflorescences and flowers. This chapter discusses the molecular aspects of *J. curcas* inflorescence with regard to reproductive-related organs/tissues such as the shoot, floral bud, and male and female flowers. Transcriptome and genomic analyses of *J. curcas* inflorescence and its related components have been identified and discussed to benefit plant breeding programs targeted for *J. curcas* yield enhancement strategies. Bioinformatics approaches such as the transcriptome data based on the differentially expressed gene analysis and gene co-expression network modelling are also addressed for the selection of candidate genes of interest in

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breeding programs. In addition, a complete view of the molecular basis that governs *J. curcas* inflorescence development and specifications is described.

Keywords Inflorescence · *Jatropha curcas* · RNA sequencing · Transcriptome

7.1 Introduction

Biodiesel has emerged as an eco-friendly and biodegradable alternative to the depleting crude oil reserves (Pan et al. 2014), and with an increasing global population, the fossil fuel supply is likely to exhaust in the long term (Raorane et al. 2010). Despite the advent of new technologies for biodiesel production (Choo et al. 2012), a shortage of raw material/feedstock has been correlated to poor biodiesel productions (Wani et al. 2006). There are about 300 oilseed species available worldwide; however, only 30 species have been listed as promising for biodiesel production (Hegde 2003). Edible oilseed crops such as rapeseed, soybean, and sunflower are suitable feedstocks for biodiesel production in developed countries since the present production is in excess of consumption (Kumar and Sharma 2008; Johnson et al. 2011; Pandey et al. 2012). In contrast, the developing countries suffer from a huge shortage of edible oil supply and yet are adopting them for biodiesel; however, few countries are now turning into non-edible oils such as that of *Jatropha curcas* for sustainable and efficient energy management.

Belonging to the Euphorbiaceae family (Tatikonda et al. 2009; Divakara et al. 2010), *J. curcas* is a monoecious, succulent perennial shrub (Borchert 1994; Foidl et al. 1996). The species is native to tropical and subtropical regions of Mexico, Central America, and South America. It thrives well in arid, semiarid, and tropical conditions and produces seeds with high (30–40%) oil content (Makkar et al. 1997; Divakara et al. 2010). *J. curcas* has a superior oil content and adapts to marginal lands and poor soils efficiently (Fairless 2007; Ye et al. 2013). In 2017, an average global seed yield of 2218 ± 148 kg/ha/year was reported although seed yield across many different regions in the world varied greatly (Lama et al. 2018). The *J. curcas* oil (JCO) is composed of saturated and unsaturated fatty acids at 8:2 ratio (Akbar et al. 2009), while the physical and mechanical properties are highly suitable for biodiesel and bio-jet fuels (Ong et al. 2011; Khalil et al. 2013). Comparatively, the biodiesel from JCO performs better than fossil diesel; it has a higher flash point, higher cetane number (fuel-burning efficiency), and excellent lubricative properties due to the presence of esters with long-chain fatty acids (Rao et al. 2008a). Despite these suitable properties, an inadequate JCO supply constraints the commercialization potential of *J. curcas*.

Plant shoot system is comprised of both vegetative and reproductive components, and the inflorescence has been the most fundamental reproductive organ that affects seed yield in *J. curcas*. Flowering, a critical component of seed development, begins with the transition of vegetative growth into reproductive growth. In plants, a switch

from vegetative to reproductive growth is only possible when the plant had acquired substantial vegetative structures. At this point, plants have reached sexual maturity and are able to flower (Copeland and McDonald 2001; Mateous et al. 2015). In general, the aboveground plant shoot system initiated by shoot apical meristem (SAM) reiteratively divides to produce lateral organs (vegetative structures). The transition of SAM into inflorescence meristem represents the hallmark to sexual reproduction. Inflorescence meristem undergoes two antagonistic processes, which are the mitotic division of stem cells (proliferation activity) and the peripheral cell recruitment (flower organogenesis) prior to floral meristem transition (Aichinger et al. 2012; Machida et al. 2013; Heidstra and Sabatini 2014). Unlike the indeterminate SAM and inflorescence meristem, the floral meristem displays a genetically programmed mode, which terminates at/after a specific developmental phase of a floral organ (Guo et al. 2015; Sun and Ito 2015; Yamaguchi et al. 2017). Sex expression and sex ratio in *J. curcas* are considered labile because complex processes with limited characterizations have been associated with its flowering mechanism (Fresnedo-Ramírez 2013). To date, the recent advance in *J. curcas* genome sequencing coupled with the improvement of technologies for gene function discoveries had enabled numerous studies on *J. curcas* reproductive biology particularly on the floral organs: gynoecium, staminate, and pistillate flowers (Gangwar et al. 2016; Hui et al. 2017). Application of high-throughput technologies to understand the genetic control and molecular events underpinning plant organ/tissue differentiation and development has greatly advanced. In this chapter we discuss findings from analyses of genomics, transcriptomics, and expression data by bioinformatics approaches (differential gene expression and gene co-expression network analyses) in an attempt to provide a resourceful molecular understanding of key genes and biological processes related to the reproductive biology of *J. curcas* inflorescences.

7.2 The *Jatropha curcas* Inflorescence

The *J. curcas* inflorescence is composed of a main peduncle bearing co-florescences of pedicels (0.6–1.0 cm in length) and flowers, which are formed terminally at the axillary region of growing new shoots (Chang-Wei et al. 2007; Wu et al. 2011). They show protandry, whereby both male and female unisexual flowers are produced within the same bunch in a simple or compound dichasial cyme pattern, i.e., a central female flower surrounded by a group of male flowers. Generally, a simple dichasial cyme has at least six or more cymes within an inflorescence. Male flowers occupy the subordinate position within the inflorescence and are found more abundant relative to the female flowers, occupying the terminal position (Fig. 7.1). In Asian countries such as India and Malaysia, an inflorescence with average flowers of 300 and a male-to-female (M/F) flower ratio of 29–22:1 yields about 10–15 fruits (Solomon-Raju and Ezradanam 2002; Noor-Alam et al. 2011; Wani et al. 2006; Gangwar et al. 2016). In contrast, plants from Central America, which is the center of

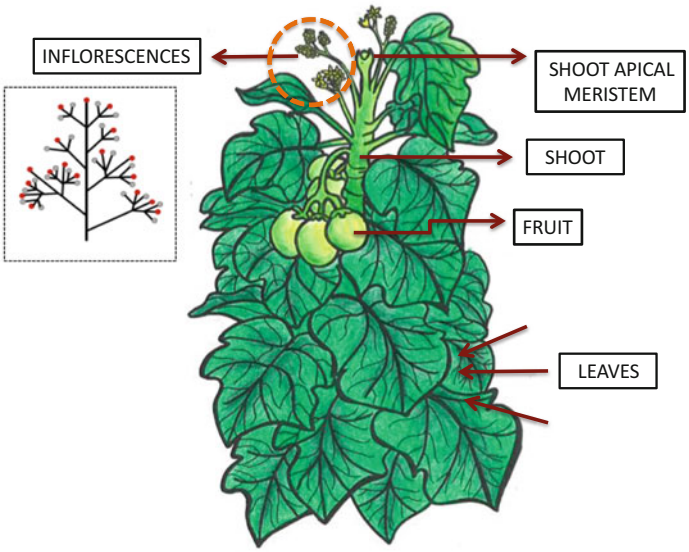

Fig. 7.1 A schematic image indicates all the aboveground organs of *J. curcas* shoot system: inflorescences, shoot apical meristems, shoots, fruits, and leaves. The dotted yellow circle indicates inflorescence, and the dotted square box shows the inflorescence dichasial cyme pattern. Red dots represent female flowers and each gray dot represents a group of six male flowers

origin of *J. curcas*, present a higher M/F flower ratio at 60:1 (Rincon-Rabanales et al. 2016). In a study conducted by Wu et al. (2011), the *J. curcas* inflorescence was categorized based on the following M/F flower ratio: female type (M/F flower ratio of 8.79), male type (all male flowers), and intermediate type (M/F flower ratio of 26.94). The inflorescence types affected not only the floral sex ratio but also the position of flowers within the inflorescence. The same study also presented the developmental process of flowering, which includes the structural characteristics of male and female flowers. Interestingly, in *J. curcas* floral development, male flower organogenesis takes place first followed by the female flower organogenesis. The female flower sustains a hermaphrodite period prior to male tissue abortion during the floral development. Most female flowers take up the terminal position (single flower), and others with the subordinate position are formed after an abortion of male tissues. In female flowers taking up the terminal position, no male tissue abortion was observed (Wu et al. 2011).

Both types of flowers open synchronously, and their mixed sexual systems include geitonogamy and xenogamy (Bressan et al. 2013; Rincon-Rabanales et al. 2016). The *J. curcas* flowers are light yellow to pale green in color with five sepals, petals, and glands each. Male flowers are about 0.75–0.9 cm in length and 0.3–0.4 cm in width with ten stamens divided into upper and lower layers. On the other hand, female flowers include three carpels and two split stigmas, which have a length and width of 0.45–0.75 cm and 0.20–0.40 cm, respectively. Generally, the male flowers open first followed by the female flowers. In a study conducted in

Malaysia, female flowers remain opened for 3–4 days, while the male flowers stay opened longer, i.e., between 8 and 10 days (Noor-Alam et al. 2012). The M/F flower ratio is a heritable character (Bhattacharya et al. 2005; Rao et al. 2008a), and significant variation in flower sex ratio has been observed among the *J. curcas* accessions in Spain (Alburquerque et al. 2017), Malaysia (Noor-Alam et al. 2012; Che-Mat et al. 2015), and North Florida (Nietsche et al. 2014). *J. curcas* has been reported to flower twice a year or even throughout the year (Noor-Alam et al. 2012; Heller 1996). The sex ratio, however, has shown tremendous variations according to the planted materials, time, climate, and nutrition conditions (Luo et al. 2007). The flowering process and its resulting seed formation are affected by multiple environmental factors (Joachim 1996; Maes et al. 2009) such as light quality (Teo et al. 2014), duration of cold temperatures, maturity, vegetative period, and exogenous application of growth regulators (Makwana et al. 2010; Pan et al. 2014). The molecular determinants of *J. curcas* racemose inflorescence (Solomon and Ezradanam 2002) lay an important foundation for floral biology, pollination ecology, seed, and fruit set improvements. In most crops, the flowering characteristics have been significantly correlated to productivity (Rao et al. 2008b; Carels 2009). In *J. curcas*, several studies have correlated flowering characteristics to plant yield components, such as fruit number per inflorescence, seed weight per plant, and oil yield per plant. In a study conducted in Indonesia by Wijaya et al. (2009), oil yield of 6-month-old *J. curcas* plants showed nonsignificant correlation to number of male flowers (−0.14), number of female flowers (0.28), total flowers (−0.11), and fruit number per inflorescence (0.09). In contrast, another study conducted in Malaysia demonstrated moderately positive correlation between the number of seeds per plant and the following traits in 1-year-old *J. curcas* plants: number of inflorescences per plant (0.57), number of flowers per inflorescence (0.66), and number of female flowers per inflorescence (0.51) (Che-Mat et al. 2015). Numbers expressed in parentheses indicate the correlation values.

A typical flower consists of four concentric whorls of floral organs: sepal, petal, stamen, and carpel. The first two whorls are not directly involved in reproduction. The outermost whorl contains sepals that protect young flower bud, and the second whorl with very attractive and copious petals is important to attract pollinators. The third whorl contains stamens (microsporophylls) that form male gametes, and the fourth whorl consists of carpels (megasporophylls) or the female reproductive cells such as stigma, style, and ovary (Araki 2001). Flowering is induced by both external (light and temperature) and internal factors (nutrients, hormone levels, and plant age). In *J. curcas*, Wu et al. (2011) presented a 12-phase event of early flowering development. Phase I–II represents the shift from vegetative to reproductive growth with the formation of inflorescence primordia. This phase completes upon the development of a floral meristem flanked by inflorescence meristems. Phase III–VI includes the appearance of sepal primordia, i.e., five calyx primordia (Phase III), five petal primordia (Phase IV), five gland primordia (Phase V), and ten stamen primordia arranged into two upper and lower layers with five stamens in each layer (Phase VI). Structural characteristics and formation patterns for both male and female flowers remain the same from Phase I–VI and start to show differences

thereafter. Phase VII of the female flower forms protuberance at the apical meristem, whereas the Phase VII of the male flower continues to develop stamen primordia. Phase VIII of the female flower shows the emergence of a carpel primordia, and Phase VIII of the male flower develops a heart-shaped stamen from an oval-shaped form. At Phase IX, female flower forms ovule primordia, with up to three ovules, whereas the male flower takes up the shallow cleft-shaped stamen primordia from a previous heart-shaped. Phase XI shows development of glands: stigma for female flower and anther for male flower. Phase XII of the female flower marks the development and maturation of the stigma, while it indicates elongation of filament and presence of pollen grains in the male flower (Wu et al. 2011).

7.3 *J. curcas* Genome

The genome size of *J. curcas* is relatively small, $C = 416$ Mb with an average 2C of 0.85 pg. The average base composition is 38.7% GC (1 pg of DNA corresponds to 0.978×109 bp). Karyotyping analysis revealed the presence of 22 small metacentric and submetacentric chromosomes (1.71–1.24 um in size) such that the haploid chromosomal number is $n = 11$ (Carvalho et al. 2008). In 2011, the Japanese researchers presented the first *J. curcas* (Palawan Island line) whole-genome sequences of 285,858,490 bp consisting of 120,586 contigs and 29,831 singlets and 40 and 929 complete and partial structures of protein encoding sequences available at http://www.kazusa.or.jp/jatropha/ (Sato et al. 2011). Following the release of the first *J. curcas* draft genome sequence, an extensive number of genetics and gene expression studies of *J. curcas* were conducted over the years targeting on reproductive organs: seed, fruit, and flower. A number of EST and genomic collections were obtained from developing seeds (Natarajan et al. 2010), flower buds (Wang et al. 2013), as well as developing and germinating endosperm (Zhang et al. 2014, 2007; Costa et al. 2010). In *J. curcas*, transcriptomes of floral organs (Xu et al. 2016; Chen et al. 2017; Hui et al. 2017), inflorescence meristems (Pan et al. 2014), roots (Juntawong et al. 2014; Natarajan and Parani 2011), shoots (Govender et al. 2017, 2018), 2-week-old seedlings (Wang et al. 2013), roots, mature leaves, flowers, developing seeds, and embryos (Jiang et al. 2012; King et al. 2011; Natarajan and Parani 2011) were established by high-throughput NGS technologies. In another *J. curcas* genome sequencing attempt, Hirakawa et al. (2012) released an upgraded genome sequence version of 297,661,187 bp with 39,277 scaffolds, a year later. In 2015, Wu et al. (2015) reported a more comprehensive version of the *J. curcas* genome assembly of an inbred cultivar GZQXo401, 320,546,307 bp of 72,474 contigs (>100 bp), and the assembled contigs were longer than the previously reported one, 23,125 scaffolds with an N50 of 0.746 Mbp. The genome sequences are available at NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

7.4 Analysis of Differential Gene Expression and Network Modeling of Co-expressed Genes

High-throughput sequencing or next-generation sequencing (NGS) approaches are widely employed by life scientists for genetics and genomics researches. Revolutionizing genomic research, the application of NGS technology has been steadily increasing in plant research owing to its feasibility, number and range of applications, as well as decreasing cost. The applications of high-throughput technologies, such as genome sequencing, target sequencing, RNA-seq, Chip-seq, RIP-seq, and methylation, provide important insights into the patterns and purpose of biological processes in living organisms. A number of NGS platforms have been developed by Life Technologies, Roche, Illumina, and Pacific Biosciences. Briefly, NGS data are obtained according to the following steps: (i) fragmentation of target sequences into desired lengths by either enzymatic (endonuclease or transposase fragmentation) or physical (sonification and acoustic shearing) method, (ii) conversion of single-stranded RNAs into double-stranded DNAs, (iii) attachment of oligonucleotide adapters to the ends of target fragments, and (iv) quantification of the fragment library prior to sequencing. RNA-seq-based transcriptome is a collection of genomic loci (coding, noncoding, antisense, and intergenic RNAs) expressed by cell(s) or a tissue in a given physiological condition at a given time. RNA-seq does not depend on pre-specified candidate probes, and therefore, beyond whole genomic loci detection and quantification, it offers a multitude of applications, such as the (i) identification of lowly expressed genes; (ii) quantification of fold changes or the differentially expressed (DE) genes between different experimental conditions; (iii) quantification of alternative splicing and different isoforms; (iv) identification of small RNA profiles such as microRNA, PIWI-interacting RNA, small nucleolar RNA, small nuclear RNA, and transfer RNA; (v) detection of chimeric transcript; and (vi) detection of novel transcripts. In *J. curcas*, RNA-seq has been established by researchers across the world primarily to investigate the plant biological processes at the molecular level (Table 7.1).

The search for DE genes or genes showing differences in the expression levels between conditions associated to a given experimental treatment and/or response is an important strategy for the dissection of phenotypic variation at the molecular level. A differentially expressed gene (DEG) analysis tool is primarily used to determine the magnitude of differential expression between experimental conditions or treatments, and the significance of the difference is detected by statistical testing. The read count obtained from a RNA-seq experiment according to a feature of interest is fed as input in a DEG analysis (Rapaport et al. 2013). Briefly, the RNA-seq count data follows the following steps under a DEG analysis: (1) library normalization, (2) estimation of model parameters, and (3) detection of DE genes (statistical test). Library normalization is performed for correcting different sources of bias such as gene size, sequencing depth, and sample size. The *Trimmed Mean of*

Table 7.1 The RNA-seq-based *J. curcas* transcriptome data available publically

Data type	Study	Accession	Platform	Layout	Institute	Date	Bio-project
Raw sequence reads	RNA-seq of *J. curcas*: flower buds 0 h after paclobutrazol treatment	SRX3562952	Illumina HiSeq 2000	Paired	Mae Fah Luang University, Thailand	15-Jan-2018	PRJNA429992
		SRX3562929					
		SRX3562935					
Raw reads	*J. curcas* leaf small RNA	SRX3218997	NextSeq 500	Single	Virani Science College, India	7-Sept-2017	PRJNA401963
Raw reads and count data	Transcriptome profile analysis reveals the regulation mechanism of floral sex differentiation in *J. curcas*	SRX3110819	Illumina HiSeq 4000	Paired	Beijing Forestry University, China	21-Aug-2017	PRJNA399175
		SRX3110818					
		SRX3110817					
		SRX3110816					
		SRX3110815					
Raw sequence reads	Transcriptome covering the whole development process of pollen in male and ovule in female *J. curcas*	SRX3082194	Illumina HiSeq 2500	Single	Guizhou University, China	9-Aug-2017	PRJNA397771
		SRX3082193					
		SRX3082192					
		SRX3082191					
		SRX3082190					
		SRX1037655					
Raw sequence reads	An attempt to understand genes upregulated in high-yielding *J. curcas* plants	SRX2248245	Illumina HiSeq 2500	Paired	Universiti Kebangsaan Malaysia, Malaysia	15-Aug-2016	PRJNA338924
		SRX2248244					
Raw sequence reads	Gynoecious and monoecious inflorescence buds, 8–9 days flower inflorescence buds	SRX2279495-84	Illumina HiSeq 2000	Paired	Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, China	26-Oct-2016	PRJNA350607
Raw sequence reads	Cadmium exposed *J. curcas*	SRX1097498	Illumina HiSeq 2500	Single	Sichuan University, China	15-Apr-2015	PRJNA281267
		SRX997124					

Raw sequence reads	Drought stress response in *J. curcas*	SRX673700-89	Illumina HiSeq 2000	Single	South China Botanical Garden, Chinese Academy of Sciences, China	9-Aug-2014	PRJNA257901
Raw sequence reads and count data	Root profiles of *J. curcas* in response to waterlogging	SRX535358-57	Ion Torrent Proton	Single	Kasetsart University, Thailand	8-May-2014	PRJNA246450
Raw sequence reads	EST sequencing: Leaf and callus samples from *Jatropha*	SRX352166	454 GS FLX Titanium	Single	University of Florida, USA	16-Sept-2013	PRJNA219354
Raw sequence reads and count data	Identification of microRNAs and transcript targets in *Jatropha* seeds	SRX346821-19	Illumina HiSeq 2000	Single	Universidade Federal do Rio Grande do Sul, Brazil	6-Sept-2013	PRJNA218216
Raw sequence reads	Normal and cold-stress treated *J. curcas*	SRX220056	Illumina HiSeq 2000	Paired	Yunnan Normal University, China	24-Jan 2013	PRJNA187138
Raw sequence reads	De novo assembly of five major tissues in *J. curcas*	SRX035761	454 GS FLX Titanium	Single	SRM University, India	20-Dec-2011	PRJNA79875
Raw sequence reads	*J. curcas* seed transcript fragment library	SRX011411	454 GS FLX	Single	Centre of Novel Agricultural Products (CNAP), University of York, UK	7-Oct-2009	PRJNA40903

Data sets presented are reported as of April 27, 2018

M-values (TMM) normalization has been widely used to correct differences in library composition between samples. Alternatively, others include Poisson goodness-of-fit statistic. For gene length bias (longer genes producing more reads than the shorter ones), mapping reads are either converted into *reads per kilobase of exon per million mapped reads* (RPKM) or *fragments per kilobase of exon per million fragments mapped* (FPKM) (Trapnell et al. 2010; Mortazavi et al. 2008). A number of programs are available publicly for the analysis of DGE: DESeq/DESeq2, edgeR, limmaVoom and Cuffdiff, baySeq, EBSeq, NOISeq, SAMseq, and ShrinkSeq. These programs differ in terms of the models and methods (parametric and nonparametric) used to compute meaningful DEs. In principle, the parametric method assumes a specific model, which describes the distribution of the RNA-seq count data, and DEs are identified between conditions that exceed the variability predicted by the model. The parametric method is highly preferred over the non-parametric method because RNA-seq experiments are generally available in small number of replicates and models (parametric method) are able to capture technical and biological variabilities present in the datasets. The parametric method has been considered as the state-of-the-art approach, and, therefore, models that are based on Poisson and negative binomial (NB) distributions have been widely employed. The NB-based methods have shown superior performance over the Poisson distributions due to their ability to capture biological variability (Soneson and Delorenzi 2013; Finotello and Di Camillo 2014; Di et al. 2011; Costa-Silva et al. 2017).

Gene co-expression networks are node-edge graphs, whereby node represents a gene and the edge connects pairs of co-expressed genes. The gene associations are measured as correlation based on empirical expression data. The construction and analysis of a co-expression network have found numerous applications in plant science, such as functional groupings of genes, identification of candidate genes underpinning a trait or a condition of interest, and elucidation of the putative gene or protein functions. In a co-expression network, the genes with similar expression are assumed to be co-expressed, and this event may possibly contribute to similar mechanism or function at the cellular level, molecular functions, and biological process (Yang et al. 2011; Silva et al. 2016). In addition, a group of co-expressed genes may also be regulated by a common transcription factor. The "guilt-by-association" principle states that a group of co-regulated genes share a common functionality (Wolfe et al. 2005). The modeling of gene co-expressions based on experimental gene expression measures is a novel approach to gain insights into the functionality of genes. It is now becoming a standard approach in systems biology to construct biologically meaningful networks of co-expression serving as models to study complex biological processes. In plants, co-expression networks are particularly useful to examine a given change in the experimental condition, developmental processes, cell division, and differentiation, among others. Co-expression networks allow the identification of candidate genes underpinning a complex trait, and the deduced gene association can potentially allow the identification of novel genes (Usadel et al. 2009). A gene co-expression network that is built upon the assumption that large complex systems follow a scale-free and small-world network topology considers the following parameters: cluster coefficient, path length, connectivity

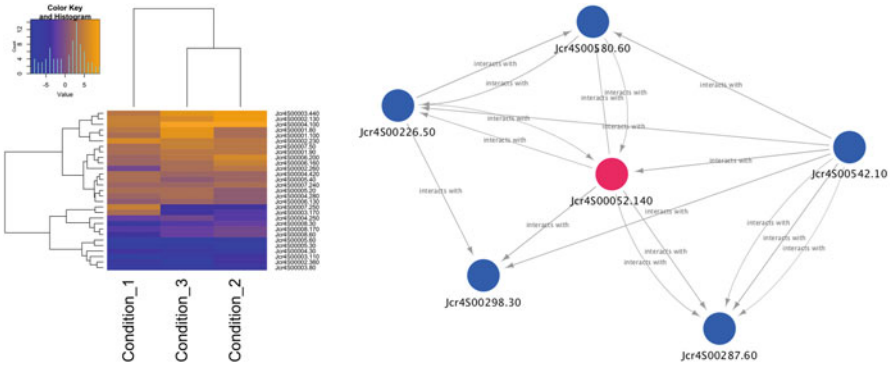

Fig. 7.2 Differentially expressed genes (DEGs) and gene co-expression network analysis of *J. curcas*. The heatmap shows DEGs of *J. curcas* obtained under three different conditions. The blue color indicates downregulated DEGs, while the orange color represents the upregulated DEGs expressed in fold change (right). A co-expression network drawn with Cytoscape 3.0 (left). Nodes represent *J. curcas* genes, and edges represent their co-expression relationships measured by Pearson correlation

degree, vertices, degree distribution, diameter, and density. The construction of a biologically meaningful gene co-expression network enables the clustering of genes in functional modules favorable to the biological process/component under investigation (Jiménez-Gómez 2014; Petereit et al. 2016; Schaefer et al. 2016) (Fig. 7.2).

7.5 Differentially Expressed Gene Analyses for the Molecular Dissection of Floral Organ Development and Differentiation in *J. curcas*

Flower buds enclose floral organs, and therefore they play an important role in the specification and developmental processes of floral organs, i.e., differentiation of the floral meristem, floral meristem identity specification and maintenance, initiation of organ primordia, floral organ identity specification, termination of floral stem cell, and maturation of floral organ (Fornara et al. 2010). In *Arabidopsis*, the floral organ specifications are influenced by floral organ identity factors, a small group of transcriptional regulators, (Wellmer et al. 2013) and most of the floral organ identity genes code for the MADS domain family-containing transcription factors (Krizek and Fletcher 2005). In addition, various genes in genetic and epigenetic pathways along with a suite of signaling molecules are expressed and regulated in a spatio-temporal manner during floral development. The ABCE model of floral organ identity specifications indicates that the four types of floral organs (sepal, petal, stamen, and carpel) are arranged in four floral whorls. The arrangement of A, B, C, D, and E functional homeotic genes specifies the floral organ anatomy. Both A and E mediate floral patterning and are involved in the specification of floral meristems; the

protein complex develops sepals in the first floral whorl. On the other hand, A, B, and E collectively specify petal in the second whorl. B, C, and E specify stamen in the third whorl, whereas C and E specify carpels in the fourth whorl. The ABCE model was evident in the floral differentiation of *J. curcas*, and a number of floral organ identity genes and transcription factors that overlap with the ABCE model genes were identified (Pan et al. 2014). Following these findings, Hui et al. (2017) extended the ABCE model to the ABCDE model. Although these genes in the ABCDE model have been characterized with specific functions in many different plants, the number of functional analyses of floral genes in *J. curcas* remains scarce. However, the dissection of biological processes corresponding to floral organ specification and development as well as the key genes involved in these processes has been rigorously studied (Wu et al. 2015; Chen et al. 2017; Hui et al. 2017).

In a study conducted by Hui et al. (2017), DEG analysis of *J. curcas* transcriptomic profiles was implemented to identify genes involved in floral sex differentiation. RNA samples from flower buds at five different developmental stages were sequenced by Illumina HiSeq 4000 platform. The five floral sex development stages in *J. curcas* were defined as follows: S1, flower buds with no sex identity (primordial stage); S2, stamen morphogenesis or primordia differentiation; S3, stamen differentiation completed (evident from the formation of ten complete stamens); S4, carpel morphogenesis or primordia differentiation; and S5, carpel differentiation and growth completed (evident from the formation of three distinct carpels). Both male and female floral differentiations were dissected at molecular level by means of pair-wise comparisons. For male flower differentiation, DEG analyses were performed for the following treatment pairs: S1 vs S2 and S2 vs S3. The DEGs identified in S1 vs S2 represented the transition event from inflorescence meristem into the floral meristem. At S1, the central zone of stem cells of the shoot apical meristem transmitted signals to peripheral primordia to initiate floral bud formation, which was previously subjected to the reiterative growth of vegetative lateral organs. Although flower bud was formed, S1 represented the absence of sexual organs, whereas the S2 and S3 showed the morphogenesis of stamen. The number of DEGs identified in S2 vs S3 was 1757, about threefold higher than the number of DEGs obtained in S1 vs S2 (620). The DEGs obtained at S1 vs S2 were mainly involved in the flavonoid biosynthesis, plant endogenous hormone signal transduction, photosynthesis, sugar metabolism, and protein synthesis pathways. A large number of DEGs were expressed simultaneously in both S1 vs S2 and S2 vs S3, and the findings suggest a continuous communication throughout the stamen development. For the female floral differentiation, which encompassed S1 vs S4 and S4 vs S5, a total of 395 and 1622 DEGs were obtained, respectively. The trend in female flower was similar to that of male flower, whereby the DEG count for developmental process (S4 vs S5) was fourfold higher than at the initiation process. These DEGs were found involved in the phytohormone signaling (brassinosteroid, IAA, cytokinin), RNA transport, protein synthesis, and plant growth pathways. In addition, about 21 DEGs related to MADS-box transcription factors were identified. Phytohormone signaling molecules have been characterized for their role in floral development and differentiation. Gibberellins (GAs) are potent inducers of floral organ differentiation that act as signals for DELLA protein degradation

(Achard and Genschik 2009; Daviere and Achard 2013) in cross talk with brassinolides. On the other hand, auxins (AA) are fundamental for male and female flower maturation, whereas the jasmonic acid (JA) plays a key regulatory role in floral organ differentiation. Cytokinins have been reported to reverse the role of GAs. In *J. curcas*, plant growth regulators such as the 6-benzyl aminopurine (6-BA), thidiazuron, and paclobutrazol have shown to promote the number of female flowers and thus have impacted positively on the overall plant seed yield performance (Ghosh et al. 2010; Pan and Xu 2011; Xu et al. 2013; Chen et al. 2014; Fröschle et al. 2017; Pan et al. 2016).

In yet another interesting study conducted on gynoecious and monoecious *J. curcas* plants in China, floral development genes and phytohormone signaling pathways were found to underpin floral sex determination (Chen et al. 2017). In this study, transcriptomes were obtained from monoecious and gynoecious genotypes at three different stages of the inflorescence development. The stages were defined as follows: Stage 1, formation of visible inflorescence buds; Stage 2, female flower development (monoecious) and male flower with an arrested development (gynoecious); and Stage 3, male and female flowers at bloom. The gibberellin-treated gynoecious genotype, introduced for the first time in *J. curcas* research, was observed to form male flowers similar to the specification process described for the monoecious genotype, and subsequently abortion takes place, paving way to female organ specification. The findings were in agreement with *J. curcas* sex differentiation reported in monoecious plants whereby female sex organ develops simultaneously on a male sex organ during the early stage of inflorescence development. The flower remains hermaphrodite for a certain period before aborting the male organs and to become a female flower (Wu et al. 2011; Xu et al. 2016). In the gynoecious plants, abortion of male organs, an important hallmark for female flowering, took place between stages 1 and 2. Between the two stages, a total of 171 co-expressed genes were found representing various biological processes that may had corresponded to male flower abortion: reproductive (GO, 0022414), reproductive developmental (GO, 0003006), and developmental regulation (GO, 0050793). A DGE analysis between stages 1 and 2 in gynoecious plants indicated the downregulation of ten genes with putative involvement in floral development, and DEs with strong role in the regulation of sex determination were identified among which one can cite *KNAT6*, *MYC2*, *SRS5*, *SVP*, *TFL1*, and *TS2*. These genes were involved mainly in phytohormone (GA and JA) signaling pathways, and some were described as involved in the floral development. In a pair-wise DEG analysis between the gynoecious and monoecious plants, the *TAA1*, *YUC4*, and *YUC8* genes (involved in the auxin biosynthesis pathway) were found to be downregulated in the gynoecious relative to the monoecious inflorescence. In parallel, the expression of auxin responsive genes [*BTB* and *TAZ DOMAIN PROTEIN 2* (BT2), *INDOLE-3-ACETIC ACID INDUCIBLE 14* (*IAA14*), *IAA29*, and *SMALL AUXIN UP RNA 20*], and auxin transport genes (*PLEIOTROPIC DRUG RESISTANCE 9* and *PIN6*) were also downregulated. Likewise, in another similar study conducted by Xu et al. (2016), flower buds collected at different developmental phases were used for the preparation of RNA-seq libraries. Six different developmental phases of flower buds

diagnosed using scanning electron characterization were identified as follows: S1, floral buds differentiated in absence of any sex determination; S2, differentiation of stamen primordia took place and up to ten stamen primordia were formed; S3, ten mature stamen primordia formed a staminate flower; S4, carpel primordia were formed and differentiation took place into three distinct carpels; S5, carpels developed; and S6, carpels fused to form pistillate flowers. The majority of DEGs identified to be involved in floral organogenesis (S3 maturation) were related to auxin and ethylene phytohormone signaling. In addition, stress hormone and metal absorption-related genes were found to be upregulated as well. The DE characterization revealed the involvement of unique biological processes in floral differentiation and development, such as the amino acid metabolism, glycan degradation, protein biosynthesis, brassinosteroid biosynthesis, terpenoid biosynthesis, phosphatidylinositol signaling system, and ubiquitin-mediated proteolysis.

7.6 Gene Co-expression Network Analysis

A gene co-expression network was constructed for *J. curcas* by Govender et al. (2018) to investigate the gene-to-gene interactions in reproductive-related shoot system. For this, transcriptomes of the aerial shoots, shoots bearing the inflorescence and inflorescence were fed into petal, an R package for gene co-expression network construction. The resultant gene co-expression network showed 718,599 associations containing 12,290 nodes (the average number of neighbors was 117). From the constructed network model, four subnetworks called vicinity networks (VN) were extracted for the molecular understanding of the gene associations underpinning overtaking traits in *J. curcas*. In the presence of the following annotated genes, the VN1 showed putative involvement in epigenetic events, signal transductions, and cell wall metabolisms: *DNA polymerase alpha catalytic subunit*, *DNA polymerase delta catalytic subunit*, *DNA mismatch repair MSH6* and *ABC transporter G family member 3, alpha-L-fucosidase 2-like* and the *alpha-galactosidase-like isoform X1, gamma-tubulin complex component 4,* the *125 kDa kinesin related*, and *myosin 17-like*. Likewise, VN2, VN3, and VN4 putatively corresponded to the biosynthesis of chlorophyll molecules and laticifers (latex-producing cells), heat stress tolerance, and flowering and signal transduction mechanism, respectively. Genes associated with each VN were described as follows: VN2, *NAD kinase chloroplastic, probable serine threonine-kinase NAK*, and *lactation elevated 1 isoform X1*; VN3, *transcription factor Pur-alpha 1*, *transcription initiation factor IIB*, and *eukaryotic translation initiation factor 2 subunit gamma-like* and *FAR-RELATED SEQUENCE 9, polyadenylate-binding 2-like, vacuolar-sorting-associated 4B-like isoform X1, Pt11-LIKE TYROSINE KINASE Atg15890*, and *adenylate isopentenyltransferase (IPT) chloroplastic-like*; and VN4, *ULTRAPETALA (ULT) 1-like, serine*

threonine-kinase D6PK-like, *serine threonine-kinase D6PKL2, calcineurin B3, Ras-related Rab11C*, *Ras-related Rab7*, and *serine hydroxymethyltransferase 4*. In each VN, although the functional analyses of most of the nodes have been described in other plant species, none have been characterized in *J. curcas*.

Understanding the gene-to-gene association with regard to a particular trait of interest would present a rather more biologically meaningful inference when both the molecular and phenotype data are attended simultaneously. As such, the weighted gene co-expression network analysis (WGCNA) offers an integration of phenotypic and transcriptome data to provide insights into the hub genes underlying a phenotype of interest (Langfelder and Horvath 2008). In soybean, the WGCNA was employed to investigate hub genes corresponding to seed storage composition (Qi et al. 2018) and seed set and size (Du et al. 2017), whereas in strawberry, the same analysis was employed to investigate the floral organ specifications (Hollender et al. 2014). The WGCNA package was made publicly available in early 2016, and to date, no application on *Jatropha* species has been reported despite the rapid growth of NGS-based big data. In breeding programs, a broad genetic diversity serves as the base for any manipulation strategies although *J. curcas* has been widely reported to have a narrowed genetic variation (Basha and Sujatha 2007; Kaushik et al. 2007). In breeding attempts targeted for yield enhancement, in-depth attention should be given on yield-related biological processes and/or metabolisms. As such the phytohormone signaling, sugar signaling and photosynthesis metabolism are among the potent aspects which are presently poorly explored for manipulation strategies in *J. curcas*.

7.7 Conclusions

With an increasing pressure on edible vegetable oil supplies, the non-edible *J. curcas* oil offers an opportunity as an alternative source for renewable energy targeted for fuel production only. The DEG analyses observe differences in the magnitude of expression levels that correspond to a given condition or response, while gene co-expression networks revealed the association of gene groups contributing to a given phenotype or trait. Therefore, integration of both DEG analysis and gene co-expression network modeling would complement each other for a comprehensive understanding of the molecular basis underlying a phenotype. In *J. curcas*, many studies have focused primarily on DE analyses due to sample size limitation. DGE and gene networks identified in *J. curcas* inflorescence for dissecting complex biological processes such as floral structure organogenesis, flowering, fruiting, and overall yield performance are likely to boost smart selective breeding strategies in the future. However, a rigorous focus on functional analysis studies is necessary to validate the inferred candidate genes.

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Chapter 8
Application of Molecular Markers in Genetic Improvement of *Jatropha*

Anoop Anand Malik and Shashi Bhushan Tripathi

Abstract *Jatropha curcas* L. has gained prominence during the past 15 years as a potential source of biodiesel. Massive plantations of *Jatropha* were raised worldwide between 2003 and 2008. Unfortunately, these plantations failed to deliver the promised yields primarily due to unrealistic assumptions and also due to the use of uncharacterised planting material. However, several initiatives have been taken towards the characterisation and genetic improvement for oil yields and other important traits. An excellent foundation in the form of genetic and genomic tools such as DNA-based molecular markers, linkage maps, genetic transformation and mapping populations has been developed in *Jatropha* so far. These resources are being used to develop *Jatropha* varieties with desirable traits such as high seed yield and oil content. Development of non-toxic varieties is also being attempted to make the oil cake usable as cattle feed.

The current chapter describes the past research and future trends in applications of various molecular marker technologies for the genetic improvement of *Jatropha*.

Keywords Genome sequencing · Interspecific hybridization · Linkage mapping · Next-generation genotyping · QTL mapping

8.1 Introduction

Biofuels have received significant attention of researchers and policy-makers in the past two decades as an alternative source of energy. In addition, they provide environmental benefits by being carbon neutral and emitting less sulphur dioxide, carbon monoxide and particulate matter in comparison with petroleum fuels (Subramanian et al. 2005). A number of plant species have been identified as sources of biofuels (both biodiesel and bioethanol). *Jatropha curcas* L. has been identified as

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a major source of biodiesel mainly due to its highly adaptive features, which are supposed to be advantageous under resource-limiting environments.

The biochemical and pharmacological properties of *Jatropha* have been a subject of intense research since almost 50 years. For a long time, researchers have been interested to characterise its various secondary metabolites such as phorbol esters (PEs) for their potential health effects and as drugs and medicines. It was only with the beginning of the current century that the biodiesel aspect of *Jatropha* started to receive attention especially in South Asia and Africa. In India, a National Mission on Biodiesel was launched by the Government in 2003 with an ambitious goal of planting *Jatropha* on 11.2 million hectares by 2012. Similar programmes were initiated in other countries of the Asian and African continents. Most of these programmes were followed by generous subsidies and financial support by governments to take the *Jatropha*-based biofuel missions forward. Consequently, large plantations of *Jatropha* were raised under various government- and industry-funded programmes between 2003 and 2008.

Unfortunately, these plantations could not deliver the promised yields and were soon to be uprooted by the farmers. This happened primarily due to the use of untested and unimproved planting material coupled with unrealistic assumptions on plant productivity. By 2012, most of the *Jatropha* programmes were closed, which resulted in the loss of confidence among different stakeholders. The farmers were the most affected segment of the biofuel production chain. The rise and fall of the *Jatropha* initiative was a global event. While the majority of the players withdrew from the initiative, a comparatively smaller number of groups persistently worked in search of sustainable solutions for *Jatropha* as a crop.

8.2 Understanding the Genetic Diversity in *Jatropha*

Genetic diversity provides the base material for genetic improvement programmes. The success of any genetic improvement programme depends largely on the levels of existing variability for the desired traits and the heritability of these traits. Accurate assessment of the levels and patterns of genetic diversity can be invaluable in crop breeding for diverse applications including analysis of genetic variability in cultivars (Smith 1984; Cox et al. 1986), identification of diverse parental combinations to create segregating progenies with maximal variability for further selection (Barrett and Kidwell 1998) and introgressing desirable genes from diverse germplasm into the available genetic base (Thompson et al. 1998). Till 2010, the majority of research projects in *Jatropha* only targeted the collection and characterisation of germplasm accessions from different geographical regions, mostly within their respective countries. This was a logical activity considering the lack of information on the extent of genetic diversity in these germplasm collections. A variety of PCR-based molecular markers such as randomly amplified polymorphic DNAs (RAPD), inter-simple sequence repeats (ISSR), amplified fragment-length polymorphism (AFLP) and microsatellite markers have been used in these studies. Basha and Sujatha (2007)

used RAPD and ISSR markers to analyse the genetic diversity in a set of 43 accessions of *J. curcas* from different geographical locations of India. Ganesh Ram et al. (2008) used RAPD markers to study the genetic diversity in eight species of *Jatropha* including *J. curcas*. Sun et al. (2008) used AFLP microsatellite markers to study the genetic relationships of 58 *J. curcas* accessions from China. The majority of these studies were based on two common pieces of inference. First, the overall genetic diversity within *J. curcas* accessions was extremely low. At least, this was true for all studies involving germplasm from countries where *Jatropha* was an exotic species, most probably introduced by Portuguese seafarers. Second, most of the observed genetic variation was found to be associated with the inclusion of one or more exotic accessions from Mexico and Central America, which form the centre of origin of *Jatropha*. This also indicated that a much greater genetic diversity of *Jatropha* was likely to be encountered in the Mexican and Central American germplasm. However, comprehensive studies on genetic diversity in germplasm from these regions were published relatively late compared to the initial excitement for *Jatropha* (Osorio et al. 2014).

Interestingly, Tatikonda et al. (2009) observed a high level of genetic diversity while analysing a collection of 48 accessions from 6 states of India. They employed 7 AFLP primer combinations, which showed an average polymorphism of 88.2% and Jaccard's similarity coefficient ranging from 0.47 to 0.93. This is the only study so far that has reported such a high level of genetic diversity within Indian accessions.

Many of the studies used *J. curcas* accessions from two or more countries (He et al. 2011; Zhang et al. 2011; Biabani et al. 2013). Basha et al. (2009) used RAPD, ISSR and microsatellite markers on a set of 72 *J. curcas* accessions from different countries. In this study, the RAPD primers revealed 42% polymorphism in Indian accessions, whereas 61.8% polymorphism was observed in the world-wide collection indicating a higher genetic diversity in accessions from diverse geographical regions. Shen et al. (2012) characterised the genetic variation among 63 populations of *J. curcas* from 10 countries in Asia, Africa and Mexico. These studies have consistently showed that the genetic diversity among accessions of *Jatropha* from India, South Asia and Africa is very low as compared to those from Mexico and Central America and thus strengthened the latter regions as the centre of origin for *Jatropha* (Trebbi et al. 2015; Li et al. 2017).

Analysis of molecular variance (AMOVA) has been carried out in several of the studies in order to understand the partitioning of the genetic diversity within and among the populations (Cai et al. 2010; Na-ek et al. 2011; Biabani et al. 2013). Cai et al. (2010) studied the genetic structure in 224 accessions from China and Myanmar using ISSR markers and observed that 12.74% and 87.26% of its genetic variance were among and within groups, respectively. Biabani et al. (2013) confirmed these results by observing that 87% of the genetic diversity was present within the populations, whereas only 13% of the total variation existed among the populations. Osorio et al. (2014) studied populations from Asia, Africa, South America and Central America and observed that the pool of Central American accessions showed very large genetic variation and the highest phenotypic variation

compared to Asian, African and South American accessions. Similar to the previous studies, AMOVA results showed that 81.7% of the variation was within the populations and only 18.3% among the populations. A higher variation within populations as compared to among populations is characteristic of open-pollinated random-mating species.

An important potential outcome of germplasm characterisation is the identification of a core germplasm through removal of redundant/duplicate accessions (Brown 1989; Jansen and van Hintum 2007). The core collection is a subsample of the whole collection which typically comprises approximately 10% of all available accessions and is intended to provide a set of genetically diverse accessions for future utilisation in conservation and breeding (Schoen and Brown 1993). In *Jatropha*, however, only few studies have reported the identification of a core germplasm. Cai et al. (2010) identified 46 core accessions out of 224 accessions, which contained 90% of the total ISSR diversity. In a recent study, a germplasm collection of 192 accessions was analysed with AFLP markers to identify 16 (8.3% of the entire collection) core accessions, which contained the entire allelic diversity of the whole collection (Sinha et al. 2015b).

8.3 Understanding the Mating System in *Jatropha*

The genetic structure of plant populations is determined by the prevalent mating system together with the mechanisms of pollen and seed dispersal (Charlesworth and Wright 2001). *Jatropha* is a monoecious plant which bears unisexual flowers in dichasial cymes where both male and female flowers are present in the same inflorescence (Raju and Ezradanam 2002). Pollination is mainly by insects as the pollen is heavy and sticky and is not compatible with wind pollination. Therefore, the floral biology of *Jatropha* seems to favour outcrossing.

Both dominant and codominant molecular markers have been widely used in the studies related to outcrossing rates (Gaiotto et al. 1997; Chaix et al. 2003; Adugna et al. 2013). Bressan et al. (2013) used five SSR markers and reported a mixed-mating reproductive system in *Jatropha* with up to 68.3% outcrossing and 13% apomixis. In another study using individuals derived from interspecific hybridization as well as SSR and AFLP markers, an outcrossing rate of 97.7% was obtained (Sinha et al. 2015a). Further, no apomixis could be detected in this study. It may be noted that the ability to detect outcrossing events in the later study was much higher due to the higher variability of the plant material owing to its interspecific nature and the higher number of marker loci used for genotyping. As there is no report of self-incompatibility in *Jatropha* so far, it is safe to assume that self-fertilisation within a plant occurs at varying levels.

8.4 Interspecific Hybridization and Prebreeding in *Jatropha*

Traditionally, *Jatropha* has been used as a live fence around the fields and houses due to its high level of drought tolerance and also because it is not eaten by the cattle. For its newer role as a biodiesel crop, however, it is desirable to have genotypes capable of producing seeds at a rate that is attractive to growers. Thus, the need for genetic improvement in *Jatropha* was realised, and selective breeding was initiated about a decade ago. Two major approaches of genetic improvement were followed by different research groups. The first line of intervention was performed by those who had access to a relatively diverse *Jatropha* germplasm from its centre of origin. Groups following this approach also included private companies such as SG Biofuels Inc., USA, and JatroSolutions GmbH, Germany. On the other hand, the researchers from Southeast Asia and India used interspecific hybridization as an alternative approach. In these countries, it was realised that the locally available genetic diversity within collections of *Jatropha* was very low and performing any intraspecific hybridization or selective breeding with these accessions would not be productive.

Interspecific hybridization is a useful method to widen the genetic diversity and generate prebreeding material for crop improvement (Stalker 1980). Further, mapping populations generated through interspecific hybridization are more efficient in development of linkage maps due to high polymorphism between the parental genotypes (Blum et al. 2002; Yi et al. 2006).

As early as in 1970, Rupert et al. (1970) obtained viable hybrid seeds from a series of crossing experiments between *J. curcas* and *J. integerrima* using *J. curcas* as the female parent. The F_1 plants so obtained were intermediate between the parental species in a number of characteristics. Interestingly, no hybrids could be obtained from the reciprocal cross (*J. integerrima* × *J. curcas*). Dehgan (1984) studied interspecific compatibilities among 11 *Jatropha* species and described the phylogenetic significance of interspecific hybridization in *Jatropha* in terms of number of flowers pollinated, number of seeds obtained, germination rate, colour and configuration of sepals, petals and leaves. It is interesting to note that most of the successful interspecific crosses reported so far in *J. curcas* involved *J. integerrima* as the male parent. The high success of this combination is likely due to the demonstrated greater genetic similarities between the two species based on morphological (Dehgan and Webster 1979; Dehgan 1984), nuclear ITS (Pamidimarri et al. 2009a), RAPD (Ganesh Ram et al. 2008; Pamidimarri et al. 2009b) and AFLP data (Sinha et al. 2014) as compared to other species. A natural hybrid between *J. curcas* × *J. gossypifolia*, named as *J. tanjorensis*, was reported from some districts of Tamil Nadu (Prabakaran and Sujatha 1999). However, no fruit set was noticed in this species which was presumably due to abnormal meiotic division in the hybrid resulting from the parental genomic differences (Prabakaran and Sujatha 1999). Dehgan (1984) had also attributed the failure of interspecific hybrid between these two species due to their greater phylogenetic distance.

A large number of studies have investigated the progenies of interspecific crosses between *J. curcas* and *J. integerrima* due to the high compatibility and success of this cross. Although the F_1 hybrid of this cross seldom bears fruits with viable seeds itself, its pollen is viable enough to be used for pollinating *J. curcas* female flowers, which enables to create backcross progenies with varied morphological traits including flower colours varying from dark pink through green or white (Parthiban et al. 2009; Sujatha and Prabakaran 2003). The F_1 hybrids between *J. curcas* and *J. integerrima* also exhibit vigorous growth with many morphological traits, which are intermediate between the two species. The hybridity of these interspecific hybrids has been confirmed using RAPD (Dhillon et al. 2009), expressed sequence tag (EST) and simple sequence repeat markers (Laosatit et al. 2014).

8.5 Genome Sequencing and Linkage Mapping

Linkage maps are valuable tools for a wide range of applications, including the identification and positional cloning of important genes (Martin et al. 1993) and marker-assisted selection (Collard and Mackill 2008). The first linkage map in *Jatropha* was constructed using a BC_1 population derived from an interspecific cross between *J. curcas* and *J. integerrima* (Wang et al. 2011). The map contained 216 EST-SSR and 290 SNP markers spanning a total of 1440 cM with an average marker-to-marker spacing of 2.8 cM. The 11 linkage groups (LGs) ranged from 84.9 to 187.5 cM. The number of markers on each linkage group ranged from 22 for LG 5 to 36 for LG 6. LG 11 possessed the highest density of markers with an average marker spacing less than 2 cM, whereas LGs 1, 4 and 8 had a relatively lower density of markers with marker spacing greater than 4 cM. A comparative linkage map between *J. curcas* and *A. thaliana* was also developed, which contained 192 loci derived from ESTs.

In another study, King et al. (2013) used intraspecific F_2 populations for linkage mapping and identification of QTLs regulating PE content in seeds. They used SSR markers and four independent F_2 populations to develop an integrated map. The integrated map contained 502 SSR loci spanning a total distance of 717 cM. In one of the mapping populations (derived from the cross, G33 × G43), a locus controlling the PE biosynthesis was identified at 41 cM on the linkage group 8. Further analysis of the data confirmed that all F_2 individuals that were homozygous for the G43 (non-toxic) allele at this locus yielded seeds without PE. Interestingly, the mean values for the PE content of the F_2 plants being heterozygous at this locus were also significantly lower than those of the plants being homozygous for the G33 (toxic) allele.

Two genome sequencing projects on *Jatropha* have been reported so far. The first *Jatropha* genome was published by Sato et al. (2010), which was 285.9 Mbp in size and contained 21,225 unigenes. This genome sequence was upgraded further with additional data in 2012 (Hirakawa et al. 2012). The size of this reference genome is about 298 Mb, which is available in the form of 39,277 scaffolds at the Kazusa DNA

Research Institute Database. Another reference genome for *Jatropha* was published by Wu et al. (2015). This reference genome is about 308 Mb and contains 6024 scaffolds containing 27,172 putative protein-coding genes. The size of both the publicly available genome sequences is consistent with the earlier reported estimated size of 410 Mbp using flow cytometry (Carvalho et al. 2008). In addition, another genome sequence has been produced jointly by Life Technologies and SG Biofuels, but it has not been made publicly available (accessed on January 30, 2015, at https://www.genomeweb.com/sequencing/life-technologies-and-sg-biofuels-sequence-jatropha-genome-solid-4). None of the available genome sequences of *Jatropha* have been annotated so far.

Efforts have been made to map these genomic sequence scaffolds on the *Jatropha* linkage map using different mapping populations. For example, 407 out of 39,277 scaffolds of the Jat_r4.5 genome, which correspond to 17 Mbp (6%) of the genome sequence, were successfully anchored on the linkage map developed by King et al. (2013). By contrast, Wu et al. (2015) succeeded to anchor 480 scaffolds, representing about 81.7% of the assembled genome, by mapping of 802 unique loci on the linkage map using an interspecific BC_1 population between *J. curcas* and *J. integerrima*.

8.6 QTL Mapping in *Jatropha*

Development of linkage maps in *Jatropha* has helped greatly in identification of QTLs for important agronomic traits. In an early study, Liu et al. (2011) investigated the expression of oleosin genes in mature seeds of *Jatropha*. SNP markers were developed for three oleosin genes, namely, *Ole*I, *Ole*II and *Ole*III, which mapped on linkage groups 5, 3 and 8, respectively. Composite interval mapping allowed the identification of 18 QTLs dispersed among all the linkage groups, except LG 3, among which 11 controlling the oil traits in *Jatropha* and 4 QTLs controlling total oil content were detected. The most effective QTL was spotted on LG 4 and explained 11.1% of the variation.

Sun and coworkers (Sun et al. 2012) identified a total of 28 QTLs for 11 growth- and seed-related traits using a BC_1 population of 296 individuals. A linkage map was constructed using 105 SSR (simple sequence repeat) markers dispersed on 11 linkage groups. Two QTLs, *qTSW-5* and *qTSW-7*, controlling total seed weight were mapped on LGs 5 and 7, respectively. Interestingly, several other QTLs controlling seed yield such as plant height, stem diameter, branch number, female flower number and fruit number were also located near the above two QTLs forming QTL clusters (five QTLs on LG 5 and four QTLs on LG 7, respectively) on these two LGs. The best alleles for different traits came from both parents, which indicate that pyramiding of positive alleles from the two parents would be required to achieve genotypes with high productivity given their environmental characteristics of growth.

Using an intraspecific F_2 population and SSR markers, Amkul et al. (2017) identified two major QTLs for seed PE content, *qPE3.1* and *qPE8.1*, on LGs 3 and 8, respectively. Together, these two QTLs explained 29.6% of the variance for PE content. It may be noted that the QTL on LG 8 was also identified earlier by King et al. (2013).

In a recent study, Xia et al. (2018) identified 13 QTLs for fruit yield in *Jatropha* using an outcrossing F_1 population and high-throughput amplified-fragment single nucleotide polymorphism and methylation (AFSM) markers (Xia et al. 2014). Ten of these QTLs, which mapped to LGs 1, 2, 3, 4, 6, 7 and 8, controlled the number of fruits. Three QTLs controlling total fruit weight were identified on LGs 1, 2 and 3.

8.7 Breeding Non-toxic *Jatropha*

For most oilseeds, oil cake obtained after oil extraction is an important by-product due to its high protein content. The oil cake can either be converted to organic manure through composting or can be used as animal feed. However, oil cake from *Jatropha* contains two major toxins, namely, PEs and curcin (a type-I ribosome-inactivating protein similar to ricin A). PEs are mainly concentrated in the seed tegmen, whereas curcin is found both in the endosperm and in the tegmen (He et al. 2011). While curcin is destroyed by the conventional heat treatment during conversion of oil cake to animal feed, PEs are not (Makkar et al. 2008). Removal of PEs requires additional processing, which increases the cost and therefore reduces the economic feasibility of oil cake use as a by-product. Consequently, an important effort is being made towards the selective breeding of elite *Jatropha* varieties lacking these toxins in the seed.

Several "edible" or "non-toxic" accessions of *J. curcas* in Mexico are known to lack PEs (Makkar et al. 1998). Using crosses between toxic and non-toxic accessions, it has been shown that the presence of PEs is controlled by a single dominant gene located around 41 cM on the linkage group 8 (King et al. 2013). Furthermore, it is suggested that PE biosynthesis in *Jatropha* may be under maternal control (King et al. 2013; Li et al. 2016). The mapping of this locus responsible for PE biosynthesis makes it possible to use marker-assisted introgression of this trait to develop novel non-toxic varieties with other desirable traits. Low PE-containing plants have also been reported from a population of interspecific backcross individuals (Popluechai et al. 2009).

An alternative RNAi-based transgenic approach has been used by Li et al. (2016) to develop *Jatropha* plants with reduced PE content. They isolated two casbene synthase (casbene is the putative precursor for PE biosynthesis) genes, JcCASA163 and JcCASD168, from *Jatropha*. These genes were used to generate RNAi gene-silencing cassettes driven by the seed-specific promotor of *Jatropha* late embryogenesis-abundant (*JcLEA1*) gene. Up to 85% reduction in seed PE content was observed in these transgenic plants. It may be noted that there are at least eight casbene synthase gene homologues in *Jatropha* (Sato et al. 2010).

A similar RNAi-based approach was used by Gu et al. (2015) to generate *Jatropha* plants with curcin-deficient seeds. In this case, a β-oestradiol-regulated Cre/loxP-mediated DNA recombination system was used to generate marker-free transgenic plants (Qiu et al. 2010). There are three curcin genes and two curcin-like genes in *Jatropha* genome (Sato et al. 2010). Of these, only the type-1 curcin gene, C1, is expressed in seeds, whereas other homologues are expressed in young leaves. The RNAi cassette to silence the C1 gene was driven by its own promoter to provide tissue specificity. Both C1 mRNA and protein levels in the endosperm of transgenic seeds were greatly reduced or remained undetectable, while there was no change in the mRNA or protein levels of another type-2 curcin gene, C2A, expressed in young leaves.

8.8 Next-Generation Genotyping and Breeding

With the development of high-throughput genotyping techniques, such as restriction site-associated genomic DNA (RAD) sequencing (Baird et al. 2008), genotyping by sequencing (GBS) (Elshire et al. 2011), the possibility of conducting genome-wide association studies (GWAS) and genomic selection is getting within the reach of plant researchers. In *Jatropha*, Anggraeni et al. (2018) used GBS markers to identify SNPs among accessions from Indonesia, Thailand, the Philippines and China. It is expected that application of these genotyping technologies will increase significantly.

Availability of genome-wide markers has now shifted the breeding strategies from marker-assisted breeding (MAS) to genomic selection. While a smaller number of loci can be easily transferred using the MAS approach, majority of agronomic traits including seed yield and oil content are expected to be controlled by a large number of genetic loci each having small effects on the phenotype. Further, the heritability of these traits is comparatively low due to greater effects of environmental factors. Peixoto et al. (2016) evaluated 179 half-sib families of *Jatropha* and observed low heritability to seed yield (0.35) and oil content (0.24). In such cases, genomic selection has become an important tool to help breeders as a prediction model by associating marker information with phenotypic information (Meuwissen et al. 2001). A pilot study on genomic selection in *Jatropha* using 1248 SNPs and Diversity Arrays Technology (DArT) markers has been recently described (Azevedo Peixoto et al. 2017).

8.9 Conclusions and Future Perspective

In the past 15 years, there has been tremendous advancement in our understanding of *Jatropha*. Due to the shift of focus towards its genetic improvement, an excellent foundation in the form of genetic and genomics resources has been created in recent

years. These resources include diverse parental genotypes for important traits as well as molecular tools such as microsatellite and SNPs, linkage maps and QTL information. TILLING (targeting-induced local lesions in genomes) populations of *Jatropha* are under development for identification of economically useful mutants (Maghuly et al. 2013; Maghuly and Laimer 2013). A number of promising genotypes were evaluated under field conditions at various research institutions (Yang et al. 2010). It is expected that the increased use of high-throughput genotyping technologies will lead to the fast development of economically attractive *Jatropha* varieties using various breeding strategies such as clone breeding, line breeding and hybrid breeding (Montes and Melchinger 2016).

There is an important need for reworking the economics of the entire *Jatropha* value chain based on realistic data. Field demonstrations in association with farmers are required to regain their confidence. Furthermore, market reforms and procurement policies, such as contract farming and public-private partnership, need to be in place for economic sustainability of this value chain.

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Chapter 9
Genomic Resources and Marker-Assisted Selection in *Jatropha curcas*

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Abstract *Jatropha curcas* L. is currently attracting much attention as an oilseed crop, which can be used as biofuel and feasible feedstock for animals. In order to improve conventional breeding strategies, DNA variants are currently being exploited. The last few years have witnessed the development of molecular markers (SSRs, SNPs, AFLPs, RTNs, etc.) that can guide selection and result in enhancement of *Jatropha* productivity. The marker discovery process has resulted in the identification of more informative markers with the availability of genome and transcriptome sequences for this species. NGS has emerged as a powerful tool to detect numerous DNA sequence polymorphism-based markers within a short timeframe growing as a powerful tool for next-generation plant breeding. A holistic approach is therefore required to establish *J. curcas* as an economically viable crop integrating all the genomic resources available with selective breeding techniques of plants. This would be an ideal strategy for the domestication of *Jatropha*.

Keywords Comparative genomics · Genomic resources · Linkage maps · Molecular markers · Transcriptomics

9.1 Introduction

Jatropha curcas L. has been established as a multipurpose plant species belonging to the Euphorbiaceae family. The advantages of domesticating this plant are very well established and more specifically concern (i) its ability to survive under diverse agroclimatic conditions, (ii) successful cultivation in degraded soils, (iii) production of high-quality oil and protein in seeds, (iv) broad adaptation, (v) short generation

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interval and (vi) large genetic variation. A recent review showed the variety of uses of this one crop along with the environmental benefits of its byproducts (Montes and Melchinger 2016). The primary interest concerns, however, its potential as a biofuel crop. *J. curcas* oil processing is relatively simple for conversion to biodiesel by chemical or biological transesterification processes (Modi et al. 2007; Berchmans and Hirata 2008).

The impressive advances in high-throughput next-generation sequencing (NGS) technologies have made its utilisation in plant breeding indispensable. *Jatropha*'s genome sequencing in 2010 led to gene annotation (Sato et al. 2011). Subsequently, a report by Natarajan et al. (2010) described the transcripts encoding diverse biological functions including oil and toxin biosynthesis by sequencing ESTs from cDNA libraries. Further, gene expression profiling and transcriptomics resulted in the establishment of full-length transcripts highly enriched in oil biosynthesis pathways (Natarajan and Parani 2011). These transcripts have also assisted in the selection of traits like higher oil production and lower toxicity. In yet another study, a functional genomics approach was taken to better understand toxin production (Sabandar et al. 2013) and fatty acid metabolism (Jiang et al. 2012) in *J. curcas*. This has helped in identification of suitable loci for improvement of economically important traits.

The fundamental breeding objectives remain as high oil yield and reduced toxicity, along with pathogen and pest resistance (Maghuly and Laimer 2013). It is worthwhile to note here that toxic *J. curcas* accessions are used as fences to protect cropping areas from animals in Africa (Jingura and Kamusoko 2018) (Fig. 9.1).

The efforts to reduce the natural toxicity of *J. curcas* include identification of accessions mitigated in phorbol esters and other toxic compounds as observed in the seed germplasm from Mexico (Goel et al. 2007). Many studies have documented an improved variety developed by crossing *J. curcas* and *J. integerrima*. In the process of identification of superior cultivars, molecular markers have been used to ascertain genetic diversity among various *Jatropha* accessions (Sudhakar Johnson et al. 2011). Despite improvements in breeding in the last decade, the full potential of *Jatropha* has not yet been exploited due to its still largely uncharacterised genome and lack of genetically improved varieties. It is also to be noted that the lack of genetic variability in *J. curcas* accessions outside its centre of origin has impaired its selective breeding.

The future in establishing *J. curcas* as a competitive biodiesel crop will require an integrated approach of diverse NGS applications (transcriptomics, proteomics and metabolomics) to help with a deeper understanding of its oil and toxin biosynthetic pathways. Further an NGS-assisted plant breeding approach can hasten the process of marker discovery and validation. This chapter summarises the genomic resources generated through the recent years for *Jatropha* along with markers which have aided in selection of desirable traits.

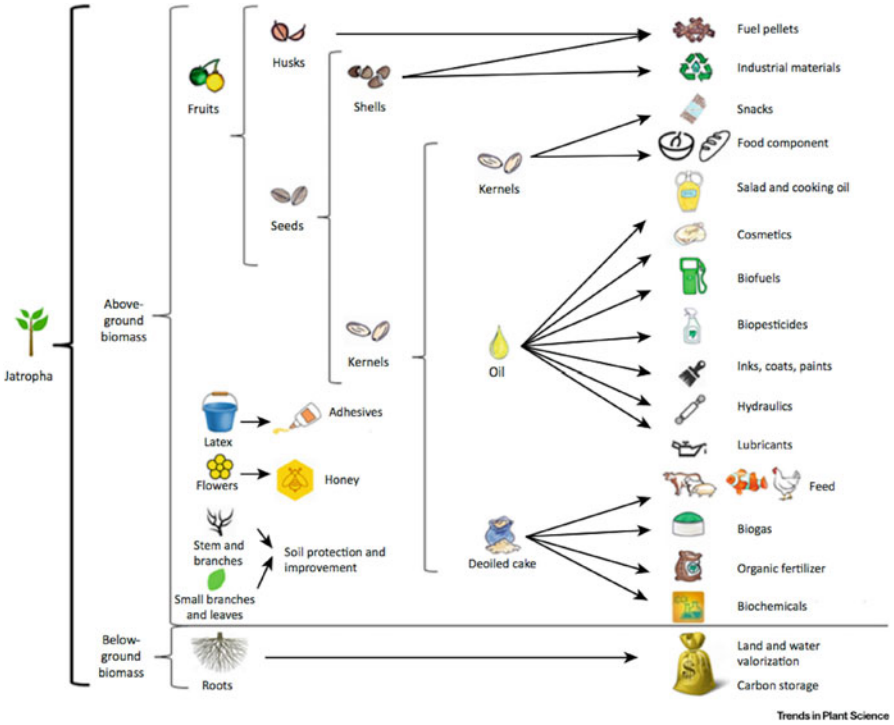

Fig. 9.1 Diverse uses of *J. curcas* with its environmental benefits: need for better breeding and domestication. (Montes and Melchinger 2016)

9.1.1 Genomic Studies in J. curcas

The first most important information about the *J. curcas* genome came in 2008 when Carvalho and colleagues presented the genome size, the base composition and the karyotype of *J. curcas*. They reported a genome size of 416 Mb with 11 chromosomes. The karyotype indicated that five of the chromosomes are metacentric (1, 2, 5, 6 and 11) and six are submetacentric (3, 4, 7, 8, 9, 10), all being relatively small in size (Fig. 9.2). These genomic features along with its ease of transformation and vegetative manipulation are considered ideal for its propagation (Carvalho et al. 2008). Marinho et al. (2018) made comparative analysis of the karyotype and genome size of six *Jatropha* species allowing the identification of chromosome markers for the genus.

The whole genome of *J. curcas* was initially sequenced using both Sanger method as well as new methods of multiplex NGS and later integrated with an assembly generated using the Illumina sequencing platform (Sato et al. 2011;

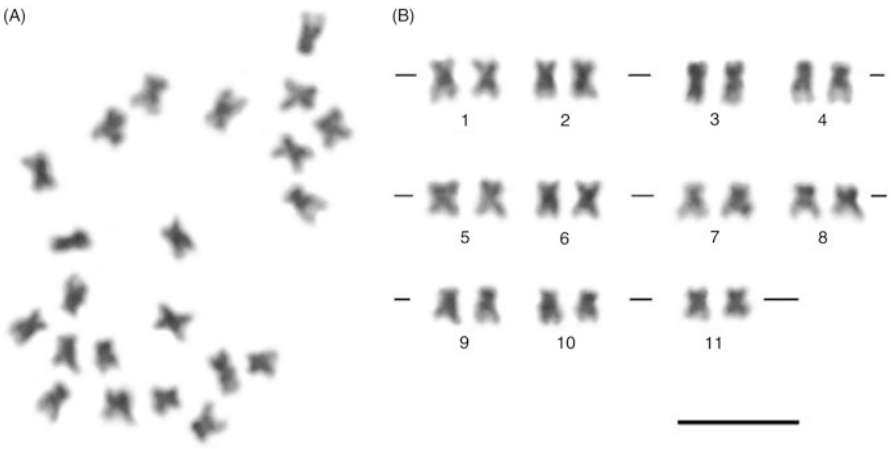

Fig. 9.2 (**a**) Metaphase spread of chromosomes from *J. curcas* root tips. (**b**) Karyotype of *J. curcas* chromosomes. (Carvalho et al. 2008)

Table 9.1 Comparison of assembly statistics (NCBI)

Parameter	Jat_r4.5	JatCur_1.0
Total sequence length	297660620	318527106
Total assembly gap	2346992	56046367
Number of scaffolds	39277	6024
Scaffold N50	15950	746835
Scaffold L50	4610	96

Hirakawa et al. 2012). The updated assembly Jat_r4.5 of Palawan accession has an assembly size of 297,660,620 bp resulting from 39,277 scaffolds accounting for 72% of the genome. This genome was sequenced to a depth of 90X using 454 GS FLX, Illumina GAII and ABI 3730xl platforms, containing as low as 0.78% gaps. Another unpublished genome assembly resource for *J. curcas* is JatCur_1.0 from an inbred line GZQX0401 that was sequenced using Illumina's technology submitted by the Chinese Academy of Sciences in 2014. The genome coverage of JatCur_1.0 is 189X, covering 76.5% of the genome size in 6027 scaffolds with an assembly size of 318,527,106 bp and 1.75% assembly gaps (Zhang et al. 2014) (Table 9.1).

9.1.1.1 Gene Identification

As an initiative to overcome the lack of cloned genes in *J. curcas*, the gene discovery project was initiated with the objective of isolating as many functional genes as possible by large-scale sequencing of expressed sequence tags (ESTs). A full-length enriched cDNA library was constructed from developing seeds of *J. curcas*, and 12,084 clones were sequenced. Thereafter, 7009 unigenes were identified, which included 6233 potential full-length genes. These genes were found to code for

diverse biological functions in *J. curcas* including oil biosynthesis, stress response, flavonol biosynthesis, etc. (Natarajan et al. 2010). Another paper published in the same time generated 13,249 expressed sequence tags (ESTs) from developing and germinating *J. curcas* seeds. The strategy adopted allowed the detection of genes related to lipid synthesis and degradation. The study also reported a larger number of transposable elements (TE) found in the ESTs of the developing seed library (800) when compared to the germinating seed library (80) (Costa et al. 2010).

Sato et al. (2011) sequenced whole genome as well as cDNAs from callus and leaf tissues and generated a total of 21,225 unigenes. Subsequently, the 454 pyrosequencing of normalised cDNAs prepared from roots, mature leaves, flowers, developing seeds and embryos of *J. curcas* yielded 14,327 new assembled transcripts. This included 2589 full-length transcripts and 27 transcripts that were directly involved in oil biosynthesis (Natarajan and Parani 2011). In another report, an EST library from salt-stressed roots of *J. curcas* was constructed for the identification of abiotic stress-responsive genes. It was found that 23 full-length CDS and a majority of transcription factors had sequence similarity to genes known to be involved in abiotic and biotic stress tolerance (Eswaran et al. 2012). Hirakawa et al. (2012) assembled all these publicly available *J. curcas* EST sequences and combined them with their own data. At present, a total of 30,203 genes, 17,575 transposons and 2124 putative pseudogenes have been determined in *J. curcas*.

9.1.1.2 Repeat Sequence Analysis

Sato et al. (2011), in their paper reporting the genome of *J. curcas*, analysed repetitive sequences in the genome. A total of 41,428 simple sequence repeats (SSRs) $\geq$15 bp were identified. The di-, tri- and tetra-nucleotide SSRs accounted for 46.3%, 34.3% and 19.4% of the identified SSRs, respectively. The SSR patterns that appeared frequently were (AT)n, (AAT)n and (AAAT)n, each representing 71% of di-nucleotide, 60% of tri-nucleotide and 58% of tetra-nucleotide repeat units, respectively. The tri-nucleotide SSRs, particularly (AAG)n and (AGC)n, were preferentially found in the exons. (AT)n, (AG)n and (AAT)n were enriched in 5′ and 3′ untranslated regions, and (AC)n frequently occurred in the introns. The repeat type of TEs constitutes 36.6% of the *J. curcas* genomic sequences. The most abundant repeat category was class I TE (29.9%), in which Gypsy (19.6%) and Copia (8.0%) LTR retroelements constituted major components (Sato et al. 2011).

In a very interesting report, the abundant EST database was explored for the presence of microsatellite repeats (Grover et al. 2014). A total of 42,477 ESTs (−22.9 Mbp) were downloaded from dbEST of NCBI and annotated into 12,358 contigs (4.76 Mbp) and 5730 singlets (6.50 Mbp). These 18,088 transcriptome sequences were searched for the presence of microsatellites, resulting in 7.91% of the sequences yielding 3557 microsatellite motifs. Most of the microsatellites were either di- or tri-nucleotide repeats, while other categories of tetra-, penta- and hexa-nucleotide repeats together constituted −4% of total microsatellites. EST-SSRs representing SSRs in coding regions are interesting since they are conserved with

Table 9.2 Classification of genomic sequence repeats from *J. curcas* (Sato et al. 2011)

	Jatropha genomic sequences		
Repeat type	Number of elements	Coverage (kb)	Percentage of sequence
Class I			
LINE	195	136.9	0.05
LTR: Copia	31,740	22,318.2	8.03
LTR: Gypsy	67,658	56,655.7	19.60
LTR: other	13,454	6436.6	2.23
Total class I	113,047	86,447.4	29.91
Class II			
Coding class II	5709	4102.9	1.42
MITE	5980	1802.8	0.62
Total class II	11,689	5905.7	2.04
Short tandem repeats	2092	148.1	0.05
Unclassified	25,997	14,953.3	5.17

a higher rate of cross species transferability compared to SSRs derived from intergenic regions. In addition, they are sufficiently polymorphic to be exploited for population genetic studies (Grover et al. 2014) (Table 9.2).

9.1.2 Transcriptomics in J. curcas

High-throughput sequencing analysis of the transcriptome of developing *J. curcas* seeds using 454 sequencing resulted in 195,692 sequences (46 Mbp) of raw sequence data. The assembly produced 12,419 contigs and 17,333 singletons (King et al. 2011). Using a combination of sequencing technologies, another group produced a transcriptome resource of 285,858,490 bp consisting of 120,586 contigs, 29,831 singlets and 40,929 complete and partial protein-encoding genes (Sato et al. 2011). The sequences and annotations were later upgraded by the addition of new data, combining the 1,025,000 reads from the conventional Sanger method and 2,312,828 reads from NGS. Based on an ab initio gene prediction analysis, 30,203 potential protein-encoding genes were identified, of which 2402 genes were ascribed to 19 metabolic pathways (Hirakawa et al. 2012).

Transcriptomics has been used in *J. curcas* to study gene expression profiles under a variety of environmental conditions with the aim of picking up candidate genes for crop improvement. In order to understand the molecular mechanisms of cold response to *J. curcas*, a high-throughput gene expression analysis was carried out. This revealed 4185 genes possibly involved with cold resistance which were then functionally annotated based on the transcriptome data (Wang et al. 2013). Many reports focus on the genes contributing to oil accumulation in the plant. Costa et al. (2010) reported EST sequences based on the identification of genes related to oil accumulation and degradation using developing and germinating seeds of

J. curcas. Global gene expression profiles of developing *J. curcas* seeds revealed key genes involved in the synthesis of storage lipids and proteins (Jiang et al. 2012). Other studies include gene expression analysis linked with impact of cytokinin on inflorescence buds (Chen et al. 2014) and metabolic pathways in drought tolerance (Sapeta et al. 2016). A more recent study compared five different transcriptomic profiles of floral sex differentiation stages using the Illumina HiSeq 4000 to reveal the underlying regulatory mechanism of male and female floral differentiation. The results showed that male floral differentiation was significantly associated with flavonoid biosynthesis process and that the female floral differentiation was significantly activated by the phytohormone signal transduction pathway (Hui et al. 2017). The availability of high-throughput data helps in *J. curcas* gene discovery and crop improvement using molecular tools.

9.1.3 Epigenetic Studies in J. curcas

J. curcas adapts differently in different climatic conditions, and therefore it is necessary to understand the germplasm variations in various adaptive environments. Currently, it is clear that genetic variations are distinguishable from the epigenetic variations inherited as preadaptations by subsequent generations in the form of epigenetic memories (Lukens and Zhan 2007; Bossdorf et al. 2008). For example, methylation of cytosine in DNA strand is an important epigenetic change associated with alteration of gene transcription leading to morphological changes and hence the altered phenotypes. These epigenetic changes usually do not alter the primary DNA sequence but are heritable (Henderson and Jacobsen 2007) during a given generation number. These conserved epigenetic markers have been found to influence many aspects of gene expression and chromosome biology, and they have characteristic genomic distribution (Doerfler 1983). Thus, genotypes exhibiting less genetic divergence might possess high epigenetic divergence (Mastan 2016). This criterion could be used as an indication for searching genotype for selective breeding that would be less susceptible to environmental variations.

A variant of methylation-sensitive amplified fragment length polymorphism (MS-AFLP) has been used to study methylation variations (epigenetic divergence) of individuals or population with respect to unmethylated, CG-methylated and CHG-hemimethylated states of specific nucleotides in the genome. MSAP has previously been used in *J. curcas* to investigate methylation levels in different accessions (Kanchanaketu et al. 2012) and during salinity stress (Mastan et al. 2012). Yet another study showed changes in methylation levels at different stages of seed development which can affect gene regulation and could be responsible for seed development and metabolism in seed tissue (Pandey et al. 2016). A recent interesting study assessed genetic and epigenetic diversity among selected elite germplasm of *J. curcas* using AFLP as genetic marker and MS-AFLP as epigenetic markers. The results clearly indicate that the genetic and epigenetic structures are not the same in *J. curcas* over the different accessions and show greater epigenetic

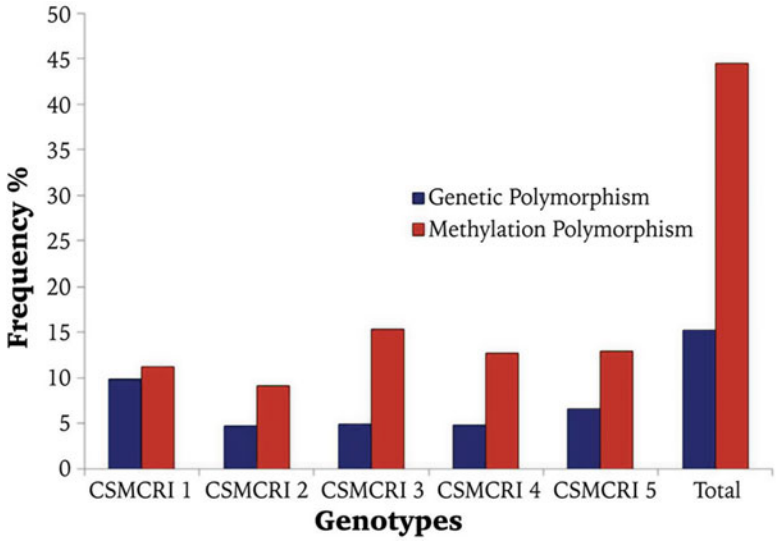

Fig. 9.3 Pictogram of epigenetic vs genetic polymorphisms across different *J. curcas* accessions. (Mastan 2016)

variation (44.4%) than genetic variation (15.2%) (Mastan 2016) (Fig. 9.3). In contrast, another study showed moderate level of epigenetic diversity in CCGG methylation and heritability of epigenetic markers over genetic diversity (Yi et al. 2010).

Thus, the phenotype variation in collections of *J. curcas* is better explained through epigenetic diversity rather than genetic diversity.

9.1.4 Metagenomic Studies in J. curcas

J. curcas has received enormous support for mass cultivation since it easily grows under harsh environment and limited nutrient availability. It is hypothesised that *J. curcas* is capable of growth in challenging environments with the help of its beneficial endophytic microorganisms. An earlier study showed the presence of nitrogen-fixing bacteria, actinobacteria and fungi from the rhizosphere of *J. curcas* (Qin et al. 2012; Madhaiyan et al. 2013).

In a recent report, the microbiome of *J. curcas* was explored and revealed *Firmicutes* as well as *Alphaproteobacteria*. The predominant genera were *Paenibacillus*, *Bacillus*, *Brevibacillus*, *Staphylococcus* and *Terribacillus*. Strains from *Rhizobium* and *Shingomonas* belonging to *Alphaproteobacteria* were also isolated. It is worth noting that the bacteria found to be enriched in the rhizosphere of *J. curcas* were plant growth promoters. The results indicated that beneficial effect of endophytes of *J. curcas* can be harnessed either by using these microorganisms as

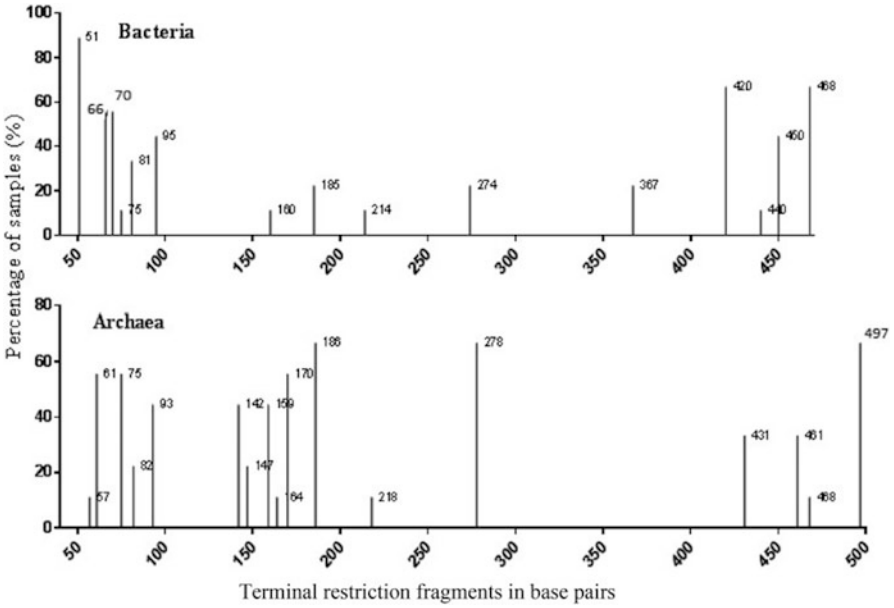

Fig. 9.4 Restriction fragment of bacteria and archaea that are prevalent in the rhizosphere of *J. curcas*. *X* axis represents base pairs, *Y* axis represents terminal restriction fragments (TRFs) abundance in the rhizosphere samples. (Dubey et al. 2016)

microbial inoculants or by intercropping with agriculturally important crops such as maize or beans (Mohanty 2017).

Another study showed the predominance of archaea in *J. curcas* rhizosphere, which is interesting data since they are known to thrive in challenging conditions. The dominant species were from the genera *Archaea* and include *Crenarchaea* and *Euarchaea* (Dubey et al. 2016) that cannot be cultured in vitro (Fig. 9.4).

9.1.5 Databases Related to J. curcas

9.1.5.1 JCDB: *J. curcas* Database

JCDB (http://jcdb.xtbg.ac.cn/) is an integrated knowledge database, which not only contains basic gene information but functional annotation of genes along with their expression profiles and network information. JCDB provides a user-friendly interface and a convenient search option allowing efficient sequence recovery, gene expression matrix, gene network and other information. JCDB also provides tools that focus on more specific purposes, such as online BLAST service, JBrowse (a genome browser), and JTools (a tool suite for genome sequence retrieval), gene ID conversion and gene network construction.

9.1.5.2 tropiTree: Tropical Tree Expressed Transcripts, SSR Markers and Primer Pairs

tropiTree (https://ics.hutton.ac.uk/tropiTree/index.html) is a resource for assembled expressed transcripts from RNA-seq study of 24 tropical tree varieties including *J. curcas*. This database contains information for all the microsatellites discovered among them and primer pairs designed for these microsatellite markers. The BLAST facility enables a search for homologous genes within the transcripts. The transcript annotation can be searched for with a specific gene name or keyword. A special search is also available to look for the number of SSRs according to the different repeat motifs discovered in *J. curcas*.

9.1.5.3 KEGG

KEGG for *J. curcas* (http://www.genome.jp/kegg-bin/show_organism?org=jcu) is a *computer representation* of *J. curcas* as a biological system. It integrates building blocks and wiring diagrams of the system—more specifically, genetic building blocks of genes and proteins, chemical building blocks of small molecules and reactions and wiring diagrams of molecular interaction and reaction networks. It is further classified into systems, genomic, chemical and health information. The systems information includes (i) PATHWAY, pathway maps for cellular and organismal functions; (ii) MODULE, modules or functional units of genes; and (iii) BRITE, hierarchical classifications of biological entities. The genomic information consists of (i) GENOME, complete genomes; (ii) GENES, genes and proteins in the complete genomes; and (iii) ORTHOLOGY, orthologous groups of genes in complete genomes. The chemical information comprises (i) COMPOUND, GLYCAN, chemical compounds and glycans; (ii) REACTION, RPAIR, RCLASS, chemical reactions; and (iii) ENZYME, enzyme nomenclatures.

9.1.6 *Comparative Genomic Studies in* J. curcas

Within the Euphorbiaceae family, the availability of complete genomes allows a comparative approach to better understand genome structure and evolution. The available genomes include *J. curcas* (physic nut), *Ricinus communis* (castor bean), *Hevea brasiliensis* (rubber tree), *Manihot esculenta* (cassava) and *Euphorbia esula* (leafy spurge).

Establishment of syntenic relationships between the species (crops) of Euphorbiaceae and *A. thaliana* (model plant) through comparative mapping has been beneficial for the identification of candidate genes contributing to agronomic traits from corresponding regions. They have also served as a resource to generate more markers for fine mapping in syntenic regions of other crops. The mapping of

192 orthologous markers identified in *J. curcas* onto the assembled whole genome sequence of *A. thaliana* enabled the identification of 38 syntenic blocks and also revealed that small linkage blocks remained well conserved, with shuffling (Wang et al. 2011). Yet another study reported that a significant degree of synteny is observed within the family Euphorbiaceae but appears to a lesser degree with the genomes of *G. max* and *A. thaliana* (Sato et al. 2011). In a comparative mapping exercise of markers between *J. curcas* and castor bean genomic sequences, a total of 410 scaffolds from the castor bean were anchored to the *Jatropha* map. These scaffolds covered a total of 189.8 Mb (about 54.1% of the total scaffold sequence) resulting in 320 well-conserved syntenic blocks containing 10,760 *J. curcas* genes collinear to castor bean (Wu et al. 2015). In a very interesting paper, a genome-wide comparative analysis revealed the specific gene expansion of aquaporin subfamilies in rubber tree and the loss of specific subfamilies in physic nut (Zou et al. 2016). Comparison of duplicated genes between *Jatropha* and cassava revealed that 36.9% of the genes are duplicated in cassava (Bredeson et al. 2016).

9.1.7 Marker-Assisted Selection in J. curcas

The basis of marker-based selection is linkage disequilibrium between a DNA marker and a gene. This can be exploited for selection as if the phenotype for the trait that is considered is caused by the marker itself. This method has some advantages: (a) simpler than phenotypic screening, which can save time, resources and effort; (b) selection can be carried out at the seedling stage; and (c) single plants can be selected (Collard and Mackill 2008). The prerequisites for the classical procedure of marker-assisted selection (MAS) are the DNA markers and linkage analysis, which enable to identify the markers that are linked to the genes controlling the trait(s) of interest. The "quality" and the number of markers have a major impact on the success of MAS. The quality of markers relates to their characteristics and to the cost and the efficiency of the genotyping process. The number of markers affects the reliability of the linkage between them and the gene(s) under consideration.

9.1.7.1 Linkage Maps

The detection of genes or quantitative trait loci (QTL) controlling traits is possible due to genetic linkage analysis, which is based on the principle of genetic recombination during meiosis (Tanksley 1993). This allows the construction of linkage maps composed of genetic markers for a specific population. Using statistical methods such as single-marker analysis or interval mapping to detect associations between DNA markers and phenotypic data, genes or QTLs can be detected in relation to a linkage map (Kearsey and Farquhar 1998). This establishes the importance of linkage maps in molecular breeding.

The first-generation linkage map generated for *J. curcas* contains 506 markers (216 microsatellites and 290 SNPs from ESTs) distributed into 11 linkage groups. This map was generated from two backcross populations with 93 progeny. The total length of the map was 1440.9 cM with an average marker space of 2.8 cM. The homologous comparison (BLAST) of 222 *J. curcas* ESTs containing polymorphic SSR or SNP markers with the EST database of NCBI revealed that 91.0%, 86.5% and 79.2% of *J. curcas* ESTs were homologous to their counterparts in castor bean, poplar and Arabidopsis, respectively (Wang et al. 2013). Subsequently, King et al. (2013) reported another genetic linkage map of *J. curcas*, created from four separate mapping populations, containing over 500 codominant (SSR and SNP) markers distributed over 11 linkage groups. They were also able to identify a locus responsible for the synthesis of phorbol esters (King et al. 2013). Following this, a high-density genetic map along with an assembled genome was reported with a total length of 320.5 Mbp containing 27,172 putative protein-coding genes. The linkage map was established using 1208 markers and harboured 81.7% of the assembled genome to produce 11 pseudo-chromosomes (Wu et al. 2015). More recently, an ultrahigh-density linkage map was published using a mapping population of 153 individuals and covering 1380.58 cM of the *J. curcas* genome, with an average marker density of 0.403 cM. The genetic linkage map consisted of 3422 SNP and indel markers, clustered into 11 linkage groups (Xia et al. 2018). Interestingly, 13 fruit-yield QTLs and two important candidate genes have been identified based on this linkage map.

9.1.7.2 Molecular Markers and Genetic Improvement of *J. curcas*

The five main considerations for the use of DNA markers in MAS as identified by one of the review papers are reliability, quantity and quality of DNA required, technical procedure for marker assay, level of polymorphism and cost (Collard and Mackill 2008).

Molecular markers have been systematically accelerating breeding programs through genetic diversity analysis, MAS, gene discovery, gene mapping, linkage map construction, QTL analysis, association analysis, gene transformation, etc. (Hyten et al. 2010; Sudhakar Johnson et al. 2011). In *J. curcas*, a variety of markers have been used to genetically distinguish local varieties in many regions including India, Africa, China and Vietnam. Examples include that of random amplified polymorphic DNA (RAPD), AFLP and inter-simple sequence repeat (ISSR) markers (Tatikonda et al. 2009; Sharma and Chauhan 2011; Rafii et al. 2012). SSR markers are considered advantageous over the above three types of markers because they are highly polymorphic, abundant, codominant, consistent and readily transferable between different plant varieties (Weber 1990). However, not many SSR markers have been described in *J. curcas*. In 2010, SSR markers from cassava were used to characterise the genetic relationships between 45 accessions of *J. curcas* (Wen et al. 2010). In another study, it was shown that 70% (211 out of 320 randomly selected) of SSRs from castor bean could be amplified in *Jatropha*, but only 7.58% showed

polymorphism in 49 *J. curcas* genotypes and eight *Jatropha* species (Sharma and Chauhan 2011). Single nucleotide polymorphism (SNP) markers are considered the most abundant type of sequence variations in genomes and are thus more informative than other markers. In 2011, a total of 1574 high-quality SNPs were identified from 11.9 Gbp of sequences, suggesting extremely low frequency of SNPs in *J. curcas*. Further, 2482 informative SNPs were discovered from *J. curcas* DNA sequences in a pool of 61 genotypes (Tian et al. 2017).

Seed oil content is governed by QTLs like many other economically important traits and other contributing factors including the environmental influence on the metabolism of oil in seeds. Molecular markers for identification of non-toxic genotypes, i.e. accessions with less phorbol esters, were identified (Sudheer Pamidimarri et al. 2009). Wide variations among biochemical (protein, oil and phorbol esters) compositions (Vasquez-Mayorga et al. 2017) and other phenotypic traits have been observed. However, molecular markers revealed narrow genetic diversity. In another interesting study, a new SNP-based multi-allelic marker system (Intra-Locus SNP Haplotype, LSH) identified multiple alleles per locus was tested and validated, increasing the genotyping power of the SNP marker system. These findings are of particular interest to better exploit the *Jatropha* genetic germplasm and to optimise the efficiency of MAS for variety improvement in selective breeding programs (Trebbi et al. 2015).

9.2 Conclusions

Genetic improvement in *J. curcas* is necessary, but its relatively small genetic diversity makes the operation challenging. Germplasm collections at its centre of origin in Central America and interspecific hybridization with *Jatropha integerrima* assisted in widening the germplasm base. However, more genetic variability needs to be added to the present founder population, and more DNA markers need to be identified by NGS in order to enable high-throughput genome-wide association analysis.

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Part II
Metabolism

Chapter 10
Fatty Acid Biosynthesis and Triacylglycerol Accumulation in the Biofuel Plant *Jatropha curcas*

Yan-Bin Tao, Xiao-Di Hu, and Zeng-Fu Xu

Abstract *Jatropha curcas* L. is recognized as one of the most promising biofuel plants because of the high oil content and the good oil composition in its seeds. Previous studies have established our understanding of the oil yield and quality of *Jatropha* in germplasm from around the world, which could be helpful for breeding programs. To obtain elite varieties of *Jatropha* with desirable traits, genetic manipulation technology can offer a feasible strategy of spatiotemporal regulation for expressing the genes involved in oil biosynthesis because *Jatropha* has the advantages of being a tree of a short generation time and an easy genetic transformation system. Based on recent studies of *Jatropha* genomics and transgenic analyses, we identified the genes involved in de novo fatty acid (FA) synthesis and triacylglycerol (TAG) assembly here, and we reviewed the gene expression profiles and transgenic manipulation of these genes in *Jatropha*. This information will be useful for *Jatropha* oil improvement via the combinatorial metabolic engineering approach.

Keywords Biosynthesis · Fatty acids · Genetic engineering · Oil content · Oil quality · Triacyl glycerols

10.1 Oil Content and Fatty Acid (FA) Composition in *Jatropha* Seeds

Due to its high content of non-edible seed oil, *Jatropha* is one of the most promising energy plants for use as a biodiesel production feedstock. For conventional breeding purposes, many studies helped in identification of superior germplasm with desirable traits such as high oil yield with superior quality. Generally, there is a positive correlation between seed oil content and seed development, indicating that the oil

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content increases linearly as the seed weight increases. During the early stages of *Jatropha* seed development, before 21 days after pollination (DAP), the seeds grow gradually, their oil content is below 10% (Sinha et al. 2015), and no oil bodies can be found in endosperm cells at 14 days after flowering (DAF) (Gu et al. 2012). After 21 DAP, during the seed filling stage, the seeds grow rapidly (Tao et al. 2014) in association with a sharp increase in oil content (Sinha et al. 2015). At seed maturity time, the oil content reaches its maximum, and the oil bodies occupy most of the space in the endosperm cells (Gu et al. 2012).

Due to different environmental and genetic factors, the highest oil contents differ among *Jatropha* germplasm accessions. *Jatropha* is native to Central America and more particularly Mexico and Guatemala, and it was presumably taken to tropical areas of Asia and Africa by Portuguese seafarers. In Mexico, the seed oil content of 17 native populations was relatively high, varying from 42.4% to 55.4% (Martinez-Diaz et al. 2017). Six *Jatropha* accessions derived from Colombia were transplanted to Spain. Three of them had seed oil content higher than 40% and up to the maximum of 42.4%, whereas the others showed seed oil content lower than 30% (Alburquerque et al. 2017). In India, Singh et al. (2016) collected 1 genotype (JCN01) from Cape Verde and 14 genotypes (JCN02-14) from India to select for elite germplasm. Most of these genotypes contained between 30% and 37.5% (JCN14) seed oil, except for one (JCN01), which contained only 27.7% seed oil. The oil yield per plant of JCN14 (0.45 kg) was five times that of JCN01 (0.09 kg). High heritability was recorded for the oil yield per plant (98.6%) followed by the seed oil content (98.2%). Thus, parental candidates for selective breeding programs should be chosen from germplasm with the largest oil yield per plant possible.

In addition to the oil content, the FA composition is also a key factor that should be considered during the germplasm selection because it determines the quality of the biodiesel. Whether a biodiesel fuel is suitable or not can be estimated by its crystallization capacity, oxidative stability, and ignition quality (Alburquerque et al. 2017). These properties have a direct correlation with the FA composition. If the number of carbons and saturation in FA chains are high, then the crystallization capacity is high, which results in the solidification of biodiesel at high temperatures. However, if the unsaturation level is too high, then the biodiesel may have a poor oxidative stability. Several parameters such as the saponification number (SN), iodine value (IV), and cetane number (CN) of the oil are used to describe these properties. SN and IV indicate the FA chain length and unsaturation levels, respectively. IV is considered as a measure of the oil stability. The CN, which is calculated on the basis of the SN and IV, indicates the ignition quality. The higher the CN, the shorter is the ignition delay time.

Most plant oils are generally composed of saturated palmitic acid (16:0) and stearic acid (18:0), monounsaturated oleic acid (18:1), and polyunsaturated linoleic acid (18:2) and linolenic acid (18:3). The major FAs in *Jatropha* oil are palmitic acid, stearic acid, oleic acid, and linoleic acid (Akintayo 2004; Kywe and Oo 2009; Wassner et al. 2016). During *Jatropha* seed development, the FA composition changes considerably. Sinha et al. (2015) found that palmitic acid accounted for over 30% of all FAs at early developmental stages but was reduced to approximately

15% in mature seeds. This reduction may be due to the fact that palmitic acid is the precursor of other FAs. Consequently, along with the seed development, the stearic and oleic acids increased from 2.14% to 6.02% and 3.90% to 44.38%, respectively. The linoleic acid increased gradually from 33.38 at 6 DAP to 47.19% at 51 DAP, but it decreased to 33.02% at maturity. Linolenic acid, which is not a major component of *Jatropha* oil, accounted for up to 26.32% of all FAs at the early developmental stages before 27 DAP, but it decreased sharply to 0.52% at 36 DAP and remained at 0.65% in mature seeds. According to the variation in the FA composition from 6 DAP to maturity, the SN and IV decreased gradually from 206.37 to 201.18 and 136.0 to 101.5, respectively, which indicated that the oil stability turned better. On the other hand, the CN increased up to 50.59 at maturity. Compared to the yellow ripe stage, a higher CN and a lower IV were present at the black ripe stage, indicating the superior ignition quality and higher oxidative stability of the oil. Therefore, harvesting *Jatropha* seeds at the black ripe stage could offer a guarantee of the oil quality.

The FA compositions were analyzed in six *Jatropha* accessions grown in Spain (Alburquerque et al. 2017). It was found that the oil quality varied among *Jatropha* accessions even when all the seeds are collected at the black ripe stage. These accessions showed the same FA composition profile but different FA amounts. The palmitic acid varied from 11.64% to 15.45%, the stearic acid varied from 3.52% to 8.18%, the oleic acid varied from 25.92% to 44.08%, and the linoleic acid varied from 33.79% to 52.20%. The higher oleic acid amounts were recorded from three accessions (4-5, 6-3, and 8-8), while their linoleic acid amounts were lower. By contrast, lower oleic acid amounts and higher linoleic acid amounts were recorded in another three accessions. A similar pattern was shown in *Jatropha* planted in India, but the variation was not as obvious (Singh et al. 2016). Biodiesel derived from oil with a high oleic acid level has excellent properties in terms of ignition quality, nitrogen oxide (NOx) emissions, and fuel stability (Graef et al. 2009). Accordingly, higher CN and lower IV were associated with seed oil of *Jatropha* accessions 4-5, 6-3, and 8-8. Additionally, the saturated FA amounts in these three accessions were higher than those in the others. Therefore, the biodiesel obtained from these accessions are also good candidates for environmentally friendly oils with lower NOx emissions because reduced NOx emissions are correlated with increasing CN values and saturation and decreasing IV. Besides the genetic component, Wassner et al. (2016) found that the temperature during the seed filling period could affect the FA composition significantly. The mature seeds harvested at cool temperatures (17.0–19.0 °C) produced oil with the largest linoleic acid content (47.5–45.4%) and the lowest oleic acid content (31.7–33.4%). However, under warm temperatures (27.9 and 28.7 °C), the opposite response pattern with the lowest linoleic acid contents (28.8% and 31.8%) and the highest oleic acid contents (47.1% and 45.4%) occurred. Consequently, a higher CN and lower IV were associated to seed oil obtained under warm conditions. This finding indicates that harvesting seeds matured under warmer temperatures helps in obtaining good-quality oil. However, the oil content was not correlated with temperature changes.

Taken together, the oil content and quality of *Jatropha* accessions vary according to genetic and local environmental factors. As a result, to select breeding candidates, a good approach is to choose the best performing plants in the location of interest. In addition to the conventional breeding approach, genetic manipulation technology can offer a feasible strategy for breeding *Jatropha* with a high oil yield and quality, especially because several efficient transformation systems have been established (Fu et al. 2015). Thus, investigating the molecular basis of oil biosynthesis during seed development becomes a prerequisite because it is essential for understanding the genetic factors that regulate the lipid biosynthesis and accumulation in *Jatropha* seeds.

10.2 Triacylglycerol (TAG) Biosynthesis

Plant oil (TAG) biosynthesis generally involves the following two pathways: de novo FA synthesis occurring in plastids (Fig. 10.1) and TAG assembly (Fig. 10.2) occurring in the endoplasmic reticulum (ER) (Ohlrogge and Chapman 2011). Plant TAGs, which are energy-dense lipids that accumulate in oilseed plants, serve as an energy reservoir for seed germination and the early growth of young seedlings. It is

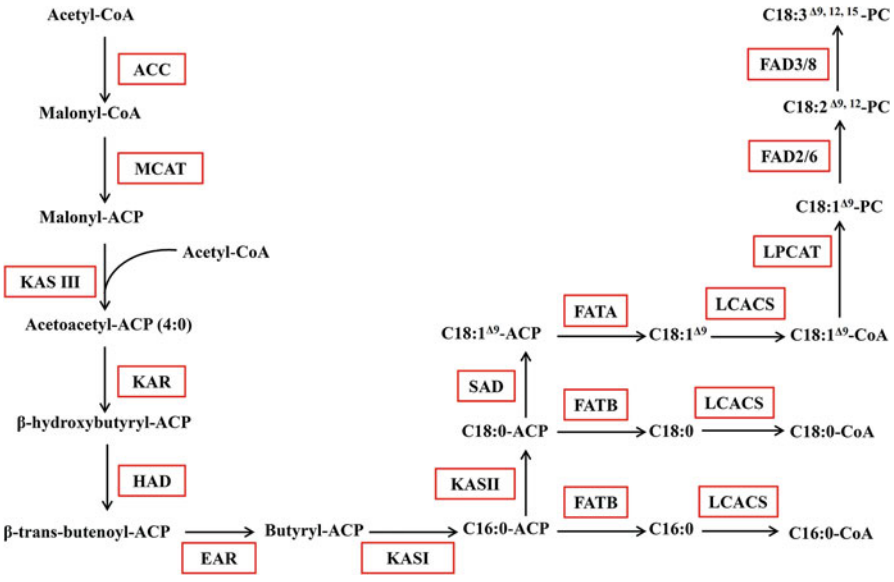

Fig. 10.1 De novo fatty acid (FA) biosynthetic pathway in *Jatropha curcas*. *ACC* acetyl-CoA carboxylase (ACCase), *ACP* acyl carrier protein, *CoA* Coenzyme A, *EAR* enoyl-ACP reductase, *FAD2*, *3*, *6*, *8*, fatty acid desaturase 2, 3, 6, 8, *FATA* acyl-ACP thioesterase A, *FATB* acyl-ACP thioesterase B, *HAD* β-hydroxyacyl-ACP dehydratase, *KAR* β-ketoacyl-ACP reductase, *KAS I*, *II*, *III*, β-ketoacyl-ACP synthase I, II, III, *LCACS* long-chain acyl-CoA synthase, *LPCAT* acyl-CoA: lysophosphatidylcholine acyltransferase, *MCAT* malonyl-CoA:ACP transacylase, *SAD* stearoyl-ACP desaturase

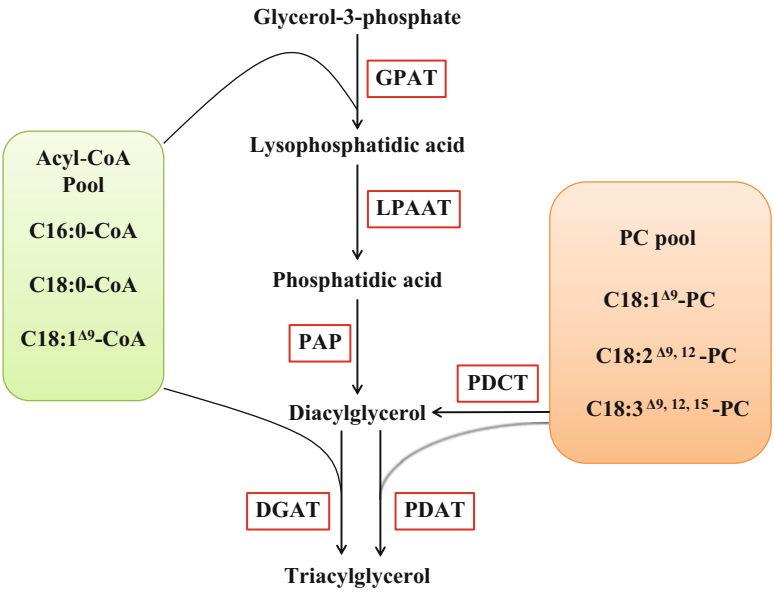

Fig. 10.2 Triacylglycerol (TAG) assembly pathway in *Jatropha curcas*. *DGAT* diacylglycerol acyltransferase, *GPAT* glycerol-3-phosphate acyltransferase, *LPAAT* lysophosphatidic acid acyltransferase, *PAP* phosphatidic acid phosphatase, *PC* phosphatidylcholine, *PDAT* phospholipid:diacylglycerol acyltransferase, and *PDCT* phosphatidylcholine:diacylglycerol cholinephosphotransferase

the major component of the plant oil. Three FA chains (usually 16 or 18 carbons long) esterified to a glycerol backbone constitute the TAGs (Durrett et al. 2008).

FA biosynthesis is initiated by the conversion of acetyl-CoA to malonyl-CoA, which is catalyzed by heteromeric acetyl CoA carboxylase (ACCase). This step is generally considered to be rate limiting. The second step is the formation of malonyl-ACP by transferring a malonyl group from malonyl-CoA to an acyl carrier protein (ACP), which is catalyzed by malonyl-CoA:ACP transacylase (MCAT). The third step is the condensation of malonyl-ACP and acyl-CoA to form acetoacetyl-ACP (4:0-ACP), which is catalyzed by β-ketoacyl-ACP synthase III (KAS III). The fourth step is the reduction of acetoacetyl-ACP to form β-hydroxybutyryl-ACP, which is catalyzed by β-ketoacyl-ACP reductase (KAR). The fifth step is dehydrating β-hydroxybutyryl-ACP with β-hydroxyacyl-ACP dehydratase (HAD) to form β-trans-butenoyl-ACP. The next reaction is the reduction of double bond in β-trans-butenoyl-ACP to form butyryl-ACP, which is catalyzed by enoyl-ACP reductase (EAR). Further chain elongation up to palmitoyl-ACP (16:0-ACP) is catalyzed by β-ketoacyl-ACP synthase I (KAS I) in six consecutive reactions. Next, the palmitoyl-ACP is converted to stearoyl-ACP (18:0-ACP), which is catalyzed by β-ketoacyl-ACP synthase II (KAS II). Stearoyl-ACP desaturase (SAD) catalyzes the first desaturation step in FA biosynthesis, converting stearoyl-ACP to oleoyl-ACP (18:1$^{\Delta 9}$-ACP). This step is also considered rate-limiting because it converts saturated FAs to unsaturated FAs. Finally, the chain elongation is terminated by acyl-ACP thioesterases that hydrolyze the

thioester bond, releasing free FAs. Thus, this step is regarded as the primary determinant of the chain length and level of saturated FAs. FATA and FATB, which are encoded by *FatA* and *FatB*, respectively, are two distinct acyl-ACP thioesterases. FATA acts on unsaturated acyl-ACPs, and FATB prefers saturated acyl-ACPs. These free FAs are then converted to acyl-CoAs by long-chain acyl-CoA synthase (LCACS) to be bound by acyl-CoA-binding proteins (ACBPs), which are able to protect them from acyl-CoA hydrolases and transport them to the ER. Chain elongation occurs in the acyl-CoA pool, whereas desaturation reactions occur in the acyl-phosphatidylcholine (PC) pool (Singh et al. 2005). Modifications that occur in the ER recruit oleoyl-CoA as the substrate that can be desaturated to polyunsaturated FAs (PUFAs). Acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) catalyzes the formation of PC ($C18:1^{\Delta 9}$-PC) from acyl-CoA ($C18:1^{\Delta 9}$-CoA) by mediating acyl-exchange between acyl moieties at the *sn*-2 position of PC and the acyl-CoA pool. Fatty acid desaturase 2 or 6 (FAD2/6) desaturates the oleic acid ($18:1^{\Delta 9}$) to form linoleic acid ($18:2^{\Delta 9,12}$) in plants. The linoleic acid can be further desaturated by FAD3/8 to produce α-linolenic acid ($18:3^{\Delta 9,12,15}$). These desaturases and their divergent forms can also edit the acyl chains to be esterified to PC.

In the ER, TAG assembly sequentially consumes the acyl-CoA using glycerol-3-phosphate (G3P) as a substrate through the Kennedy pathway, which involves four steps. G3P, the precursor of the glycerol backbone, is catalyzed by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA), which is further catalyzed by lysophosphatidic acid acyltransferase (LPAAT) to produce phosphatidic acid (PA). The next step converts the PA to diacylglycerol (DAG), and it is catalyzed by phosphatidic acid phosphatase (PAP). Finally, diacylglycerol acyltransferase (DGAT) converts DAG to TAG by using an acyl-CoA as acyl donor. To transfer PUFAs-PC to form TAG, two routes are available. The PC substrate for this process is provided by the DAG produced in the Kennedy pathway, which can be converted to PC by the catalyzation of CDP-choline:DAG cholinephosphotransferase (CPT). PC backbones carrying PUFAs can be converted into DAG through the removal of the phosphocholine headgroup through the action of phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Ichihara and Suda 2003; Singh et al. 2005). This process allows PUFAs to form TAG through the Kennedy pathway. The second route involves the phospholipid: diacylglycerol acyltransferase (PDAT)-mediated transacylation of PUFAs from PC to the DAG to form TAG (Dahlqvist et al. 2000). Various TAGs are then assembled in oleosins to form oil bodies in the seeds.

10.3 Expression Analysis of FA Biosynthetic Pathway Genes During Seed Development

Using the *Jatropha* genome sequences (Wu et al. 2015) uploaded in NCBI (https://www.ncbi.nlm.nih.gov/), we first retrieved all the genes and proteins that are related to oil synthesis in *Arabidopsis thaliana* from the TAIR database (http://www.

arabidopsis.org/) based on previous reports. The cDNA and protein sequences of *Jatropha* were aligned against known *A. thaliana* FA genes and proteins using BLASTN and BLASTP with E-values of $1e^{-10}$ and $1e^{-5}$, respectively (Altschul et al. 1990). All predicted proteins were then subjected to PFAM analysis, and the candidate proteins containing certain domains of the respective gene families were checked manually and verified with the results of the BLASTP. The genes encoding the key enzymes involved in *Jatropha* FA biosynthesis are listed in Table 10.1.

ACCase catalyzes the first step of FA biosynthesis, which comprises the following four subunits: a carboxyltransferase α-subunit (CTα) encoded by *accA*, a biotin carboxyl carrier protein (BCCP) encoded by *accB*, a biotin carboxylase (BC) encoded by *accC*, and a carboxyltransferase β-subunit (CTβ) encoded by *accD* (Gu et al. 2011). In *Jatropha*, single copies of *ACC1-LIKE*, *accA*, *accC*, and *accD* were each found. The *ACC1-LIKE* encodes a cytosolic ACCase. There are two *accB* genes, which are designated as *accB1* and *accB2*, with *accB1* encoding ACC-BCCP 1 and *accB2* encoding ACC-BCCP 2. ACC-BCCP 1 has two isoforms, which are formed by mRNA alternative splicing. So, there are three ACC-BCCP proteins in *Jatropha*. During seed development, *ACC1-LIKE* was highly expressed during late stages (40 and 50 DAP), while its expression levels were low at early stages (Xu et al. 2011). Consistently, *accA*, *accB*, and *accD* were predominantly expressed at 42 DAP, while *accC* was expressed at low levels during whole seed development. In addition, *accD* was also highly expressed in the leaves (Gu et al. 2011). In the second reaction, a single *MCAT* was found in *Jatropha*. *MCAT* began to be expressed at 14 DAP, and its expression levels gradually reduced down to the minimum at 42 DAP, but it increased sharply in the mature seeds (56 DAP) (Gu et al. 2012).

KAR, HAD, and EAR are involved in the formation of common intermediates in FA biosynthesis. *KAR* and *KAR-LIKE* were found in *Jatropha*. *KAR* was significantly expressed during seed development except in mature seeds (Gu et al. 2012). A single *HAD* started being expressed at 14 DAP and reached its maximum at 28 DAP, and its levels decreased gradually in the later stages (Gu et al. 2012). Two *EAR* genes (*EAR1* and *EAR2*) are present in *Jatropha*. EAR2 encoded by *EAR2* has two isoforms, which are formed by mRNA alternative splicing. The *EAR1* was primarily expressed during the mid to late stages (28–42 DAP) (Gu et al. 2012).

The KAS gene family includes *KAS I*, *KAS II*, and *KAS III*. Two *KAS I* genes (*KAS I-1* and *KAS I-2*) and single copies of *KAS II*, *KAS III*, and *mtKAS* were found. KAS I and KAS II are responsible for the synthesis of palmitic acid (16:0) and stearic acid (18:0), respectively. KAS III is involved in controlling the initial reaction to form C4:0-ACP. The strongest expression of *KAS I* was detected at mid stage (28 DAP) (Gu et al. 2012), which may lead to a high content of palmitic acid before 36 DAP (Sinha et al. 2015). *KAS II* was predominantly expressed at 42 DAP (Gu et al. 2012), resulting in the highest content of stearic acid exhibited at that stage (Sinha et al. 2015). By contrast, *KAS III* was constitutively expressed in *Jatropha* (Li et al. 2008), and its expression levels during seed development were the strongest at the mid-stage (Gu et al. 2012).

SAD is responsible for the synthesis of oleic acid ($18:1^{\Delta 9}$), which is the first desaturation step in FA biosynthesis. We found four *SAD* genes, which were designated as *SAD5a*, *SAD5b*, *SAD6*, and *SAD7* according to their homologs in

Table 10.1 Summary of fatty acid (FA) biosynthetic genes in *Jatropha curcas*

Gene	Gene ID	Protein ID	Annotation
ACC1-LIKE	105638840	NP_001295714.1	Acetyl-CoA carboxylase 1-like, cytosolic ACCase
accA	105649954	XP_012092191.1	ACC- CTα methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial
accB-1	105644890	XP_012085781.1	ACC-BCCP1 X1 biotin carboxyl carrier protein of acetyl-CoA carboxylase 1, chloroplastic isoform X1
		XP_012085782.1	ACC-BCCP1 X2 biotin carboxyl carrier protein of acetyl-CoA carboxylase 1, chloroplastic isoform X2
accB-2	105644147	NP_001295674.1	ACC-BCCP2 biotin carboxyl carrier protein of acetyl-CoA carboxylase 2, chloroplastic
accC	105643965	NP_001292959.1	ACC-BC biotin carboxylase 1, chloroplastic
accD	7564871	YP_002720121.1	ACC- CTβ, chloroplastic
MCAT	105639234	NP_001306855.1	Malonyl-CoA-acyl carrier protein transacylase, mitochondrial
KAS III	105645991	NP_001292956.1	Beta-ketoacyl-ACP synthase 3, chloroplastic
KAR	105644771	XP_012085628.1	Ketoacyl ACP reductase
KAR-LIKE	105640730	XP_012080517.1	Low-quality protein: beta-keto acyl-carrier protein reductase, chloroplastic-like
HAD	105645386	XP_012086368.1	Hydroxyacyl-ACP dehydratase
EAR1	105646511	XP_012087759.1	Enoyl-[acyl-carrier-protein] reductase 1[NADH], chloroplastic
EAR2	105634472	XP_012072726.1	Enoyl-[acyl-carrier-protein] reductase 2[NADH], chloroplastic isoform X1
		XP_020535082.1	Enoyl-[acyl-carrier-protein] reductase 2[NADH], chloroplastic isoform X2
KAS I-1	105633975	NP_001292958.1	Beta-ketoacyl-ACP synthase I, chloroplastic
KAS I-2	105645646	XP_012086687.1	Beta-ketoacyl-ACP synthase I, chloroplastic
KAS II	105638018	NP_001292950.1	Beta-ketoacyl-ACP synthase II, chloroplastic precursor
mtKAS	105640881	XP_012080672.1	Beta-ketoacyl-ACP synthase, mitochondrial
FATA	105644367	NP_001292940.1	FATA, oleoyl-acyl carrier protein thioesterase, chloroplastic
FATB1	105639285	XP_012078682.1	Palmitoyl or stearoyl acyl-ACP thioesterase, chloroplastic
FATB-Like1	105636910	NP_001292931.1	Palmitoyl acyl-ACP thioesterase, chloroplastic-like
FATB-Like2	105634942	XP_020535202.1	Palmitoyl acyl-ACP thioesterase, chloroplastic-like
FATB-Like3	105634943	NP_001292949.1	Palmitoyl acyl-ACP thioesterase, chloroplastic-like
SAD5a	105644924	XP_012085823.1	Stearoyl-[acyl-carrier-protein] 9-desaturase 5, chloroplastic
SAD5b	105644925	NP_001295684.1	Acyl-[acyl-carrier-protein] desaturase, chloroplastic-like

(continued)

Table 10.1 (continued)

Gene	Gene ID	Protein ID	Annotation
SAD6	105629158	XP_012066083.1	Stearoyl-[acyl-carrier-protein] 9-desaturase 6, chloroplastic
SAD7	105628257	NP_001292942.1	Acyl-[acyl-carrier-protein] desaturase 7, chloroplastic
FAD2–1	105646514	NP_001295707.1	Omega-6 fatty acid desaturase, endoplasmic reticulum isozyme 2
FAD2–2	105650891	XP_012093240.1	Bifunctional desaturase/conjugase FADX
FAD3	105630235	NP_001292929.1	Omega-3 fatty acid desaturase, endoplasmic reticulum-like
FAD4	105632931	XP_012070789.1	Fatty acid desaturase 4, chloroplastic
FAD6	105633504	NP_001292968.1	Omega-6 fatty acid desaturase, chloroplastic
FAD8a	105646096	NP_001292928.1	Omega-3 fatty acid desaturase, chloroplastic
FAD8b	105646580	NP_001295746.1	Omega-3 fatty acid desaturase, chloroplastic-like
FAD8c	105631214	XP_012068645.1	Omega-3 fatty acid desaturase, endoplasmic reticulum
LACS1	105642647	XP_012082936.1	Long-chain acyl-CoA synthetase 1
LACS2	105631770	XP_012069337.1	Long-chain acyl-CoA synthetase 2
LACS4a	105645675	XP_012086723.1	Long-chain acyl-CoA synthetase 4
LACS4b	105645676	XP_012086726.1	Long-chain acyl-CoA synthetase 4
LACS6	105632205	XP_012069918.1	Long-chain acyl-CoA synthetase 6, peroxisomal
LACS7	105647541	XP_012089058.1	Long-chain acyl-CoA synthetase 7, peroxisomal
LACS8	105641541	XP_012081513.1	Long-chain acyl-CoA synthetase 8
LACS9	105638933	XP_012078234.1	Long-chain acyl-CoA synthetase 9, chloroplastic

A. thaliana. *SAD* was predominantly expressed in mature seeds (56 DAP) (Gu et al. 2012), which is consistent with the high amount of oleic acid noted at the same stage (Sinha et al. 2015). The next desaturations rely on fatty acid desaturase (FAD). Oleic acid ($18:1^{\Delta 9}$) as a substrate is catalyzed by FAD2 or FAD6 to form linoleic acid ($18:2^{\Delta 9,12}$), which is further desaturated by FAD3 or FAD8 to produce linolenic acid ($18:2^{\Delta 9,12,15}$). Only two *FAD2-1* and *FAD2-2* and a single *FAD6* genes were identified for FAD2 and FAD6, respectively. The expression levels of these genes were detected at early stages up to the maximum at the mid stages and then subsequently reduced (Gu et al. 2012). A single *FAD4* was identified, and its substrate remains unknown. There are four *FAD* genes, *FAD3*, *FAD8a*, *FAD8b*, and *FAD8c*, which belong to the omega-3 fatty acid desaturase family.

FATA and *FATB* encode two distinct acyl-ACP thioesterases that terminate FA chain elongation. A single *FATA* gene encodes the FATA enzyme, which catalyzes oleoyl-ACP, releasing free oleic acid. The expression levels of *FATA* increased gradually during seed development up to the maximum at 42 DAP, but they were reduced sharply in mature seeds. *FATB* encodes FATB, which prefers the saturated palmitoyl-ACP (16:0) and stearoyl-ACP as substrates. The four *FATB* genes *FATB1*,

FATB-Like1, *FATB-Like2*, and *FATB-Like3* were found. The *FATB1* expression levels increased during later developmental stages (Wu et al. 2009). The free FAs released from acyl-ACPs are converted to acyl-CoAs by LCACS. Here, we identified the following eight *LCACS* genes: *LCACS1*, *LCACS2*, *LCACS4a*, *LCACS4b*, *LCACS6*, *LCACS7*, *LCACS8*, and *LCACS9*. The expression pattern of *LCACS* is almost the same as that of *HAD* (Gu et al. 2012).

10.4 Expression Analysis of TAG Biosynthetic Pathway Genes During Seed Development

Using the same approach, we found genes encoding the key enzymes involved in *Jatropha* TAG assembly, and they are listed in Table 10.2.

At the beginning of TAG assembly, GPAT catalyzes glycerol-3-phosphate, the precursor of the glycerol backbone, to LPA. There are 12 *GPAT* genes in *Jatropha*, namely, *GPAT1*, *GPAT2a*, *GPAT2b*, *GPAT3a*, *GPAT3b*, *GPAT5*, *GPAT6a*, *GPAT6b*, *GPAT6c*, *GPAT8*, *GPAT9*, and *GPAT-LIKE*. The predominant expression levels of *GPAT* were detected at the early to mid stages (14–28 DAP) (Gu et al. 2012) because this enzyme is involved in the first step of TAG assembly. LPAAT and PAP are involved in the formation of intermediates. The six *LPAAT* genes *LPAAT1*, *LPAAT2a*, *LPAAT2b*, *LPAAT4*, *LPAAT5*, and *LPAAT-LIKE* were identified. The expression patterns of *LPAAT1*and *LPAAT2* are similar. They were expressed during seed development, with the highest levels at mid stage. *LPAAT5* was also highly expressed at the mid stage, but the levels at other stages were very low. By contrast, the expression of *LPAAT4* was increased gradually from the early stages to maturity. Four *PAP* genes, *PAP2a*, *PAP2b*, *PAP2c*, and *PAP3*, were identified. *PAP* started being expressed at the early stage and reached its maximum level at 28 DAP and then subsequently decreased considerably in the later stages (Gu et al. 2012).

DGAT catalyzes the final step of TAG biosynthesis, and its level is given with respect to TAG accumulation. We identified three *DGAT* genes, *DGAT1*, *DGAT2*, and *DGAT3*, in *Jatropha*. *DGAT1* and *DGAT2* are generally known (Hobbs et al. 1999; Oelkers et al. 2000), whereas *DGAT3* (*cytoDGAT*) has been identified recently and is involved in the cytosolic TAG biosynthetic pathway (Saha et al. 2006). DGAT1 incorporates the usual FAs into TAG, whereas DGAT2 prefers unusual FAs such as ricinoleate and vernolic acid. *DGAT1* exhibited high expression levels at 28 and 42 DAP (Gu et al. 2012). The expression of *DGAT2* also detected in leaves was much higher than in seeds (Xu et al. 2011). There have been no reports about the expression pattern of *cytoDGAT* in *Jatropha* thus far. In peanuts, *AhDGAT* (*cytoDGAT*), which prefers oleoyl-CoA as the acyl donor, was detected only in immature seeds (Saha et al. 2006).

To transfer PUFAs-PC into TAG, PDAT and PDCT mediated the process in two distinct ways. The three *PDAT* genes (*PDAT1*, *PDAT1-LIKE*, and *PDAT2*) and a

Table 10.2 Summary of triacylglycerol (TAG) biosynthetic genes in *J. curcas*

Gene	Gene ID	Protein ID	Annotation
GPAT1	105629551	XP_012066550.1	Glycerol-3-phosphate acyltransferase 1
GPAT2a	105638856	XP_012078122.1	Probable glycerol-3-phosphate acyltransferase 2
GPAT2b	105638854	XP_012078119.1	Probable glycerol-3-phosphate acyltransferase 2
GPAT3a	105642646	XP_012082935.1	Probable glycerol-3-phosphate acyltransferase 3
GPAT3b	105647808	XP_012089426.1	Probable glycerol-3-phosphate acyltransferase 3 isoform X1
		XP_020540395.1	Probable glycerol-3-phosphate acyltransferase 3 isoform X2
GPAT5	105647393	XP_012088851.1	Glycerol-3-phosphate acyltransferase 5
GPAT6a	105646620	XP_012087892.1	Glycerol-3-phosphate 2-O-acyltransferase 6
GPAT6b	105642423	XP_012082634.1	Glycerol-3-phosphate 2-O-acyltransferase 6
GPAT6c	105649803	XP_012091984.1	Glycerol-3-phosphate 2-O-acyltransferase 6
GPAT8	105642972	XP_012083373.1	Probable glycerol-3-phosphate acyltransferase 8
GPAT9	105630072	NP_001295680.1	Glycerol-3-phosphate acyltransferase 9
GPAT-LIKE	105640970	XP_012080786.1	Glycerol-3-phosphate acyltransferase, chloroplastic-like
LPAAT1	105633522	XP_012071517.1	Lysophosphatidic acid acyltransferase 1, chloroplastic
LPAAT2a	105634720	NP_001295696.1	Lysophosphatidic acid acyltransferase 2
LPAAT2b	105648809	XP_012090707.1	Lysophosphatidic acid acyltransferase PLS1
LPAAT4	105633742	XP_012071774.1	Probable lysophosphatidic acid acyltransferase 4
LPAAT5	105631921	XP_012069535.1	Probable lysophosphatidic acid acyltransferase 5
LPAAT-LIKE	105632950	XP_012070810.1	Lysophosphatidic acid acyltransferase isoform X1
		XP_012070815.1	Lysophosphatidic acid acyltransferase isoform X2
PAP2a	105636922	NP_001295641.1	Phosphatidic acid phosphatase 2
PAP2b	105638770	XP_012078018.1	Phosphatidic acid phosphatase 2 isoform X1
		XP_020536804.1	Phosphatidic acid phosphatase 2 isoform X2
		XP_012078019.1	Putative phosphatidic acid phosphatase 2, chloroplastic isoform X3
PAP2c	105636725	XP_012075457.1	Phosphatidic acid phosphatase 2
PAP3	105636923	XP_012075719.1	Phosphatidic acid phosphatase 3 isoform X1
		XP_012075720.1	Putative phosphatidic acid phosphatase 3, chloroplastic isoform X2
DGAT-1	105637897	NP_001292926.1	Diacylglycerol O-acyltransferase 1
DGAT-2	105646335	NP_001292973.1	Diacylglycerol O-acyltransferase 2
DGAT-3	105642702	XP_012083005.1	Diacylglycerol O-acyltransferase 3, cytosolic
PDAT1	105631411	XP_012068907.1	Phospholipid:diacylglycerol acyltransferase 1
PDAT1-LIKE	105637933	XP_020536294.1	Phospholipid:diacylglycerol acyltransferase 1-like isoform X1
		XP_020536295.1	Phospholipid:diacylglycerol acyltransferase 1-like isoform X2

(continued)

Table 10.2 (continued)

Gene	Gene ID	Protein ID	Annotation
PDAT2	105637340	XP_012076169.2	Putative phospholipid:diacylglycerol acyltransferase 2
PDCT	105634846	XP_012073167.1	Phosphatidylcholine:diacylglycerol cholinephosphotransferase

single *PDCT* were found. High expression levels of *PDAT* were found in leaves and in the late developmental stages of the seeds (Xu et al. 2011). Studies on *Jatropha PDCT* are lacking. In flax, two *PDCT* genes, *LuPDCT1* and *LuPDCT2*, displayed similar expression profiles, which were expressed at very low levels in vegetative and floral tissues but at high levels in the mid to late stages of embryo development (Wickramarathna et al. 2015). The seed-specific expression of *LuPDCT1* and *LuPDCT2* in *A. thaliana* resulted in increases in C18-PUFAs, with an attendant decrease in the oleic acid content. Therefore, to increase the proportion of oleic acid in *Jatropha*, which helps to improve the biodiesel quality, a reduction in the *PDCT* expression would be a suitable approach.

Altogether, the FA and TAG biosynthetic genes that we identified here can facilitate an understanding of the oil biosynthesis mechanisms in *Jatropha*.

10.5 Genetic Engineering of FA Composition and TAG Accumulation in *Jatropha*

Despite the high potential of *Jatropha*, an available elite germplasm with desirable traits for this plant is still under selective breeding at present, and thus, unreliable oil yields and quality limit its use in the biodiesel industry. Therefore, genetic improvement has become an imperative for *Jatropha* breeding, especially Sood and Chauhan (2015) found that genes from FA and TAG biosynthetic pathway were expressed at a higher level in accessions with large oil contents than in accessions with low oil contents. The understanding of spatiotemporal regulation of the genes involved in the process of oil biosynthesis in *Jatropha* is essential for the improvement of oil production from *Jatropha*.

As we mentioned above, biodiesel derived from oil with a high monounsaturated FA content has excellent properties with respect to the ignition quality, NOx emissions, and oxidative stability. However, *Jatropha* oil contains 30–52% PUFAs (primarily linoleic acid), which negatively impacts its biodiesel quality, although FA unsaturation relieves the solidification of fuel at a cold temperature. One available approach is to reduce the conversion of oleic acid to linoleic acid. FAD2 is the key enzyme responsible for linoleic acid production in plants. Qu et al. (2012) isolated two *FAD2* genes from *Jatropha* and downregulated the expression of *JcFAD2-1* in a seed-specific manner through RNA interference (RNAi). The resulting transgenic plants exhibited a significant increase in oleic acid (>78%)

and a corresponding decrease in PUFAs (<3%) in the seed oil, while the control *Jatropha* contained approximately 37% oleic acid and 41% PUFAs. As a result, the oil of the transgenic *Jatropha* produced a cetane number as high as 60.2, which is similar to the required cetane number (CN) for conventional diesel fuels (60) in Europe. It is well-known that the CN value is perhaps the most important factor for biodiesel use because it indicates the ignition quality of diesel fuels and also negatively correlates with the NOx emissions (McCormick et al. 2001). The higher the CN, the shorter the ignition delay time and the lower are the NOx emissions. The CN value can be increased by increasing the chain length and the saturation level of FA (Vaknin et al. 2011). KAS II in FA biosynthesis catalyzes the conversion of palmitoyl-ACP (16:0-ACP) to stearoyl-ACP (18:0-ACP). The overexpression of the *Jatropha KAS II* (*JcKASII*) gene under the *CaMV35S* promoter in *A. thaliana* resulted in decreases in the C16:0 and increases in the C18:0 in transgenic plants (Wei et al. 2012). This result indicates that *JcKASII* could promote the conversion of C16:0 into C18:0 FA and increase its accumulation in *Jatropha* seed oil. As outlined above, a *FATB* gene designated as *JcFATB1* was isolated from *Jatropha*. When the seed-specific expression of *JcFATB1* was induced in *A. thaliana* by transgenesis, it led to a three- to fourfold increase in accumulated palmitic acid and moderate increases in other saturated FAs, along with reductions in the unsaturated FAs (Wu et al. 2009). This result confirms that JcFATB1 has a higher affinity for the catalysis of saturated acyl-ACPs, especially palmitoyl-ACP. These manipulations can be performed in *Jatropha* to increase the chain length and saturation of FAs to improve the ignition quality and reduce NOx emissions.

In addition to modifying the FA composition, the efforts toward increasing the oil content in seeds are considerable. GPAT catalyzes the first step in TAG assembly. Two *GPAT* genes were isolated from *Jatropha* and were then characterized in *A. thaliana* (Misra et al. 2017). The expression levels of *JcGPAT2* were higher than the *JcGPAT1* levels during *Jatropha* seed development. The oil contents in transgenic *A. thaliana* overexpressing *JcGPAT1* under the *CaMV35S* promoter or the seed-specific promoter increased by 13% and 20% more than the control, respectively. On the other hand, the oil contents in transgenic *A. thaliana* overexpressing *JcGPAT2* under the same two promoters increased by 42% and 60%, respectively, more than the control. It appears that *JcGPAT2* plays a more important role than *JcGPAT1* in TAG biosynthesis. These results were consistent with the fact that the oil content in seeds of antisense transgenic lines of *JcGPAT1* and *JcGPAT2* under the control of the *CaMV35S* promoter was lower than in the control. This result indicates that increasing the intermediates in the TAG biosynthesis pathway can regulate oil accumulation. In the final step of TAG biosynthesis, the overexpression of *JcDGAT1*and *JcDGAT2* in transgenic tobacco resulted in seed oil content increases of 32.8% and 31.8%, respectively (Xu et al. 2014). The FA composition was also different in transgenic plants modified for *JcDGAT2*. The proportion of linoleic acid in the *JcDGAT2* transgenic lines increased significantly compared with that in the *JcDGAT1* transgenic lines and the control, and correspondingly, the oleic acid significantly decreased. These results suggest that *JcDGAT1* is a better choice for the improvement of oil content compared to *JcDGAT2*, since *JcDGAT2* could entail a

decrease in the oil quality. Recently, the *A. thaliana DGAT1* gene *AtDGAT1* was used to improve oil accumulation in *Jatropha*. This gene successfully increased the oil content by 20–30% in seeds and 1.5- to 2.0-fold in the leaves of *Jatropha* through genetic engineering and entailed a correlated increase in oleic acid and a decrease in PUFAs (Maravi et al. 2016).

Another way to accumulate oil content is by preventing the degradation of TAG by TAG lipases. During seed germination, TAG lipases hydrolyze TAG into glycerol and free fatty acids (FFAs) (Ma et al. 2017). *Jatropha* oil contains a high FFA level up to 27% (Kywe and Oo 2009; Kim et al. 2014). However, the FFA content must be as low as possible to avoid acidic transesterification and improve the biodiesel storage stability. The *sugar-dependent 1*(*SDP1*) gene encodes SDP1, which is specifically responsible for the first step in TAG degradation. The downregulation of *JcSDP1* expression in *Jatropha* was achieved by RNAi technology, and the *JcSDP1*-RNAi transgenic plants accumulated 13–30% higher total lipids, along with a 7% compensatory decrease in the protein content, than the control (Kim et al. 2014). Moreover, the FFA content in the seed oil decreased from 27% in the control plants to 8.5% in the transgenic plants. The considerable reduction in FFA resulting from this approach will be of great value when dealing with the high FFA problem of oil from *Jatropha* for biodiesel production.

In addition to genes that encode the key enzymes involved in oil biosynthesis, transcription factors (TFs) are also involved in regulating lipid biosynthesis. However, in contrast to the many functional data on the oil biosynthetic pathway in *Jatropha*, TFs have been rarely functionally characterized. At present, only a *JcWRI1* encoding WRINKLED1 (WRI1), which is a member of the large plant-specific APETALA2 (AP2) family of TFs involved in controlling seed oil biosynthesis, was characterized in *Jatropha*. The overexpression of *JcWRI1* increased the seed oil contents in both transgenic *A. thaliana* and *Jatropha* (Ye et al. 2018). In contrast to oil biosynthesis genes, increase in seed oil synthesis induced by the *JcWRI1* in transgenic lines did not appear to be correlated with a modified FA composition; however, this functional difference may depend on TF and target genes that are considered.

10.6 Conclusions and Future Perspective

Recently, increasing the oil content in non-seed tissues has been proposed as a novel platform for meeting global vegetable oil production needs. The TAG accumulation in vegetative tissues has the potential to outyield current oilseed crops, especially when engineered in high-biomass crops (Ohlrogge and Chapman 2011; Weselake 2016). In *Jatropha*, excessive vegetative growth is one reason for low oil yields. Increasing the oil content in leaves is one way to improve the oil yield. Maravi et al. (2016) reported that the overexpression of *AtDGAT1* effectively increased the oil content by 1.5- to 2.0-fold in the leaves of *Jatropha*. In addition, the thick pericarps could be another candidate platform to test by directing oil biosynthesis genes using

pericarp-specific promoters. However, the increased levels of TAG in non-seed tissues are still lower than they are in seeds. This pattern can largely be attributed to the single-gene strategy of most studies. To address this problem, a variety of combinatorial metabolic engineering approaches have been established to increase the storage of lipids through the de novo fatty acid ("Push") and TAG assembly ("Pull") pathways while stabilizing the cytosolic lipid droplets ("Package") and minimizing lipid turnover ("Protect"). With this strategy, transgenic tobacco accumulated until 30–33% TAG in the leaves (Vanhercke et al. 2017), and there was a greater than 100-fold increase in TAG accumulation, to levels up to 3.3% of the tuber dry weight of transgenic potatoes (Liu et al. 2017). Because a great deal of functional information about oil biosynthesis in *Jatropha* has been revealed, the combinatorial metabolic engineering approach would be optimal for *Jatropha* oil improvement.

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Chapter 11
Proteomic Studies in *Jatropha curcas* Seeds

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Abstract *Jatropha curcas* (L.) has gained interest when it is realized as a potential source of vegetable oil for biodiesel production, a process that results with a press-cake rich in protein. However, press-cake cannot be used without processing due to its high content of toxic and antinutritional compounds. The *Jatropha* genetic resources remain poorly characterized; however in Mexico exists in the wild a non-toxic *J. curcas* genotype with high potential as a source of information for plant breeding in order to obtain varieties with increased oil contents and low amount of toxic compounds that will increase the use of press-cake as feedstock. Then it is necessary to unravel the mechanisms of triacylglycerol and antinutrients biosynthesis pathways. Proteomics is a powerful tool that has been used to identify the proteins that are accumulated in the different seed tissues, e.g. endosperm, integument, oil bodies, and plastids, with the aim to generate information about key enzymes that could be potential targets for the development of new strategies for the selective breeding of *Jatropha.*

Keywords Endosperm · Integuments · Non-toxic genotypes · Oil bodies · Seed storage proteins · Shotgun analysis · Two-dimensional gel electrophoresis

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11.1 Introduction

Jatropha curcas (L.), a perennial plant belonging to the Euphorbiaceae family (Kumar and Tewari 2015), is native from Mexico and Central America (Ovando-Medina et al. 2011). Nowadays it is distributed worldwide in Central and South America, Africa, India, and Southeast Asia (Achten et al. 2010). The increased interest in *Jatropha* is due to its high potential for biofuel production; however, *Jatropha* has not yet been domesticated. Although some plant improvement by conventional breeding is being performed, oil yield is not yet sufficient for *Jatropha* to be considered as a sustainable crop for biodiesel production (Yue et al. 2013).

On one hand, efforts have been made to improve the yields of *Jatropha* oil extraction, through improvement of the biochemical engineering processes (Ceasar and Ignacimuthu 2011; Moniruzzaman et al. 2016). On the other hand, the expected increase in *Jatropha* for biodiesel production has generated concern about the uses of its by-products such as press-cake, which could be a valuable source of proteins. *Jatropha* proteins have been used as food and therapeutic applications (Mandal and Mandal 2000). However the presence of toxic compounds such as phorbol esters can limit the use of press-cake as food and feed resources. The existence in Mexico of a wild non-toxic *Jatropha* genotype has generated hope for the selective breeding of potential improved cultivars with increased oil contents and non-toxic press-cake. However, a better understanding of the biology, molecular biology, and the mechanisms of oil, antinutrients biosynthesis, and accumulation in *Jatropha* seeds is still necessary.

Jatropha molecular breeding is still in development, but huge efforts have been done to assess its genetic diversity (Wen et al. 2010; Wang et al. 2011) and to map the genetic factors that control *quantitative trait loci* (QTL) such as growth, seed yield, and oleosins (Sun et al. 2012; Liu et al. 2011a) using DNA markers. Transcriptome analysis had enabled identification of some genes involved in the fatty acid biosynthesis (Gu et al. 2012). But while genomic and transcript-profiling studies have provided information about several processes related with plant development, there is growing information describing that the abundance of mRNA transcripts does not always correlate with the levels of protein accumulation and that post-translational processes play an important role as mechanism of regulation (Maghuly and Laimer 2013). The availability of *Jatropha* genome and transcriptome in public databases (Costa et al. 2010; JCDB at http://jcdb.xtb.ad.cn) opens new approaches of study with proteomics as one of the most promising tool.

Proteomics is a systems biology-based approach investigating the whole accumulated proteins at a given time and under certain conditions (Wilkins et al. 1995). Proteomics has been shown to have potential to gain insights about complex biochemical processes and is being used in various fields of modern botany and agriculture like the search of plant biomarker for disease resistance *diseases* as well as drought and water stresses.

Proteomics tools have been used to study *Jatropha* seeds with the aim to identify the spatio-temporal accumulation of proteins that are important for fatty acid

biosynthesis as well as the key enzymes involved in the synthesis of toxic compounds (Raorane et al. 2013). Proteomic data generated will be very useful to implement new strategies for *Jatropha* breeding in order to increase the oil content, to optimize fatty acid composition, and to obtain new varieties with low or null levels of toxic compounds.

The methods of protein separation based on polyacrylamide gel electrophoresis in one or two dimensions (1-DE and 2-DE) are and will be classical methods widely used for proteomic studies because besides being reasonably quantitative, they provide important information about proteins that cannot be obtained by gel-free methods, such as changes in protein size, p*I*, and post-translational modifications (PTMs) (Rogowska-Wrzesinska et al. 2013). *Jatropha* proteomics have been carried out using 1-DE, 2-DE, and quantitative shotgun analysis for the study of seed development as well as for the analysis of whole seed and individual tissues or organelles (embryo, endosperm, gerontoplasts, integument, and plastids).

11.2 Seed Morphology

Jatropha seeds are formed within capsules; each capsule contains three seeds (King et al. 2009). Seed maturation occurs about 3–4 months after pollination. Mature seeds of Mexican non-toxic genotypes are smaller than toxic ones. Typically, a seed of *J. curcas* is made up of 50–60% shell, which is an indigestible coat that protects the seed against biotic and abiotic stresses (Fig. 11.1).

Jatropha seeds are rich in oil (20–40%) and protein (22–35%) that contains essential amino acids (Saeed et al. 2017), but its consumption is limited due to the presence of toxic and antinutritional factors (Makkar and Becker 2009). The *Jatropha* toxicity is mainly attributed to phorbol esters (PEs) (Makkar et al. 1997; Kumar et al. 2016), curcin, and in minor grade to phytates, lectins, and trypsin inhibitors (Martínez-Herrera et al. 2006; Devappa et al. 2012). The quality and quantity of the oil synthesized and stored in *Jatropha* seeds depend on several factors within which the environmental conditions are those that most affect the seed quality (Xu et al. 2011). Environmental factors such as biotic and abiotic stresses can influence the biosynthesis and accumulation of lipids within seeds (Rotundo and Westgate 2009).

Jatropha seeds are derived from one ovule, an organ that houses the female gametophyte, which in turn is surrounded by the nucellus and one or two integuments (Fig. 11.2a). After fertilization, the gametophyte differentiates into the diploid embryo and triploid endosperm. The seed coat develops from the ovule integuments. During the seed development, the cells of the nucellus degenerate, while the cellular endosperm, the major seed storage organ, expands and begins to accumulate reserves. It has been proposed that the destruction of the nucellus and the integument of the seeds is a developmentally programmed cell death (Greenwood et al. 2005). Mature *Jatropha* seeds contain an embryo of the new plant and endosperm that accumulates the nutrients necessary for the embryo's development during

Fig. 11.1 *Jatropha* seeds from Mexican non-toxic (upper panel) and toxic (lower panel) genotypes

germination (Fig. 11.2b). The endosperm is essentially nucleus-free from 5 to 10 days post-anthesis, and it becomes primarily cellular by 15 days post-anthesis (Greenwood et al. 2005).

11.2.1 Seed Endosperm and Embryo Proteome

The first proteomic studies were performed focusing on the analysis of the embryo and endosperm in order to compare the protein accumulation between these two tissues (Liu et al. 2009). These comparisons were approached by using the total protein extracted from embryo and endosperm and performing 2-DE mapping in the

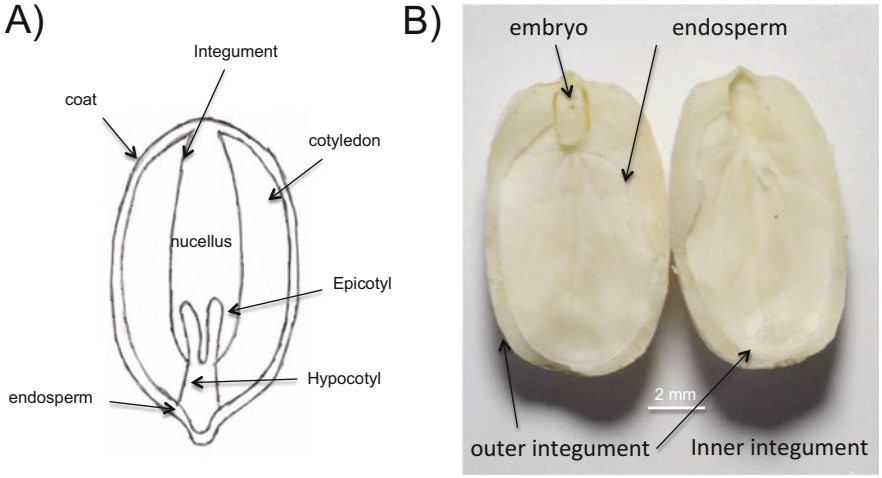

Fig. 11.2 *Jatropha* seed morphology. (**a**) Schematic representation of *Jatropha* seed under development. (**b**) Mature *Jatropha* seed

range of pH 4–7. Results showed that embryo and endosperm presented a similar 2-DE pattern. Although 380 and 533 protein spots were detected in embryo and endosperm, respectively, only 180 protein spots were similar in both tissues, which were expected because of the functional differences. Only 15 proteins were successfully identified indicating that the lack of a reference genome available was a limiting at the time of the experiment. Liu et al. (2011b) improved the protein separation reporting 66 differentially accumulated proteins between embryo and endosperm, but only 28 were successfully identified. These proteins were associated with nine functional groups. Most of the stress-related proteins identified were more abundant in embryo than in endosperm, meaning that stress-related proteins protect embryo of the mature dry seeds. Whereas in endosperm the abundant proteins were related with catabolism-related enzymes, while the embryo was characterized by the presence of anabolism-related proteins. This reinforces the knowledge that the endosperm is ready to provide nutrition for the embryo during germination and embryo is ready to start signal transduction for seed germination in response to environmental stimuli.

Taking advantage of the newly available *J. curcas* genome, Liu et al. (2013) increased the amount of detected proteins using the 2-DE proteomics approach applied to seeds in developing phases at 5, 10, 15, 20, 25, and 30 days after flowering (DAF). Proteins were extracted under mild conditions (Tris, sucrose, EGTA, PMFS, 2-ME, and Triton X-100), and the results showed that the number of resolved spots increased in the interval of 5–30 DAF. From the differentially accumulated protein spots, 24 were qualitatively different, whereas 80 were quantitatively different. Proteins involved in glycolysis and oxidative pentose phosphate pathways were

the major routes for producing the carbon flux, and the glucose-6-phosphate and triose-phosphate were the major carbon sources for fatty acid synthesis. Proteins involved in fatty acid synthesis were accumulated in the late seed development except the two proteoforms of the heteromeric acetyl-CoA biotin carboxylase protein. The main finding of this study was that the carbon source is converted into acetyl-CoA for fatty acid synthesis in the late seed development. Shah et al. (2015) performed a shotgun gel-based label-free quantitative proteomic analysis of the developing endosperm. *Jatropha* seeds were collected at 30, 32, 35, and 37 days after pollination (DAP), and mature seeds were also analysed. Endosperm was isolated from seeds avoiding any contamination with other seed tissues such as inner integument and embryo. The identified proteins in the five stages were up to 1760 and were grouped in classes related to seed storage proteins, metabolism of fatty acids, carbohydrates, toxic components, and proteolytic processing. Although the identified proteins include some related to the diterpenoid biosynthesis, the synthase/cyclases involved in the synthesis of phorbol esters were not detected. Three curcin isoforms were detected, one (Jcr4S12813.10) was only identified at 30 DAP, whereas the other two were detected at 32, 35, 37 DAP, and mature seeds.

Proteins are responsible for most metabolic processes, but they are also important as structural components in the cytoskeleton, part of membranes, and cell wall. Hence the dynamic range for protein amount in a system may be as high as 10^{10}–10^{12}, whereas the dynamic range for the analytical methods is limited to about 10^{3}–10^{4} (Hortin and Sviridov 2010; Miernyk and Johnston 2013). In order to compensate the limit detection of proteins by analytical methods, studies focused on protein pre-fractionation; hence, proteins in a seed can be studied by tissue separations (embryo, endosperm) or by subcellular compartments such as mitochondria, chloroplasts, and oil bodies.

One approach to study endosperm is based on the separation of highly abundant proteins in order to focus on the detection of the low-abundant ones (Miernyk and Hajduch 2011). In this regard, León-Villanueva et al. (2018) analysed the whole seed proteome using 2-DE and the protein pre-fractionation approach based on the solubility properties of seed storage proteins, i.e. the water-soluble fraction or albumins, the salt-soluble fraction of globulins, and the highly hydrophobic proteins, which include membrane proteins and glutelin fractions. The water-soluble proteins were better separated in the range of linear pH 5–8, whereas globulins and glutelins were resolved in the whole range of pH 3–10. The analysis was performed using LC-MS/MS, and the rate of successful protein identifications was increased considerably (Tables 11.1 and 11.2).

11.2.2 Proteomic Analysis of Seed Integument

In order to explore the process of *Jatropha* seed development and the mechanism responsible to generate the carbon and nitrogen sources necessary to feed the growing embryo and endosperm, Soares et al. (2014) analysed the proteome of the

Table 11.1 Proteomic approaches used for identification of proteins in endosperm and embryo of *J. curcas* from developing and mature seeds

Approach	Protein	Sample	Protein spots	References
2-DE gels (p*I* 4–7)	Total protein from embryo and endosperm	Mature seeds	14	Liu et al. (2009)
2-DE gels (p*I* 4–7)	Total protein from embryo and endosperm	Mature seeds	28	Liu et al. (2011b)
2-DE gels (p*I* 4–7)	Total proteins	Developing seeds	104	Liu et al. (2013)
1-DE/shotgun label-free	Total proteins	Developing seeds	1517; 1256, 1033, 752, 307[a]	Shah et al. (2015)
2-DE gels	Pre-fractionation of SSPs	Mature seeds	919	León-Villanueva et al. (2018)

[a]Proteins identified at 30, 32, 35, 37 DAP, and in mature seeds. SSPs stands for seed storage proteins

Table 11.2 Endosperm and embryo proteins identified in *J. curcas* by LC-MS/MS

Biological process	Unique proteins	%
Amino acids biosynthesis	7	4.8
Defence/stress	13	8.9
Detoxification	7	4.8
Gluconeogenesis	8	5.5
Glycolysis	15	10.3
Growth	4	2.7
Heat shock	14	9.6
Krebs cycle	6	4.1
Lipids metabolism	13	8.9
Nutrient reservoir	21	14.4
Oxide reduction	15	10.3
Pentose phosphate	3	2.1
Peroxisomes	1	0.7
Photosynthesis	1	0.7
Pyrimidine biosynthesis	4	2.7
Signalling	2	1.4
Translation	12	8.2
Total	146	100

Data obtained from Lui et al. (2009, 2011b, 2013) and León-Villanueva et al. (2018)

inner integument dissected into two sections named distal and proximal, as well as from the whole integument. The approach used was shotgun proteomics that enabled the detection of 1526, 1192, and 1062 proteins from proximal, distal, and whole inner integument, respectively.

This work reinforced the knowledge that the differentiation of the integuments into seed coat is carried out by two types of programmed cell death (PCD) triggered

by vacuole collapse (Hara-Nishimura and Hatsugai 2011; Soares et al. 2017). As reported by Soares et al. (2014), PCD starts with the mobilization and accumulation of several proteases, carbohydrases, and lipases in the proximal region of the inner integument whose function is to degrade biomolecules (proteins, carbohydrates, lipids), mainly from vacuole destruction to generate the carbon and nitrogen sources necessary for the development of the embryo and endosperm. The identification of seed storage proteins (SSPs) in the inner integument provided additional evidence that the seed coat serves as a reservoir of nutrients for the growing embryo and endosperm.

The identification of several classes of peptidases (Table 11.3), particularly of γ-VPE and KDEL-tailed CP, highlighted the role of developmental PCD in the developmental biology of *Jatropha* seeds.

11.2.3 Proteome of Inner Integument Gerontoplast and Plastids from Developing Seeds

During senescence of green tissues, chloroplasts differentiate into gerontoplasts (Fig. 11.3); this transition is accompanied by dismantling of chlorophyll and thylakoid membranes, which release large amounts of fatty acids and phytol (Rottet et al. 2015). With the aim to understand the proteome dynamics of this transition, Shah et al. (2016) analysed the gerontoplasts isolated from the inner integument, a non-photosynthetic tissue, from developing (25 DAP) *Jatropha* seeds. The inner integument was separated using a surgical spatula, and gerontoplasts were isolated through Percoll gradient. Proteomics approaches used were 1-DE combined with an *in solution* shotgun protein analysis by LC-MS/MS. The subcellular fractionation resulted in the identification of 1923 proteins; with this data set, the whole set of enzymes involved in metabolic pathways such as glycolysis, oxidative pentose phosphate, and TCA cycle were identified. In agreement with Soares et al. (2014), a group of hydrolases (peptidases, lipases) was also observed, which supports the progressive recycling of proteins, lipids, and carbohydrates to provide carbon and nitrogen sources to the growing embryo and endosperm.

Chloroplasts are essential organelles of photosynthetic eukaryotes; in non-photosynthetic seeds, chloroplasts transform to plastids – the organelles that play important role in carbon and nitrogen flow and are the sites of the biosynthetic pathways for fatty acids, amino acids, growth regulators, and secondary metabolites (Huang et al. 2013). The interest in plastids of *Jatropha* is based on the fact that diterpenoids, which are precursors of phorbol esters (PEs), are synthesized in these organelles.

By focusing on subcellular proteomics, Pinheiro et al. (2013) studied the plastids isolated from the endosperm of developing *Jatropha* seeds. In order to avoid contamination, endosperm of seeds was extracted using spatula, and plastids were

Table 11.3 Peptidases and peptidase inhibitors identified in distal and proximal regions as well as in intact inner integument of developing *J. curcas* seeds

Protein ID*	Description	Protein ID*	Description
Aspartic		Threonine	
Jcr4S00063.130	Aspartic proteinase	Jcr4S00385.110	26s PRS 6a a-like
Jcr4S00223.70	Aspartyl protease	Jcr4S01199.20	26s PRS 6b homologue
Jcr4S00739.60	Aspartic proteinase nepenthesin-1	Jcr4S00131.160	26s PRS 7-like
Jcr4S02642.50	Aspartic proteinase nepenthesin-2	Jcr4S11084.40	26s PRS 7-like
Cysteine			
Jcr4S02147.10	Cathepsin b-like	Jcr4S00296.90	26s PRS 10b b-like
Jcr4S00066.90	Cysteine proteinase (RDEL)	Jcr4S00110.140	26s PN-ATPse RS
Jcr4S01104.40	Cysteine proteinase (KDEL)	Jcr4S17106.10	26s PN-ATPse RS 11-like
Jcr4S00024.130	Cysteine proteinase inhibitor	Jcr4S00721.10	26s PN-ATPse RS 14-like
Jcr4S01597.40	Cysteine proteinase rd19a	Jcr4S00699.10	26s PN-ATPse RS 1-like
Jcr4S01609.40	Cysteine proteinase rd21a	Jcr4S00823.20	26s PN-ATPse RS 2
Jcr4S02110.50	Latex-abundant protein (metacaspase)	Jcr4S01446.20	26s PN-ATPse RS 4-like
Jcr4S00265.140	Vacuolar-processing enzyme-like (ϒ VPE)	Jcr4S05822.30	26s PN-ATPse RS rpn12a
Metalloprotease			
Jcr4S05049.10	Aspartyl aminopeptidase	Jcr4S08174.20	26s PRS 4 homologue a-like
Jcr4S00343.100	Leucine aminopeptidase	Jcr4S12171.10	26s PRS 4 homologue a-like
Jcr4S01697.60	Mitochondrial-processing peptidase a-like	Jcr4S25727.20	26s PRS 4 homologue a-like
Jcr4S01168.90	Oligopeptidase a	Jcr4S00100.60	26s PN-ATPse RS 6-like
Jcr4S02605.20	Peptidase m20 m25 m40 family protein	Jcr4S00843.120	PS-a
Jcr4S00865.10	Probable Xaa-pro aminopeptidase p-like	Jcr4S01752.50	PS-a
Jcr4S02821.10	Proliferation-associated protein 2 g4-like	Jcr4S00995.10	PS-a type-1-a-like 1
Jcr4S02294.40	Puromycin-sensitive aminopeptidase-like	Jcr4S02802.40	PS-a type-2-b
Jcr4S05166.10	Puromycin-sensitive aminopeptidase-like	Jcr4S03336.20	PS-a type-3-like
Serine			
Jcr4S01708.20	a-amylase/subtilisin inhibitor	Jcr4S05570.10	PS-a type-5-like
Jcr4S03455.30	Inhibitor of trypsin and Hageman factor	Jcr4S07728.10	PS-a type-7-like
Jcr4S03167.30	Lysosomal pro-x carboxypeptidase-like	Jcr4S00603.30	PS-b

(continued)

Table 11.3 (continued)

Protein ID*	Description	Protein ID*	Description
Jcr4S05848.10	Prolyl carboxypeptidase-like	Jcr4S00632.50	PS-b
Jcr4S00057.260	Proteasome subunit b type-1	Jcr4S01671.20	PS-b
Jcr4S03455.20	Proteinase inhibitor	Jcr4S02302.40	PS-b
Jcr4S03773.40	Serine carboxypeptidase-45	Jcr4S17767.10	PS-b
Jcr4S00378.10	Serine carboxypeptidase-49	Jcr4S00057.260	PS-b type-1
Jcr4S00079.140	Serpin-zx-like	Jcr4U29488.10	PS-b type-1-like
Jcr4S01323.10	Subtilisin-like protease-like	Jcr4S08435.20	PS-b type-2-a-like
Jcr4S01500.10	Tripeptidyl-peptidase 2-like	Jcr4S00852.50	PS-b type-3-b
Jcr4S02309.10	Xylem serine proteinase 1	Jcr4S11633.20	PS-b type-5-like
Jcr4S04612.10	Zeamatin precursor, putative	Jcr4S01070.40	PS-b type-6

*Protein identification according to the *J. curcas* genome database (release 4.5 May 2014). *PRS* Protease Regulatory Subunit, *PN-ATPse* Proteasome non-Atpase Regulatory Subunit, *PS* Proteasome Subunit

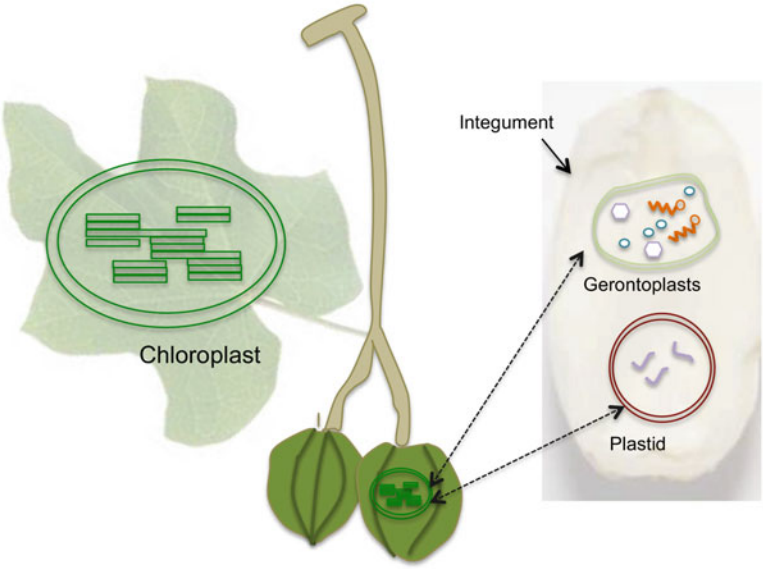

Fig. 11.3 Transition of chloroplasts from green tissues to gerontoplasts or plastids in seeds

isolated through Percoll gradient centrifugation. Plastid proteome was analysed using 2-DE proteomics approach and LC-MS analysis. This approach led to the identification of 1103 proteins. Proteins were grouped into amino acid metabolism, followed by carbohydrate, energy, and lipid metabolisms. Small and large Rubisco

subunits were identified. Although attention was taken in the enzymes involved in the biosynthesis of diterpenoids, however, the terpene synthase/cyclases, the key enzymes responsible for phorbol synthesis, were not detected. The failure to detect those enzymes, suggests that PEs occur in other plant tissues and then are translocated to the developing seed for storage.

11.3 Proteins Involved in Secondary Metabolism for Synthesis of Toxic Compounds

Because of the increased interest in *Jatropha* as biofuel plant source and the concomitant production of protein rich press-cake, the goal of removing PEs from *Jatropha* seeds has been performed through a biotechnological process using organic solvents, which is a complicated and expensive process. Consequently, the use of non-toxic Mexican varieties is an invaluable source of material for plant breeding, but plant bioengineering approaches were also proposed for the manipulation of PE biosynthesis. Of course, the characterization of genes involved in PE synthesis is of vital importance in order to successfully inhibit PE biosynthesis in the low- or non-toxic accessions of *Jatropha* resulting from green biotechnology (Li et al. 2016).

The biosynthetic pathway of PEs is not fully understood, and the only presumed step is the conversion of the geranyl geranyl diphosphate (GGPP) to a monocyclic diterpene, the casbene: a reaction that is catalysed by the casbene synthase. Eight homologues of casbene gene were reported in *Jatropha* genome (Sato et al. 2011); however, there is a lack of functional evidence linking casbene with PE biosynthesis.

The set of enzymes that were identified to be involved in biosynthesis of diterpenoids is divided into three major steps summarized in Table 11.4. In the first step, the isoprene unit isopentenyl diphosphate is synthesized via the non-mevalonate 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway from which four out of seven enzymes were identified: 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 2- C-methyl-D-erythritol 4-phosphate cytidylyltransferase, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase, and (E)-4- hydroxy-3-methylbut-2-enyl-diphosphate synthase. This successful protein annotation indicates that the DOXP pathway is fully operational in plastids as has been previously suggested by a transcriptomic study in developing seeds (King et al. 2011). In a second step, isoprenyl diphosphate synthases catalyse the synthesis of the intermediate diphosphate precursors, namely, geranyl diphosphate, farnesyl diphosphate, and GGPP. The enzyme GGPP synthase, which is responsible for the synthesis of GGPP, a precursor of several diterpenoids, was identified, but the enzymes involved

Table 11.4 Proteins involved in terpenoid metabolism identified in gerontoplasts from the inner integument of developing *J. curcas* seeds

Protein ID	Description
Jcr4S00089.150	1-deoxy-D-xylulose 5-phosphate reductoisomerase, chloroplast precursor
Jcr4S00528.60	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, chloroplast precursor
Jcr4S01018.60	3-ketoacyl-thiolase
Jcr4S15816.20	3-ketoacyl-thiolase peroxisomal-like
Jcr4S05483.10	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
Jcr4S01187.50	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
Jcr4S00049.110	7-dehydrocholesterol reductase-like
Jcr4S00742.70	Acetyl-CoA acetyltransferase
Jcr4S07308.30	Carotenoid cleavage dioxygenase
Jcr4S09217.20	Coproporphyrinogen-iii chloroplastic-like
Jcr4S14333.10	C-sterol isomerase
Jcr4S00345.110	Cycloartenol synthase
Jcr4S00111.120	Cycloeucalenol cycloisomerase
Jcr4S03868.30	Delta-aminolevulinic acid dehydratase
Jcr4S03988.40	Delta-sterol reductase-like
Jcr4S05771.10	Dihydroxypolyprenylbenzoate methyltransferase
Jcr4S00030.70	Ent-kaurene oxidase
Jcr4S06457.10	Ent-kaurene synthase
Jcr4S00092.40	Geranylgeranyl hydrogenase
Jcr4S07015.40	Geranylgeranyl pyrophosphate synthase-related protein
Jcr4S01820.40	Glutamate-1-semialdehyde-aminomutase
Jcr4S01592.30	Diphosphomevalonate decarboxylase-like
Jcr4S05363.10	Homogentisate phytyltransferase
Jcr4S00228.60	Homogentisate solanesyltransferase
Jcr4S02733.20	Homogentisate-dioxygenase
Jcr4S00523.60	Hydroxymethylglutaryl- synthase
Jcr4S00947.40	Lycopene beta-cyclase
Jcr4S01162.50	Mitochondrial protoporphyrinogen oxidase
Jcr4S00651.50	Monooxygenase
Jcr4S02359.30	2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase 2
Jcr4S00580.50	Phytoene desaturase
Jcr4S01302.90t	Protoporphyrinogen oxidase, chloroplast precursor
Jcr4S00004.100	S-adenosyl-l-methionine:delta24-sterol-Cmethyltransferase
Jcr4S26264.20	Tocopherol cyclase
Jcr4S01529.60	Zeta-carotene desaturase

Accession numbers according to the *J. curcas* genome database (release 4.5 May 4, 2014)

in the synthesis of other precursors of GGPP were not. In the third step, the precursors act as substrates for terpene synthases/cyclases giving rise to diverse diterponoids. The proteomics works from whole seed, embryo, endosperm, and organelles were unable to identify any terpene synthase in seeds, which could be responsible for the synthesis of casbene from GGPP. Interestingly, Li et al. (2016) have cloned two casbene synthase homologues (*JcCASA1663* and *JcCASD168*) from *Jatropha* genome, whose transcripts are highly expressed during seed development. Using a platform of RNAi-mediated silencing, the casbene synthase genes were silenced in a seed-specific manner by using a seed-specific promoter. Transformed seeds showed a decrease in PEs accumulation, and such trait was heritable and co-segregated with the transgene.

Taken together, the main results of subcellular proteomics indicate that endosperm of developing seeds are not the site for PEs synthesis. This observation is supported by studies carried out by He et al. (2011) who indicated that the main site of PEs accumulation is the tegmen, a maternal tissue that is originated from the inner integument of seeds, so further work should be performed on the proteome analysis of the tegmen.

11.4 Proteome of Oil Bodies and Synthesis of Fatty Acids in Seeds

In seed plants lipids are generally stored in oil bodies in the form of triacylglycerols (Bewley et al. 1994). During imbibition and germination, biological utility of oil bodies changes from a sink to source organelles (Shimada et al. 2018). Lipids, starch, and proteins stored in seed embryos and endosperms are mobilized to support germination and the establishment of seedlings (Popluechai et al. 2011) where physical interactions between glyoxysomes and oil bodies activate the degradation of oil body lipids (Shimada et al. 2018). Oil bodies comprise a monolayer of phospholipids stabilized by proteins that determine their size and nature (Popluechai et al. 2011). In *Jatropha* proteomic studies performed by protein separation through SDS-PAGE and band analysis by LC-MS/MS demonstrated that oleosin, caleosin, and steroleosin are the most abundant components in oil bodies and are similar to those found in seeds from other species (Popluechai et al. 2011). Further studies have applied the comparative proteomics approach to study the protein profiles in oil bodies with different lipid contents (Liu et al. 2015).

The use of high-throughput sequencing technology has allowed genomic and transcriptomic analyses in *Jatropha* with the aim to reveal important genes related with metabolic networks linked to seed development in a spatio-temporal context of gene expression associated with the accumulation of seed lipids (Costa et al. 2010;

Gu et al. 2012; Jiang et al. 2012; Wu et al. 2015). Proteomic analysis of oil bodies has been limited by the difficulties to isolate pure fractions of these cellular compartments as well as the absence of commercial tools (e.g. antibodies) to check their purity or to determine contaminating proteins extracted together with the oil bodies (Liu et al. 2015). Analysis by 2-DE the protein profiles of oil bodies from mature *Jatropha* wild-type seeds in the pH range of 3–10 revealed that most of the associated proteins (>85%) were of low molecular weight (<45 kDa), whereas protein identification by LC-MS/MS and homology database search allowed the successful identification of 83 oil bodies associated protein species grouped in four classes including germination related, oil bodies stabilization, oil bodies synthesis, and other proteins that were diagnosed as clear contaminants (e.g. curcin and seed storage proteins). It is a challenging task to determine if all isolated and thus identified proteins by proteomics approaches are contaminant proteins or they really belong to oil bodies. Improvements in oil body isolation techniques are required so that in conjunction with the large amount of available genomic and transcriptomic data for *Jatropha* obtained in the last years (Costa et al. 2010; Gu et al. 2012; Jiang et al. 2012; Wu et al. 2015), the use of proteomic tools will make possible to unequivocally identify proteins present specifically in the oil bodies. The same authors evaluated the differential accumulation of proteins isolated from preparations of oil bodies extracted from low-lipid, high-lipid, and wild-type mature *Jatropha* seeds. They also found 28 differentially accumulated protein spots related with oil bodies stabilization, oil bodies biosynthesis (including transport and signalling, protein and lipid biosynthesis), and germination-related proteins. Transient expression analysis in *Nicotiana benthamiana* revealed that alongside oleosins (the most abundant protein species in seed oil bodies), calcium-binding protein and a glycine-rich RNA-binding protein were directed to oil bodies (Liu et al. 2015). The use of proteomic approaches to investigate the major and other associated proteins in oil bodies as well as the study of changes in protein accumulation during seed development are essential for understanding the biology and biotechnological improvement of lipid content in *Jatropha* seeds. Proteins involved in the biosynthesis of the fatty acids identified from plastids in developing *J. curcas* seeds are listed in Table 11.5.

11.5 Conclusions

Efforts are being made to obtain a proteome catalogue of *Jatropha* seeds, which could constitute a valuable resource for studies addressing the developmental biology of these seeds. Proteome analysis uncovered a pattern of proteins involved in sugars and lipid synthesis and degradation. Data show a set of differentially accumulated protein isoforms in different tissues. The knowledge of protein accumulation and distribution patterns in developing and mature seeds of *J. curcas* deserves special attention. So far, proteome data provided information about the regulatory mechanisms of seed development; however the lack to find key enzymes related with

Table 11.5 Proteins involved in the biosynthesis of the fatty acids identified from plastids of developing *J. curcas* seeds

Protein	Accession No.
16.9 kDa oleosin	AAM46777.1
3-ketoacyl-ACP reductase	gi|255634733
3-oxoacyl-ACP reductase	Jcr4S01274.20,Jcr4U30974.10, Jcr4S00273.200
Acetyl-CoA carboxylase	Jcr4S01232.50, Jcr4S03449.40, Jcr4S25260.10, Jcr4S00416.90, Jcr4U31237.20, Jcr4S09385.30, Jcr4U32160.10, gi|225544133, Jcr4U29862.20
ACP-S-malonyltransferase	Jcr4S04735.20
Acyl-ACP desaturase	Jcr4S03070.40, Jcr4S13936.20, Jcr4S01370.20, Jcr4S03070.30, Jcr4S03522.10. gi|134943
Acyl-CoA-binding protein, partial	Jcr4S00983.40
Beta-ketoacyl-ACP synthase I	gi|116248667
Biotin carboxylase	Jcr4S25260.10, Jcr4S03449.40,
Caleosin	gi|6478218, CAO71462
Dihydrolipoyl dehydrogenase-like	gi|449460949
Enoyl-ACP reductase (NADH)	Jcr4S03253.10
Heteromeric acetyl-CoA biotin carboxylase	gi|238837063
Hydroxyacyl-ACP Dehydrase	Jcr4S03305.10
Oil body-associated 2B-like	Jcr4S00805.80
Oleosin 1	ABW90148
Oleosin 2	ABW90149
Oleosin 3	ABW90150
Oleoyl-ACP hydrolase	Jcr4S20073.10, Jcr4S00539.70
Plastidlipid-associated protein	NP 193955
Stearoyl-ACP desaturase	NP 197128
Steroleosin	EEF34360, AAX49394
β-ketoacyl-ACP synthase I	Jcr4S00903.20, Jcr4S04655.30, Jcr4S27123.10, Jcr4S02541.50, Jcr4S08397.10
β-ketoacyl-ACP synthase III	Jcr4S00903.20

Accession numbers according to UniprotKB, NCBI, and the *J. curcas* genome database (release 4.5 May 4, 2014), *ACP* Acyl Carrier Protein

PEs synthesis indicates that these compounds are synthesized in other tissues and then translocated to the seed endosperm. An alternative explanation would be to consider that improved technologies are still needed to enhance the detection of low-abundant proteins.

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Chapter 12
Pervasive System Biology for Active Compound Valorization in *Jatropha*

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Abstract Physic nut (*Jatropha curcas* L.) is a tree in the family Euphorbiaceae whose members have been known for the production of important natural compounds for therapeutic applications. Physic nut is also one of the few important plant species whose genome has been fully sequenced under high scrutiny, mostly because it is a potential source of oil, which could contribute to alleviate the worldwide energy crisis. However, for being a new crop, *J. curcas* is not yet domesticated to the point of being industrially productive, and a long way is being undertaken to improve it by selective breeding. During the last decade, the scientific community has performed a huge effort to aggregate knowledge to this plant species. The challenge around *J. curcas* constitutes a fertile ground to look for natural compounds that may serve as scaffolds for new drug applications. In this chapter, we review the principal conceptual strategies that may be taken to valorize natural compounds in the genus *Jatropha*.

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12.1 Introduction

After an initial enthusiasm in the year 2008–2010, several *Jatropha* plantations were grown worldwide without any previous technical knowledge on the crop features, appropriate germplasm, and price politics (Carels 2012, 2013; Sunil et al. 2013; Li et al. 2014; Ehrensperger et al. 2014; Jingura and Kamusoko 2015; Soto et al. 2018). The use of genotypes with a low seed productivity (Yang et al. 2010) typically between 1 and 1.5 t/ha per year at 32–34% oil content drawn from a germplasm with narrow genetic variability disseminated worldwide by Portuguese settlers (Sunil et al. 2013; Ovando-Medina et al. 2013; Li et al. 2017a) and the ignorance of the specific needs of *Jatropha* generally brought pioneers into economic losses (Sun et al. 2008; Basha et al. 2009; Rosado et al. 2010). However, worldwide spread research groups understood that the bottleneck was in the inappropriate germplasm selection (Laviola et al. 2018) and bravely started to identify the genetic and agronomic features necessary to optimize *Jatropha* as a crop (Ghosh et al. 2010; Joshi et al. 2011; Vaknin et al. 2018) or as a bioengineering system (Valdés-Rodríguez et al. 2013; Giadrossich et al. 2016; Campos et al. 2016). Using molecular markers, research groups from Central America showed that the center of origin of the species was in Southern Mexico (Dias et al. 2012; Salvador-Figueroa et al. 2014; Montes Osorio et al. 2014; Vásquez-Mayorga et al. 2017), in particular in the region of Chiapas, which is at the border with Guatemala. This region is now considered as an important place for *Jatropha* improvement because of its high level of genetic diversity for *amplified fragment length polymorphism* (AFLP) markers and important agronomic traits (Pecina-Quintero et al. 2011, 2014). Asian groups brought major contributions, releasing the first version of Jatropha's genome sequence on the public platform (Sato et al. 2011) and a first genetic map (Wang et al. 2011) allowing the characterization of *quantitative trait loci* (QTL), important for selective breeding (Yue et al. 2013).

In parallel to these scientific achievements, some members of the corporate sector succeeded in significant developments toward sustainable crop models for *Jatropha*. For instance, SGB (http://www.sgbinc.com/) proved that there is still a future for *Jatropha* as a commodity for biofuel production. Thus, at the moment the most important issue is still to understand as fast as possible how to integrate physiology, genetics, and agronomical features in order to optimize the oil production and, more generally speaking, the economical returns of *Jatropha* on a worldwide basis. The threshold of seed sustainable production has been recognized to be 2.5 t/ha (Mmopelwa et al. 2017). SGB demonstrated that hybrids with a seed productivity between 2 and 7 t/ha per year at 35–38% oil content could be reached by using the Central American germplasm (http://www.hardmanagribusiness.com/wp-content/uploads/2016/01/SGB-Inc-Company-Assets.pdf). Of course, the seed threshold of 2.5 t/ha depends on market rate of fossil oil, but market fluctuations must be absorbed by government politic strategies (Mmopelwa et al. 2017; Soto et al.

2018) to warrant that it may benefit from a diversified array of energy resources in the long term to protect itself against aggressive geopolitical speculations. The rate of 2.5 t/ha is well below that of 7 t/ha, which means that with the SGB's production system, a sustainable production is already available even if 7 t/ha can be considered as a peak of productivity difficult to reach on the average. The point is that the potential exists in the germplasm as available from Central America; the rest is selective breeding, biotechnology, investment, and time.

The SGB germplasm is based on 12,000 genotypes derived from ~550 initial accessions. SGB was the first corporation to produce sequences from genome and transcriptome for *genome-wide association study* (GWAS), *simple sequence repeat* (SSR), and *single nucleotide polymorphism* (SNP) markers as well as for the evaluation of its germplasm, proved to be structured in 18 clades. The significant traits initially observed in its germplasm collection were captured as F_3 progenies derived from crosses between different accession lines. Many of these traits segregate as apparent recessive traits and were stabilized into families by inbreeding (Montes and Melchinger 2016).

For the purpose of hybrid seed production, SGB propagates its inbred female parental lines clonally by stem cuttings (Mat et al. 2016), which are then interplanted with adjacent rows of a monoecious inbred male line according to a scheme similar to that followed for maize. The asset sell includes thousands of inbred lines that segregate for the recessive female traits selected for their productivity, flowering time, plant architecture, and overall vigor as well as tolerance to biotic and abiotic stresses. Hybrids perform better than open-pollinated lines because of their heterosis that impacts positively on yields, vigorous germination, seedling establishment, vigorous growth, and stress tolerance while providing the uniformity that is important for crop production and mechanization.

Traits that were improved by SGB's breeders are larger fruit clusters, larger fruit and seed size, oil content, variety in plant architecture (Giadrossich et al. 2016), disease resistance, and insect tolerance over a range of biotic and abiotic pressures. Improved traits for flowering time, synchronous flowering, branch number and branch erectness (One et al. 2014a), leaf size and shape, seed size and color, as well as overall productivity were fixed in breeded parental lines such that a benefit return based on an oil production of 1.5 t/ha (~5 t seeds/ha), a protein meal of 1.3 t/ha, and a biomass of 20 t/ha can be warrant to the producer. Other corporate competitors such as JOil (Yi et al. 2014; http://www.joil.com.sg/), JatroSolutions (http://www.jatrosolutions.com/), Jatro Power (http://www.jatropower.ch/), and Bionor (http://www.bionor.es) are on the same track as SGB (http://www.hardmanagribusiness.com/hardman-img/2015/05/21st-Century-Jatropha-Mach-2015.pdf), which means that a clear path to *Jatropha*'s economical success starts to emerge. These pieces of technico-economical evidence were a necessary motivation to sustain the *Jatropha*'s research worldwide.

Among the traits that can be considered for the selective breeding of a plant species, one may cite secondary metabolites for cosmetic (Warra 2012) or drug development. However, the market segment that can be reached by such product is completely different from that of commodities such as biofuel or protein meal for animal feeding. Actually, the overproduction of secondary metabolites would most likely compete with animal feeding as already occurs with toxic (Kumar et al. 2012)

and non-toxic (Herrera et al. 2012) *Jatropha* accessions considering phorbol. The issue of choosing one among both the strategies, i.e., producing for biofuel and protein meal or for biofuel and cosmetics, would depend on local opportunities and market access.

Several potentially valuable secondary metabolites were described in the genus *Jatropha*; they cover antimicrobial, molluscicidal, insecticidal, anti-inflammatory activities, and tumor inhibition (Sahidin et al. 2011; Katagi et al. 2016) that were found in all parts of the plant (Shibata et al. 2017; de Sant'Anna et al. 2013; Pullaiah and Bahadur 2013). The secondary metabolites described in *J. curcas* belong to the families of terpenes, alkaloids, tannins, saponins, flavonoids, phenolics, HCN, and phytate (Harry-Asobara and Samson 2014). The genus is rich in compounds of these families (Nwokocha et al. 2011; Rumzhum et al. 2012). Euphorbiaceae are generally rich in terpenes, and this is also the case of the genus *Jatropha* (Devappa et al. 2011) with, in particular, (i) diterpene compounds such as jatrophone, jatrophatrione, spruceanol, cleistanthol, curcusones, and japodagrol are antitumoral in vitro, while others such as jatrophalactam, faveline derivatives, multifolone, curcusone, and jatrophone derivatives are cytotoxic, and japodagrin, jatrogrossidione, and jatropholone are antimicrobial. Phorbol esters have a wide range of toxicity due to their competition activity for the diacylglycerol receptor (Goel et al. 2007); (ii) triterpenoids and sesquiterpenoids are also isolated from the genus *Jatropha* (Sutthivaiyakit et al. 2009; de Sant'Anna et al. 2013; Yang et al. 2013a). Calenduladiol (a triterpenoid derivative) shows antiretroviral activity to HIV-1 in vitro (Barroso-González et al. 2009). Preussomerins and deoxypreussomerins are compounds with a large range of biological activities that were also found in *J. curcas*' plant stems (Ravindranath et al. 2004). These examples are just a small drop within at least 12,000 metabolites that *J. curcas* could house (Ohtani et al. 2012), among which some could benefit human health (Thomas et al. 2008; Sabandar et al. 2013).

The integration of mass spectrometry data with gene-coexpression derived from six transcriptomes of flower and fruits showed that flavonoid oligomers decrease from fruit stage 1 to 3 and increase in stage 4 because of polymerization and incorporation of low-molecular-weight metabolites (Shibata et al. 2017).

Often, when a secondary metabolite is recognized as valuable and with low cost for chemical synthesis, the metabolic pathway leading to its synthesis is transferred to another domesticated organism rather than produced from the original plant system itself. The exercise of metabolic engineering for successful metabolic pathway transfer to a microorganism for industrial production requires a precise knowledge of that pathway that is often beyond the tradition of selective breeding techniques. However, a technique like GWAS may significantly contribute to identifying QTLs for secondary metabolites in medicinal plants together with the help of mass spectrometry and molecular markers. Biotechnological investigations as well as mathematical and computational modeling of a pathway of interest are additional steps generally necessary for metabolic engineering that we review hereafter.

12.2 Genome and Genetic Maps

12.2.1 Genome

The diploid genome of *J. curcas* is divided into 2 × 11 chromosomes with flow cytometry analysis of *J. curcas* root tips indicating an average 2C value of 0.85 pg and an average base composition of 38.7% GC (guanine + cytosine). In terms of bp, the data of flow cytometry would be equivalent to a genome size of 416 Mb, which is roughly similar to the size of the rice genome. The karyotype of *J. curcas* is made up of 22 relatively small metacentric and submetacentric chromosomes whose size range from 1.71 to 1.24 μm. The morphometric similarities observed between chromosomes from heterologous pairs suggest that *J. curcas* is an autotetraploid species (Carvalho et al. 2008). The 5S and 45S rDNA banding by FISH in *J. curcas*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica*, and *J. mollissima* suggested a relatively conserved karyotypes and genomes size varying betwen 2C = 0.64 and 0.86 pg (Marinho et al. 2018).

The draft sequence of the whole genome of *J. curcas* was published online at the end of 2010 (Sato et al. 2011) and further upgraded by the addition of new data in 2012 (Hirakawa et al. 2012). Briefly, the total length of the obtained sequences was 297,661,187 bp, consisting of 28,665 *supercontigs* and 10,612 *unassembled contigs*, which represent ~70% of the genome size estimated by flow cytometry (Carvalho et al. 2008). The 30% difference being supposedly due to the repeated sequences in the pericentromeric regions is known to be difficult to account for. The GC content was 33.7%, on average, with 43% exons and 32% for introns, on average (Sato et al. 2013). The discrepancy of GC content between flow cytometry data and sequencing data in the range of 5% can also be explained by a bias in the sequencing of GC-rich repeat sequences. These discrepancies should vanish with improved genome sequence version for the expected ~30% representativity of pericentromeric and telomeric regions in the genome. It is worthwhile to note here that a systematic bias between genetic and physical maps is observed in heterochromatin regions because of their low recombination rate, which means that positioning contigs and scaffolds in these regions is also more difficult. As a result of ab initio gene prediction by AUGUSTUS (Stanke et al. 2004), a total of 50,313 genes were detected in the genome sequences with 22,088 having significant sequence similarity of the translated amino acid of their coding sequences to TrEMBL from which 2402 could be assigned to metabolic pathways (Sato et al. 2013). The factor two of gene number redundancy is a signature of a past event of genome diploidization that confirms that *J. curcas* is indeed an autotetraploid species (Carvalho et al. 2008).

The first set of 42,477 expressed sequence tags (EST) currently available from GenBank in 2010 (Costa et al. 2010; Gomes et al. 2010; Natarajan et al. 2010; Natarajan and Parani 2011; Jiang et al. 2012; Zhang et al. 2015a) and the first genome sequence by Sato et al. (2011) and Hirakawa et al. (2012) publically released soon after in 2011 by Kasuza enabled subsequent research by the international community for molecular markers that could be used for linkage analyses and

genetic mapping. For instance, the EST repository has been used to search *simple sequence repeats* (SSR), which led to 6108 markers of this type (Grover et al. 2014). However, the bulk of SSR markers has been found by scrutinizing the genome sequence itself with 40,000 di-, tri-, and tetranucleotide SSRs equal to or greater than 15 bp being identified, and thus, the frequency of occurrence of these SSRs could be estimated to be one SSR in every 7.0 kb (Sato et al. 2011; Hirakawa et al. 2012).

According to Gupta et al. (2012), the frequency of SNP is much larger with an average of one per 100 bp taking 148 genotypes over India, North America, South America, and Africa into account. Considering the genome (Maurya et al. 2015), *J. curcas* showed relatively more SNPs than InDels, with a bias toward transition base substitution over transversion. However, the SNP frequency was much lower in coding sequences (Silva-Junior et al. 2011). SNPs can be arranged in arrays and annotated as either coding or regulatory for maximizing the detection of genes associated polymorphism (Kumar et al. 2015).

The use of some of these markers enabled to show that Mesoamerican regions (Guatemala and Mexico) are indeed the center of origin of the *J. curcas* species because a greater genetic diversity was found at SSR and SNP loci in plant accessions from these regions compared to those derived from Asia and Africa, where no significant difference was observed (Raposo et al. 2014; Osorio et al. 2014).

12.2.2 Genetic Maps

Because *J. curcas* outside its center of origin has been recognized to be largely homozygous, a first attempt to study its genome structure through genetic mapping was to cross it with *J. integerrima*. While the two *J. curcas* individuals were homozygous at all 245 microsatellites loci, 65% of these microsatellites were heterozygous in the *J. integerrima* individual, and every individual of the *J. curcas* × *J. integerrima* F_1 hybrid progeny was heterozygous at all 245 microsatellite loci (Ye et al. 2017). Thus, a first-generation linkage map could be constructed from two backcross populations with 93 progenies. Five hundred six markers (216 microsatellites and 290 SNPs from ESTs) were mapped into 11 linkage groups. The total length of the map was 1440.9 cM with an average marker space of 2.8 cM (Wang et al. 2011). Another genetic map obtained through hybridization of *J. curcas* and *J. integerrima* was obtained by Wu et al. (2015). The total genetic distance covered by this linkage map was 1655.8 cM, with an average marker density of 2.1 cM for unique loci. This linkage map was used for anchoring the scaffolds of the latest genome assembly. A total of 480 scaffolds covering 261.8 Mb (81.7% of the total scaffold sequences) were anchored to the map to produce 11 pseudochromosomes. Despite chromosome incompatibilities (Fukuhara et al. 2016), interspecific hybridization between *J. curcas* and *J. integerrima* is feasible (Sujatha et al. 2013; Marques et al. 2013) and enables to locate and to uncover the genetic basis of interspecific variability for phenotypic characters such as important

agronomic traits relative to growth (One et al. 2014a; Putranto et al. 2014) and seed traits (One et al. 2014b; Sun et al. 2017) through genetic mapping. In addition, King et al. (2013) constructed a genetic map from the F_2 from *J. curcas* parental lines displaying differences in a range of traits, in particular, toxic and non-toxic seeds. The linkage map that emerged from that cross accounted for 502 codominant markers distributed with a mean marker density of 1.8 cM. Phorbol ester biosynthesis was associated to a QTL spanning 41 cM over a single linkage group, and other seed traits were described by Ye et al. (2014). Later on, Xia et al. (2018) constructed an ultrahigh genetic map whose marker density is one per 0.403 cM, on average. This map consists of 3422 SNPs and covers 1380.58 cM; it enabled to identify ten QTLs that control the number of fruits and three that control the total weight of fruits, respectively.

12.3 Selective Breeding

12.3.1 Genotype and Phenotype Spaces

Understanding phenotypic variability requires understanding as to how genotypic changes translate into phenotypic changes (Chen et al. 2014; Tjeuw et al. 2015; Giadrossich et al. 2016). Most mutations that affect an organism's genotype are deleterious and produce inferior phenotypes. To find new superior phenotypes, one may have to explore many genotypes. However, evolution preserves existing, well-adapted phenotypes. In other words, organisms have to be conservative and explore many new phenotypes at the same time. Genotype space and the phenotypes therein share two organizational features of genotype space that facilitate phenotypic variability and evolutionary innovation. These are the existence of genotype networks and of a great phenotypic diversity in different neighborhoods of genotype space. Two genotypes are neighbors in genotype space if they differ in a single character (their coding for a metabolic reaction, for instance). A neighborhood of a genotype comprises all the neighbors that differ from it in a single character. However, this genotype diversity does not mean phenotype diversity and a vast set of genotypes share the same phenotype (Wagner 2013). Genotypes differing by one character can be connected through a sequence of steps forming circuits corresponding to the same phenotype (Ciliberti et al. 2007; Cotterell and Sharpe 2010).

In sum, two properties can be seen in the genotype-phenotype relationship: (i) genotypes that have the same phenotype are typically organized in large genotype networks that reach far within the genotype space, and (ii) small neighborhoods around different genotypes typically contain different phenotypes even if the genotypes do not differ greatly.

Somewhere in genotype space, a superior phenotype may exist. Natural selection eliminates any mutants that have not preserved the old phenotype or replaced it with a superior phenotype. One can view such a population as a cloud of points that diffuses on a genotype network through genotype space. Without genotypic

diversity there is no genotype network in the population, which means that selection cannot explore different neighborhoods in the genotype space; it is exactly the case of the worldwide population of *J. curcas* outside its center of origin. In addition, if the neighborhoods of different genotypes contained mostly identical new phenotypes, the existence of genotype networks is irrelevant to the exploration of novel phenotypes. The reason is that even though a population's genotypes could change during evolutionary exploration of a genotype network, the changing genotypes would have access to the same unchanging spectrum of phenotypes (Wagner 2013). These are the reasons why selective breeding outside the center of origin is not fruitful and needs application of biotechnological approaches.

12.3.2 Breeding Value

The growth and yield of *J. curcas* can be greatly affected by various biotic and abiotic stresses (Sastry and Francis 2015), with seed production being lower under drought conditions (Niu et al. 2012). The biological mechanisms behind the tolerance of *J. curcas* to drought (Liu et al. 2012; Silveira et al. 2013; Sapeta et al. 2013, 2016; Winter and Holtum 2015) or other stresses (Silva et al. 2015) are still poorly understood. The small genetic diversity detected in African and Asian *J. curcas* contrasts with its considerable phenotypic variations, which suggests that epigenetic factors are likely responsible for this variation (Montes Osorio et al. 2014). Such putative epigenetic factors are responsible for unexpected plant response to the environment with the fact that an unfavorable year for *J. curcas* production may be "kept in memory" well over the next year. Thus, the relationship between epigenetic factors and phenotypic variation is needed to be understood to proceed with the species domestication and epigenetic control is surely a factor to take into consideration within a selective breeding project. For example, variations in genomic methylation were demonstrated to be associated with leaf shape and photosynthetic traits in *Populus simonii* (Ci et al. 2015), and changes in morphology from 1 year to another or from one place to another are commonly observed in *J. curcas*. As a consequence, trait data should be collected over multiple years and locations along with environmental conditions to better understand the genetic basis of phenotypic plasticity (Valladares et al. 2000; Trabucco et al. 2010; Srinivasan and Shanthi 2017).

The estimation of the correlation between traits and their heritability can help to determine to what extent genetic improvement is possible through selection. Quantitative traits show a normal distribution due to an underlying genetic distribution attributed to the polygenic model and underlying environmental distribution. Thus, the phenotypic variation results from the sum of two contributions: the genetic and the environmental ones. The genetic contribution can be further split in *additive* and *nonadditive*. Among these components, only the *additive genetic effects* can be

passed on to progeny. It is these additive genetic effects that are referred to as *breeding value* (BV). Thus, the breeding value is the value of genes that are passed on to the progeny, and the *genetic value* is the value of genes to self, which includes nonadditive effects, such as *dominance*, that cannot pass on to progeny because it results in specific allele combinations that are disrupted from one generation to the other. When the genetic effect is additive, considering one locus where the heterozygote is with one dose of each allele, there is a linear relationship between the three possible allele combinations (genotypes) and their induced phenotypes. In case of dominance, the phenotype of the heterozygote is equal to one of the homozygotes. The difference between genetic and breeding value is a matter of *dominance deviation*, i.e., when the effect of one allele (A) on phenotype masks the contribution of a second allele (a) at the same locus. The dominance deviation can be incomplete or complete. Thus, the breeding value is the sum of average effects of alleles. If considering one locus, it is the average deviation of individuals receiving a given allele from one parent with the other allele coming at random from the population. The prediction of BV is obtained through the heritability (h^2), which measures how much of the genetic value of a parent will be transferred to its progeny with another parent. Since the contribution of each effect to the phenotype is proportional to the variance explained by the effect, the BV is equal to the ratio of the variance of the allele (V_A) in the progeny to the variance of the phenotype (V_P) in that progeny multiplied by the phenotype variance (P) of the parents. Thus, $h^2 = V_A/V_P$ and $BV = h^2 P$ with $0 \leq h^2 \leq 1$. If $h^2 = 1$, the heritability is total, and the entire character value is transferred to the progeny. Thus, the regression coefficient (b) of the linear relationship between P and BV is equal to h^2 and by definition of b, $h^2 = \text{cov}(BV,P)/V_P$, which enables to obtain h^2 directly from experimental data. Thus in theory, by associating genetic markers used as alleles, the analysis of their association with the phenotype distribution in the population enables to determine their heritability (Zhang et al. 2017). However, there is an important distinction between ${h_S}^2_{NP}$ and the narrow-sense h^2 defined as the proportion of phenotypic variance explained by the additive effects of all causal variants. Strictly speaking, the marker association is not a method to estimate trait heritability using SNP data because SNPs are not causal variants, and they are unlikely to be able to perfectly tag all causal variants for a trait. When the data are from a study involving a substantial proportion of close relatives, the estimate of ${h_S}^2_{NP}$ is very similar to the estimate of h^2 from a pedigree analysis, because in this case, the pedigree is reconstructed using genome-wide SNP markers, and the estimate of ${h_S}^2_{NP}$ is dominated by its close relatives. Therefore, the estimate ${h_S}^2_{NP}$ derived from family data cannot be interpreted as the variance explained by all the SNPs of a population because it could be confounded with some possible shared environmental effects and miss the effects of causal variants that are not tagged by the SNPs but captured by the pedigree structure. The estimate of ${h_S}^2_{NP}$ from a sample of unrelated individuals is an unbiased estimate of h^2 only if the genetic relationship is generated from all causal variants (Yang et al. 2013b).

12.3.3 Quantitative Trait Loci

The phenotype of polygenic characters varies continuously in populations. These *quantitative trait loci* (QTL) can be explained within the framework of discrete genetics as being the result of segregating polygenes. The QTL mapping is based on the systematic search of *linkage disequilibrium* (LD) between markers and causal loci. LD is the nonrandom association of alleles at different loci in a given population. Loci are said to be in linkage disequilibrium when the frequency of association of their different alleles is higher or lower than what would be expected if the loci were independent and associated randomly. Since causal loci are unknown a priori, their detection occurs through the linkage of marker loci with the phenotype through their implicit LD with causal loci (Mulualem and Bekeko 2016). To perform such an exercise, it is necessary to (i) have a segregating progeny in order to access the relation between LD and genetic distance between markers, (ii) determine the alleles at the genetic markers (genotype) for the considered trait of each individuals of the progeny, (iii) measure the phenotype at the QTL for each individuals of the progeny, (iv) search the loci whose genotype is correlated with the QTL, and (v) estimate the genetic parameters of the detected QTL. For such estimation, one can use (i) the heritability when it is large enough, (ii) the average BV from the progeny obtained by autofecundation of an individual, or (iii) the backcrossing with a reference line, which is a strategy used when producing hybrid varieties. If considering codominant markers individually, one may follow the segregation of the three alleles for each locus and measure the average value of the trait considered corresponding to each genotype. A variance analysis is then carried out to verify if the differences of means are statistically significant or not (Kujur et al. 2016). Each marker produces a specific partition of individuals in the progeny or population in such a way that it is possible to filter out the genotypes that show an association with the QTLs they represent. Since markers for a given QTL are in the same linkage group, the fact of classifying individuals according to their genotypes at marker loci also has the consequence of classifying them according to the QTL. Generally, QTL and markers are not entirely linked, which means that individuals homozygous at the markers are not necessarily homozygous to the linked QTL due to the recombination rate. Thus, the additive effect is estimated at the marker loci; its representativeness of the QTL depends on the strength of the linkage relationship between the marker loci and the QTL.

Recombination rate is larger in outcrossing species as in maize compared to species that exhibit self-fertilization, such as *Arabidopsis*, rice, soybean, and wheat, which leads to a slower LD decay. Larger recombination rate induces a larger genetic diversity and offers a higher likelihood to precisely identify QTL location because the faster LD decays, the better it enables to restring the prediction of the QTL extension (Zhong 2008). Publicly available information on LD decay in *J. curcas* is still very limited to date (Li et al. 2017b; Xia et al. 2018). The alleles at a QTL locus are likely to be in LD with a number of marker loci, to varying extents. Ideal marker data would exhibit high LD between the alleles at the QTL and alleles at nearby marker loci and that LD would rapidly diminish with genomic

distance from the marker locus. Even in that ideal situation, the QTL effect is likely to be distributed across a number of the marker loci. However, these individual locus effects are collectively capturing the same QTL effect.

12.4 Genome-Wide Association Study

Although marker-assisted selection has played an important role in plant breeding, its application in the improvement of QTL such as seed yield and seed oil content is challenging because those traits are controlled by numerous loci with small effects easily affected by the environment, which result in small to moderate heritability. Darvasi et al. (1993) showed that the power of QTL detection and location depends more on the population size than on the marker density. In addition, it is better to promote one repetition over many different genotypes than fewer genotypes with more repetitions (Knapp and Bridges 1990). This evidence led to the mapping of QTL within segregating outcrossing populations. As proposed by Meuwissen et al. (2001), a *genome-wide association study* (GWAS) can be used as a prediction model by associating marker information with phenotypic information to find novel QTLs or candidate genes for various traits simultaneously over large populations through space and time, which can lead to the identification of useful genes that make different contributions and can allow their functional characterization (Korte and Farlow 2013; Gupta et al. 2014).

GWAS involves a genome-wide search for chromosome fragments with significant association with phenotype; it directly examines the statistical association between genetic markers and phenotypes in a broader germplasm context than linkage mapping. GWAS combine *linkage* and LD information. The linkage or QTL mapping information is generated by recombinations within the pedigree, detected by marker genotyping of parents and their offspring. In the LD or *association mapping*, information is generated by ancestral recombination associations between individuals at population level. Instead of generating an imputed marker value from haplotypes and predicting trait value from the imputed marker value, one can simply predict the trait directly from haplotypes if considering a large population with significant phenotype variation. When marker spacing is substantially less than the extent of LD, there is a potential benefit from a multi-locus analysis. GWAS applies equally well to the linkage disequilibrium in a population history than in families. The main difference being that in an association study performed in a population, the extent of LD and hence potential resolution is much less. Thus, it is only necessary to adapt the size of the genomic window to fit LD in each case (Marchini et al. 2007; Servin and Stephens 2007).

Bayesian multiple-regression methods are being successfully used for genomic prediction and selection. These regression models simultaneously fit many more markers than the number of observations available for the analysis. Thus, the Bayes theorem is used to combine prior beliefs of marker association with the phenotype, which are expressed in terms of prior distributions, with information from data for

inference. The best posterior distribution is supposed to match the genotype QTL association. The Bayesian method is additive in the sense that the posterior of one analysis may be used as prior for another; by this way, the information can be extracted from heterogeneous data over time and space (Fernando and Garrick 2013). One Bayesian approach to GWAS makes inferences using samples from the posterior distribution of genotypic effects on the phenotype obtained in the training phase of genomic prediction. The approach to GWAS of estimating the effect of one genotype at a time to find its association to a phenotypic trait is prompt to bias induced by population stratification (Hao et al. 2010), such as the admixture of different founder groups in different relative frequencies. Thus, associations due to LD between a marker and causal locus induced by recent population structure are considered spurious if the marker is not located close to a causal variant. Such *non-causal* correlations have limited benefit in forming predictive models or finding causal variants. However, the simultaneous fitting of genotypes at different loci simultaneously accounts for known or unknown population structure even in admixed populations (Toosi et al. 2010).

Bayesian GWAS requires genotypic and phenotypic information as well as a model that describes the factors that influence phenotype and specification of prior distributions in order to derive the posterior distributions that can be used for inference (Sorenson and Gianola 2002). It is the model and its parametrization that link prior and posterior probability distributions given the data. Large population size is necessary to warrant that posterior distribution is not influenced by the prior one. The occurrence of a rare allele also poses a problem in a GWAS because of the lack of statistical significance (Gupta et al. 2014). This problem can be addressed by using a combination of QTL analysis or linkage mapping with association mapping and a large population size in which the rare allele may be present in a sufficient number in order to detect the missing heritability (Li and Leal 2008; Zhu et al. 2011; Gibson 2012).

The best markers for GWAS are biallelic in order to be able to securely associate a breeding value; however, SNPs are sufficient to uncover LD. One of the major interests from a GWAS is the identification of markers that have the best association with the observed variation of a QTL. There are a number of approaches for ranking the covariates to identify the loci with the largest effects. In models that fit dense SNP maps simultaneously, the contribution of each marker to explain the phenotypic trait tends to be small because most of them will be in close LD. In such cases, most markers will have at least some high LD counterparts, and the markers tend to fit simultaneously. Markers might lack contrasting posterior probability because they are not well associated with the trait or because there are many other markers with which they are in very high LD. Inference about individual effects from a model that fits markers simultaneously is therefore difficult. SNPs located in genic regions explain more variation than those in intergenic regions, which is consistent with the fact that the recombination rate is larger in euchromatin than in heterochromatin (Garrick and Fernando 2013).

The underlying concept of a genomic breeding value (Burgueño et al. 2012) is that it results from the sum of the additive effects across the QTL, but without the

knowledge of the QTL it treats the marker loci as the QTL itself, and sum up the marker effects to get the genomic breeding value (Alves et al. 2015). Thus, to optimize the likelihood of this association, one may reduce the marker density in order to increase their prior odds in subunits of the genome or a smaller particular window over the genome (Garrick and Fernando 2013). Commonly used marker densities would typically result in 500–1000 at any particular 1 Mb genomic window. The majority of GWASs have been conducted using single nucleotide polymorphism (SNP) markers, but some association studies have used simple sequence repeat (SSR) markers (Zhang et al. 2015b).

Rather than to apply a costly genotyping of all genome markers in a large population, it is clever to restrict the genotyping campaign to QTL regions by mapping LD in the population. A by-product of this approach is that most spurious associations due to population structure will be eliminated by the QTL mapping study. Typically, the windows that are significant at a p-value <0.05 in terms of genetic variation for the character considered and the genetic variation larger than expected under an infinitesimal model are considered to explain the target QTL. Under the infinitesimal model, quantitative traits of the offspring follow a normal distribution around the average of the parents (Yang et al. 2013b). This distribution has a variance that is independent of the parental trait value, and, in a large outcrossing population, the variance remains constant despite selection. With inbreeding, the variance decreases in proportion to relatedness. Under the infinitesimal model, the distribution of genetic components within families remains normal with the variance evolving in a way that is entirely determined by relatedness (Barton et al. 2017).

In *J. curcas*, GWASs have proven successful in predicting and obtaining a selection gain on the grain yield and weight of 100 seeds using a varying number of markers, from 2 to 1248, because the genetic variance between evaluated genotypes was significant. A training model of 1000 and 800 markers was proven to be sufficient to capture the maximum genetic variance and, consequently, maximum prediction ability of grain yield and weight of 100 seeds, respectively (Azevedo Peixoto et al. 2017). The combination of GWAS population with inter-crossing lines from Central American germplasm (Laviola et al. 2018), *next-generation sequencing* (NGS), and high-density marker resources (Xia et al. 2018) promises to accelerate the process of selective breeding in *J. curcas*.

12.5 Biotechnology for Selective Breeding

The selective breeding processes outlined above have a long history of victories by bringing many food plants in the range of industrial production sustaining the increase of human populations worldwide to reach the unprecedented number of >7 billion people. However, depending on the recombination rate, unrelated genes can be inherited in the same QTL, and it can be difficult to isolate one trait from another (Eshed and Zamir 1995). Actually, the power of QTL detection depends not

only on the additive effect at the QTL but also on the genotypic variance, the environmental effects, and the epistatic effect of other QTLs in the same region. With the eruption of molecular biology since the 1980s, biotechnological approaches were developed that may help to unravel the relationship between genotype and phenotype. Forward genetics is the approach of determining the genetic basis responsible for a phenotype, which was initially performed by using naturally occurring mutations or inducing mutants with radiation, chemicals (Na Chiangmai et al. 2014), or insertional mutagenesis (e.g., transposable elements). This process of mutation induction was typically followed by subsequent mutant isolation and gene mapping. However, QTL cloning can only be thought about if localized at a scale well below one centimorgan in the range of the size of a fragment cloned in a *bacterial artificial chromosome* (BAC). In addition, confirming that the isolated gene effectively encodes the QTL considered needs transformation (Kumar et al. 2013; Ye et al. 2013; Franco et al. 2016), which may cause other technical problems. An example of such process has been the study of biological mechanisms behind the tolerance of *J. curcas* to drought with transgenic *Arabidopsis* or tobacco plants improved for their tolerance against drought or salinity stresses by transforming them with several *J. curcas* genes (Liang et al. 2013), but few attempts of genetic transformation were made to improve the stress tolerance of *J. curcas* itself (Tsuchimoto et al. 2012). Another use of transformation refers to insertional mutagenesis (Feldmann 1991) with several cases described in plants.

QTLs generally depend on the additive effects of polygenes; the transfer of several genes by synthetic biology and metabolic engineering is a difficult task. An alternative is to transform the plant with only one representative gene regulated by a strong promoter (Deikman et al. 2012); it is indeed the strategy that is generally followed. An example of this sort on the way is the expression of key genes in the auxin pathway including ARF and IAA families and downstream effectors to control seed size in *J. curcas* (Ye et al. 2014; Sun et al. 2017).

In the case where a character is encoded by few main genes, *expression QTL* (eQTL) can be searched in the transcriptome. Then, by PCR amplification in line with a broad genetic context, one may isolate SNPs for the corresponding amplicons. Finally, these SNPs can be used as probes for location on a genetic map (Gomes et al. 2010). Forward genetics can be thought of as a counter to reverse genetics, which determines the function of a gene by analyzing the phenotypic effects of altered DNA sequences.

Reverse genetics is one of the most important tools of functional genomics, which in contrast to forward genetics can identify phenotypes for specific genes much faster. Several reverse genetic approaches have been developed in order to understand gene function including homologous recombination, antisense or RNA interference (RNAi) suppression, target gene inactivation, and target-induced local lesions in genomes (TILLING) (Maghuly et al. 2013). Finally, genome editing through CRISPR/Cas system allows the targeted cleavage of genomic DNA guided by a customizable small noncoding RNA, resulting in gene modifications by both nonhomologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms (Belhaj et al. 2013).

The detection of genes involved in biosynthetic pathways is an imperative element of medicinal plant biotechnology. In addition to the techniques just outlined, this process has traditionally been performed by means of labeling (Fernie and Morgan 2013). For example, condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate led to the production of all terpenoids (Poulter et al. 1981; Dudareva et al. 2005). This process can benefit from the use of various elicitors (Zulak et al. 2005) since secondary metabolite synthesis is often activated in stress conditions such as pathogen invasion. Alternatively, the production of secondary metabolites in specific plant organs (Lommen et al. 2006) can be investigated through differential gene expression using transcriptome data. Reverse genetics has become central to various functional genomics strategies involving RNA profiling (transcriptomics), protein profiling (proteomics), and metabolome study (metabolomics) for the prediction of pathways involved in secondary metabolite production (see Khatri et al. 2017 for a review). Thus, biotechnology is an important tool for the validation of system biology inferences.

12.6 Pathway Modeling

Soon after its completion about one decade ago, it was realized that the human genome was much more than its genes. Cells are tightly regulated in time and space; they are externally decorated by an array of receptors that are specific to their tissue location and function. These receptors receive chemical signals in the form of small organic compounds (hormones) and contact interaction with neighboring cells. The signal is transmitted internally all the way to the nucleus through the signaling network. The endpoints of signaling network are transcription factors that interact according to a Boolean algebra with gene promoters on the genome DNA. Activated genes transcribe their DNA code in protein products that self-regulate and regulate others through several types of feedback loops. Some of these actions activate or repress genes involved in several structural, signaling, or/and enzymatic functions. Enzymes move the cell into its functional program within the context of the whole body regulation. In the case of plants, which are sessile organisms, the management of environmental response to trigger proper cellular response is essential. Thus, cellular and molecular modelings, which were possible thanks to high-throughput techniques of genome, transcriptome, metabolome, and other -ome characterizations, constitute another layer of information that may help to better understand the relationship of genotype and phenotype. Such information is especially valuable when metabolic engineering is envisaged.

System biology is the computational and mathematical modeling of complex biological systems. It is a biology-based interdisciplinary field of study that focuses on complex interactions within biological systems, using a holistic approach to biological research. One of the aims of systems biology is to model and discover emergent properties of cells, tissues, and organisms functioning as a system; it typically involves cell networks. For instance, Grover et al. (2014) showed in

J. curcas that according to a gene ontology (GO) analysis, 931 unigenes were related to fatty acid or lipid metabolism pathways, and those overrepresented were classified into the GO terms "fatty acid metabolic process" and "fatty acid biosynthetic process."

12.6.1 Signaling Networks

Cellular networks are graphs whose interacting species, be them genes, proteins, or other biological components, are represented by vertices and their interactions by edges, which are drawn between them. It is common to divide networks in protein interaction network, genetic network, transcriptional network, and the metabolic network (Costanzo et al. 2010). Data regarding other networks taking part in the cellular processes, such as sRNA and RNAi-mediated networks, is still lacking because their knowledge is currently little.

12.6.1.1 Interactome

In a constant effort to explain the genotype-phenotype relationship, modeling has extended its activities from classical genetic approaches to molecular descriptions. Biological entities are involved in intricate and dynamic interactions, which form complex systems. Knowledge cannot translate into new mechanistic understanding by just considering the oversimplified *one-gene/one-enzyme/one-function* representation of the genotype-phenotype relationship. QTLs provide compelling pieces of evidence of the complexity between genotypes and phenotypes. GWAS revealed many more contributing loci than originally anticipated, with some loci contributing as little as a few percents to the heritability of the phenotype(s) of interest (Carvunis et al. 2013).

Most individual proteins execute their biological functions by interacting with one or several other proteins. Typically, molecular interactions are represented by graphs termed *interactomes*. In graphs representing signaling networks, physical interactions between vertices are best described as undirected edges. A protein may be involved in more than one binary interaction with its neighbors; when the number of interacting neighbors is high (typically larger than 50), a vertex is said to be a *hub* (of connections). Graphs of signaling network are strongly heterogeneous in the distribution of edges per vertex (*degree*) that follow a power law and are named *scale-free*. In networks characterized by such a degree distribution, most vertices have only a few links, whereas a few nodes (hubs) have a disproportionately large number of links. These hubs are the glue that binds the majority of low-degree vertices together in such a way that each vertex of a scale-free network can be reached by a path of a few steps from any other one (Barzel et al. 2013). The robustness of cellular networks relies strongly on the hub vertices and

consequently works as a double-edged sword because despite allowing the networks to withstand a large number of random failures, it makes them extremely vulnerable to intentional interventions. The removal of just a small number of key hubs will cause a scale-free network to break down into isolated dysfunctional clusters (Albert et al. 2000; He and Zhang 2006).

Protein interactions can form large protein complexes, such as molecular machines (ribosomes, proteasome, etc.), performing functions that no single protein can assume, which demonstrates that protein-protein interactomes exhibit emergent properties beyond the sum of all individual protein interactions. Protein complexes typically contain five to six different proteins, within a wide range from two to hundreds (Gavin et al. 2002).

Owing to cooperative and allosteric effects, a protein may have a high affinity for two others simultaneously, so that the tripartite protein complex is considerably more stable than the sum of its component affinities (Williamson 2008). The characterization of entire protein complexes, as they assemble in cells, is a necessary route to gather information on gene function and biological systems (Fraser and Plotkin 2007; Gavin et al. 2006; Wang and Marcotte 2010). When two proteins belong to the same protein complex, they may not necessarily be in direct physical contact. Hence, edges in interactomes involving protein complex representation have a very different meaning than edges in networks just representing protein-protein (binary) interactions. The working hypothesis is that interactomes exhibit local and global properties that are related to genotype-phenotype relationships.

Densely connected subgraphs corresponding to topological modules in interactome networks most likely coincide with specific biological processes or functions (Barabási and Oltvai 2004). It follows that identifying modules containing genes or proteins of both known and unknown function can help to assign functions to uncharacterized genes or proteins (Jansen et al. 2003; Bader and Hogue 2002; Bar-Joseph et al. 2003; Ihmels et al. 2002; Stuart et al. 2003; Tornow and Mewes 2003). Because (i) protein interactions mediate protein functions as well as (ii) protein interactions tend to connect genes involved in related phenotypes just as they tend to connect genes and proteins with related functions, it is suggested that protein interactions can be used to predict the genes of given traits. Actually, GWAS provide an emerging example where predictions can be attempted without training examples. Although GWAS serve to identify a genomic locus associated with a trait, it often cannot pinpoint which genes among several or many of those residents within the locus are the actual genes encoding the trait concerned. By contrast, the subset of genes encoding interacting proteins is likely to contemplate the causal genes of the trait under consideration (Franke et al. 2006). If interactome networks underlie genotype-phenotype relationships, then edges (protein interactions) should be associated with functions and phenotypes just as vertices (proteins) are. Actually, there are unbiased pieces of evidence for a correlation between degree, essentiality, and functional pleiotropy (Yu et al. 2008). In addition, proteins

associated with cancer are preferential hubs in the human interactome (Goh et al. 2007; Tilli et al. 2016; Carels et al. 2015).

A central hypothesis of systems biology is that intricate molecular networks within cells are also governed by natural selection. Hence, understanding the principles driving the evolution of molecular networks would contribute to a deeper understanding of genotype-phenotype relationships. If protein-protein interactomes were evolutionarily stable systems, interactions between orthologous protein pairs from distinct species should be largely conserved. However, the observed fraction of conserved interactions is low across species (Beltrao and Serrano 2007; Levy and Pereira-Leal 2008; Shou et al. 2011). Conservation of biological interactions is maintained between species but mainly at the module level rather than at the level of individual genes or proteins (Kapitzky et al. 2010; Zinman et al. 2011). If a vertex has many edges, it is more likely to acquire new edges, creating a state where the rich gets richer. The result is that the more connected vertices gain new edges more readily and eventually emerge as hubs. This process is rooted in that of gene duplication (Barabási 2016) and is clearly responsible for network growth, as duplicated genes produce duplicate proteins and thus introduce new vertices into the network. A vertex that was introduced early in the history of the network will have more time to accumulate edges and, by the rich-get-richer mechanism, enhance its chances of becoming a hub (Bellay et al. 2011).

Different types of protein interactions are rewired at different rates. Transient interactions appear more evolutionarily volatile than the more lasting interactions forming protein complexes (Teichmann 2002; Roguev et al. 2008; Shou et al. 2011), and protein-peptide interactions appear to change more rapidly than interactions between large proteins (Beltrao and Serrano 2007). In response to extracellular perturbations, protein complexes generally remain stable, but the functional connections between these complexes are substantially reorganized, as reflected by genetic interaction changes (Bandyopadhyay et al. 2008; Hannum et al. 2009; Ideker and Krogan 2012).

12.6.1.2 Transcriptome

The representation of protein interactions available through protein-protein interactome maps is still mostly static as in psimitab (ftp://ftp.ebi.ac.uk/pub/databases/intact/current/psimitab/), for instance. However, a real interactome is dynamic, which is needed to represent the interactions that may occur in all cell locations, times, and environments. The development of *nucleic acid programmable protein array* (NAPPA, Ramachandran et al. 2004) allows the mapping of until 12,000 real-time interactions at once (Yazaki et al. 2016). A simple approximation is to use bioinformatics to integrate interactome maps and expression profiles. This process may enable to identify biological conditions, whereby two proteins that can interact, according to an interactome map, are also co-expressed, according to their expression profiles. Physically interacting proteins are more likely to exhibit similar expression patterns than would be expected by chance (Ge et al. 2001; Jansen

et al. 2002; Yu et al. 2008). These quantitative genetic interaction profiles enabled the construction of a global network in which genes with similar interaction patterns are located next to one another, while genes sharing less similar interaction profiles are further apart in the network (Costanzo et al. 2010). The resulting network provides a multiscale view of the functional organization within a cell. Globally, genes displaying tightly correlated profiles form large and readily discernable clusters corresponding to distinct biological processes. The relative distance between these clusters appeared to reflect shared functions highlighting the interdependencies of general cellular processes and the inherent functional organization of the cell. In general, this local network structure reveals genes that belong to the same functional module and the genetic relationships between separate modules that share partially redundant functions. Compromising the function of either nonessential module leaves the cell viable but simultaneously compromising both results in cell death. Redundancy between genes appears to be more frequently the result of module-level compensation rather than single-gene buffering (Bellay et al. 2011). However, most interacting proteins are not co-expressed and some pairs are even anticorrelated in expression. Interactome dynamics, therefore, appear to be under tight transcriptional control, with most protein interactions being transient.

Network perturbations such as those induced by RNA interference (RNAi) or treatment by inhibitors or elicitors may help in the characterization of gene interaction through analysis of gene co-expression. Transcriptional signatures resulting from the loss/reduction of individual network components by RNAi have been used to infer the flow of information through proteins that are interconnected within a cellular network (Boutros et al. 2002).

The integration of interactome and transcriptome data has not yet been described for *J. curcas* even if these data exist independently as shown above.

12.6.2 Genetic Networks

Protein-protein or protein-DNA interaction networks define connections that support local, molecular-level interactions, whereas genetic interactions capture more general information revealing how genes are broadly organized into modules that support cellular function. The main distinction is that genetic interactions capture functional consequences of (combined) genetic perturbations, whereas most other mapping efforts highlight physical interactions, such as protein-protein (Gavin et al. 2006; Krogan et al. 2006; Tarassov et al. 2008; Yu et al. 2008) or protein-DNA (Harbison et al. 2004; Rhee and Pugh 2011) interactions.

As mentioned above, geneticists have long recognized that genetic interactions are important for shaping the phenotypic landscape of a population. The term *epistasis* used to describe a specific type of genetic interaction whereby one mutation masks the effects of another mutation has been expanded to include any multi-locus mutant effect that deviates from the additive combination of the corresponding individual loci. Today, epistasis is often used to generally define a genetic interaction

as an unexpected phenotype that cannot be explained by the combined effects of the individual mutations. For instance, negative genetic interactions describe more severe phenotypes than expected and are interesting because they often occur between genes that impinge on a common essential biological function (Tong et al. 2001). By contrast, positive interactions describe mutant phenotypes less effective than expected.

The prevalence of modular structure in both negative and positive genetic interactions provides a rich basis for associating genes with a common function and broadly characterizing the functional organization of genomes. Each specific *between-pathway* set of interactions defines two specific functional modules and highlights their compensatory relationship. Interestingly, genes sometimes appear in several different between-pathway structures where each structure is composed of a unique set of genes, enriched for a distinct function. This observation highlighted the utility of genetic networks for identifying multifunctional or *pleiotropic* genes (Bellay et al. 2011). Network hubs tend to be pleiotropic and interact with many functionally diverse sets of genes (Costanzo et al. 2010). Hubs may represent general buffers of phenotypic variation because they are capable of enhancing the phenotypic consequences associated with mutations in numerous different genes.

In addition to the between-pathway structure, another type of genetic interaction network motif includes a set of negative interactions that connect a common set of genes (a *clique-like* structure in graph theory terms). In this structure, known as *within-pathway*, a set of genes all exhibit negative genetic interactions with each other, however, they appear to be much less abundant than between-pathway structures and to correspond to essential protein (Kelley and Ideker 2005; Baryshnikova et al. 2010; Bandyopadhyay et al. 2008) complexes or groups of co-regulated genes (Bellay et al. 2011). Like negative genetic interactions, positive genetic interactions also exhibit modular structure, although to a lesser extent.

There is little direct overlap between genetic interactions and physical networks. Only 10–20% of protein-protein interaction pairs were found to also share a negative or positive genetic interaction. Thus, the large majority of either type of genetic interaction, negative or positive, do not reflect direct physical binding events among the corresponding gene products (Costanzo et al. 2010; Baryshnikova et al. 2010). The relatively low overlap between genetic and physical interaction networks is closely related to the observation that most negative and positive genetic interactions occur in between-pathway network structures, which reflect two distinct modules of genes bridged by a large number of genetic interactions between them. In fact, protein complexes often appear within the sets of genes on either side of these between-pathway structures (Kelley and Ideker 2005; Bellay et al. 2011), suggesting that genetic and physical interactions are largely orthogonal to one another.

Genetic studies do not distinguish between direct and indirect effects, and therefore it is not clear where the different genes identified may act in a network. Understanding how they contribute to the overall structure of cellular signaling requires the integration of genetic data with other datasets such as protein-protein interaction networks (Kulkarni and Perrimon 2013).

12.6.3 Regulation Networks

12.6.3.1 Gene Regulatory Network

Cells have evolved a repertoire of sophisticated regulatory mechanisms that measure and process environmental input in order to exert the appropriate biological output. In addition to RNA polymerase II, a set of general transcription factors (TFs) is required for the modulation of all eukaryotic genes transcription at short DNA sequences located within gene promoters, enhancers, or other types of regulatory sequences. TFs are proteins that control the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequence. The core promoter is ~100 bp region, made up of a succession of several TF binding sites that span 3–8 bp, whose function is to provide a docking site for the transcription complex and to position the start of transcription relative to CDSs (Lee and Young 2000). Clusters of approximately 6–15 binding sites for 4–8 different transcription factors may operate as functionally coherent segments termed enhancers (Arnone and Davidson 1997). The terms enhancer, booster, activator, insulator, repressor, locus control region, upstream activating sequence, and upstream repressing sequence all refer to various types of regulatory sequences with specific features that function as Boolean (off/on) or scalar (quantitative) elements whose interactions have predictable, additive effects on transcription (Tuch et al. 2008). Deleting one of these regulatory sequences often eliminates a specific aspect of the expression profile without disrupting the remainder.

The function of TFs is to regulate, turn on, and turn off genes in order to make sure that they are expressed in the right cell at the right time and in the right amount throughout the life of the cell and the organism (Wray et al. 2003). TFs are a fundamental part of a regulation network. Most transcription factors directly regulate a few percents of the genes. Genetic networks are therefore highly connected, and each node that is represented by a transcription factor is linked to many other nodes. This high degree of connectivity may be responsible in large part for the classical genetic phenomena of epistasis, polygeny, and pleiotropy. The expression profile of a gene is a system property; thus, even if a mutation in a promoter region alters transcription, the network of functionally interacting genes and gene products may modulate this effect. Feedback loops are rather common components of gene networks (Lee et al. 2002) and may mask some functionally significant mutations in promoters.

Gene regulatory network (GRN) followed a *hierarchical* organization that can lead to signal amplification and confers adaptability (Ravasz et al. 2002; Jothi et al. 2009). Interestingly, the TFs that reside in different GRN layers have distinct attributes. For instance, TF hubs that regulate large numbers of downstream targets are rarely found at the top of the hierarchy. Similarly, bottleneck TFs are mostly found in the middle layers. Studies agree that the TFs occurring in the top layer are most highly conserved.

A high degree of modularity has been proposed to facilitate a quick response to external cues and has been observed in several metabolic networks and GRNs. In GRNs, two types of modularity can be observed: TF modules that are characterized by sharing target genes and gene modules that share interacting TFs (Vermeirssen et al. 2007). Redundant TFs are expected to reside in similar modules and may share target genes by binding highly similar DNA sequences.

GRNs can be of different types of sub-circuits or building blocks that provide different types of information flow and logic of gene control. A special type of such a building block is called a *network motif*, which is defined as a type of circuit that is overrepresented in real networks compared to randomized networks (Bulyk and Walhout 2013).

In some examples, specific types of GRN circuitry were shown to be responsible for discontinuous, hysteretic responses to different levels of signal input (Saka and Smith 2007; Goentoro et al. 2009; Davidson 2010), which may be the general explanation for the often observed Boolean regulatory response to graded signaling ligand distributions. Because transcriptional regulation conditions the way in which genotype is converted into phenotype, functional genetic variations in promoter sequences within populations are sorted by selection. Many mutants that have emerged from genetic screens involve quantitative effects on transcriptional regulation and are constituents of QTL.

Studies on different tissues dissected from *A. thaliana* roots examined by gene expression profiling have uncovered gene expression patterns important in plant root development that will contribute for deciphering the GRNs in plants (Brady et al. 2007; Moreno-Risueno et al. 2010).

12.6.3.2 Regulatory Factors in *J. curcas*

Among the genetic regulatory factors described in *J. curcas*, one may cite noncoding RNAs (ncRNA) in addition to TFs. A genome-wide search has enabled to discover 1481 putative TF-encoding gene models that were classified into 61 TF families (Mochida and Tran 2017; http://treetfdb.bmep. riken.jp/index.pl). Because of the hierarchical regulatory structure of TFs and their multiple gene targets in a transcriptional regulatory network, TF-encoding genes are often key genes of certain biological functions, such as physiological response and metabolism. Molecular interactions of TFs with other transcriptional regulators or with noncoding RNAs are also crucial in the transcriptional regulatory network. In *Arabidopsis*, nearly 1500 TF-encoding genes were identified from its 26,000 annotated genes (Riechmann et al. 2000). A number of TFs have been characterized in developmental regulations such as phytohormone-signaling pathways (Ohashi-Ito et al. 2013; Aya et al. 2014). In crop species, particular mutation of TFs involved in plant morphology was selected during the domestication and breeding (Doebley et al. 2006; Sakuma et al. 2011).

NcRNA can be small (smRNA) or long (lncRNAs) and can act by interference on genes expression as part of complex regulatory circuits. Genomic loci of many lncRNAs are associated with histone modifications and DNA methylations suggesting an epigenetic regulation of these loci. In addition, some sense and antisense double-stranded RNAs involving lncRNA partners are processed by the RNA interference machinery into *short-interfering RNAs* (siRNAs; Jin et al. 2013). SiRNAs and *microRNAs* (miRNAs) act to regulate endogenous genes and to defend the genome from invasive nucleic acids (virus, mobile elements). The effects of smRNAs on gene expression and control are generally inhibitory, and the corresponding regulatory mechanisms are referred to as RNA silencing.

In *J. curcas*, miRNAs were described in successive experiments: (i) 52 miRNAs were identified to control genes in fruits and seeds (Wang et al. 2012); (ii) 78 potential genes categorized into three miRNA families were proposed to regulate cell growth and development, signaling, and metabolism of oil synthesis (Vishwakarma and Jadeja 2013); and (iii) 180 conserved miRNAs, 41 precursor miRNAs (pre-miRNAs), and 16 novel pre-miRNAs were identified from three libraries of *whole transcriptome shotgun sequencing* (RNA-seq) of immature, intermediate, and mature seeds (Galli et al. 2014). These miRNAs are valuable information to better understand the QTL they control.

12.6.4 Metabolic Network

A living cell can be abstracted as a self-replicating system (Sousa et al. 2015; Barenholz et al. 2017; Hordijk et al. 2018): a group of molecules that, complemented by a small set of imported nutrients, serves as substrates for their own production following a series of transformations promoted by enzymatic chemical reactions.

One can give a network perspective to the metabolic capabilities of a cell with reactions as vertices and (directed) edges that reflect the existence of one or more metabolites serving both as a product in the former reaction and as a substrate in the latter. Provision of metabolic demands from specific nutrient sources can be visualized as a flow of metabolites through a sub-network of selected nodes. Since enzymes and transporters result from the expression of particular sets of genes, one might obtain, from accurate genome annotation, valuable information to assess organism-specific genotype-phenotype interactions. Genome-scale metabolic reconstructions (GEMs) are cross-disciplinary efforts (Wang et al. 2017) that generate, following refined creation protocols (Thiele and Palsson 2010), organism-wide libraries of biochemical transformations, usually accompanied by gene-protein-reaction (GPR) relationships linking genomic information to enzymatic reactions and transport processes. Available to a large number of organisms,[1] they constitute

[1]Check http://systemsbiology.ucsd.edu/InSilicoOrganisms/OtherOrganisms for an updated list of available reconstructions.

the best representation of an organism's metabolic capabilities, creating well-defined mathematical objects that pave the way to quantitative predictions derived from powerful analytical tools (Bordbar et al. 2014; Maranas and Zomorrodi 2016; Vazquez 2017; Yilmaz and Walhout 2017).

12.6.5 Network Modeling

In addition to a network structure in vertices and edges, it is desirable to describe the dynamics of mass or information transfer through it. Dynamic models characterize the vertices by states, such as concentration or activity, which change in time according to the interactions encoded in the network wiring. Continuous dynamic models use sets of differential equations to capture the detailed variation of concentrations of key substances in the system. However, parameter estimation in large-scale systems is often impossible to set. To circumvent parameter estimation, one may perform discrete dynamic modeling such as Boolean networks (Li et al. 2006) and Petri nets (Chaouiya 2007). To be representative, the model has to match common situations and be refined iteratively. Discrete dynamic models generate insightful analysis about the interweaving of system components and the cascade of information flow; it enables experiments of in silico vertex knockout that produce informative predictions about the system.

12.6.5.1 Boolean Modeling

Discrete dynamic models have been successfully implemented in numerous biological systems, facilitating the study and greater understanding of biological processes such as flower development (Mendoza et al. 1999; Espinosa-Soto et al. 2004) and hormone signaling in plants (Li et al. 2006; Diaz and Alvarez-Buylla 2006). Boolean network modeling of biological systems is the simplest of the discrete dynamic models. Each vertex can have one of two discrete states, namely, 0 and 1. The state of a system that has N nodes is therefore represented by an N-dimensional vector with each value being 0 or 1. The network must be complemented with rules that specify the ways in which all upstream components are combined. A natural and economical method is to use the Boolean operators AND, OR, and NOT whose combination can describe most possible relationships or reactions between substances and components in a biological system.

The state of the system at a time step is determined by its predecessor state (s) through Boolean transfer functions. The calculation of a vertex state at a time step based on system state(s) at earlier step(s) is called updating. Depending on the updating scheme used, a number of vertices, ranging from 1 to N, are updated, thereby obtaining the system state at a new time step. As time evolves, the system state (N-dimensional vector) goes through the state space, and after a finite number of transient states, it settles into an attractor. Two types of attractors are possible:

(i) after hitting a certain state, any future updating results in the same state, hence the system reaches a steady state invariant of time (attractor); or (ii) there exists a small set of states that the system keeps revisiting over time (Sun and Albert 2013).

Boolean dynamic modeling of a biological system is comprised of the following steps:

1. To reconstruct the network based on biological knowledge. Extensive literature searches and compilation enable to denote the relevant elements of a system by vertices and their pairwise relationships by edges.
2. To determine the Boolean transfer functions. The Boolean transfer function expresses the way the states of the input regulators of a given vertex are combined through the Boolean operators AND, OR, and NOT. If two inputs functions are largely independent, they will not exhibit synergy and can substitute one another, then the Boolean OR function (rather than AND otherwise) should be used. The more inputs a node has, the more possible ways exist to combine all the input states, and it is needed to be careful in obtaining the rule that is able to generate dynamic simulation results that best fit the experimental data.
3. To choose an updating scheme among two major types: synchronous and asynchronous (Papin et al. 2005). For synchronous updating every vertex is updated once at each time step, using the states of the input vertices at earlier time step as inputs to the transfer functions. Synchronous updating overlooks the differences of time scales on which biological processes are taking place, which can range from milliseconds for protein phosphorylation and posttranslational modifications to hundreds of seconds for transcription and transcriptional regulations (Papin et al. 2005). Asynchronous updating (Thomas 1973), which provides more detailed tracking of time scales and temporal orders (Li et al. 2006), is devised in order to account for the diversity in the duration of biological processes.
4. To determine the initial state of the model. The initial state of each vertex is determined such that it is consistent with known biological facts.
5. To analyze the model, including its attractors and state space. The system will have the same steady states under both synchronous and asynchronous updating, owing to the fact that a steady state repeats itself infinitely, making the order in which the nodes are updated irrelevant. In the case of oscillation of a subset of vertices, the attractor for the state vector of the system will be a limit cycle.
6. Validate the model according to the biological reality (e.g., does a given network wiring or perturbation leads to a fixed level of a metabolite of interest?). An important step toward obtaining a successful model is to examine the dynamic sequence of system states in detail and compare it with biological pieces of evidence.

12.6.5.2 Petri Net

The Petri net formalism combines an intuitive, unambiguous, qualitative bipartite graphical representation of arbitrary processes with a formal semantics. Petri nets are

built from basically four different building blocks, which are (i) *places* that represent passive vertices that refer to conditions, local states, or resources; (ii) *transitions* that represent active vertices that describe local state shifts, events, and activities in the system; (iii) *tokens* that are variable elements that represent current information on a condition or local state; and (iv) *directed arcs* that are connectors (edges) that specify relationships between local state and local action by depicting the relation between transitions and places. Tokens transit across the model along the arcs.

The dynamics of the system is orchestrated by a firing rule that enable tokens to move around a Petri net. A transition is considered to be enabled when tokens are present on all its inputs at the moment of the firing (clock ticking). When it occurs, tokens are consumed from the input places and produced (fired) on the output places.

The intuitive and graphical presentation of Petri net makes easier for biologists to describe biochemical reaction systems, where tokens are interpreted as single molecules or bits of information (events) (Koch et al. 2005, 2011). Moreover, the Petri net formalism provides a natural framework that integrates qualitative (given by the static structural topology) and quantitative (given by the time evolution of the token distribution) aspects tightly integrating different methods for model simulation and analysis as shown in Peleg et al. (2005). There are numerous successful application illustrating the versatility of Petri nets and their use for metabolic (Doi et al. 2004), gene regulatory (Chaouiya 2007), and signaling (Sackmann et al. 2006; Hardy and Robillard 2008) networks. Moreover, the Petri net formalism can provide an integration of models able to represent biological behavior at different levels, i.e., molecular, cellular, organism, and process level, in a multiscale hierarchical structure.

There are several types of Petri net implementations; the standard class is the place/transition Petri nets or *qualitative Petri nets* (QPNs), is discrete, and has no association with time or probability. In QPNs, systems are analyzed in terms of causalities and dependencies, without any quantification.

In *continuous Petri nets* (CPN), the discrete values of the net are replaced by continuous (real) values to represent concentrations over time and no longer an integer. The mark is called token value and can be considered as concentration, where one token value is assigned for each place. The instantaneous firing of a transition takes place like a continuous flow, and the firing event is determined by continuous deterministic rate functions, which are assigned to each transition.

In *stochastic Petri nets* (SPN), the firing delay is a random variable following an exponential probability distribution. The semantics of an SPN with exponentially distributed firing delays for all transitions are described by a *continuous-time Markov chain* (CTMC) (Mura and Csikász-Nagy 2008).

The *hybrid Petri nets* (HPN) combines both discrete and continuous components in one model. It enables to represent biological switches in which continuous elements are turned on/off by discrete elements (Alla and David 1998). The HPN capture the randomness and fluctuation of the discrete stochastic model and allows a reasonable computation performance.

Colored Petri nets (CoPN) is a Petri net modeling concept that extends quantitative and qualitative Petri nets by distinguishing tokens and arcs according to colors in order to specify which token can flow over specific arcs. Moreover, transitions can constraint by additional Boolean expressions (guards).

The integration of modeling and bench experimentation is essential in the creation of a computational system that can simulate events and predict outcomes of biological behavior. Since bench research can be costly, time-consuming, or ethically infeasible, in silico modeling is an important aspect of system biology (Carvalho et al. 2018).

12.6.5.3 Bayesian Modeling

Bayesian networks offer a useful tool for the analysis of many kinds of data. They represent models of dependencies between various variables that can help to understand the mechanism that generated these dependencies and the flow of causality in the system of interest.

Biological systems consist of many components that interact with each other in a consistent manner. At different moments, the system is in different states. If the system being deal with is a cell, the state of that cell can be determined in terms of the number of ligands that bind to receptors on its membrane, the concentration of mRNA (or proteins and metabolites) molecules in cytoplasm compartments, etc. Hence, if considering random sampling, some system states are more probable than others. The likelihood of a state can be deduced from the joint probability between node pairs on all of the cell components. So, by modeling the system as a joint distribution over a collection of random variables that describe the states of a Bayesian network, answers can be given to a wide range of questions (Friedman et al. 2000).

Bayesian networks allow the inference of gene/protein interactions and key biological features of cellular systems from biological data such as gene expression profiles and SNP data. Additional nodes representing other attributes that affect the system, such as exogenous cellular conditions, temporal indicators, and experimental conditions, can be added to the network. As a result, one can model the biological mechanisms of interest and the external conditions that influence them in one single broad network.

The graphical structure of a Bayesian network G=(V,A) is a directed acyclic graph (DAG), where V stands for the nodes that represent random variables and A are the arcs or edges, which are arrows that represent the relationships among nodes that are often (but not always) causal; so they represent the probabilistic dependencies between them.

Joint probability is the probability of two events happening together, whereas the joint probability distribution is the probability of every possible event as defined by the combination of the values of all the variables. Bayesian networks define the probability distribution over *n* random variables. Instead of enumerating all possible combinations of these *n* random variables, the Bayes network is defined by

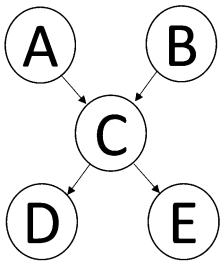

Fig. 12.1 Example of a Bayesian network. A and B are merely defined by P (A) and P(B) because they simply do not have incoming arcs, while C is conditionally dependent on A and B, P(C|A,B), and further determines D and E from P(D|C) and P(E|C)

$$P(A,B,C,D,E) = P(A) \times P(B) \times P(C|A,B) \times P(D|C) \times P(E|C)$$

probabilities distributions that are inherent to each individual node. Bayesian networks reach compactness by factoring the joint probability distribution into local, conditional distributions for each variable. The form of the factorization is given by the Markov property of Bayesian networks, which states that every random variable directly depends on its parents.

Hence, in order a Bayesian network suitably model a probability distribution, the following must be true: each node variable in a Bayesian graph is only conditionally dependent on the value of all its directly connected parent nodes (Fig. 12.1).

In Bayesian network modeling, selection algorithms associate node dependencies to network topologies (structure learning algorithms). Since an exhaustive search associated to the undirected graph underlying the network structure is computationally unfeasible for most data sets, all learning algorithms use some kind of optimization, such as restricting the search to the Markov blanket at each node. The Markov blanket of a node comprises the parents of this node as well as the children of this node including their own parents. The directions of the arcs that are part of a *V* structure are set according to $Xj \rightarrow Xi \leftarrow Xk$. Undefined edge directions are finally set up to satisfy the acyclicity constraint of Bayesian networks. It should be noted that automated network structure inference may not work perfectly, because (i) it may not include all the expected edges and (ii) it may infer some of them in the wrong directions. So the best practice is to combine algorithmic network structure learning with manual editing.

Then, local probabilities are associated to nodes under topological restrictions by setting up a conditional probability table (CPT). The type of local probability can follow (i) a *multinomial model* in which each variable is discrete and depicted as the probability of each possible state of a child variable given the state of its parents or (ii) a *linear Gaussian model* in which a linear regression model is applied to a child variable given its parents.

There are three types of structure learning algorithms. *The constraint-based algorithms* that learn a network structure by (i) analyzing the conditional independence relationship between nodes and (ii) constructing a graph that satisfies the corresponding D-separation statement. $X \subset V$ is d-separated from $Y \subset V$ given $Z \subset V$ if every path from *X* to *Y* in the graph is blocked by *Z*. The resulting models are often

interpreted as causal models even when they are learned from observational data (Pearl 1988). *Score-based algorithms* assign scores to each candidate Bayesian network in order to find the best candidate. Constraint-based methods can be more effective than score-based approaches, particularly with a great number of samples. However, they may not assign a direction to every edge. Hence, the score-based approach is generally preferred when dealing with small sample size and noisy data. *Hybrid algorithms* combine both constraint-based algorithms to reduce search space and score-based algorithms to maximize the choice of the optimal network.

The bnlearn package for R (available online from www.bnlearn.com) is one of the most used packages for Bayesian network inference. It implements several different algorithms. Among the constraint-based learning algorithms, the package uses the following functions: Grow-Shrink (GS); PC (the *stable* version); Incremental Association Markov Blanket (IAMB); Fast Incremental Association (Fast-IAMB); Interleaved Incremental Association (Inter-IAMB); Max-Min Parents and Children (MMPC); and Semi-Interleaved Hiton-PC (SI-HITON-PC). Alternatively, GeNIe is also a very effective and user-friendly package for Bayesian modeling that has the benefit of an easy to understand tutorial and intuitive graphical interface (https://www.bayesfusion.com/).

The bnlearn package also implements (i) score-based structure learning algorithms (Hill Climbing, HC; Tabu Search, Tabu); (ii) hybrid structure learning algorithms (Max-Min Hill Climbing, MMHC; General 2-Phase Restricted Maximization, RSMAX2); (iii) local discovery algorithms (Chow-Liu; ARACNE); and (iv) *Bayesian network classifiers* (naive Bayes; Tree-Augmented Naive Bayes – TAN).

12.6.5.4 Flux Balance Analysis

The organism-wide library of biochemical processes (enzymatic reactions, transport processes, and other metabolite-driven cellular processes like biomass accumulation) provided by a genome-scale metabolic reconstruction (GEM) defines a mathematical object, called stoichiometric matrix S, describing the contribution of each participating metabolite on the reactions from the reconstruction: $S_{ij} = s(-s)$ if s units of metabolite i are produced(consumed) on reaction j (zero if not present). Replication, abstracted as the result of metabolic processes leading to accumulation of biomass precursors, can be accounted for in S as a pseudoreaction having protein, lipids, vitamins, DNA, RNA, and a few other metabolites as substrates with stoichiometries given by their respective amounts in cellular composition. The rate of this reaction is directly compared to experimentally measured values of growth rate (Feist and Palsson 2010), and, in particular, the physiological strategy adopted by an organism in a given context should be associated to one (out of possibly many) metabolic pathway(s) built with reactions coming from its metabolic reconstruction (Feist and Palsson 2010; Yilmaz and Walhout 2017; Bartell et al. 2017).

Avoiding the (practically unfeasible) numerical integration of hundreds to thousands coupled differential equations describing how metabolite concentrations change in time from knowledge of reaction rates defined by kinetic models (available to a small number of enzymatic reactions), constraint-based methods generate

valuable predictions of experimentally verifiable flux pathways connecting metabolite demands to available nutrients with biochemical transformations derived from a metabolic reconstruction (O'Brien et al. 2015; Maranas and Zomorrodi 2016) by means of systematic reduction of the space of feasible solutions and following restrictions imposed by biologically plausible assumptions.

Flux balance analysis (FBA) is a powerful method (Orth et al. 2010) that relies on three main assumptions: (1) mass balance, provided in cells growing with fixed growth rate Neidhardt et al. 1990; Neidhardt 1999) along a reaction pathway in which all internal metabolites are produced and consumed at the same rate, mathematically expressed as

$$\sum_{j=1}^{N} S_{ij} f_j = 0 \quad \forall i \in (1 \ldots M)$$

where S_{ij} is the stoichiometry of metabolite i on reaction j and f_j the reaction's flux (usually defined in mmol/L/h). Since the number of metabolites M is usually smaller than the number of reactions N, there are multiple flux configurations (reaction pathways), which satisfy this set of equations. (2) Physically limited uptake rates that, as noted by Jacques Monod (Monod 1949), constrain growth rates to finite values. (3) Logic association between cellular physiology and optimization of metabolic demands, provided by the definition of an objective function, $H(\{f\})$, with values that quantify the demand provided by each set of reaction fluxes satisfying (1) and (2). Fluxes from the set with largest H are expected to be experimentally detected in optimal phenotypes that are reached following mutation and selection events.

FBA hypotheses have been successfully confirmed on experiments with *E. coli* cells (Ibarra et al. 2002), where maximum growth rates in common carbon substrates were predicted from the metabolic reconstruction as the constrained solutions of FBA with the flux of biomass production reaction as objective function. Selective consumption of substrates or diauxic growth, first predicted by J. Monod (Monod 1942, 1947), has been predicted with FBA extended by an additional constraint on the allocatable volume for proteins promoting each metabolic process (Vazquez 2017; Beg et al. 2007).

12.6.5.5 Modeling Pathways for Secondary Metabolites

It is only recently that biotechnology brought new approaches to disease therapies, but natural products have been the most important sources of lead molecules for drug discovery since the dawn of humanity and its interest is renewed by the availability of high-throughput technologies (http://medicinalplantgenomics.msu.edu/). Microbial resistance, for instance, is projected to be one of the major global challenges to be overcome for the maintenance of our future health systems.

The secondary (or specialized) metabolism is composed of a set of metabolic reactions not essential to organisms' biomass production and tends to be specific to

each species. Plants synthesize a multitude of compounds that contribute to adaptation to their ecological niches. Such compounds serve as attractants of other living organisms beneficial to plants or as defenses against other biotic as well as abiotic agents. Selection for increased fitness, a never-ending process, has resulted in each plant lineage synthesizing a distinct set of specialized metabolites appropriate to its environment. The number of specialized metabolites made by plant species has been estimated at roughly 200,000, which is probably a gross underestimation. The total number of specialized metabolites found in the plant kingdom far exceeds the capacity of any one plant genome to encode the necessary enzymes, and just as a plant lineage acquires the ability to make new specialized compounds during evolution, it also loses the ability to make others. It is clear that each plant species can synthesize only a small fraction of the total number of specialized metabolites found throughout the plant kingdom (Pichersky and Lewinsohn 2011). Among the 26,000 genes encoding proteins in *Arabidopsis*, approximately 20% of the genes are annotated as being involved in the biosynthesis of secondary metabolites (The Arabidopsis Genome Initiative 2000). Lineage-based groupings of enzymatic reactions revealed how metabolic functionality diverged after important bifurcations in plant evolution. Algae were enriched in hormone-related reactions, whereas early land plant reactions were enriched in carbohydrate metabolism, and reactions unique to the angiosperms were enriched for specialized metabolism (Chae et al. 2014). In angiosperms, expansion of gene families occurred mainly through gene duplication even if whole genome duplications have occasionally occurred repetitively in the course of their evolution (Chae et al. 2014; Zhao et al. 2013).

Considering the specialized metabolism to be under selection driving the expansion of gene families coding for specific enzymatic processes in plants (in contrast to genes underlying the function of primary metabolism), it appears that the driving forces acting in that evolutionary process favor organisms that can produce effective chemicals at low energy cost (Firn and Jones 2000). Despite the huge diversity of plant secondary metabolites, the number of enzyme systems involved in their biosynthesis is quite small, and the biosynthetic principles are highly conserved for many secondary metabolites (Weber and Kim 2016). The precursors of secondary metabolites are derived from primary metabolic pathways, and the dedicated biochemical pathways for the production of some secondary metabolites are short (Zhao et al. 2013). In addition, enzymes involved in secondary metabolism have low substrate specificity with a single mutation in an enzyme susceptible to broadly change the spectrum of a family of secondary metabolites (Firn and Jones 2000; Zhao et al. 2013). High specificity will more usually be gained by subsequent selection. Actually, potent biological activity is a rare property for any molecule; thus a great many chemical structures have to be generated (most of which will possess no useful biological activity) before some fitness benefit can be gained by the producer. This large low-cost metabolite production by angiosperms must be advantageous since it has been maintained through their extensive radiation, which is obvious when considering their role in ecological adaptation, physiology, and defense to biotic and abiotic stresses. The low proportion of potent chemicals in the

secondary metabolite space also justifies why very large compound libraries have to be screened in order to find drug leads (Firn and Jones 2000).

It is the biological activity of secondary metabolites that motivates scientists to model their pathway of biosynthesis with the background idea of transferring them into organisms adapted to industrial (Julsing et al. 2006) production for health but also for ecological, industrial, and commercial applications. However, the genome-scale reconstruction of metabolism in plants is much more challenging than in bacteria because of the unknown functions of thousands of genes and hundreds of enzymatic and transport functions for which no gene is yet identified (Seaver et al. 2012).

Winzer et al. (2012) performed a transcriptomic analysis, with the aim of elucidating the biosynthetic pathway of noscapine (an antitumor phthalideisoquinoline alkaloid from opium poppy), and characterized the main genes involved. In addition, the authors analyzed an F2 mapping population and showed that these genes were tightly linked and confirmed the gene cluster for noscapine synthesis by BAC cloning and sequencing. Several works of this type let to the notion that a significant proportion of genes for secondary metabolites is organized in clusters in plants (Moumbock et al. 2017). Actually, one-third of the metabolic genes in *Arabidopsis* (30.1%), soybean (30.2%), and sorghum (30.5%) and one-fifth of the genes in rice (22.4%) were found to be clustered and with patterns differing across species and classes of specialized metabolic compounds (Chae et al. 2014).

Since there is a set of enzyme families, which are often very specifically associated with the biosynthesis of different classes of secondary metabolites, rule-based approaches have been used to identify gene clusters encoding known biosynthetic routes with high precision. Such rule-based search strategies are, for example, implemented as an option in the pipeline *antibiotics and secondary metabolite analysis shell* (antiSMASH), which provides detection rules for 44 different classes and subclasses of secondary metabolites. A problem that may occur with gene cluster detection based on rules is the inaccurate prediction of their borders; in that particular case, the correlation of gene expression levels of biosynthetic enzymes with the occurrence of secondary metabolites may be necessary to confirm. The power of combining large-scale genome and metabolome data was explored along with computational approaches to identify novel secondary metabolites. Unfortunately, rule-based algorithms cannot detect novel pathways that use a different biochemistry and enzymes. To avoid this limitation, rule-independent methods such as ClusterFinder and EvoMining were developed. These tools use machine-learning approaches or automated phylogenomics analyses to perform their predictions. In any case, sequence similarity comparison (BLAST or PSI-BLAST) and hidden Markov models (HMM) work very well with low false-positive rates for many different classes of genes involved in secondary metabolite biosynthesis (Weber and Kim 2016).

The accumulated knowledge concerning the metabolic processes of living organisms spans decades of research. With the help of curators scrutinizing the literature, pathway boundaries were defined as well as pathways classified within the frame of a broad ontology in order to maximize their utility and the efficiency of pathway

prediction by software. Extensive revisions of bioinformatic tools and databases for secondary metabolite mining are given in Moumbock et al. (2017) and Weber and Kim (2016). This accumulated knowledge is being stored in bioinformatic systems, such as MetaCyc (Caspi et al. 2012) and KEGG (Kanehisa et al. 2010), among others. To give an example, at moment, MetaCyc contains 2642 pathways from 2941 different organisms (https://metacyc.org/). The metabolic annotation of newly sequenced genomes is based largely on protein homology. In essence, servers propagate by homology initial annotations made in a few model organisms by expert curators. Curators use literature resources to make functional assignments, group sets of reactions into pathways, and propose new functional assignments for previously unknown proteins. Species-specific databases include AraCyc, MaizeCyc, and KEGG-ath. Generic plant databases include PlantCyc (https://www.plantcyc.org/) and KEGG (Seaver et al. 2012). KEGG and MetaCyc differ by their organization, content, complexity, tools, and the way they define the pathway boundaries. In KEGG, different routes are typically combined into a single pathway, while MetaCyc curates a different pathway for each known route. MetaCyc enables genome-scale pathway prediction base on the content of its database through Pathway Tools/PathoLogic. A metabolite can be traced through several pathways using tracer (Caspi et al. 2013). With MetaFlux in the Pathway Tools software, scientists can also use the data files available for each Pathway/Genome Database to generate their own networks outside of the Pathway Tools software. While there are some ready-made tools to facilitate this process (such as the BioCyc plug-in for the Cytoscape program, released in 2010), Pathway Tools can paint the resulting fluxes onto the cellular overview for visual analysis. In addition, Pathway Tools guides the user in producing a complete functional model that produces all metabolites in the biomass equation (Seaver et al. 2012). PlantCyc provides pathways in over 350 plant species that can be simulated with MetaFlux, but *J. curcas* is not yet included.

FBA also allows the prediction of secondary metabolite overproduction with important industrial applications, like antibiotics and biodiesel compounds, following modulation of selected reaction rates that lead to new optimal states (Fondi et al. 2017; Kim et al. 2014). A large number of species-specific secondary metabolites produced by plants can be harnessed through heterologous biosynthesis in organisms engineered at the molecule level, but challenges need to be overcome (Bolger et al. 2017). A major goal of post-genomic biology is to reconstruct and model in silico the metabolic networks of entire organisms. The work, well advanced on bacteria, is now underway for plants with AraGEM (http://www.ebi.ac.uk/biomodels-main/MODEL1507180028) and other coming models. Genome-scale modeling in plants is much more challenging than in bacteria. The challenges come from specific features of higher organisms (subcellular compartmentation, tissue differentiation, circadian and seasonal regulation) and also from gene functions that remain undiscovered. Consequently, steady-state FBA modeling approaches have a utility that is still limited to cell culture. The metabolic modeling of these systems, therefore, demands multiscale approaches (Morgan and Rhodes 2002; Baghalian et al. 2014). Kinetic modeling approaches are limited by lack of kinetic information, and stoichiometric modeling approaches are limited by their

reference to a steady state. Yet, if a stoichiometric modeling approach delivers information about potential perturbation sites in metabolism, this will enable systematic in-depth analysis. Actually, the perturbation sites between two different metabolic states can be calculated hinting a significant regulatory event, e.g., the change of enzymatic reaction rates due to environmental perturbations. In principle, using this approach it is possible to connect a large metabolomics experiment with many samples and thousands of variables directly with the predicted genome-scale metabolic network to calculate biochemical regulation in the investigated biological system (Nägele and Weckwerth 2012). Multiorgan FBA model has also been combined with a dynamic whole-plant multiscale functional plant model. Dynamic FBA was performed by partitioning a selected plant growth phase into several time intervals and by computing a static FBA at the beginning of each time interval. To include dynamic processes, exchange fluxes that had been predicted by the functional plant model and are also time dependent were used to constrain the static FBA within each time interval (Grafahrend-Belau et al. 2013). However, network gaps and mass balance errors resulting from incomplete genome annotation and reaction stoichiometry errors may severely affect the predictive power of network models. To cope with this issue, model validation by quantitative measurement through the integration of mass spectrometry with gas chromatography (GC-MS) or liquid chromatography (LC-MS) has to be performed. Metabolome dataset from 36 distinct tissues suggesting many secondary metabolites was produced in a tissue-specific manner. Some recent metabolomic studies also revealed the dynamic production of secondary metabolites in response to a large range of environmental stresses, such as the increase of tannin in poplar upon herbivore attack, pathogen infection, and drought (Zhao et al. 2013).

At moment, a complete interaction map of the factors involved in the regulation of *Arabidopsis* secondary metabolism is not available; the only group of plant secondary metabolites whose regulation is more or less completely understood and can be visualized in the *Arabidopsis* interactome is that of flavonoid biosynthesis. The biosynthetic pathway starts with general phenylpropanoid metabolism, which involves phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL).

In *Arabidopsis*, anthocyanins (red pigment) are produced only in the light, and their levels are further increased by biotic and abiotic stress factors. The modeling of the flavonoid pathway showed that MYC2 (a helix-loop-helix TF) has a major role in the regulation of jasmonic acid (JA) signaling and cross talk with ethylene, gibberellin, and light-signaling pathways. The most interesting components in MYC2 signaling are the MYC2-JAZ-COI1 and MYC2-DELLA interactions. Briefly, JAZ initiates transcriptional reprogramming of cells by switching the basal developmental programs to the stress-response programs. COI1 is required for the JA-induced expression of PAP1, PAP2, and anthocyanin biosynthesis promoter through cytokinin signaling. Gibberellins (GAs) regulate many developmental processes in plants via DELLA proteins, such as stem elongation, leaf expansion, and flowering (Hui et al. 2018). DELLAs have been described as regulators of plant secondary metabolism. MYC2 activates the expression of sesquiterpene synthase genes in

Arabidopsis, and DELLAs inhibit MYC2 activity, negatively affecting sesquiterpene biosynthesis. DELLAs are also important for maintaining high levels of anthocyanins under phosphate starvation conditions. In contrast, GA represses sucrose-induced anthocyanin accumulation, most likely through the GAI-mediated signaling pathway. Thus, MYC2 mediates the cross talk of hormonal signaling and ensures the accumulation of various groups of secondary metabolites as a function of plant growth and a wide range of developmental processes (see refs in Bulgakov et al. 2017).

In *J. curcas*, the elicitation of leaves by JA in toxic and non-toxic accessions boosts a threefold increase in flavonoids and anthocyanins. Flavonoids play a major role in plant responses due to their antioxidant function as scavengers of reactive oxygen species (ROS). In addition, anthocyanins have the capacity to reduce the potential for oxidative damage by means of light attenuation and reducing ROS through scavenging and metal chelation. Flavonoids form part of the antioxidant complex network that regulates plant growth and stress tolerance and involves other molecular signals such as jasmonates, ROS, and antioxidant enzymes (superoxide dismutase, catalase). Upon biotic or abiotic stress, jasmonates can induce the production of H_2O_2, superoxide anions (O^{2-}), and hydroxyl free radicals (HO^{-}), which have deleterious effects on pathogens and can act as a secondary messenger inducing the expression of a variety of plant defense-related genes. Different cell compartments may activate different defensive systems to reduce ROS excess, using antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidases (POD), and glutathione reductase (GR), among others, as well as nonenzymatic components such as ascorbate, glutathione, carotenoids, phenolic, and flavonoid compounds. The antioxidant effects of flavonoids explain why they are proposed to have a preventive effect on cancer. The difference in the anthocyanin content in the two *J. curcas* accessions might be related to different levels of resistance to environmental stresses and also by the selective domestication processes toward the non-toxic accessions. Phorbol esters have been suggested to be involved in biotic and abiotic stress responses through the activation of plant protein kinases, which are key components of JA signaling. It has been proposed that this mechanism could be responsible for the induction of secondary metabolite accumulation in cultured cells of carrot and *Sanguinaria canadensis* elicited with phorbol esters (see refs in Lucho-Constantino et al. 2017). As for leaves, when elicited with JA, cell cultures of *J. curcas* in the late growing phase triggered a threefold triterpene accumulation with respect to the untreated control (Zaragoza-Martínez et al. 2016).

12.7 Designing Drugs from Active Compound Scaffolds

We noted above that GWAS can help us to identify key genes involved in the functional mechanism of a particular process. We also saw that biotechnology and system biology may help in unraveling the genetic architecture of a trait and provide

us with a new genetic approach by engineering sets of functional genes. Furthermore, it can provide a genetic basis for selecting parents for QTL analysis and may suggest candidates for mutagenesis or genetic transformation, including genome editing.

Thus, in the absence of complete characterization of secondary metabolic pathways, a prerequisite is still selective breeding of native plant resources. However, system biology and molecular genetics are increasingly improving our understanding of secondary metabolic pathways (Wilkinson and Micklefield 2007). Thus, molecular farming, which is an approach that includes (a) the production of particular compounds in plants or (b) the transfer of a pathway to other plants or microbes (Crozier et al. 2009), has promoted their production.

Natural products are a major source of drugs used by humans since all times. Because their chemical synthesis is not always available (Krivoruchko et al. 2011) and their bioengineering in vivo difficult to obtain, an attractive hybrid solution is to proceed with molecular farming of a natural compound scaffold followed by steps of chemical synthesis on that scaffold. Since this solution is target oriented, it must be assisted by a recurring process of in silico design and in vitro validation. During the step of in silico design, the natural compound scaffold is tentatively improved in order to reduce its toxicity and increase its specificity.

In this process, the protein target of a natural product is supposed to be known. Thus, its tridimensional (3D) structure needs to be established in order to deduce the interaction the protein target can have with the natural product under study. Structural elucidation of proteins by X-ray diffraction is an expansive process, fortunately, an alternative has been found for three-dimensional (3D) model creation via molecular homology modeling in silico. Homology modeling takes advantage of a 3D model by X-ray diffraction obtained for a sequence in another biological species that has homology to the sequence under study. The homologous sequence is used as a template to model the studied sequence on the basis of the alignment of the two amino acid (template and experimental) sequences. When a protein has no homology to any sequence with 3D model available, this procedure is impossible to implement. In this case, de novo modeling strategies can be applied, but these strategies are still insufficiently accurate or require computational resources out of access in normal condition (Nayeem et al. 2006; Xiang 2006; Vyas et al. 2012).

After the creation of a 3D model, this model is subjected to a refinement step where its conformation is tested to determine the accuracy of the structure in relation to the structure of the template. This process is usually initiated by minimizing its energy via molecular dynamics, based on the nature of the force field used to model the protein. The molecular dynamics (MD) is a process that enables to study the evolution of the 3D structure of a protein in silico given its physico-chemical characteristics. MD allows the new model to loosen the constrains introduced by the modeling process in reference to the template. The force field is the set of parameters used to describe the movement of each amino acid as a function of time during MD (Ohlson et al. 2004; Floudas et al. 2006; Vyas et al. 2012).

After various steps of model creation and refinement, one reaches the validation stage whose purpose is to identify the coherence of the expected model properties

such as stereochemical, angular accuracy, NMR measurements, and stability. According to the type of problem and type of protein, other properties may, of course, be taken into account (Pecoul et al. 2016).

The medicinal chemists in the computational area are interested in high-performance computing since the in silico modeling methodologies have huge computational costs, especially in the absence of resolved structures for molecular targets of therapeutic interest, which require a high degree of confidence. For example, the screening of inhibitors in silico, which is a first step for the identification of prototypes of new drugs, consists in the prediction of conformations and orientations of ligands within a specific region of thousands of compounds. By ligands, one means the potential inhibitors originated from a database or a library of natural products with resolved chemical structure, interacting (docking) with the active site of a protein or an enzyme. Docking investigations need an accurate 3D model of the protein target. The identification of molecular characteristics responsible for certain biological functions with the purpose of finding compounds that best act on the target is a complex challenge (Taylor et al. 2002; Alonso et al. 2006).

However, when an inhibitor is known to be (i) already complexed to the target, (ii) described in the literature, or (iii) derived from another drug, it enables to drive the investigation in such a way as to find the best orientation or conformation of the derived ligands in the active site of the target. The flexibility of ligands and of their macromolecular targets and the possible interactions of both molecules with water in the medium and electronic distributions are difficult to describe in silico in the context of docking. The general idea of in silico docking is to create a considerable range of possible conformations of the ligands and to order their interaction with the protein target by scores based on their energy affinity and stability. The chemical recognition process between a ligand and its target is fundamentally dictated by the electrostatic potential (van der Waals force) involving them. However, the strength of the electrostatic potential decays quickly with the distance between the surface of the target and the ligand under consideration (Höltje and Folkers 2008).

The main feature of docking is the ability to reproduce in silico the chemical interactions between a ligand and a target protein on the basis of another protein-ligand complex as a reference when it exists. However, when the reference is absent, the position of the ligand in the target is most likely to provide interactions as predicted by the program used given the main amino acids present in the active site (Schneider and Fechner 2005).

In addition, novel inhibitors may be investigated by optimizing the interaction of ligand fragments in the target binding site to improve functional group positioning. Indeed, the binding optimization of ligand fragments may aid at the construction of a more effective inhibitor for the target protein considered because of spatial constraints due to steric hindrances of the original inhibitor. In contrast, the fragment-based inhibitor construct considers the affinity of each functional group in each region of the binding site (Verdonk et al. 2003).

This strategy has already proven to be effective in the inference of inhibitors such as (i) a Kv.1.5 channel inhibitor with the TOPAS, (ii) a hepatitis C helicase inhibitor with LigBuilder, and (iii) a new binding FKBP-12 based on the core structure of the

FK506 protein with the LUDI. Thus, this technique proposes the discovery of new drugs, which bind more strongly to a given target enzyme or protein than their natural substrate (Bohm 1992; Wang et al. 2000; Schneider and Fechner 2005).

A potential inhibitor can be further optimized with the aid of bioinformatics, according to its toxicity, solubility, the number of hydrogen donors or small (<5) receptors, or even a low molecular weight (<200 g/mol), by the insertion of functional groups to improve one or more of these criteria. This approach enables to derive existing ligand, such as natural products, by using them as a scaffold for additional chemical synthesis (Kapetanovic 2008).

12.8 Metabolic Engineering

Because of the limitations associated to molecular farming in plants (Gandhi et al. 2015), there is an increasing interest in developing new manufacturing platforms based on model microorganisms that are easy to manipulate and are usually able to reproduce numerous enzymatic steps in mild conditions, which are more environmentally friendly than chemical synthesis. For example, pharmaceutical products such as flavonoids have been produced by yeast more efficiently as compared to prokaryotic hosts. Yeast has been involved with the production of chemicals that may range from commodity chemicals (1,2-propanediol) to fine chemicals (resveratrol). The most emblematic example is the bioengineering of yeast for cost-effective industrial production of artemisinin, a naturally antimalarial drug synthesized by *Artemisia annua*, making the malaria treatment affordable in developing countries (Paddon and Keasling 2014). Other valuable compounds that were considered for synthesis through metabolic engineering in yeast are opioids (Smolke et al. 2014; Galanie et al. 2015). Terpenoids such as Taxol (Ding et al. 2014), which is widely recognized for its chemotherapeutic properties (Zhuang and Chappell 2015), are synthesized chemically, but the process is often costly and inefficient.

Yeast engineering can be achieved by either introducing a new pathway in yeast or by altering the native pathway. The potential pathway with a higher likelihood of success is not necessarily a well-known pathway in nature, and it may be a combination of genes from different organisms. The best pathway is usually chosen based on the specificity of enzymes for the desired reaction, the minimum number of enzymes involved in that pathway, and its thermodynamic favorability (Henry et al. 2010). However, multi-enzymatic pathways from different species may not bring optimized functions in the desired host. Causes for low or no production of the desired molecule are often multifactorial and can be due to (i) inherent failure of the pathway, (ii) intracellular accumulation of secondary metabolites, (iii) generation of toxic intermediates, (iv) inappropriate metabolic flux, (v) cofactor availabilities, (vi) spatial localization, and (vii) incompatible codon usage, among others. There are several bioinformatic packages to optimize these variables (Garcia-Ruiz et al. 2018).

Promoters and terminators play an indispensable role in metabolic engineering for controlling gene expression. These critical elements are involved in regulating both the strength of transcription and the longevity of transcripts. Optimizing microorganisms for chemical production via metabolic engineering requires the use of these elements to create highly regulated intracellular flux (Blazeck and Alper 2010). Promoters for complex eukaryotes have largely been discovered via high-throughput screening methods such as *promoter trapping* (Pontiller et al. 2008, 2010; Chen et al. 2013). Random mutagenesis is a powerful approach to increase promoter effectiveness without explicitly requiring extensive knowledge of sequence-to-function mapping. Eukaryotic promoters, although more complex and less rigidly defined than prokaryotic counterparts, can be broken down into a core promoter (Juven-Gershon et al. 2006, 2008) and upstream enhancer element (s) (Struhl 1984, 1995). It is possible to combine disparate elements in a *hybrid promoter engineering* scheme, which are often stronger than the core scaffold (Blazeck and Alper 2010). Unlike prokaryotic promoters, eukaryotic promoters are largely enhancer limited, meaning that the addition of enhancer elements can both regulate and amplify promoter activity (Blazeck et al. 2012). The best promoters for metabolic engineering are those that are completely decoupled from the host regulatory network; they are called orthogonal promoters (Myronovskyia and Luzhetskyy 2016). Library of orthogonal core promoters was obtained for yeast by screening native promoters over a wide range of growth conditions in order to find a promoter scaffold that would exhibit the least amount of natural regulation (Blount et al. 2012).

Terminators serve as important control points when tuning expression in circuits and pathways (Du et al. 2009, 2012). Unlike promoters, terminator cataloging has not been as extensive until recently. In fact, most commonly used terminators have been relics from past experiments and are not often the most efficient (Deaner and Alper 2018).

In yeast, when multiple genes need to be expressed together in an optimally way, multiple pairs of promoter and gene associations are shuffled (Lu and Jeffries 2007). Changing the spatial configuration of pathway enzymes is another strategy for redirecting fluxes toward the desired product. It can avoid the loss of intermediates by degeneration or diffusion (Albertsen et al. 2011). FBA has been a useful method for such purpose (Bro et al. 2006). When changes to a network are required, further validation through targeted approach must be performed. This can be done by *multiplex automated genome engineering* where many selected genes can be altered simultaneously (Fehér et al. 2012).

12.9 Conclusions

The continuous development of new technologies of high-throughput data generation together with new algorithms of bioinformatics and mathematic modeling shed a new light on the complexity of biological systems. System biology also opened

new avenues for the reprogramming of cell activities in a sense that is useful to humans in controlled conditions. This chapter discusses an integrated view of how system biology may help to extract information from biological systems in order to produce miracle compounds interesting for health, agricultural, or industrial purposes like biodiesel from *J. curcas*.

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Part III
Physiology and Plant Production

Chapter 13
Agronomy of *Jatropha curcas* in Mexico

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Abstract *Jatropha curcas* L. is considered an ideal plant to produce biodiesel due to the quantity and quality of the oil contained in its seeds. It is considered a native plant in Mesoamerica, where there is a tradition of its use and consumption. However, despite its uses and potential to produce biodiesel from the oil extracted from its seeds, there is little information available for its cultivation in Mexico. In this work, we describe the main technological advances for its cultivation in Mexico, including information on agronomic activities for planting and establishment, varieties, nutrition, weed control, pest and disease control, harvesting, and post harvest of jatropha. The information presented is based on the results of research and validation of technological packages by various research institutions in Mexico.

Keywords Jatropha · Biodiesel · Agronomic management

13.1 Introduction

Physic nut, *Jatropha curcas* L. (hereafter referenced to as jatropha), is a perennial species belonging to the Euphorbiaceae family and is considered an ideal raw material for biodiesel production because of the high oil content of its seeds. The oil from the nut, from which biodiesel can be made, can contribute to reducing dependence on petroleum and excessive use of fossil fuels (Prueksakorn et al. 2010). Jatropha is also a new option for biofuel producers to diversify and can help increase profits, contribute to sustainable development, and mitigate emission of polluting gases. However, although jatropha is a species native of Mesoamerica and there is a

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tradition of its use and consumption in several regions of Mexico (Pecina-Quintero et al. 2014; Hernández-Nicolás et al. 2018), there is little information regarding its cultivation. For this reason, this chapter presents advances related to production technology of this crop in Mexico.

13.1.1 Field Preparation

Preparing the soil, known as tillage, aims to create favorable conditions for crop development, i.e., seed germination, root and shoot growth, and, in most cases, fruit formation and significant increase in production (Márquez 2001; Leyva 2009). Jatropha is highly susceptible to moisture; thus preparing the land begins with maximum leveling as much as possible to prevent puddling in the rainy season. Land preparation should be done 2 months before the beginning of rains as listed and described below.

13.1.2 Weed Control

This activity aims to cut weeds present in the field, to incorporate them into the soil as a source of organic matter, and also to make the use of agricultural implements easier.

13.1.3 Tillage

Tilling the soil entails the breaking of the superficial soil layer that is compacted by rain impact and promotes better conditions of aeration as well as moisture retention for root development. Two passes are made with a disc plow or moldboard plow at a depth of 20–30 cm (Zamarripa-Colmenero and Solís 2013b). This practice allows oxygenation and exposure to sun of 0–30 cm of the top soil, destroying the bulk of immature stage insect pests, and helps to control some weeds, especially perennial species (González et al. 2011).

13.1.4 Harrowing

It is a necessary practice to level the soil, pulverize organic residues, and refine the top soil. This activity aims at fluffing the soil; it should be done 1 or 2 weeks after plowing with 2 cross passes of a 24-disc harrow at a depth of 10–20 cm, depending on the soil texture and characteristics (Zamarripa-Colmenero and Solís 2013b).

13.1.5 Furrowing

For the three INIFAP varieties, which are medium stature genotypes, a 3 m plant spacing is suggested. Depth of the furrows depends on the variety to be planted, which can be 20–25 cm when planting seedlings or cuttings (Zamarripa-Colmenero and Solís 2013b).

13.2 Establishment and Plantation Management

In Mexico, INIFAP has developed three clone varieties of jatropha designated as "Don Rafael," "Gran Victoria," and "Doña Aurelia" to meet the industrial demand for both plants and oil for the energy market of Mexico, the Mesoamerican region, and the Caribbean. The varieties are registered by the National System of Inspection and Certification of Seeds (SNICS, *Sistema Nacional de Inspección y Certificación de Semilla*) in Mexico. During 2014 and 2016, plant breeder's rights (*Títulos de Obtentor*) were granted in accordance with the decree *Ley Federal de Variedades Vegetales* and the International Union for the Protection of New Varieties of Plants (UPOV). These varieties are outstanding in that the plants of the varieties "Gran Victoria" and "Doña Aurelia" have 100% feminine flowers, while "Don Rafael" has a larger proportion of masculine flowers (20 masculine flowers for every feminine flower on average) and is thus the pollinator variety. The clonal varieties have grain yield of 0.9–1.98 t/ha in the fourth year of cultivation and reach 1.9–3.6 t/ha at maturity with a 50% oil content and excellent fatty acid proportions especially for linoleic (32–40%) and oleic acids (22–33%).

To maintain the positive characteristics of the new INIFAP jatropha varieties, they should be asexually propagated with cuttings. Propagation by seed is not recommended because the selected traits of the mother plant would be lost (Zamarripa-Colmenero et al. 2009). The two forms of plant production that have greater potential are propagation by cuttings from clone gardens and in vitro propagation by somatic embryogenesis in liquid medium. Nevertheless, the two propagation techniques should be optimized to increase efficiency and lower production costs.

To increase massive availability of vegetative material for production in the medium and long term in Mexico, new *J. curcas* varieties were established by INIFAP in 0.25 ha plots in four states of the republic: Michoacán, Yucatán, Tamaulipas, and Chiapas (Zamarripa-Colmenero 2012). In a second phase, in 2014, four multiplication plots were established with the three jatropha varieties to satisfy the demand for vegetative material to establish more commercial plantations. The multiplication plots were established in four experimental fields of INIFAP at (i) Rosario Izapa, Tuxtla Chico, Chiapas; (ii) Valle de Apatzingán, Michoacán; (iii) Las Huastecas, Altamira, Tamaulipas; and (iv) Mocochá, Uxmal, Yucatán (Iracheta et al. 2015).

Fig. 13.1 Clone garden of Mexican varieties of jatropha for biofuel production (left) and open-air nursery of cuttings of clones in Chiapas, Mexico (right)

For vegetative propagation, Zamarripa-Colmenero et al. (2009) recommend harvesting straight cuttings 0.8–1.0 m long and approximately 2–4 cm in diameter after the rainy season. Cuttings should be planted in 20 × 20 × 20 cm holes overhanging the soil surface by 0.6 or 0.8 m; this procedure favors the emission of vegetative shoots which form the plant structure. With this method of propagation, more than 95% root and shoot emergence was observed 18 days after planting under conditions of the wet tropics (Zamarripa-Colmenero et al. 2009). It is worth noting that the percentage of rooting depends on the genotype. In experiments conducted with 17 accessions of jatropha, using 40-cm-long cuttings, it was observed that in the third month after planting, percentage of rooted cuttings varied between 7% and 85% (Zamarripa-Colmenero and Solís 2013a). To establish a commercial monoculture as a polyclonal variety, the cuttings can be 40–50-cm-long with a minimum diameter of 3 cm. Planting can be done in seedbeds, in bags, or directly in the field. When planted in bags, it is recommended to use a loam or sandy loam substrate, preferably mixed with organic fertilizer. The plants take 5–8 weeks to reach the ideal condition for their establishment in the field (Fig. 13.1).

13.2.1 Planting

It is recommended that planting be done at the beginning of the rainy season in 20 × 20 × 20 cm holes. An average of 1833 cuttings should be considered for establishing 1 ha of *J. curcas*. Emergence of shoots is observed from 14 to 18 days after planting in conditions of the wet tropics. On day 25 after planting, about 5% loss is to be expected and might be replanted to keep the optimal plantation density (Zamarripa-Colmenero and Solís 2013b).

Regarding plant spacing, former experience in India and other countries indicates that a density of 2500 plants per hectare (separated 2 × 2 m) can and should be considered as optimal (Hooda and Rawat 2005). According to Mayorga (2006),

Table 13.1 Relationship between plantation density and yield of jatropha in Mexico

Planting distances (m)	Plants/ha	Height (m)	Raceme number	Yield (kg/ha)
2.0 × 2.0	2500	1.65a	4.8a	703a
2.5 × 2.5	1600	1.50a	4.6a	565a
3.0 × 3.0	1111	1.20b	3.0b	250b
3.5 × 3.5	816	1.10b	2.0b	60c

Means with different letters in a column are statistically different (*t*-student $p \leq 0.05$)

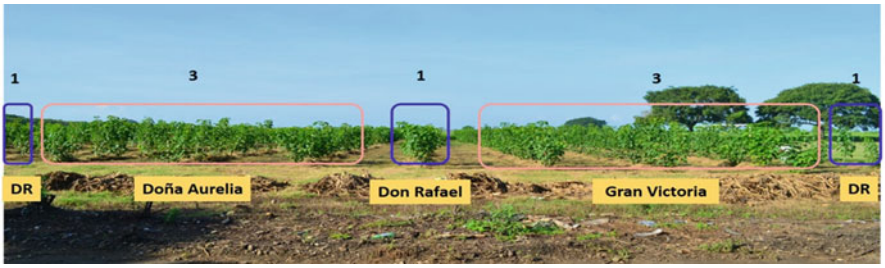

Fig. 13.2 Planting arrangement of three jatropha clones following a 1-3-1-3-1 scheme in the state of Chiapas, Mexico

planting can be performed following a quincunx arrangement, in a 2 × 4 m planting frame, which enables intercropping with an annual crop in the first year. In Mexico, experiments were conducted on population densities in different environments. The densities evaluated varied from 816 up to 4444 plants per hectare in different planting frames: 1.5 × 1.5 m, 2 × 2 m, 2.5 × 2.5 m, 3 × 3 m, and 3.5 × 3.5 m. The best densities fluctuated between 2500 and 4444 plants per hectare because lower densities produced lower yields. For hotter environments, such as the state of Chiapas, the recommended densities are 1666–2222 plants per hectare in 3 × 1.5 and 3 × 2 m arrangements, respectively (González et al. 2011; Teniente et al. 2011; Zamarripa-Colmenero et al. 2011; Hernández et al. 2012; Zamarripa-Colmenero and Solís 2013b).

Table 13.1 shows the results of productive behavior in four evaluated densities. The greatest phenological and productive development was observed in the density of 2500 plants per hectare. It is important to note that the results correspond to the first year of production when competition among plants is low.

For the clonal varieties of jatropha released by INIFAP, monoculture of the polyclonal variety with a 3 × 2 m planting arrangement and a population density of 1666 plants per hectare is recommended. It is suggested to plant 27% "Don Rafael" (DR), which serves as pollinator, at a rate of 450 plants distributed in 9 rows with 50 plants per row interspersed with the female varieties "Doña Aurelia" and "Gran Victoria" at rates of 600 plants of each distributed in 12 rows of 50 plants per row in a 1-3-1-3-1 arrangement (Fig. 13.2).

Fig. 13.3 Plantation of Mexican jatropha in monoculture in the Soconusco coastal region of the state of Chiapas, Mexico

Fig. 13.4 Plantation of Mexican jatropha intercropped with maize in the Soconusco coastal region of the state of Chiapas, Mexico

13.2.1.1 Production Systems

Jatropha is a perennial crop whose production stabilizes in the fourth year. During the first years, yields are low and are not sufficient to generate profits for the farmer when it is mono-cropped (Fig. 13.3). However, polyculture with three successive annual crops can be a viable option to compensate for the first low yields of jatropha.

In Mexico, the production system recommended for jatropha production is intercropping with maize (*Zea mays* L.) and a succession of bean (*Phaseolus vulgaris* L.) in rainfed conditions (Fig. 13.4). Four rows of maize can be planted between the rows of jatropha as soon as the land is prepared. Once the maize is harvested, seven rows of beans may be planted. Jatropha is often intercropped with maize, beans, squash, or some other regional crop. It is recommended that this system be used with distances of 3–4 m between paths and 2 m between jatropha plants.

In Chiapas, Mexico, the jatropha-maize production system with succession of bean was evaluated in rainfed conditions to obtain better plantation arrangement aiming to optimize productivity and an efficient soil use. Planting distances were 2×2, 2×4, and 4×4 m with densities of 2500, 1250, and 650 plants per hectare,

Table 13.2 Results of the experiment of Mexican jatropha associated with maize in Chiapas, Mexico

Planting distances (m)	Jatropha plants/ha	Maize plants/ha	Maize yield (t/ha)	Bean plants/ha	Bean yield (kg/ha)
2 × 2	2500	32,166	1.4b	71,050	615
2 × 4	1250	60,416	4.2a	109,200	1018
4 × 4	625	60,416	4.4a	84,000	720

Means with different letters in a column are statistically different (Tukey $p \leq 0.05$)

Fig. 13.5 Succession of jatropha in association with maize (left) followed by beans (right) in the state of Chiapas, Mexico

respectively. Trials of one or four rows of maize were planted between rows of jatropha at these densities. In succession, two, five, and seven rows of bean were planted between rows of jatropha at the distances mentioned. In accordance with the obtained results, it is recommended to establish a plantation of Mexican jatropha in a 2 × 4 m arrangement with four rows of maize succeeded by five rows of beans in the paths (Table 13.2), using short stature maize and bush-type beans. These results show that the association of jatropha-maize-bean is technically feasible.

The association of jatropha with basic crops is recommended because it can generate income and/or food for the producer from the first year while waiting for jatropha to reach its productive stage.

Studies on the feasibility of the jatropha-maize-bean production system showed that the annual average profit rate was 102% (Fig. 13.5) (Zamarripa-Colmenero and Solís 2013b).

Efficient use of land through association of Mexican jatropha with basic crops improves its productivity and diversification of production with 3.39 t maize, 0.9 t beans, and 450 kg jatropha seeds per hectare, respectively. Today, many of these systems are managed by traditional farmers, who have integrated this type of production into their agricultural system. They have broad empirical experience, which they have acquired over many years and transmit orally or through practice, sustaining their production by polyculture.

Fig. 13.6 Jatropha-maize-squash production systems in the Frailesca of the state of Chiapas, Mexico

As an example, in Frailesca of Chiapas, jatropha growers have begun these polyculture systems with regional crops, such as maize, squash, peanuts, and medicinal plants (Fig. 13.6). In this region farmers predominantly use oxen to work the land.

13.2.1.2 Nutrition

One of the great myths about jatropha is that it adapts to poor low-fertility soils and does not require fertilization. However, investigations have shown that a jatropha plant that does not receive fertilizer, chemicals, or organics will be undernourished and weak and will tend to have low production (Zamarripa-Colmenero et al. 2011; Zamarripa-Colmenero and Solís 2013a). In fertilization assays in different edaphoclimatic environments of Mexico, strong response to chemical and organic fertilization was found during the first and second year of the plantation (González et al. 2011; Teniente et al. 2011; Zamarripa-Colmenero et al. 2011; Hernández et al. 2012; Zamarripa-Colmenero and Solís 2013a). Fertilization with nitrogen (N) and phosphorous (P) significantly increased yield from 400% to 800% and favored vegetative development (number of branches, number of leaves, leaf diameter, etc.). In the case of Chiapas, Mexico, all the treatments with N increased yield, relative to the unfertilized control. The formula 60-40-20 was the one that best promoted seed yield, with a rate larger than 400% (Zamarripa-Colmenero and Solís 2013b).

Fertilizing jatropha is recommended in the first year of crop establishment that included two applications of formula 60-40-20. The first dose should be applied at 30 days after planting and can be obtained with a physical mixture of 130.43 kg urea, 86.95 kg triple superphosphate, and 33.33 kg potassium chloride. The second dose should be applied 1 month after pruning with the same fertilizing scheme or alternatively through foliage nutrients.

In the second year after crop establishment, it is recommended to apply the fertilizer formula 80-40-20 twice during the year. The dosage can be obtained with

Fig. 13.7 Circular furrow for applying fertilizer in a crop of jatropha in Chiapas, Mexico

the physical mixture of 117.6 kg of the formula 17-17-17, 130.4 kg urea and 43.4 kg triple superphosphate. The first dose should be applied at the beginning of the rainy season. In addition to this fertilizer application, 1 L per ha foliar fertilizers should be applied to promote flower and fruit set. The second dose should be applied after productive pruning, and 1 month later foliar fertilizer should be applied again as just described. For fertilizer application, make a small circular furrow (or half circle depending on the topographical conditions of the terrain) around the plant half a meter from the trunk; the fertilizer mixture is placed in the furrow and covered with the soil that is dug out of the furrow and leaf litter (Zamarripa-Colmenero and Solís 2013b) (Fig. 13.7).

It is highly recommended to complement chemical fertilizer with manure, which contain nutrients that are essential for plant growth. They can be applied with the same technique described above, with a dose of 2–3 kg per plant, divided in two or three applications during the year.

13.2.1.3 Pruning

Pruning is done to form the ideal plant architecture, to promote early production and obtain better, more competitive, constant production over time, and to prolong the productive life of the plantation. By pruning Mexican jatropha plants, we aim at (1) regulating their stature and balance in function of crop needs; (2) adapting the plantation to the system of plant formation; (3) improving their aeration and illumination; (4) eliminating damaged, unproductive, or diseased parts; (5) favoring flowering; and (6) increasing plantation yield (Zamarripa-Colmenero and Solís 2013b).

Fig. 13.8 Pruning delimited to 80 cm (left) resulting in architecture shaping of jatropha (right) in Chiapas, Mexico

13.2.1.4 Shape Pruning

Because the plantation was established with cuttings of improved INIFAP varieties, which at planting should have a height that favors emission of vegetative shoots, pruning should be done to give shape to the plant structure. The entire plantation should have homogeneous formation, maintaining the same distance from the ground. After planting, exuberant sprouting occurs. Generally, four branches will form the jatropha structure from the emerging sprouts. When the sprouts become more lignified, the branches that are not growing in the desired direction are eliminated. Shape pruning is performed on the primary branches, making a cut at 80 cm from the stem neck (Fig. 13.8), which promotes the formation of around 20 secondary branches. This activity is done at the end of harvest or after the rainy season during the vegetative phase.

13.2.1.5 Productive Pruning

Plants are pruned to maintain the stature of the jatropha plant and promote emergence of productive shoots. This activity should be done 1 month after fruit harvest and in agreement with the physiological cycle of the plant; otherwise, the productive cycle can be affected in the following year. In the conditions of the Chiapas state, the best time for productive pruning is October and November, which are the months that coincide with the end of the rainy season and fruit harvest and where jatropha enters into dormancy. When pruning is performed in this period, the development of productive shoots is induced in agreement with the physiological cycle of jatropha. Height should be limited to 1.2–1.4 m to facilitate fruit harvest (Zamarripa-Colmenero and Solís 2013b). Cuts should be made diagonally to prevent accumulation of rainwater and rotting of branches during the rainy season (Fig. 13.9). After pruning, lime dissolved in water, copper oxychloride, or oil-based paint should be applied to the cut area to promote rapid scarring and prevent damage from pests and

Fig. 13.9 Productive pruning of jatropha in Chiapas, Mexico

Fig. 13.10 Jatropha response to productive pruning in Chiapas, Mexico

diseases. One month after productive pruning, vegetative shoots grow and the first floral buds appear (Fig. 13.10).

It is worth mentioning that the response to pruning of the three varieties registered by INIFAP is very favorable, with a yield increase by as much as between 20% and 30%. However, every genotype from INIFAP germplasm collection or resulting from advanced selective breeding did not respond favorably or positively in terms of grain yield despite shape and productive pruning. Pruning the selected varieties has enormous agronomic advantages, which are reflected in their high grain yield and prolonged productive life (Fig. 13.10).

13.2.1.6 Weed Control

Weeds have a great capacity for resisting long periods of drought, to produce large quantities of seeds that easily disperse and to adapt to diverse environmental conditions. For these reasons, they are difficult to control. Weeds compete for water, light, space, and nutrients. They can also modify the plantation's microclimate to favor incidence of some pests and diseases that can damage the crop. Moreover, an excessive weed population makes cultural activities, such as

Fig. 13.11 Local manual weeding of a jatropha plantation to reduce water competition

harvesting, fertilization, and pest and disease control, more difficult. Weed control should be appropriate and scheduled to decrease its negative effects. However, excessive control can also entail adverse consequences, such as soil loss from water and wind erosion (Zamarripa-Colmenero and Solís 2013b).

The weeds commonly present in the Soconusco region of Chiapas, Mexico, are elephant grass (*Pennisetum purpureum* Schumacher), gherkin (*Cucumis anguria* L.), chaff flower (*Achyranthes aspera*), spiny amaranth (*Amaranthus spinosus*), sensitive pea (*Chamaecrista trichopoda*), zarza (*Mimosa somnians*), touch-me-not (*Mimosa pudica*), redroot (*Portulaca oleracea*), Johnson grass (*Sorghum halepense*), and melampodium (*Melampodium divaricatum*), among others.

Weed control is much more important during the first 3 years after establishing the jatropha crop. It is during jatropha's slower initial growth that weeds are a problem due to water competition and weed control should be more frequent. During this period weeds in the paths can be kept low, but the area above the jatropha roots should be kept clean by manual weeding. For the case of the wet tropics, weeding is done integrally by hand 1 month after crop establishment. One week later, commercial weed-specific herbicide is applied in the morning when wind is not strong. This activity should be carried out at least twice a year (Fig. 13.11).

13.3 Pests and Diseases

Despite its toxicity, jatropha is infested by many insect pests and frequently exhibits symptoms of attack by fungi, bacteria, and viruses. Significant losses have been caused by insects and phytopathogenic microorganisms (Grimm 1996, 1999; Grimm and Maes 1997; Kaushik et al. 2007; Sharma and Srivastava 2010; Torres-Calzada et al. 2011; Quiroga-Madrigal et al. 2013). Although Mexico is considered as the center of origin of jatropha (Li et al. 2017), the information available on insect pests and pathogenic microorganisms is limited. Among the insects considered as pests of jatropha, one may cite *Pachycoris klugii* Burmeister, *P. torridus* (Scopoli)

(Heteroptera: Scutelleridae), *Leptoglossus zonatus* (Dallas) (Heteroptera: Coreidae), *Ectomylois mursicis* (Dyar) (Lepidoptera: Pyralidae), *Liothrips jatrophae* Mound (Thysanoptera: Phlaeothripidae), *Stomphastis thraustica* (Meyrick) (Lepidoptera, Gracillariidae), *Psapharochrus* sp., and *Lagocheirus undatus undatus* (Voet) (Coleoptera: Cerambycidae) (Tepole-Garcia et al. 2012; López-Guillén et al. 2012, 2013; Gómez-Ruiz et al. 2015a; Mound et al. 2016). Diseases reported in Mexico that commonly affect jatropha are caused by fungi and bacteria, such as *Pythium aphanidermatum* in non-toxic jatropha seedlings and seeds cultivated in the state of Veracruz (Valdez Rodríguez et al. 2011) and in Sinaloa (*Alternaria alternata*) that attacks the inflorescences causing symptoms of necrosis (Espinoza et al. 2012) and production loss. Other diseases of *J. curcas* reported in Mexico include *Colletotrichum gloeosporioides*, *C. circinans*, *C. siamense*, *C. trucatum*, *Phakopsora arthuriana*, *Pestalotiopsis* sp., *Fusarium solani*, *F. equiseti*, *Botryodiplodia* sp., *Ralstonia solanacearum*, *Pythium aphanidermatum*, *Colletotrichum capsici*, *Lasiodiplodia theobromae*, *Curvularia lunata* and *Alternaria alternata*, *Chaetomium cupreum*, *Glomerella cingulata*, and *Phomopsis* sp. y *Pestalotia* sp. (Valdez Rodríguez et al. 2011; Espinoza et al. 2012; Quiroga-Madrigal et al. 2013; Pabón-Baquero et al. 2015; Gómez-Ruiz et al. 2015b; Herrera-Parra et al. 2017; Uc-Várguez et al. 2018) (Figs. 13.12 and 13.13).

13.3.1 Pest and Disease Control

In Mexico, the entomopathogenic fungus *Beauveria bassiana* has been evaluated for its potential in the biological control of *P. torridus* and *L. zonatus*. For example, Gómez-Ruiz et al. (2015b) found that the *B. bassiana* strains Bb-19, Bb-15, and Bb-Rhy and a commercial product (GHA BotaniGard® 22WP) caused 52.94, 55.88, 70.58, and 67.64% mortality, respectively, in adults of *P. torridus*. Barrera et al. (2016) reported 85.71% mortality of *L. zonatus* with the *B. bassiana* strain BB01.

The potential of microbial antagonists for control of *J. curcas* phytopathogens has been evaluated in the laboratory. Hernández-Guerra et al. (2016) found that the bacteria *Bacillus subtilis*, *B. mojavensis*, *B. thuringiensis*, and *Lysinibacillus sphaericus* inhibit mycelial growth of *Fusarium verticillioides* by up to 55%. In addition, Pabón-Baquero et al. (2015) reported that chitosan at concentrations of 0.5, 1.0, 2.0, and 4.0 mg/ml inhibited mycelial growth of *F. equiseti* and *C. lunata*.

13.4 Harvest and Post harvest

The uneven ripening of jatropha fruits in Mexican varieties makes it difficult to determine when to harvest, which prevents mechanical harvesting. For this reason, harvesting is manual: 2 days of work are needed to pick 500 kg of fruit per hectare in

Fig. 13.12 Main pests of *J. curcas* in Mexico. Adult of *P. torridus* (**a**), *P. klugii* (**b**), and *L. zonatus* (**c**), respectively, sucking a green fruit. A larvae of *E. mursicis* feeding in seeds of *J. curcas* (**d**). A pupae of *L. undatus undatus* in stem of *J. curcas* (**e**). An adult (white arrow) of *L. jatrophae* sucking a leaf (**f**)

each harvest period. A variety is considered good if fruit set and ripening are uniform, to minimize the number of picking operations. The genetic diversity present in Mexico should favor the search for more uniform varieties that mature homogeneously (Fig. 13.14).

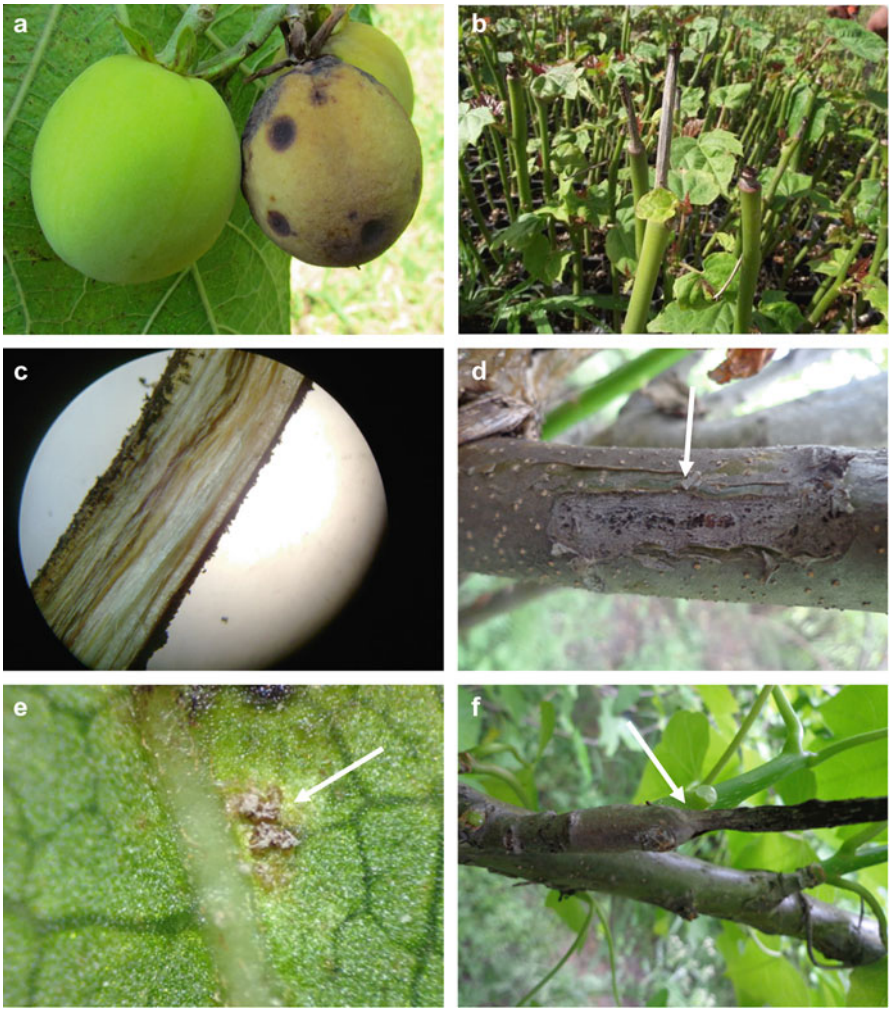

Fig. 13.13 Main diseases of jatropha in Mexico. Fruits of jatropha with symptoms of *C. gloeosporioides* (**a**) and *Phomopsis* sp. (**b**), stem with symptoms of *F. equiseti* (**c**), stem of *J. curcas* (white arrow) with symptoms of *C. cupreum* (**d**), leaf with symptoms and signs (white arrow) of *P. arthuriana* (**e**), branch of jatropha (white arrow) with symptoms of *C. siamense* (**f**)

The pickers should be trained so that damage and waste are minimized at harvest. They should be able to recognize ripe jatropha fruits and detach them with as much care as possible by cutting or pulling carefully to not damage almost ripe fruits. Containers, bags or baskets, should have openings that permit good ventilation.

Fig. 13.14 Ripening stages of jatropha fruits in Chiapas

Fruits are processed with a manual husker. Average seed yield is about 20% of the harvested fruits. After harvesting, the grain should not be exposed directly to the sun to avoid heating and possible damage by solar radiation. After harvest and dehusking, the jatropha seeds should be put into 50 kg sacks to be stored until sale.

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Chapter 14
Phenology and Photosynthetic Physiology of *Jatropha curcas* L. Grown Under Elevated Atmospheric Carbon Dioxide in a Semiarid Environment

Sumit Kumar, Shalini Mudalkar, and Ramachandra Reddy Attipalli

Abstract Climate change at the global scale has emphasized the need for identifying plants with stable productivity under unfavorable abiotic conditions. *Jatropha curcas* is a fast-growing species that thrives with minimum inputs, demonstrates drought tolerance, and has gained worldwide attention as an oilseed suitable for alternative fuel. However, there is limited knowledge on its phenological and physiological behavior under different geographical realms, which imperatively need to be understood before any selective breeding for growth and productivity can be initiated in a particular geographical region. In this chapter, we present a comprehensive information with systematical experimentation on growth and photosynthetic physiology of *J. curcas* with particular emphasis on elevated CO_2 concentration in semiarid conditions. Furthermore, the morphophysiological status is elaborated in the context of photosynthetic efficiency, source-sink interaction, and reproductive phenology in *Jatropha* grown under CO_2-enriched atmosphere.

Keywords Drought · Elevated CO_2 concentration · Morphophysiology · Photosynthesis · Reproductive phenology

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14.1 Introduction

Climate change is one of the prominent issues of our time that needs global attention. The alarming pace with which this change is occurring in recent times is a matter of concern as it has already started to show its ill impact on environment and biosphere. The adverse climate change has been attributed to abnormal rise in world population, fossil fuel consumption, changing land use practices, elevated levels of atmospheric carbon dioxide (CO_2) concentration, and increased temperature components of global warming affecting economic and social development. The atmospheric CO_2 concentration was stable at ~270 μmol/mol for at least 1000 years prior to the start of the industrial revolution. Since that time, CO_2 has been accumulating in the global atmosphere at an accelerating pace. Today, the atmospheric CO_2 concentration is at ~410 μmol/mol, ~50% higher than at any time in the last 20 million years [source – Mauna Loa Observatory, Hawaii (Scripps UCSD)]. The present rate of increase at an average of 1.5 μmol/mol CO_2/year over the last two decades will probably continue for the next two decades, and the atmospheric CO_2 might approach ~550 μmol/mol by the middle or second half of this century (Prentice et al. 2001). Climate change, particularly global warming, imposes a severe impact on the terrestrial ecosystem. One such environmental setback is the declining water resources, which are sensitive to climate change and variability, especially in arid and semiarid regions (Mehran et al. 2017). Moreover, prediction of long-lasting droughts under the present climate change scenario by Intergovernmental Panel on Climate Change (IPCC) has further intensified the importance of drought among other abiotic stresses (IPCC 2014). Mean precipitation is expected to decrease in many mid-latitude and subtropical dry regions, and mean world ocean temperatures will continue to increase throughout the twenty-first century, with the strongest warming trend projected for tropical and subtropical regions in the Northern Hemisphere. Low water availability to plants poses to be the most deleterious abiotic stress factor causing considerable loss in crop yield. As predicted by the IPCC (2014) report, decreases in food production and quality, mainly resulting from heat and drought stresses, are considered a major future risk in many areas (Zandalinas et al. 2017). A possible effort to alleviate this dual situation of elevated CO_2 concentration and exacerbated abiotic stress is the promotion of plant species that have stable production under high temperature and drought conditions. Furthermore, these plants with improved performance under sub-optimal environmental conditions are potential sources of genes for breeding of agricultural and forestry crops.

Jatropha curcas L. is a perennial, semievergreen large shrub or small tree belonging to Euphorbiaceae, which can be grown in varied soil conditions with minimal irrigation input. It is native to Central America but now grows naturally in most tropical areas of the world. Portuguese introduced *J. curcas* in Asia and Africa as an oilseed plant. In India, it occurs in wild, semi-wild, and cultivated states in almost all biogeographical zones from the coastal areas to the outer Himalayan ranges (Kumar and Tewari 2015). It can reach a height of 3–5 m in 6–9 months, but under favorable conditions it can attain a height of 8 or 10 m within 1–2 years of

growth (Kumar and Sharma 2008). Flowering in *J. curcas* begins within its first 3–4 months of growth, and the first seed yield can be obtained after only 6 months, i.e., the time from anthesis to seed maturity is about 45–50 days. Flowering can be year round under optimum temperature and rainfall conditions. However, the full potential of seed yield is realized only after 3 years of growth. Seeds contain 46–58% of oil on the basis of kernel weight and 30–40% on the basis of whole seed weight (Subramanian et al. 2005). *J. curcas* has drawn attention as a source of oil that can provide an economically viable substitute to fossil diesel upon transesterification and is regarded as a second-generation biofuel crop. In addition, *J. curcas* offers many advantages as a crop, since it can be grown in poor soils with minimal input enabling wasteland and degraded land reclamation. For instance, it is used for preventing soil erosion and improving soil fertility by sequestering atmospheric CO_2 and enhances the water capacity of the soil (Wani et al. 2012). Because of these features, *J. curcas* has been considered as a crop that could be cultivated under semiarid and poor soil conditions without competing with food production for land use (Fairless 2007; Divakara et al. 2010; Sapeta et al. 2013). It also responds well to pruning and coppicing, which enhance biomass production (Bailis and McCarthy 2011). Thus, a comprehensive evaluation and understanding are required to predict the agronomic performance of *J. curcas* in response to climate change scenario in various geographical realms, especially in semiarid zones in terms of growth (biomass) and seed yield traits. The improved knowledge on *J. curcas* physiology is needed to support breeding programs, selecting superior genotypes, and optimization of crop management practices.

In this chapter, we summarize the ecophysiological responses of *J. curcas* grown under elevated CO_2 concentration and drought conditions by focusing on morphology and physiology in relation to growth and photosynthetic potential.

14.2 Growth and Photosynthesis of *J. curcas* Under Elevated CO_2 Concentration and Drought

Increase in atmospheric carbon dioxide and rapid depletion of fossil fuels are components of a double-edged sword. To meet energy demand, deforestation for agriculture has led to reduction in the green cover. Depletion of limited fossil fuel reserves has emphasized the importance of alternative and renewable sources of energy. One such source is the oil derived from oilseed plants including *J. curcas*, which is being investigated for the production of biodiesel in order to alleviate or even replace the consumption of fossil diesel. Carbon credits should help this species to be economically feasible, which is needed for its large-scale cultivation. Renewable sources of energy are expected to allow the mitigation of excess atmospheric CO_2 and lessen the strain on fossil fuel reserves. Thus, managing carbon sinks to offset the potential increasing atmospheric CO_2 concentrations has become crucial for the reduction in greenhouse gas emissions in the atmosphere. We recorded the

morphophysiological response of *J. curcas* grown under the elevated CO_2 concentration of ~550 ppm during 1 year and summarize our findings in terms of photosynthetic potential and source-sink relationships. Furthermore, the effect of a drought stress of 21 days on growth and photosynthesis of *J. curcas* is also discussed.

Plant growth and photosynthesis are influenced by various internal and external stimuli inducing morphological and biochemical adjustments within the plants to ensure sustained development. Significant variations were recorded for leaf gas exchange characteristics of *J. curcas* grown in CO_2-enriched conditions when compared to ambient CO_2 conditions during successive production cycles. Light-saturated net CO_2 fixation rate (A_{sat}) of *J. curcas* was maintained in an environment enriched by ~50% of CO_2 concentration when compared to ambient CO_2 conditions during 1 year. Stomatal conductance (g_s) and transpiration rate (E) decreased when plants were grown in elevated CO_2 concentration (Fig. 14.1). These responses were in agreement with the numerous reports on the effects of elevated CO_2 concentration on leaf assimilation physiology, and the existing information shows a primary response in increased photosynthetic rates and reduced stomatal conductance

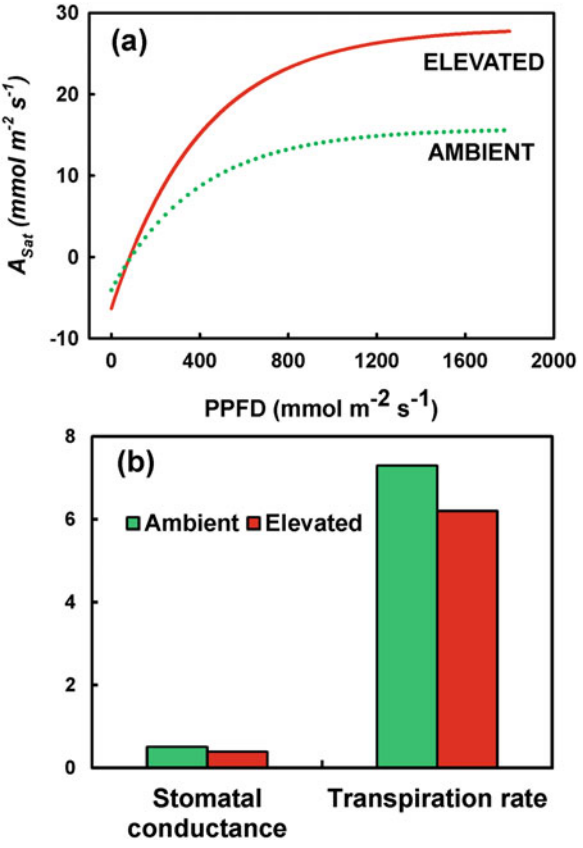

Fig. 14.1 Foliar gas exchange characteristics measured in upper canopy leaves of *J. curcas* grown under ambient (~390 ppm) and elevated CO_2 concentration (~550 ppm) by infrared gas analyzer. Representative graphs of (**a**) A/Q curve analysis (relationship between the CO_2 assimilation rate and photosynthetically active radiation) at saturating light intensities of 0, 300, 600, 900, 1200, 1500, 1800, and 2000 μmol photons m^{-2} s^{-1} and (**b**) stomatal conductance to CO_2 (g_s) and transpiration rate (E) are presented. The values of g_s and E are presented as mean of two growth seasons from a sample size of $n = 15$. (Data from Kumar et al. 2014)

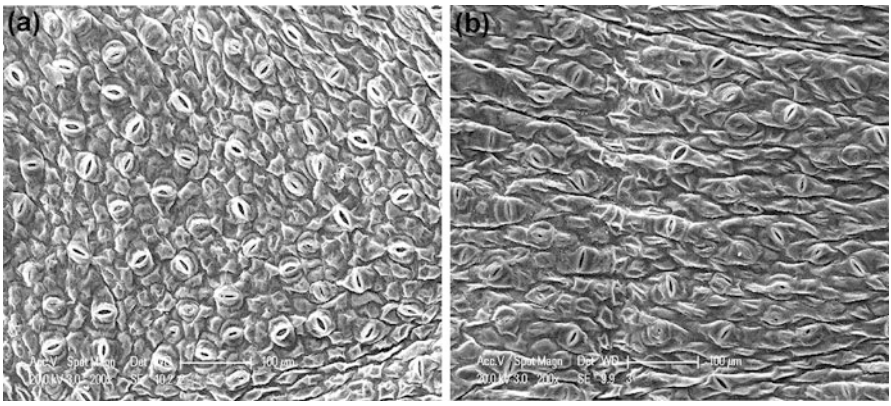

Fig. 14.2 Stomatal opening in the leaves of *J. curcas* plants grown at (**a**) ambient and (**b**) elevated CO_2 concentration. A larger number of partially open and closed stomata can be observed in elevated CO_2 concentration. (Data from Kumar et al. 2014)

(Ainsworth and Long 2005; Ainsworth and Rogers 2007; Kimball 2016). The rate of CO_2 exchange between the plants and its external environment is tightly regulated by stomatal dynamics and stomatal conductance that imposes limitation on CO_2 assimilation, particularly when plants are exposed to enriched CO_2 atmosphere (Wullschleger et al. 2002; Ainsworth and Rogers 2007). Stomatal conductance generally decreases under elevated CO_2 concentration and depends on the stomatal opening and stomatal density. It is however reported that changes in stomatal aperture rather than density determine the response of g_s to elevated CO_2 concentrations (Tricker et al. 2005; Ainsworth and Rogers 2007) as highlighted by a significant variation in number of open and closed stomata of *J. curcas* plants grown under elevated CO_2 (Fig. 14.2). The responses of g_s to elevated CO_2 were variable and subject to environmental feedback (Leakey et al. 2006). Elevated CO_2 concentrations also reduced transpiration rate due to partial stomatal closure, a direct response of guard cells to increased intercellular CO_2 concentration (Paoletti and Grulke 2005). This adaptation allows the plants to preserve greater soil moisture contents in CO_2-enriched environments with a positive feedback on plant growth (Leakey et al. 2009). Moreover, enhanced biochemical activity in leaves can lead to the increase of A_{sat} despite the decreased g_s in elevated CO_2 conditions (Herrick et al. 2004). The rates of instantaneous photosynthesis increased in many C_3 species exposed to a high level of atmospheric CO_2 is due to the increased carboxylation efficiency of RuBisCO, but the CO_2 concentration comes into competition with its oxygenation function at the active site of the enzyme (Urban 2003; Ainsworth and Rogers 2007). Elevated CO_2 also induced significant changes in morphological components associated with growth and productivity of *J. curcas*. The *J. curcas* plants grown in elevated CO_2 atmosphere were ~34% taller in comparison with those from the field or grown at ambient CO_2 during successive production periods, which is coherent with the relative growth rates of these plants (Fig. 14.3). This

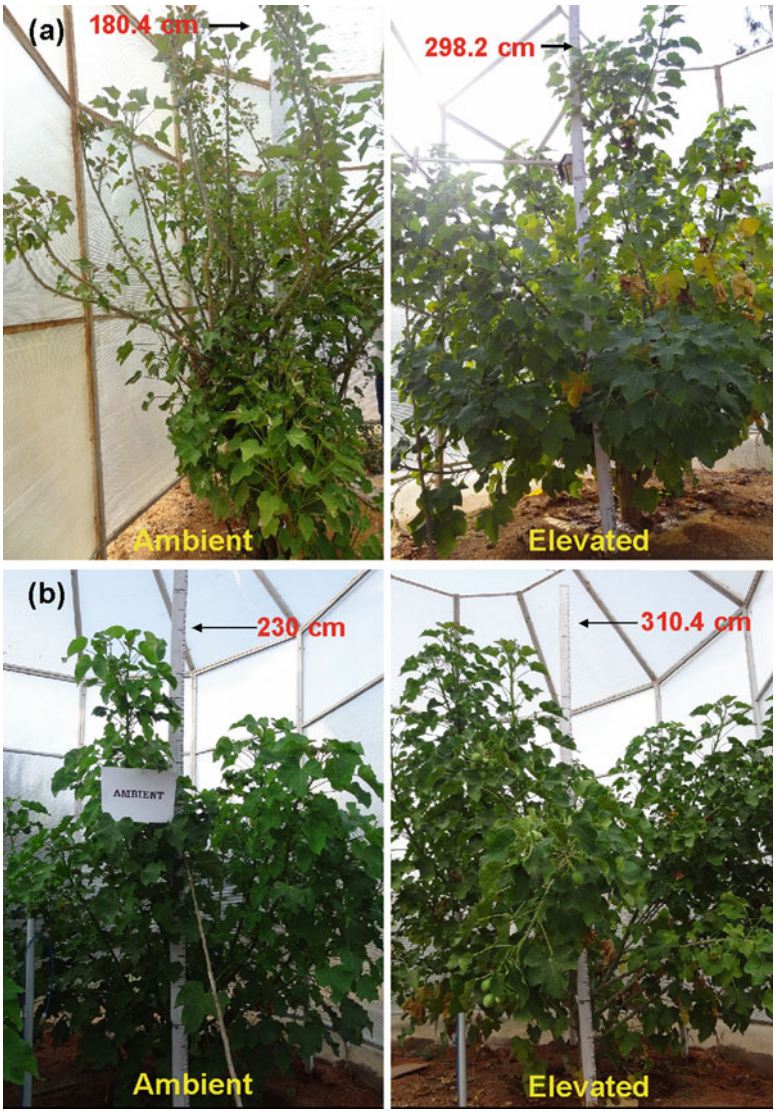

Fig. 14.3 Comparative analysis of plant height of *J. curcas* measured at the end of two growing seasons of 6 months each in ambient (left) and elevated CO_2 concentration (right). (Data from Kumar et al. 2017)

short-term growth stimulation by elevated CO_2 has been described in certain plant and tree species (Zhang et al. 2006; Rasineni et al. 2011a). Nonetheless, these enhanced growth rates under high CO_2 were only transient in many plant species as they reprogram their photosynthetic processes to better use the available resources (Long et al. 2004). Many reports have suggested that a possible reason for

photosynthetic downregulation in plants under elevated CO_2 is the accumulation of soluble leaf carbohydrates and starch in the absence of any available potential sink. The ability of plants to grow under elevated CO_2 not only depends on enhanced photosynthetic ability but also on the optimal allocation of available resources to sink organs (Moore et al. 1999; Reddy et al. 2010). Significant increase in foliar carbohydrate content including starch and soluble sugars under elevated CO_2 conditions in *J. curcas* did not lead to photosynthetic downregulation, which suggested the availability of potential sinks for better resource utilization (Kumar et al. 2014). Growth of *J. curcas* under high CO_2 atmosphere was accompanied by significant increase of tertiary branches, which positively connoted improved resource allocation when compared to ambient CO_2-grown plants (Fig. 14.4). In addition, the increased leaf area in *J. curcas* under elevated CO_2 concentration implies the improvement of light capture and photochemical efficiency as demonstrated by fluorescence measurements of chlorophyll a (Fig. 14.4 and Table 14.1).

Due to the recurring drought cycles in the natural growth habitats of *J. curcas*, it is more prone to drought and other abiotic stress factors. Of late, several groups had

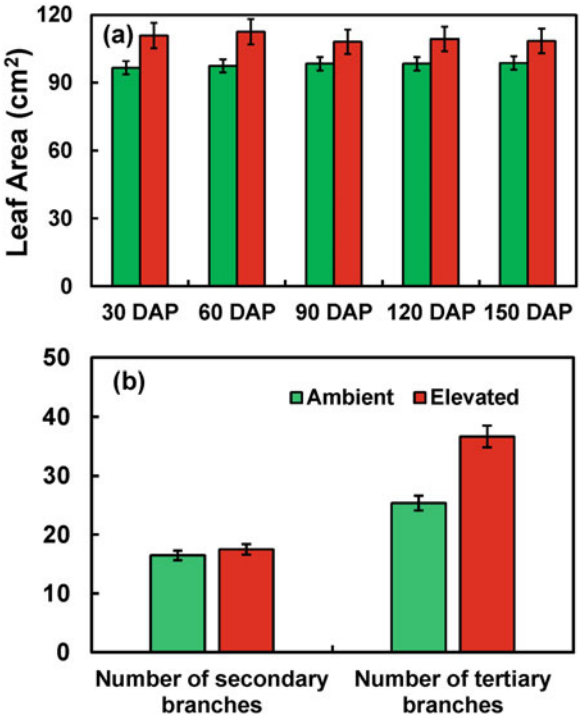

Fig. 14.4 Morphophysiological parameters, (**a**) leaf area, and (**b**) number of secondary and tertiary branches in ambient and elevated [CO_2] grown *J. curcas* plants. The third leaf was used for leaf area measurement of upper canopy ($n = 15$). The data represents average of two growth seasons (6 months each). (Data from Kumar et al. 2014)

Table 14.1 Measurements of chlorophyll a fluorescence from leaves of *J. curcas* grown under ambient and elevated CO_2 concentration with mini-PAM

	Ambient CO_2 concentration	Elevated CO_2 concentration
Maximum quantum yield of photochemistry (F_v/F_m)	0.723	0.8
Effective quantum yield of photochemistry ($\Delta F/F_{m'}$)	0.687	0.756
Electron transport rate	149.4	169.52
Non-photochemical quenching	0.577	0.435
Photochemical quenching	0.725	0.814

Data from Kumar et al. (2017)

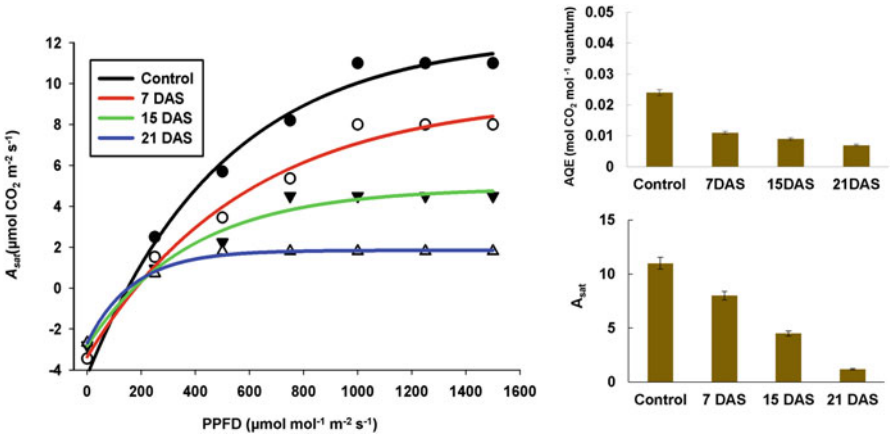

Fig. 14.5 A/Q curves and calculated parameters during progressive drought stress in *J. curcas*. (Unpublished data from Mudalkar et al.)

identified the drought tolerance potential of *J. curcas* under controlled as well as field conditions at both seedling and mature plant stages. After the initial sensing of water limitation through root meristematic tissue, the oxidative damage is translocated to photosynthetic pigments, thereby affecting the photosystems and subsequently photosynthetic carbon fixation process. Recent studies have shown the active resistance of photosystem and related pigments in *J. curcas* under progressive drought stress. The photosynthetic rate as a function of leaf gas exchange physiology was sustained till 7 days after stress (DAS) but decreased by 83% at the end of 15 DAS and by 92% at the end of 21 DAS. The delay of photosynthetic decay under drought stress was strengthened by the light curve (A/Q) analysis, which is a reliable indicator of photosynthetic rate as a function of increasing light intensities (Fig. 14.5). The A/Q provides a precise understanding of photorespiration and maximum light-saturated photosynthesis when working with leaf gas exchange physiology. The apparent quantum efficiency (AQE) was significantly reduced after 15 DAS indicating the dominance of photorespiration in

J. curcas during long-term drought stress. Photosynthesis is the prime factor influenced by any form of abiotic stress. However, the decrease in photosynthetic rate depends on the level of tolerance of a plant to a particular abiotic stress. Osmotic or ionic stress affects the photosynthetic rate in a complex manner where either it lowers the carboxylation capacity of RuBisCO or it decreases the regeneration capacity of RuBisCO by lowering the metabolism of ATP under the conditions of mild and severe stresses, respectively. Severe drought stress significantly affects the growth of *J. curcas* both during seedling and mature stages. The morphological parameters including plant height and number of nodes and branches were negatively affected by severe drought stress. However, under mild stress up to 15 days, the plants were not deviated significantly from the well-watered control as far as growth and morphology were concerned. In a different study conducted by Maes et al. (2009), the growth of different accessions of *J. curcas* seedlings under constraint water regimes had the effect of reducing the leaf area as well as relative growth, thereby lowering the water content for seedlings. Moreover, the decreased growth of leaf, stem, and root tissues was higher in aboveground tissues compared to roots, thereby increasing the root to shoot ratio in water-stressed *J. curcas* seedlings. Thus, the physiological and growth parameters of *Jatropha* under drought stress provide a comprehensive understanding of its drought-tolerant behavior.

14.3 Biomass Accumulation in Relation to Leaf Photosynthesis in *J. curcas* Under Elevated CO_2 Concentration and Drought

Under elevated CO_2 concentration, the flower and fruit yields in the additional tertiary branches were significantly higher than in normal condition, which means that these organs might act as potential sinks of carbohydrates allowing *J. curcas* to increase its photosynthesis rate (Hogy and Fangmeier 2009; Rasineni et al. 2011b). Subsequently, increased metabolic activity promotes fast growth and thereby sink, which in all likelihood enabled *J. curcas* to escape from photosynthetic downregulation under elevated CO_2 concentrations (Idso et al. 2002; Wittig et al. 2005; Körner 2006). Furthermore, the enhanced biomass accumulation and increase in morphological components of growth like tertiary branches and reproductive tissues in *Jatropha* under elevated CO_2 suggest efficient regulation in carbon capture and storage (Fig. 14.6). This efficient carbon sequestration in *Jatropha* attributed to ecophysiological traits like increased gas exchange rates, efficient leaf area development (both individual leaf area and total tree leaf area), and development of sylleptic branches able to capture additional solar radiation per mass unit contributes to tree growth (Dillen et al. 2010; Chen and Setter 2012). The main sink of biomass allocation was found to be stems suggesting that they are preferential organs for carbon storage.

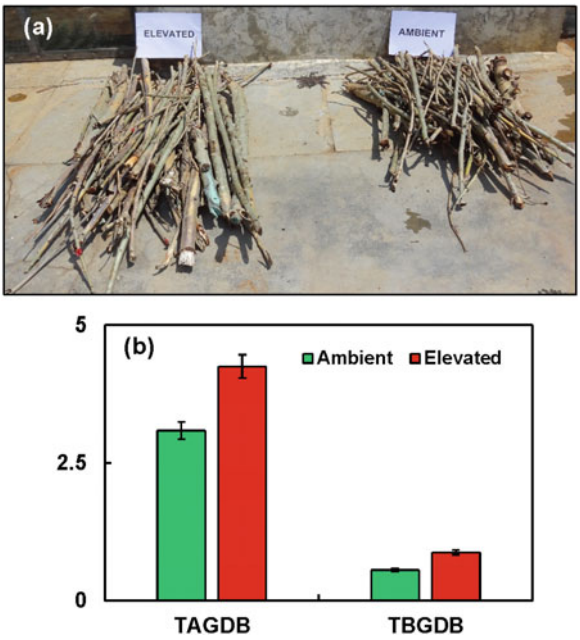

Fig. 14.6 (**a**) A representative image showing the number of secondary and tertiary branches harvested from *J. curcas* plants grown under ambient and elevated CO_2 concentration. (**b**) Total aboveground dry biomass (TAGDB) and total belowground dry biomass (TBGDB) after harvest of ambient and elevated CO_2-grown *J. curcas* plants. The data represent the averages of two growing seasons of 6 months each. (Data from Kumar et al. 2014)

Drought induced a decrease in leaf dry weight as well as in stem and root biomass (Fig. 14.7). However, this decrease became more significant with time, which clearly indicates efficient drought tolerance mechanism operating in *J. curcas* wherein the available water content is optimally utilized to maximize plant growth.

14.4 Flowering Phenology in *J. curcas* Under Elevated CO_2

An important phenological response noted was that the flowering time of *J. curcas* under elevated CO_2 was accelerated by ~8 days (Kumar et al. 2014). The timing of flowering is an important stage of development during the life cycle of any plant as the seed number would be determined at this stage. Early flowering has been reported in many short-day and long-day plants under elevated CO_2 (Springer and Ward 2007). Early flowering is an adaptation strategy allowing a plant to ensure resource accumulation before a starvation period induced by a dry or cold season (Roux et al. 2006). However, the mechanisms controlling this response are not clearly understood yet. Flowering time can be modulated in plants by carbohydrates, which act as regulatory signal molecules during the course of growth and allow the physiological as well as developmental adaptation of plants according to their environmental constraints (Rolland et al. 2006). The increased growth rates due to

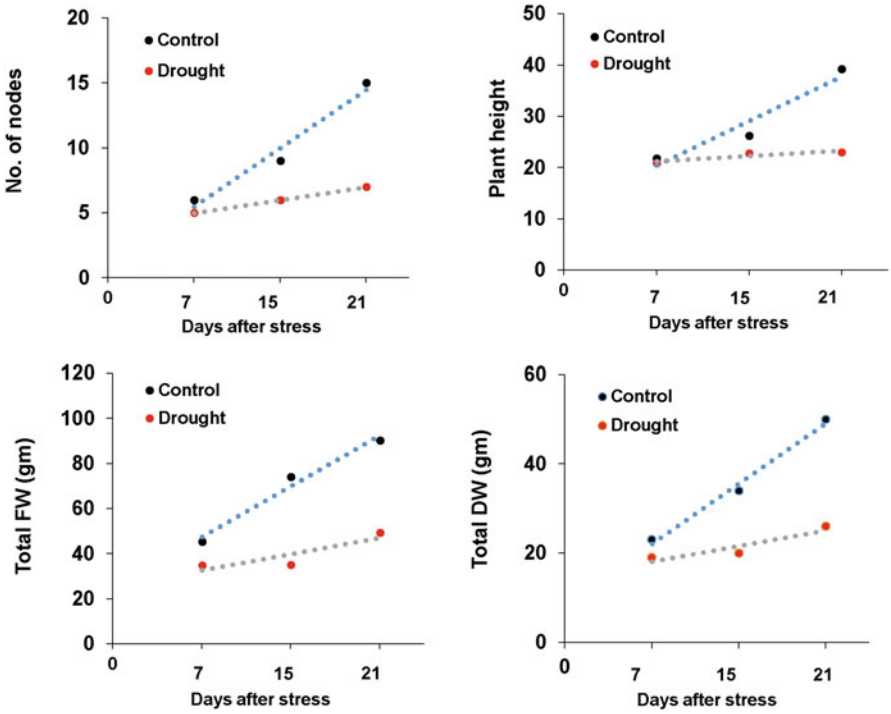

Fig. 14.7 Plant morphology and biomass accumulation of *J. curcas* under progressive drought stress. (Unpublished data from Mudalkar et al.)

stimulated photosynthesis and the foliar carbohydrate accumulation in *J. curcas* grown under elevated CO_2 concentration may act in the signaling for early flowering initiation.

Seed yield in *J. curcas* depends on floral morphology and development, which relies on the number of flowering branches, number of female flowers, and most importantly on the ratio of male to female flowers within an inflorescence (Wu et al. 2010). *J. curcas* demonstrated significant increase in most of the characters that contributed to the improvement of fruit and seed yield under elevated CO_2 concentration. Seasonal variations of fruit and seed yields of *J. curcas* have been observed in semiarid environments (Fig. 14.8). The flowering corresponding to a seed harvest between the summer months of April to June resulted in a minimal fruit setting even though the number of flowers per branches in ambient CO_2 conditions was sizable. By contrast, *J. curcas* plants grown during the same period under elevated CO_2 concentration demonstrated significantly larger fruit and seed yields (Fig. 14.8). The yield significantly improved in ambient CO_2 conditions when plants were grown in the rainy season of autumn, but still plant grown under elevated CO_2 concentration performed better in terms of fruit and seed yields (Fig. 14.8). *J. curcas* is a

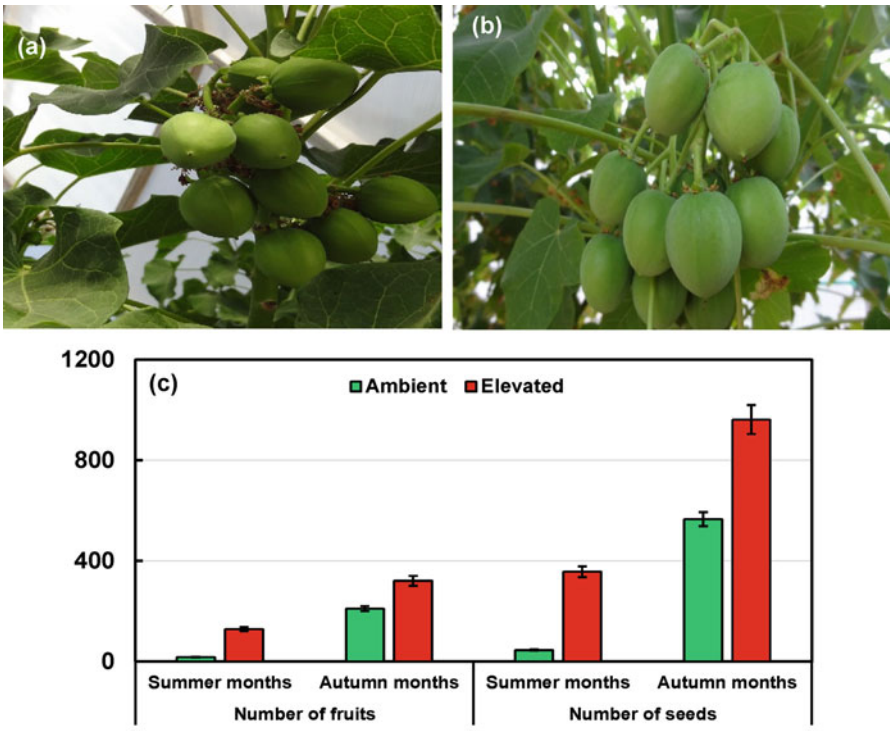

Fig. 14.8 Representative image of a fruit bunch from a *J. curcas* plant grown in (**a**) ambient and (**b**) elevated CO_2 concentration during autumn. (**c**) Fruit and seed yields after harvest of *J. curcas* plants grown at ambient and elevated CO_2 concentration for two seasons. (Data from Kumar et al. 2014)

day-neutral species (Heller 1996), which means that any interaction between photoperiod and flowering can be ruled out. Temperature and rainfall are the known factors which govern flower development and fruit formation in *J. curcas* (Brittaine and Lutaladio 2010). An optimum temperature of 20–28 °C and a watering of 500–600 mm applied during 6–7 weeks before flowering are sufficient for *Jatropha* to flower year round (Kumar et al. 2014). At high temperatures (>35 °C), the vegetative growth of *J. curcas* is unaffected, while the reproductive development may be repressed (Gour 2006). During summer months, when flowering was initiated, the daily maximum temperatures ranged from 40 °C to 44 °C, which might have hindered the female flower development and subsequently the fruit setting in many of the branches that flowered under ambient CO_2 concentration. However, plants grown under elevated CO_2 concentration had a lower ratio of male to female flowers in each flowering branch, which resulted in a higher fruit setting and greater seed number. Thus, elevated CO_2 concentration might have played a role in offsetting the inhibitory effect of high temperatures on floral development (Hamilton et al. 2008; Wang et al. 2008). During rainy and autumn months, the mean daily maximum temperatures ranged from 22 °C to 37 °C, and the plants grown in ambient

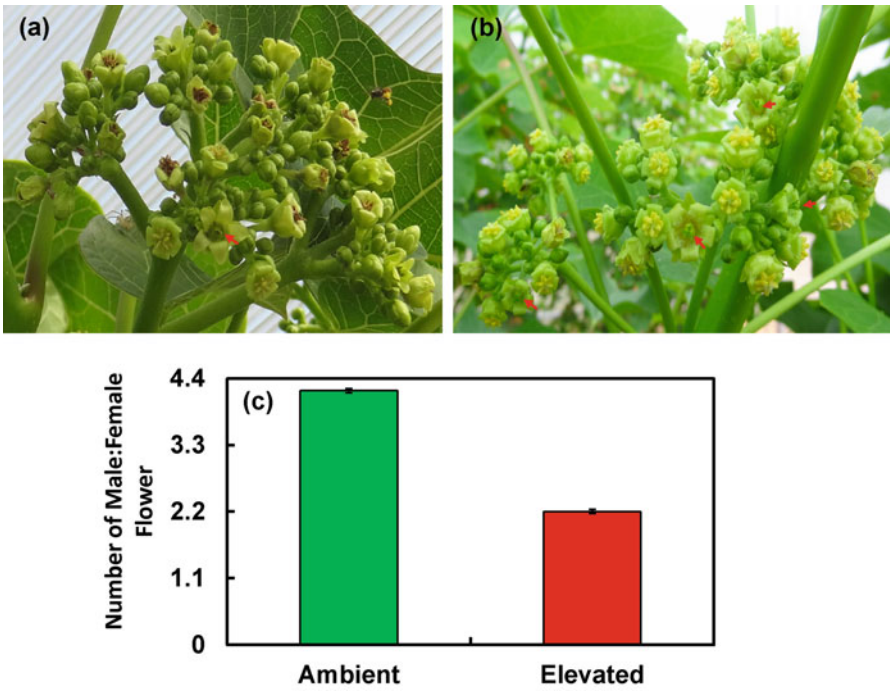

Fig. 14.9 Flowering morphology with respect to the number of open female flowers (indicated in red arrows) in a single inflorescence *J. curcas* plants grown in (**a**) ambient and (**b**) elevated CO_2 concentration. There were larger numbers of open female flowers observed in plants grown at elevated CO_2 concentration at any given time point of flowering stage than in ambient conditions as clearly observed in (**c**) where the ratio of male to female flowers in ambient was larger than in elevated CO_2 concentration. (Data from Kumar et al. 2014)

and elevated CO_2 concentrations exhibited normal flowering and fruit development. However, plants grown in elevated CO_2 atmosphere, again, outperformed their counterparts grown in ambient CO_2 atmosphere, in terms of fruit and seed yields. One of the possible reasons attributed to this observation is that the ratio of male to female flowers in an inflorescence was significantly low in plants grown under elevated CO_2 concentration (Fig. 14.9). The increased number of female flowers might have led to increased pollination and subsequently larger fruit and seed yields.

14.5 Conclusions

Elevated CO_2 can effectively enhance biomass productivity, higher seed and fruit yield in *J. curcas*. This behavior can be attributed to efficient coordination between photosynthesis and organ sinks suggesting an efficient regulation in carbon capture and storage. Interestingly, elevated CO_2 concentrations were able to improve the

negative effects of temperature on the reproductive yields of *J. curcas*, which otherwise was observed in the ambient conditions. Overall, the fast growth and efficient carbohydrate translocation toward branches and fruit sink likely enabled *J. curcas* to avoid photosynthetic downregulation under elevated CO_2 concentration during 1 year growth. The drought-induced symptoms were delayed in *J. curcas* during prolonged drought stress imposition indicating that drought tolerance mechanisms are operating at physiological and molecular levels. The genetic basis of the physiological response of *J. curcas* to elevated CO_2 concentration and drought clearly indicates its potential in terms of fast and efficient adaptability for selective breeding programs to cope with future climate change scenarios. It is proposed that the adaptability of *J. curcas* makes it a suitable candidate for climate-smart agriculture models.

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Chapter 15
Can One Use Chlorophyll *A* Fluorescence as a Physiological Marker of *Jatropha curcas* L.?

Diolina Moura Silva, Ramon Negrão Santos Jr., and Pedro Corrêa Damasceno Jr.

Abstract The *Núcleo de Estudos da Fotossíntese* (NEF) of the Universidade Federal do Espirito Santo studied the regulation of photosynthesis in *J. curcas* accessions from different regions of Brazil and the world. The effects of environmental variations of stress factors, such as rainfall and temperature, on the kinetics of chlorophyll *a* fluorescence (CF) induction in leaves of three genotypes, Janaúba (NEF 01), CPATSA 1501 (NEF 02), and CPATSA C2/10 (NEF 03), were investigated for 4 years. High performance of photosystem II, transpiration rate, and rate of net CO_2 assimilation were observed mainly in the NEF 02 accession. Since it was necessary to understand the dependence of tolerance mechanisms to diverse environmental stresses, the NEF team followed the development of these plants in several locations. The coastal region presents warm humid tropical climate, in contrast with an inland region where the temperature is very hot in the summer and cold in winter with extremes around 8 °C. The development of *J. curcas* plants was affected by the level of photosynthetically active radiation, seasonality of temperature and precipitation, casting doubt on the agroclimatic zoning, which meant that physiological variables needed to be considered. The plants with the best yield were those grown in the inland region, although the photochemical efficiency (PI_{ABS} and PI_{TOTAL}) and the *net assimilation of* CO_2 (*A*) of the plants in the coastal region were higher. The molecular mechanisms underlying the species adaptability may serve for modeling plant traits in order to maximize biofuel production and improve the agronomic features.

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Keywords Net CO^2 assimilation · Photosynthetically active radiation · Photochemical efficiency · Rainfall · Temperature

15.1 Introduction

The Espirito Santo State was the first Brazilian state to officially create a program to support *Jatropha curcas* L. (here referred to as *Jatropha*) as a biofuel source. The government instituted the *Pinhão-manso Pole* to give sustainability to the Program for Research and Production of Potential Oleaginous Cultures and to attend to the National Program for the Production and Use of Biodiesel-PNPB (Collares 2012).

According to Pezzopane et al. (2012), about 78% of the *Capixaba* (adjective relative to things belonging to the Espirito Santo State) territory was suitable for the cultivation of this species because of its low relief with only few mountains and its climate favorable to cultivation. However, the plant material that was planted in the Espirito Santo State was genetically unknown, ununiform, and without minimum guarantee regarding environmental adaptability and productivity (Laviola et al. 2010). It is necessary to register, here, the lacuna existing at the time, in the understanding of the biology of *Jatropha*; the basic knowledge of the interactions of the physiological, biochemical, and molecular processes; and the effects of environmental factors acting on them. Such knowledge, if available at that time, would probably have increased the success of management techniques adopted for *Jatropha*'s culture in a more adequate and productive way (Machado and Laviola 2011).

It is well known that crop productivity results from a complex chain of events and processes in interaction with climatic factors, such as efficiency of water and nutrient use as well as photosynthesis (Prado 2007). Therefore, evaluating genotypes interaction with the environmental features of several regions is of utmost importance for suitable recommendations concerning cultivar performance under specific conditions as well as genotypes stability and adaptability.

The Espirito Santo State lies between meridians 39°38′ and 41°50′ west longitude and between parallels 17°52′ and 21°19′ south latitude. It represents one of the four units that integrate the southeastern region of the Brazilian territory. It currently has 78 municipalities covering a total area of 46,184.1 km^2, and its boundaries are the Atlantic Ocean to the east, Bahia to the north, Minas Gerais to the west and northwest, and the state of Rio de Janeiro to the south (SEAG 2009).

The agroclimatic zoning for the *Jatropha* culture in the state of Espirito Santo, based on parameters of temperature, rainfall, and altitude, suggested that 14.10% (corresponding to 6513.54 km^2) of the state area is suitable for *Jatropha* culture (Toledo et al. 2009). The suitable areas are relocated mainly in the south and metropolitan macro regions but also cover a small part of the northeast of the state, with predominance of cold, rugged, rainy, and low-fertility soils. In these

regions, it is recommended to use additional irrigation during periods of water deficit, which are usually less than 2 months. However, for *Jatropha*, the authors emphasize the dispensation of irrigation once the crop has a good development in conditions of water deficit. Inadequate areas of the state's territory account for 85.9% and are mainly on the coast and in the north. In the coastal region, the conditions considered unsuitable for the cultivation of *Jatropha* are plains, with high temperatures, in transition from the rainy climate to the dry under tidal influence, possibly susceptible to drought, with soils tending to low fertility (Feitosa et al. 2009). Due to the climatic conditions in these regions, it is imperative to use irrigation for 6–-8 months in a year. In summary, the water deficit associated with low altitudes was the limiting factor for the inadequate recommendations of larger areas in the cultivation of *Jatropha* in the Espirito Santo State.

The *Núcleo de Estudos da Fotossíntese* (NEF) of the Universidade Federal do Espirito Santo started in 2008 studied the regulation of photosynthesis in accessions of *Jatropha* coming from different regions of Brazil and the world. The team evaluated the ways in which plants respond to different levels of light, particularly to excess of light in conjunction with temperature variations and water stress.

The present studies aimed to fill important information gaps on *Jatropha* culture in tropical regions in a short term. Photosynthesis is one of the physiological events most affected by environmental conditions (Deng et al. 2003; Souza et al. 2017) that received special attention in studies aiming at the selection of species and/or varieties adapted to adverse environments, since it is the process that generates organic materials and energy for the growth and production of plant biomass (Santos 2008).

Photosynthesis is a major determinant of plant production (Flood et al. 2011) and therefore is a character of interest in selective breeding for improved productivity with reduced inputs. It is also a complex trait, whose developmental and adaptation responses are poorly understood at a genetic level, but offers large potential for further yield increase (Long et al. 1994). The chlorophyll *a* fluorescence (CF) makes it possible to analyze the genetic determinants of numerous properties associated to the photosynthetic process. As most abiotic stresses affect the photosynthetic activity, CF measurements are a potential phenotyping technique for monitoring plant performance under stress conditions (Rungrat et al. 2016).

In tropical regions, photosynthesis can be limited by water, light, and temperature stresses, among others, that restrict diffusion through stomata or indirectly alter the photosynthetic apparatus through oxidative stress.

Noninvasive measures of gas exchange and CF enable to build a dataset and correlate it with photosynthetic process in species of agribusiness interest subject to environmental stress. These methodologies provide important information about the functionality of the photosynthetic apparatus, which, combined with other physiological, biochemical, and molecular investigations, help to understand the molecular mechanisms of plant response (Guidi and Calatayud 2014).

Techniques of gas exchange analysis and CF kinetics have been fundamental for analyzing the effect of changes in environmental conditions. Considering that the growth and accumulation of biomass can be affected by any environmental factors

that affect photosynthesis, it is possible to understand why these techniques have been widely used.

15.2 Chlorophyll *A* Fluorescence and Gas Exchange

Light, the primary source of energy for photosynthesis, together with the availability of CO_2, is the determinant of plant development, growth, and productivity (Osmond 2014), but when the photosynthetic apparatus absorbs more light energy than it can use in photochemical conversions, the excess of energy must be dissipated to keep the cells under normal physiological conditions. The dissipation of non-photochemical energy occurs in the form of heat and fluorescence (Stirbet et al. 2018). The fluorescence emitted by the chlorophyll *a* is mainly associated to *photosystem II* (PSII) and allows the measure of the photosynthetic capacity and physiological condition of a plant. Another important element influenced by the different levels of solar radiation is the opening variations of stomata (Schock et al. 2014), which directly affect photosynthesis.

CF is one of three fates of light, following interception by a leaf (and other photosynthetic organs). Alternative fates of light following interception are dissipation (i.e., heat or non-photochemical quenching) and photosynthesis (photochemistry). Importantly, these fates are linked since a change in one of them results in a change in the other two (Maxwell and Johnson 2000) and it is this balance that makes CF measurements so useful. CF measurements provide information on the status and function of PSII reaction centers and antenna on the donor (P_{680}) and acceptor (pheophytin) sites (Kalaji et al. 2016). While this information may seem highly specialized, it has been used to measure, and in some cases to categorize, a range of stress-impacting photosynthetic processes (Parker and Mohammed 2000).

CF is therefore a noninvasive method, easy and simple to implement, and provides basic information on the functional and structural state of the photosynthetic apparatus (Strasser et al. 2000). The curve of fluorescence emission kinetics can be divided into two phases – a fast or transient phase that lasts for approximately 1 s after the onset of illumination and a slow phase occurring for the next few minutes until fluorescence stability is reached. By using measurements of fluorescence kinetics, it is possible to characterize, quantify, and detect plant stress before its symptoms become visible on leaves (Christen et al. 2007).

When a leaf is exposed to the dark followed by a saturation pulse of about 3000 μmol of photons m^{-2} s^{-1}, the fluorescence increases from its minimum level (F_0) to its maximum level (F_M). This fast transition when plotted on a logarithmic time scale shows a polyphase curve (Fig. 15.1) characterized by several key fluorescence levels, which are sequentially labeled as O-J-I-P: (i) O is for origin (the dark-adapted minimum fluorescence F_O); (ii) J and I are for two inflections at 2 ms and 30 ms (F_J and F_I), respectively; and (iii) P is for the peak (F_P, or F_M) when the fluorescence is maximal, which is usually in the ~300 ms range. In addition to these

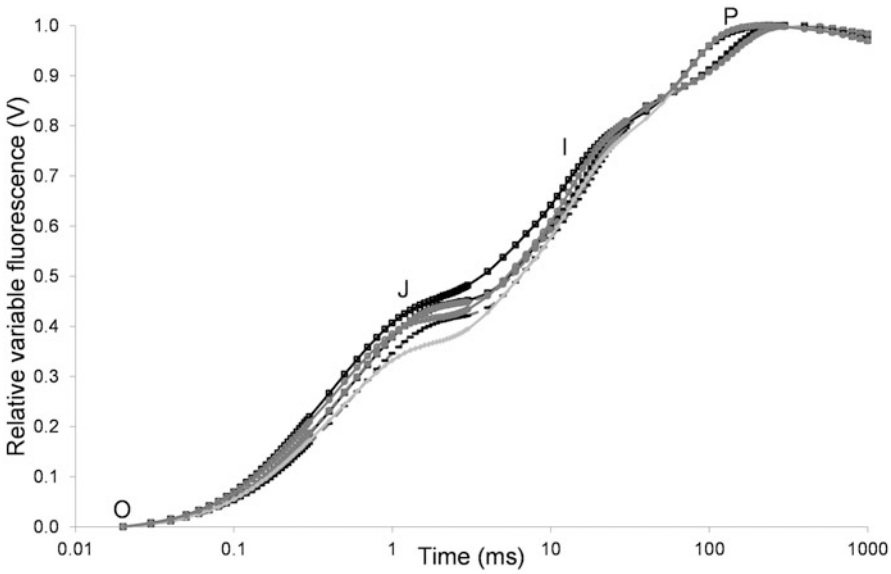

Fig. 15.1 Chlorophyll *a* fluorescence transient measured in leaves of *J. curcas* plotted on a logarithmic time scale. The O, J, I, and P steps are marked in the figure, where O is for the origin (the minimum fluorescence F_O); J and I are for the intermediary fluorescence levels at 2 ms and 30 ms (F_J and F_I); and P is for the fluorescence peak (F_P)

fluorescence levels, sometimes a K level can be observed at 0.3 ms in plants exposed to high temperature (Strasser and Govindjee 1991; Schansker et al. 2013).

The first phase of photochemical reactions (the upward curve O-J) is related mainly to the reduction of primary plastoquinone electron acceptor (Q_A) and strongly depends on the number of photons absorbed. At the end of this step, Q_A is completely reduced. The next increases (J-I-P) constitute the so-called thermal phase. The phase J-I represents the reduction of the *plastoquinone pool* (PQ-pool) by PSII, and the point of inflection I is reached when the oxidation rate of PQ-pool is near its maximum. The phase I-P represents the complete reduction of the electron receptors, $NADP^+$ and ferredoxin, in the *photosystem I* (PSI). According to Stirbet and Govindjee (2012), Q_A continues to be photoreduced until the fluorescence reaches its maximum yield ($F_P = F_M$) during the thermal phase (J-I-P). In parallel, the PQ-pool is reduced by generating a transmembrane pH gradient.

Strasser and Strasser (1995) introduced a test that translates the changes observed in the *fluorescence transient* (an expression used to describe the variation of chlorophyll *a* fluorescence when exhibited upon illumination of a dark-adapted photosynthetic sample by saturating light) leading to quantitative changes derived from the energy flux theory. The quantitative analysis of the fluorescence transient is called the JIP-test, which can be used to explain the stepwise flow of energy through PSII at the *reaction center* (RC) as well as the level of a PSII *cross section* (CS). The parameters of the fluorescence transient model are interrelated by probabilities that

Table 15.1 Parameters, formulae, and definition of terms of the JIP-test used for the analysis of the chlorophyll *a* fluorescence transient O-J-I-P emitted by dark-adapted photosynthetic samples, according to Strasser et al. (2010) and Stirbet et al. (2018)

Accession code	Acronym original	Region of origin	Matrix
NEF 01	JANAUBA	MG	IFES-STeresa
NEF 02	CAPTSA 1501	EMBRAPA	IFES-STeresa
NEF 03	CAPTSA C2/10	EMBRAPA	IFES-STeresa
NEF 04	NOV/01	PB	IFES-ITAPINA
NEF 05	PRT	PB	IFES-ITAPINA
NEF 06	RPB	PB	IFES-ITAPINA
NEF 07	INC1	PB	IFES-ITAPINA
NEF 08	INC2	PB	IFES-ITAPINA
NEF 09	INC 3	ES	IFES-ITAPINA
NEF 10	JFT	ES	IFES-ITAPINA
NEF 11	IFT01	MG	IFES-ITAPINA
NEF 12	IFT02	MG	IFES-ITAPINA
NEF 13	IFT03	PB	IFES-ITAPINA
NEF 14	IFT04	PB	IFES-ITAPINA
NEF 15	IFT05	ES	IFES-ITAPINA
NEF 16	IFT06	ES	IFES-ITAPINA
NEF 17	IFT07	MS	IFES-ITAPINA
NEF 18	IND	?	Novabra Energia ES, S.A.
NEF 19	AL 5	?	Novabra Energia ES, S.A.
NEF 20	AL 6	?	Novabra Energia ES, S.A.

define exciton trapping and electron transport (Table 15.1). Although the JIP-test is an oversimplification of the energy flux theory, it nevertheless incorporates the in situ complexities of antenna structure (pigment arrangement, exciton migration, and connectivity). Besides considering possible damages to PSII functionality and to the electron transport chain, the JIP-test parameters can give additional insights in the understanding of oxidative stress, i.e., role of PSI to prevent photodamage of PSII and changes in the PSII\PSI ratio (Kalaji et al. 2016).

Transferring a leaf from the dark to light results in the progressive turning off of its PSII reaction centers, which is followed by CF quenching due to two types of mechanisms. We highlight (1) the parameter of *real quantum yield* of energy conversion in PSII (φPSII) that measures the proportion of light absorbed by the chlorophyll associated with PSII, which is actually used at the beginning of the photochemical stage, and (2) the parameter of quenching or *non-photochemical quencher* (NPQ), which is derived from the Stern-Volmer equation and used mainly to indicate the excess radiant of energy dissipated as heat in PSII (Schreiber et al. 1986; Genty et al. 1989; Maxwell and Johnson 2000).

The method based on this concept is extremely simple: at any given illumination state, Q_A can be fully reduced by a saturation pulse of light, such that photochemical quenching is completely suppressed. During the saturation pulse, a maximal

fluorescence state, F_M', is achieved, which generally shows a value lower than the dark state (F_M) and serves as a reference. According to the assumption that non-photochemical quenching does not change during a short saturation pulse, the reduction of F_M can be used as a measure of non-photochemical quenching. A useful fluorescence parameter derived from saturation pulse method is the efficiency of PSII photochemistry, which is calculated as $\Phi PSII = (F_M' - F_t)/F_M'$. The parameter that describes energy dissipation, F_V'/F_M', is an estimate of the PSII quantum efficiency if all PSII reaction centers are in the open state; it is calculated as $F_V'/F_M' = (F_M' - F_0')/F_M'$. Since ΦPSII is the quantum yield of PSII photochemistry, it can be used to determine linear electron transport rate (ETR) as described by Genty et al. (1989): ETR = ΦPSIIxPPFDx0.5, where PPFD (*photosynthetic photon flux density*) is the absorbed light and 0.5 is a factor that accounts for the partitioning of energy between PSII and PSI.

When combining gas exchange measurements with CF kinetics, one has the opportunity to estimate the forms of photosynthetic regulation and a large amount of photosynthesis parameters such as mesophilic conductance (g_m), the CO_2/O_2 ratio which shows the relative specificity of the carboxylase and oxygenase activity of *ribulose-1,5-bisphosphate carboxylase/oxygenase* (RuBisCo), and the proportion of the flux density of photons absorbed by the photosynthetic pigments channeled into PSII (Stirbet et al. 2014).

15.3 Results Obtained with the NEF Germplasm Bank of *Jatropha*

Twenty clonal accessions of *Jatropha* (Table 15.2) obtained by means of cuttings from experimental field matrices of INCAPER (*Capixaba* Institute of Research, Technical Assistance and Rural Extension); EMBRAPA Bioenergy Germplasm Bank, Experimental Area of NOVABRA Bioenergy Company (located in São Gabriel da Palha, ES, Brazil); and Instituto Federal do Espirito Santo (IFES), campus Itapina (19°32′22″S, 40°37′50″W) and Santa Teresa (19°47′58″S, 40°40′25″W), were evaluated over the last 10 years. Each accession was given a number by NEF to facilitate its identification during the experiments.

Some of these results are described below, and they point out (i) on the need of research with *Jatropha* not only to fill the gap in our knowledge regarding the physiology of the species and how it can be exploited efficiently and economically but also (ii) on the peculiarities that accessions may show in different regions. The mechanisms of tolerance to environmental stresses should be better studied to provide support to breeders.

Table 15.2 *J. curcas* accessions used by the *Núcleo de Estudos da Fotossíntese* (NEF) and place of origin of cuttings obtained from matrix plants

Technical fluorescence parameters		
F_0	$F_{20\mu s}$	Minimal fluorescence, when all RCs are open
F_M	F_p	Maximal fluorescence, when all RCs are closed
F_V	$F_M - F_0$	Maximal variable fluorescence
N	S_m (M_0/V_J)	Number of times the Q_A is reduced until it reaches the maximum fluorescence
V_i	$(F_I - F_0)/(F_M - F_0)$	Relative variable fluorescence at the I step
V_j	$(F_J - F_0)/(F_M - F_0)$	Relative variable fluorescence at the J step
Specific energy fluxes		
ABS/RC	M_0 ($1/V_J$) ($1/\varphi P_0$)	Absorption flux per RC (also a measure of PSII apparent antenna size)
TR_0/RC	M_0 ($1/V_J$)	Trapping energy flux per RC
ET_0/RC	M_0 ($1/V_J$) ($1 - V_J$)	Electron transport flux per RC
RE_0/RC	M_0 ($1 - V_J$) ($1 - V_I$)	Electron flux reducing end electron acceptors at the PSI acceptor side, per RC
DI_0/RC	ABS/RC – TR_0/RC	Dissipation energy flux per RC
Phenomenological fluxes		
ABS/CS_0	Chl/CS_0	Absorption flow by CS at $t = 0$
ET_0/CS_0	φP_0 Ψ_0 (ABS/CS)	Electron transport flux by CS at $t = 0$
TR_0/CS_0	φP_0 (ABS/CS)	Flow of energy transport by CS at $t = 0$
Quantum efficiencies or flux ratios		
φE_0	ET_0/ABS	Quantum yield for electron transport
ΨE_0	ET_0/TR_0	Efficiency/probability that an electron moves further than Q_A^-
φP_0	$F_V/F_M = TR_0$/ABS	Maximum quantum yield of primary photochemistry
φD_0	F_0/F_M	Quantum yield of energy dissipation
φR_0	RE_0/ABS = $\varphi P_0(1 - V_I)$	Quantum yield for reduction of end electron acceptors at the PSI acceptor side
δR_0	$RE_0/ET_0 = (1 - V_I)/(1 - V_J)$	Efficiency of electron transfer between intermediate carriers to the reduction of end electron acceptors of PSI
DI_0/ABS	$1 - TR_0$/ABS	Quantum yield of energy dissipation in PSII antenna
RC/ABS	ϕ_{P0} (V_J/M_0)	Density of photosynthetically active PSII reaction centers (reduction of Q_A per PSII reaction center)
$\varphi P_0/(1 - \varphi P_0)$	TR_0/DI_0 = kP/kN = F_V/F_0	Maximum efficiency of the photochemical process in PSII
$\Psi E_0/(1 - \Psi E_0)$	$ET_0/(dQ_A^-/dt_0)$	The ability to feed electrons into the electron chain between PSII and PSI
$\delta_{R0}/(1 - \delta_{R0})$		Performance of oxy-reduction reactions of PSI
Performance indexes		
PI_{abs}	(RC/ABS) ($\Phi P_0/-1\Phi P_0$) ($\Psi E_0/1 - \Psi E_0$)	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptor
		Potential performance index of PSII
PI_{total}	PI_{abs} $\delta R_0/(1 - \delta R_0)$	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors
		Total photochemical performance index

15.3.1 Effects of Environmental Stresses

Our first experiment was carried out with the objective of investigating the effects of environmental variations, such as rainfall and temperature variation, on the kinetics of CF induction in leaves of three *Jatropha* accessions. These three accessions were Janaúba (NEF 01), CPATSA 1501 (NEF 02), and CPATSA C2/10 (NEF 03) (the first preselected by farmers, the second and third from the germplasm bank of EMBRAPA Agroenergy). For 4 years we have followed the development of these genotypes in the experimental field of IFES, Santa Teresa, ES.

CF emission data were collected between 7 and 9 am in fully expanded young leaves, preadapted to dark for 40 min, using a portable fluorometer (HandyPea, Hansatech Instruments Ltd., UK). The relative quantification of chlorophyll was performed with a portable chlorophyllometer SPAD-502 (Minolta Camera Co. Ltd.), and plants were characterized by measurements of stem height and diameter as well as the number of branches, flowers, and fruits counted. The net assimilation rate of CO_2 (A, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}), transpiration (E, mmol m^{-2} s^{-1}), and intercellular CO_2 concentration (C_i, μmol^{-1}) were measured under artificial saturating photosynthetic active radiation (1300 μmol m^{-2} s^{-1}) with an infrared gas analyzer (LCIPro$^+$ ADC Bioscientific Ltd., England, UK), operating in the open system and with an airflow of 380 ppm. Whenever possible, all measurements of CF, gas exchange, and total chlorophyll were performed in the same time and on the same leaves.

The measures of our first experiment were performed during the acclimatization period, outside the seedling nursery, in October 2009 (Fig. 15.2). In November, after 40 days of acclimatization, the plantlets were transferred to the field (Galazzi 2011; Galazzi and Silva 2011). Plants of the genotype Janaúba (NEF 01) were chosen as control because this genotype was recommended for planting in all regions suitable for cultivation of *Jatropha* in the Espirito Santo State. In the first year of analysis, heavy rains occurred in the summer of December 2009 and January 2010. The IFES experimental area of Santa Teresa was flooded for 2 weeks, and several plants of the experiment were lost. The results presented in Fig. 15.2 (January 2010) were performed with plants under flooding conditions. However, the excess water in the soil did not change the *potential performance index* of PSII (PI_{ABS}) nor *total performance index photochemical* (PI_{TOTAL}) of the accessions NEF 02 and NEF 03 when compared to the genotype NEF 01 taken as a reference and set equal to unity. Furthermore, it can be seen from Fig. 15.2 (May 2010 and August 2010) that the plants of the genotype NEF 03 presented the best PI_{TOTAL}. This result was reached because there was a greater efficiency in the reduction of the PSI electron acceptors $[\delta R_0/(1 - \delta R_0)]$, which parameter is one of the four of the PI_{TOTAL}. PI_{TOTAL} is related to the spatial and functional organization of photochemical steps of photosynthesis and, therefore, demonstrate the functional state of the photosynthetic apparatus (Chen et al. 2011). It is also observed that the genotype NEF 02 presented the lowest PI_{TOTAL} in most of the evaluated months analyzed. This result was evidenced by the low RC/ABS, $\varphi P0/(1 - \varphi P0)$, and $\psi E0/(1 - \psi E0)$

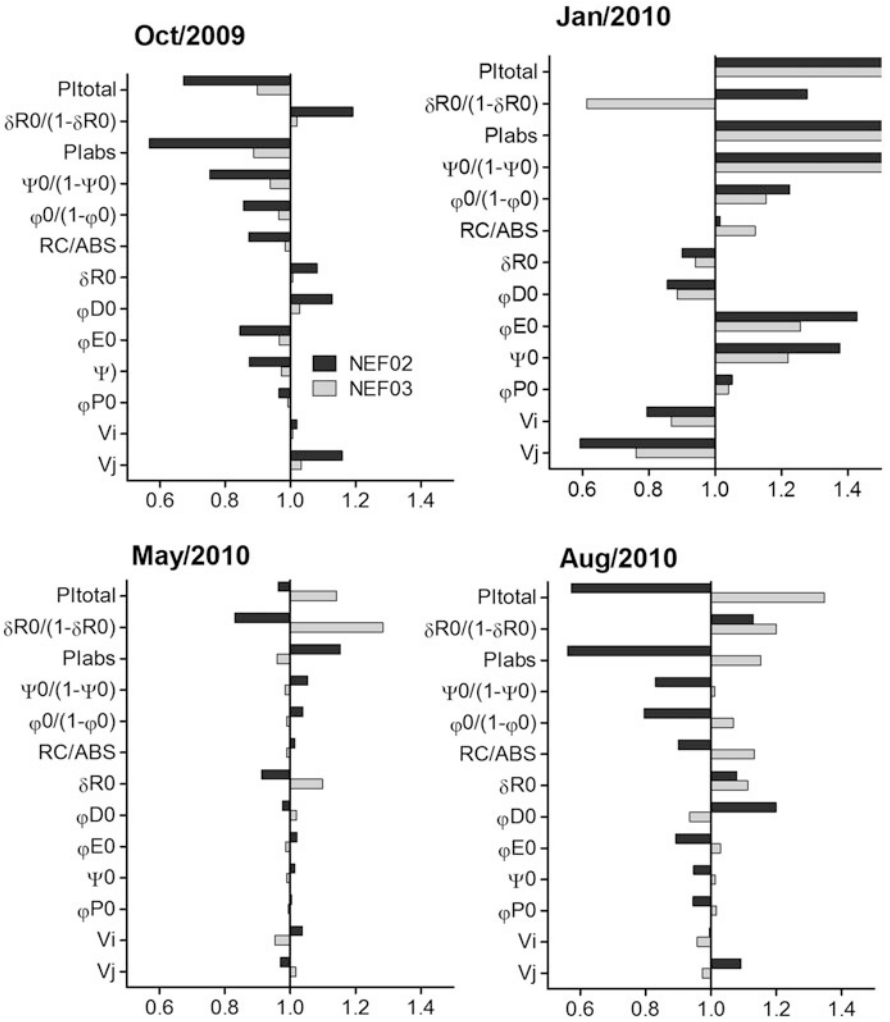

Fig. 15.2 Selected JIP-test parameters of three *J. curcas* accessions, NEF 01, NEF 02, and NEF 03. The average value of NEF 01 was used as reference and set to unity. See Table 15.2 for parameter description. Relative values are calculated from averages of 120 measurements

values. The NEF 02 plants also presented a high quantum yield of energy dissipation (φD_0), a lower rate of electron moving further than Q_A^- (ΨE_0) accompanied by a smaller maximum quantum yield primary photochemistry (φP_0) (Santos et al. 2012) suggesting a photoinhibition of the photosynthetic process (Ohada et al. 2011). The absence of precipitation in the month of August 2010 in the region could explain, in part, the results obtained. It has been observed that the presence of much water in the soil did not alter the vitality of the NEF 02 plants, but the drought registered in August resulted in a greater sensitivity of this genotype.

Table 15.3 Chlorophyll index obtained with chlorophyllometer SPAD-502 in leaves of *J. curcas* plants grown in the experimental area of the Santa Teresa campus of the Instituto Federal do Espirito Santo, during 2011 and 2012

Chl index							
	Aug	Sept	Nov	Dec	Feb	Mar	May
NEF 01	18.19 ns	16.717 b	42.23 ns	34.84 b	41.57 ns	40.17 b	51.75 ns
NEF 02	21.57 ns	24.999 a	44.20 ns	39.12 a	43.00 ns	43.08 ab	49.39 ns
NEF 03	22.47 ns	25.284 a	41.56 ns	34.44 b	44.75 ns	45.78 a	48.52 ns

The averages followed by the same letter in the column do not differ statistically according to Tukey test 5% ($n = 10$)

Table 15.4 Number of branches, height, and diameter of shoot in triplicate clones of *J. curcas* genotypes cultivated in the experimental area of the Santa Teresa campus of the Instituto Federal do Espirito Santo

Growth							
		Aug	Oct	Dec	Feb	Mar	May
Number of branches	NEF 01	2.6 a	4.2 a	3.9 b	5.8 a	5.4 a	5.6 a
	NEF 02	4.7 a	3.3 a	5.8 a	6.3 a	6.0 a	6.3 a
	NEF 03	4.8 a	3.5 a	3.1 b	6.4 a	5.2 a	4.9 a
Shoot height	NEF 01	117.0 a	114.5 a	163.2 ab	173.6 a	204.4 a	198.9 b
	NEF 02	120.9 a	123.7 a	174.9 a	178.8 a	226.1 a	235.0 a
	NEF 03	127.0 a	118.5 a	157.4 b	172.4 a	228.3 a	228.3 b
Shoot diameter	NEF 01	15.4 b	21.0 a	6.6 b	10.5 a	9.1 a	10.9 b
	NEF 02	19.7 a	23.5 a	7.9 a	9.6 a	9.6 a	13.4 a
	NEF 03	18.0 ab	20.7 a	5.9 b	10.7 a	12.8 a	9.0 b

The averages followed by the same letter in the column do not differ statistically according to Tukey test 5% ($n = 10$)

New analyses were performed between August 2011 and May 2012. There were significant differences in the chlorophyll index of the accessions (Table 15.3).

Analyses of the morphological characteristics of the three accessions growing in the experimental area of the IFES of Santa Teresa revealed significant differences in the number of branches formed during the month of December 2011. The three accessions presented better growth in respect of height in December 2011 and May 2012 with larger stem diameters in August 2011, December 2011, and May 2012. In addition, growth data showed that the plants developed better for NEF 02 in December 2011 and May 2012, while NEF 01 and NEF 03 performed similarly (Table 15.4).

As already mentioned, the CF induction curves (O-J-I-P) were analyzed according to the JIP-test (Tsimilli-Michaelli and Strasser 2008; Strasser et al. 2010), which allowed the calculation of several phenomenological and biophysical expressions that quantify the energy flow through the electron transport chain (Fig. 15.3). It was observed that in August, the best PI_{ABS} and PI_{TOTAL} occurred in both NEF 02 and NEF 03 accessions, which means that they had better performances in all redox reactions of the electron transport chain of the photochemical

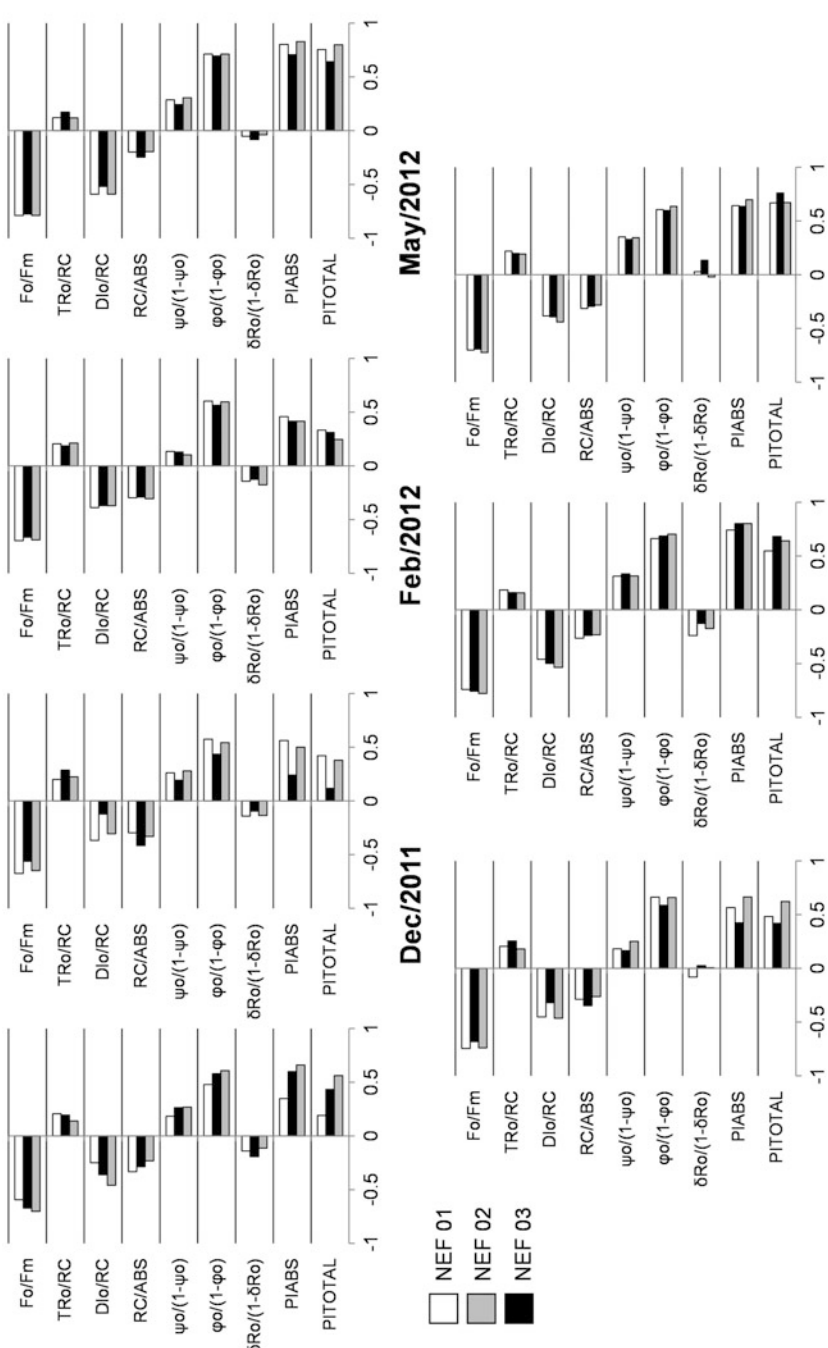

Fig. 15.3 Selected JIP-test parameters of three *J. curcas* accessions, NEF 01, NEF 02, and NEF 03. For each parameter, the average value of 120 measurements is used as the basis for normalization (take as control = 0). See Table 15.2 for parameter description

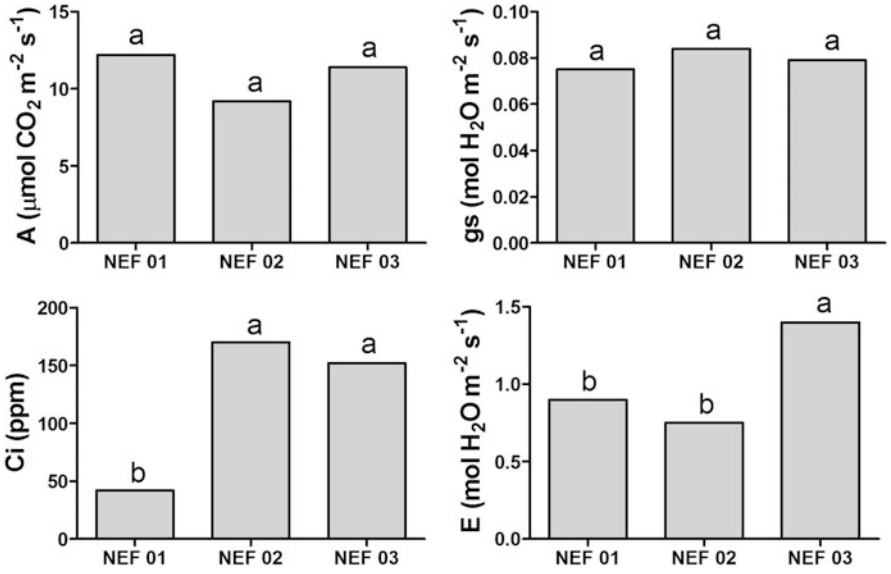

Fig. 15.4 Net assimilation rates of CO_2 (A), internal CO_2 in the sub-stomatal cavity of plants (C_i), stomatal conductance (g_s), and transpiration (E) of the three *J. curcas* accessions. Values with the same letter are statistically equal with Turkey test ($n = 10$)

steps of photosynthesis compared to the NEF 01 accession. When comparing the activity of the electron transport chain of these plants during the first and second year of cultivation, it was observed that the performance indexes, PI_{ABS} and PI_{TOTAL}, remained high. Even though the *density of photosynthetically active PSII reaction centers* (RC/ABS) varied, the *performance of oxy-reduction reactions of PSI* [δ_{R0}/($1 - \delta_{R0}$)] decreased, and there were variations in the *quantum yield of energy dissipation* (φD_0). Thus, it was necessary to disentangle the plausible mechanisms of tolerance to the diverse environmental stresses that these plants had undergone, which were subjected to dry, cold, rainy, and high-temperature conditions.

Measurements of gas exchange were only made in May 2012, and no significant difference was observed in the *net assimilation of CO_2* (A) nor in the stomatal conductance (Fig. 15.4). These results validate those found in the photochemical stage, where high PSII performance on the entire electron transport chain was observed, mainly in the NEF 03 accession, associated with higher transpiration rate and high A (Santos et al. 2012).

In 2013, the plants from the three genotypes completed 4 years, and reproductive phenological phases could be recorded from November 2012 to April 2013. A larger percentage of inflorescences were observed in November, December, and February. The number of open flowers was larger in NEF 02 and NEF 01 than in NEF 03. For NEF 02, the months where the largest number of opened flowers was observed were February and March. This genotype also had the highest number of female flowers, whereas NEF 01 had the largest number of male flowers (Fig. 15.5). The greatest

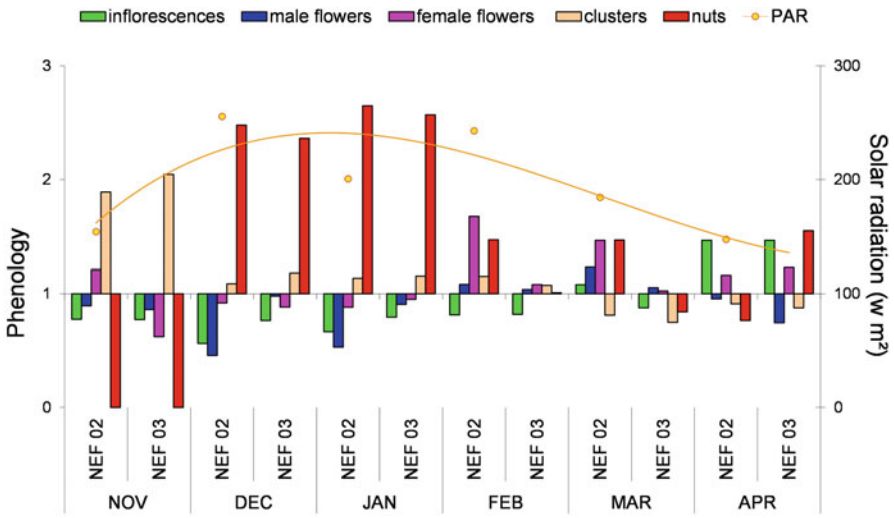

Fig. 15.5 Reproductive phenological phases from November 2012 to April 2013 of three *J. curcas* accessions grown in the experimental area of IFES, Santa Teresa, ES, since 2009. The data shown are mean values of at least ten repetitions

number of developing fruits and cluster formation was observed in November. The NEF 02 and NEF 03 accessions showed a larger number of ripe fruits and nuts in relation to those of NEF 01 in December, January, and April; in the other months, no difference was observed. In March and April, there was an increase in the percentage of female flowers, although the percentage of male flowers in all accessions was larger than the percentage of female flowers (Fig. 15.5).

15.3.2 Effects of Temperature

The performance of the same three accessions NEF 01, NEF 02, and NEF 03 was evaluated for efficiency in the capture of light energy in response to alternating temperature and luminosity. Cuttings were planted in September 2011 in plastic pots of 12 L, containing humus and sand in the proportion of 1:2, and kept in a greenhouse with irrigation every 2 days, under natural light and temperature. Transient fluorescence of chlorophyll *a* was used as the main tool in the detection of plant stresses, and gas exchange was used to validate the results obtained.

After 3 months of growth, a first experiment was performed where five plants of each genotype were analyzed in three schedules (9 am, 1 pm, and 4 pm) to record the effect of temperature variations throughout the day on the photochemical efficiency of the plants. The minimum and maximum temperatures were 21°/31°, 23°/31°, and 28°/31°, at 9 am, 1 am, and 4 pm, respectively. Using the JIP-test, it was observed that the lower energy use by NEF 01 plants (taken as reference and equal to unit)

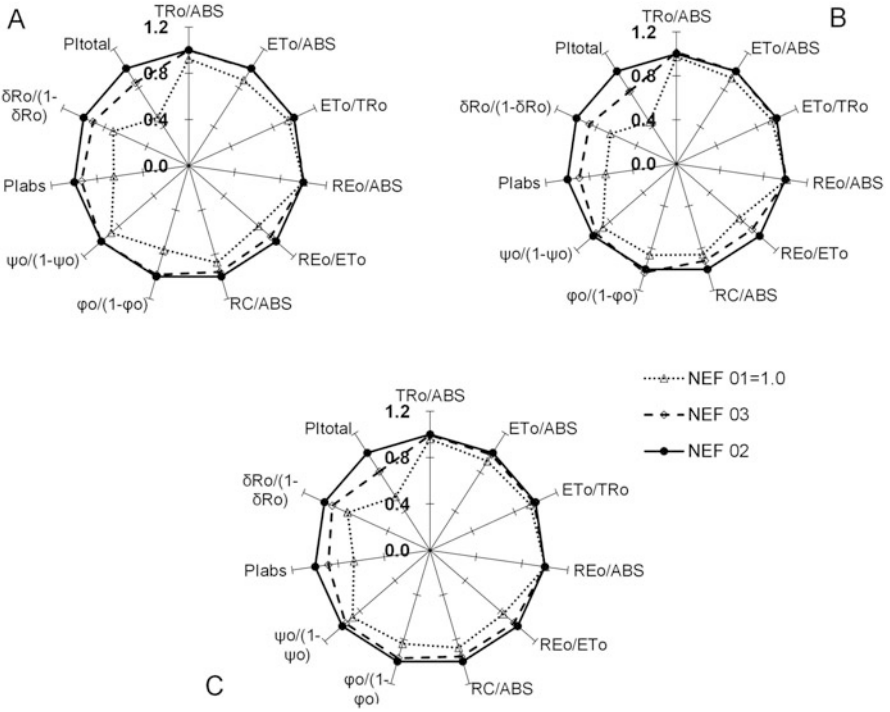

Fig. 15.6 Selected JIP-test parameters of three *J. curcas* accessions, NEF 01, NEF 02, and NEF 03. The average value of NEF 02 was used as reference and set to unity. (**a**) Measurements were taken at 9 am, (**b**) 1 pm, and (**c**) 4 pm. Data represent the mean value from five replicates

compared to NEF 02 and NEF 03 was due to the lower density of its photosynthetically active PSII reaction centers (RC/ABS), lower maximum efficiency of the photochemical process in PSII [$\varphi P_0/(1 - \varphi P_0)$], lower transport of excited electrons from PSII to PSI [$\Psi E_0/(1 - \Psi E_0)$], and lower rates of oxidation-reduction reactions in PSI [$\delta R_0/(1 - \delta R_0)$] in the three times of the day where the measurements were taken (Fig. 15.6). Consequently, the PI_{ABS} and PI_{TOTAL} in plants of the NEF 02 and NEF 03 accessions presented values significantly higher than those of the NEF 01 genotype, evidencing a lower efficiency in light energy use in relation to these accessions. The lower efficiency of light energy utilization was also associated with higher dissipation energy flux per RC (DI_0/RC) shown by NEF 01 plants (Tessari et al. 2012).

It is important to record that the plants from the NEF 01 genotype (Janaúba) were chosen as control throughout the comparisons of our investigations because this genotype was recommended for planting in all regions suitable for the *Jatropha* cultivation in the Espirito Santo State. However, in culture under greenhouse, the NEF 01 genotype presented a lower efficiency in the use of light energy in the three

periods evaluated. The temperature during this period had a greater variation in the morning (10 °C) than in the afternoon, which was very small (3 °C). The genotype preselected by the farmers (NEF 01) had a lower index of total photochemical performance (PI_{TOTAL}), showing less efficiency in the photosynthetic machinery, throughout the day.

A second experiment was performed simulating the fall in temperature that occurs frequently in the months of July and August in regions of higher altitudes in the Espirito Santo State. The sensitivity of *Jatropha* to night temperatures below 15 °C (dark chilling) is reflected in the changes that occur in metabolism, growth, development, and yield (Brestic et al. 2012). Plants of the three accessions were submitted to low night temperature in cold room for 3 days (25/10 °C), while control plants were kept at room temperature (25/19 °C). The following morning of each cold night, the gas exchanges and the CF kinetics were measured. After 15 days, the analysis was repeated with the purpose of verifying how plants could recover from a cold stress according to their genotype. The results showed that the applied cold stress at night did not cause significant changes in NEF 01 (Tessari et al. 2012). In NEF 02, changes were observed only after 2 days of treatment, while in NEF 03, the *A* decreased over 3 days, accompanied by a drop in g_s and an increase in C_i/C_a. Thus, stomatal conductance may have decreased due to the direct inhibition of their opening by cold with the side effect of raising the internal CO_2 concentration (C_i), as suggested by Allen et al. (2000), which explains the high value of the increasing C_i/C_a ratio despite the constant ambient CO_2 concentration (C_a) (Fig. 15.7).

The decrease in night temperature for 3 days did not inhibit the maximum quantum yield primary photochemistry of PSII ($TR_0/ABS = F_V/F_M$) in any of the three accessions, a feature that has also been observed by Neuner and Larcher (1991) with oleaginous soybean and *Jatropha* cultivars. There was a decrease in the quantum yield of energy dissipation in PSII antenna (DI_0/ABS) on the first day after low night temperature. There was even an increase in performance indices, especially in NEF 01. The other parameters of the photochemical stage were not affected, evidencing that there was no photochemical participation in the fall of *A* in NEF 01 (Fig. 15.8).

In summary, it was clear that the three accessions of *Jatropha* subjected to cold stress had different behaviors in their primary metabolism (primary photochemistry and gas exchange). These results are encouraging for the selective breeding of genotypes with improved survival to nocturnal cold stress. Studies with young plants of *Jatropha* under cold stress had already shown that seedlings maintained higher antioxidant enzymatic activities (SOD, APX, CAT, and GR) and antioxidant levels (AsA and GSH) as well as higher osmolyte contents (proline and betaine) when compared to the control. This behavior indicates that antioxidants and osmolytes play an important role in cold tolerance induced by low-temperature shock in seedlings of *Jatropha* (Ao et al. 2013; Li et al. 2013).

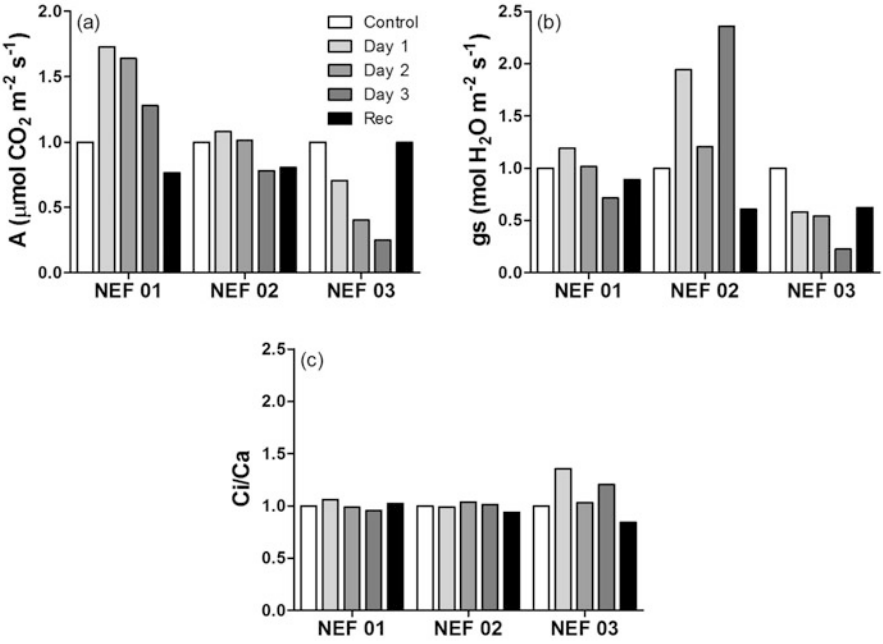

Fig. 15.7 Net assimilation rates of CO_2 (A), stomatal conductance (g_s), and ratio of leaf intercellular CO_2 concentration to that in the surrounding atmosphere (C_i/C_a) of plants of the three *J. curcas* accessions submitted to low night temperature for 3 days 25/10 °C and after 15 days of recovery. Control plants were kept at room temperature 25/19 °C. For each accession, the average value of control is used as a reference data for normalization (equal to 1.0)

15.3.3 Effects of Large Radiation Intensities, Wind, and Nutritional Deficit

Plants of the genotype NEF 01 were also cultivated in farms throughout the Espirito Santo State. The team from the *Núcleo de Estudos da Fotossíntese* of the Universidade Federal do Espirito Santo followed the development of these plants in several locations. The coastal region of Pontal do Ipiranga (latitude, 19°23.28′S; longitude, 40°04.20′W) of the municipality of Linhares presents warm humid tropical climate, with rains in the summer, dry winter, and average temperature around 24 °C. In contrast, the climate varies significantly due to the great topography variation in the municipality of Itarana (latitude, 19° 55.319′S; longitude, 40° 50.838′W), which corresponds to an inland region. On average, the temperature is hot in the summer (average of 28 °C) and cold in winter with extremes of 8 °C. Photosynthesis measurements were performed during 18 months (the first measure was performed 5 months after planting, the second measure 6 months after planting, the third measure 8 months after planting, and the fourth measure at 18 months after

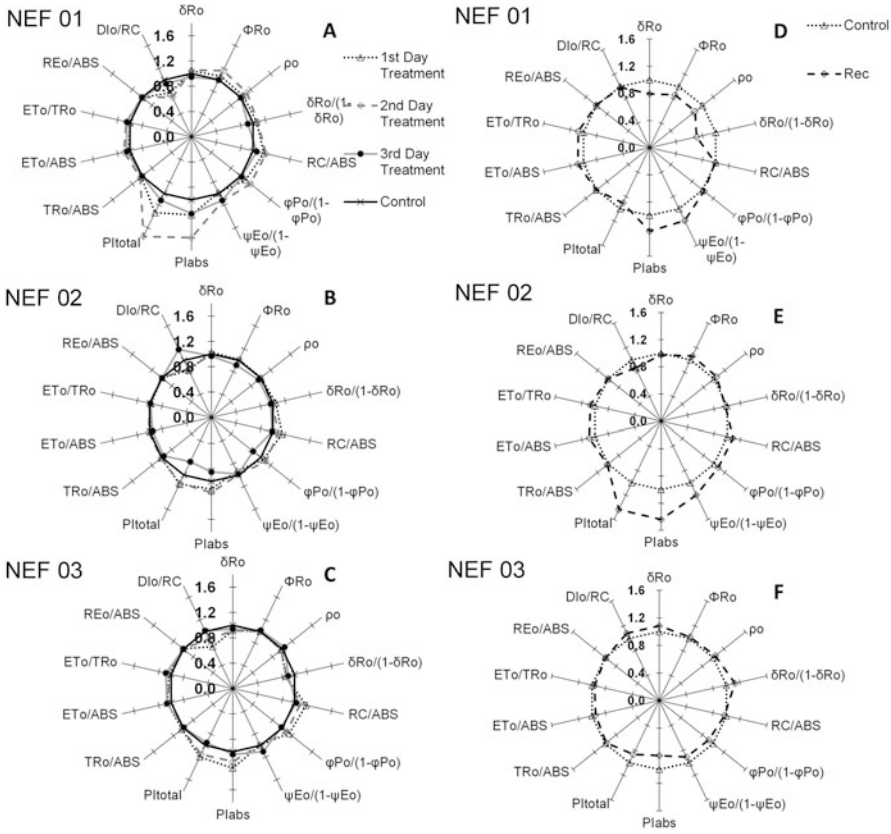

Fig. 15.8 Radar plot of several parameters calculated by the JIP-test and plotted on logarithmic scale of plants of three *J. curcas* accessions, submitted to low night temperature (25/10 °C) for 3 days 25/10 °C (**a**–**c**) and after 15 days of recovery (**d**–**f**). Control plants were kept at room temperature 25/19 °C. For each accession, the average value of the control was used as a reference for data normalization and set to unity ($n = 10$)

planting in 2010). The fluorescence emission of chlorophyll *a* was measured in fully expanded young leaves. The rates of *A* were measured between 8 and 9 am at a CO_2 concentration of 380 ppm, an O_2 of 21%, and moderate temperatures between 32 and 35 °C, with an IRGA analyzer. The values of all analyzed parameters had the inland region as a reference because it is recommended for planting *Jatropha* in the Espirito Santo State.

The specific flows of absorbed (ABS/RC), captured (TR_0/RC), and transported (ET_0/RC) energy were slightly higher in the littoral plants in the first measurements, but the energy dissipated (DI_0/RC) in the PSII when the plants had 6 *months after planting* (MAP) compromised PI_{ABS} and PI_{TOTAL}. When the plants reached

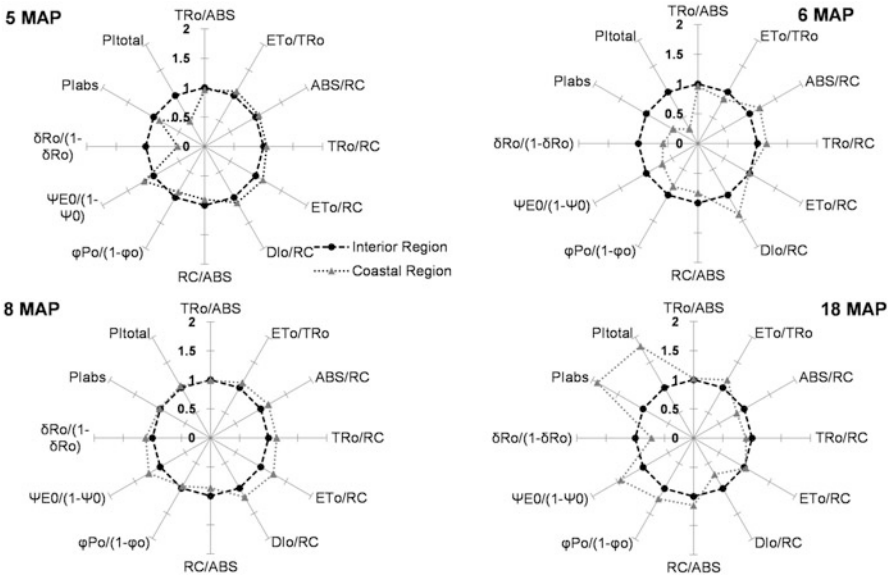

Fig. 15.9 Radar plot of several parameters calculated by the JIP-test and plotted on logarithmic scale of plants from the genotype NEF 01 cultivated in commercial farms in the inland (Itarana) and coastal (Pontal do Ipiranga) regions of the Espirito Santo State. Averages of the inland region plants were taken as reference and were used for normalization (equal to 1.0). First measure = 5 months after planting (MAP); second measure = 6 MAP; third measure = 8 MAP; fourth measure = 18 MAP in 2010 ($n = 10$)

18 MAP, a higher photochemical performance was observed in plants grown in the littoral region, but with only a little growth in height and number of branches and leaves, suggesting the reduced utilization of light energy by the plants from this area (Fig. 15.9).

Therefore, at 5 MAP, lower values in the performance of the redox reactions in the PSI [$\delta R_0/(1 - \delta R_0)$] caused a smaller efficiency of the photochemical reactions of PSII. According to Oliveira et al. (2018), different water supply regimes can cause different effects to photosynthesis and interfere with metabolic processes and crop yield. Indeed, in the coastal region, the availability of water is much lower than in the inland region. The total photosynthetic performance index PI_{TOTAL} translates information from the light energy absorption of the PSII electron acceptors to the final reduction in PSI across the energy cascade. It is known that PI_{TOTAL} allows to infer the ability of PSII to convert light energy into redox energy, given the entire electron transport chain of photosynthesis. In the coastal region, which is characterized by a combination of abiotic stresses, including, among others, high wind, sandy soil, and high salinity, a drop in PI_{TOTAL} was observed at 6 months after planting, possibly indicating an increase in the regulation of efficiency of photosynthetic machinery (Fig. 15.9).

In the second year (18 MAP), the specific flows of absorbed (ABS/RC), captured (TR_0/RC), and transported (ET_0/RC) energy were similar in both coastal and inland regions. There were a decrease in energy dissipation (DI_0/RC) and an increase in the density of active reaction centers (RC/ABS) in the plants of the coastal region, which, consequently, caused a greater efficiency of the photochemical reactions of the PSII. In addition, greater efficiencies were observed in the photochemical process of PSII [$\varphi P_0/(1 - \varphi P_0)$] and in the reactions of the intersystem [$\Psi E_0/(1 - \Psi E_0)$]. Thus, PI_{ABS} and PI_{TOTAL} in redox energy over the entire transport chain of photosynthetic electrons presented significant differences in relation to those obtained in the plants from the mountainous regions. These results are very interesting, since they mean that the impact of abiotic stresses in both regions can be experimentally predicted by the fast and noninvasive technique of transient fluorescence O-J-I-P (Gasparini et al. 2015). This argument may not be entirely reasonable, since the rate of CO_2 assimilation can also be regulated by modifications in the RuBisCo activity, which cannot be recognized through transient fluorescence measurements, that is, when RuBisCo is inactive. However, it should be considered that in the in situ experiments, two types of adaptation are handled: plants adapted to dark (in measurements through transient fluorescence) and adapted to light (in measurements with the IRGA) in addition to the data on climatic variations as well as water and nutrients availability during the measurements (Fig. 15.10). Nevertheless, it can be observed that in both measures the PI_{TOTAL} and A were higher in the plants from the coastal region.

Measurements were, then, performed between December 2012 and April 2013 on *Jatropha* plants grown in Itarana (inland region) and Pontal do Ipiranga (coastal region). The plants, which were planted in January 2010, already presented flowers and fruits. Data from reproductive phenology, gas exchange as well as chlorophyll fluorescence to transient and modulated chlorophyll index were evaluated (Santos

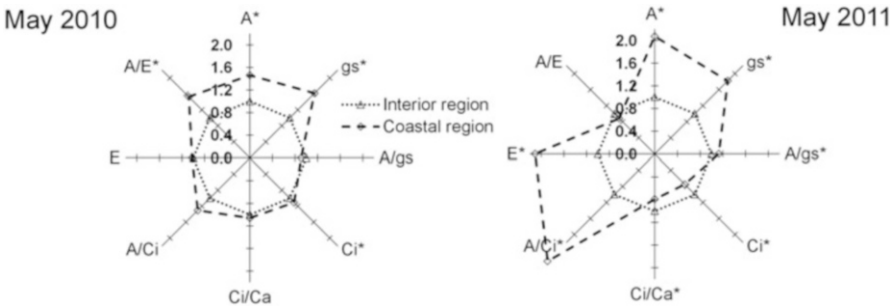

Fig. 15.10 Net assimilation rates of CO_2 (A), stomatal conductance (g_s), intrinsic water-use efficiency (A/g_s), internal CO_2 in the sub-stomatal cavity of plants (C_i), ratio of leaf intercellular CO_2 concentration to that in the surrounding atmosphere (C_i/C_a), instantaneous carboxylation efficiency (A/C_i), transpiration (E), water-use efficiency (A/E) in plants of NEF 01 grown in May 2010 and May 2011 in commercial farms in the inland (Itarana) and coastal (Pontal do Ipiranga) regions of the state of Espirito Santo ($n = 10$). * indicates significant values according to the Tukey test ($p < 0.05$)

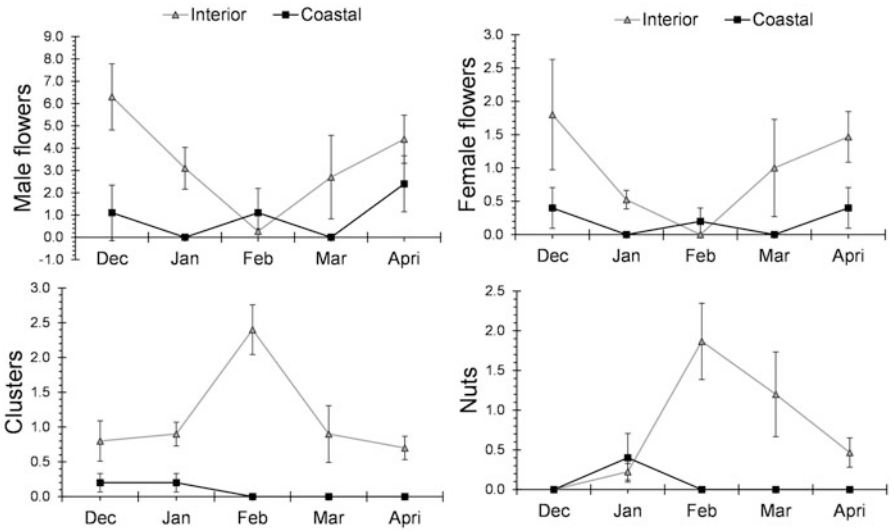

Fig. 15.11 Reproductive phenological phases from December 2012 to April 2013 of the NEF 01 accession grown in the inland and coastal regions of Espirito Santo State. Values are means ± SD ($n = 10$)

et al. 2014). The largest blooming season of plants grown in Itarana was observed during the assessment period in December and January, which had the largest record number of nuts in March. Plants grown in Pontal do Ipiranga showed lower growth of shoots, inflorescences, as well as flower and fruit numbers (Fig. 15.11).

A drop of older leaves was observed during the period of water stress. This was followed by a regrowth after the first rains in the region of Pontal do Ipiranga, while in the region of Itarana, there was no leaf drop during the analysis period (dry period). Gas exchange (December, January, and February) showed the highest rates in Itarana at the beginning of the reproductive period and gradually fall over the successive months, while in the region of Pontal do Ipiranga, the reverse occurred, and the highest rates of net photosynthesis took place at the end of the fruiting period (Fig. 15.12).

To compare the samples from both regions according to O-J, O-I, and I-P phases (see Sect. 15.2), we normalized the transient fluorescences in relative terms (V): $V_{OJ} = (F_t - F_0)/(F_J - F_0)$, $V_{OI} = (F_t - F_0)/(F_I - F_0)$, and $V_{IP} = (F_t - F_I)/(F_P - F_I)$. In addition, we also calculated the kinetic differences, $\Delta V = V - V_{ref}$ ("ref" is for plants from the inland region), considering that the agroclimatic data were more favorable to the cultivation of *Jatropha* in these regions than in the littoral area.

The kinetic differences between O, J, I, and P steps of the raw or normalized transients revealed bands that are usually hidden (Gama et al. 2013). The kinetic differences ΔV_{OJ} revealed the K-band (at about 300 μs) which, when positive, is considered to reflect either an inactivation of the oxygen evolving complex or an increase of the functional PSII antenna size. After heat treatment, or at high

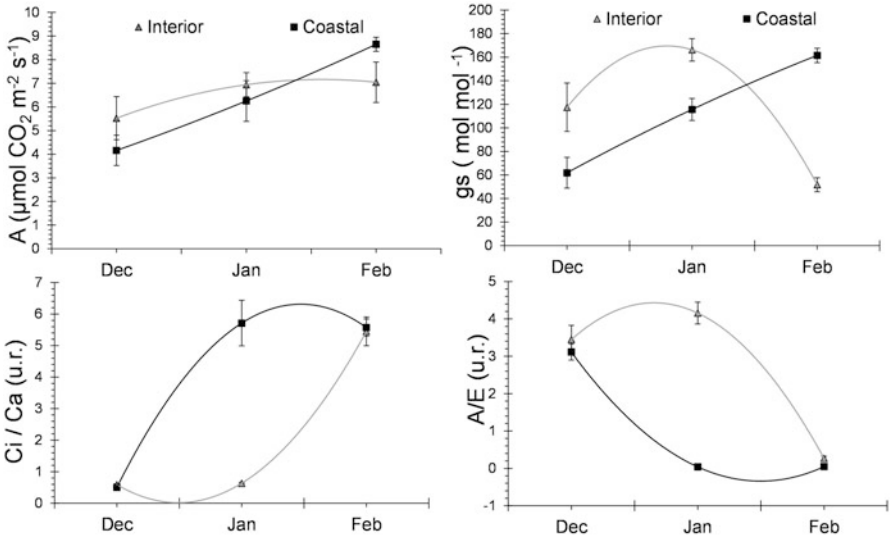

Fig. 15.12 Net assimilation rates of CO_2 (A), stomatal conductance (g_s), ratio of leaf intercellular CO_2 concentration to that in the surrounding atmosphere (C_i/C_a), water-use efficiency (A/E) in plants of NEF 01 from December 2012 as well as January and February 2013 in commercial farms in the inland (Itarana) and coastal (Pontal do Ipiranga) regions of the Espirito Santo State. Values are means ± SD ($n = 10$)

temperature, when inactivation of the oxygen evolving complex is induced (see, e.g., Falqueto et al. 2017), the K-band appears even in direct fluorescence transients (then called OKJIP). The L-band (Stirbet and Govindjee 2012) at about 150 μs is an indicator of the energetic connectivity (grouping) of the PSII units, being higher when the connectivity is lower (Strasser and Govindjee 1991). An activation of FNR (ferredoxin-NADP reductase) can affect considerably the O-J-I-P transient. For example, it was assumed that the two peaks, labeled as G and H, which were observed after the I step in *Trebouxia*-containing lichens, are due to FNR activation (Ilik et al. 2006; Morales-Flores et al. 2013). A different explanation for the G peak was given by Lazár (2013); based on his PSI fluorescence model, he has suggested that the G peak may be a manifestation of PSI variable fluorescence. In his opinion, this idea is also supported by experimental data showing that PSI can emit significant variable fluorescence under strong reducing conditions.

The analysis of the kinetic differences of fluorescence emission curves between stages O (20 μs) and P (300 ms) using the equation $\Delta V_{OP} = V_{OP(Pontal)} - V_{OP(Itarana)}$ indicates positive variations in the leaves of plants grown in Pontal do Ipiranga (Fig. 15.13a), which clearly demonstrate the inhibition of electron transport between PSII and PSI in the first 3 months (December to April).

Furthermore, fluorescence data were normalized between the steps O and J (2 ms), as $V_{OJ} = (F_t - F_0)/(F_J - F_0)$, and plotted with the ΔV_{OJ} between the samples from Pontal do Ipiranga and Itarana in the 0–300 μs range revealing the

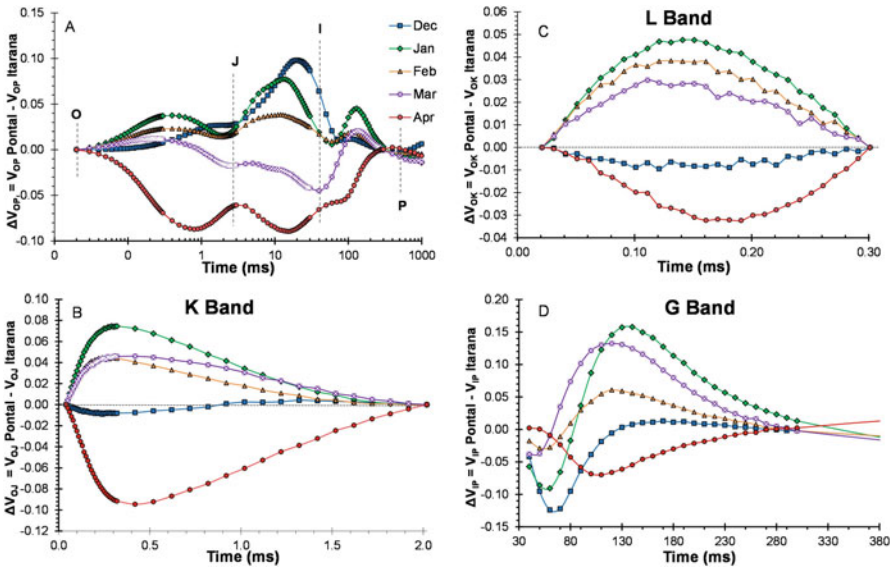

Fig. 15.13 Kinetic differences of chlorophyll *a* fluorescence normalized between the steps O and P [$\Delta V_{OP} = V_{OP\ (Pontal)} - [V_{OP\ (Itarana)}]$ (**a**); between the steps O and K [$\Delta V_{OK} = V_{OK\ (Pontal)} - V_{OK\ (Itarana)}$] (**b**); between the steps O and J [$\Delta V_{OJ} = V_{OJ\ (Pontal)} - V_{OJ\ (Itarana)}$] (**c**); and between the steps I and P [$\Delta V_{IP} = V_{IP\ (Pontal)} - V_{IP\ (Itarana)}$] (**d**) in plants of NEF 01 cultivated in the inland (Itarana) and coastal (Pontal do Ipiranga) regions from December 2012 to April 2013. See Table 15.2 for parameter description. All results are the averages of three independent measurements with ten repetitions

K-band (Fig. 15.13b). An increased slope (positive K-band) indicates an increased reduction rate of quinone (Q_A), the primary electron acceptor of PSII, from Q_A to Q_A^-, which could mean that the oxygen evolving complex (OEC) becomes leaky and offers access to non-water electron donors (Chen et al. 2011). A positive K-band (at about 300 μs) suggests that the OEC is either inactivated or there is an increase in the functional PSII antenna size (Stirbet 2013). The appearance of a positive K-band confirms the suggestion of damage in the process of photooxidation of water in the water-splitting complex, which decreased the connectivity of the units that form the PSII reaction center (Fig. 15.13b).

To further evaluate differences between the plants cultivated in Itarana and Pontal do Ipiranga, fluorescence data were normalized between O (20 μs) and K (300 μs) steps, as $V_{OK} = (F_t - F_0)/(F_K - F_0)$, and plotted as $\Delta V_{OK} = V_{OK\ (Pontal)} - V_{OK\ (Itarana)}$ in the time range of 50–300 μs revealing the L-band (Fig. 15.13c). The L-band has been suggested to be an indicator of the energetic connectivity (grouping) of the PSII units. The G-band was indicative of damage at the level of the plastoquinone pool, which impaired the efficiency of PSI in the plants of Pontal do Ipiranga in January, February, and March (Fig. 15.13d). Whereas the development of Jatropha culture was affected by the level of photosynthetically active radiations,

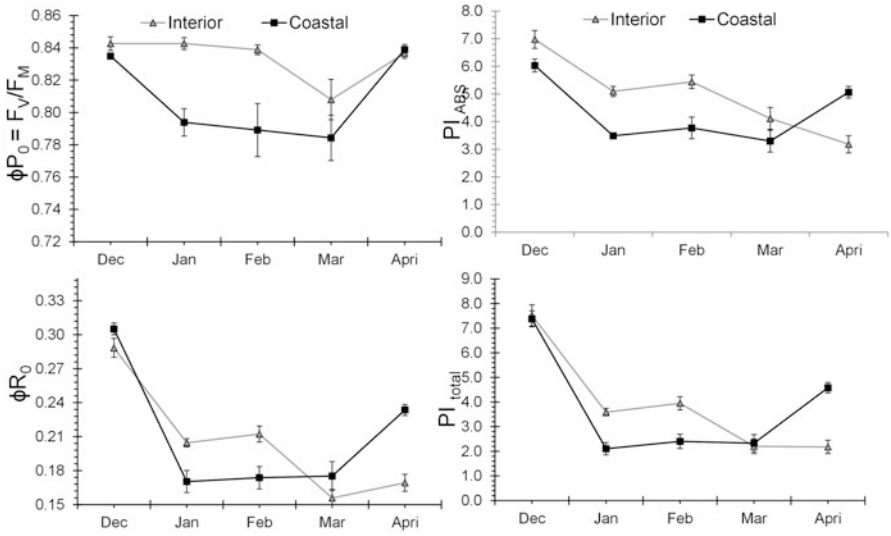

Fig. 15.14 Parameters calculated by the JIP-test, expressed in relative units of the chlorophyll *a* fluorescence, of plants of the genotype NEF 01 cultivated in commercial farms in the inland (Itarana) and coastal (Pontal do Ipiranga) regions of the Espírito Santo State (from December 2012 to April 2013). See Table 15.2 for parameter description. Values are means ± SD ($n = 10$)

seasonality of temperature, and rainfall, the effect of agroclimatic zoning on physiological variables also needed to be considered within the model. As a result, we found that the plants which possessed better crop performance were those grown in the municipality of Itarana.

The results obtained using the JIP-test also enabled us to observe that the efficiency of photosystem II (φP_0) and photosystem I (φR_0) started to decrease from the beginning of the year until March and started to recover in April. Confirming the results presented above, a similar trend was observed for the energy transport efficiency within the electron transport chain (PI_{ABS} and PI_{TOTAL}) in the coastal region, while in the inland region (Itarana), the downward trend continued in April (Fig. 15.14).

15.3.4 Effects of Shading

In a farm above a hill in the municipality of Itarana (latitude 19°52′26″S and longitude 40°52′31″W), we considered, in 2013, two conditions according to whether (i) plants received more hours of sunlight in the morning and (ii) plants received more hours of sunlight in the afternoon. The plants receiving morning light were directed to the east and received direct radiations from 6 am to 3 pm (solar

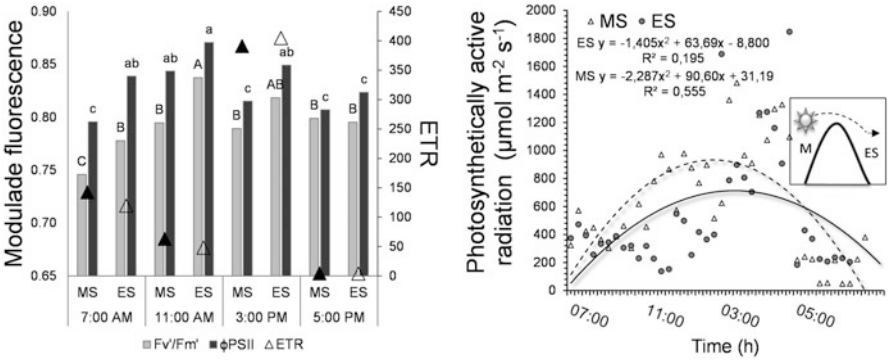

Fig. 15.15 Efficiency of excitation capture by open PSII reaction centers ($F_{v'}/F_{m'}$), actual PSII efficiency (φPSII), apparent electron transport rate (ETR), and photosynthetically active radiation during the day of measurements in plants of NEF 01 in commercial farms in the inland region (Itarana) of Espirito Santo State. Morning sun = plants that received more hours of sunlight in the morning; evening sun = plants that received more hours of the sunlight in the afternoon ($n = 10$)

time), while those receiving afternoon light began to receive direct radiation after 9 am until twilight. The measurements were performed both in young and fully developed leaves throughout the day, in two conditions of sun exposure in four schedules (7 am, 11 am, 3 pm, and 5 pm).

We used the *pulse-amplitude modulated* (PAM) fluorometry techniques to study the induction and quenching of chlorophyll fluorescence in physiological studies.

We observed that efficiency of excitation capture by open PSII reaction centers ($F_{v'}/F_{m'}$) and the actual PSII efficiency (φPSII) reached their maximum at 11 am. At this time the apparent electron transport rate (ETR) was very low and only reached its maximum at 3 pm. The ETR remained low in both treatments throughout the day; however, it was statistically different between the plants that received the highest light intensity in the morning compared to plants that received higher intensity in the afternoon. We observed that photochemical activity increased throughout the morning in both sets of plants but always showed lower values in plants that were exposed to direct solar radiation in the morning (Fig. 15.15).

The chlorophyll index measured in the new and fully expanded leaves did not differ significantly when considering those that received sunlight in the early morning or those that received sunlight for a greater number of hours in the afternoon (data not shown). However, the gas exchange analysis showed that plants exposed to the sun in the morning, except at 11 am, presented higher *A* and A/C_i throughout the day. These results suggested higher photoassimilate production, since these parameters are directly associated with CO_2 fixation (Fig. 15.16).

The ratio of the internal and external CO_2 concentration was consistent with carboxylation efficiency, i.e., lower in plants exposed to the sunlight in the early hours of the morning when the rate of photosynthesis was high. However, it was also observed that the intrinsic efficiency of water use was lower in plants that received

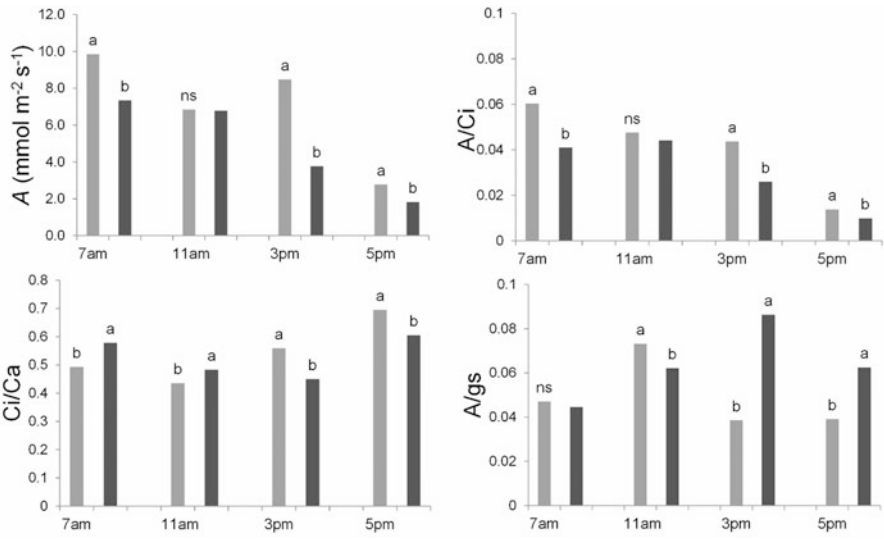

Fig. 15.16 Net assimilation rates of CO_2 (A), instantaneous carboxylation efficiency (A/C_i), ratio of leaf intercellular CO_2 concentration to that in the surrounding atmosphere (C_i/C_a), and intrinsic efficiency of water use (A/g_s) in plants of NEF 01 in commercial farms in the inland (Itarana) region of Espírito Santo State. MS = plants that received more hours of sunlight in the morning; AS = plants that received more hours of sunlight in the afternoon ($n = 10$)

more hours of sunlight in the morning (MS), suggesting greater sensitivity to high radiation and heat.

15.4 Evidence for a Physiological Marker

It can be concluded from the description of the behavior of the three accessions submitted to different environmental stresses that the parameters of transient fluorescence validated by the data of gas exchanges and the modulated fluorescence have a fine sensitivity to the environmental variations.

In December 2014, we visited the experimental area of IFES at the campus of Itapina, ES, where 15 accessions of *Jatropha* were grown. Branches were collected from each of these matrices, and cuttings were planted in 15 L pots in the experimental area in the botany department, in Vitória. The clones were kept in open-air conditions with automatic irrigation twice a day to maintain them at field capacity. In the first 2 months, 50 ml of Hoagland and Arnon nutrient solution, ½ ionic strength, was given every 15 days. Irrigation volume and frequency did not vary between the months of February and June when data collection occurred. A transient chlorophyll *a* fluorescence assessment was performed during summer (February, average temperature 30/35 °C) and winter (June, average temperature 20/30 °C) (Fig. 15.17) and

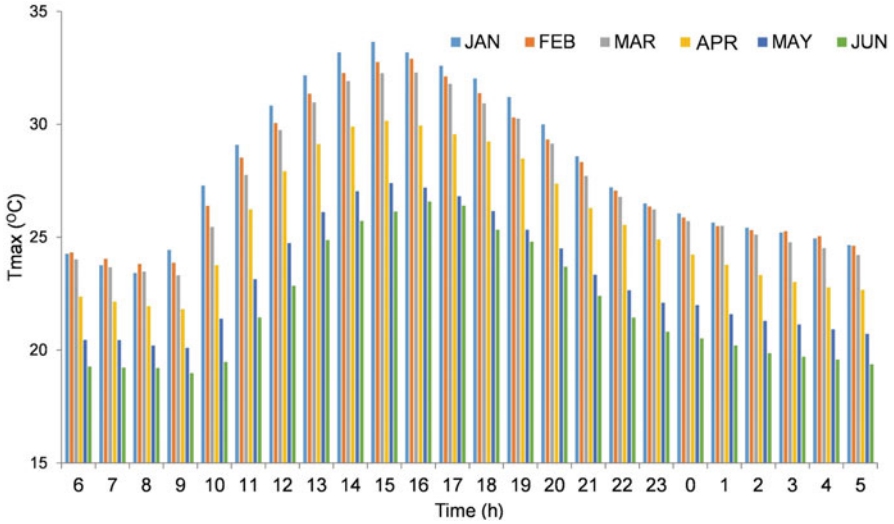

Fig. 15.17 Variation of maximum daily temperatures during the months of January to June 2015 in Vitória, ES, Brazil

showed differences in the tolerance to high temperatures of nine accessions. The *principal component analysis* (PCA) was used to reduce the dimensionality of chlorophyll fluorescence parameters obtained by the JIP-test, which allowed the grouping of sample data according to the two main sources of variance.

The PCA score graph enabled to distinguish two groups of samples. The first one corresponded to photosynthesis during February (morning and afternoon) and the second one to photosynthesis in winter (morning and afternoon) indicating the same trend of differential photosynthesis efficiency between summer and winter for all accessions throughout the day, either in condition of greater or lower temperature alternation (Fig. 15.18). However, the variation associated to some genotype between winter and summer was lower than for others, which indicated their better performance during winter time.

The parameters calculated by the JIP-test that best contributed to the discrimination of the *Jatropha* accessions in the months of February (high temperatures) and June (milder temperatures) were DF_{total} (driving forces, i.e., the performance of PSII and of specific electron transport reactions), PI_{ABS}, $\delta R_0/(1 - \delta R_0)$, and PI_{TOTAL} (Fig. 15.19). In addition, the PIs (PI_{ABS} and PI_{TOTAL}) were positively correlated with genotype discrimination in February and June.

Strasser et al. (1999) defined the *driving force* (DF) to be equal to log(PI), which was suggested to estimate the global driving force of the processes evaluated by the corresponding PI. However, Stirbet et al. (2018) argue that the analogy between DFs and the Nernst equation should be seen only as a mathematical formalism since DFs do not represent similar physical laws nor real potentials or real driving forces. Therefore, the definition of DF as the sum of partial potentials for energy

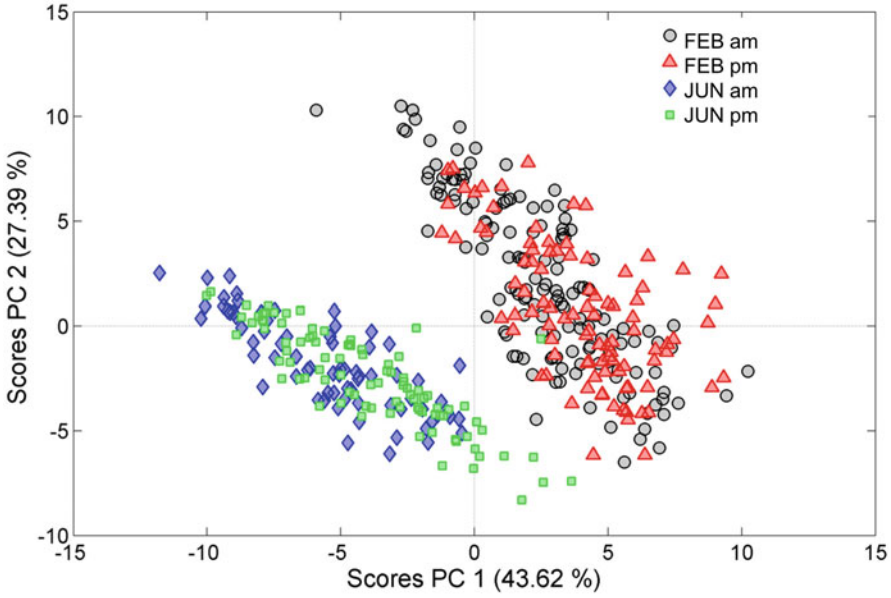

Fig. 15.18 PCA scores for 9 *J. curcas* accessions plotted according to the 52 parameters of the JIP-test evaluated during the months of February and June in the morning and the afternoon

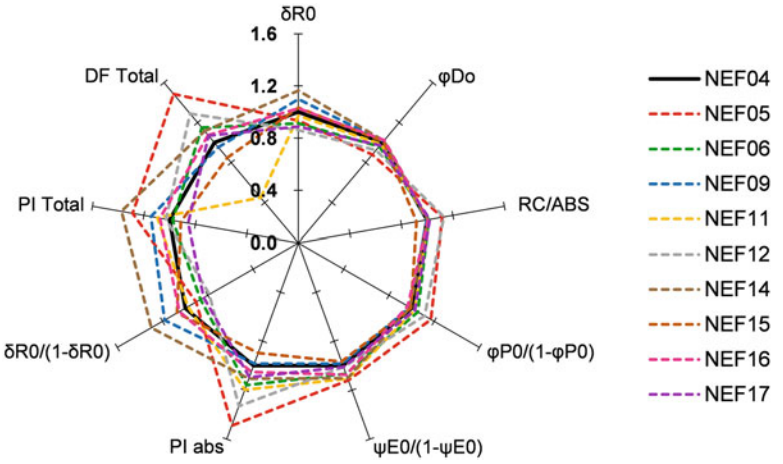

Fig. 15.19 Radar plot of several parameters calculated by the JIP-test, from chlorophyll *a* fluorescence induction curves, expressed as relative values calculated from averages of 100 measurements of plants of the ten *J. curcas* accessions, cultivated from cuttings in the experimental area of the Universidade Federal do Espirito Santo, Vitória, Brazil. The average value of NEF 04 was used as reference and set to unity. See Table 15.2 for parameter description

conservation should not be taken literally. Moreover, since DF is simply another mathematical expression of PI [i.e., DF = log(PI)], it may be considered redundant as an index, since it does not bring any new information beyond that provided by PI.

Summing up, we initially used three accessions that we call NEF 01, NEF 02, and NEF 03, respectively, from the mass selection of independent farmers in Minas Gerais and two hybrids from EMBRAPA Bioenergy's germplasm bank. From 2014, we started investigating the ecophysiology of photosynthesis using the accessions of the germplasm bank of the IFES. Different experiments with cuttings were performed, and the results generated several questions giving greater impetus to new investigations.

The PCA data relative to transient fluorescence parameters showed interesting and divergent results, however, always pointing to some parameters that can be used as biological markers. Thus, for high temperatures, we observed that the performance index of PSII (PI_{ABS}) can be a useful marker. New experiments have been developed and have evidenced significant differences of PI_{ABS} in NEF 04, NEF 05, NEF 06, NEF 07, NEF 08, NEF 09, NEF 10, NEF 11, NEF 12, NEF 13, NEF 14, NEF 15, NEF 16, and NEF 17 accessions, in the efficiency of reduction of final receptors of PSI electrons ($\delta R_0/(1 - \delta R_0)$ and PI_{TOTAL}.

Analyses based on *inter simple sequence repeat* (ISSR) molecular markers indicated that every genotype analyzed was different (Fig. 15.20). The largest distance between them was estimated to 0.59 (between the NEF 04 and the other genotypes) and the lowest at 0.35 (between NEF 16 and NEF 17), respectively.

In all, among the 13 genotypes analyzed, four statistically different groups were formed, according to the methodology proposed by Kelley et al. (1996). The largest group was made of NEF 10, NEF 11, NEF 12, NEF 13, NEF 14, NEF 15, NEF 16, and NEF 17. This group represented 62% of the analyzed genotypes. Three other groups were also formed, two of them containing two genotypes (NEF 07 and NEF 09; NEF 05 and NEF 06) and one containing only the genotype NEF 04. Based on

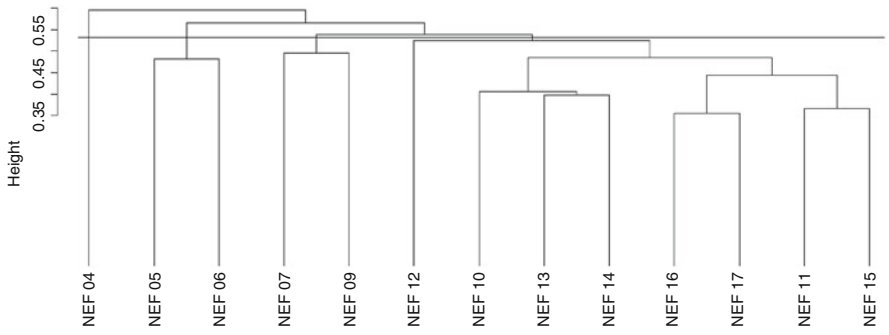

Fig. 15.20 UPGMA dendrogram made from the analysis of 8 primers ISSR, which shows the genetic distance via Jaccard index among 13 *J. curcas* accessions belonging to the germplasm collection of the Universidade Federal do Espírito Santo Vitória, Brazil. The dendrogram shows the formation of four distinct groups of genotypes. The cophenetic correlation between the phenetic and cophenetic matrix was estimated in 0.76 significant by the Mantel test to 1% of probability

the greater distance observed between NEF 04 and the other genotypes as well as on the number of groups formed, it was noted that the ISSR primers used were able to discriminate the analyzed genotypes according to the specific fingerprint they revealed.

Polymorphism is quite common among genotypes from the center of origin of a species. Singh et al. (2010), after analyzing the genetic diversity in *Jatropha*, concluded that, in general, the literature reports a low genetic diversity among the analyzed genotypes worldwide. However, Chen et al. (2011) failed to identify a relationship between oil content and genotypes grouped according to the molecular polymorphism of RAPD and ISSR markers. Incidentally, Tanya et al. (2011) selected five SSR primers, made from ESTs that were able to distinguish between toxic and non-toxic genotypes from Mexico and Asia, respectively.

Even if the primers used here were obtained randomly across the genome and may not correspond to regions coding for the analyzed traits (i.e., the subunits of PSII and PSI, the complexity of evolution of O_2, or the substances that constitute the intersystem), some bands may, however, be directly related to chloroplasts.

The results of the case studies presented here clearly show that increased activity, increased energy conservation efficiency, and increased stress stability are the results of the species' plasticity and resilience to the environment conditions where it is thriving. From the experimental point of view, these results demonstrate that biophysical phenomena, which are based on integration of analysis of plant vitality and JIP-test, provide a powerful tool for the in vivo and in situ investigation of plant physiology according to its complex interactions with environmental conditions.

To conclude, CF is a technique with numerous benefits: (i) it provides an early diagnosis of changes in vitality that can be applied to leaves as well as any green part of a plant, (ii) CF measurement is fast and can be performed in vivo or in situ and anywhere in the field or in greenhouses, and (iii) CF analysis is not destructive and, above all, is quite affordable. In addition to the technical-experimental benefits, the expressions of every biophysical parameters presented here are derived from a solid basis that results from on a long experimental history (see Strasser et al. 2000, 2004; Kalaji et al. 2016; Stirbet et al. 2018).

Our results show that PI_{ABS} is the most sensitive parameter used so far to understand *Jatropha*'s behavior in the field. In addition, PI_{ABS} is coherent with the behavior of its constitutive parameters, which validate its efficiency.

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Chapter 16
Jatropha: From Seed to Plant, Seed, Oil, and Beyond

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Abstract *Jatropha curcas* (Jatropha), one of the most popular biofuel crops, is also one of the most controversial crops. It is generally hailed as biofuel mandate crop in Asia and Africa, but its low genetic diversity in these continents has failed most of the crop improvement programs initiated so far. Breeding through the utilization of germplasm from Mexico is one of the priority areas for its genetic improvement. Nevertheless, proper agricultural practices, which may vary from region to region, are also important, not only to obtain optimum yields from improved germplasm but also the best yield from the germplasm introduced in different agroecological systems from the wild around the Jatropha belt. It is desired that Jatropha plantations sustain themselves, and resources meant for agriculture for food shall not be diverted towards agriculture for fuels. Irrigation, nevertheless, plays an important role in ensuring economically beneficial yields from jatropha cultivation. Similarly, application of fertilizers (NPK) is important for obtaining a good harvest from Jatropha fields. While, biodiesel, obtained through transesterification, is the major fuel obtained from Jatropha, many other fuels like biogas, fuel briquettes, Fischer-Tropsch (FT) diesel, ethanol, etc. can also be obtained as by-products. Utilization of biodiesel blended with fossil diesel is least technologically challenging with regard to Jatropha-based economy. However, availability of desired quantities of fuel is a challenge. Despite all pros and cons associated with Jatropha, it is still considered as an ideal feedstock for biodiesel, which doesn't compete with food crops, and returns are offered through several by-products.

Keywords Agrotechnology · Biodiesel · Genetic diversity · Origin · Toxicity

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16.1 Introduction

The use of fossil fuels as a source of energy is discouraged because of global warming and fluctuating market prices. Therefore, there is an urgent need for a renewable source of energy. While renewable energy may refer to solar, wind, hydrogen, hydroelectric, geothermal, bioenergy, etc., for most of the practical purposes, bioenergy alone, if available in sufficient quantities, would be able to substitute the petroleum fuel. Further, utilization of bioenergy has other advantages like mitigation of carbon dioxide through photosynthesis and creation of jobs, especially in third world countries.

Presently, bioethanol and biodiesel are the most popular biofuels, out of which, biodiesel is environmentally more sustainable and chemically similar to petroleum diesel. Biodiesel is technically a mixture of monoalkyl esters of long fatty acids derived from vegetable oils or animal fats (Yang et al. 2012). The major biodiesel producers currently are the USA, Brazil, Germany, Indonesia, and Argentina (Statistica 2018). Conversely, most of the biodiesel today is derived from oils obtained from corn, soybean, rapeseed, palm, coconut, and Jatropha (Bergmann et al. 2013; Supranto 2013).

Jatropha (*Jatropha curcas* L.) is a multipurpose non-edible oil yielding semi-woody perennial tree (Fig. 16.1), originated in Central America (Divakara et al. 2010; DIBER 2017). Presently, it occurs throughout the tropics and sub-tropics (Heller 1996) and adapted to a variety of rainfall and edaphic conditions (Francis

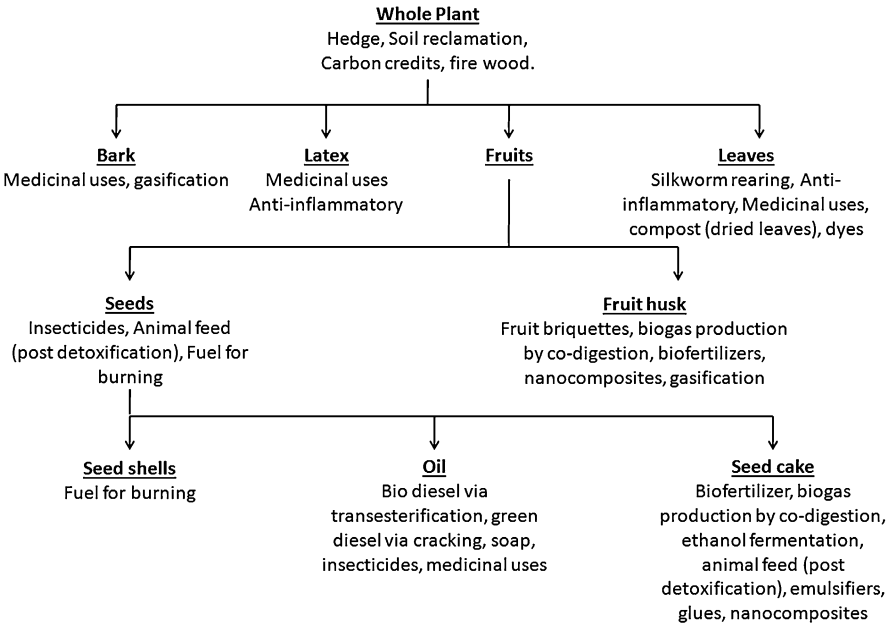

Fig. 16.1 Multi-utility of Jatropha plant, which prompted its recommendation as biofuel plant

et al. 2005). To meet the biodiesel mandate, it is currently planted on approximately 1.8 million ha in Indonesia, China, Brazil, India, and Africa (Carels 2013). The plant can live for 30–40 years in fruit-bearing stage and can survive even in low-fertility and low-moisture soils. None of the plant parts go to waste, and a number of products like dyes, medicines, bio-fertilizers, bio-insecticides, biopesticides, etc. are derived from tissues like bark, seed cake, leaves, etc. (Agbogidi et al. 2013; Sabandar et al. 2013; Bueso et al. 2016; DIBER 2017). The oil itself has a number of medicinal uses (Rug and Ruppel 2000).

Jatropha, as a biofuel crop, has been claimed to have many desirable characteristics such as rapid growth, easy propagation, drought tolerance, insect and pest resistance, and particularly seeds with high oil content (27–40%) and desired quality for biodiesel and biokerosene fuel production (Pandey et al. 2012; Dias et al. 2012; Edrisi et al. 2015; DIBER 2017). Because Jatropha oil is rich in unsaturated fatty acids (up to three-fourths fatty acids in seeds) (Sarin et al. 2007; Maghuly and Laimer 2013), it serves as an ideal feedstock for biodiesel in compliance with the international standards (Tiwari et al. 2007). However, the global aura around Jatropha has not resisted to the test of time, which led to the closure of several Jatropha biodiesel projects. There have been many reasons for that, but the main factor has been the failure to obtain the desired yields in the absence of optimized agricultural practices. Nevertheless, there are several success stories also, which are scattered and isolated from each other. In this review, we attempt to bring about the state of the art at each of the life stages of Jatropha.

16.2 Origin, Diversity, and Breeding

J. curcas (physic nut or Ratanjot; Fam. Euphorbiaceae, tribe Joannesieae) is native to Central America and related closely to the castor plant, *Ricinus communis*. The 416 Mb Jatropha genome is organized in 22 chromosomes (Grover et al. 2013). It is a monoecious outcrossing species with a male-to-female flower ratio being skewed as 25:1, respectively, (DIBER 2017) and pollinated by bees.

During the last decade, Jatropha plantations have been encouraged worldwide to support biofuel programs in different countries. However in most of the cases, efforts at the development of improved germplasm were rather limited, and seeds derived from locally available plants have been planted in the arid and semiarid regions without any concern for genetic potentiality and agronomical practices. Consequently, many commercial plantations ended up due to poor productivity, and several projects have, thus, been abandoned (Singh et al. 2014; Van Eijck et al. 2014a; Von Maltitz et al. 2016).

The primary focus of Jatropha improvement projects is to identify and develop superior germplasm with high seed yield and oil content. In addition, development of non-toxic (edible) varieties is also sought for. The success of selective breeding efforts has been often prevented by the limited germplasm information of the species. Genetic diversity in Jatropha has been studied using molecular markers

like random amplified polymorphic DNA (RAPDs), inter-simple sequence repeats (ISSRs), amplified fragment length polymorphism (AFLPs), and simple sequence repeats (SSRs) and was proved to be narrow outside its center of origin. Highly polymorphic microsatellites were many times shown to be less polymorphic in Jatropha (Yi et al. 2010; Yue et al. 2014). Assessment of germplasm from the Old World, i.e., China, India, Southeast Asia, and Africa, concluded low levels of genetic diversity (Basha and Sujatha 2007; Ranade et al. 2008; Sun et al. 2008; Mastan et al. 2012; Kumari et al. 2013). Germplasm from Brazil also displayed low genetic diversity (Sun et al. 2008; Rosado et al. 2010). This is actually contrary to the general expectations for an outbreeder species. Montes Osorio et al. (2014) even observed high levels of homozygosis in the Jatropha gene pool. Such conditions can occur if current populations in a given geographical area have originated from relatively few progenitor individuals in not so distant past. Montes Osorio et al. (2014), however, suggested that a high level of self-fertilization is also prevalent in Jatropha. Other factors such as vegetative propagation, artificial selection, etc. also account for lower genetic diversity in Jatropha germplasm in the Old World.

The highest genetic diversity in Jatropha is recognized to be present in the germplasm that originated from Central America and especially from Guatemala and Mexico (Basha et al. 2009; Tatikonda et al. 2009; Ambrosi et al. 2010; Ovando-Medina et al. 2011, 2013; Montes Osorio et al. 2014; Pamidimarri and Reddy 2014; Raposo et al. 2014; Pecina-Quintero et al. 2011, 2014; Avendano et al. 2015; Vásquez-Mayorga et al. 2017), with Chiapas Central Depression in Mexico to be the likely center of origin of Jatropha (Li et al. 2017). In addition to be considered as center of diversity of Jatropha, Mexico is thought to be its center of domestication (Heller 1996; Openshaw 2000; Pamidimarri et al. 2008; Abdulla et al. 2009; Basha et al. 2009; Tatikonda et al. 2009; Pecina-Quintero et al. 2011, 2014; Dias et al. 2012; Pamidimarri and Reddy 2014; Salvador Figueroa et al. 2015). Migration of Jatropha from the New World to the Old World has probably occurred from the Port of Veracruz (Li et al. 2017).

In the present scenario, using only local resources to develop high-yielding varieties in Asia and Africa has limited scope, due to the narrow gene pool that is found in these areas. Understandably, a limited number of genotypes were brought to Asia and Africa by Portuguese seafarers, and interbreeding within this gene pool is not likely to create many variants, which resulted in the so-called founder effect. Interestingly, African and Asian germplasms are closer to each other than to the Mexican germplasm (Basha et al. 2009). It has been suggested that low seed yield in African and Asian genotypes is genetically determined (Li et al. 2017). Further, the initial selection of Jatropha plants was based on its utility as live fence and medicines in the New as well as the Old World; no particular human intervention was made for identifying and selecting high-yielding genotypes (Agbogidi et al. 2013; Sabandar et al. 2013; Li et al. 2017). Epigenetic mutations, if any, occurring in the germplasm in Asia and Africa under local natural selection pressures and adoption to local environment would have been favored and caused only minor phenotypic variations (Montes Osorio et al. 2014; Yue et al. 2014), and therefore,

sporadic reports on high-yielding or high oil yielding genotypes of Jatropha are made. Thus, characterization and conservation of Jatropha germplasm from the New World are important to sustain selective breeding efforts in Jatropha.

In recent years, as seed yield and oil content in seeds have become important for the scientific world, concerted efforts have been made both by selection and breeding to obtain Jatropha varieties with high yield, high oil content, non-toxicity, early maturity, dwarfness, etc. Many of these traits are controlled by QTLs (King et al. 2015). Genetic variation in seed traits is important for Jatropha, as seed is the principal economic part of the plant. Fortunately, in Jatropha, seed traits like seed length, breadth, and thickness are positively correlated (Galapia et al. 2012). Nevertheless, seed traits may also often end up being influenced by environmental factors, and therefore developing a universally performing line of selective breeding is a strong challenge. Even under similar agroclimatic conditions, variations in seed characteristics (morphogenic as well as biochemical) are not uncommon (Kaushik et al. 2007; Basha et al. 2009; Yi et al. 2010). Even the scientifically controlled breeding efforts end up displaying considerable morphogenic diversity in the progeny including the agronomic traits (Rao et al. 2008).

Propagation by seed would always create genetic variation in offspring; therefore there has been a trend to propagate improved varieties by vegetative methods including tissue culture (DIBER 2017) to maintain the genetic uniformity in the offspring. However, selective breeding has also produced inbred lines whose F_1 hybrids showed homogeneous heterosis; thus inbred line progenies can be sold as seeds, which provide farmers with plants having better rooting system. Further, it becomes very important to prevent cross-pollination within the population, to keep a stock of "true-to-type" material.

Interestingly, both public and private sectors have shown interest in the cultivation and improvement of Jatropha. In India, for example, JOil(S) Pvt. Ltd., Reliance Life Sciences, and Labland Biotech India Ltd. are few of the companies investing in crop improvement of Jatropha. Worldwide companies such as D1 Oils plc (www.d1plc.com), Viridas plc (www.viridasplc.com), and Energem Resources Inc. (www.energem.com) are mainly involved in Jatropha crop improvement. Unfortunately, very limited information is available on quantitative genetic variation in Jatropha, seriously affecting the breeding programs. It is a well-known fact that germplasm diversity is the key factor in choosing parents for hybridization – the more diverse the parents would be, the higher would be the chance of larger heterotic expression in segregating generations.

16.2.1 Toxicity of Jatropha Seeds

The seed kernel contains about 30% protein by weight (Marasabessy et al. 2011), which can potentially be used for food, feed, and non-food applications (Gubitz et al. 1999; Lin et al. 2003; Martinez-Herrera et al. 2006; Lestari et al. 2010; DIBER 2017) after extraction of oil. However, the presence of toxalbumin curcin protein and

diterpenoid phorbol esters (Devappa et al. 2010a) along with trypsin inhibitors, phytic acid, saponins, and glucosinolates makes the seeds and seed kernel toxic (Devappa and Swamylingappa 2008). Sequential extraction using acid can reduce curcin toxicity (DIBER 2017), while trypsin inhibitors and lectins can be destroyed by heat treatment (Makkar 2016). Curcin is a ribosome-inactivating protein (RIP, type I), which depurinates rRNA, arresting protein synthesis (Qin et al. 2005). Management of such type I RIPs and their detoxification is an easier task. This institute (DIBER) has attempted to create curcin-free Jatropha plants by silencing curcin precursor gene (Patade et al. 2014). Phorbol esters pose a relatively difficult challenge, as their structures remain unaltered even after being heated (Devappa et al. 2010a). Jatropha seeds treated with alkali and heat have been shown a 90% reduction in phorbol ester content but are still toxic (Goel et al. 2007; Rakshit et al. 2008). Similarly, chemical treatments like neutralization with NaOH, bleaching, etc. with or without heat can also reduce the level of phorbol esters in seed oil to 40–60% but still remain toxic (Haas and Mittelbach 2000; Chivandi et al. 2004; Ahmed and Salimon 2009; Diwani et al. 2011). High-polarity solvents like methanol, ethanol, petroleum ether, and isopropanol can also be used for removing phorbol esters from the deoiled cake (Chivandi et al. 2004; Oskoueian et al. 2011; Devappa et al. 2012; Punsuvon and Nokkaaew 2013). It has been suggested that phorbol esters below 3 mg/kg treated residues can be considered safe for animal feeding as an alternative protein source to laboratory animals (Makkar 2016). This should however be supplemented with phytase enzymes and lysine (Makkar 2016). Importantly, phytases can be derived from Jatropha oil cake itself (Kannoju et al. 2017). Incubation with clay or black soil under sunlight for varying periods has also been demonstrated to degrade phorbol esters (Yunping et al. 2012).

In addition to chemical treatments, physical and biological processes have also been reported for detoxification of phorbol esters (Gomes et al. 2018). Yu et al. (2016), for example, reported effective decomposition of phorbol esters by treatment of oil cake at 195 °C and 19.1 MPa for 15 min. Sadubthummarak et al. (2013) combined heat treatment (at temperatures 120 °C and 220 °C for 1 h) with chemical treatments using 10% adsorbing bentonite, nanoparticles of zinc oxide (100 μg/g), and 4% $NaHCO_3$ prior to a 4-week incubation to succeed in decomposing phorbol esters to a final concentration of 0.04–0.05 mg/g. High moisture (130 g/kg) over prolonged periods (21 days) has also been reported to degrade phorbol esters at room temperature or higher (Devappa et al. 2010b). Gamma irradiation and ozone treatments can also significantly reduce (70–75%) phorbol esters in deoiled cakes (Diwani et al. 2011; Gogoi et al. 2014).

Obviously, chemical and physical methods are fast and easy to scale up, while biological methods that include press cake utilization as a solid-state substrate for the growth of fungi provide the advantage of being more efficient and specific (Zhang et al. 2016; Gomes et al. 2018). *Pseudomonas aeruginosa* PseA strain and *Streptomyces fumicarius* YUCM were demonstrated to degrade phorbol esters (>97%) under solid-state fermentation conditions (Joshi et al. 2011; Wang et al. 2013).

Other fungi like *Bjerkandera adusta*, *Phlebia rufa*, *Cunninghamella echinulata* CJS-90, *Trichoderma harzianum*, *Paecilomyces sinensis*, *Cladosporium cladosporioides*, *Fusarium chlamydosporum*, etc. can also degrade phorbol esters with varying efficiencies; the maximum being reported is 99.7% (de Barros et al. 2011; Najjar et al. 2014; Sharath et al. 2014). These detoxified products obtained through biological methods may not only reduce the phorbol esters to more than 90% but may also enhance the nutritive value of Jatropha cake by increasing the available protein content and reducing the crude fat, thereby deemed fit for animal consumption and plant growth (Wang et al. 2013; Zhang et al. 2016). Kannoju et al. (2017) used deoiled cake from Jatropha as a substrate in submerged culture for the growth of *Candida parapsilosis* while successfully reducing the concentration of phorbol esters to less than 0.5%. Purified enzymes can also be used for degradation of phorbol esters. For example, Hidayat et al. (2014) used lipase isolated from Ciherang rice bran for degradation of phorbol esters.

Plant varieties with a non-toxic seed cake would be more readily accepted by local farmers because sub-products of the oil extraction process could be utilized for animal feeding (Makkar and Becker 2010). There are reports of the occurrence of non-toxic Jatropha plants in nature, particularly in Mexico (Dias et al. 2012), and the use of these plants in breeding Jatropha for non-toxicity (King et al. 2013; Vasquez-Mayorga et al. 2017). However, such plants and their hybrids typically show a low grain yield (Montes Osorio et al. 2014), calling for rigorous assessment of genetic basis of phorbol esters for proper utilization of non-toxic varieties.

16.3 Seed to Plant (Agrotechnology)

Evidently, the focus of scientific community revolves around seed yield and high oil content in seeds of Jatropha, and any value above 30% w/w for seed oil is generally appreciated (Ginwal et al. 2004; Kaushik et al. 2007; Singh et al. 2013). However, if high oil content is not correlated to the seed size, number of seeds, and number of fruits per plant, the oil content remains an overenthusiastic parameter (Singh et al. 2013). It is generally agreed that a seed yield of 4–5 tons/ha is the threshold for commercial viability of Jatropha (Gopinathan and Sudhakaran 2010; Li et al. 2014). However, the actual yield of Jatropha generally falls much below this level (Everson et al. 2013; Singh et al. 2013; DIBER 2017), even with the best genotypes and agronomic practices (Terren et al. 2012; Tikkoo et al. 2013).

Thus, suitable agrotechnologies are required even for the improved variety to ensure not only high oil content but also high seed content and large seed size. Interestingly, genetically similar Jatropha genotypes from different parts of the world may exhibit variations in seed size and oil contents (Kumari et al. 2013; Singh et al. 2013).

16.3.1 Land Preparation and Soil Reclamation

Though plantation can be done without any cleaning activities, it is advisable to partly clean up the area. Tall trees can be left as such and bushy plants should be cleaned. Jatropha has low fertilizer and water demand and does not even require tillage for soil preparation. It can grow even in crevices of rocks but does not thrive in wetland conditions. However, wherever soil depth is less than 15 cm, plantation of Jatropha should not be taken up as a commercial crop.

Jatropha plantation is carried out in appropriate size of pits. The pit size ideally depends on the slope of the land, availability of water, and quality of soil. The planting density in fertile soils should be lower than in soils with low fertility. The pits should be dug with proper layout to manage plantations. A pit size of $30 \times 30 \times 30$ cm (DIBER 2017) is ideal for plantation in soils fairly rich in nutrients.

The litter of leaves that fall in winter months forms mulch around the base of the plant. The organic matter from litter has been observed to enhance earthworm activity in the soil around the root zone of the plants, which is a fair indicator for improvement in microfauna and fertility and soil texture (DIBER 2017).

16.3.2 Propagation

Jatropha is propagated through seeds, stem cuttings, and in vitro regeneration. The propagation through seeds does not give population identical to the parental stocks due to cross-pollination. Vegetative propagation through stem cuttings maintains the purity of parental stocks due to clonal propagation but increases the risk of disease spread. Therefore, there is constant emphasis to develop in vitro protocols to produce large number of quality material to fulfill the need of mass propagation.

Quality seeds (attractive black color) should be selected for growing. Freshly harvested seeds should be kept over a month time at ambient temperature to overcome dormancy and achieve good germination rate. Dry seeds will normally germinate readily without pre-treatment. However, before sowing, seed should be soaked overnight in water, and the next morning after the removal of water, seed has to be spread on gunny cloth bag. It has been observed that direct seed sowing done at the beginning of the rainy season helps in the development of healthy taproot system. Seeds may be planted without any treatment, but pre-treatment of seeds, particularly with 150 ppm gibberellic acid (GA_3), has been shown to enhance not only the germination percentage but also the post-germination growth of plants significantly (DIBER 2017).

Seed rate of 6–8 kg/ha is required. For nursery raising, line-to-line and seed-to-seed distance has to be kept within 5–10 cm and 3–5 cm, respectively, with a depth of 4 cm (DIBER 2017). Seed treatment is done with 2 g of mixtures (Bavistin + vitavex + Thiram)/kg seed. Soil treatment with carbofuran/thimet is

recommended for the soils where insects are abundant. Light water showering is required at every alternate day till germination and later on based on the need.

Plantlets raised on soil beds in nursery can be taken out and transported at bare root even to long distances. The transportation should be done on rainy days or packing in water soaked media. Soil bed is prepared in field by raising soil about 3–4 in. to avoid water flood/logging in soil bed.

Sowing of Jatropha seeds in polybags is a cost-effective method compared to sowing in soil bed. Polythene bags of 10 × 20 cm or 15 × 25 cm have been found suitable (DIBER 2017). The saplings can be maintained for 3 months in these bags. The overnight pre-soaked seeds germinate in 6–10 days in hot humid weather, whereas the process continues for 10–15 days. It takes more time in low temperature conditions. The nursery should be irrigated as and when needed.

The tree is pruned after maturation of stems and branches. For vegetative propagation, these stems should be cut into pieces measuring 20–25 cm depending on their length, and planting should be done in soil bed or polybags. After 25–30 days, lateral roots initiate, and one of these transforms into taproot. Treatment of fresh stem cuttings of diameter less than 5 mm with 2.5 ppm IBA can enhance sprouting and rooting by 63.3% and 43.3%, respectively (DIBER 2017). In general, hormonal treatments can significantly increase sprouting and rooting percentage in all types of cuttings.

For better establishment of saplings, monsoon season should be preferred for planting. Pits are filled with 500 g farmyard manure and 100 g neem cake or vermicompost to ensure profuse rooting in nutrient-deficient soils. Before transplantation, 1 kg FYM/vermicompost, 10 g urea, 20 g phosphate, and 5 g potash are mixed with soil and poured until three-fourths of pit height. Transplanting in the field should be done preferably in the evening. The saplings removed from nursery should be kept in a shaded place to avoid wilting. Plants transplanted with bare roots have to be kept in soil where shady place is available and depending upon the requirements watering is done if transportation has been delayed.

16.3.3 Plant Geometry

For transplantation, plant-to-plant and row-to-row distance may be followed according to soil type, rainfall pattern, and irrigation facilities available. A spacing of 2 × 2 m is generally used to accommodate 2500 plants per ha, which is the density normally required under nonirrigated conditions (Table 16.1).

16.3.4 Manure and Fertilizers

Although Jatropha is adapted to low fertility site and alkaline soils, better yield is obtained on poor-quality soil if fertilizers with small amounts of calcium,

Table 16.1 Plant density and land types

Spacing (m)	Plants/ha	Types of land/conditions
2 × 1.5	3333	Nonirrigated/low rainfed
2 × 2	2500	Nonirrigated/rainfed
2 × 2.5	2000	Rainfed
3 × 2	1666	Rainfed
3 × 3	1111	Rainfed for intercropping
4 × 3	833	For tractor operation and grass collection
0.5 × 0.5	4000	For bio-fencing

magnesium, and sulfur are used (Marasabessy 2015). Mycorrhizal associations have been observed with Jatropha. Madhaiyan et al. (2013) reported endophytic *Enterobacter* sp. R4-368 in Jatropha, contributing in nitrogen fixation. In general, application of superphosphate (150 kg/ha) followed by one dose of NPK (40:100:40 kg/ha) at half yearly intervals is reported to improve yield (DIBER 2017). Earlier, Singh et al. (2013) suggested 2 kg of farmyard manure (organic manure) per plant and NPK ratio of 1:2:1, respectively. The application should coincide with rainy season or followed by proper irrigation immediately after the application of fertilizers. From fourth year onward, 10% extra superphosphate should be added to compensate for fruit harvest. The quantity of fertilizer ensures good plant performance.

Seed oil content generally responds positively to increased nitrogen supply as nitrogen plays a central role in many physiological and biochemical processes (Nurcholis et al. 2015). Higher application of potassium increases the yield, probably due to higher rate of photosynthesis and increased respiration, which are essential for protein synthesis and for fruit formation. Tikkoo et al. (2013) reported that the application of 90 kg N/ha and 60 kg K_2O/ha to 3-year-old plantation, in combination with two irrigations, could increase seed yield to 472.51 kg/ha, nearly threefold compared to the control, oil content to 34.5%, and oil yield to 163.31 kg/ha, about 3.5 times to that of control.

16.3.5 Protective Irrigation

The seedlings require irrigation especially during the first 2–3 months of planting. In Indian conditions, Singh et al. (2013) suggested irrigation requirement at an interval of 30 days.

Behera et al. (2010) and Srivastava et al. (2011) opined that the irrigation frequency does not affect significantly the Jatropha growth. But other investigators like Openshaw (2000) and Santos et al. (2016) demonstrated that plants grow better with irrigation and may even produce three to four harvests per year. In fact, certain investigators have proposed that irrigation may be a limiting factor for seed yield

(Singh et al. 2013). Irrigation indeed has a positive impact on plant growth, yield, and other agronomic characters, whatever the stage at which it is applied (Santos et al. 2016).

It may be argued that irrigation would disrupt the overall ago-economic scenario that is often advocated to recommend Jatropha cultivation. Life cycle assessment suggests that irrespective of the use of irrigation, there is still substantial reduction in greenhouse gas emissions, and there is no substantial difference in net energy ratios compared to the rainfed Jatropha plantations (Kumar et al. 2012).

16.3.6 Pruning

Pruning of Jatropha plants is a relatively ignored area linked to the seed yield with overall impact on biofuel scenario (Rajaona et al. 2011), even though it has empirically been shown to affect various yield parameters in Jatropha (Santos et al. 2016). Limited trials indicate that pruning may result in net seed yield initially (Singh et al. 2013; Tjeuw et al. 2015), but most studies till date suggest that pruning contributes to the plant canopy structure development and in long-term helps in better yields. To ensure maximum branching, pruning should be done when the tree sheds its leaves and enters into a period of dormancy preferably during winter seasons. In addition, pruning also reduces disease and insect incidence and improves the air flow within the canopy (Yarborough 2006; Oliveira and Beltrão 2010; Pescie et al. 2011; Santos et al. 2016). The trees are kept in short height by pruning, so as to manage cultural operations, pesticide application, and picking of mature capsules manually (Alam et al. 2011; Brasileiro et al. 2012; Everson et al. 2013; Santos et al. 2016).

Pruning should be carried out in such a way that a plant should have 15–25 branches, so as to obtain optimum production and productivity (DIBER 2017). Crop architecture plays an important role in a plant like Jatropha where proper pruning will help in producing more branches, healthy inflorescences to enhance good fruit set, and ultimately better yield. The pruning of terminal stems is essential at a height of 45–60 cm and 6 months age to induce secondary branching. Likewise the secondary and tertiary branches are to be pruned at the end of the second year. Usually two prunings are required at 6 months interval; however, a third pruning can be performed in 6–8% of the plants that bear a lower branch number. Periodical pruning can be done depending on the vegetative growth of plants to avoid overcrowding and crisscrossing of branches.

16.3.7 Interculture Operations

The standard cultural practices involve timely weeding, proper fertilization, surface ploughing, and pruning. Most agricultural scientists have ignored documentation of interculture operations in Jatropha (Rajaona et al. 2011).

The field should be kept free from weeds at all times. Two weedings in the initial period are enough to keep the field free from weeds until the crop crosses the height of weed/grass. Light harrowing is beneficial during the early growth stage. Power tiller and tractor are most suitable for intercultural operation in big plantations with plant-to-plant and row-to-row distances of 3 × 3 m or 3 × 2 m.

16.3.8 Flowering and Fruiting

Plant flowers during the rainy season and two flowering peaks are often observed. In humid regions, flowering occurs throughout the year, but flowering peak occurs during July to August. The plant produces yellowish green flowers in racemose inflorescences with dichasial cyme pattern. Numerically, 10–15 female flowers and 25–90 male flowers are produced per inflorescence. Female flowers are quite similar to male flowers in shape but are relatively larger. The flowering depends on the location and agroclimatic conditions. Inflorescence in Jatropha is terminal having unisexual flowers with male and female ratio of 25:1 and the inflorescences emerging at stem tips.

Flowers are pollinated by insects, especially honeybees. Usually flowering initiates in rainy season and bears fruiting in winter; however this character varies according to the geo-climatic conditions. Gibberellic acid (100 ppm) can be sprayed to promote early flowering (DIBER 2017).

Non-domesticated (wild) genotypes of Jatropha take 2–3 years to initiate fruiting and reach full maturity in the fifth year. Only few genotypes start fruiting in the first year of plantation, but with a production rate lower by about 35–45% than that obtained at maturity, however, this production loss can be mitigated with irrigation.

Fruits are gray-brown capsule, 4 cm long, and generally formed of three compartments, each comprised of one seed. Seeds are black and about 2 cm long and 1 cm thick. In India, fruits generally reach maturity by September to October within 3 months after flowering. When fruits begin to open, seeds are mature. Seeds cannot be stored for long time as they lose viability within 6 months.

16.4 Plant to Seed (Harvesting and Post-harvest Technology)

Jatropha shows asynchronous maturity; thus fruit picking is not possible at one time. Total picking of fruits takes around 2 months duration. Capsules that turn yellow are harvested along with brown matured capsules, and picking of green fruits is avoided. The fruits are collected manually and seeds are separated mechanically or manually. The seeds for planting purpose are dried in a shed, while for oil purpose, they are dried in the sun for 4 days till the moisture level comes to 8–10% (DIBER 2017).

The harvested fruits should be spread in a single layer on hard or cemented threshing floor for drying. Fruits must be kept free from small stones or other solid impurities to prevent damage to the shelling equipment (Lim et al. 2015).

Deshelling is an important post-harvesting practice, which eases the process of expelling (Pradhan et al. 2011). Jatropha seeds (two to three seeds in each fruit) are covered by shells (the outer layer of fruit) in each fruit. Usually, there are three seeds per fruit (DIBER 2017), though variations have been observed (Singh et al. 2013). The seed itself consists of husk (the outer coating of the seed) and kernel (the nucleus of the seed). The fruits contain 35–40% of shells by weight and 60–65% of seeds by weight, while the seeds consist of 40–42% husks and 58–60% kernels by weight (Pandey et al. 2012).

Moisture content has its crucial effect on the process of shelling rate or decortication (Lim et al. 2015).

16.5 Fuels from Jatropha

16.5.1 Biodiesel

Biodiesel is the main product from Jatropha, which is obtained from the oil derived from its seeds. Oil is typically extracted using cold press or a mechanical expeller. Steam may be used during the process to maximize the recovery. A high-quality oil is one without any free fatty acids (FFA). It is not unusual to find up to 15% FFAs in crude Jatropha oil, which can be brought to <1% by esterification with methanol and sulfuric acid as a catalyst. Subsequently, NaOH is used as a catalyst in alkaline transesterification reaction in the presence of methanol (Tiwari et al. 2007; Berchmans and Hirata 2008).

Often oil purification steps involving degumming, deacidification, dewaxing, dephosphorization, dehydration, etc. are performed prior to its transformation in biodiesel especially when it has to be used as a straight fuel. Besides these operations, iodine value (IV), i.e., the measure of the degree of unsaturation in the oil, and saponification value, i.e., the chain length of the fatty acids present in the oil, are also important parameters to assess the oil quality (Table 16.2). Together, IV and saponification values are indicative of the cetane number of the derived biodiesel. The molecular weight of Jatropha oil is 872 g/mol (DIBER 2017). The molecular

Table 16.2 Generally accepted ranges of various physicochemical properties of crude Jatropha oil

S. no.	Characteristics	Unit	Acceptable values
1.	Acid value	mgKOH/g	10.1–12.6
2.	Free fatty acid (as oleic)	%	5.1–6.3
3.	Iodine value	gI_2/100 g	103.6
4.	Saponification value	mgKOH/g	193.0

weight is taken into consideration in the empirical calculation of the quantity of methanol required for transesterification.

Many of the properties of biodiesel from Jatropha stand superior to fossil diesel, while some of course are inferior. The net properties of fossil diesel and biodiesel blends are advantageous for commercial use. Biodiesel typically has a higher kinematic viscosity (>4.9 cS at 38 °C) and density (~0.88 kg/l) compared to diesel (~3.1 cS and 0.84, respectively) (Shambhu et al. 2012; DIBER 2017). Biodiesel also has higher flash, cloud, and pour points compared to fossil diesel (Shambhu et al. 2012; DIBER 2017), which can be considered as an advantage for security issues of fuel handling and transportation.

The process of transesterification is simple compared to crude fossil oil distillation; it actually converts any crude vegetable oil, including that of Jatropha, into a mix of methyl esters (Shambhu et al. 2012) with lower viscosity and density than the oil itself, which increases the fluidity of the resulting fuel, ensuring complete combustion without ignition delay.

While biodiesel is the main product in the reaction, a number of by-products like glycerol, soap, and potassium sulfate are also obtained as a result of esterification and transesterification processes. The method is effective, and up to >90% conversion can be obtained using this method (DIBER 2017). Alternately, composite and heterogeneous catalysts like zeolite or mesoporous materials like AlMCM or microporous ZSM-5 have also been used for transesterification. Transesterification by homogeneous and heterogeneous catalysts leads to high-quality biodiesel (Poddar et al. 2017), but the homogeneous process is still preferred because it is cheaper and simpler.

16.5.2 Bioethanol

Fruit by-products like husk, seed cake, etc. are rich in protein and carbohydrates (Marasabessy 2015). These materials are particularly rich sources of pentoses and hexoses and ideal raw materials for fermentation. With a minor pre-treatment using dilute sulfuric acid for 30 min at 140–180 °C, these materials can be fermented to produce bioethanol (Marasabessy et al. 2011).

16.5.3 Biogas

Fruit husk, fruit coat, hulls, shell, seed cake (deoiled), peel, fruit capsules, and sludge of the crude Jatropha oil can be co-digested with inocula from cow dung to decompose them into biogas (Praptiningsih et al. 2013; DIBER 2017). In fact, biogas production from seed cake is higher than that from the cow dung (Singh et al. 2008;

Ali et al. 2010). However, capsule husk, due to its low density float on the surface of the slurry, ends up clogging inlet and outlet of the biomethanation chamber (Makkar and Becker 2009). Nevertheless, a two-step biomethanation process, which includes a first acid fermentation step in a different reactor, has been demonstrated to effectively utilize capsule husk as substrate for biomethanation (Hendroko et al. 2013; Praptiningsih et al. 2014). In addition, retention time enhancement to up to 7 days has shown to increase the degradation of capsule husk in a biomethanation chamber as well (Hendroko et al. 2014). Alternatively, additives like 3% urea and 5% crude Jatropha oil have also been demonstrated to enhance biogas production using capsule husk as a substrate (Praptiningsih et al. 2013).

16.5.4 Fuel Briquettes

Fuel briquettes are prepared from the husks of Jatropha fruits for utilization as fuel (DIBER 2017). Its calorific value is as good as that of coal (Ghosh et al. 2007) and can be used to fuel boilers.

16.5.5 Bioelectricity and Fischer-Tropsch Diesel

Jatropha biomass can suitably be gasified or pyrolyzed to yield high calorific value gases and connected to gas genset for the generation of electricity. Further, these gases can be converted to green diesel or Fischer-Tropsch (FT) diesel using Fischer-Tropsch synthesis (Portugal-Pereira et al. 2015; DIBER 2017).

16.6 Business Models and Environmental Benefits of Using Jatropha-Based Fuels

The paradox with Jatropha promotion in most countries has been the fact that while governments have funded large plantations of Jatropha, they also have encouraged and advocated smallholder models. The sustainability of both models deserves debate. Smallholders' model ensures wider public participation and land rights. Because agro-practices employed are mostly manual, there is less GHG impact, and this ends up being economically viable (Kagathi et al. 2017). On the other hand, plantation model creates more employment (Van Eijck et al. 2014a, b). Many of these plantations have been found economically unviable due to low seed yields, high cost of production, delayed production, and uncompetitive feedstock prices (Kagathi et al. 2017). Nevertheless, management and implementation of either of the

models can have relative positive or negative impacts, and the single most important factor remains the current yields of Jatropha plants. Indeed there were around 150 projects running on Jatropha biofuels, whose numbers have consistently declined in the last 10 years. Such discontinuations were due to the loss of incomes for the farming communities (van Eijck et al. 2014b).

Though the main end product of Jatropha is biodiesel, several energy coproducts are obtained along different production pathways, as discussed above. Under optimized agronomic practices and post-harvesting techniques, whichever route is taken for the conversion of Jatropha biomass into biofuels, net environmental benefits have been recorded. It has been suggested that the life cycle impact of Jatropha production and use yields benefits in terms of non-conventional renewable energy utilization and greenhouse gas emission reduction (Portugal-Periera et al. 2016). Local environmental effects in terms of terrestrial acidification potential and respiratory inorganic effects are even higher (Portugal-Periera et al. 2016). Bioelectrogenesis using gasification is a more beneficial route than following the FT diesel synthesis route for energy recovery, though less pollution is caused in obtaining FT diesel synthesis route.

Interestingly, Jatropha can withstand heavy metal or salt concentrations and even low pH in the soils (Badoni et al. 2016; Singh et al. 2015). Therefore, Jatropha is also an attractive option in the implementation of phytoremediation for reclamation of degraded lands.

16.7 Biodiesel Applications and Uses

16.7.1 Usage in Railways

Considering the physicochemical properties of methyl esters (biodiesel) derived from Jatropha, it makes an ideal fuel for blending with fossil diesel of up to 20% (DIBER 2017). World over, many trials have been conducted to assess the suitability of the blended diesel in different vehicles and machineries. Many of these trials have been protracted. For example, Amtrak Corp. conducted year-long trials on a P-32 passenger locomotive with B20 blend in the year 2010, which was included in *Time*'s list of "The 50 Best Inventions of 2010" (Smith and Surland 2013; Indian Railways Organization for Alternate Fuels 2013). However, the source of the biodiesel for these trials was not Jatropha (Smith and Surland 2013). Similarly, in Disneyland, some tourist trains were operated on blends of biodiesel obtained from cooking oil in 2007. However, their use was discontinued later due to non-availability of the fuel. Virgin Trains had conducted a 6-month trial using B20 blend, and Indian Railways has performed several trial runs and regular runs using Jatropha biodiesel blend B5 (Indian Railways Organization for Alternate Fuels 2013).

16.7.2 Usage in Aircrafts

Jatropha biokerosene blends have also been used to run aircrafts. Baroutian et al. (2013) suggested that up to 20% blends do not affect jet performance.

On December 30, 2008, Air New Zealand flew the first successful test flight with a Boeing 747 running one of its four Rolls-Royce engines on a 50:50 blend of aviation grade biofuels derived from Jatropha oil and jet A-1 fuel.

16.7.3 Vehicular Use

Many automobile manufacturers have now started accepting biodiesel blends suitable for their automobile models. The list of such automobile manufacturers includes Chrysler, Volkswagen, Mercedes Benz, Tata Motors, Chevrolet, etc. The blends tolerable in their models vary from 5% to 100% according to the manufacturer. In many cities around the world, for example, Bengaluru in India, public transport systems have started converting their fleets to biodiesel. Karnataka State Road Transport Corporation (KSRTC) in India runs luxury interstate buses on pure biodiesel after minor engine modifications.

Further, more and more academic trials are being conducted around the world using Jatropha biodiesel blends. DIBER has already carried out trials of B20 blend for nearly 50,000 km in different vehicles (fully laden) and under different geographical conditions (DIBER 2017). University of Botswana has run protracted trials using B10 blend.

16.7.4 Usage in Generator Sets

In parallel to trials in vehicles, DIBER has also tested B20 blends in diesel generator sets. In all, more than 700 h of testing has been carried out in generator sets of capacities up to 450 kW (DIBER 2017). Globally, UC Riverside had installed a 6 MW generator system running on 100% biodiesel in 2001.

16.7.5 Other Potential Uses

The biodiesel from Jatropha can also potentially be used as a heating fuel in boilers like any other hydrocarbon or any other biodiesel from a different feedstock. Combustion system of boilers is simpler than compression ignition of diesel engines,

making biodiesel use in boilers much easier. However, it has been observed that fuel consumption increases significantly with blends of biodiesel in larger proportion than B20 (Komariah et al. 2013). B5 or lower blends can be used as heating biofuels as per ASTM 396 standards, but higher blends of up to B20 are also used by several consumers. It is further notable that blends in larger proportions than B10 have better emission characteristics when used in boilers as heating fuels. These blends emit lesser CO, SO_2, and CO_2 and NO_X but higher SO_2 than diesel (Ghorbani et al. 2011).

Biodiesel can also be used for cleaning oil spills due to its solvent properties (Fernendez-Alvarez et al. 2007).

16.8 Current Trends

Despite its huge ecological benefits, Jatropha did not release any economic benefits to its investors, so far. As a result, more and more entities are pulling out of Jatropha ventures. Last year, Mozambique and Rwanda pulled out of Jatropha projects. In India, all major oil marketing companies, i.e., Indian Oil, Bharat Petroleum, and Hindustan Petroleum, pulled out of Jatropha projects, abandoning 1,800,000 ha of plantations because of economic inviability.

Because prospects of easy manufacturing and availability of biofuels have diminished, the prospects of new investments on biodiesel are also vanishing. Nevertheless, the fact is that (i) none of the biofuels is currently available at competitive prices and (ii) fluctuating fossil fuel prices forced several countries to reconsider the biodiesel of Jatropha only as a potential source but still needing research investment to be feasible. The decision by Ethiopian and Nigerian governments, taken in 2015 and 2016, respectively, to re-look at Jatropha ventures is a prominent example of such a trend. Recent developments led to issue elite Jatropha genotypes that promise to be economically favorable and may revive the prospects of the crop.

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Part IV
Feeding Use

Chapter 17
Influence of the Acid Soils of Tabasco Mexico in the Physicochemical Composition of Xuta or Edible Mexican Pinion (*Jatropha curcas* L.)

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Abstract The composition of the soil affects the physicochemical properties of oil and seed of *pinion*; this has been evidenced in studies done in seeds of the states of Morelos, Sinaloa, Veracruz, and Tamaulipas, among others; however, there are no reports of evaluation of this species in Tabasco, a state with potential for the intensive production of this species. The physicochemical characteristics of the seeds and the oil of seeds harvested in the localities of Huimanguillo and Mantilla, Cunduacán, Tabasco, were evaluated. The seeds from Huimanguillo showed better physical characteristics: viz., seed weight (0.94 ± 0.099 g), length (19.7 ± 0.6 mm), and width (10.92 ± 0.3 mm); in addition their protein and oil content are higher than in the seeds harvested in Mantilla. However, the concentration of protein presented was lower than in any of the seeds grown in other states. These said materials could

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be recommended for the biodiesel industry because of its high oil content and valuable physicochemical characteristics, which supports that the state of Tabasco has ideal ecoclimatic characteristics suitable for cultivation. The results obtained from the oil analysis show no significant difference in the values of acidity, saponification, peroxide, iodine, viscosity, and specific density compared to the oils obtained in the different locations of this study. The fatty acid profile indicated a greater quantity of oleic and linoleic acid (34–36.5%) and a low content of myristic acid (1.1–1.5%) and palmitoleic acid (1.3–2.4%).

Keywords Fatty acids · Food uses · *Jatropha* · Physicochemical characteristics · Tabasco

17.1 Introduction

Jatropha curcas L., a plant belonging to the Euphorbiaceae family, is native to Mexico and Central America and is considered a wild plant with potential for various industrial and food applications. The species has great potential for the production of biodiesel due to its high content of kernel oil (40–60%) and proteins (20–30%). The production of oil from the seed of *J. curcas* is near to 1590 L per hectare per year, exceeding the production of vegetable oils from commercial sources such as castor oil, sunflower, rapeseed, and soybeans that have a production of 1320, 890, 1100, and 420 L per hectare per year, respectively. Two ecotypes have been reported, the toxic and the non-toxic types (Makkar et al. 2007). Due to the above and due to its adaptability, *J. curcas* is a great agricultural production alternative for Mexico and especially for the state of Tabasco, where *J. curcas* is known as Mexican *pinion* or *piñoncillo*.

The interest of investigating the pinion production is that it has certain characteristics that favor the development of new research since it has been previously reported that it can be used as animal feed or for human consumption, and an additional advantage is that it has biochemical properties that hold it as an energy crop (Martínez et al. 2010). In addition, there is little information about the physical and chemical characteristics of the residual paste obtained after pressing, which has impaired the development of new by-products from the press cake. Studies have been conducted to fortify tortillas, with protein-rich non-conventional sources as *J. curcas* meal, and concluded that non-toxic *J. curcas* flour is an excellent option for increasing the protein value of tortillas (until 10.85%); this is an important food in the diet of Mexicans (Arguello et al. 2017). Studies performed by González et al. (2015) indicate that Tabasco is a region presenting favorable soil and climatic conditions for the development of *J. curcas* cultivation, which covers an area of 833,181 ha over four municipalities with greater potential: Balancán (256,201 ha),

Huimanguillo (131,596 ha), Tenosique (130,708 ha), and Cárdenas (68,267 ha). The acid soils of the savanna of Huimanguillo are characterized by high phosphorus fixation; deficiencies of zinc, boron, calcium, magnesium, and potassium; low rate formation of ammonium and nitrates; as well as a high percentage of aluminum saturation. These restrictive conditions of fertility are manifested in foliar deficiencies that affect the yield and fruit quality of common food crops, as, for example, in citrus and pineapple (Salgado et al. 2017).

Preliminary assessments made in the experimental fields of the INIFAP in Tabasco, Mexico, suggest that *J. curcas* cultivation could be economically profitable due to its potential in the production of biodiesel or other applications; however, it is necessary to study other characteristics that could enable diversification in new food and pharmaceutical products. It is important to mention that, in the municipality of Comalcalco, *pinion* almond were covered with chocolate to make candies with great acceptance by consumers. However, there are few reports about the use of *pinion* in the state of Tabasco even if it exists natively in some municipalities as Huimanguillo, Cardenas, Centla, Comalcalco, Cunduacán, and Macuspana (Martínez et al. 2010). Given the favorable climatic characteristics of temperature and humidity of the Tabasco state, foliage sprouting and flowering start as soon as in March and fruiting starts in May, June, July, and August, extending to the months of September and even October. Because of this, Tabasco is one of the few states, where fast growth and prolonged production is observed compared to other states from Mexico such as Morelos, Puebla, Veracruz, Michoacán, Yucatan, Oaxaca, and even Chiapas.

The objective of this report is to characterize the influence of the savanna soil of Huimanguillo and Cunduacán on the physicochemical properties of oil and seed in order to optimize the crop management of *J. curcas*. Therefore, we investigated the impact of quantitative and qualitative variables, at plant level, on (i) the physical and chemical properties of seeds, (ii) the physicochemical composition of oil (density, viscosity, acid number, saponification, peroxide number, and iodine index) obtained by physical (pressing) and chemical (solvent) extraction, and (iii) the profile of fatty acids in the two locations of this study.

17.2 Seed Source, Experimental Locations, and Agronomic Management

The edible seeds collected in 2005 in the city of Pueblillo, municipality of Papantla, Veracruz, Mexico (20°15′21″ LN, 97°15′33.9″ LW), were found to be non-toxic and rich in oil and protein, and these seeds were planted in the municipality of Mazatepec, Morelos (Martínez et al. 2006). The material for the establishment of the experimental plots in Huimanguillo and Cunduacán was obtained from this municipality (Mazatepec, Morelos).

17.2.1 Experimental Locations

The first plot was located in the community of Mantilla, belonging to the municipality of Cunduacán (18°02′46.1″ LN 93°18′38.6″ LW), and the second in INIFAP CE Huimanguillo (17°50′51.9″ LN, 93°23′47.3″ LW) in the municipality of Huimanguillo. The plantation in Mantilla was established in June 2012 as part of a pilot project for the commercial evaluation of *J. curcas* in Tabasco, while the plantation in Huimanguillo was established in June 2015 in order to evaluate aspects of agronomic management such as pruning and fertilization, among others. In both the locations, the altitude was less than 50 m above the sea level. The climates were Am (f) and A (f) and were prevailing on 80.32% and 19.68% of the municipal area, respectively. The predominant land uses in terms of municipal area were seasonal agriculture (43.24%), cultivated pasture for livestock (32.49%), evergreen forest (9.38%), and hydrophilic vegetation (7.64%) (INEGI 2011).

17.2.2 Agronomic Management

The two experimental plots were established from seeds that were germinated in nurseries at the INIFAP CE Huimanguillo. When sufficiently vigorous (approximately 30 cm), seedlings were transplanted considering a distance of 2 m between each plant in a row and 2.5 m between rows. To date, pruning is performed to maintain plant height, as well as to increase the number of outbreaks. In the year of harvest (2016), the soil fertilization was performed with 100 g of N/P/K (17:17:17) per plant and foliar application of Energyplant® at the dose of 1 L per 100 L of water during flowering. During the same year, it was necessary to irrigate two times, during flowering and seed filling, both in Huimanguillo and Mantilla. Other agronomical practices such as pruning, pests and diseases control, seed harvesting, and handling were performed in 2016 as recommended by Pérez-Vázquez et al. (2013) and Díaz-Fuentes et al. (2015).

17.3 Soil Analyses in the Municipalities of Huimanguillo and Cunduacán

The soil samples were obtained from the experimental sites in the month of June 2016. From each experimental site, five random samples were taken at a depth of 30 cm; the samples were homogenized, and a subsample of 1 kg of soil was obtained, which was the basis for each determination. The variables addressed were pH, electrical conductivity (EC), cationic exchange capacity (CEC), exchangeable acidity, organic matter (OM), total nitrogen (TN), texture, magnesium (Mg), calcium (Ca), phosphorus (P), boron (B), sulfur (S), iron (Fe), zinc (Zn), manganese

Table 17.1 Soil analysis in the municipalities of Huimanguillo and Cunduacán

Parameters	Origin	
	Huimanguillo	Mantilla, Cunduacán
pH (H_2O)	7	7
EC (ds/m)	0.123	0.064
OM (%)	1.5	1.2
N (%)	0.5	0.3
P-Olsen (mg/kg)	66.9	30.3
K (mmol/kg)	0.55	0.25
Ca (mmol/kg)	35	17.9
Mg (mmol/kg)	1.08	0.27
Na (mmol/kg)	0.17	0.12
CEC (mmol/kg)	18.6	35
Fe (mg/kg)	91.18	70
Zn (mg/kg)	3.0	2.5
Mn (mg/kg)	36.7	81.2
B (mg/kg)	32.7	13.6
Texture (%)	Sandy-loam	Loam-clay

(Mn), and molybdenum (Mo) taking the official Mexican standard NOM-021-SEMARNAT-2000 (DOF 2002) as a reference. The obtained results were presented according to NOM-021-SEMARNAT-2000, which enabled to determine the soil characteristics and the influence that its composition may have on the physicochemical properties of seeds.

Both locations presented soils considered suitable for planting of *J. curcas* (González et al. 2015). These soils favored the availability of the main elements; however, the Huimanguillo soils had a higher concentration of essential nutrients compared to those from Mantilla, Cunduacán (Table 17.1). In order to favor the physicochemical quality of *J. curcas* seeds, it was necessary to apply nitrogen, phosphorus, and potassium as suggested by Díaz-Fuentes et al. (2015). Foliar application of fertilizers during flowering and fruiting as proposed by these authors promotes intensive seed production and allowed the proper fruit tie-down, which led to increased yields per plant.

17.4 Determination of Physical Analysis of *J. curcas* Seeds

The extraction of seeds from fruits was done manually on the same day of harvest and with the help of a tweezer and by breaking shells for peeling. The extracted seeds were placed in trays for prolonged drying of 4 days, allowing storage without formation of mold due to excess of moisture. The tree harvests performed at both localities (Huimanguillo and Mantilla) were analyzed independently. In Mantilla, the fruit harvests were performed on July 15 and 29 and August 12 of 2016, while in Huimanguillo they were performed on July 22 and August 5 and 19 of the same year.

Table 17.2 Physical parameters of the *J. curcas* seeds

Parameters	Origin	
	Huimanguillo	Mantilla
Number of seeds per kilogram	1514	1750
Seed weight (g)	0.94 ± 0.09	0.82 ± 0.07
Seed size (mm)	19.7 ± 0.6	19.9 ± 0.9
Seed width (mm)	10.9 ± 0.3	10.7 ± 0.4

At the end, 1 kg of seeds with moisture of 8–9% was obtained for each harvest at each locality. These seeds were stored in a cool and dry room in paper bags to avoid damage by fungus or pests.

The physical characteristics of seeds analyzed were number of seeds per kg, weight of 100 seeds (g), and length and width (mm) of 10 healthy seeds randomly taken from the samples of each harvest (30 seeds in total by location). The oil density was determined according the location, taking as reference the standard NMX-F-075-1987, at a temperature of 25 °C which is recommended for vegetable oils. Viscosity on its part was quantified at room temperature (~26 °C) with a flow ramp within the shear rate range from 10 to 500 s^{-1} using a Brookfield controlled stress rotational rheometer model RST CCT (Mildelboro MA USA) and a concentric cylinder geometry CCT-25.

Studies conducted by Peralta (2010) indicate that in Morelos, Mexico, the average weight per seed is 0.48 ± 0.05 (60% corresponds to the kernel and 40% to the shell), and the seed average dimensions are 12 ± 1 mm long and 4 ± 1 mm wide. In Sinaloa, Mexico, the length, thickness, and weight of seeds according to López (2008) were 18–18.7 mm, 8.6–8.7 mm, and 0.68–0.72 g, respectively. These values are lower than those found in the present study (Table 17.2), which is not indicative of genetic superiority since there is an interaction with environmental factors, even though Henning (2009) argued that the size of the seed depends on its origin. However, it can be indicative of good agronomic management according to the recommendations of Pérez-Vázquez et al. (2013) and Díaz-Fuentes et al. (2015).

In three collections of Sinaloa (Station Dimas, Quelites, and Campana), no significant differences were observed ($p < 0.05$) according to length, width, and weight of seeds (Araiza et al. 2015). The variation of data extended between 15.1 and 15.9 mm for the length, 7.4–7.9 mm for the width, and 0.61–0.63 g for the weight, respectively. The number of seeds per kilogram varied in the three populations between 631 and 632. The Araiza et al. (2015) materials and those evaluated in the present study have different origins; therefore, the genetic and environmental factors influence the morphological aspects of the seed. Consequently, the morphological differences of the *J. curcas* seed may be attributable to its genetic origin and agronomic management. Therefore, to evaluate the environmental effect on the phenotype, it is necessary to homogenize the genetic factor to be able to select outstanding materials (Vertel et al. 1999).

17.5 Chemical Composition of *J. curcas* Seeds in Huimanguillo and Cunduacán

Non-defatted seed flour showed an average of 8.6 ± 0.06 of crude protein (%) considering the three harvests, and significant differences were observed for the harvest from Mantilla (Table 17.3). In Huimanguillo, the protein content was 3% higher (11.47%) attributable to the environment and agronomic management performed in this locality. Protein content of seeds from different states of Mexico ranged from 18% to 28%, which has been reported to be similar to previous data obtained from other countries such as Nigeria (27.8%), Nicaragua (25.6%), India (24.2), Kenya (25%), and Cabo Verde (25.6%) (Castil et al. 1991; Makkar et al. 1997; Martínez et al. 2006, 2010). Based on the above considerations, it was proved that the seeds coming from the state of Tabasco have low protein content with respect to other sources, and this may be due to the genetic expression of the plant material under these local conditions.

On average, the lipid content of seeds from Huimanguillo was 7.2% higher than the average obtained from Cunduacán (Table 17.3). These values are lower than those reported in other studies such as Castil et al. (1991), Makkar et al. (1997), and Martínez et al. (2006) whose results showed that the average percentage of lipids in the seeds of Mexico (Veracruz, Morelos, and Papantla) is 56% oil, which was similar to materials from Nigeria, Nicaragua, India, Kenya, and Cabo Verde with average values of 55%. It is probable that the highest values in lipid content were those reported by Bautista (2010) with values between 57% and 69% in edible seeds from the region of Totonacapan, Mexico. The discrepancy in fatty acid contents among these studies can be due to the genetic diversity of the *J. curcas* accessions from Mexico and/or that to its expression under the local growth conditions. For instance, Bautista (2010) reported that many *J. curcas* trees from which he collected seeds standed near houses and were constantly irrigated or fertilized indirectly by organic waste. Alternatively, the discrepancies among results could also be due to the use of different protocols of lipid extraction or result reporting, among these studies.

Table 17.3 Chemical analysis of whole flour from *J. curcas*

Origin	Variable	Harvest		
		1	2	3
Huimanguillo	Protein (%)	8.2 ± 0.03	8 ± 0.1	9.70 ± 0.06
	Lipids (%)	41.52 ± 0.5	44.49 ± 0.2	45.92 ± 0.02
	Moisture (%)	3.70 ± 0.07	3.80 ± 0.01	3.80 ± 0.03
	Ash (%)	4.50 ± 0.1	4.80 ± 0.1	5.00 ± 0.2
Mantilla, Cunduacán	Protein (%)	11.21 ± 0.06	11.5 ± 0.09	11.7 ± 0.09
	Lipids (%)	46.50 ± 0.02	52.39 ± 0.04	54.79 ± 0.04
	Moisture (%)	4.00 ± 0.07	4.40 ± 0.07	4.60 ± 0.2
	Ash (%)	3.60 ± 0.1	3.80 ± 0.2	4.50 ± 0.01

±Standard deviation

In the case of the plantations of Tabasco, Mexico, the agronomic management was done according to the recommendations of specialists; unfortunately the soil type and characteristics did not favor the expression of the physicochemical properties of *J. curcas* seeds. Therefore, although the climatic conditions are ideal (González et al. 2015), the characteristics of soils should be part of the criteria that must be considered when planning the establishment of a commercial plot, with the view to obtaining higher concentrations of oil in seeds.

The moisture content and ashes in the flour of the seeds harvested in the three periods at each location (Table 17.3) were in the ranges previously reported in other studies for materials of different origin and management (Castil et al. 1991; Makkar et al. 1997; Martínez et al. 2006).

17.6 Characterization of the Chemical Composition of *J. curcas* Oil

The quantification of the oil was performed using a Soxhlet; the protein content, humidity, and ashes were determined by the AOAC method (1995). These analyses were made on the samples of the three harvests at each location. The oil extracted by Soxhlet and by manual pressing was used to determine the saponification and acidity index as recommended by AOAC (1995). The peroxide and iodine indices were determined according to Standard UNE 55.011. All analyses were performed in triplicates for each location.

17.6.1 Acid Number

The acid number is the mass of potassium hydroxide (KOH) that is required to neutralize 1 g of chemical substance. In the Huimanguillo samples, the acid numbers of oil were 7.93 and 10.09 mg KOH/g when extracted by Soxhlet and mechanical pressing, respectively. In the Mantilla samples, the difference between both methods was lower, and the acid number was higher in oil extracted by mechanical mean by only 1.55 mg KOH/g (Table 17.4). These values are higher than those reported by Araiza et al. (2015) in materials from Sinaloa, Mexico, which were between 0.7 and 0.79 mg KOH/g. The high values of oil acidity found in the two localities and by the two extraction methods may cause problems during the transesterification process, because acidities greater than 4 mg KOH/g requires acid esterification to decrease the percentage of free fatty acids, followed by an alkaline transesterification for the production of biodiesel with effectiveness greater than 90%.

The high values of acidity observed in the oil of *J. curcas* seeds harvested in Tabasco have also been reported in seed oil from countries such as Cuba with

Table 17.4 Physicochemical analysis of *J. curcas* oil

Origin	Variable	Extraction method	
		Pressing	Soxhlet
Huimanguillo	Acidity (mg KOH/g)	10.09 ± 0.1	7.93 ± 0.01
	Saponification (mg KOH/g)	195.78 ± 1	190.33 ± 2
	Peroxide (meq O_2/kg)	4 ± 1	10 ± 2
	Iodine (cg I/g)	95.67 ± 1	89.9 ± 0.1
Mantilla, Cunduacán	Acidity (mg KOH/g)	9.85 ± 0.05	8.3 ± 0.1
	Saponification (mg KOH/g)	195.7 ± 3	192.98 ± 5
	Peroxide (meq O_2/kg)	2 ± 0.01	8 ± 0.4
	Iodine (cg I/g)	91.83 ± 0.01	98.29 ± 1

5.92 mg KOH/g (Pérez et al. 2012) and Colombia with 9.6 mg KOH/g (Río et al. 2015). Situation that decreased during oil refining (Pérez et al. 2012) or by hydrogenation for the saturation of double bonds existing in its constituent triglycerides (Río et al. 2015).

Zanahua et al. (2009) determined that the increase in acidity in *J. curcas* oil is due to storage without any pretreatment, which favors hydrolysis, polymerization, and oxidation reactions, due to the presence of moisture and oxygen, a situation that does not match with what was observed in this research. Thus, it is possible that the acidity of *J. curcas* oil may be of genetic or environmental origin.

17.6.2 Saponification Value

Saponification value is expressed by potassium hydroxide in mg required to saponify 1 g of fat. Studies conducted by Ortiz (2012) indicated that the saponification value of oils obtained from biotypes originating from Mexico varies between 63 and 172 mg KOH/g, while Yate (2013) reported that in the case of Colombian seed oil, this presented values of 175.41 mg KOH/g (Soxhlet) and 201.72 mg KOH/g (mechanical extraction). Material from Costa Rica obtained values of 180 mg KOH/g, Malaysia (193.55) (Akbar et al. 2009). Except for the values reported for Mexico, the saponification value determined for the two localities of Tabasco (Table 17.4) is in the range that has been reported in other countries.

The values of the saponification value are indicative that the oil obtained has a higher presence of low molecular weight fatty acids and that it is possible to use it in the industry (minimum value 185 mg KOH/g) or for food (values between 184 and 196 mg KOH/g) (Lafont et al. 2011). Due to the origin of the materials, this value is important, since the seeds are free of phorbol esters, with low presence of saponins and phytates; therefore, its comestible use is possible.

17.6.3 Peroxide Value

The peroxide value is the most widely used. It gives a measure of the extent to which an oil sample has undergone primary oxidation and is determined by measuring the amount of iodine which is formed by the reaction of peroxides, formed in oil upon primary oxidation, with iodide ion. The iodine liberated is titrated with sodium thiosulfate. Belén et al. (2005) considered that oils with peroxide values higher than 2 meq O_2/kg are prone to rancidity, so the oil extracted from the seeds of *J. curcas* harvested in the two experimental sites is highly prone to become rancid regardless of the extraction method. Another adverse effect concerns its industrial use since with these values it would cause the formation of polymers. These levels of peroxidation are within the range that have been reported for different samples from Mexico (Ortiz 2012); however, they are superior to the materials from Malaysia (1.93 meq O_2/kg) and Brazil (0.34–0.63 meq O_2/kg) reported by Akbar et al. (2009) and Brossard et al. (2010), respectively.

17.6.4 Iodine Index

The iodine index is a measure of the degree of unsaturation of oils (Lafont et al. 2011). It is a constant value for oil, but it depends on the extraction technique used. A solution of iodine is yellow/brown in color; when it is added to a solution to be tested, C=C double bonds react with iodine and reduce the magnitude of the color by taking iodine out of solution. The color change can be measured by spectrophotometry and used to measure the unsaturation rate of oils. The higher degree the unsaturation the oil is, more prone to. When the iodine value is low (26–48 g/100 g), the oil is saturated and tends to solidify, while for high values (94–135 g/100 g), the level of unsaturation increases; therefore, the oil remains in liquid state at lower temperature and has a lower viscosity (Araiza et al. 2015). Under this criterion, the oil extracted by the pressing method from the town of Mantilla is prone to solidification at relatively high temperature. This can be attributed to the extraction method rather than to the genetic variation or the response to the environment. The rest of the values were close to those reported in other studies such as that of Guevara et al. (2016) who found iodine indices greater than 100 g/100 g in samples from Tamaulipas, Mexico. In other countries, such as Costa Rica and Malaysia, iodine indices ranged from 77.14 to 103.62 (Akbar et al. 2009).

17.6.5 Specific Density and Viscosity

The specific density of the *J. curcas* oil was 0.91 ± 0.003 and 0.916 ± 0.007 kg/m^3 for Mantilla (Soxhlet extraction) and Huimanguillo (extraction by mechanical

Table 17.5 Fatty acid profile of *J. curcas* of oil samples from Huimanguillo and Cunduacán

	Origin	
Fatty acid	Mantilla, Cunduacán	Huimanguillo
Myristic acid (C 14:0)	1.1	1.1
Palmitic acid (16:0)	13.1	12.9
Palmitoleic acid (16:1)	2.4	1.3
Stearic acid (18:0)	6.3	6.8
Oleic acid (C18:1)	36.4	35.7
Linoleic acid (C18:2)	35.4	34.4

The saturated fatty acids represent less than 25% of the total fatty acids, which means that 75% of oil is made of unsaturated fatty acids

pressing). These values are on the upper limit of the range of 0.86–0.90 g/cm^3 required for the production of biodiesel (ASTM D6751) making the oils obtained from the seeds of *J. curcas* harvested in Tabasco still suitable for the production of biodiesel, regardless of the extraction method. The values obtained in this work are similar to those reported for castor oil (Benavides et al. 2007), although it is necessary to consider as the interaction of specific density with temperature, which is closely correlated to oil density (Benjumea et al. 2006).

The viscosity of 50–52 mm^2/s in the municipalities of Huimanguillo and Cunduacán are similar to those reported in Cuba (51.37 mm^2/s) by Lafargue et al. (2012). As for specific density, temperature influences oil viscosity. For example, the viscosity values reported by Rodríguez et al. (2012) for castor bean (252 mm^2/s) and *J. curcas* (33.89 mm^2/s) determined at 40 °C, while for 100 °C these values were 19.90 and 7.59 mm^2/s, respectively, for each species. These values and those found in oil from *J. curcas* harvested in Tabasco, Mexico, have the same tendency with respect to temperature.

17.6.6 Fatty Acid Profile in J. curcas *Oil*

Fatty acid profiles were determined in an Agilent Technologies Gas Chromatograph (7890A GC System) coupled to an Agilent Technologies mass spectrometer (5975C V2-MSD with triple-axis detector), with an auto sampler, an injector G4513A, and a spectroscopy mass detector. The range of temperature gradient in the column was 60–255 °C, and the gas flow rate was 1.39 mL/min. The fatty acid standard was injected, and the retention times were used to identify the peak of the samples. The fatty acid levels were estimated as a percentage of the total area of the methyl ester peak. The sample used for this quantification was the one extracted by Soxhlet at each location.

The extraction method did not show any change in the chemical composition of the *J. curcas* seed oils harvested in the two locations (Table 17.5).

The dominance of oleic and linoleic fatty acids is a good indicator for the production of good-quality biodiesel; however, this feature also applies to human nutrition. Although, far from having the properties of olive oil (oleic acid content larger than 65%; Salas et al. 1997), *J. curcas* oil is within the ranges of commercial oils, such as those from sunflower and canola seeds.

When comparing the profile of fatty acids between oils of *J. curcas* from Mexico to those of other countries, Ortiz (2012) found, in samples from Sinaloa and Puebla, Mexico, amounts of oleic acid ranging from 44.8% to 46.5%, which are larger to those reported by Raja et al. (2011) in oils (34.4%) from Tabasco. The importance of determining the profile of fatty acids lies in the final use for the oil. For example, Mudhaffar et al. (2013) suggested that if *J. curcas* oil is rich in oleic acid (>43.3%) and linoleic (>36.7%) as it is the case for Malaysian samples, it can be used as biolubricant.

17.7 Effects of Soil on Seeds and Oil in Tabasco, Mexico

The weight and size of seeds is an indicator of physiological quality; large or heavier seeds show better germination and vigor; however, the knowledge of genetic characteristics of seeds and their response to environmental variations is necessary. Given this fact, Suárez et al. (2011) considered that for the production of *J. curcas* seeds, several factors should be considered such as the genetic identity of the material, the available humidity, the agronomic management, the type of soil, and climate conditions, in addition to other factors related to seed production. The validity of these assumptions is demonstrated in this work, since the highest values for most of the variables considered were found in seeds harvested in Huimanguillo, which has better agroclimatic characteristics compared to Mantilla.

Pérez-Vázquez et al. (2013) and Díaz-Fuentes et al. (2015) produced information manuals whose objective is to help farmers to obtain better yields of *J. curcas* seeds in Mexico. Among the basic recommendations made in both the works is nitrogen fertilization, as well as pruning at strategic times. Machuca (2007) documented that seed yields of *J. curcas* from Salvador are close to 6 ton/ha, and oil production was estimated at 2.31 m^3/ha.

According to Heller (1996), *J. curcas* cultivation is tolerant to poor soils, is fast growing under conditions of low soil fertility, is resistant to drought, and adapts to the conditions of tropical and semiarid regions. However, Córdova et al. (2015) concluded that the natural distribution area of the species in Mexico is in the areas with greater precipitations, in which spreads some doubt on its supposed resistance to drought. On the other hand, González et al. (2015) showed that the state of Tabasco has 833,181 ha that is optimal for the establishment of plantations of *J. curcas* and that the municipalities with greater areas for this activity are in Balancán (256,201 ha) and Huimanguillo (131,596 ha).

17.8 Conclusion

The physicochemical characterization of *J. curcas* in Tabasco, it is concluded that this species has the potential to be a profitable and competitive crop for biodiesel production. However, much remains to be investigated concerning the benefits that can be drawn from coproduct of the whole plant, such as those for applications in pharmaceutical, chemical, and food industries, especially with regard to the non-toxic accessions.

In contrast to fatty acid profile and physical properties of oil, the physical parameters as well as oil and protein contents of seeds were influenced by the agronomic management of the crop.

The oil of *J. curcas* presented a high acidity and peroxide index, which represents a drawback for biodiesel production because polymers that may form during oil storage are expected to affect the transesterification process. To cope with this problem, a step of acid esterification is required prior to the process of alkaline transesterification, which implies an additional cost to the production of biodiesel. However, the use of oil can be diversified into multiple applications beyond the production of biodiesel alone. In addition, proteins from seed flour or seed cake can be used for human and animal consumption.

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Chapter 18
Applications of *Jatropha curcas* Cake

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Abstract *Jatropha curcas*, a tropical and subtropical shrub/tree, has been emerging as a promising biodiesel crop because of its high oil content and ability to grow in marginal lands. The kernel cake is the major by-product of the Jatropha biodiesel chain, rich in protein and has potential to be used in livestock feed; however, the presence of anti-nutritional factors and phorbol esters limits its use. Thus, this report discusses on phorbol esters toxicity and risks and strategies for cake detoxification. Besides feeding application of *J. curcas* cake – that we see as a possibility of integration with biodiesel and food chains – we presented some other alternative uses.

Keywords Animal feed · Biodiesel · Detoxification · Phorbol esters · Press cake

18.1 Introduction

Research during the last decade has shown that *Jatropha curcas* L. oilseed offers considerable potential for use as raw material in biodiesel production (Makkar et al. 2009; Adebayo and Ameen 2017). This potential is attributed to both the seed and oil yields, which is typically 1500 kg/ha/year for oil and larger than observed in traditional oilseeds (Quirino et al. 2014). In addition, the physicochemical characteristics of oil, namely, viscosity and high-quality fatty acids, are favorable for biodiesel production (Jongschaap et al. 2007; Becker and Makkar 2008; Kumar and Sharma 2008; Koh and Ghazi 2011; Duraes et al. 2012).

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In order to obtain 1 ton of *J. curcas* oil, mechanical extraction requires 2.85 tons of seeds as input (Makkar and Becker 2009). During this process, in addition to the production of the oil, press cake (JCC) is generated in abundance and represents about 70% of seed mass (Gomes et al. 2018).

Press cake is, therefore, a by-product of oil production that is rich in nitrogen, phosphorus, potassium, and carbon in addition to the hemicellulose fraction. It also shows a high protein content (at least 16%) including all amino acids (except lysine) considered to be essential for growth and animal health (Makkar et al. 1998; Belewu et al. 2009).

Despite its volume and nutritional characteristics, animal feeding with JCC is hampered due to the presence of bioactive compounds, such as anti-nutritional factors (trypsin inhibitors and phytates), toxins (curcin and phorbol esters), and allergens (2S proteins).

Phorbol esters are the main toxic components present in JCC and, when ingested, may act in the body either acutely (intense inflammatory response) or chronically (tumor induction). As they are liposoluble, most phorbol esters are extracted together with the oil. However, the minimal amounts that remain in the JCC are capable of causing damage to various animal species (Goel et al. 2007; Makkar et al. 2009; Devappa et al. 2010).

The lack of adequate destination and value aggregation for JCC, associated with the low agronomic information on this species across different regions of Brazil, has resulted in a lack of interest in its planting. Given the current commercial disinterest in this crop, research is ongoing in Brazil into the development of cost-effective methods for degradation of phorbol esters to enable application in animal supplementation.

Despite the benefits of *J. curcas* for biodiesel production, this species is still under domestication, with many research challenges to be overcome. *J. curcas* is poorly genetically characterized, and there is little information about production levels in the different regions of the country. Agronomical production systems are not completely validated, and more information regarding propagation, plant density, maintenance, nutrition, and pest management are still necessary to enable industrial scale production. Nevertheless, *J. curcas* genotypes that do not produce phorbol esters in their seeds have been identified, which may enable the subsequent development of non-toxic commercial cultivars whose JCC might be used for animal nutrition (Quirino et al. 2014). Unfortunately, these non-toxic genotypes are more susceptible to pests and diseases and, therefore, with lower yields. As such, JCC detoxification is still an economical attractive as a viable alternative for animal feeding.

Although detoxification of JCC is an important bottleneck for its use in animal feed supplementation, a number of additional applications also exist for its valorization that are discussed below.

18.2 Biodiesel in Brazil

Brazil is at the forefront, along with few other countries, in the diversification of the energy matrix. A central pillar in this industry is the production and application of biodiesel. The blending of 2% biodiesel into fossil diesel (B2) became mandatory in Brazil in 2008. This same law (N.11.097/2005) also stated that the percentage of biodiesel blended into petrodiesel should increase to 5% by 2013, but this goal was reached in 2010. A second law (N.13263/2017) set that by March 2019, the biodiesel addition should reach 10% (B10). Considering the expansion of biodiesel in the Brazilian energy matrix and the National Energy Policies as well as its economical, social, and environmental benefits, the government decided to implement B10 as mandatory as soon as in March 2018 (CNPE Resolution N° 11, of 1.3.2017 – Union Official Journal). The current projections estimate that by the end of 2018, a total of 5.4 billion liters of biodiesel will have been consumed in Brazil.

The creation of the Social Fuel Stamp, an instrument of tax exemption, was an important mechanism for achieving the goals established by the National Program for the production and use of biodiesel. This stamp consists of a certification that provides a series of tax and commercial benefits to companies that produce biodiesel from oilseeds cultivated by smallholder farmers (family agriculture). In 2016, from a total of 48 industries capable of commercially production of biodiesel in Brazil, 92% were holders of the Social Fuel Seal (MME 2017).

Another state policy action that will likely boost the sector is the RenovaBio program. In December 2017, a National Biofuel Policy was launched by the Federal Government (Law 13,576/2017), with the objective to expand biofuel production in Brazil, based on predictability, environmental sustainability (reduction of greenhouse gases, in the marketing and use of biofuels), and socioeconomic compatibility with market growth. CBios (decarbonization credits) will be sold by biodiesel producers to be bought by fuel distributors (that make mixtures of petrodiesel with biodiesel). This will stimulate the use of new raw materials, especially those with better carbon footprints, in order to earn more credits.

Biodiesel production occurs across all regions of Brazil, with an installed capacity of 7.7 million m^3/year. However, due to the availability of raw materials, production is mainly concentrated in the south (40.1%) and central west regions (44.6%) (MME 2017).

The main oilseeds and raw materials employed in Brazil for biodiesel production comprise soybean (76.4%), animal fats (15.7%), cottonseed (1.1%), and other minor sources such as sunflower, palm, castor, and peanut oil as well as residual fats. As such, the *Programa Nacional de Produção e Uso do Biodiesel* (PNPB) is basically dependent on vegetable raw materials, with soybean being the main source for biodiesel production (MME 2017).

The diversification of raw materials for the production of biodiesel will depend on the success of government programs and research that solve problems in productive

chains. This diversification is fundamental, considering that soybean is unsuitable for planting in many regions of Brazil due to issues of climatic aptitude or agroecological zoning. *J. curcas* is a high energy density crop (considering the amount of oil produced per hectare) and is well suited for family farming, which would enable companies to obtain the Social Fuel Seal by buying from smallholder farmers.

In the case of *J. curcas*, in addition to the challenges of genetics for crop improvement and the development of economically viable production systems, destination of the remaining JCC after oil extraction requires optimization. This material, although presenting high protein content, cannot be used directly in animal feed because of its toxic compounds.

18.3 Phorbol Esters

Phorbol esters (PE) are natural chemical compounds that occur in plant species belonging to the Euphorbiaceae and Thymelaeaceae families (Makkar et al. 1997, 2009; Makkar and Becker 2009). These toxic compounds are tetracyclic diterpenes with tigliane skeleton structures, with hydroxyl groups on neighboring carbon atoms esterified with fatty acids. A number of different phorbol ester compounds have been identified, varying on the basis of ester linkages and the acid fraction, and hydroxylation of the central structure (Goel et al. 2007; Oskoueian et al. 2012; Devappa et al. 2013a). A total of six distinct phorbol esters compounds have been characterized in *J. curcas*, which all possess a core primary structure of 12-deoxy-16-hydroxyphorbol. These have been named as C1-C6 Jatropha factors according to side chain carbon distribution (Haas et al. 2002; Raja Krishna Kumar et al. 2013).

These diterpenes can have an acute effect on the body, inducing an intense or chronic inflammatory response that can result in the promotion of tumor growth. The toxicity of these compounds in the affected organism depends on the particular chemical structure (Makkar et al. 1997). The biological activity of each phorbol ester is structure-specific and may cause intoxication even at low concentrations (Goel et al. 2007). Different forms of phorbol esters (PE) can activate different pathways in the cellular response, causing different symptoms in each organism, which include tumor development, inflammation, differentiation, and apoptosis in animal tissues (Oskoueian et al. 2012).

PEs, which show an affinity to phospholipid membrane receptors, act analogously to diacylglycerol by activating protein kinase C (Goel et al. 2007). During normal cellular signal transduction process, diacylglycerol functions as an activator of protein kinase C (PKC). This enzyme is fundamental in the transduction of signals induced by various hormones and regulates cell proliferation. Prolonged activation of this enzyme, however, can result in a myogenic response, which can increase the efficacy of other carcinogenic substances. As such, PEs are considered cocarcinogens (Goel et al. 2007), promoting tumor growth without any direct involvement in

initial tumor formation (Li et al. 2010). The inflammatory effect of PEs is one of the first symptoms of acute *J. curcas* intoxication, with mobilization of phospholipids releasing arachidonic acid and subsequent secretion of prostaglandins. Inflammation will occur in the contacted area and in the eyes, while in the case of ingestion, inflammation will mainly be in the gastrointestinal tract (Strair et al. 2002; Devappa et al. 2010, 2013b).

The concentration of PEs present in JCC will vary according to the genetic makeup of the *J. curcas* genotype. Factors such as climate, altitude, and genotype also influence PE content in seeds as well as oil residue associated with the solid material after seed processing (Rakshit et al. 2008). Varieties of *J. curcas* free of PEs or with minimal amounts (<0.09 mg/g) are known to occur in Mexico and are considered to be non-toxic (Makkar et al. 1998; He et al. 2011). Even if PEs are lipophilic, they remain strongly bound to the residue matrix even after oil extraction, which means that approximately 30% of the PEs present in seeds remain in the JCC (Makkar and Becker 2009). Concentrations of PE typically range from 0.82 to 3.85 mg/g in JCC and from 2 to 4 mg/g in oil (Makkar et al. 1997; Becker and Makkar 2008; Li et al. 2010).

18.3.1 Toxicological Effect of Phorbol Esters in Animal Feed

A number of experiments on ruminants (cattle and goats) and non-ruminants (mice, rats, chickens, fish) have shown that seeds can be toxic and, depending on the dose, can cause death (Rakshit et al. 2008; Devappa et al. 2010; Li et al. 2010; Honorato et al. 2017). In one study, for example, acute doses of 2.5 g seed/kg of animal weight/day and chronic doses of 0.025 g seeds/kg of animal weight/day were evaluated over a 14-day period in ruminants. Both doses resulted in animal death following development of symptoms that included diarrhea; dyspnea; dehydration; hemorrhagic effects in organs, such as the rumen, lungs, kidneys, and heart; as well as pulmonary congestion and edema (Goel et al. 2007). Similarly, Li et al. (2010) reported LD50 values of PEs in mice of 27.34 mg/kg body weight, with DL5 and DL95 values of 18.87 and 39.62 mg/kg body weight, respectively. While histopathological analyses showed no significant damage to organs at a concentration of 21.26 mg/kg, severe lesions, especially in lung tissues, together with diffuse hemorrhaging and kidney atrophy, were evident in animals that received a higher PE dose at 32.4 mg/kg. Exposure to PEs at 36 mg/kg resulted in multiple detachment of cardiac muscle fibers and cortical neuron discoloration (Li et al. 2010). Barros et al. (2015) also described the negative effects of untreated JCC on poultry, with symptoms of intoxication including weight loss, increased heart weight, and testicular atrophy. PEs can also cause negative effects on animal reproduction, as reported by Teixeira Sousa Moura et al. (2017). In their study, in comparison to control groups, healthy rats that were crossed with rats fed on JCC (1.01 mg/g PE) resulted in a decrease in the number of living fetuses and an increase in placental weight.

Given such toxicological effects of PEs in animal feed, efficient methods for detoxification of JCC are an essential step to add value to this by-product. Several approaches for PE degradation have been developed and can be classified as chemical, physical, biological, and combined methods. Recently Gomes et al. (2018) published a review summarizing all PE degradation methods and the benefits and drawbacks of each process (Table 18.1).

While chemical treatment of PEs can reduce PE concentrations, the treated JCC may not be suitable for animal feed yet. For example, although the JCC treatment with NaOH and $Ca(OH)_2$ was shown to reduce its PE concentrations from 2.14 to 0.32 g/kg and 0.36 g/kg, respectively (Katole et al. 2013), goats fed with treated substrate showed a decrease in nutrient intake and digestibility. In addition, results of blood metabolite analyses indicated that hemoglobin, globular volume, serum urea, triiodothyronine, and testosterone levels were reduced (Katole et al. 2013).

Similarly, while combined treatment (alkaline and thermal) of JCC for PE degradation has been shown to reach a reduction of 89% (Rakshit et al. 2008), rats fed on this treated substrate over a 12-day period showed severe signs of intoxication such as diet rejection, diarrhea, loss of motor activity, and mortality.

18.4 Applications of *J. curcas* Cake

18.4.1 Animal Feed

The composition of JCC from toxic and non-toxic seeds is similar in terms of essential amino acid content (except lysine), and appropriate for application in feed, in accordance with the references indicated by FAO (Makkar et al. 1998; Devappa et al. 2013a). In studies evaluating non-toxic JCC, Oliveira et al. (2013) reported no negative effect when incorporated up to 300 g/kg in sheep fed over a 70-day period, with no deleterious effects on animal development or physiological alterations. Similarly, Félix-Bernal et al. (2014) reported inclusion of non-toxic JCC in the diet of sheep at up to 140 g/kg, suggesting that it is suitable as a potential replacement for soybean meal and dry-rolled corn. Such studies indicate that JCC offers potential as a substitute of soy protein concentrate in animal nutrition, with widespread inclusion limited only by the presence of PEs. These toxins, on the other hand, can be efficiently degraded using suitable treatments. Several studies that have been successful in the detoxification and application of JCC in animal supplementation are described in the literature and will be briefly presented.

Detoxification by solid-state fermentation with the fungus *Aspergillus niger* has been reported, with no toxicity symptoms observed in rats following incorporation into the diet over a 4-week period (Faoziyat et al. 2014). According to these authors, there was no observable difference in weight gain between a control group fed with soybean meal and the group fed with detoxified JCC. Similarly, Zhao et al. (2018) showed that proteins isolated from detoxified JCC can be used effectively as a

Table 18.1 Comparison of physical, chemical, and biological methods for degradation of phorbol esters

Treatment	Degradation method	Initial PE level	Final PE level	% reduction
Physical	Ozone	12 ppm	PE decreased to non-detectable level	100
	Gamma radiation (125 kGy)	0.29 mg/g	0.012 mg/g	96
	Sunlight, room temperature (oil)	2.09 g/L	1.04 g/L	49
	Sunlight, room temperature (oil)	2.3 g/L	PE decreased to non-detectable level	100
Chemical	Hydrogen peroxide oxidation was established and optimized	0.52 mg/g	PE decreased to non-detectable level	100
	Methanol solvent extraction	3.6 mg/g	0.10 mg/g	97.30
	Ethanol solvent extraction	3.07 mg/g	PE decreased to non-detectable level	100
	Surfactants	1.45 mg/g	0.27 mg/g	80
	Alkaline ($NaHCO_{3)}$	1.29 mg/g	0.70 mg/g	55
	Alkaline and heat	1.78 mg/g	0.13 mg/g	92
Combined	Alkaline and heat ($Ca(OH)_2$ and NaOH)	135 mg/g	16 mg/g and 13.6 mg/g	89
	Alkaline and heat (NaOH and $Ca(OH)_{2)}$	2. 14 g/kg	0.32 g/kg and 0.36 g/kg	85 and 83.2
	Bjerkandera adusta and *Phlebia rufa*	0.82 mg/g	0.05 mg/g and 0.02 mg/g	91 and 97
	Pleurotus ostreatus	0.63 mg/g	0.0063 mg/g	99
Biological	*Ganoderma lucidum*, *Trametes zonata*	1.072 mg/g	PE decreased to non-detectable level	100
	Pleurotus ostreatus, *Pleurotus sajor-caju*	1.072 mg/g	0,28 mg/g and 0.37 mg/g	73 and 65
	Pleurotus sapidus, *Pleurotus florida*, and *Phanerochaete chrysosporium*	1.072 mg/g	0.27 mg/g, 0.38 mg/g and 0.6	74, 64, and 45
	Trametes hirsuta, *Trametes versicolor*, and *Trametes gibbosa*	1.072 mg/g	0.2 mg/g, 0.15 mg/g and 0.08 mg/g	81, 86, and 92
	Aspergillus niger, *Penicillium chrysogenum*	0.13 mg/g	0.03 mg/g and 0.11 mg/g	76 and 15
	Rhizopus oligosporus, *Rhizopus nigricans*, and *Trichoderma longibrachiatum*	0.13 mg/g	0.12 mg/g, 0.10 mg/g and 0.11 mg/g	7, 23, and 15
	Aspergillus versicolor	0.832 mg/g	0.158 mg/g	81
	Trichoderma harzianum, *Paecilomyces sinensis*, and *Cladosporium cladosporioides*	2.78 mg/g	0.05 mg/g, 0.03 mg/g and 0.08 mg/g	99.7, 98.9, and 96.9

(continued)

Table 18.1 (continued)

Treatment	Degradation method	Initial PE level	Final PE level	% reduction
	Morganella morganii	Not specified	Not specified	92
	Pseudomonas aeruginosa	Not specified	PE decreased to non-detectable level	100
	Bacillus sp.	0.60 mg/g	PE decreased to non-detectable level	100
	Bacillus licheniformis	0.1199 mg/g	0.04 mg/g	62
	Acinetobacter calcoaceticus	Not specified	PE decreased to non-detectable level	100
	J. curcas lipases	1.05 mg/g	0.06 mg/g	94
	Pleurotus ostreatus	0.5 mg/g	0,005 mg/g	99

From Gomes et al. (2018)

protein substitute in the diet of animals at levels below 40% with no histopathological changes observed in kidneys or livers in rats that received this protein diet from JCC.

Detoxification by chemical treatment has also been reported to be suitable for subsequent application in feed. Teixeira Sousa Moura et al. (2017), for example, treated JCC with 5 M NaOH and reached detoxification of PE to levels lower than 0.06 mg/g. When added at 5% to the diet of male rats for 60 days, animals did not display any signs of toxicity, in contrast to rats fed on JCC without chemical treatment, where histopathological symptoms included diffuse degeneration of the liver and edema around the pulmonary vessels.

Toxicological analysis of detoxified JCC has also been conducted on animals of greater economic importance, such as shrimp, fish, pigs, and sheep. For example, Harter et al. (2011) evaluated detoxified JCC in shrimp diets (*Litopenaeus vannamei*) with weight gain, specific growth rate, and metabolic growth rate higher in the group fed with detoxified JCC. Data indicated the potential in detoxified JCC for replacement of fishmeal in the shrimp daily diet. In fishes, Shamna et al. (2015) analyzed the effect of incorporating JCC detoxified by *Aspergillus niger* in up to 20% of the diet of *Labeo rohita*, with no harmful effects related to weight gain and physiological and immunological responses observed. Similarly, Krome et al. (2014) reported safe application of detoxified JCC in up to 75% of the diet of Nile tilapia (*Oreochromis niloticus*).

Detoxified JCC has also been evaluated in the pig diet over a 28-day period with no negative effects related to weight gain, feed conversion, or biochemical

parameters of livers and kidneys detected over the time period evaluated (Wang et al. 2011). Based on these results, the authors suggested that detoxified JCC can substitute 50% of soybean meal in pig diets.

Similar results were recently reported by Li et al. (2018) who also claimed that detoxified JCC can be used to replace crude soybean meal proteins by up to 30% in swine diet formulations with no effect on growth performance and nutrient utilization. In their study, no traces of PEs were detected in animals fed on detoxified JCC, providing evidence for meat safe for consumption (Li et al. 2018).

According to Patil et al. (2015), lambs fed on a diet that had soybean meal replaced by 37.5% detoxified *J. curcas* meal also showed no adverse effects on carcass yield or meat quality.

Efficient biodetoxification of JCC using edible macrofungi, such as *Pleurotus ostreatus*, has also been described with increased nutritional quality of JCC following fungal growth enabling inclusion of up to 20% in goat diets (Kasuya et al. 2012).

18.5 Production of Enzymes of Industrial Interest

In addition to application in animal feed, JCC can also be used as a substrate for microbial colonization and secretion of microbial enzymes appropriate for industrial application. These have included cellulolytic and xylanolytic enzymes from *Aspergillus niger* (Ncube et al. 2012); lipases and proteases from *Pseudomonas aeruginosa*; xylanases from *Scytalidium thermophilum* (Joshi and Khare 2011); amylase, cellulase, lipase, pectinase, protease, and xylanase from *Bacillus* sp. (Chang et al. 2014); and cellulases from *Paecilomyces variotii* (Pathak et al. 2016). Under optimized conditions, JCC has also been used as a substrate for the production of Pullulan by *Aureobasidium pullulans*. This exopolysaccharide is an industrially important enzyme with numerous potential applications in the food, pharmaceutical and cosmetic industries (Choudhury et al. 2012).

18.6 Antimicrobial and Antiparasitic Action

Given their toxicity, extracts from JCC and other plant tissues, which contain PEs, have been cited as potential antimicrobial, antiparasitic agents and antiviral agents (Insanu et al. 2013). Bioinsecticide and molluscicide activities have been reported (Ratnadass and Wink 2012) with methanol extracts from *J. curcas* leaf tissues and successfully employed in the biological control of the insect pest *Culex quinquefasciatus* (Kovendan et al. 2011). Recently, protein extracts from JCC were also shown to inhibit growth and infection of the parasite *Toxoplasma gondii*

(Soares et al. 2015). According to Devappa and collaborators (2013a, b, c), extraction and recovery of PEs with methanol are simple and can be scaled up without major difficulties. However, the form and storage period are important for successful application of PEs, as they are degraded by auto-oxidation. Although cold storage can reduce such degradation, loss of biological activity will be proportional to storage time (Devappa et al. 2013b).

18.7 Additional Applications of *J. curcas* Cake

In addition to biodiesel and non-toxic residual vegetable biomass as fertilizers or feed, additional potentially viable applications of *J. curcas* include a number of chemical compounds released during the degradation of the plant cell wall. For example, Watanabe et al. (2014) described the possibility of applying *J. curcas* in a biorefinery system, where several plant tissues (seed coat, kernel, stem, xylem, bark, and leaf) were submitted to torrefaction under different temperature regimes. Compounds such as oligosaccharides, glucuronoxylan, maltodextrin, syringyl, guaiacyl from cellulose degradation, hemicellulose, and lignin were detected, with PEs completely degraded at 300 °C.

Given the decrease in natural wood supplies, there is a need for particle boards free of synthetic adhesives. In this context, JCC as a raw material for the production of binderless boards has also been described (Hidayat et al. 2014).

While most applications require the removal of PEs, their recovery from plant tissues and oil can also have application in the synthesis of prostratin (Devappa et al. 2013a). The applications of JCC are summarized in Fig. 18.1.

18.8 Integration of Productive Chains (Biodiesel and Fungiculture)

Many basidiomycete fungal species can efficiently colonize and sporulate on lignocellulosic substrates due to secretion of hydrolytic and oxidative enzymes released into the extracellular medium. A result of this can be the degradation of lignocellulose and the corresponding production of vegetative biomass. The cultivation of edible macrofungi is a developing industry in full expansion in Brazil, given the medicinal and culinary applications and improved economical conditions of the country in the recent years.

We have been developing a line of research for application of JCC as a substrate for mushroom cultivation. Here, toxic JCC is used for the cultivation of macrofungus that detoxifies the substrate by fungal enzymes secreted during its growth. The remaining biomass formed by the fermented JCC and its constitutive mycelium, after the production and harvest of edible mushroom, is called *spent mushroom*

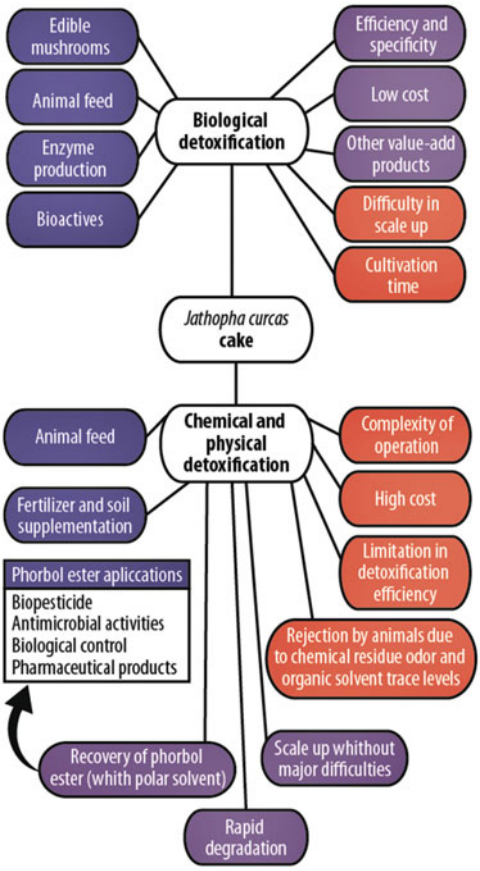

Fig. 18.1 Applications of *J. curcas* by-products that offer added value according to detoxification method (in blue), benefits (in purple), and drawbacks (in orange)

substrate (SMS). SMS can be used as an input in ruminant feed with increased protein content of biomass because PEs were degraded by prior fungal fermentation. In addition, the edible mushrooms (with no trace of phorbol esters) may be used for human consumption.

Not all macrofungi have the ability to produce enzymes that detoxify JCC. Even different strains from the same species may be different in their detoxifying capacity. In the present work, following screening of candidate macrofungal species, a strain belonging to the genus *Pleurotus* was identified with high efficiency in PE degradation, with a 60-day cultivation period resulting in a 99.9% degradation of the toxin and edible mushrooms free of PEs (unpublished data) (Fig. 18.2). Subchronic toxicological tests on rats, using feed supplemented with 8.5, 24, or 37% SMS (composed of 80% detoxified JCC), revealed no signs of toxicity, with weight gain not significantly different from control groups (unpublished data). Serum blood chemistry and biochemistry also showed no differences between groups, with the exception of total cholesterol content, which was lower in the group exposed to

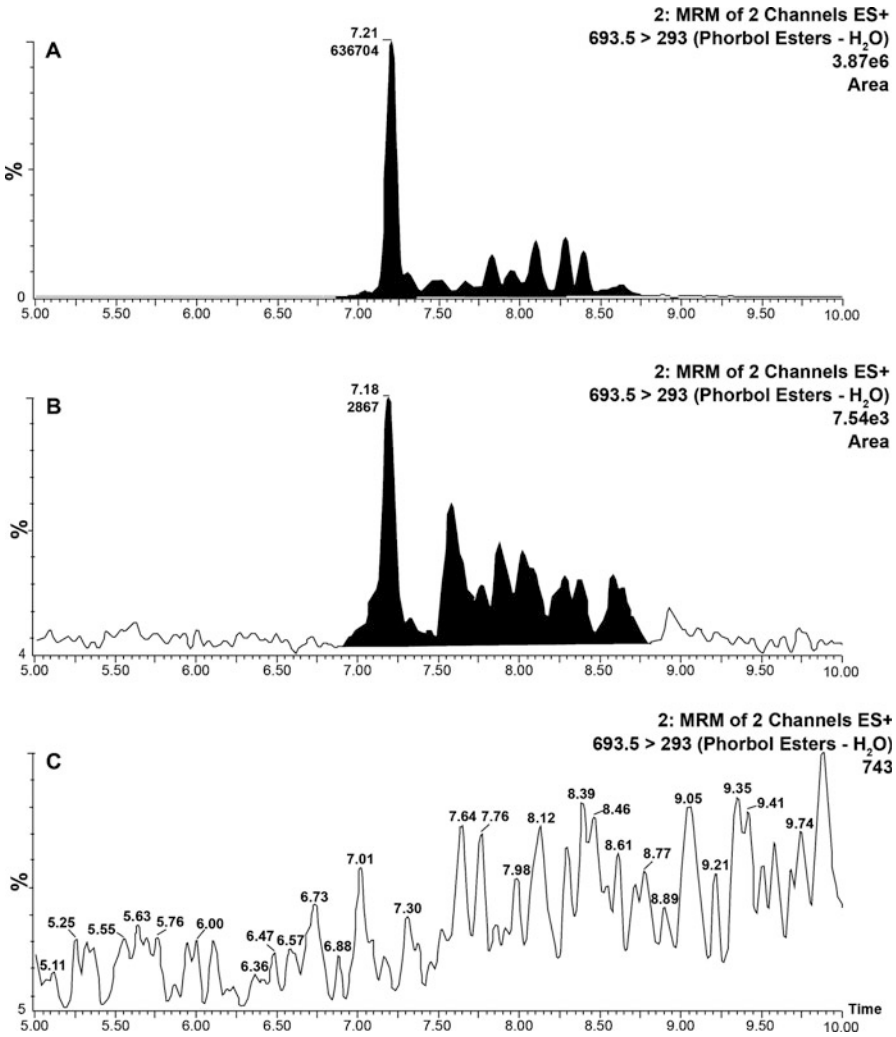

Fig. 18.2 Chromatograms referring to phorbol ester quantification through MS detection. (**a**) J. curcas cake in natura (2.17 mg/g phorbol esters); (**b**) bio-detoxified J. curcas cake (0.007 mg/g phorbol esters); (**c**) mushrooms produced on J. curcas cake (base-line noise only, indicating an absence of phorbol esters)

detoxified JCC SMS. This can indicate that bioactive substances are present due to fungal growth.

This same by-product is currently being tested for potential use in swine diet formulations. Given this initial data, it appears that potential exists in the integration of *J. curcas* production and mushroom production chain, where JCC is used as

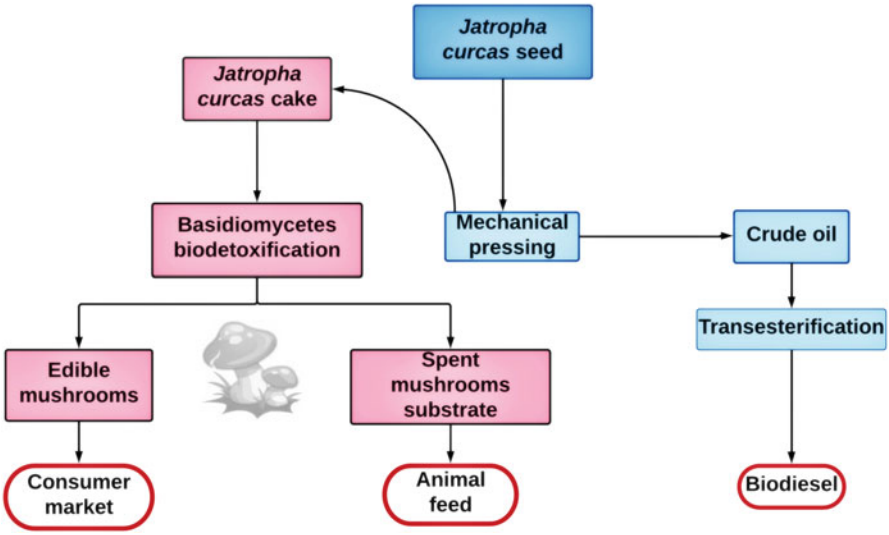

Fig. 18.3 Integration of biodiesel, food, and feed chains using *J. curcas*

substrate for mushroom production, with cultivation degrading the toxic compound and SMS destined for animal feed supplementation (Fig. 18.3).

18.9 Conclusions

Jatropha curcas represents an important crop for biodiesel production due to high oil yield. Besides agronomic issues to establish production chain, it is also necessary to add value to byproducts; otherwise, oil would not be economically viable to fuel production. Toxic compounds are present in JCC and the choice of method for detoxification ultimately depends on the final destination of the crop residue, whether in animal feed, fertilizer, mushroom, bioactive extracts, and/or enzyme production. We presented macrofungal biodetoxification as a possibility for safe integration of biofuel, food, and feed chains.

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Part V
Coproducts

Chapter 19
Conversion of Glycerine into 1,2-Propanediol for Industrial Applications

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Abstract Glycerine is a by-product from biodiesel production. After transesterification using methanol, the oil from *Jatropha* seeds produces high amounts of stearic and palmitic methyl esters and about 10%wt. in glycerol. This chapter deals with the different aspects of the valorization of glycerine for the production of propylene glycol (1,2-PD). After introducing the subject, we evaluate the glycerol and 1,2-PD markets, particularly for pharmaceutical use. We then describe the processes of aqueous-phase hydrogenolysis (APH), aqueous-phase reforming (APR), and catalytic hydrogen transfer (CTH) applied to glycerol and describe some thermodynamics aspects and metal catalysts applied to these processes. Finally, we discuss some detailed kinetic models and application of molecular modeling to this reaction.

Keywords Biodiesel · Glycerine hydrogenolysis · Metal catalysts · Molecular modeling

19.1 Introduction

Since Carl Wilhelm Scheele accidentally discovered it in 1779, by mixing olive oil and lead monoxide, glycerine found its way as a household chemical, whose fame was epitomized by nothing less than a song at the peak of the Billboard of 1995

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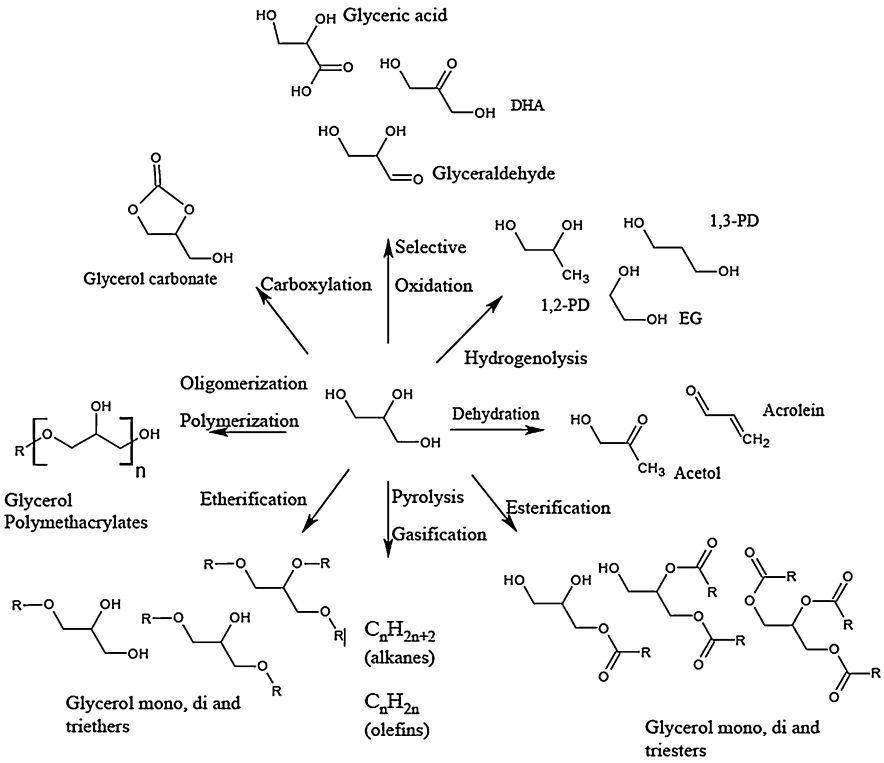

Fig. 19.1 Processes for the conversion of glycerol

(SDA 1990; BILLBOARD 1995). More recently, the biodiesel industry did the job of bringing such glamor into secular widespread mass production of billions of metric tons of "the sweet principle of fat," as the German-Swedish chemist originally called it. After giving its name from the Greek word *glykys* (sweet), in 1811, French chemist Chevreul thoroughly investigated its properties, opening ways for thousands of uses for glycerin in the years that ensued. As the biodiesel industry enters steady state, chemical transformation of glycerol opens the door for ingenious numeral other possibilities, especially in pharmaceutical applications.[1]

Glycerates thus derived can be phosphorylated and converted to 3-phosphoglycerate [9], which has major pharmacological interest, since it is a precursor in the biosynthesis of the amino acid l-serine, whose deficiency may lead to severe neurological disorders. Figure 19.1 shows some of the main glycerol-derived compounds of interest for industrial applications. Some of the

[1]A subtle distinction in nomenclature is in order: although glycerin and glycerol are widely used interchangeably, the term glycerin (or glycerine) refers to glycerol solutions, typically containing over 95% glycerol, while the term glycerol refers to the propane-1,2,3-triol molecule ($C_3H_8O_3$) or to the pure compound.

most important catalytic processes in glycerol valorization are its selective oxidation (Carrettin et al. 2002), dehydration (Corma et al. 2008), etherification (Karinen and Krause 2006), oligomerization (Barrault et al. 2004), carboxylation (Aresta et al. 2006), and esterification (Kale et al. 2015). Selective oxidation of glycerol using an external source of oxygen leads to compounds with a C:O ratio greater than 1. For instance, glyceric acid can be produced over Au/grafite at 333 K and 3 bar of O_2 in the presence of NaOH (Carrettin et al. 2002). Glycerates thus derived can be phosphorylated and converted to 3-phosphoglycerate (Kolb and Orgel 1996), which has major pharmacological interest, since it is a precursor in the biosynthesis of the amino acid l-serine, whose deficiency may lead to severe neurological disorders. Carboxylation of glycerol and subsequent oligomerization of glycerol carbonate leads to oligo-(glycerol carbonate-glycerol) esters (Holmiere et al. 2017), which are rich in linear carbonate groups suitable for creams and emulsions, substituting fossil-based ethoxylated surfactants.

A complete description of all the possible routes and applications for glycerol-derived substances would require entire books.

This chapter presents some aspects involved in the intertwined reactions of aqueous-phase hydrogenolysis (APH) and aqueous-phase reforming (APR) of glycerol, which have considerable interest in the production of propylene glycol (PG, 1,2-propanediol, or 1,2-PD), ethylene glycol (EG), and others. Many good reviews have been written on the subject of glycerol APH using heterogeneous catalysts, and we advise the reader to be acquainted to some of them (Zhou et al. 2008; Ten Dam and Hanefeld 2011; Nakagawa and Tomishige 2011; Ruppert et al. 2012; Martin et al. 2013; Feng and Xu 2014; Vasiliadou and Lemonidou 2015; Clark et al. 2015; Wang et al. 2015).

Besides its use as an antifreeze, 1,2-PD is also used in various pharmaceutical applications, mainly as an excipient, applied as solvent, stabilizer, humectant, and preservative. Alongside these applications, it should be highlighted that, differently from 1,3-PD, its diol isomer, 1,2-PD possesses a chiral carbon and therefore can participate in asymmetric syntheses of important chemicals. For example, (R)-1,2-PD is converted into (R)-propylene carbonate, which is an intermediate in the production of tenofovir, an AIDS drug used in the inhibition of reverse transcriptase from the HIV virus (Zhou et al. 2008).

This chapter presents a critical review on glycerol conversion to 1,2-PD, including a marketing analysis, especially in terms of pharmaceutical use. Also this chapter addresses scientific issues of glycerol conversion to 1,2-PD such as catalysis, kinetics, and molecular modeling.

19.2 Glycerine Market

19.2.1 Glycerine and Biodiesel Relations

The development of new catalysts, as well as the amount of kinetic studies involving conversions of glycerol in higher added value products, is explained by the exponential growth of biodiesel production by the transesterification process of vegetable oils and animal fats. As crude glycerol is a by-product, the last decades registered the increasing of the supply disconnected from the demands for this product. The biodiesel industry has always relied on the commercialization of crude glycerol as a way to remain economically viable and competitive. The entire industry relies on the stabilization of crude glycerol prices to continually grow, in order to match the demands of the new world energy matrix based on environmental low-impact fuels.

The biodiesel market has always been stimulated worldwide by a series of government subsidies. In the Brazilian scenario, the public policies related to fuel began with the creation of the Inter-ministerial Executive Committee on Biodiesel (CEIB) and the Management Group (GG). In 2004, the National Program for the Production and Use of Biodiesel (PNPB) was launched by the federal government, with the main objective of building the national energy matrix for biodiesel. During this period, the legal and regulatory bases for the sector were developed, including the gradual growth plan of the minimum biodiesel fractions in the composition of the national diesel. Since 2008–2014, the evolution of biodiesel levels inside Brazilian fossil diesel was determined by Sect. 19.2 of Statute 11,097/2005. In that period, these percentages increased from 2% to 7%. Statute 13,263/2016 confirmed the schedule for increasing the biodiesel content to a mandatory 10% value until 2019 (ANP 2018a).

These national subsidies, coupled with the increase of the international biofuels market, have been developing the entire Brazilian biodiesel production chain. In 2017, the production of biodiesel reached values about 4.3 million m^3. Figure 19.2 shows the increment of Brazilian biodiesel production since 2005, as well as the production of crude glycerol as a consequence of process technology (ANP 2018b). Currently, soybean oil is the main raw material for biodiesel production in the country. Other oleaginous used include native Brazilian seeds such as macuba, copaiba, palm, and dry coconut (MCCB 2017). In all this scenario, *Jatropha curcas* is also included as an alternative for biodiesel production and is the purpose of a rational selective breeding process by *Empresa Brasileira de Pesquisa Agropecuaria* (Embrapa).

Associated with the rise in the production of biodiesel in the last few years, the production of glycerol reached amounts around 430 thousands m^3. Due to these production volumes, Brazil currently occupies the position of the largest exporter of glycerol in the world, accounting for 14% of the world market and handling 51 million dollars (FOB). Meanwhile, China is the largest importer of crude glycerol, taking 33% of imports, trading at 119 million dollars (FOB) (OEC 2016). In the

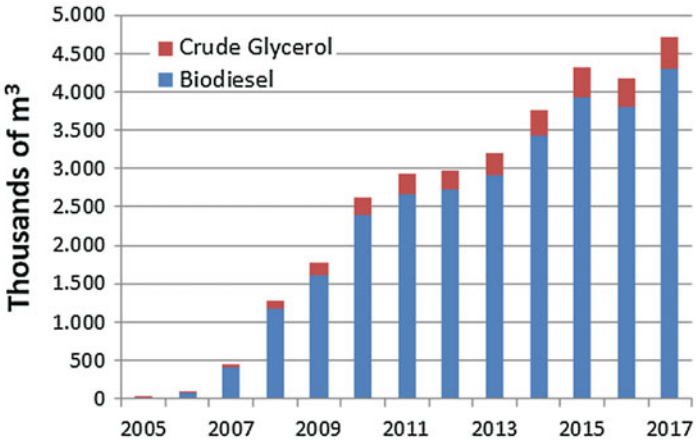

Fig. 19.2 Brazilian historical production of biodiesel and crude glycerol. (Adapted from ANP 2018b)

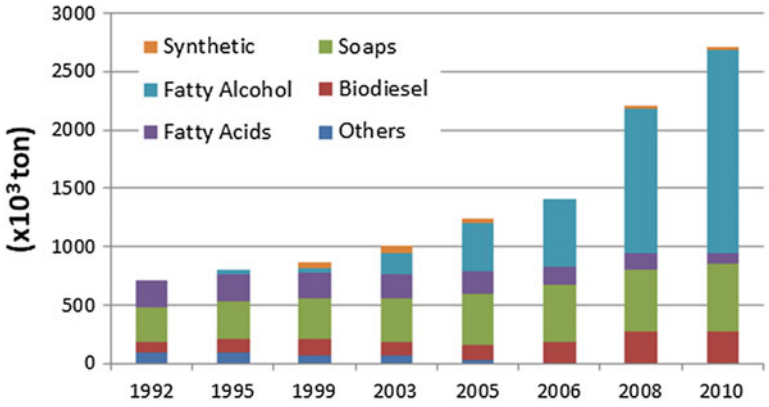

Fig. 19.3 Consumer market profile of glycerol. (Adapted from Beatriz et al. 2011)

last two decades, the world growth of biodiesel production has led to a change in the profile of the main sources of crude glycerol, as may be seen in Fig. 19.3.

In the early 1990s, the soap and grease industries were accountable for the major source of glycerol. In this same period, the biodiesel industry has gone from a non-existent role in the supply of glycerol to held 64% of the sector's demands in 2010. Currently, the largest producers of glycerol from biodiesel are the United States and European Union countries, particularly France and Germany. These countries are accompanied by South American producers, led by Brazil and Argentina, and by the Asian market, led by China and Indonesia (Ayoub and Abdullah 2012). The coproduction of glycerol plays a strategic role for the biodiesel industry. Usually, the average cost of producing the fuel is about US $ 500/m^3 and is mainly

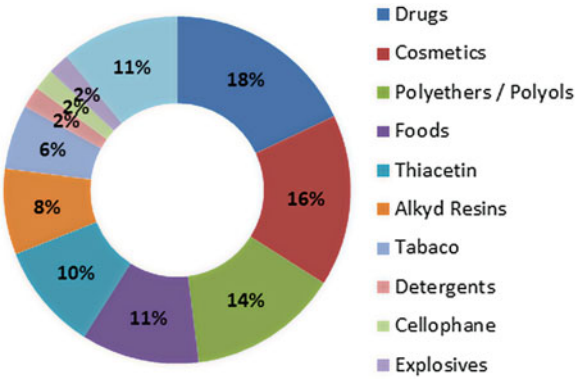

Fig. 19.4 Changes in the crude glycerol profile sources. (Ayoub and Abdullah 2012)

dependent on the costs of the industrial transesterification process coupled with the variations in agricultural input's prices. As a comparison, these costs are 1.5 times higher than the production costs of diesel from petrochemical origin (Ayoub and Abdullah 2012). In addition to government incentives, sales of raw glycerin may potentially reduce by 13–14% of biodiesel production costs. These sales's credits of crude glycerol might linearly reduce the production costs of biodiesel as prices in the international glycerol market increase.

The price fluctuations for crude glycerol are, in turn, influenced by its main consumer markets. Glycerol is a versatile feedstock, mainly after the elimination of water and other typical impurities from the biodiesel production process. Figure 19.4 shows the worldwide profile of the glycerol market.

Historical variations in crude glycerol prices put the competitiveness of the biodiesel production chain at risk. As shown in Fig. 19.4, of all sectors, drugs and cosmetics are the most strategic, accounting for 34% of the market. In addition, the glycerol consumed in these cases needs to meet special purity criteria, which creates opportunities for higher-profit margins. In recent decades, crude glycerol prices have ranged from US$ 330/ton (in 2001) to US$ 130/ton (2010), with historical lows of US$ 44/ton (Ayoub and Abdullah 2012). At particular moments there were decreases down to negative values for crude glycerol, which obligated biodiesel producers to pay for the disposal through incineration of the by-product. These variations are both the result of the constant growth in biodiesel production and the variations in demand for glycerol, mainly in the Asian market, which comprises more than 44% of imports of the product (MCCB 2017).

19.2.2 Glycerine Market: New Opportunities

This scenario of supply and demand's relations for glycerol and its strategic influence on the competitiveness of the biofuel sector are the main incentive factors for developing new applications for glycerol. Current proposals include expanding its

direct application in animal feed, using it as fuel in thermoelectric plants, liquid reform for hydrogen production (APR), and other catalyzed reactions or biological processes. The Brazilian company H_2O, since 2008, takes advantage of the national availability of crude glycerol for its application as a dust suppressor in the storage of ores and vegetal and mineral charcoal, among others. This glycerol-based product forms an adhesive film without altering the properties of the stored material, reducing losses, water consumption, and improving local air quality (H_2O 2018).

At first, new applications are developed directly for crude glycerol and its purified versions in order to immediately explore the product offerings. Thereafter, the development of new technologies capable of adding substantial value to the product becomes interesting. Many researchers have been developed in the last decades with different proposals of glycerol application. In this chapter, conversion possibilities of glycerol via heterogeneous catalysis are presented, particularly the conversion of glycerol to 1,2-PD for pharmaceutical market.

Glycerol is already used directly in the production of medicines in its purified version. Crude glycerol produced by the biodiesel industry has a glycerol mass content of 60–80%, with other components like water, ash, and soap. Meanwhile its purified version has a glycerol content of 99.1–99.8% (ALICEWEB 2018). Thus, after biodiesel production, crude glycerol is then decanted and might undergo distillation steps. The purified product is finally applied in the pharmaceutical and cosmetic sectors, mainly as a solvent or as an emulsifier. In the last 5 years, purified glycerol price on the international market averaged around US$ 490/ton (FOB), 1.48 times higher than the crude glycerol (ALICEWEB 2018).

Comparatively, the margins of pharmaceutical 1,2-PD are more advantageous. Over the last 5 years, international market prices for high-purity 1,2-PD have ranged from US$ 1500 to 2200/ton (FOB), with an average value 5.6 times higher than crude glycerol in periods of high price (Chiu 2006). Allied to industrial process and catalysis technologies, these price differences are a stimulus to using crude glycerol as the basic input for the production of 1,2-PD.

According to data from the Brazilian National Association of Essential Medicines (RENAME), more than 18% of the country's strategic medicines have 1,2-PD as emulsifying solvent for active ingredients and other components (ABIQUIFI 2017). More than 20,000 tons/year (FOB) of 1,2-PD are imported by Brazil, with expenditures of US$ 24 million/year (ALICEWEB 2018). Generally, Brazil is deficient in pharmaceutical inputs, which affects the competitiveness and expansion capacity of the drug production chain. Figure 19.5 presents the evolution of spending on national pharmaceutical inputs over the last 10 years.

Due to these import expenditures and competitiveness risks, the Brazilian Development Bank (BNDES) has invested R$ 4.3 billion over the past 10 years in modernizing and developing the national industrial chain of medicines production (BNDES 2018). In this sense, exploring technologies of heterogeneous catalysis to produce 1,2-PD from glycerol is an interesting solution, from the increasingly supply by national biodiesel production to the demands for 1,2-PD from a strategic sector like the pharmaceutical one.

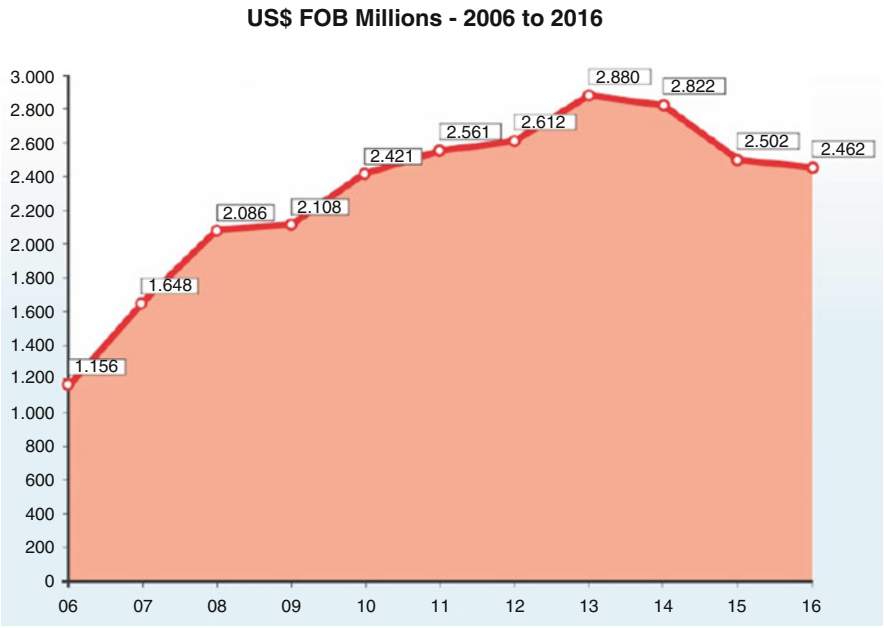

Fig. 19.5 Imports of pharmaceutical inputs by the Brazilian industry. (ABIQUIFI 2017)

Besides its pharmaceutical use, 1,2-PD has applications in cosmetics, in animal feed, as an intermediate in polymers industry, in dyes, and as a refrigerant fluid. Overall, the 1,2-PD market globally moves more than US$ 13 billion/year, growing at a rate of 4%/year. The high-purity 1,2-PD market alone moves about US$ 2.6 billion/year (Chiu 2006). Germany is the largest exporter of 1,2-PD, accounting for 28% of that market. Imports of 1,2-PD are more evenly distributed between the Asian (32%) and European (47%) markets (MCCB 2017).

Dow Chemical® is the world's largest producer of 1,2-PD, supplying more than 409,000 tons/year through the industrial process based on petrochemical propylene oxide. Dow's main 1,2-PD-based products are Dow PuraGuard Propylene Glycol, intended for more demanding pharmaceutical markets, such as the US, and propylene glycol USP/EP, that meets the minimum legal requirements of most important health organizations in several countries (Dow 2000).

Since 2007, Dow Chemical® has been announcing the development of industrial process technology for the production of 1,2-PD from biodiesels crude glycerol (Ahlich and Shah 2007). Following this same market trend, Johnson Matthey® offers process technology and heterogeneous catalysis licensing with similar objectives (Matthey 2018). None of these companies provides more detailed information about their technologies. In addition to studying the catalytic conversion reaction, Chiu also has proposed an industrial process for the 1,2-PD production in two stages, with irreversible dehydration of glycerol to acetol at first and then with hydrogenation of acetol into 1,2-PD (Chiu 2006).

The incentives to 1,2-PD production from glycerol are the combined results of market forces from both inputs and the development of new catalysts coupled with industrial process technologies. Particularly in Brazil, crude glycerol supply has been growing, while the pharmaceutical sector faces the deficit stagnation in inputs such as 1,2-PD. Movements in the business segments related to the products and the biodiesel industry also support this trend. Thus, catalytics, reaction mechanisms, and industrial process studies concerning glycerol are strategic for any player interested in advancing the future of high value-added chemical inputs.

19.3 Glycerol Hydrogenolysis Using Metal Catalysts

19.3.1 APH, APR, and CTH

The first use of the word hydrogenolysis referred to the cleavage of the C–O bond in oxygenated organic compounds, followed by hydrogenation of the generated fragments (Zhou et al. 2008). In their review on the subject, Ten Dam and Hanefeld (2011) provide an accurate definition of the term hydrogenolysis, meaning that a true hydrogenolysis consists of the homolytic cleavage of the C–O bond parallel to the homolytic cleavage of H–H, followed by the joining of the fragments, which results in water and a dehydroxylated compound. Figure 19.6 depicts this definition. On the other hand, the widespread use of the term hydrogenolysis encompasses more than that definition, actually meaning in many cases the process of an elimination followed by a hydrogenation. Still on the topic of terminology, the word hydrodeoxygenation (HDO) is also commonly used but is associated mainly with the deoxygenation of biomass (Nakagawa and Tomishige 2011). In this work, glycerol hydrogenolysis is the process by which it is possible to transform glycerol into different products by use of an external source of hydrogen, and APH is the process in which hydrogenolysis happens when water is the solvent.

The use of water or an alcohol as a solvent is typical, and hydrogen pressurizes the vessel where the reaction takes place. Even though the reaction is thermodynamically downhill, it needs an active catalyst to drive it. The main products of APH are 1,2-PD, 1,3-propanediol (1,3-PD), 1-propanol (1-PrOH), 2-propanol (2-PrOH or i-PrOH), propanone (acetone), hydroxyacetone (acetol), EG, ethanol, methanol, and glyceraldehyde. Other compounds that may possibly appear among the products are acetic acid, propionic acid, 3-propionaldehyde, allylic acid, acrolein, lactic acid,

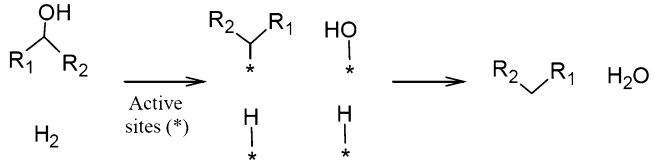

Fig. 19.6 Scheme for a "true" hydrogenolysis process

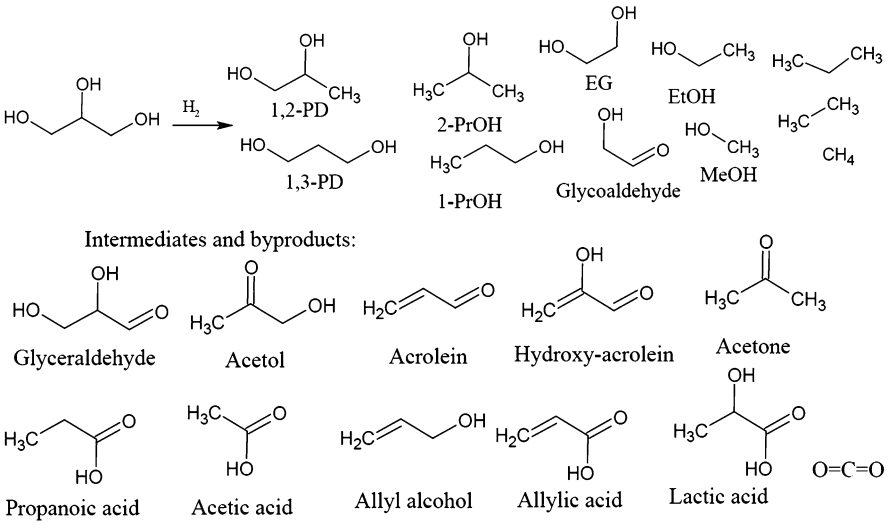

Fig. 19.7 Main products, intermediates, and by-products from glycerol APH and APR

pyruvaldehyde, and 2-hydroxy-acrolein. Figure 19.7 shows the main products, intermediates, and by-products from APH and APR of glycerol.

Aqueous-phase reforming (APR) of glycerol is closely related to APH, since many of the products from the former reaction are the same as from the latter. The first works related to APR aiming at hydrogen production from biomass are related to the steam reforming (Antal 1975), reaction with supercritical water (Yu et al. 1993), or with compressed water (Minowa et al. 1998) of organic substances, some present in waste effluents. This is clear evidence that at that point the physical state of water was uncertain for the reaction conditions. The term APR became relevant in a seminal work done by Cortright et al. (2002) aiming to describe the production of hydrogen from sugars and alcohols in a single pressure vessel over supported platinum catalysts. Thus, APR can be defined as the process of hydrogen production from organic molecules in aqueous solutions under inert gas pressures as high as 60 bar and temperatures up to 540 K.

Therefore, although kinetics and thermodynamics differ from one process to the other, the key difference between APH and APR is also due to an operational aspect. In APH, H_2 comes from an external source; in APR an inert gas (such as He, Ar, or even N_2) pressurizes the vessel, and typical hydrogenolysis products are formed from reaction with the H_2 generated by reforming.

Some thermodynamic aspects are relevant to discuss the differences and similarities between APH and APR. When applying APR with varying catalysts for the conversion of different polyols, such as sorbitol ($C_6H_{14}O_6$), glycerol ($C_3H_8O_3$), EG

($C_2H_6O_2$), and methanol (CH_4O), it was observed that H_2 selectivity increases as the number of carbon atoms decreases (Cortright et al. 2002; Huber et al. 2006). For the same C:O ratio, a higher number in carbon atoms leads to higher selectivity in the formation of alkanes. For polyols, the general reaction of APR is:

$$C_nH_{2n+2}O_{n(l)} + nH_2O_{(l)} \rightleftharpoons (2n+1)H_{2(g)} + nCO_{2(g)} \tag{19.1}$$

In the case of glycerol, $n = 3$. The decomposition of liquid glycerol should follow the stoichiometry (Cortright et al. 2002; Martin et al. 2013):

$$C_3H_8O_{3(l)} \rightleftharpoons 3CO_{(g)} + 4H_{2(g)} \quad \Delta H_{513\,K} = 321.0\ \text{kJ/mol} \tag{19.2}$$

In aqueous solutions, vapor or liquid, water gas shift reaction (WGSR) is expected to occur. For steam, it is:

$$CO_{(g)} + H_2O_{(g)} \rightleftharpoons H_{2(g)} + CO_{2(g)} \quad \Delta H_{513\,K} = -42.1\ \text{kJ/mol} \tag{19.3}$$

For applications below the critical temperature with high pressures, water is available for the reaction as a hot liquid. Actually, it is likely that the APR reaction occurs after dehydrogenation and C–C cleavage processes on the glycerol molecule, which implies that dissolved CO may enter in contact with liquid H_2O on the surface of the catalyst. Thus:

$$CO_{(g)} + H_2O_{(l)} \rightleftharpoons H_{2(g)} + CO_{2(g)} \quad \Delta H_{513\,K} = 10.6\ \text{kJ/mol} \tag{19.4}$$

Combining the aforementioned reactions leads to the aqueous-phase reforming of glycerol, which is endothermic:

$$C_3H_8O_{3(l)} + 3H_2O_{(l)} \rightleftharpoons 7H_{2(g)} + 3CO_{2(g)} \quad \Delta H_{513\,K} = 352.6\ \text{kJ/mol} \tag{19.5}$$

Therefore, APR requires more energy than steam reforming ($DH_{513K} = 225.8$ kJ/mol, for $H_2O_{(g)}$). However, the selective production of H_2 poses a challenge, since carbon monoxide or dioxide may promptly react with hydrogen to form hydrocarbons. The formation of methane, for instance, is:

$$CO_{2(g)} + 4H_{2(g)} \rightleftharpoons CH_{4(g)} + 2H_2O_{(l)} \quad \Delta H_{513\,K} = -162\ \text{kJ/mol} \tag{19.6}$$

Thermodynamically, glycerol APR contrasts with the APH reaction for the formation of 1,2-PD, because the latter is exothermic:

$$C_3H_8O_{3(l)} + H_{2(g)} \rightleftharpoons C_3H_8O_{2(l)} + H_2O_{(g)} \quad \Delta H_{513\,K} = -51.5\ \text{kJ/mol} \tag{19.7}$$

This is also the case of total hydrogenation of glycerol:

$$C_3H_8O_{3(l)} + 5H_{2(g)} \rightleftharpoons 3CH_{4(g)} + 3H_2O_{(g)} \quad \Delta H_{513\,K} = -261.9 \text{ kJ/mol} \quad (19.8)$$

Therefore, although APH and APR generate many of the same products, they are overall opposite reactions, regarding the energy flow. This has serious implications for the reaction temperature, since increasing the temperature leads to higher rates in both APR and APH of glycerol but also thermodynamically drives APR preferably. Hence, selective APH is highly dependent on catalyst performance at low temperatures, while selective APR ideally occurs with the lowest possible CO_2 production.

One last term commonly used is the catalytic transfer hydrogenation (CTH), which departs from the donor-acceptor rationale, having the hydrogen atom (or a proton) as the object of transfer. This view is very useful from a chemical perspective, but also from an engineering point of view, since liquid hydrogen-rich substances offer a significant advantage over H_2 storage.

19.3.2 Catalysts

As occurs in many hydrogen-related reactions, cobalt, nickel, copper, ruthenium, rhodium, palladium, and platinum are some of the most prominent active catalysts used in APH. However, one of the most important features of these metal catalysts is the material used as the support, which affects greatly both activity and selectivity. One of the first and most important works with supported metal catalysts for APH of glycerol was done by Montassier et al. (1988). They showed results for the APH of glycerol, xylitol, and sorbitol, using Co, Ru, Rh, Ir, and Pt supported on SiO_2, and Ni and Cu-Raney, under pressures in the range of 30–40 bar and temperatures between 453 and 533 K. In a sequel, their experiments with modified Cu-Raney catalysts, by use of Ru and Pt as promoters (Montassier et al. 1991a, b), proved to be in foresight, since supported Ru-Cu catalysts are promising materials for high-selectivity 1,2-PD processes. The approach from Montassier et al. (1988) paved the way for many future efforts. Besides showing that the size of Ru particles has an effect on C–O and C–H bonds cleavage, their more significant contribution remains in the identification of a mechanism consisting of dehydrogenation followed by dehydration of glycerol as prior steps to the actual hydrogen consumption. Figure 19.8 shows (a) the 1,3-PD general dehydration-hydrogenation mechanism, (b) the dehydration-hydrogenation mechanism for the production of 1,2-PD, and (c) the dehydrogenation-dehydration-hydrogenation mechanism for the production of 1,2-PD.

A decade after the work from the French group, Chaminand et al. (2004) investigated Rh, Pd, and CuO/ZnO catalysts, presenting the dehydration-hydrogenation mechanism, with acetol as an intermediate, as seen in Fig. 19.8 b. This same mechanism was independently presented by Dasari et al. (2005), by use of Ru, Pd, Pt supported on carbon, Ni-Raney, Cu-Raney, and copper-chromite ($Cu_2Cr_2O_5$). Those authors found that copper-chromite was the best catalyst. A later work was done by Chiu et al. (2006) in which $Cu_2Cr_2O_5$ is used in semi-batch

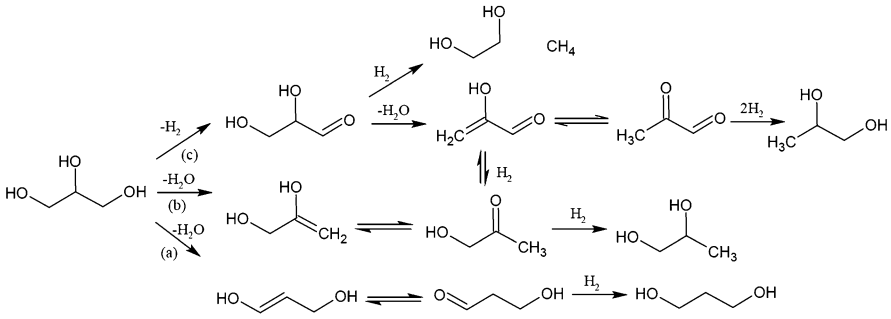

Fig. 19.8 Mechanisms for glycerol hydrogenolysis towards 1,2-PD and 1,3-PD

experiments for dehydration of glycerol and acetol production, which points to the fact that a great part of the catalyst activity in APH is actually due to the first steps, which do not involve hydrogenation. Glycerol is transformed by protonation of its terminal hydroxyl, which forms the enol prop-1-en-2,3-diol, which tautomerizes to form acetol. After dehydration, the final hydrogenation of the carbonyl yields 1,2-PD. According to this rationale, 1,3-PD may analogously come from the dehydration of glycerol, by elimination of the secondary hydroxyl, which generates the enol prop-1-en-1,3-diol, which tautomerizes to form an aldehyde (3-hydroxy-1-propanal), followed by subsequent hydrogenation.

At this point, a question arises: what drives such selectivity for dehydration?

An attempt to suitably answer this question involves the nature of the acid sites that cause dehydration. Brønsted sites are able to protonate, while Lewis sites are prone to accept electrons. Moreover, basic sites cannot be ignored, since they may induce a dehydrogenation. Thus, the dehydration of glycerol to form acetol can be viewed as a straightforward Brønsted acid site protonation of the primary hydroxyl. Since there are two terminal hydroxyls but only one secondary hydroxyl in glycerol, formation of 1,2-PD should be more probable than of 1,3-PD. Figure 19.9 shows how Brønsted sites can activate and participate in the non-hydrogen-consuming steps of the reaction.

Notwithstanding the fact that normally Brønsted sites are depicted as * – OH, metal impregnation cannot be ignored for the creation of Brønsted and Lewis acid sites.

Nonetheless, high Brønsted acidity alone does not cause high 1,2-PD selectivity.

Experiments for hydrogenolysis of glycerol over Ir-ReOx/SiO_2 performed by Amada et al. (2011) showed that increasing the concentration of H_2SO_4 does not increase conversion. By using other probe molecules and the same conditions in parallel tests for hydrogenolysis of hydroxylated ethers, there was no diol and hydrocarbon formation, which would be the expected products, but only monohydroxyl alcohols. Therefore, it has been suggested that a hydride is formed on the catalyst surface in connection with Ir-Re systems, which is responsible for attacking a carbon atom. Thus, the C–O bond is easier to cleave because of the attack on the

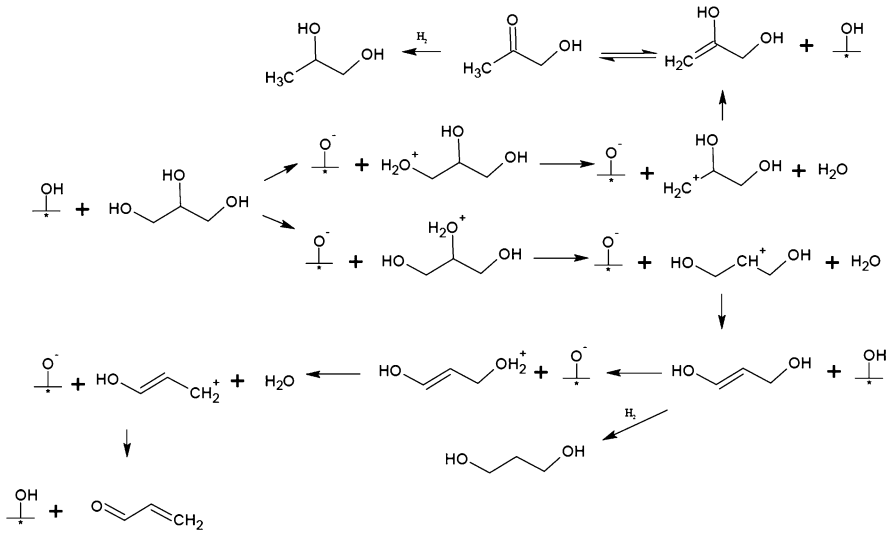

Fig. 19.9 Dehydration-hydrogenation of glycerol over Brønsted sites

carbon atom, rather than by sole protonation of the hydroxyl. After interaction of a terminal hydroxyl group with part of the catalyst particle, the glycerol molecule is adsorbed, and the secondary carbon should be more electron deficient and, therefore, available for a hydride attack. Similar results, presented by the same group (Nakagawa et al. 2010), using also Rh as active metal (Shinmi et al. 2010), seemed to confirm the actual hydride mechanism for 1,3-PD.

On the other hand, Lewis acid sites are connected with the formation of 1,2- PD (Ruppert et al. 2012), as seen in Fig. 19.10. In a nutshell, both Brønsted and Lewis sites attack on the terminal hydroxyl, leading to acetol, but C–C cleavage for the generation of EG ensues from an additional interaction with the catalyst after a Lewis attack on the secondary hydroxyl. The reaction can also begin by interaction with basic sites, by the generation of a carbanion and cleavage of the C–C bond. The nature of the acidity has a lot to do with the support but also with the content of active metal. Alhanash et al. (2008, 2010) have worked with Ru over modified cesium salts (CsPW). These authors have found that dehydration using CsPW occurs mainly because of Brønsted acid sites protonating the secondary and terminal hydroxyls for formation of acrolein, while Lewis acid sites act by a dehydration at the secondary carbon, which leads to the formation of the enol. This will generate acetol, as the most stable tautomer in aqueous solution. They have also observed that although conversion may increase with increasing hydrogen pressure over a narrow range, in the long range catalyst, activity decreases as pressures go higher. This kinetic description matches the behavior of Pt/Fe3O4 catalysts, which presents almost exclusively Lewis acid sites (Soares et al. 2017).

Maris et al. (2007) have performed experiments with Ru/C and Pt/C (Gandarias et al. 2012b) and have observed that bases play an important role in the reaction

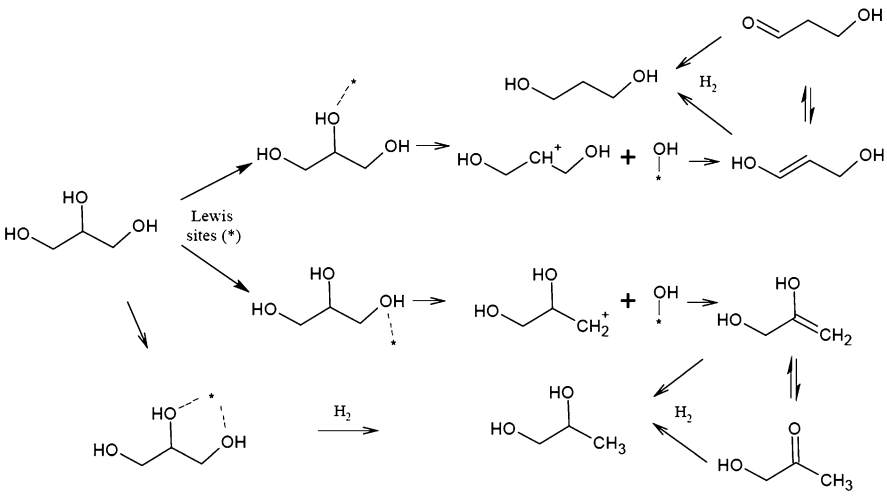

Fig. 19.10 Dehydration-hydrogenation of glycerol over Lewis sites

mechanism. By using NaOH and CaO, they have proposed that these bases act in concert with the active metal sites. Dehydrogenation and hydrogenation should happen on metal sites, while the base catalyzes the dehydration and cleavage of the C–C bonds. Further exploring bimetallic Pt-Ru/C and Au-Ru/C catalysts, the authors describe the same promoting effect of bases (Maris et al. 2007).

Among the noble metals used in glycerol APH and APR, Ru, Rh, Pd, Ir, and Pt appear as suitable catalysts, while Ag and Au present unfavorable results. Pamphile-Adrián et al. (2016) tested Ir as the active metal supported on SiO_2, ZrO_2, and Al_2O_3 and have observed that Ir/ZrO_2 is favorable to C–O cleavage, while Ir/Al_2O_3 favors C–C cleavage, which is due to the metal-support interaction in each system.

Salazar et al. (2014) investigated titanium oxide-supported catalysts and found that for bimetallic $Ru\text{-}Cu/TiO_2$, selectivity in the formation of 1,2-PD is favored by increments in Cu content. Works using Ru-Cu catalysts supported on Al_2O_3 and ZrO_2 also show that $Ru\text{-}Cu/ZrO_2$ is less active than other systems, such as monometallic Ru/Al_2O_3, but it is highly selective for 1,2-PD (Soares et al. 2016b; Liu et al. 2012). In the context of pharmaceutical grade 1,2-PD, these studies show promising ways for large-scale production.

Miyazawa et al. (2006) have done experiments with Ru/C, Rh/C, Pd/C, and Pt/C, all with 5%wt. active metal content, showing that the activity is as follows: Rh < Pd < Pt < Ru. They report that addition of H_2SO_4 or HCl lower the catalyst activity. Similarly, Kusunoki et al. (2005) have shown almost identical results, identifying Amberlyst as a suitable resin to substitute acids such as H_2SO_4. Furikado et al. (2007) have also compared the same metals over different supports (C, SiO_2, and Al_2O_3) and noted that Rh/SiO_2 is superior. This Japanese group has used mild temperatures (393 K) in their experiments, which is highly desirable. Wang et al. (2013) compared the activity of the same active metals supported on monoclinic

zirconia, m-ZrO_2, and found that for a similar particle size (2 nm) the rate decreased in the sequence Ru >> Rh > Pt > Pd at 473 K and 6.0 MPa. The inverse order (Ru < Rh < Pt < Pd) was found for the selectivity of bond cleavage ratio (C–O)/(C–C). While the selectivity of C–O bond cleavage is optimal around 480 K for Ru/m-ZrO_2, but at 450 K, the cleavage is optimal for Pt/m-ZrO_2.

The matter of catalyst activity cannot be settled by mere selection of an element. For instance, Dasari et al. (2005) have found superior 1,2-PD yield when using Pt/C rather than Ru/C. Van Ryneveld et al. (2011) have tested monometallic Ru, Pd, and Pt supported on C, Al_2O_3, and SiO_2 and have reported that Ru is the most active. Kurosaka et al. (2008) tested Pt/WO_3 supported on TiO_2, HY, AIMCM-41, SiO_2-Al_2O_3, Al_2O_3, and ZrO_2 and have reported that 2% wt.Pt/20% wt.WO_3/ZrO_2 was the best performing catalyst. After testing Ru, Rh, Pd, and Pt on WO_3/ZrO_2, they have found that the Pt catalyst showed better results than the Ru catalyst at the same conditions, which is due to the effect of Pt-WO_3 interaction. WO_3 was also used, and Pt/WO_3/TiO_2/SiO_2 performs more actively than Pt/WO_3/TiO_2 for 1,3-PD production (Gong et al. 2010). On the same lines, mesoporous WO_3 was used as a support for Pt, which showed conversion four times higher than when using commercial WO_3 (Longjie et al. 2012).

Supports based on Ti and W show promise. Tuning of Ti and W in the composition of the oxide supports leads to an optimization in the content of active acid Brønsted and Lewis sites (Zhang et al. 2013). Characterization of Pt/TiO_2 and tests for APR of glycerol show that this system contains more Lewis acid sites when compared with other catalytic systems, such as Al_2O_3 and Al_2O_3-SiO_2 (Delgado et al. 2013). Catalytic activity was high even for a system with a low number of Lewis acid sites. In a later work, Delgado et al. (2014) have also shown that 1,2-PD is actually consumed faster than glycerol when Pt/TiO_2 is used in aqueous phase. Given that TiO_2 is a known photocatalyst, APR experiments at room temperature using Pt/TiO_2 show that it is feasible to produce H_2 by photocatalysis (Panagiotopoulou et al. 2013). Therefore, pharmacological applications that use both TiO_2 and 1,2-PD at reaction-inducing conditions, such as the case of sunscreens, should be carefully monitored.

Transition metal catalysts in general show lower activity than noble metal catalysts.

Nonetheless, there has been considerable investigation on cobalt-supported (Guo et al. 2009, 2011; Liu et al. 2010a; Cao et al. 2010), nickel-supported (Banu et al. 2011; Grilc et al. 2014; Yu et al. 2010; Liu et al. 2010a), and copper-supported (Chaminand et al. 2004; Wang and Liu 2007; Yuan et al. 2011; Balaraju et al. 2008; Huang et al. 2008) catalysts. As Guo et al. (2009) have noted, the interaction between Co and MgO can be adjusted in Co/MgO catalysts by calcination temperature. For 15.0% wt.Co/MgO calcined at 873 K, conversion was as high as 44%, and selectivity for 1,2-PD was 42.2%, after 9 h, by use of 200 g glycerol solution/g_{cat}, 10%wt. glycerol solution, 2.0 MPa H_2 and 473 K, which can be considered as mild conditions. This activity could be due to the presence of Mg-Co-O solid solution and $MgCo_2O_4$ as active phases, which is indicative of the role of active metal-support interaction, as key for catalyst development. Also, CoNi nanowires perform as

suitable catalysts for APH of glycerol (Liu et al. 2010a). Although Ni is not very active for the transformation of glycerol in aqueous phase, when impregnated with a promoter, it can show non-negligible conversions. For instance, Ni-Ce/C (Yu et al. 2010) can be used as a catalyst for relatively mild conditions (6 h, 5 MPa H_2, 473 K, 25% glycerol solution), from which a conversion of 90% can be achieved. The improvement in catalytic activity is due to the addition of Ce, resulting in lower temperature of reduction of higher Ni oxidation states and stabilization of active particles. Synthesis of homogeneously dispersed Cu over hydrotalcite (HTL) can form a bifunctional layered solid catalyst, with very desirable results: high selectivity for 1,2-PD (98%) with 80% conversion (Yuan et al. 2011).

Exploring different supports is an interesting way to adjust catalyst activity. In experiments using Pt supported on MgO, HTL, Al_2O_3, HZSM-5, H-Beta, and carbon, 2%wt.Pt/HTL performed best for 1,2-PD selectivity (Yuan et al. 2009). This is directly linked to the large number of weak basic sites on the support, which is corroborated by a similar result for HTL (Pendem et al. 2012).

There are different methodologies for the execution of hydrogenolysis reactions that use other substances as sources for hydrogen. For instance, Jin et al. (2013) have used Pt, Ru, Rh, Pd, Raney Ni, Raney Co, and Cu catalysts for the reforming of glycerol, xylitol, and sorbitol in order to obtain hydrogen from dehydrogenation and subsequent hydrogenolysis. Vasiliadou et al. (2015) have used methanol reforming as a source for hydrogen in the hydrogenolysis of glycerol in the presence of Cu:Zn:Al catalysts with varying ratios of the three metals. Gandarias et al. (2012a) have performed experiments in which 2-PrOH (Gandarias et al. 2011) and formic acid were used as hydrogen donors over Ni-Cu/Al_2O_3 catalysts. From these studies, it has been seen that low-content Cu catalysts favor the formation of 1,2-PD, whereas the increment in Ni content enhances activity. Thus, the selection of such materials involves the tuning of an optimal Ni/Cu ratio. This tuning is paramount to environmentally friendly processing, since the alloy formation between Ni and Cu leads to C-C bond cleavage inhibition, and the dehydrogenation of formic acid leads to CO_2. 2-PrOH can also be used as a hydrogen source when Pd/Fe and Pd/Co catalysts are used (Mauriello et al. 2015), provided that Fe or Co are maintained in low oxidation states, since the catalysts activity is improved when Fe is at a low oxidation states (as in Fe_3O_4, or FeO, for instance), possibly by the creation of dehydration sites. One interesting way of conducting a CTH reaction is to use glycerol as a source for hydrogen in parallel processes. Liu et al. (2010b) have used Fe_2O_3 as a dehydration catalyst for the production of acetol and acrolein but have also observed the formation of allyl alcohol. According to those authors, allyl alcohol is produced by the hydrogen transfer from an alcohol (possibly glycerol) to acrolein, by means of the selective hydrogenation of the C=O bond.

Bimetallic catalysts should be focused, since it is well known that these systems can perform better than monometallic catalysts. For instance, Pt-Ru/C performs better than Ru/C does (Maris et al. 2007), and for glycerol APR, a physical mixture of Ru/Al_2O_3 and Pt/Al_2O_3 is more active than is each of the individual monometallic catalysts (Roy et al. 2010).

When using Pt-Sn/Al_2O_3 for APR of glycerol in a continuous flow tubular reactor, Brandner et al. (2009) found a considerable drop in catalyst activity by the addition of Sn. In a different study, also using Sn as a co-catalyst for SiO_2-supported Pt catalysts, Barbelli et al. (2012) have shown that there is an optimal ratio of Sn/Pt around 0.2. Above this proportion, incremental Sn does affect the activity negatively. When using Pt-Cu/MgAlO catalysts for the APR of glycerol, Boga et al. (2013) observed high selectivity of H_2 due to the bimetallic interaction between Pt and Cu. This is also responsible for the suppression of methane production. The synergetic effect is also observed in Pt-Ni/Al_2O_3 catalysts (Huai et al. 2015), which are selective for the formation of EG, in view of monometallic Pt and Ni catalysts. The use of Pt and Ni over CZA (a support consisting mainly of Al_2O_3, but modified by fractions of CeO_2 and ZrO_2) is likely to form bimetallic systems presenting nanoparticles with the alloy phase $PtNi_3$ (Barbelli et al. 2014). When preparing Pt-Ni/Al_2O_3 systems by the sol-gel method, Ni particles form in such a way that makes it possible for the occurrence of Pt clusters on top of them. The interaction of Pt-Fe was also studied in depth for bimetallic catalysts supported on Al_2O_3 (El Doukkali et al. 2013; Huber et al. 2006; Soares et al. 2016a). Fe shows a consistent promoting effect for Pt, both in APH and APR, due to the presence of reduced Fe atoms on the bimetallic nanoparticles. As the temperature increases in APH, selectivity for 1,2-PD decreases and for 1-PrOH increases, up to a limit. Optimal atomic Pt:Fe ratio is around 2:1 in Pt-Fe/Al_2O_3. The turnover frequency for Pt-Fe/Al_2O_3 systems in APH ranges from around 0.03 to 0.3/s for temperatures between 493 and 513 K, respectively.

When comparing Pd/Fe_3O_4 and Pt/Fe_3O_4, the former performs better as an APR catalyst, while the latter is preferable in APH (Soares et al. 2017). But Pd activity can be tuned when a promoter is used, as well as a different support, as in the case of Pd-Zn/ZnO-Al systems, prepared under different calcination temperatures (Li et al. 2018). When calcined at 623 K, this system performs at high conversion and selectivity for the formation of 1,2-PD (70% and 92% respectively). The influence of higher calcination temperatures in decreasing catalyst activity is remarkable. Catalyst deactivation and stability are important aspects for viable processes. For Pt/CeO_2 and Pt/La_2O_3, the main deactivation mechanisms are leaching and the formation of undesired organic compounds material responsible for site blocking and modification of the structure of the metallic surface (Checa et al. 2015). The conduction of experiments for the hydrogenation of acetol reveals that the presence of the active metal is paramount for the suppression of oligomerization or polymerization of acetol. Besides leaching, in Pt/-Al_2O_3 the main factor for catalyst deactivation is the transformation of -Al_2O_3 into AlO(OH) (El Doukkali et al. 2014). The transition from alumina to boehmite is hampered by the presence of Pt in a way that increasing the content of Pt stabilizes the catalyst for more time (Ravenelle et al. 2011, 2012).

19.4 Kinetic Models for Glycerol Hydrogenolysis

19.4.1 A Review on Proposed Models for Copper and Ruthenium Catalysts

Comprehensive kinetic studies on the hydrogenolysis of glycerol are still scarce, considering the diversity of products obtained under different reaction conditions and the amounts of distinct mechanisms proposed for this process. The main researches on the subject focus in simplified power law or Langmuir-Hinshelwood-type models to determine the reaction rate expressions that might match complex experimental data (Vasiliadou and Lemonidou 2015).

One of the first main contributions on this subject was carried out by Lahr and Shanks (2003) who assumed zero order reaction for the degradation products of hydrogenolysis, suggesting the catalyst surface would be saturated with these substances. However, their first model attempt yields a negative initial rate for glycerol consumption, which was later substituted by a different reaction rate expression that included an estimated reaction order of 1.5 with respect to the glycerol concentration, without a deeper theoretical analysis. Degradation products are the results of a greater extent of hydrogenolysis, and this phenomena is mainly observed with catalysts whose metallic sites are more prone to excessive cleavage of C-C bonds, in the case of Ru (Vasiliadou and Lemonidou 2015; Wang et al. 2013), or when hydrogenolysis is conducted under alkaline conditions (Wang et al. 2015). This is clear evidence for insertion of degradation steps in the Langmuir-Hinshelwood model to obtain an expression for the reaction rate more accurate and consistent with the experimental data. Lahr and Shanks (2003) also demonstrated the competitive adsorption between EG and 1,2-PD by active sites, and the results indicated a higher competitiveness of EG over Ru/C. Both species can undergo subsequent hydrogenolysis and generate degradation products, with higher catalyst surface coverage of EG, but at faster rates of 1,2-PD consumption.

Zhou et al. (2010) investigated the behavior of hydrogenolysis of glycerol with Cu-ZnO-Al_2O_3 catalysts and proposed a Langmuir-Hinshelwood-type kinetic model with two different types of active sites, presented in Fig. 19.11. Competitive adsorption between glycerol, acetol, and 1,2-PD was assumed at active sites on the surface of the support and dissociative adsorption of hydrogen at metal sites, in agreement with previous research (Goddard et al. 1992; Singh and Vannice 2001; Abdelrahman et al. 2017; Chen et al. 2007). Copper catalysts are known for their ability to suppress the excessive attack on C-C bonds, while promoting the cleavage of C-O bonds (Feng et al. 2016), which usually yields high-selectivity values for C3 products, such as 1,2-propanediol (about 93.6%). Thus, the formation of degradation products by hydrogenolysis of C3-diols could be neglected.

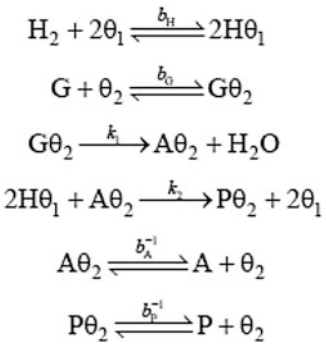

Fig. 19.11 Kinetic model for hydrogenolysis of glycerol over Cu-ZnO-Al_2O_3 (Zhou et al. 2010). The θ_1 and θ_2 denote the active sites for hydrogen and organic molecules, respectively. The bH, bG, bA, and bP are the adsorption constants of hydrogen, glycerol, acetol, and 1,2-PD, respectively. Then, k_1 and k_2 represent the rate constants of glycerol dehydration to acetol and acetol hydrogenation to 1,2-PD, respectively

The constant presence of acetol in all streams of the studied process, reported by the authors, led Zhou al. (2010) to consider the dehydration-hydrogenation mechanism to build the kinetic model. The adsorption and desorption steps were considered at quasi-equilibrium, and the dehydration reaction of glycerol and hydrogenation of acetol were considered the rate determinant steps (RDS). Activation energy for dehydration of glycerol over Cu-ZnO-Al_2O_3 catalyst calculated by the model, 86.56 kJ/mol, was much higher than the activation energy for the hydrogenation of acetol, about 30 kJ/mol, indicating that the second stage of the mechanism is faster and more likely to occur than the first one. In addition, the adsorption rate constants of glycerol estimated by the authors indicate strong attachment of the glycerol on the surface of the catalyst, compared to 1,2-propanediol, which is easily desorbed (Zhou et al. 2010; Vasiliadou and Lemonidou 2015).

Vasiliadou and Lemonidou (2013) published a research on the kinetics of the hydrogenolysis of glycerol with Cu/SiO_2 catalysts through a simplified power law-type model. In spite of the several parallel and consecutive reaction steps that may occur during hydrogenolysis process, the authors suggested a simplified version of this reaction network, due to the high selectivity of the catalyst to 1,2-propanediol and 1,3-propanediol (about 95%) and unsignificant selectivity to gaseous degradation products and EG (about 2%). The model consists of two parallel and irreversible formation reactions for the main 1,2-propanediol and 1,3-propanediol products. The experimental data indicates low dependence of the rate of consumption of glycerol with its initial concentrations and apparent first-order reaction to the hydrogen. This behavior is justified by the active sites being mostly filled with adsorbed glycerol, according to the authors. However, the formation rate of 1,2-PD showed little dependence on the initial concentrations of glycerol, although it is strongly associated with the hydrogen concentrations in the liquid phase (first-order

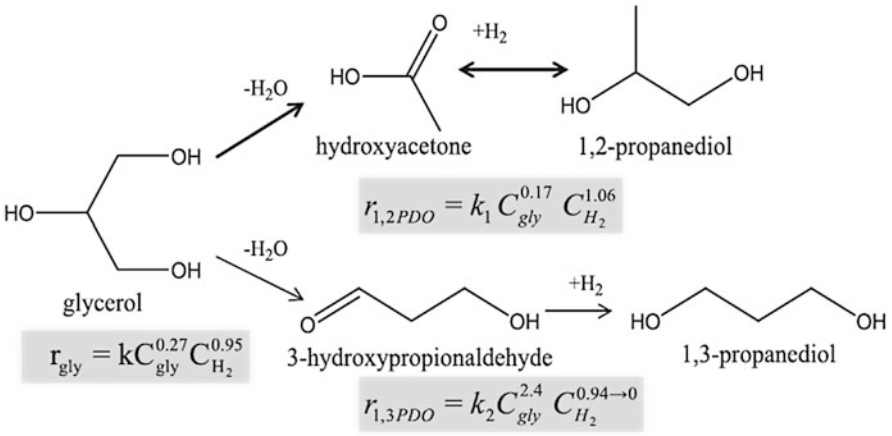

Fig. 19.12 Mechanism for the hydrogenolysis of glycerol over Cu/SiO_2 catalyst. (Vasiliadou and Lemonidou 2013)

apparent). The formation of 1,2-PD is more dependent on the hydrogenation phase of the two step model, and its selectivity is higher when hydrogenolysis is carried out under lower temperatures.

The 1,3- propanediol formation pathway provides a production rate more sensitive to the initial concentrations of glycerol and less dependent on hydrogen concentrations in the liquid phase, especially at pressures over 6 MPa. Vasiliadou and Lemonidou (2013) suggested that one of the reasons why the reaction rate relies on these initial concentrations is in the reaction mechanism itself, because the elimination of primary or secondary – OH groups depends on different configurations of glycerol adsorption, and for 1,3-propanediol formation an ensemble of Cu active sites is needed. The low selectivity for the production of 1,3-propanediol may also be justified by the instability of its reaction intermediate, 3-hydroxypropionaldehyde, obtained for copper catalyst, as shown in Fig. 19.12.

Recent researches, such as published by Rajkhowa et al. (2017) using commercial copper catalyst, demonstrated high selectivities for 1,2-PD (at least 90%) and acetol, whose values are consistent with those obtained by other authors for similar catalysts. Despite the evidences for choosing two different types of active sites kinetic model (Goddard et al. 1992; Singh and Vannice 2001; Abdelrahman et al. 2017; Chen et al. 2007), the researchers opted to design a single-site model for simplification purposes. The model is based on Langmuir-Hinshelwood kinetics, with some reaction steps at quasi-equilibrium and two others as the reaction determining steps (RDS) (Neurock 1994), and follows the mechanism of dehydration-hydrogenation to build the elementary reactions. Dehydration of glycerol is considered the RDS step to generate acetol, and hydrogenation of acetol is the second RDS, to generate 1,2-PD.

The activation energy obtained for the dehydration of glycerol is approximately 84.2 kJ/mol, consistent with previous studies and higher than that obtained for the hydrogenation of acetol, approximately 59.3 kJ/mol, which explains the greater sensitivity to the variation of temperature observed experimentally by the authors. The low selectivity of this Cu catalyst for degradation products is justified by the low rate coefficient values calculated by the transition state theory (Chorkendorff and Niemantsverdriet 2017; Davis and Davis 2012) and indicates high activation energy values for the degradation of 1,2-PD. It was also observed the low surface coverage of 1,2-PD and the presence of glycerol as (most abundant surface species) MARS, with high coverage even in higher conversions, which corroborates the phenomenon of low reaction order (Vasiliadou and Lemonidou 2013; Chiu et al. 2006).

The majority of kinetic studies on hydrogenolysis of glycerol are focused on highly selective catalysts, such as Cu, but there are still some investigations carried out with other catalysts such as Ru, Pd, Pt, and other bimetallics (Xi et al. 2010; Jin et al. 2016; Kim et al. 2012; Sun et al. 2017). Torres et al. (2010) evaluated the kinetics of the hydrogenolysis reaction on Ru/C and Ru-Re/C catalysts and registered a selectivity of only 18.9% for 1,2-propanediol and 39.8% for EG, propanol, and ethanol together for the Ru/C catalyst. According to the authors, the use of the bimetallic Ru-Re/C suggests the action of Re as a promoter for selectivity of 1,2-PD, which increased from 18.9% to 36.6%, and from the other products of liquid phase, altered from 39.8% to 52.3%. Still, many degradation products, especially in the gas phase, are formed due to high Ru activity for cleavage of C–C bonds (Wang et al. 2013). Thus, the authors proposed a simplified mechanism, presented in Fig. 19.13, in order to include all species involved in the direct and consecutive product formation reactions. For each obtained product, an irreversible formation reaction was attributed, and thereafter, a rate expression is based on the power law-type model that despite the lacking information about intermediates, the model achieves a good parity with the experimental data.

19.4.2 Future Perspectives

One of the main reasons for the scarcity of studies about the kinetics of glycerol hydrogenolysis is the complex network of parallel and consecutive reactions that take place during the process, already proven by other studies (Nakagawa and Tomishige 2011; Wang et al. 2013; Vasiliadou and Lemonidou 2015). In addition, depending on the selectivity level of the studied catalyst, developing a kinetic model that is computationally feasible and consistent with experimental data can be a very complex task (Rajkhowa et al. 2017).

Comprehensive descriptions of reaction mechanisms tend to detail many steps for a more accurate analysis of the influence of each species on the process

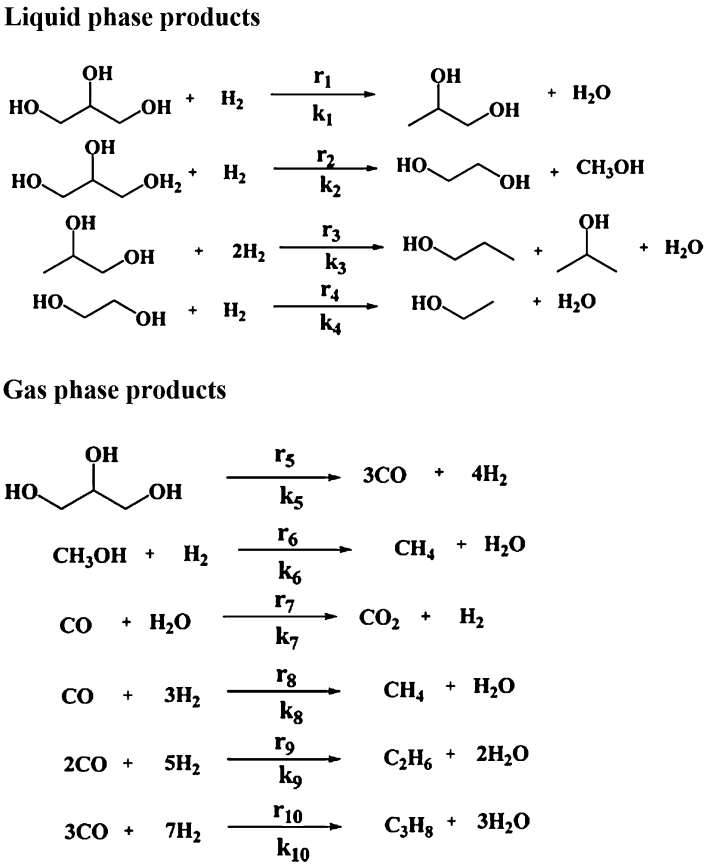

Fig. 19.13 Kinetic model for hydrogenolysis of glycerol over Ru-Re/C catalyst. (Torres et al. 2010)

(Rajkhowa et al. 2017; Zhou et al. 2010; Pandhare et al. 2017). An expected step forward for the development of new kinetic models is the microkinetic approach of the mechanism, where each elementary step, including reactional intermediates, is considered reversible and with a distinction between the types of active sites available in the catalyst (Neurock 1994). A good fit for the reactional parameters – such as pre-exponential factors of the rates, activation energies, and adsorption heats – can reveal more details about the thermodynamics of the reaction and allow later evaluation of the elementary steps associated with the degree of rate control over the complete process, according to Stegelmann's research (Stegelmann et al. 2009).

Therefore, with a very detailed and coherent kinetic model, it is possible to obtain a better degree of predictability for hydrogenolysis in terms of process scale increase, to infer new studies with catalysts containing promoters, among other possibilities.

19.5 Molecular Modeling of Glycerol Hydrogenolysis

Accurate simulations of the properties of solids and molecules where the predictions can be made with a reasonable level of confidence require an excellent representation of the behavior of such systems. The classical approximations using parameterized simple models, such as interatomic potential (Keating 1966) or "charge-binding" models (Phillips 1968), have been used in the past to study problems involving collections of atoms with some success.

Nowadays, systems consisting of thousands of atoms are simulated using these classical methods. In the last decades, quantum mechanics methods have become well established for the study of systems containing dozens of atoms (Hirunsit et al. 2015).

Non-relativistic simulations of the electronic and structural properties of systems containing up to about 1000 atoms have become routine in recent years in various laboratories. Such systems require an accurate solution of the Schrödinger equation. The Schrödinger equation itself is easily constructed for a system of many bodies. However, it is impossible to solve it directly beyond the simplest systems without making any approximations (Parr 2012).

19.5.1 Methods in Molecular Modeling

The resolution of Schrödinger's equation gives the exact solution to the system of many particles which is the case of an atom or the system of several atoms (see Cohen et al. 1973 for an example). However, the computational cost to find a solution to this equation is so great that it is eventually unfeasible (Sakurai and Napolitano 2017).

One of the best options for solving this problem is to obtain approximate functions that can describe the system as a whole, in order to simulate the true wave function (more costly solution). There are several methods to propose these approximate functions following different algorithms (Foresman and Frisch 1996).

The Gaussian functions used are usually obtained from quantum calculations in atoms (e.g., "Hartree-Fock" calculation). Frequently, the exponents are varied until the minimum energy of the atom is obtained (Sakurai and Napolitano 2017). In some cases, the exponents are optimized individually. In others, exponents are related to each other by some equation, and the parameters of this equation are optimized (e.g., even tempered and well-tempered basis functions) (Foresman and Frisch 1996).

The primitive Gaussian functions derived from these calculations describe isolated atoms, but cannot describe with precision the deformations of the atomic orbitals due to the presence of other atoms in the molecule. Basic functions for molecular calculations are usually obtained with other specific functions. For molecular calculations, Gaussian primitives must be contracted, or some linear combination of these functions will be used with a base. The term "contraction," in this case,

means a linear combination of primitives to be used as the basis function. Such a basis function will have fixed exponents and coefficients (Cohen et al. 1973; Foresman and Frisch 1996).

It is also necessary to use a specific method to perform the molecular modeling calculations. This method can be the solution of the Schrodinger's equation, the use of the Hartree-Fock method, semiempirical methods, or methods that are based on the electronic density of the system. The latter is based on the density functional theory (DFT), which proposes to replace the complicated function of waves of several electrons by the electronic density of the analyzed system and to create a functional that describes the system as a whole (Viana et al. 2004).

The use of a functional as a method of calculation is much more advantageous due to the computational cost savings and the large number of available functional appropriate to each system, which takes into account all relevant effects. According to the size, charge, electronic state, relativistic effects, and other thermodynamic parameters of the system, one chooses the most appropriate functional, previously validated with the experimental data, that allows the description of the system with great accuracy (Viana et al. 2004).

19.5.2 The Molecular Modeling Literature of Hydrogenolysis

Currently, many authors have proposed new uses for molecular modeling. Rather than explaining the experimental data, its main application has been to create models over observed patterns, extrapolating the experimental results and predicting the behavior of the chemical systems. For this, several programs that use algorithms based on quantum mechanics can be used to determine the Gibbs free energy, the most stable geometry of the molecules as well as the more stable isomers (Jensen 2017).

In his work, Salciccioli et al. (2010) elaborated a comparative table of group additivity calculated by DFT (at 298 K) for the oxygenation of $C_2H_xO_2$ and $C_3H_xO_3$ and was able to show that this energy grows linearly. Knowing this growth profile helped in the prediction of reaction mechanisms that involve oxygenation and dehydration of small-chain organic molecules.

Also the pre-exponential factors for the kinetic study of the reaction can be calculated via DFT and approximate based on the type of reaction. Neurock (1994) estimated reaction orders based on the type of surface interaction and on these pre-exponential factors calculated by molecular modeling. Even the diffusion barriers of surface intermediates can be estimated from the binding energy (Nilekar et al. 2006).

As an illustration, glycerol, 1,2-propanediol, and 1-hydroxy-2-propanone molecules, which were calculated using the B3LYP functional and the basis set function 6-31G(d,p), are shown in Fig. 19.14.

To assist in the proposition of a coherent kinetic mechanism for the production of 1,2-propanediol, 1-hydroxy-2-propanone, and other important products from

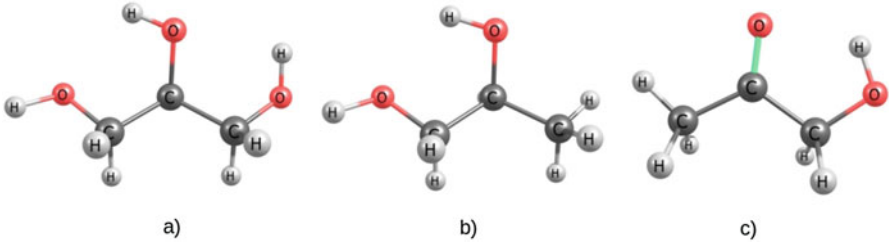

Fig. 19.14 Geometry of glycerol molecule (**a**) 1,2-propanediol molecule (**b**) and 1-hydroxy-2-propanone molecule (**c**) calculated with the B3LYP functional and 6-31G(d,p) basis set

glycerol hydrogenolysis, molecular modeling calculations can be made. This is even a useful tool for the elucidation of intermediate structures and energies, aiming to map the thermodynamically favorable reaction paths and corroborate with the experimental data in the elaboration of a reaction model.

The computational study of the hydrogenolysis reaction of glycerol agreed with the experimental results. If a high pressure of molecular hydrogen or a good hydrogen donor, such as formic acid (methanoic acid), is used, for example, the production of 1,2-propanediol can be maximized, and the separation among glycols derivatives becomes the new challenging step (Salciccioli et al. 2011).

By using molecular modeling studies, Hirunsit et al. (2015) showed that the use of alumina-supported transition metal catalysts significantly alters the selectivity of the hydrogenolysis reaction toward the formation of 1,2-propanediol. This has been a new route to obtain this specific product (Auneau et al. 2011).

At this point, it may be noted that the study of the heterogeneous catalysis is being improved by the use of molecular modeling. In their review, Salciccioli et al. (2011) analyzed the literature of reactions catalyzed by metals at different scales and proposed new perspectives for the study of reaction mechanisms looking into the different influences at the micro and macro levels. A new, more rigorous approach concerning both aspects related to fluid dynamics and intramolecular interactions that occur in the crystalline network of solids and surface phenomena is being undertaken.

19.6 Conclusions

The market and chemical aspects explored in this chapter clearly indicate that glycerol is an important pharmaceutical commodity for environmentally friendly process. The case of propylene glycol production from glycerol poses an even more interesting use of biomass, assuming present profit margins. Furthermore, glycerol-derived propylene glycol and other hydrogenolysis products offer new opportunities for drug synthesis, many of which are still to be developed. Theoretical and experimental studies for the understanding of the hydrogenolysis and reforming of

glycerol are welcome efforts toward process optimization. For pharma grade 1,2-PD, special attention should be given to high-selectivity catalysts, preferably to low-cost transition metals. In due course, biomass-derived glycerine expands from its traditional applications into core processes in the pharmaceutical industry.

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Chapter 20
Jatropha: Phytochemistry, Pharmacology, and Toxicology

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Abstract *Jatropha curcas*, a non-edible oilseed species with several uses and extensive lucrative prospective, is considered as a potential biofuel plant. Even though the genus *Jatropha* comprises *ca*. 200 species, to date just a few species have been investigated for their chemical constituents. There are still many species that have not received much attention on phytochemical and biological actions. On the other hand, few *Jatropha* species have been recognized for the pharmacological action of different crude extracts, proteins, peptides, and isolated compounds as antimicrobial, antifungal, antioxidant, anti-inflammatory, antidiarrheal, antihypertensive, antidiabetic, anticoagulant, and anticancer agents.

Keywords Bioactive compounds · *Jatropha* · Medicinal properties · Nutraceutical

20.1 Introduction

Jatropha species constitute a rich source of phytochemicals and are used as conventional medicine to cure several infirmities in Africa, Asia, and Latin America. In these continents, the plants are used as a traditional health restorative and as energy crops (Pullaiah and Bahadur 2013; Sabandar et al. 2013). Earlier findings reported that the *Jatropha* species have pharmacological applications and are therapeutic targets for various diseases as they contain phytochemicals such as steroids, alkaloids, saponins, tannins, phenolic acids, terpenoids, functional proteins, and peptides (Zhang et al. 2009). Extracts from different *Jatropha* species are reported to have antimicrobial, antioxidant, antihypertensive, anti-inflammatory, anticancer,

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antifungal, antidiabetic, hepatoprotective, and antineoplastic activities (Sharma and Singh 2012). *J. curcas* proteins and peptides have been studied for their therapeutic and functional roles in the plant's own metabolic activities.

J. curcas kernel meal contains a rich source of proteins and is comparable to soybean meal (Makkar et al. 2008a). The kernel meal cannot be used as animal feed due to the presence of antinutrient components such as trypsin inhibitor, phytates, saponins, phenolics, tannins, and lectins in high amount (Makkar et al. 2008a). To reduce the toxicity of antinutrients, traditional processing methods like thermal degradation, soaking in distilled water/$NaHCO_3$/$CaOH_2$, germination, and methanol extraction were applied (Aderibigbe et al. 1997; Ramakrishna et al. 2006; Magdi 2007; Yasmin et al. 2008). Moreover, there is very less information available regarding the phytochemical prospective and pharmaceutical application of *Jatropha* species. The review aims to provide a comprehensive account of the chemical composition, biological activity, traditional uses, toxic constituents, and detoxification methods for use of seed meal as animal feed to improve the economic value of *Jatropha* plant.

20.2 Phytochemical Constituents

In Euphorbiaceae family, terpenoid compounds are mostly present. Among the terpenes, diterpenoids have been isolated from most of the *Jatropha* species. These diterpenoids have unique chemical structure and pharmaceutical value (Devappa et al. 2011). The diterpenoids possess biological activity such as antimicrobial, anti-inflammatory, antihypertensive, antioxidant, anticancer, and antiretroviral (Kapil et al. 1993; Bruneton 1999; Niu et al. 2002; Kumari et al. 2003) (Table 20.1). Jatrophine, jatropham, jatrophone, and curcain compounds are isolated from latex of different *Jatropha* species. Among them, jatrophine and jatropham

Table 20.1 Carbohydrate composition of *J. curcas* seed endosperm. %-values refer to the total carbohydrate content of *J. curcas*

Carbohydrate monomer	*J. curcas* (mol%)
Fucose	1.1
Rhamnose	9.9
Arabinose	27.9
Galactose	33.4
Glucose	11.9
Mannose	4
Xylose	3.5
Ribose	4.9
Galacturonic acid	2.7
Glucuronic acid	0.7

Source: Zippel et al. (2010)
Carbohydrates as determined by HPAEC-PAD after TFA hydrolysis against external standard calibration

Table 20.2 Phytochemicals isolated from different parts of *J. curcas* plant

S. no	Various parts	Chemical composition	References
1.	Aerial parts	Organic acids (*o*- and *p*-coumaric acid), *p*-OH benzoic acid, protocatechuic acid, resorcylic acid, saponins, and tannins	Hemalatha and Radhakrishnaiah (1993)
2.	Stem bark	β-Amyrin, β-sitosterol and taraxerol saponins, steroids, tannin, glycoside, alkaloids, and flavonoids	Mitra et al. (1970) and Igbinosa et al. (2009)
3.	Leaves	Cyclic triterpenes stigmasterol, stigmast-5-en-3β, 7β-diol stigmast-5-en-3β, 7α-diol cholest-5-en-3β, 7β-diol cholest-5-en-3f1, 7α-diol campesterol, β-sitosterol, 7-keto-β-sitosterol, as well as the β-D-glucoside of β-sitosterol. Flavonoids apigenin, vitexin, isovitexin. Leaves also contain the dimer of a triterpene alcohol ($C_{63}H_{117}O_9$) and two flavonoidal glycosides. Alkaloids, saponins, steroids, tannins	Mitra et al. (1970), Khafagy et al. (1977), Hufford and Oguntimein (1987), and Akinpelu et al. (2009)
4.	Latex	Curcacycline A, a cyclic octapeptide	Van den berg et al. (1995)
		Curcain (a protease)	Nath and Dutta (1991)
5.	Seeds	Curcin, a lectin	Stripe et al. (1976), Adolf et al. (1984), Makkar et al. (1997), and Staubmann et al. (1999)
		Phorbol esters	
		Esterase (JEA) and lipase (JEB)	
6.	Kernel and press cake	Phytate, saponins, and trypsin inhibitor	Aregheore et al. (1998), Makkar and Becker (1997b), and Wink et al. (1997)
7.	Roots	β-Sitosterol and its β-D-glycoside, marmesin, propacin, the curculathyranes A and B and the curcusones A–D, diterpenoids and jatropholones A and B, the coumarin tomentin, the coumarino-lignan jatrophin as well as taraxerol	Naengchomnong et al. (1986, 1994)

Source: Kumar and Sharma (2008), Igbinosa et al. (2009), and Akinpelu et al. (2009)

have anticancer properties (Schmook and Serralta-Peraza 1997; Graham et al. 2000; NIIR 2006).

Jatropha species are rich in phytochemicals. The steroids, alkaloids, saponins, tannins, 5-hydroxypyrrolidin-2-one, pyrimidine-2,4-dione, and phenolic acids such as quercetin, coumarin, kaempferol, and catechin have been isolated from *J. curcas* leaves (Staubmann et al. 1999; Rejila and Vijayakumar 2011; Rejila et al. 2012) (Table 20.2). Xu and Tan (2012) isolated 14 phenolic compounds from *J. curcas* stems. In addition, stem bark extracts contain tannins, steroids, saponins, glycosides,

flavonoids, and alkaloids which have pharmaceutical activity. Further, *Jatropha* seed protein has functional properties such as high emulsifying and foaming properties (Lestari et al. 2010). On the other hand, the fraxetin compound isolated from *Jatropha* species is present only in Euphorbiaceae family. The fraxetin isolated from *J. gossypifolia* (Das and Kashinatan 1997), *J. ciliata* (Okuyama et al. 1996), and *J. glandulifera* (Parthasarathy and Pardha Saradhi 1984) is a chemotaxonomic feature of the *Jatropha* species.

20.3 Functional Proteins and Peptides

The rapid growth of *J. curcas* in dry weather condition is due to the presence of the functional protein – aquaporin. It regulates the water content of cells in all kingdoms of life (Amiry-Moghaddam et al. 2005). The *J. curcas* plants are resistant to abiotic stresses (drought, heat, and salt) due to the increased expression of JcPIP2 and JcBD1 genes (Zhang et al. 2007, 2008). The thermostable esterase enzyme present in *J. curcas* has an industrial application (Owusu and Cowan 1989). In addition, the *J. curcas* latex contains functional proteins such as curcain and curcin 2. The curcain is a proteolytic enzyme, and it has wound healing property, and the plant expresses curcin 2 under stress condition (Wei et al. 2005a; Huang et al. 2008).

On the other hand, the functional cyclic peptides with pharmacological actions are identified in *J. curcas* (Craik et al. 2004; Joullie and Richard 2004; Sarabia et al. 2004) (Table 20.3). The cyclic peptides containing 7–10 hydrophobic amino acids are present more in *Jatropha* species. From the nutritional view point, the *Jatropha* peptides are more economical compared to the soybean proteins besides possessing pharmacological applications. The functional peptides isolated from distinct parts of the plants have biological activities such as cytotoxic (Mongkolvisut et al. 2006), immunosuppressive activity (Morita et al. 1997), antimalarial (Baraguey et al. 2000), vasorelaxant activity (Morita et al. 2005), and cyclooxygenase, acetylcholine esterase, and tyrosinase inhibitory (Morita et al. 1994; Yahara et al. 1989).

20.4 Nutritional Potential of *J. curcas*

20.4.1 Health-Related Attributes of J. curcas

20.4.1.1 Antioxidant Activity

The plant-derived products (edible and non-edible) include a mixture of phenolic compounds that possess biological activity including antioxidant activity. Diwani et al. (2009) reported that different parts of *J. curcas* have significant antioxidant activity based on antioxidant assays like radical scavenging and reducing power activity. Extracts from various plant parts of *J. curcas* are a potential source of

Table 20.3 Biological activities of *Jatropha* proteins and peptides

S. no	Functional proteins	Biological activity	Source	References
1.	Aquaporins	Drought resistance	*J. curcas*	Zhang et al. (2007)
2.	Betaine aldehyde dehydrogenase	Drought resistance	*J. curcas*	Zhang et al. (2008)
3.	Esterase and lipase	Hydrolysis of triglycerides	*J. curcas*	Staubmann et al. (1999) and Abigor et al. (1985, 2002)
4.	Curcain	Wound healing property	*J. curcas*	Nath and Dutta (1989, 1988, 1991, and 1992)
5.	Curcin	Inhibits protein synthesis, immunotoxins	*J. curcas*	Stripe et al. (1976), Lin et al. (2002), and Weike et al. (2006)
6.	β-glucanase	Antifungal activity	*J. curcas*	Wei et al. (2005b) and Jin-Xia et al. (2005)
Cyclic peptides				
1.	Mahafacyclin	Antimalarial activity	*J. mahafalensis*	Baraguey et al. (2000, 2001)
2.	Labditin and biobollein	Immunomodulatory activity	*J. multifida*	Altei et al. (2008), Pieters et al. (1999), and Rudi et al. (1993)
3.	Jatrophidin	Antifungal activity	*J. curcas*	Altei et al. (2008)
4.	Chevalierins	Antimalarial activity	*J. chevalieri*	Baraguey et al. (1998)
5.	Cycloglossine	No biological activity	*J. gossypifolia*	Horsten et al. (1996) and Auvin et al. (1997)
6.	Podacyclin	No biological activity	*J. podagrica*	Van den Berg et al. (1996)
7.	Pohlianins	Antimalarial activity	*J. pohliana*	Auvin-Guette et al. (1999)
8.	Curcacycline	Antimalarial activity, inhibits cell proliferation and classical pathway of human complement	*J. curcas*	Baraguey et al. (2001), Auvin et al. (1997), and Van den Berg et al. (1995)
9.	Integerrimides	Antiproliferative against tumor cells	*J. integerrima*	Mongkolvisut et al. (2006)

natural antioxidants, due to the presence of different bioactive compounds with high antioxidant activity (Igbinosa et al. 2011; El-Baz et al. 2014). Nithiyanantham et al. (2013) reported that the raw and processed *J. curcas* kernel meal contains high levels of polyphenolic compounds which contribute to the active antioxidant activity.

The methanolic extract of *J. unicostata*, *J. macarantha*, and *J. gaumeri* showed strong antioxidant activity (Mothana 2011; Desmarchelier et al. 1997; Sãnchez-Medina et al. 2001). Kharat et al. (2011) reported that *J. gossypifolia* leaves have antioxidant activity based on the presence of phenolic compounds, tannins and

flavonoids. Pompelli et al. (2010) reported the activities of oxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, and glutamine synthetase. The results of this study provided sufficient baseline information for further use of *Jatropha* species as nutraceutical and value added products.

20.4.1.2 Antihypertensive Activity

Abreu et al. (2003) reported that *J. gossypifolia* has hypotensive effect based on its vasorelaxant action. Furthermore, the contractile response was induced by norepinephrine or $CaCl_2$ in rats and repressed in a dose-dependent manner.

20.4.1.3 Anti-inflammatory Activity

The root methanolic extract of *J. curcas* exhibited systemic and significant anti-inflammatory activity in rats (Mujumdar and Misar 2004). Olukunle et al. (2011) reported that *J. curcas* has anti-inflammatory and analgesic activity. Chhabra et al. (1990) revealed that *J. curcas* leaves contain anti-inflammatory compounds such as flavonoids, apigenin, and its glycosides vitexin and iso-vitexin, the sterols stigmasterol, β-D-sitosterol, and its β-D-glucoside. *J. unicostata* and *J. zeyheri* extracts have moderate anti-inflammatory activity in a dose-dependent manner (Luseba et al. 2007; Mothana 2011). On the other hand, *J. platyphylla* extracts have strong anti-inflammatory activity (Ambriz-Perez et al. 2016). Extracts from different parts of *J. gossypifolia* exhibited significant systemic acute and chronic anti-inflammatory activity (Panda et al. 2009; Bhagat et al. 2011; Purohit and Purohit 2011).

20.4.1.4 Antimicrobial Activity

The extracts from different parts of *Jatropha* have significant antimicrobial activity. Latex of *J. curcas* exhibited strong antimicrobial activity against gram positive and gram negative bacteria (Oyi et al. 2002). Further, extracts from distinct parts of *J. curcas* showed strong growth inhibition against pathogens (Solsoloy and Solsoloy 1997; Aiyelaagbe 2007; Igbinosa et al. 2009). The crude extract of *J. multifida* leaves and different parts of *J. podagrica* extracts revealed active antimicrobial activity (Kupchan et al. 1970; Sievers et al. 1949; Irvine 1961; Aiyelaagbe et al. 2000; Rampadarath et al. 2014). Bahadur et al. (1997) reported the antimicrobial activity of eight *Jatropha* species. The macrocyclic diterpene jatrophenone isolated from *J. gossypifolia* is reported to possess a significant antimicrobial activity against *S. aureus* (Ravindranath et al. 2003).

20.4.1.5 Antifungal Activity

The ethanolic extract of *J. curcas* seed cake exhibited strong antifungal activity, and the extract consists of phorbol esters mainly responsible for antifungal activities (Saetae and Suntornsuk 2010). The leaf extracts of *J. curcas* were used to control the fungal pathogens *Sclerotium* sp. and *Colletotrichum musae* (Thangavelu et al. 2004). *J. curcas* proteins have strong antifungal and biological activities (Rakshit et al. 2010). Aiyelaagbe et al. (2000) reported that *J. podagrica* root bark and root wood exhibited antifungal activity against *Candida albicans*.

20.4.1.6 Antitumoral Activity

Curcin is a toxic lectin (Stripe et al. 1976) with N-glycosidase activity that has been extracted from *J. curcas* leaves and has been shown to have antitumor properties (Langdon 1977; Lin et al. 2003). Extracts from different parts of *J. curcas* showed strong anticancer properties due to the presence of bioactive compounds like diterpenes, tannins, phytates, phenolics, flavonoids, and saponins (Li and Wang 2003; Singh et al. 2003; Oskoueian et al. 2011).

20.4.1.7 Antipyretic, Wound Healing, and Analgesic Activity

Curcain, a proteolytic enzyme isolated from *J. curcas* latex, and arabinogalactan protein isolated from *J. curcas* seeds exhibited promising wound healing property than nitrofurazone (Nath and Dutta 1992, 1997). Extracts from different parts of *J. curcas* exhibited strong wound healing property (Hodek et al. 2002; Luseba et al. 2007; Shetty et al. 2006). Additionally, jatrophine isolated from *J. curcas* latex showed wound healing (Esimone et al. 2009) and biological activities (Van-den et al. 1995). The phenylquinone and acetic acid are used to induce writhing response in lab animals (Berkenkopf and Weichman 1988). Olukunle et al. (2011) reported that aqueous extract (39%) of *J. curcas* leaves showed inhibition of writhing response compared to the control drug paracetamol (57.9%). The analgesic activity of methanol extract of *J. gossypifolia* leaves and fruits showed highly significant inhibition of writhing response (Apu et al. 2012, 2013), and the latex was used to dress sores and ulcers (Iwu 1993; Jongschaap et al. 2007).

20.4.1.8 Anticoagulant Activity

The *J. curcas* latex showed strong anticoagulant activity and significant reduction in clotting time of human blood. Furthermore, diluted latex also exhibited strong anticoagulant activity (Osoniyi and Onajobi 2003). The *J. gossypifolia* latex and

leaves are widespread as a hemostatic agent for preventing bleeding disorders (Oduola et al. 2005).

20.4.1.9 Antidiabetic Activity

The aqueous ethanolic extract of *J. curcas* leaves showed antidiabetic activity in allaxon-induced rats (Mishra et al. 2010).

20.4.1.10 Antiulcer Activity

The methanol extract of *J. curcas* and latex showed antiulcer activity (Jongschaap et al. 2007; Kannappan et al. 2008).

20.4.1.11 Antidiarrheal Activity

The methanolic extract of *J. curcas* root showed significant antidiarrheal activity (Mujumdar et al. 2001). Furthermore, *J. gossypifolia* leaves and fruits exhibited strong antidiarrheal activity (Apu et al. 2012, 2013).

20.4.1.12 Hepatoprotective Activity

Methanolic fraction of *J. curcas* exhibited hepatoprotective activity (Balaji et al. 2009). In addition, extracts of different vegetative parts of *J. gossypifolia* showed strong hepatoprotective action (Panda et al. 2009).

20.4.1.13 Insecticidal Activity

The methanol and ethanol extracts of *J. curcas* oil revealed high insecticidal activity against larvae of *Lipaphis erysimi*, *Pieris rapae*, *Sitophilus oryzae*, *S. zeamais*, *Busseola fusca*, and *Sesamia calamistis* (Makkar et al. 2007). Jatropherol-I, a diterpene isolated from *J. curcas* oil, showed insecticidal activity against *Bombyx mori* L., *L. erysimi*, and *P. rapae*; it acted on *B. mori* and *P. rapae* (Solsoloy 1995). The methanol and n-butanol extracts of unripened seeds of *J. gossypifolia* exhibited high insecticidal activity against two freshwater snails (Sukumaran et al. 1995). In addition, extracts of *J. podagrica* root showed moderate insecticidal activity against *Helicoverpa zea* (Aiyelaagbe and Gloer 2008).

20.4.1.14 Other Effects

The *Jatropha* oil is commonly used to cure skin disease. Singh et al. (2007) reported a dark blue dye which is produced from bark of the *Jatropha* plant that has been used for coloring cloth and fishing nets. The *Jatropha* leaves can be used as food for silkworms and burnt root ash as a salt substitute (Morton 1981). Kirtikar and Basu (1991) reported that fresh leaf juice with lemon is used to cure fever. In parts of Asia, *J. curcas* root is used as an antidote for snakebite, and in parts of Africa, *J. curcas* kernel is used for the termination of unwanted pregnancies (Makkar and Becker 2009a). Fagbenro-Beyioku et al. (1998) reported that *Jatropha* leaves have antiparasitic activity. The cyclic octapeptide (Gly-Leu-Leu-GlyThr-Val-Leu-Leu-Gly) has been isolated from *J. curcas* latex, and it regulates classical pathway and proliferation of T-cells (Van den berg et al. 1995).

The *Jatropha* leaves contain bioactive compounds which are in combination with other factors and used against muscular pains (Agbogidi and Ekeke 2011). Mpiana et al. (2009) reported that *J. curcas* anthocyanins crude extract revealed a significant in vitro normalization of sickle cell erythrocytes. In addition, the *J. curcas* roots yield a yellow oil with strong antihelminthic properties (Sirisomboon et al. 2007; Karaj and Muller 2010). The anti-HIV effect of 12-deoxyphorbol-13-phenyl acetate, a compound from *J. curcas* phorbol esters, inhibits HIV entry into target cells (Wender et al. 2008).

20.5 Seed Cake Toxicity

Toxic and non-toxic genotypes of *J. curcas* are reported. The non-toxic genotypes are available only in Mexico. The toxic genotype seed contains high oil and crude protein content, but unsuitable for human or animal consumption due to its toxicity (Liberalino et al. 1988). Becker and Makkar (1998) reported that consumption of seeds showed signs of giddiness, vomiting, and diarrhea. The *J. curcas* seeds contain high amount of phorbol esters and are considered as primary toxic substances (Adolf et al. 1984). In addition, it includes trypsin inhibitor, phytate, saponins, and lectin (Cano-Asseleih et al. 1989). Lectin is not the toxic compound in *Jatropha* meal, and it is reported that lectin and trypsin inhibitor activity can be downregulated by traditional processing methods (Aderibigbe et al. 1997; Aregheore et al. 1998). The phytate and saponin contents of toxic and non-toxic genotypes are almost similar (Table 20.4). The addition of phytase enzyme mitigates the adverse effects of phytate (Makkar et al. 2008b), and saponins did not possess hemolytic activity (Makkar and Becker 2009b). Aderibigbe et al. (1997) reported that tannins, condensed tannins, cyanogens, glucosinolates, and amylase inhibitors were not detected. The seed cake contains hemicelluloses, cellulose, and lignin in the percentages of 26.8, 13.5, and 12.4, respectively. Lime pretreatment was effective in removing lignin and hemicelluloses (Liang et al. 2010).

Table 20.4 Important phytochemicals in seed meal of toxic and non-toxic varieties of *J. curcas*

S. no	Component	Toxic variety	Non-toxic variety
1.	Phorbol esters (mg/g kernel)	2.79	0.11
2.	Total phenols (% tannic acid equivalent)	0.36	0.22
3.	Tannins (% tannic acid equivalent)	0.04	0.02
4.	Phytate (% dry matter)	9.40	8.90
5.	Saponins (% diosgenin equivalent)	2.60	3.40
6.	Trypsin inhibitor (mg trypsin inhibited per g sample)	21.30	26.50
7.	Lectins (1/mg of meal that produced hemagglutination per ml of assay medium)	102	51

All data are on a dry matter basis, Source: Makkar et al. (1998)

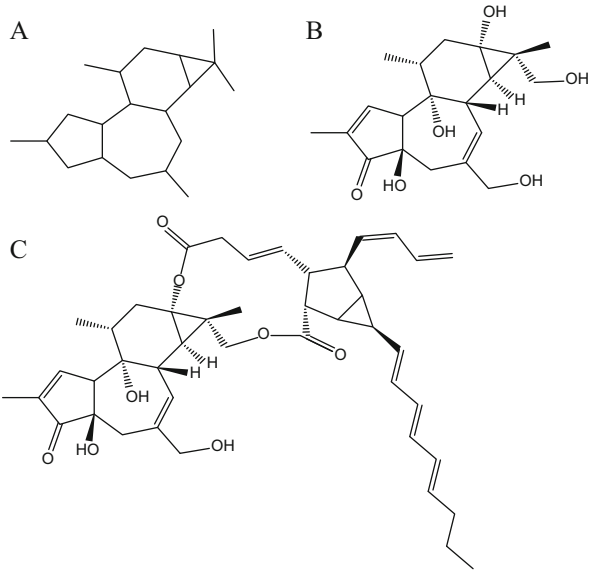

Fig. 20.1 (**a**) 5-7-6-3 tigliane ring structure common to all phorbols. (**b**) 12-hydroxy-16-deoxylphorbol structure common to all phorbol esters from *J. curcas* (**c**) *J. curcas* factor C_1 is one of the six phorbol esters identified in the seeds of *J. curcas*. (Source: Haas et al. 2002)

20.5.1 Phorbol Esters

Phorbol esters are mainly present in members of Euphorbiaceae and Thymelaeaceae families. They are tetracyclic diterpenoids (Evans 1986), and structures of six phorbol esters were identified through NMR (Haas et al. 2002). Among them, one of the structures of phorbol esters and 12-hydroxy-16-deoxy phorbol is depicted in Fig. 20.1. The phorbol esters suppressed the action of signal transduction pathways and skin-irritant activities. Goel et al. (2007) reported that phorbol esters have strong insecticidal, molluscoidal, and antimicrobial properties. Hirota et al. (1988) reported that phorbol 13-myristate 12-acetate from *Croton tiglium*, *J. curcas* oil, and phorbol

Table 20.5 Phorbol esters in different parts of the toxic *J. curcas* plant

S. no	Parts	Phorbol esters (mg/g dry matter)
1.	Kernel	2.0–6.0
2.	Leaves	1.83–2.75
3.	Stems	0.78–0.99
4.	Flower	1.39–1.83
5.	Buds	1.18–2.10
6.	Roots	0.55
7.	Latex	Not detected
8.	Bark (outer brown skin)	0.39
9.	Bark (inner green skin)	3.08
10.	Wood	0.09

Source: Makkar and Becker (2009a, b)

esters showed tumor-promoting effects and activate Epstein-Barr virus (Macneil et al. 2003). All parts of *J. curcas* plant contain phorbol esters at different levels (Table 20.5).

The non-toxic genotype of *J. curcas* could be an excellent protein source for animals. Following detoxification, the toxic genotype of *J. curcas* protein can also be utilized for animal consumption. Aderibigbe et al. (1997) reported that phorbol esters are heat stable, and hence, heat treatment is not an effective method to detoxify the toxins in the seed meal. Haas and Mittelbach (2000) reported that traditional oil refining methods like deacidification and bleaching can decrease the phorbol esters content of seeds by about 50%. The phorbol esters content has been reduced by several chemical treatments (Aregheore et al. 2003). A wide range of methods have been applied to reduce the toxicity of seed meal, and extraction with 80% ethanol or 92% methanol for four times reduced 95% toxicity (Makkar and Becker 1997a), and petroleum ether extraction suppressed 67.7% toxicity (Chivandi et al. 2004). Extraction with polar organic solvents, and combined heat/$NaHCO_3$ treatments, resulted in 48% reduction (Martinez-Herrera et al. 2006). Rakshit and Bhagya (2007) reported 90% reduction in toxicity by using chemicals. Moreover, Joshi et al. (2011) reported that solid state fermentation (SSF) could be a viable approach for the complete degradation of the toxic phorbol esters paving the way for use of seed meal in animal feeding.

20.5.2 Trypsin Inhibitors

Trypsin inhibitors (TI) are antinutrient substances present in many plants (Norton 1991). The role of TI is in reduction of protein digestibility. In toxic and non-toxic genotypes, TI levels are identical, ranging from 18.4 to 27.3 mg trypsin inhibited/g

(Makkar et al. 1997). *J. curcas* kernel meal fed to carp (*Cyprinus carpio*) failed to show any marked differences in growth performance, which indicated that fish can tolerate high levels of TI (Makkar and Becker 1999). In addition, feeding monogastric animals, such as poultry, pigs, and fish other than carp, with unheated *J. curcas* kernel meal may produce adverse effects.

20.5.3 Saponins

Saponins are steroids present in most of the plants. Saponins may serve as antifeedants, bitter in taste, and reduce plant palatability in livestock (Sen et al. 1998). It can affect animal performance and metabolism in a number of ways, namely, hemolysis, reduction of blood cholesterol, depression of growth rate, bloat (ruminants), inhibition of smooth muscle activity, enzyme inhibition, and reduction in nutrient absorption (Cheeke 1971). Belmar et al. (1999) reported that saponins alter cell wall permeability and produce toxic effects when ingested. Johnson et al. (1986) showed that saponins bind to the cells of the small intestine, affecting the absorption of nutrients. The saponin contents of toxic and non-toxic genotypes of *J. curcas* are similar. The saponin content of *J. curcas* kernel meal from different accessions ranged between 1.8% and 3.4% (as diosgenin equivalent) (Makkar et al. 1997, 1998).

20.5.4 Phytate

In plant seeds, the phosphorus is stored in the form of phytate or phytic acid. When phytates are consumed along with the diet, mineral ions such as Ca^{2+}, Mg^{2+}, Zn^{3+}, Cu^{3+}, and Fe^{3+} become unavailable for consumers (Duffus and Duffus 1991). Non-ruminants cannot degrade phytate (Liener 1989), and it forms complexes with proteins, thus a reduction in digestibility (Richardson et al. 1985). On the other hand, ruminants can digest phytate because phytase is produced by rumen microorganisms. *J. curcas* kernel meal from both toxic and non-toxic *J. curcas* genotypes contains phytates ranging from 7.2% to 10.1% (Makkar et al. 1997). The effects of *J. curcas* phytate in animals have not yet been studied. Since the levels of phytate in the kernel meal are high, efficient utilization as feed for monogastric animals would require a preprocessing of fermentation or the addition of phytase to the diet.

20.5.5 Lectins

Lectins are glycoproteins and are ubiquitous in nature. It alters protein, carbohydrate, and lipid metabolism, promotes enlargement of internal organs, and brings changes

in the hormonal and immunological status. The growth and health of the animals are affected when lectins are consumed at high concentration in their diet (Vasconcelos and Oliveira 2004). The toxic and non-toxic genotypes of *J. curcas* kernel meal contain 102 and 51 (1/mg of meal that produced hemagglutination per ml of assay medium) lectins (Makkar et al. 2007).

20.5.6 Animal Feed

The *Jatropha* seeds are rich in protein. The protein composition of *J. curcas* kernel meal contains all the essential amino acids except lysine (Makkar et al. 1998). Hence, the kernel meal can be utilized as animal feed, and it improves the economic value of *Jatropha*, due to the dual role as fuel and feed. The non-toxic genotype lacks phorbol esters but has trypsin inhibitors, saponins, phytates, and lectins similar to the levels in the toxic genotype (Makkar et al. 1998). Aregheore et al. (1998) reported that the seeds of the non-toxic genotype are roasted and the kernels are consumed by humans in Mexico. The raw and heat-treated non-toxic variety was fed to carp, and both groups grew to an almost identical extent (Makkar and Becker 1999). These results suggest that the non-toxic genotype is a good protein source for fish and livestock animals. The carp is highly sensitive to toxins and can detect phorbol esters at a level of 15 ppm (Becker and Makkar 1998). Kumar et al. (2008) reported that the defatted *J. curcas* kernel meal from toxic variety fed to carp showed no changes in enzyme activity, blood parameters, and histopathological lesions in the organs. The defatted *J. curcas* meal can replace 50% fish meal protein in carp diets and rainbow trout without compromising on the growth and nutrient utilization of fish. In fish muscle tissue, the phorbol esters were not detected, and it shows that the fish fed on *J. curcas* meal is safe for human consumption (Kumar et al. 2011a, b).

Studies with animals (Adam 1974; Ahmed and Adam 1979; Liberalino et al. 1988; El-Badwi et al. 1995) have shown that mice (Adam 1974) and rats (Stripe et al. 1976) are good models in mammals to investigate toxicity of *J. curcas*. The toxic variety of *Jatropha* nuts was fed to rats, and within 23 days (raw nuts), 14 days (roasted nuts), and 68 days (cooked nuts), rats died. Abd-Elhamid (2004) reported that acetonitrile extract of *J. curcas* (seed or oil) when fed to albino rats (50 mg/kg) showed mild toxicological and histochemical changes. Cai et al. (2010) reported acute toxicity of phorbol esters when fed to Swiss Hauschka mice (IP) which showed prominent lesions in lungs and kidneys.

A high mortality in chicks and calves fed with toxic genotype *Jatropha* seeds has been reported (Ahmed and Adam 1979; El-Badwi et al. 1995). Rakshit and Bhagya (2008) reported enlargement of organs due to the deficiency of essential amino acids in *J. curcas* meal. The mortality of animals was not only due to the toxicity of phorbol esters but also probably due to high contents of hull or antinutritional constituents present in the *J. curcas* meal.

20.6 Conclusions

The different parts of the *Jatropha* plant contain phytochemical components which find place in pharmacological applications like diabetes, hypertension, cancer, malaria, inflammation, cardiovascular, ulcer, wound healing property, hepatic infections, and several other diseases. In conclusion, the review described about the phytochemicals and the pharmacological values of these compounds from *Jatropha* plant. The SE bioactive compounds can be exploited as herbal drugs in complementary and alternative medicine. The utilization of by-products, coproducts, and secondary metabolites can increase the economic value of *Jatropha.* The functional proteins and peptides that are present in *Jatropha* species carry strong biological activities. In the future, the action of these bioactive compounds needs to be elucidated and explored for their potential use in agro-pharmaceutical industries.

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Chapter 21
Jatropha curcas L. Latex Production, Characterization, and Biotechnological Applications

Luciane Madureira Almeida, Fábio Santos Matos, Elisa Flávia Luiz Cardoso Bailão, and Pablo José Gonçalves

Abstract Latex is a fluid that flows out of some plants when injured and has been explored mainly for the rubber production and drug development. Particularly, latex obtained from *Jatropha curcas* L. is not used to obtain rubber but presents metabolites and bioactive compounds that possess potential medicinal applications. However, this latex was until now poorly scientifically investigated. In this chapter we will focus on its harvest, yield, chemical composition, and biotechnological applications of the *J. curcas* latex. Regarding the production, *J. curcas* latex is strongly influenced by environmental factors (water, light, nutrients, and temperature) that interfere in photosynthetic activity and endogenous conditions (fruit production and leaf senescence) determining the allocation of carbon for primary or secondary metabolisms. The main compounds found in *J. curcas* latex are curcain, curcacyclines A and B, and jatrophidin I, which present medicinal properties, such as wound healing, anticancer, and antioxidant. In addition to isolated molecules, the crude latex also presents antimicrobial, antiviral, antinematode, anti-inflammatory, procoagulant, and anticoagulant activities. *J. curcas* latex also has shown nanotechnological potential due to its capacity of reducing and capping agents and of avoiding nanoparticle aggregation. Since its use is quite recent and still little is explored, new possibilities for the development of bio-based products can arise and add more commercial value to this crop.

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21.1 Introduction

21.1.1 Plants and Their Exudates

Exudates released from plants consist of complex mixtures of organic and inorganic molecules such as latex, sap, gums, resins, seed, and root exudates (Licá et al. 2018). The latex is an organic milky fluid that flows out of some plants after a tissue injury. The general functions of this exudate in plants are associated with the excretion of waste metabolites, coverage of damaged tissue, and defense against pathogens (Konno 2011). The latex production occurs in highly specialized cells called laticifers, which are secretory cells that produce latex as secondary metabolites. The laticifers usually are spread through the plant tissues, being present in roots, stems, petioles, and leaves (Hagel et al. 2008). Laticifers may be divided into articulated laticifers, which are composed of a series of cells joined together, or non-articulated laticifers, which consist of one long coenocytic cell and idioblastic (Krishnamurthy et al. 2013). In the case of the articulated laticifers, the cells can be connected (anastomosing) or not connected (non-anastomosing). In the case of the non-articulated laticifers, the cells have a continual elongation and growth throughout the lifetime of the plant. These cells can reach up to tens of centimeters long and can be branched or unbranched (Fig. 21.1). The origin, distribution, and structure have been well described earlier (Krishnamurthy et al. 2013).

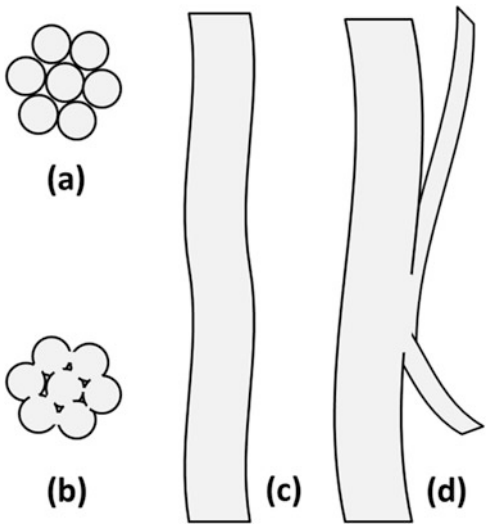

Fig. 21.1 Schematic representation of plant laticifers. (**a**) Articulated non-anastomosing, (**b**) articulated anastomosing, (**c**) non-articulated unbranched, and (**d**) non-articulated branched

Generally latex is a white sap, but there are exceptions, and in some cases, it is clear or with distinct color (Konno 2011). The latex composition varied between species, but in general the latex is composed of carbohydrates, proteins, alkaloids, starches, tannins, oils, resins, and rubber (Domsalla and Melzig 2008). Latex has been reported in approximately 20,000 species of flowering plants in over 40 families, being most commonly found in Euphorbiaceae and Apocynaceae (Konno 2011).

The latex has been biologically and biotechnologically explored for centuries with different purposes. For example, the latex obtained from *Hevea brasiliensis* is the main source of natural rubber over the world. The main use of rubber is in automotive industry, particularly in the manufacture of pneumatic tires. In addition, there are an infinite amount of products composed by rubber, such as toys, gloves, elastic products, insulation for electrical wiring, waterproof clothing, and many other consumer products (Beilen and Porier 2007). Other major area of latex use is for medicinal purposes. One of the most ancient uses of the latex is to obtain opium from *Papaver somniferum*. Many alkaloids are extracted from opium poppy, which are used in several narcotic drugs such as morphine, codeine, thebaine, papaverine, and noscapine. Several studies presented the high medicinal potential of plant latex, such as analgesic (Dewan et al. 2000), anti-inflammatory (Fernandez-Arche et al. 2010), antioxidant (Chaudhary et al. 2015), antifungal (Ramos et al. 2015), antiviral (Nothias-Scaglia et al. 2015), antimicrobial (Sharma et al. 2016), antiulcer (Bharti et al. 2010), angiogenic (Almeida et al. 2014), antiangiogenic (Rebouças et al. 2012), anticancer (Mousinho et al. 2011), insecticidal (Ramos et al. 2010), and antinociceptive (Soares et al. 2005). Despite the pharmacological activities associated with plant latex, very few lactiferous species were scientifically investigated. It is believed that less than 1% of lactiferous plant species have been studied for their pharmacological potential (Almeida et al. 2015).

Here, we are particularly interested in reporting the general characteristics of the *J. curcas* latex and to give some highlights of its application in medicinal and nanotechnological fields. This chapter will be divided in the following sections: *J. curcas* general information, ecophysiology of latex production in *J. curcas*, chemical compounds of *J. curcas* latex, *J. curcas* latex medicinal applications, and *J. curcas* latex nanotechnological applications.

21.2 *J. curcas* General Information

J. curcas is a perennial lactiferous species belonging to the Euphorbiaceae family. It is also popularly known as physic nut, humble, or hardy tree. *J. curcas* is a rustic plant with high edaphoclimatic adaptability and may thrive on soils of marginal fertility, of increased salinity, and in drought conditions (Matos et al. 2014). It is easy to cultivate because it grows relatively quickly and keeps yielding seeds for about 50 years. It also can be used to prevent and/or control soil erosion or to reclaim

Fig. 21.2 *J. curcas* latex flows out from cut ends of stems and branches

exhausted land. These characteristics have prompted the expanding cultivation of *J. curcas* mainly in regions unsuited to drought-sensitive crops (Matos et al. 2018).

Regarding its morphology, *J. curcas* is a shrubby tree, usually 3–5 m high, but can reach a height of up to 8 or 10 m under favorable conditions (Malviya et al. 2011). The leaves are green to pale green, broad and usually simple, angular, and deeply palmately three- to five lobed with spiral phyllotaxis. Inflorescences are formed terminally on branches in the leaf axil, with petiole length between 6 and 23 mm. *J. curcas* has soft wood, smooth gray bark that gives off a whitish colored watery latex when injured (Fig. 21.2). In this species, the laticifers are mainly distributed in the cortex and phloem, whereas few laticifers are found in xylem of stem and vein, as well as in pith of stem, petiole, pedicel, and fruit stalk. In relation to the laticifer's morphology, *J. curcas* presents all the four types of lactiferous vessels: articulated anastomosing and non-anastomosing and non-articulated branched and non-branched (Huan-Fang et al. 2006).

Regarding its geographical distribution, *J. curcas* is a species native to Central America, which was spread by Portuguese sailors to Africa and Asia (Fairless 2007; Maghuly and Laimer 2013). Extensive areas have been planned worldwide for cultivation of *J. curcas*, particularly in the semiarid and arid areas of African and Asian countries (Fairless 2007). In addition, studies have reported two main lineages of *J. curcas*, which refer to toxic and non-toxic genotypes (Makkar et al. 1998). The non-toxic genotypes have been reported to be exclusive from Mexico (He et al. 2011). Independently of toxic potential, both lineages contain a range of interesting metabolites and bioactive compounds, which is receiving attention for research as a medicinal plant (Sabandar et al. 2013).

People in almost all parts of the world have been using this plant against alopecia, ascites, burns, convulsions, cough, dermatitis, diarrhea, dropsy, dyspepsia, fever, syphilis, tumors, ulcers, yaws, and others (Debnath and Bisen 2008) because a wide range of pharmacological activities were associated with *J. curcas* extracts, such as anti-inflammatory, antioxidant, antimicrobial, antiviral, molluscicide, larvicidal,

anticancer, antidiabetic, procoagulant, anticoagulant, hepatoprotective, analgesic, healing, and abortifacient (Abdelgadir and Van Staden 2013; Bahadur et al. 1997; Pullaiah and Bahadur 2013; Sharifi-Rad et al. 2017; Mwangi et al. 2017; Mbele et al. 2017; Rampadarath et al. 2016). Besides the medicinal application, *J. curcas* also has attracted attention due to its high oil content that is easily extracted from the seeds (30–35% of oil per seed dry mass). Many researchers consider that *J. curcas* is one of the most promising oilseeds for biofuel production (Maghuly and Laimer 2013; Pereira et al. 2018). Thus, the extraction of natural biocompounds from *J. curcas* would add commercial value to this cultivar, which could be used to produce medicines and biodiesel. Among the various biological extracts and exudates obtained from *J. curcas*, the latex has been shown to have high biotechnological potential and will be highlighted in the next sections.

21.3 Ecophysiology of Latex Production

Except for data obtained for *H. brasiliensis*, there is currently little information in the literature regarding latex harvest and production methods in different latex-producing species. Regarding *J. curcas*, there is a sole article in the literature that assesses only two different harvesting methods; in one method the latex was extracted through a vertical cut in the stem region, while in the other method, the cut was made on the branches. The results showed that there were no significant differences in latex volume obtained by the two methods, which was on average 1 ml per cut/plant. The small amount of latex obtained in each extraction is evident, especially when compared to *H. brasiliensis*, which is from the same botanical family. The differences in latex composition and volume are certainly related to the different ecological roles of latex in both species (Matos et al. 2018).

Latex is a product of the secondary metabolism and composed of phenols, terpenes, carbohydrates, amino acids, mineral nutrients, and others. Secondary compound production in leaves, stems, and roots, with toxic and allelopathic effects, is ecologically important for perennial species to survive pest and disease attacks and has a competitive advantage for the same resources in the environment with other plant species. In spite of the important role of the secondary metabolites in plant protection, the synthesis of these compounds is a deviation of carbon from the primary metabolism. The shikimic acid pathway, the main pathway for phenol production, accounts for about 20% of the carbon assimilated during photosynthesis (Taiz and Zeiger 2017). It can be stated that secondary compound synthesis can, under specific circumstances, compete with the primary metabolism and consequently interfere in *J. curcas* fruit and seed production.

The high phenotypic plasticity of *J. curcas* allows easy adaptation to different edaphoclimatic conditions, but the species is very sensitive to low temperatures and frosts. Innumerable reports show that the suitable temperature for cultivation of *J. curcas* is in the interval between 18 °C and 35 °C. Air temperature is an important factor for *J. curcas* survival, growth, reproduction, and distribution, which is

sensitive to temperatures lower than 10 °C that trigger leaf senescence; nutrient removal, especially nitrogen; alterations in nitrate reductase activity; and decrease in the relative growth rate. In addition, many cell components are degraded during leaf senescence as a direct result of low temperatures, and regardless of plant age, low temperatures delay growth and reduce photosynthesis (Matos et al. 2012).

In the Brazilian Central West conditions where rainfall concentrates from October to March and high temperatures from September to March, the appearance of flowers, fruit filling, and ripening start in the 3rd week after the start of the rains in October, which stop about 4–5 months later. It is a reproductive process totally asynchronous in space and time, which means that there are plants in the same area and equal age with mature fruits, beginning the fruiting or flowering and others ending the vegetative stage. The reproductive inequality represents an ecological strategy of perpetuation of the species that under sporadic stress presents plants in different phenological stages with different potentials of overcoming the adverse conditions and producing seeds. In an anthropocentric view, these characteristics hinder the economic return by the high demand of manpower. Despite the lack of uniformity in fruit ripening, there is an ecological syncretism between the reproductive process and environmental factors, especially temperature, because the initiation of the cold season almost always coincides with the end of fruiting. In the cold period, the plant without leaves and fruits certainly directs part of its reserves to latex production. The absence of assimilate draining by fruits in the cold season certainly contributes to larger latex production during this period. The continuous flowering in parallel to fruit ripening on a same plant together with the long reproductive period increases the duration of competition between latex and fruit production (Matos et al. 2012; Taiz and Zeiger 2017).

Latex production in *J. curcas* plants may be correlated with nutrient re-mobilization during leaf senescence. The most obvious event that characterizes leaf senescence is leaf yellowing and chlorophyll degradation. The main target of leaf senescence is the chloroplast, and a large part of the leaf nitrogen is in this organelle. Nitrogen is quantitatively the main nutrient required by *J. curcas*, and about 75% of this element present in the mesophyll cells is in the chloroplasts (Taiz and Zeiger 2017). The presence of nitrogen and amino acids in *J. curcas* latex may originate in the re-mobilization process of these elements during leaf senescence. In addition, it is pointed out that in the peak of latex production, plants have no leaves, and there is certainly less transpiration and soil solution absorption, which are conditions that prevent significant nitrogen absorption.

In *J. curcas*, latex production decreases after the appearance of the flowers in November and continues to fall until it reaches a minimum in February when trees have their maximum fruit load (Fig. 21.3). Starting in March, when the last fruits are in their final stage of ripening, the latex volume starts to increase. In the following months, the leaf senescence process starts as the air temperature decreases, and in May, when temperatures are much lower for the species, leaf senescence is complete. The plants remain without leaves until the middle of August when the shoots start sprouting.

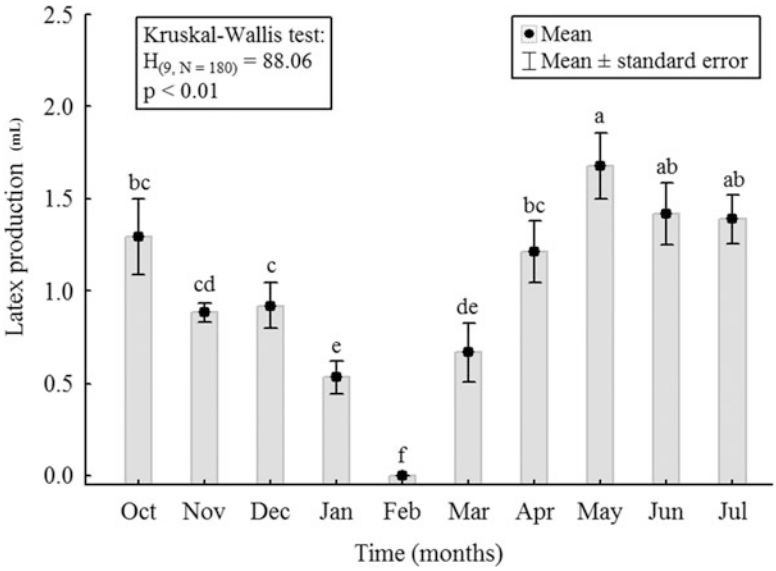

Fig. 21.3 Average latex production in *J. curcas* compared over 10 months, using the Kruskal-Wallis test. Means followed by the same letter do not differ by the Kruskal-Wallis test. (Matos et al. 2018, license: CC BY 4.0)

It is important to emphasize that the increase in latex production may not be directly related to low temperature, but rather to the leaf senescence process, because in the cold period, the reduced metabolism and absence of source organs (leaves) limit assimilate production to support the secondary metabolism; however, the availability of reserves derived from the re-mobilization is certainly sufficient for the supply of carbon skeletons and respiration in order to support the energy production for the metabolism needed in the cold season.

Latex production depends on the availability of reserve carbohydrates, amino acid, and protein biosynthesis. These minimal conditions are supplied after leaf senescence, but, during the warm period with the presence of leaves, flowers, and/or fruits, the seasonal fluctuations in light availability, temperature, air humidity, and soil can affect photosynthetic activity and, consequently, latex production. From this point of view, it can be stated that leaf senescence would have the primary effect on latex production, and air temperature would exercise a secondary role. Furthermore, the succulent stem typical of the species permits tissue turgidity maintenance even during periods of drought and enables the biochemical reactions necessary for secondary metabolite production.

J. curcas trees have vigorous growth, and the stem represents 30–60% of the total dry matter. The succulent stem is an important organ for water and nutrient storage that sustain the operation of leaves, and its development directly affects latex production. During the cold season and leaf senescence, the re-mobilized products

Table 21.1 Multiple regression analysis to evaluate the influence of fruit productivity, plant height, and crown diameter on latex production in *J. curcas* (Matos et al. 2018)

Variables	Beta	Standard deviation from beta	*T* value	CC
Intercept	1.3965621	0.3143246	4.443**	0.28
Fruit productivity	−0.0026198	0.0004736	−5.532**	−0.33
Plant height	0.4601745	0.0955994	4.814**	0.28
Crown diameter	−0.7600980	0.1442738	−5.268**	−0.31

Beta = estimated regression coefficient, that is, slope of the regression line. *T* value = *t* test for the significance of betas
**Significant at 1% probability by t test, *CC* correlation coefficient

are partially stored in the stem and are directed to latex production. In this way, the increase in stem diameter or plant height may be correlated with latex production (Table 21.1).

J. curcas shows intense competition between the vegetative and reproductive development phases since simultaneously branch growth and flowering occur at least for 5 months. The *J. curcas* flowers and fruits are produced at branch tips and compete for the same resources that derive from photosynthesis throughout the reproductive period. Branch growth depends on tissue turgidity and on metabolites' flow for biosynthesis of cellulose, hemicellulose, and microfibrils. Then, metabolites driven to fruits and branches certainly compete with latex production during the reproductive period. Under this condition latex production is a secondary drain and is inversely correlated with fruit production (Table 21.1).

The crown diameter represents the architecture and dimension of the photosynthesis potential because it reflects the number of leaves and leaf area of the photosynthetic apparatus. It can be stated that fructification depends on the production potential of assimilates from the crown, and, because of this, the number of fruits and crown diameter present positive correlation. In the fructification period, *J. curcas* directs the largest part of its metabolites to seeds that are priority, and the crown diameter is the variable that correlates directly with fruit yield. However, crown diameter correlates negatively with latex production, i.e., the largest the crown diameter, the lower the latex production (Table 21.1).

Physiologically the assertion that latex production decreases with increase in the photosynthesizing leaf area and crown diameter is incoherent because carbon assimilation is the probable source of compounds for the primary and secondary metabolisms. However, fruit and latex production are negatively correlated because of competition for metabolites. The crown diameter that analogically represents the photosynthesis industry correlates directly with the priority drains (fruits) and indirectly with latex production at the reproductive stage of the plants. Certainly, latex production at the vegetative stage (absence of fruits) would present direct relationship with the crown diameter.

Generally, the environmental factors that guide the production of assimilates (light, water, nutrients, and temperature) interfere decisively in latex production along with endogenous factors such as leaf senescence (that make resources

available for latex production), flowering, and fructification (that when present are priority drains and compete with latex biosynthesis).

Light: The availability of sufficient amount of active photosynthetic radiation is essential for the supply and conversion of light energy to sugars. Intense competition for metabolites between the vegetative, reproductive, and secondary metabolism stages requires high photosynthetic activity.

Water: Plentiful water availability during the reproductive period enables plant growth, reproductive organ formation, and latex production. Water storage in the stem in low rainfall periods certainly enables biochemical reactions, especially latex production.

Nutrients: Nitrogen is the main nutrient required by *J. curcas*, and proper availability of this mineral element is important in latex production because it has amino acids and nitrogenated compounds in its composition.

Temperature: Air temperature plays a determining role in latex production, because when it falls below 10 °C, the leaf senescence process is triggered on, which re-mobilizes nutrients and makes them available for storage and latex production.

21.4 Chemical Compounds of the Latex

Plant latex contains a variety of (i) proteins, such as peptides, proteases, protease inhibitors, lectins, chitinases, and oxidases, and (ii) secondary metabolites, such as isoprenoids (rubber, cardenolides, terpenoids, etc.), alkaloids, anthraquinones, and phenolic compounds. So far, curcain is the only protease that was isolated from *J. curcas* latex. Curcain (~22 kDa) was purified by chromatography on carboxymethyl cellulose and gel filtration on Sephadex G-200. The protease activity of this molecule was determined using azoalbumin as substrate (Nath and Dutta 1991). However to date, there is no structural information about this molecule.

Three cyclic peptides, viz., jatrophidin I (PubChem CID: 101873937) and curcacyclines A (PubChem CID: 10462870) and B (PubChem CID: 101702231), were also isolated from *J. curcas* latex (Fig. 21.4). The main source of cyclic peptides in *Jatropha* species is the latex (Sabandar et al. 2013). Cyclic peptides form a ring usually via amide ester or disulfide bonds. This structure combines favorable properties that make them potential therapeutic agents. Over 40 cyclic peptides are currently in clinical use, and about one new cyclic peptide drug enters the market every year. The vast majority of clinically approved cyclic peptides are derived from natural products (Zorzi et al. 2017). The main characteristics that make cyclic peptides so successful as therapeutic agents are (i) a large surface area that favors improved target binding and affinity, (ii) a limited conformational flexibility that improves target selectivity and molecular stability, (iii) the chemical nature (amino acids composition) in favor of a low toxicity, and (iv) a relative structural

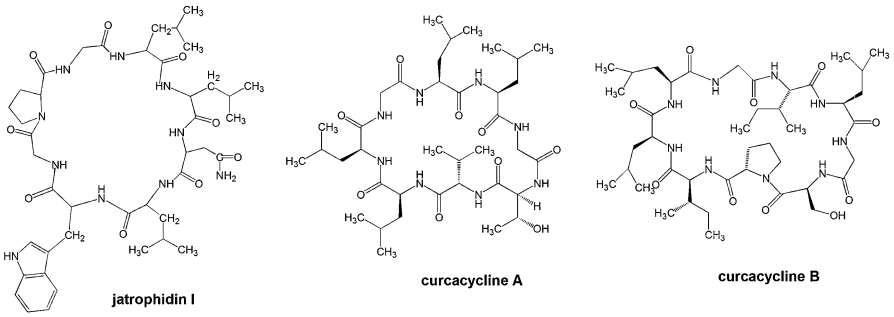

Fig. 21.4 Molecular structures of the three important compounds from *J. curcas* latex

simplicity (simple to modify, handle, and characterize), which improves chemical synthesis (Zorzi et al. 2017).

Jatrophidin I, a cyclic octapeptide, was purified from *J. curcas* latex using column chromatography followed by high-performance liquid chromatography (HPLC), and its structure was elucidated by extensive two-dimensional nuclear magnetic resonance (2D NMR) spectroscopic analysis (Altei et al. 2014). In silico investigation using PASS (prediction of activity spectra for substances) (Goel et al. 2011), SEA (similarity ensemble approach) (Keiser et al. 2007), and SwissTargetPrediction (Gfeller et al. 2014) suggested that jatrophidin I could be a wound healing agent, binding to endothelin-1 receptor (Khimji and Rockey 2011) (Table 21.2), and could bind to histone deacetylase enzymes (HDACs) (Table 21.2). HDACs are emerging cancer drug targets since they regulate gene expression by removing acetyl groups from histones, resulting in chromatin condensation and, then, in transcriptional repression (Watson et al. 2012). However, this putative function of jatrophidin I remains to be elucidated.

Curcacycline A, also a cyclic octapeptide, was first isolated from *J. curcas* latex using an alcohol extraction-based protocol followed by column chromatography (van den Berg et al. 1995). The structure of the compound was completely elucidated in 1995 and confirmed 17 years later. *In silico* investigation suggested that curcacycline A could (i) bind to endothelin-1 receptor, to calpain, as well as to aspartic and cysteine proteases and (ii) act as an immunosuppressant and antibiotic (Table 21.2). The potential of curcacycline A as immunosuppressant and antibiotic was demonstrated in vitro (Insanu et al. 2012; van den Berg et al. 1995). However, the possible involvement of this compound in wound healing, due to its potential to bind to endothelin-1 receptor and calpain (Jiang et al. 2012; Khimji and Rockey 2011), remains to be investigated.

Curcacycline B, a cyclic nonapeptide, was first purified from *J. curcas* ethyl acetate-latex fraction using column chromatography followed by HPLC (Auvin et al. 1997). The structure of the compound was fully elucidated in 1997 and confirmed with more sophisticated bioanalytical techniques 15 years later. Curcacycline B presents some structural features in common with cyclosporin A, an

Table 21.2 Top-ranked in silico predictions of bioactivities (PASS) and targets (SEA and SwissTargetPrediction) of compounds present in *J. curcas* latex

Compound	Rank	PASS[a]	SEA[a]	SwissTargetPrediction[a]
Jatrophidin I	1	Antibiotic glycopeptide-like	Endothelin-1 receptor	Substance-K receptor
	2	Nootropic	Histone deacetylase	Histone deacetylase 3
	3	Uterine relaxant	Substance-K receptor	Histone deacetylase 1
	4	Somatostatin 2 agonist	Histone deacetylase	Histone deacetylase 2
	5	Oxytocin antagonist	Integrin alpha-3	Melanocortin receptor 4
	6	Antineoplastic (non-Hodgkin's lymphoma)	Substance-K receptor	Melanocortin receptor 5
	7	Opioid dependency treatment	Melanocortin receptor 5	Melanocortin receptor 3
	8	Interleukin-2 agonist	Nuclear receptor corepressor 2	Melanocyte-stimulating hormone receptor
	9	Neurokinin-2 antagonist	Substance-K receptor	Adrenocorticotropic hormone receptor
	10	Wound healing agent	Somatostatin receptor type 5	Renin
Curcacycline A	1	Antibiotic glycopeptide-like	Endothelin-1 receptor	Renin
	2	Immunosuppressant	Galanin receptor type 1	Cathepsin D
	3	Antiischemic, cerebral	Galanin receptor type 2	Napsin-A
	4	CYP3A5 substrate	Motilin receptor	Calpain-1 catalytic subunit
	5	Proteasome ATPase inhibitor	Integrin beta-5	Cathepsin B
	6	Membrane integrity antagonist	Integrin beta-3	Calpain-2 catalytic subunit
	7	P-glycoprotein 1 inhibitor	Integrin alpha-3	Calpain-8
	8	CYP3A3 substrate	Integrin alpha-V	Calpain-9
	9	Muramoyltetrapeptide carboxypeptidase inhibitor	Integrin alpha-IIb	Calpain-3
	10	CYP3A substrate	Prolactin receptor	Calpain-11

(continued)

Table 21.2 (continued)

Compound	Rank	PASS[a]	SEA[a]	SwissTargetPrediction[a]
Curcacycline B	1	Antibiotic glycopeptide-like	Histone deacetylase	Tumor necrosis factor receptor superfamily member 10A
	2	Membrane integrity antagonist	Histone deacetylase	Tumor necrosis factor receptor superfamily member 10B
	3	Immunosuppressant	Endothelin-1 receptor	Tumor necrosis factor receptor superfamily member 10D
	4	Na^+-transporting two-sector ATPase inhibitor	Galanin receptor type 1	E3 ubiquitin-protein ligase XIAP
	5	Uterine relaxant	Galanin receptor type 2	Baculoviral IAP repeat-containing protein 3
	6	Nootropic	Histone deacetylase 6	Baculoviral IAP repeat-containing protein 2
	7	P-glycoprotein 1 inhibitor	Histone deacetylase 5	Baculoviral IAP repeat-containing protein 8
	8	Antifungal	Histone deacetylase 8	Renin
	9	P-glycoprotein inhibitor	Oxytocin receptor	Cathepsin D
	10	CYP3A5 substrate	Vasopressin V2 receptor	Napsin-A

[a]The criteria adopted to use PASS online (http://www.pharmaexpert.ru/passonline/index.php) results was Pa>Pi; to use SEA (http://sea.bkslab.org/) results was e > −15; and to use SwissTargetPrediction (http://www.swisstargetprediction.ch/) results was probability >50%

immunosuppressant. It has been suggested by in silico investigations that curcacycline B could act as an immunosuppressant and could bind to the tumor necrosis factor receptors (Table 21.2). In fact, curcacycline B was able to enhance the rotamase activity of human cyclophilin B, the cyclosporin A-target (Auvin et al. 1997). Moreover, in silico studies suggested that curcacycline B could act as an antibiotic and antifungal agent and that this cyclic peptide could bind to HDACs and to endothelin-1 receptor (Table 21.2).

The putative potential of curcacycline B against microorganisms, to bind to HDACs or to act in wound healing process (due its putative potential to bind to endothelin-1 receptor) (Khimji and Rockey 2011), is not yet elucidated in vitro or in vivo and should be investigated.

The geometry optimization of curcacycline A and curcacycline B was performed through molecular modeling at the semiempirical level (Bar et al. 2009). These authors found different size pockets within the cavity of curcacyclines with a radius in the range of about 20–35 nm, which is interesting to nanotechnological

applications in particular for synthesis of nanoparticles. The authors suggested that these cavities are able to entrap metal atoms into the core structure of curcacyclines reducing and stabilizing these metals in situ by the amide group of the host peptide. This result is important for the development of eco-friendly synthetic route for nanoparticles, which will be described below.

Besides the isolation of some proteins from the *J. curcas* latex, proteomic profiles of this exudate were recently obtained by two different protein extraction methods (phenol or trichloroacetic acid/acetone). In total, 459 proteins were identified, being only 65 proteins commonly isolated by both methods. Around 2.26 times more proteins were identified by the phenol method, indicating that this technique worked better for *J. curcas* latex samples (Yang et al. 2017). The proteomic profile contributed to reinforce that *J. curcas* latex is actually a specialized cytoplasm for containing membrane-bound organelles and particles. Moreover, this latex is very important for plant metabolism for containing large parts of the catalytic activities, including the production of the secondary metabolites.

Regarding the secondary metabolites, the methanol fraction of *J. curcas* latex contains alkaloids, saponins, tannins, glycosides, phenolic compounds, and flavonoids, while terpenoids and steroids were not identified. Compared to other *J. curcas* exudates, latex presented a high content of total phenolics, but low content of total flavonoids (Sharma et al. 2016). This high phenolic content corroborates the good antioxidant potential of *J. curcas* crude latex ($IC_{50} = 0.87$ mg/ml) when compared with different antioxidant controls such as BHT ($IC_{50} = 0.20$ mg/ml), BHA ($IC_{50} = 0.10$ mg/ml), ascorbic acid ($IC_{50} = 0.09$ mg/ml), and tannic acid ($IC_{50} = 0.04$ mg/ml) (Bailão et al. submitted). This finding suggests that the latex could act as a good free radical scavenger.

21.5 Medicinal Applications of Latex

21.5.1 Traditional Medicine

Because of its peculiar chemical composition, *J. curcas* latex has been used in traditional medicine for the treatment of different human and veterinary illnesses (Table 21.3).

Besides the wide use, some folk communities report several side effects when using this plant, such as nausea, impotency, sterility, dizziness, and hallucination. Most of *J. curcas* latex folk medicinal properties need to be scientifically investigated once its effectiveness, pharmacological and toxic potential, the molecular action, and proper dosage remain to be elucidated.

Table 21.3 Folkloric employment of *J. curcas* latex

Folk medicine employment	References
Against bee and wasp sting	Watt and Breyer-Brandwijk (1962)
Toothache, burns, hemorrhoids, ring-worm, ulcers	Perry and Metzger (1980)
Dressing wounds, inflamed tongues, and ulcer	Perry and Metzger (1980) and van den Berg (1995)
Anticancer and disinfectant	Duke and Ayensu (1983)
Help tooth come out in children and carious teeth treatment	Iwu (1993)
Leucorrhagia, urethritis	Neuwinger (1996)
Toothache, styptic	Heller (1996) and Kaushik and Kumar (2008)
Internal wounds such as gastric ulcers	Villegas et al. (1997)
Purgative and laxative	Aminul Islam et al. (2012)
Genital itchiness	Ganesan et al. (2004), and Samuel and Andrews (2010)
Disinfectant in mouth infections to treat mouth sores	Asase et al. (2005) and Kumar and Sharma (2008)
Toothache, mouth ulcer, cracked lips	Jain and Srivastava (2005), Verma and Chauhan (2007), and Silja et al. (2008)
Skin diseases, piles	Kumar and Sharma (2008)
Sores in domestic livestock	Kumar and Sharma (2008)
Eczema, scabies, wounds, burns, cancer	Deka et al. (2008) and Yesodharan and Sujana (2007)
Headache	Rajendran et al. (2008) and Tripathi and Srivastava (2010)
Cease bleeding, against infection	Wole and Ayanbode (2009)
Against leishmania	Odonne et al. (2011)
Cold flu and cough, otorrhoea	Meena and Yadav (2010) and Nath and Choudhury (2010)
Bleeding wounds (coagulation effects on blood)	Aminul Islam et al. (2012)

21.5.2 Scientific Evidence of Medicinal Properties

In addition to use in folk medicine, the pharmacological potential of crude *J. curcas* latex or of some of its isolated compounds has been confirmed (Table 21.4). The *J. curcas* crude latex presents some biological potential. *J. curcas* latex in high concentrations showed a procoagulant activity, reducing the clotting time, when the prothrombin and thromboplastin time coagulation were analyzed. Diluted solutions of this latex, on the other hand, prolonged the clotting time (Osoniyi and Onajobi 2003). These results suggested both procoagulant and anticoagulant activities of *J. curcas* latex, corroborating the folkloric use.

The diluted *J. curcas* latex (10% and 15%) was also used in the development of a cream formulation, which was tested in mice wound healing process. The results showed a moderate immune reaction of macrophages on wound healing as assessed through CD68 protein expression. Based on this data, the authors suggested that the

Table 21.4 Biological and/or pharmacological properties of *J. curcas* latex based on scientific literature

Biological and/or pharmacological activity	Biological agent	References
Procoagulant and anticoagulant	Crude latex	Osoniyi and Onajobi (2003)
Wound healing and anti-inflammatory	Latex cream	Salim et al. (2018)
Antimicrobial properties against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, and *Candida albicans*	Crude latex	Thomas (1989)
Antimicrobial properties against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Candida albicans*, *Trichophyton* sp.	Crude latex	Oyi et al. (2007)
Antimicrobial activity against *Shigella flexneri*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis*	Methanolic fraction of latex	Sharma et al. (2016)
Inhibition of watermelon mosaic virus	Crude latex	Tewari and Shukla (1982)
Inhibition of *Haemonchus contortus* larval motility	Dried crude latex	Muhamad et al. (2017)
Wound healing	Curcain	Nath and Dutta (1991)
Protease inhibition activity	Jatrophidin I	Altei et al. (2014)
Antiproliferative	Curcacycline A and B	Devappa et al. (2010)
Cytotoxic activity against ovarium cancer cells	Curcacycline A	Insanu et al. (2012)
Apoptosis modulation and suppressing ROS in sheep oocytes	Curcacycline A and B	Barakat et al. (2016)

latex cream of *J. curcas* possesses anti-inflammatory activity in wound healing process of mice skin (Salim et al. 2018).

Other example of crude latex potential refers to its antimicrobial activity. It was demonstrated that *J. curcas* latex is active against the bacterial species, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Enterococcus faecalis*, and against the fungus species, *Candida albicans*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, and *Trichophyton* sp. (Thomas 1989; Oyi et al. 2007; Sharma et al. 2016).

J. curcas latex also has been applied in the agronomical and veterinary field. For example, the inhibition of watermelon mosaic virus was demonstrated in plants by *J. curcas* latex (Tewari and Shukla 1982). In addition, *J. curcas* latex also inhibited the larval motility of the nematode *Haemonchus contortus* suggesting that *J. curcas* latex can be used to control gastrointestinal nematodes of small ruminants (Muhamad et al. 2017).

Regarding the isolated compounds from *J. curcas* latex, the proteolytic enzyme curcain showed wound healing properties in mice (Nath and Dutta 1991). The curcain accelerated the wound healing process by increasing the tissue granulation, wound contraction, and collagen and hydroxyproline levels.

When the cyclic peptides were biologically investigated, jatrophidin I presented a moderate aspartic protease inhibition activity, but antimalarial, antifungal, and antioxidant activities were not detected (Altei et al. 2014). Curcacycline A exhibited anti-inflammatory activity, inhibiting the activation of the classical complement pathway and the human T-cell proliferation (van den Berg et al. 1995).

Moreover, curcacycline A was active against *Bacillus subtilis* and *Pseudomonas aeruginosa*. Due to structural similarities, as highlighted previously, curcacycline B may present an immunosuppressant activity (Auvin et al. 1997). It has also been demonstrated that curcacyclines A and B protected sheep oocytes against heat stress through the modulation of the apoptosis pathway by altering the expression of apoptotic genes and suppressing reactive oxygen species (ROS) generation (Barakat et al. 2016).

Besides their pharmacological potential, the crude latex and the curcacyclines demonstrated toxicity in different biological systems. The latex cytotoxicity was demonstrated against human cell lines, such as HT-29 (colon adenocarcinoma) and Chang liver cell (cervix carcinoma) in a dose-dependent manner (Oskoueian et al. 2011). Moreover, toxic, cytotoxic, and genotoxic effects of *J. curcas* latex were observed on the *Allium cepa* model (Ciappina et al. 2017). Cytogenotoxic activity of *J. curcas* latex was also observed on mouse bone marrow, but, surprisingly, antigenotoxicity effect occurred in the presence of doxorubicin (Bailão et al. submitted). In addition, curcacyclines A and B did not show any mutagenicity in the Ames assay, even if both presented significant toxicity in the brine shrimp lethality assay and cytotoxicity against ovarian cancer cells (Insanu et al. 2012).

These data indicate that more cellular models should be tested to characterize the *J. curcas* crude latex and its compounds regarding their biological activity and safety, once they could be used as therapeutics in the future. Moreover, the *J. curcas* latex dual role (mutagenic and antimutagenic) should be deeply investigated to safeguard the well-being of patients, since there is a lack of data on herb-drug interactions (Bailão et al. submitted).

21.6 Nanotechnological Applications of *J. curcas* Latex

Nanotechnology has become an important issue for scientific exploration in different technological areas such as catalysis, energy conversion, optical and electronic devices, nano-drug delivery systems, medical and antimicrobial preparations (Daniel and Astruc 2004; Peer et al. 2007; Sun et al. 2008; Penon et al. 2017). Nanotechnology employs nanostructures in the nanoscale range (1–100 nm) that can exhibit unusual physical, chemical, and biological properties different from the characteristics of the bulk phase. These properties are directly related to nanostructure size and shape (El-Sayed 2001).

Recently several synthetic protocols have been developed to obtain nanoparticles. Generally, they are prepared by a variety of methods, which are quite expensive and use reactants that are hazardous to the environment. In addition, surface passivation reagents may be required to avoid agglomeration of nanoparticles, which can also be toxic to the environment. Biosynthesis of nanoparticles has received considerable attention as an eco-friendly route; these novel routes are called green synthesis. Also, by means of biosynthetic routes, it is possible to obtain nanoparticles with a better morphology and with a better defined size than some other chemical and physical methods (Raveendran et al. 2003; Hutchison 2008). In this sense, microorganisms, plant extracts, fruits, and others (Singaravelu et al. 2007; Guidelli et al. 2011; Luangpipat et al. 2011; Rajesh et al. 2012; Mittal et al. 2013) have been used as a substrate to produce nanoparticles free of environmental contamination.

A novel green route to biosynthesize silver nanoparticles (AgNPs) employing *J. curcas* latex was originally reported in 2009 (Bar et al. 2009). The authors presented an important alternative to chemical synthesis, since this procedure is pollutant-free and a bio-friendly synthetic route. The mixture containing diluted latex and aqueous silver nitrate solution was prepared and produced silver nanoparticles with diameters in the range of 20–40 nm with mostly spherical shapes and other larger uneven shapes. The smaller particles reduce Ag^{+} to Ag^{0} and stabilize Ag nanoparticles in situ by the amide group within the cavity of the cyclic peptides (curcacyclines A and B), and the larger ones with uneven shapes are reduced and stabilized by the curcain enzyme.

Patil et al. (2012) reported the one-step solvent-free synthesis of AgNPs using latex from a few Euphorbiaceae plants and assessed their potential as antimicrobial agents. AgNPs synthesized by *J. curcas* latex presented an average size diameter of about 73 nm and zeta potential of −25.2 mV. Biochemical contents of the dried latex were evaluated, and proteins, proteases, saponins, phenolics, terpenoids, and alkaloids were found. AgNP solutions showed stability for over 90 days, which may be attributed to their negative zeta potential. The long stability was attributed to the electrostatic repulsion between the negatively charged particles, which avoids their agglomeration and gives stability to nanoparticles. AgNPs showed high antimicrobial activity against *S. aureus* and *E. coli* that are Gram-negative and Gram-positive bacteria, respectively. These findings may be valuable for application of AgNPs as enzyme inhibitors, biocontrol agents, and medical as well as antimicrobial preparations.

Kumar et al. (2017) reported another route for the synthesis of a stable silver nanocolloid using *J. curcas* latex. The nanoparticles obtained were 47 nm in diameter, on average, with high stability even after 1 year of storage and a low cytotoxicity against red blood cells. The nanocolloid exhibited a low minimum inhibitory concentration (MIC) against planktonic *C. albicans* and also low minimum biofilm eradication concentration against *C. albicans* biofilm, suggesting its uses as a nano-drug to combat fungal infections.

The low-cost green synthesis of lead, ZnS, and TiO_2 nanoparticles employing *J. curcas* latex has been described (Joglekar et al. 2011; Hudlikar et al. 2012a, b). Using an aqueous extract of latex in the range of 0.3–0.5%, monodisperse of ZnS and lead nanoparticles were obtained, measuring 10 nm and 10–12.5 nm,

respectively. On the other hand, larger size TiO_2 nanoparticles were obtained in the range 25–100 nm. The authors also noted that the cyclic peptides (curcacyclines A and B) possess a crucial role in reducing as well as stabilizing nanoparticles. ZnS semiconductor nanoparticles are formed by reduction of zinc acetate to Zn and then ZnS by the combined action of curcacyclines A and B and curcain. The latex acts as a source of sulfide ions that are donated to zinc ions. Residues of cysteine or thiol present in curcain may be donating the sulfide ions. Carbonyl groups from amino acid residues and peptides of protein reduce $TiO(OH)_2$ to TiO_2 and Pb^{+2} to Pb^0. TiO_2 nanoparticles could find potential applications in biomedical, bioengineering, electronic, and environmental systems; ZnS nanoparticles are attractive for applications in areas such as optical devices and fast optical switching devices, while lead nanoparticles could be applied in lead batteries, catalysis, etc.

Latex from *J. curcas* was also used to produce stable gold nanoparticles (AuNPs) (Patil et al. 2016). AuNPs presented a crystalline nature with irregular shapes and sizes ranging from 20 to 50 nm. The authors observed that capping latex metabolites (mainly proteins) around a nanosurface plays an indispensable role in the synthesis and stabilization of AuNPs, preventing aggregation and maintaining the AuNPs in a stable colloidal state for a long time. AuNPs inhibited the enzymatic activity of trypsin, the most important protease in animals and insects, essential for digestion, for insecticide resistance, and in several disease conditions. The authors suggested that AuNPs bind to trypsin at its catalytic site and inhibit its activity by disorganizing its tertiary structure. The inhibition of trypsin activity might serve as a potent agent in insect biocontrol.

21.7 Conclusions

The latex obtained from *J. curcas* has been used as a novel route for low-cost green synthesis of nanoparticles due to its native compounds acting as reducing or capping agents that prevent aggregation of particles and increases their stability as well as their long-term activity. Although the properties of *J. curcas* latex presented in this chapter suggest its potential uses in several nanotechnological applications, these scientific investigations are quite recent, and the potential of these techniques is explored to a limited extent. Thus, in the near future, new developments in bio-based products may emerge and add commercial value to *J. curcas* and thereby improving the sustainability of the crop.

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Chapter 22
The Characterization and Technologies for the Use of *Jatropha curcas* L. By-Products as Energy Sources

Sergio Peres

Abstract This chapter reports on the characterization of the by-products of oil extraction in *Jatropha curcas* L. and their usage as energy sources. The biomass and the technology, such as thermoconversion (combustion, pyrolysis, and gasification) and biological routes (anaerobic digestion and alcoholic fermentation), available to convert these by-products in energy and other types of fuels, are presented. The oil extracted from seeds accounts for ~35.1% of their dry weight. The de-oiled cake presented the following characteristics: 8.64% moisture, 39.38% cellulose, 21.18% hemicelluloses, 9.67% lignin, 93.55% volatiles, 0.86% fixed carbon, and 5.59% ashes. The higher and lower heating values (HHV and LHV) were 23.08 MJ/kg and 22.87 MJ/kg, respectively. The de-oiled press cake pyrolysis was performed in a bench-scale fixed bed pyrolyzer at 700 °C, 800 °C, and 900 °C in argon atmosphere. Pyrolysis gaseous fuel showed high hydrogen and methane yields, ranging from 41.10% to 43.47% (mol/mol) and 21.82–23.71% (mol/mol), respectively. The lower heat contents of the gases produced by pyrolysis were 16.76, 16.43, and 16.53 MJ/m^3 at 700, 800, and 900 °C, respectively. A total energy balance of the *J. curcas* products and by-products was performed, and the results showed that the best energy route was to produce biodiesel from seeds and energy from direct combustion of residual biomass to generate heat. However, other routes can be used depending on the applications.

Keywords By-products · Energy sources · Feedstock · Pyrolysis

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22.1 Introduction

The worldwide use of *Jatropha curcas* L. (referred to as JCL hereafter) to produce biodiesel is not widespread yet. However, the interest in using JCL seeds to produce biodiesel has been increasing because they contain approximately 35% of nonedible oil. In addition, JCL is a perennial plant, resistant to drought, and able to grow in marginal lands; hence, it is seen as an oilseed that should not compete with other oleaginous crops. According to Drumond et al. (2016), JCL seeds have oval geometry with their size ranging from 1.5 to 2.0 cm in length and from 1.0 to 1.3 cm in width. Their oil content varies from 33% to 38% of the seed weight (Jourabchi et al. 2014). The objective of this work was to characterize the JCL residual biomass as well as the composition and heating values of the gases formed when the cake is pyrolized in a bench-scale fixed bed at 700, 800, and 900 °C in an argon gas atmosphere (Silva and Peres 2017).

22.2 Feedstock

JCL seeds were supplied by EMBRAPA – Cerrados, located in Brasilia-DF, Brazil. The experiments were carried out at the Fuel and Energy Laboratory of the University of Pernambuco, Recife, state of Pernambuco in the northeast of Brazil. To prepare the biomass for its characterization through pyrolysis, the oil was extracted mechanically in a manually driven hydraulic press MARCON MPH-60. Then the residual oil of the press cake was chemically extracted with hexane in a Soxhlet apparatus. The resulting cake was grounded using a knife mill MARCONI, model MA-48, and then sifted through an internal mesh 20 (0.841 mm) screen. Moisture, volatiles, fixed carbon, and ash contents were determined using a Shimadzu TGA/DTG-60 according to the standard method ABNT NBR 8.112/1986. The cellulose, hemicellulose, and lignin contents were determined following the Van Söest and Robertson method (1991). The heating values were obtained using an IKA WERKE C2000 calorimeter based on the ABNT NBR 8.633/1984 method. All the experiments were done in triplicates.

22.3 Pyrolysis

The pyrolysis rig apparatus consisted of a stainless steel reactor, electrically heated by an external source, in which dimensions were 260 mm (length), 51 mm (internal diameter), and 5 mm (wall thickness) (Fig. 22.1). The resistances of the heat source (3000 W, 220 V) consisted of two electrical bracelets made of Nikrothal® 80 by Kanthal (Brazil). The wire characteristics of these resistances were a diameter of 6 mm, a maximum operating temperature of 1200 °C, and a resistivity at 20 °C, of 0.9 Ω (mm^2/m). The temperature was measured using a thermocouple type K.

Fig. 22.1 The POLICOM bench-scale pyrolysis rig. Source: Silva los and Peres (2017)

22.4 Oil and By-products

No seed de-shelling was performed prior to the analysis in order to obtain the rough energy generated by the whole product. Subsequently, this total energy was divided into its by-product components, following an existing literature survey. Figure 22.2 is an attempt to represent the JCL by-products that result from biodiesel production. According to several authors, the seed weight varied from 59% to 79% of the dry fruits, and the oil extracted by means of press and solvent represent about 25% and 38% of the dry seed weight, respectively. Oil is the product of highest added value because it is used to produce biodiesel by transesterification and/or esterification. Seeds also contain about 36.6–42.0% husk and 58.0–63.4% kernels (Martin et al. 2010; Nithiyanantham et al. 2012; Pandey et al. 2012; Lim et al. 2015; Drumond et al. 2016). According to the extraction results of our extraction experiments performed at the Fuel and Energy Laboratory (POLICOM-UPE), we found a rate of JCL oil of 13.75% by mechanical pressing and of 21.34% by additional solvent extraction of the press cake, which sum up to 35.09% of the seed weight. This oil extraction rate is in accordance with the reported values as outlined above. Thus, besides the nonedible oil that can be used to produce biodiesel, there are three by-products, shells, press cake, and glycerin, that can be used as energy sources (Fig. 22.2).

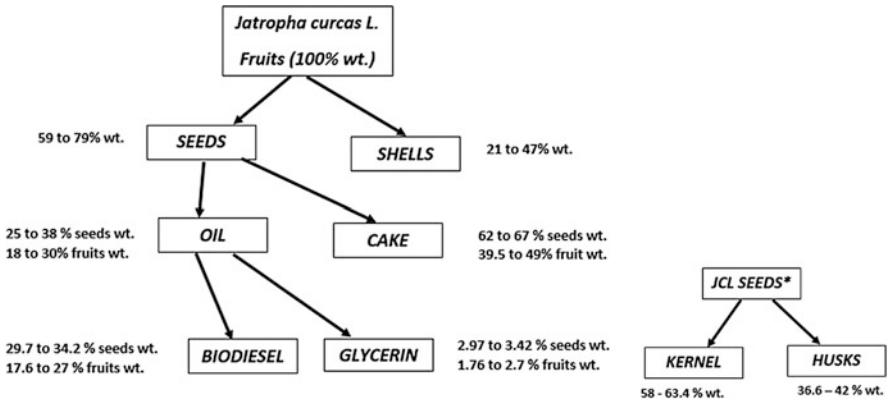

Fig. 22.2 From JCL seeds to biodiesel. Origin of by-products

22.5 Quantification of the Energy Released from By-products

This section presents the data from the literature and the results obtained from JCL by-product characterization using the POLICOM.

22.5.1 Calorific Value

The heating value is the amount of heat released during combustion of a specified amount of a substance. It can be expressed in terms of higher heating value (HHV) and lower heating value (LHV) in MJ/kg. The HHV, also known as gross calorific value (GCV), is the energy necessary to bring all products of combustion back to the original pre-combustion temperature, which is typically 15 °C. LHV is determined by subtracting the heat of vaporization of the water from the HHV. LHV assumes that the water component of a combustion process is in vapor state at the end of combustion, as opposed to HHV, which assumes that all of the water generated as product of the combustion process is in a liquid state after the combustion is completed. Thus, LHV is the actual energy that can be obtained when combusting the fuel. The data are illustrated in Table 22.1.

The product with the highest added value of the JCL fruits is obviously the biodiesel with an energy content that ranges from 39.23 to 41.72 MJ/kg, which is almost twice the energy content of the residual biomass. These results were also found in the literature (Kumar and Sharma 2008; Oliveira et al. 2009; Nithiyanantham et al. 2012; Jourabchi et al. 2014; Gonzáles 2016). The JCL biodiesel energy content represents between 93.4% and 99.3% of the fossil diesel (42 MJ/kg), and it is much higher than the energy content of any other by-product.

Table 22.1 Heating values of the JCL products

Feedstock	Seed[1]	Shells[2]	Husks[3]	Oil[4]	Biodiesel[5]	Bio-oil[6]	Cake[7,8]	Cake biochar[9]
HHV (MJ/kg)	17.2–24.0	14.5–17.2	14.3–16.93	36.7–40.7	37.8–42.05	15.1	19.1–21.44 23.08[8]	26.1
LHV (MJ/kg)	NA	NA	NA	NA	NA	NA	19.6–22.9[8]	NA

Sources: [1]Staubmann et al. (1997), Openshaw (2000), Kongkasawan et al. (2016); [2]Vale et al. (2001), Openshaw (2000), González (2016); [3]González (2016); [4]Staubmann et al. (1997), Jongschaap et al. (2007), Kumar and Sharma (2008), Oliveira et al. (2009), González (2016); [5]Kumar and Sharma (2008), Oliveira et al. (2009), Nithiyanantham et al. (2012), Jourabchi et al. (2014), Gonzáles (2016); [6]bio-oil is a liquid fuel produced from biomass pyrolysis (Jourabchi et al. 2016); [7]Vale et al. (2011), Openshaw (2000), Jourabchi et al. (2014), Kongkasawan et al. (2016); [8]experimental data obtained at the Fuel and Energy Laboratory (POLICOM); [9] Vale et al. (2011)
NA not available

Table 22.2 Proximate analysis data (% by weight)

Feedstock	Seed[1]	Shells[2]	Husks[2]	De-oiled cake[1,3]	De-oiled cake[4]
Moisture	7.5	12.35	10.75	3.31–6.5	8.64
Volatiles	77.1	68.73	71.04	70.98–73.0	93.55
Fixed carbon	9.4	16.38	24.99	19.72–11.03	0.86
Ash	5.9	14.88	3.97	5.99–9.20	5.59

Sources: [1]Kongkasawan et al. (2016); [2]Gonzáles (2016); [3]Jourabchi et al. (2014); [4]Results from POLICOM analysis

22.5.2 Proximate Analysis

Proximate analysis aims at the analysis of organic materials by thermal degradation in an inert atmosphere to obtain the values of moisture, volatiles, and fixed carbon. The ash content is determined using an oxidative atmosphere. The results are shown in Table 22.2.

22.5.3 Ultimate Analysis

The ultimate analysis aims at the determination of carbon, hydrogen, nitrogen, and oxygen contents by prescribed methods (Table 22.3).

22.5.4 Chemical Composition of Fibers

The chemical composition of the press cake fibers is illustrated in Table 22.4.

Table 22.3 Ultimate analysis results (% by weight)

Feedstock	Seed[1]	Shells[2]	Press cake[3]	De-oiled cake[4]
Carbon	53.7	48.29	45.75	43.3
Hydrogen	8.0	5.59	6.24	5.8
Nitrogen	4.5	0.59	3.56	5.0
Oxygen[a]	27.9	45.54	6.24	36.7

Sources: [1]Kongkasawan et al. (2016); [2]Wever et al. (2012); [3]Jourabchi et al. (2014)
[a]By difference

Table 22.4 Chemical composition of fibers from JCL press cake (% by weight)

Feedstock	Pressed cake[1, a]	Pressed cake[2]	Press cake[3, b]
Cellulose	48.9	44.8	56.5
Hemicellulose	13.0	12.3	29.7
Lignin	25.0	42.9	13.9
Cellulose C1	6.5	NA	NA
Aliphatic	6.5	NA	NA

Sources: [1]Jourabchi et al. (2014); [2]Grimsby et al. (2013); [3]Results from POLICOM analysis
[a]^{13}C NMR
[b]The fiber contents were obtained using the Van Söest and Robertson Method (1991)

22.6 Using JCL as an Energy Source

The main purpose of extracting a nonedible oil is to produce biodiesel, as it does not serve for food. However, the residual biomass of the oil extraction, such as shell, husks, press cake, and glycerin as well as the residual biomass of the agricultural management of JCL plantation, can be used as energy source in both biological and thermochemical processes. The technologies and by-products that can be used to produce energy using JCL as source of raw materials are shown in Fig. 22.3.

Several authors have been investigating the use of the residual JCL biomass for the production of energy (Peres 1997; Staubmann et al. 1997; Openshaw 2000; Jongschaap et al. 2007; Kumar and Sharma 2008; Martin et al. 2010; Vale et al. 2011; Visser et al. 2011; Nithiyanantham et al. 2012; Pandey et al. 2012; Wever et al. 2012; Grimsby et al. 2013; Gracia et al. 2014; Huerga et al. 2014; Jourabchi et al. 2014, 2016; Lim et al. 2015; Kongkasawan et al. 2016; Gonzáles 2016; Jablonski et al. 2017; Silva and Peres 2017). The options of JCL biomass recycling include combustion; pyrolysis to produce biochar, bio-oil, and combustible gases; as well as anaerobic digestion and alcoholic fermentation of the cake (Peres 1997; Staubmann et al. 1997; Kumar and Sharma 2008; Visser et al. 2011; Garcia et al. 2014; Huerga et al. 2014).

As illustrated in Fig. 22.3, all the JCL parts can be used as sources of energy. Thermoconversion technologies, such as combustion, gasification, and pyrolysis,

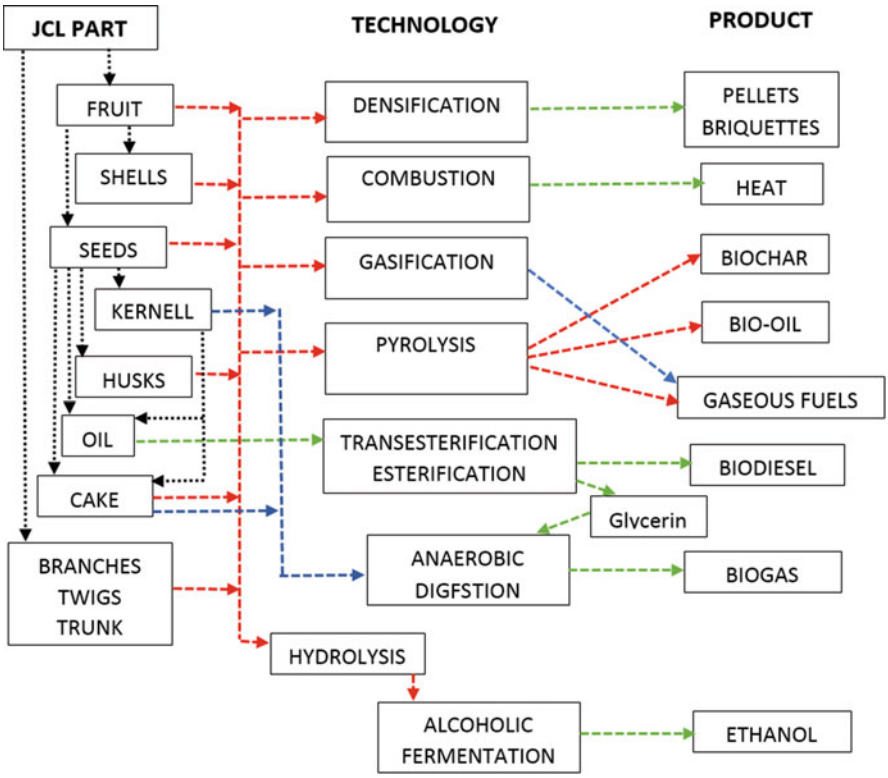

Fig. 22.3 Technology and products for the use of JCL as energy source

can use almost all types of JCL biomass to produce heat, biochar, bio-oil, and combustible gases. Besides, biomass with high cellulose content, sugar, and starch can be used in a hydrolysis process (either enzymatic or acidic saccharification) to be fermented for ethanol production.

Staubmann et al. (1997) reported that 1 kg of JCL seedcake can produce 355 L of biogas containing 70% methane. The nutshells from seeds only gave 37 L of biogas per kg of dry weight. Grimsby et al. (2013) reported that the press cake produced 289 L/kg VS methane with a methane concentration ranging from 60% to 65%. Similar results were obtained by Jablonski et al. (2017) with biogas (281 L/kg) produced using JCL press cake. Since methane has HHV and LHV of 35.6368 MJ/m^3 and 32.08431 MJ/m^3 (Peres 1997), respectively, 1 kg of press cake, if used in a biodigester, can provide 12.65 MJ (HHV) and 11.39 MJ (LHV). Huerga et al. (2014) reported that seedcake (meal) and glycerin produced 285 $L.kg^{-1}$ when a substrate in a proportion of 24.23% for glycerin and 75.76% for de-oiled cake diluted according to a ratio of 5.2 parts water for 1 part substrate.

Kumar and Sharma (2008) stated that the JCL wood burns too fast and for this reason is not popular as a fuel. In addition, these authors stated that the conversion of JCL seed shells into biochar is only feasible if there is a large amount of seed shells available. Further studies are needed to determine whether the JCL wood would be suitable for gasification and pyrolysis to produce gaseous fuel. According to Sotolongo et al. (2009), *J. curcas* wood is light, porous and soft, and not good for fuel as it burns quickly with energy content of 15.5 MJ/kg. It is commonly used as energy source in developing countries and has the potential to supply 1.3 PJ energy.

Visser et al. (2011) stated that cellulosic ethanol could be produced using by-products of oil extraction. In addition, the authors reported a theoretical potential of JCL ethanol production of 40 m^3 km^{-2} using enzymatic hydrolysis and fermentation. Garcia et al. (2014) reported a study on the conversion of JCL shells by means of acids and enzymes. However, in their studies, the shells to ethanol yields were not released.

Openshaw (2000) stated that the importance of using JCL residual biomass maximizes energy production if all by-products are not all used. In addition, Openshaw stated that in terms of weight unit, the kernel has 40% more energy than the whole fruit.

22.6.1 JCL Cake Pyrolysis

Pyrolysis is a thermochemical process that occurs in the absence of oxygen gas (Jourabchi et al. 2014). Pyrolysis produces biochar, liquid products (bio-oil), and gases in different proportions depending on the reactor temperature, as illustrated in Fig. 22.2. In most of the processes involving pyrolysis for the production of bio-oil, the temperature varies between 250 °C and 500 °C (Jourabchi et al. 2014); for production of combustible gases, as was the case for the work performed at the Fuel and Energy Laboratory (Recife), the temperature of the reactor ranged from 700 to 900 °C. The gas compositions at three heating temperatures are given in Table 22.5.

Table 22.5 shows that the gaseous fuels with highest LHV by volume were obtained at 700 °C. However, a more complete analysis in which the volume of gases generated at each temperature is still being performed. According to its pyrolysis investigations, Peres (1997) using sugarcane bagasse as a feedstock showed that the amount of gaseous fuel generated increased with temperature. The yield of gases for 700 °C was 0.36 m^3/kg, while it was 0.42 m^3/kg at 800 °C and 0.55 m^3/kg at 900 °C. Similar results would be expected for the JCL seedcake. For this reason, even though the heating value of the gaseous fuels was higher at 700 °C, it is expected that the total energy (LHV in MJ/m^3) produced by unit of press cake (m^3/kg) would be much higher performing pyrolysis at 900 °C. Hence, it could be concluded that the higher the temperature, the more energetic is the gaseous fuel produced by pyrolysis.

Other researchers have been investigating pyrolysis technologies that use temperatures between 400 °C and 550 °C to convert residual JCL biomass into bio-oils

Table 22.5 Pyrolysis of JCL press cake pyrolysis and gas compositions at three heating temperatures

Gases composition (% mol/mol)	Pyrolysis temperature (°C)		
	700	800	900
H_2	43.47	43.22	41.10
CH_4	21.82	21.38	23.71
CO_2	22.04	24.13	25.69
CO	4.90	4.20	3.50
C_2H_6	6.57	5.05	3.19
C_3H_8	0.45	0.95	1.73
n- C_4H_{10}	0.38	0.34	0.96
I- C_4H_{10}	0.38	0.73	0.12
HHV (MJ/kg)	78.39	77.34	74.98
LHV (MJ/kg)	67.27	66.33	64.37
HHV (MJ/m^3)	18.76	18.39	18.50
LHV (MJ/m^3)	16.76	16.43	16.53

and other products. Jourabchi et al. (2014) reported that flash pyrolysis of JCL cake, in an electrically heated fluidized bed reactor in nitrogen atmosphere, produced a maximum oil yield of 62.25% by weight at 500 °C and that this oil had a HHV of 19.66 MJ/kg (Jourabchi et al. 2014).

22.6.2 JCL Energy Balance

In order to assess an energy balance of JCL, it is necessary to determine the amount of oil that can be extracted from seeds and, then, to take into account the energy content of each of its by-product. Table 22.6 illustrates the JCL fruit energy balance for a 100 kg sample, using the mean value of triplicates.

The cake provides the highest energy outcome, even though the biodiesel (the second major total energy product) is the main product of JCL.

As illustrated in Fig. 22.2, there are several technology routes to produce fuels and energy using the JCL residual biomass. In Table 22.6, the total energy was determined using 3 separate routes: Route 1, total energy of seeds and shells without biodiesel production; Route 2, total energy of biodiesel and de-oiled cake and shells; Route 3, the total energy available in the JCL biodiesel and the de-oiled cake biogas and shells; and Route 4, the total energy of the JCL biodiesel and the press cake bio-oil and shells. The best technology route is Route 2 that produced 2463 MJ, Route 1 produced 2120 MJ, and the use of the cake for either biogas or bio-oil seemed to be the worse energetic option. However, the residue of the biodigestion process can be used as fertilizer in JCL plantations for improving their productivity.

Table 22.6 Energy balance of JCL by-products (100 kg sample)

JCL fruit	Mass (kg)	HHV (MJ/kg)	Energy (MJ)	Ratio to TE[a] 1 (%)	Ratio to TE 2 (%)	Ratio to TE 3 (%)	Ratio to TE 4 (%)
Seeds	66	24.0	1584	74.7	64.3	78.2	78.9
Biodiesel (BD)	24	39.9	958.6	45.2	38.9	47.3	47.7
Shells (S)	34	15.8	535.5	25.3	21.7	26.4	26.7
Cake (CK) for combustion	42	23.1	969.4	45.7	39.4	47.87	48.3
Cake for biogas (BG)	42	12.7	531.3	25.1	21.6	26.2	26.5
Cake for bio-oil (BO)	42	19.7	514.1	24.3	20.9	25.4	25.6
Total energy 1 (seeds + shells)	100	21.2	2120	100	86.1	104.7	105.6
Total energy 2 (BD+CK+S)	100	24.6	2463	116.2	100	121.6	122.6
Total energy 3 (BD+BG+S)	100	20.3	2025	0.96	82.2	100	100.8
Total energy 4 (BD+BO+S)	100	20.1	2008	0.95	81.5	99.2	100

[a]TE is for total energy

22.7 Conclusion

To conclude, every part of the JCL can be used to produce energy. Discarding of any part implies in losing its corresponding available energy. There are several routes that can be used in order to use all JCL parts. Even though the JCL biodiesel is the most valuable and much needed product, it is not the one that carries the highest level of energy. The seedcake is the most energetic part of the JCL biomass. The JCL parts can be used as feedstock in thermochemical or biological processes. The most common use of the residual biomass as feedstock is through combustion to provide heat. However, the residual biomass can be used to produce more advanced fuels such as bio-oil, biochar, and gaseous fuels. The use of cake, kernel, and glycerin in anaerobic digestion can produce a significant amount of biogas that can be used in the JCL biodiesel industry. In addition, the lignocellulosic residues can produce ethanol through enzymatic and acidic saccharification hydrolysis. Hence, the use of the JCL residual biomass for energy production is a way to reduce operational costs and to reach environment and energy sustainability.

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Part VI
Processing

Chapter 23
Biodiesel: A Survey on Production Methods and Catalysts

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Abstract Biodiesel is a biodegradable biofuel, which is non-toxic and free of sulfur and aromatics. In addition, the CO_2 emitted during combustion is absorbed by plants during photosynthesis. It is a substitute for petroleum diesel and can be used without the need for the adaptation of existing engines and presents good lubrication capacity, prolonging the life of the engine and reducing the need for maintenance. The direct use of vegetable oil as biofuel is not applied due to its high viscosity and low volatility; the transesterification appears as the most used method for the production of biodiesel. The reaction of biodiesel production can be carried out with acidic, basic, or enzymatic catalysts, in addition to using homogeneous or heterogeneous catalysts. This chapter lists the different types of catalysts used in current scientific work, along with their advantages and disadvantages.

Keywords Biofuel · Catalytic biodiesel · Transesterification · Triglycerides

23.1 Introduction

Biodiesel is a biofuel derived from renewable biomass for use in internal combustion engines with compression ignition. It is a biodegradable and environmentally friendly fuel, registered in the Environmental Protection Agency (EPA) of the United States as fuel and fuel additive. It can be used in mixtures with petroleum diesel. According to the ASTM (American Society of Testing and

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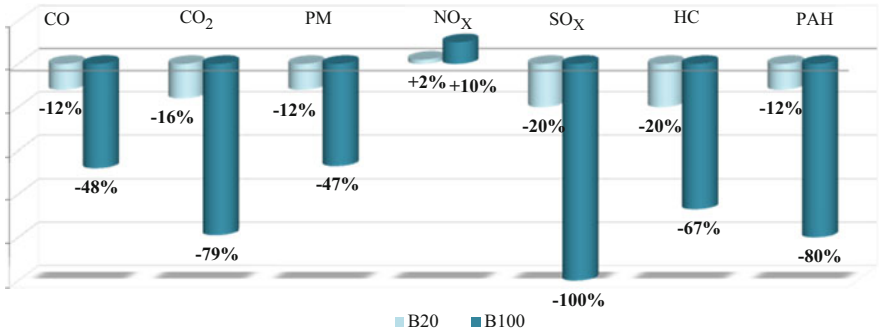

Fig. 23.1 Average emissions of biodiesel compared to diesel

Materials) D6751/2008, biodiesel is defined as mono-alkyl esters of long chain fatty acids, produced from vegetable oils or animal fats, for use in diesel cycle engines (Luque et al. 2010).

The use of biodiesel provides many environmental advantages. It reduces the emissions of carbon monoxide (CO) by 48%, carbon dioxide (CO_2) by 79%, particulate matter (PM) by 47%, sulfur oxides (SO_x) by 100%, hydrocarbons (HC) by 67%, and polycyclic aromatic hydrocarbons (PAH) by 80%. However, the emissions of nitrogen oxide (NO_x) are increased up to 10% when compared to petroleum diesel (Fig. 23.1) (Kiss et al. 2006).

Different raw materials can be used in the production of biodiesel, including non-edible oils such as *Jatropha curcas* L., and residual feedstocks, such as frying oil and tallow, which were previously discharged and are now used as raw material for the production of this biofuel. In addition, the price of waste cooking oil is 2.5–3.5 times less than the price of refined vegetable oils (Hasan and Rahman 2017).

The oil content in *J. curcas* seeds is approximately 300–400 g/kg. The presence of some toxic components, such as curcin and phorbol esters as well as free fatty acids, makes *J. curcas* oil not recommended for edible purposes. *Jatropha* oil contains, mainly, linoleic, stearic, oleic, palmitic, and arachidic acids, which can be converted to methyl esters through transesterification. Thus, the *Jatropha* oil presents an interesting and sustainable alternative for the production of biodiesel. Figure 23.2 shows the number of scientific papers published, since 2010, having *J. curcas* and biodiesel as keyword. Great interest in the topic is observed, in view of the increasing number of publications over the years, confirming the relevance of the subject (Kamel et al. 2018).

The high viscosity of vegetable oils and animal fats prevents their direct use as biofuel. However, this can be solved through chemical modifications. There are several techniques for producing biodiesel by transesterification via homogeneous, heterogeneous, or enzymatic catalysis, as well as under supercritical conditions. Esterification and hydroesterification are also alternative processes, especially when the fatty acid content of the raw material is high (Farobie and Matsumura 2017).

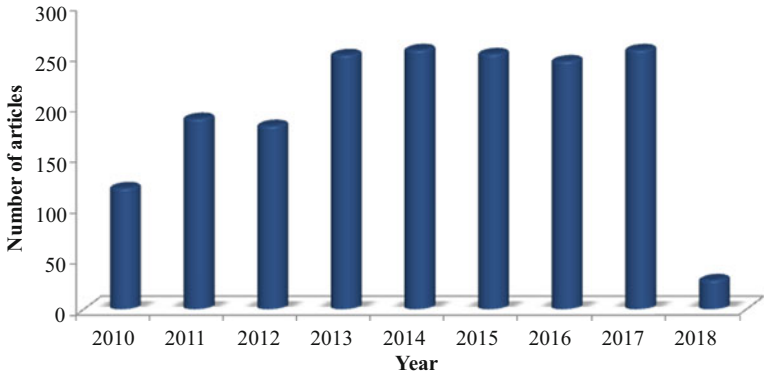

Fig. 23.2 The number of published scientific articles with *J. curcas* and biodiesel as keywords

23.2 Transesterification

Transesterification is the most used method for the production of biodiesel. The process consists of three reversible and consecutive reactions, in which monoglycerides and diglycerides are intermediates, yielding alkyl esters and glycerol (Borges and Díaz 2012).

Several factors influence the transesterification process such as the type of catalyst (basic, acid, or enzyme), molar ratio, temperature, reaction time, and purity of the reagents. Theoretically, 1 mole of triglyceride reacts with 3 moles of alcohol to afford 3 moles of the alkyl esters and 1 mole of glycerol. In practice, excess of alcohol is added to shift equilibrium and increase the yield of biodiesel. Figure 23.3 shows the transesterification of a triglyceride with methanol (Ma and Hanna 1999). The reaction preferably takes place with low molecular weight alcohols such as methanol, ethanol, propanol, or butanol. However, methanol is highly used because of its low cost, higher polarity, and easy separation from glycerol. The catalysts employed may be of acidic, basic, or enzymatic nature and can be homogeneous or heterogeneous (Aransiola et al. 2014).

Some undesirable reactions may occur during the production of biodiesel:

1. The presence of water in the reaction medium can lead to hydrolysis of the esters forming free fatty acids and, consequently, soap, which causes problems in the purification of biodiesel (Fig. 23.4).

2. Triglycerides with high concentration of free fatty acids neutralize the basic catalyst and form soap, reducing the yield of biodiesel (Fig. 23.5).

The production of biodiesel through base catalysis is the most attractive from an industrial point of view. The reaction is fast, and the catalysts used are cheap, widely

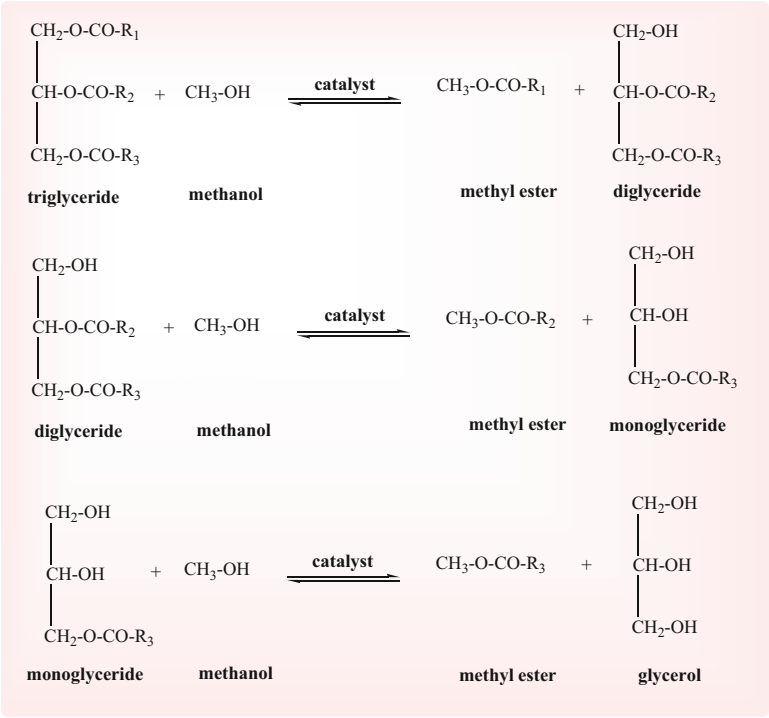

Fig. 23.3 Transesterification of triglycerides with methanol

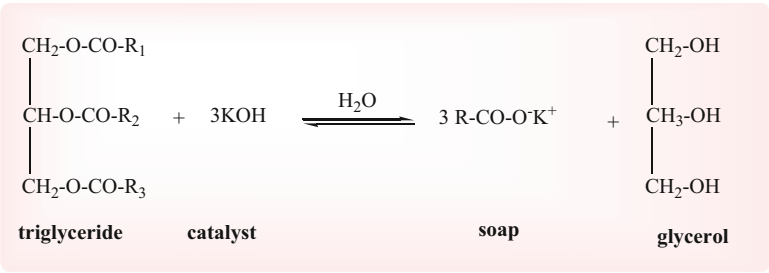

Fig. 23.4 Saponification of triglycerides

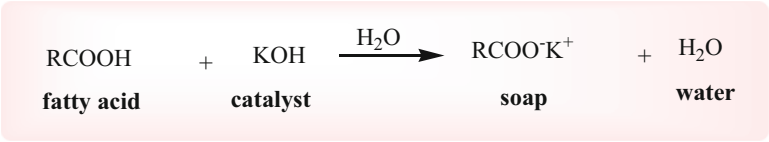

Fig. 23.5 Soap formation as a result of free fatty acid neutralization

available, and non-corrosive. Among them, sodium hydroxide (NaOH), sodium methoxide ($NaOCH_3$), and potassium hydroxide (KOH) are commonly used. However, base catalysis can only be applied in the presence of high-quality raw materials, because the presence of high levels of free fatty acids can neutralize the catalyst and promote soap formation. The content of free fatty acids and humidity are important parameters in the transesterification of plant oils and animal fats. They must be below 1%, because the higher the acidity of the raw material, the lower is the conversion to biodiesel (Helwani et al. 2009; Lee et al. 2014).

Acid catalysts are an alternative when raw materials with high levels of free fatty acids are used. It is possible to obtain high yields of biodiesel, but at usually longer reaction times. The acid-catalyzed transesterification is slower when compared to the base-catalyzed one. There are still problems associated with corrosion when using homogeneous acid catalysts. In addition, their removal from the reaction medium is problematic and expensive, requiring neutralization steps and laborious washing that results in the formation of emulsions and soap (Lotero et al. 2005).

Solid acid catalysts are insensitive to moisture and high FFA contents and can use cheaper and readily available raw materials without the need of pre-treatments. In this type of catalyst, transesterification and esterification can occur simultaneously. For use in industrial processes, the catalysts must have high stability under operating conditions and high selectivity and catalytic activity. There are several works in the literature that report the use of heterogeneous catalysts for the production of biodiesel, such as acidic zeolites, metal oxides, inorganic salts, ion exchange resins, heteropoly acids, and ionic liquids, among others (Su and Guo 2014).

The use of heteropoly acids supported on Nb_2O_5 as acidic solid catalyst in simultaneous esterification and transesterification of palm oil to produce biodiesel was studied (da Conceição et al. 2017). The catalyst based on $H_3PMo_{12}O_{40}$ (HPMo) supported on niobium oxide was prepared by impregnation. The simultaneous esterification and transesterification of palm oil was carried out at 700 rpm for 4 h. The maximum ester content (99.5%) was obtained at 200 °C, 120:1 ethanol/oil molar ratio, and 20 wt% of catalyst. The catalyst was reused in three successive cycles, just washing the material with tert-butyl alcohol between the cycles and drying at 120 °C for 2 h. The ester content gradually decreased achieving 88.6% after the third cycle. Deactivation may be associated with the leaching of the active phase, because the acidity of the material also decreased by 32% of the original one upon reuse.

Ghesti et al. (2007) studied the activity of HUSY and Ce/HUSY zeolites in the transesterification of soybean oil with ethanol for biodiesel production. The incorporation of cerium into the zeolite decreased the number of acid sites but resulted in greater structural stability compared with HUSY. The biodiesel production was carried out with alcohol to oil molar ratio of 30:1 and 0.001 mol of catalyst, at 1000 rpm, 20 bar, and 24 h at 200 °C. Under these conditions, the conversion was over 99% for both the zeolites. The catalysts were recycled after calcination in air at

550 °C during 8 h. Zeolite HUSY showed a decrease in conversion to 96.4% upon three reuses, whereas the zeolite Ce/HUSY practically maintained the same initial conversion (Borges et al. 2013; Mansir et al. 2017).

The use of enzymes gives high yields of biodiesel under mild conditions, with no parallel reactions. However, the process is expensive and some enzymes are inactive in the presence of methanol. Thus, this pathway is not normally used in industrial biodiesel production (Christopher et al. 2014).

The use of heterogeneous catalysts provides a number of advantages compared with the homogenous ones. They reduce purification steps, thus diminishing the effluents. In addition, they can be easily separated from the reaction medium and reused, also allowing the production of biodiesel in continuous flow operation. The use of heterogeneous catalysts for biodiesel production has considerably increased in the last decade. However, the synthesis of these catalysts is still somewhat expensive, and they usually deactivate upon reuse (Calero et al. 2014). Table 23.1 shows the advantages and disadvantages of each type of catalyst used in transesterification (Tan et al. 2013; Sbihi et al. 2015; de Lima et al. 2016).

The reuse of heterogeneous catalysts is a matter of extreme importance. Most of the materials mentioned in the literature undergo deactivation upon reuse, which are primarily caused by (i) leaching of the active phase and (ii) poisoning of the active sites by impurities in the raw material. In cases where the catalysts are resistant to high temperatures, regeneration can be carried out by heat treatment (Abdullah et al. 2017).

23.3 Esterification

Esterification is another process to produce biodiesel. The reaction takes place between a fatty acid, present in plant oil or even pure, and an alcohol to afford an alkyl ester (biodiesel) and water (Fig. 23.6). Esterification normally occurs via acid catalysis. This process is indicated for plant oils with high levels of free fatty acids, where base catalysis is virtually impossible. The reaction is reversible, but slower compared with base catalysis, requiring higher temperatures and longer reaction times to achieve good yields. Esterification can be catalyzed by inorganic acids, like sulfuric acid (H_2SO_4). It is a reversible reaction and an excess of one of the reactants, or the removal of water is normally undertaken to shift reaction equilibrium toward biodiesel accumulation (Liu et al. 2015).

Esterification is an alternative route when the free fatty acid content in the feedstock is high as in the case of *J. curcas* oil, residues of animal fat, and other low-quality raw materials. These sources have lower cost and reasonable availability. Thus, destination to biodiesel production is more convenient than recycling, because such materials are often disposed in inappropriate places (Melero et al. 2009).

Table 23.1 Advantages and disadvantages of various types of catalysts used in the transesterification of triglycerides

Type of catalyst	Examples	Advantages	Disadvantages
Basic and homogeneous	NaOH	Fast reactions and high conversion rates	Oils with less than 0.5% of free fatty acid (FFA)
	KOH	Mild reaction conditions	Formation of soap if the FFA content is greater than 2%
		Low costs	Laborious purification of the products: biodiesel and glycerol
		Widely available	
Basic and heterogeneous	CaO	Faster than acid catalysis	Sensitive to oils with high levels of FFA
	MgO	Easy catalyst separation	Formation of soap if the content of FFA is greater than 2%
		Possibility of catalyst reuse and regeneration	Leaching of catalyst active sites
		Reduces washing steps to purify the products	
Acid and homogeneous	H_2SO_4	Insensitive to free fatty acid and water content in the oil	Slower than base catalysis
	HCl	Use of low-quality triglyceride sources	More severe reaction conditions required
			Corrosion problems
			Problematic separation of catalyst from reaction medium
Acid and heterogeneous	ZrO_2	Insensitive to free fatty acid and water contents in the oil	Expensive synthesis procedures
	TiO_2	Use of low-quality triglyceride sources	Drastic reaction conditions
	SnO_2	Easy separation of the catalyst	Long reaction times required
	Zeolite	Possibility of catalyst reuse and regeneration	Leaching of catalyst active sites
Enzyme	*C. antarctica*	Insensitive to free fatty acid and water contents in the oil	Long reaction times required
	Mucor miehei	Use of low-quality triglyceride sources	High cost
		Mild reaction conditions	Sensitivity to methanol, capable of deactivating the enzyme
		Simple purification steps	

23.4 Hydroesterification

This process allows the use of any raw material, independent of the acidity and humidity they have. Tallow, vegetable oil, and used frying oil, among others, can be used as feedstock in hydroesterification (Aguieiras et al. 2014).

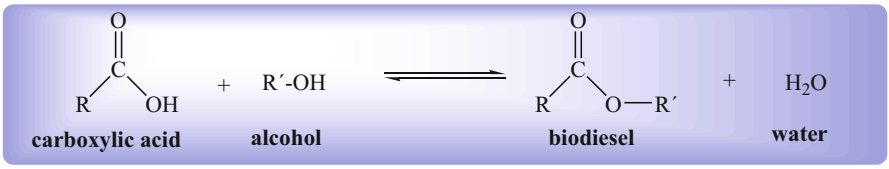

Fig. 23.6 Esterification of carboxylic acid

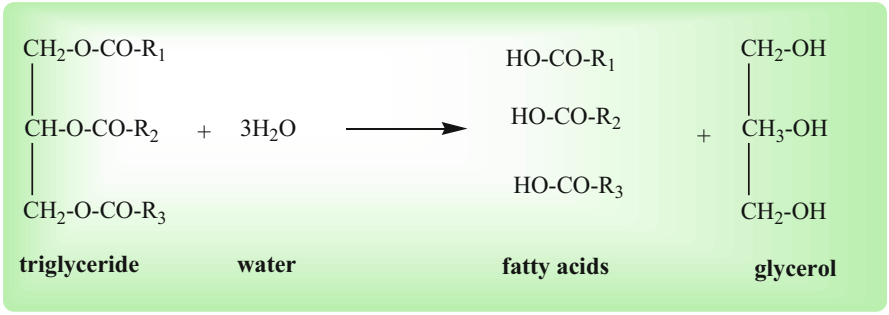

Fig. 23.7 Triglyceride hydrolysis

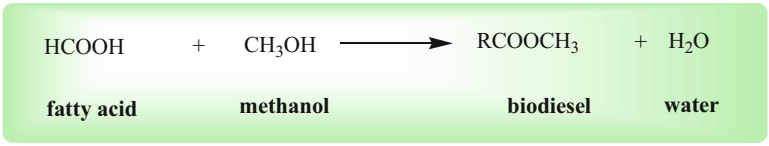

Fig. 23.8 Fatty acid esterification

The process consists of two consecutive reactions: hydrolysis followed by esterification. The hydrolysis consists of a chemical reaction between the triglyceride (oil or fat) and water, in the presence of a catalyst, affording fatty acids and glycerol (Fig. 23.7). After glycerol separation, fatty acids are reacted with an alcohol (methanol or ethanol), in the presence of an acidic catalyst, to yield an ester (biodiesel) plus water (Fig. 23.8). It is important to stress that in this process, there is no contact of the glycerol (already removed in the hydrolysis step) with the biodiesel (produced in esterification step). Only water is generated as by-product of esterification, and it is returned to the hydrolysis process. A high-purity biodiesel is obtained without the need of excessive washing procedures (Ngaosuwan et al. 2009; de Mello et al. 2017).

Hydrolysis can be accomplished with basic, acidic, or even enzymatic catalysis, whereas esterification is carried out under acid catalysis, as previously described. The process has some advantages compared with the traditional transesterification,

Table 23.2 Critical conditions of the alcohols normally used in supercritical transesterification

Alcohol	Critical temperature (°C)	Critical pressure (MPa)
Methanol	239	8.1
Ethanol	243	6.4
1-Propanol	264	5.1
1-Butanol	287	4.9

because it can use cheap raw materials, of high free fatty acid content, and yields - high-purity glycerol, which can be further commercialized for many uses, and only water is generated as a final by-product of esterification. However, a biodiesel production plant using hydroesterification technologies requires high initial investments, as two separated units need to be constructed: one to run the hydrolysis and another to run esterification, meaning more capital expenditure (de Mello et al. 2017).

23.5 Supercritical Conditions

The production of biodiesel under supercritical conditions is also known as a non-catalytic process, because the reaction occurs between the oil and the alcohol at high temperatures and pressures. The kinetics of the reaction is extremely rapid, and the separation of the products at the end of the reaction is facilitated due to the absence of catalysts, consisting only in the separation of glycerol and removal of the excess of alcohol (Minami and Saka 2006).

Transesterification under supercritical conditions is conducted at pressures and temperatures above the critical point of the alcohol, generally methanol or ethanol. Table 23.2 shows the critical temperatures and pressures of some alcohols (Shah et al. 2015).

Although promising, high temperatures and pressures are a disadvantage. To cope with the process conditions, cosolvents such as propane and carbon dioxide may be used. The commercial scale production of biodiesel using supercritical conditions requires specific equipment to support the high temperatures and pressures, usually in the range of 300–350 bar and 400 °C. These conditions increase the production costs, as well as the capital expenditure (Cao et al. 2005; Imahara et al. 2009).

Table 23.3 shows the comparison between the basic transesterification process and the supercritical method for the production of biodiesel from methanol and plant oil. As noted, the supercritical process leads to simplified purification procedures.

Table 23.3 Comparison between the processes of catalytic biodiesel and supercritical conditions

Reaction conditions	Catalytic	Supercritical conditions
Alcohol	Methanol	Methanol
Catalyst	Base (NaOH, $NaOCH_3$)	Absent
Temperature (°C)	30–65	Above 250
Pressure (MPa)	0.1	10–25
Esters (%)	96.5	98
Purification	Methanol, catalyst, glycerol, soaps	Methanol

23.6 Heterogeneous Basic Catalysts

The advance of the technologies to produce biodiesel involves the development of heterogeneous basic catalysts, which can be reused or run the process in continuous flow. Therefore, it is worthwhile to briefly discuss the most common heterogeneous basic catalysts being studied.

23.6.1 Metal Oxides and Mixed Oxides

There are several studies in the literature on the use of CaO as a catalyst for biodiesel production. High yields may be obtained under mild reaction conditions. However, CaO activity is affected by poisoning caused upon the contact of the catalyst with atmospheric air and CO_2, which promote the formation of $CaCO_3$ (Kesić et al. 2016). Calcination at high temperatures may regenerate the catalyst, decomposing the carbonate. Another problem is leaching, which leads to a gradual loss of activity (Banković-Ilić et al. 2017).

Colombo et al. (2017) studied the transesterification of soybean oil with methanol using commercial CaO as catalyst, 6:1 molar ratio of methanol to oil, 3 wt% of catalyst loading, and 75 min of reaction time at 65 °C, obtaining high yield of biodiesel. Nevertheless, the concentration of calcium in the biodiesel was 127.9 mg/kg, which is well above the limit of 5 mg/kg set by regulations (Colombo et al. 2017).

CaO/SiO_2 was synthesized by the sol-gel method and tested on the transesterification of corn oil with methanol, using 16:1 molar ratio of methanol to oil and 6 wt% catalyst loading at 60 °C for 8 h at 600 rpm, resulting in 85.6% conversion to biodiesel. The catalyst was reused in five catalytic cycles, washed with methanol, and dried at 90 °C before reuse. The conversion decreased to 62.6% after the fifth run, due to leaching of calcium upon reuse (Moradi et al. 2014).

Mixed oxides were prepared by impregnation of the respective metal oxide (Zn, Fe, Mn, and Al) over CaO. The catalyst was tested in transesterification of *Jatropha* oil with methanol to oil molar ratio of 12:1 and 5 wt% catalyst loading, at 65 °C and 500 rpm. The yield of the biodiesel ranged from 96% to 98%. The higher basicity of

the mixed oxides relative to pure CaO is due to the synergistic action of multi-metal ions that increase the basicity in the active site of the catalyst (Joshi et al. 2015).

Sudsakorn et al. (2017) synthesized a CaO/MgO catalyst doped with Sr^{2+}. The catalyst was prepared by the coprecipitation method and evaluated in the transesterification of *Jatropha* oil (initial acidity, 5.974 mg KOH per g of oil). Reactions were carried out at 65 °C for 2 h, using methanol to oil molar ratio of 9:1 and 5 wt% catalyst loading. The methyl ester produced at these conditions reached 99.6%. Between the reuse cycles, the catalyst was washed with cyclohexane, filtered, and dried at 140 °C for 4 h prior to reuse in the next reaction. The results showed a loss of catalytic activity from 99.6% to 91.2% in the fourth cycle, due to the leaching of Sr and Ca. Comparison of the synthesized Sr^{2+}/CaO/MgO catalyst (methyl ester content, 99.6%) with CaO/MgO (methyl ester content, 6.1%) showed that Sr^{2+} played a key role in the catalytic performance.

23.6.2 Hydrotalcites

Hydrotalcites are natural or synthetic materials with a layered structure. The general formula is $[M^{II}_{1-x} M^{III}_{x} (OH)_2]^{x+} A_{x/n}{}^{n-} \cdot n\, H_2O$, where M^{II} accounts for a metallic divalent cation such as Mg, Co, or Zn; M^{III} accounts for a metallic trivalent cation such as Al or Fe; A^{n-} is an inorganic or organic anion such as carbonate, chloride, nitrate, adipate, or malonate; and $x = M^{II}/(M^{II} + M^{III})$ ranges from 0.1 to 0.5 (Hájek et al. 2017; Sikander et al. 2017).

These materials have been widely used as catalysts for biodiesel production due to their basicity. Thermal treatment before use leads to the loss of water molecules and decomposition of hydroxide and carbonate ions, leading to the formation of mixed oxides. The strong interaction and the synergistic effect between the elements explain the good stability of this type of catalyst.

Nowicki et al. (2016) prepared Zr-dopped Mg-Al hydrotalcites with different Zr/Mg molar ratios. The catalysts were prepared by the coprecipitation method, calcined at 500 °C for 3 h, and tested on the transesterification of rapeseed oil. Biodiesel yield of 88% was observed using methanol to oil molar ratio of 12:1, at 100 °C, 8 wt% catalyst loading, and 6 h. The modification in the structure of the Mg-Al hydrotalcite lamellae with Zr^{4+} tetravalent cation significantly increases the catalytic activity of the material, leading to 99.8% conversion.

Hydrotalcites are susceptible to deactivation, and one of the possible causes is the poisoning of the active sites by the products of the reaction. Another possibility is the loss of active cation sites (Zr^{4+}, Mg^{2+}, and Al^{3+}), which causes a gradual destruction of the hydrotalcite structure. To evaluate the reusability of the catalyst, three catalytic cycles were carried out. The catalyst was washed with methanol and dried at 110 °C for 5 h between the cycles. The conversion decreased from 99.5% to 54.2% (Nowicki et al. 2016).

Di Serio et al. (2012) evaluated commercial Mg/Al hydrotalcite as catalyst in the transesterification of soybean oil in a continuous packed bed reactor. Before the

reaction, the catalyst was calcined at 400 °C for 18 h. The catalytic tests were performed at 220 °C, 55 bar of pressure, a feed rate of soybean and methanol of 2.6 cm^3/min each, 27.4 g of catalyst, and residence time of 6 min. A rapid deactivation of the catalyst was observed; after 15 h, the yield of biodiesel dropped from 85–80% to 60–65%. To verify the possibility of regeneration of the catalyst, a stream of pure methanol at 100 °C and then acetone at 70 °C were fed during 4 h each. The regenerated catalyst presented similar catalytic activity to the fresh one.

In the absence of calcination, the hydrotalcites do not present catalytic activity for transesterification. The calcination temperature is an important parameter that affects the basicity of the catalyst as well as the accessibility. The activity of hydrotalcites can be completely restored after use, upon washing with solvent to remove the residual triglycerides followed by a new calcination procedure (Liu et al. 2007).

Helwani et al. (2013) produced biodiesel from the *J. curcas* oil using synthetic hydrotalcite recrystallized from mixed oxides. The conversion to biodiesel was 75.2% when the reaction was carried out at 65 °C, with methanol to oil molar ratio of 12:1, at 6 h and 4 wt% of catalyst loading. The biodiesel produced from *J. curcas* oil met the required biodiesel standard by ASTM D6751.

The catalyst was tested for reuse in subsequent reactions. It was verified that prolonged reaction times cause strong adsorption of triglycerides on the surface of the catalyst, but the activity of the hydrotalcites was completely restored by re-calcination after extensive washing with methanol to remove the residual triglycerides. Moreover, leaching of the Mg^{2+} and Al^{3+} species was not observed, since the elemental analysis showed that the Mg/Al ratio remained unchanged before and after the transesterification (Helwani et al. 2013).

23.6.3 Hybrid Materials

Hybrid materials are formed by the combination of organic and inorganic moieties, resulting in materials with unique characteristics and properties that are not found in the individual parts. Such singular properties are not the result of adding the organic molecule into the inorganic support, but rather due to the synergisms between the two systems at molecular level. There are several works in the literature on the functionalization of organic compounds, such as amines, on various inorganic supports. High functionalization with uniform distribution of functionalized groups is necessary to warrant large catalytic performance materials (Minella et al. 2017).

Mesoporous silica nanoparticles such as MCM-41 (Mobil Composition of Matter number 41) and SBA-15 (Santa Barbara Amorphous number 15) have gained considerable attention in recent years. They are mesoporous materials formed by siloxane groups in the interior and silanol groups on the external surface that are responsible for the silica reactivity. In addition, they are considered excellent supports for heterogeneous catalysts due to the hexagonal matrix of uniform pore size, high surface area, and thermal stability (Fig. 23.9) (Xie and Fan 2014).

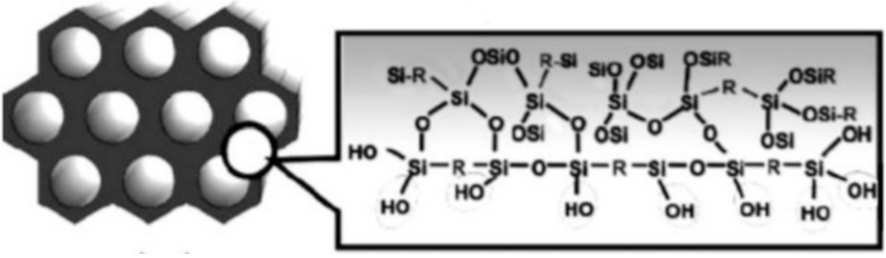

Fig. 23.9 Organized pore structure of the MCM-41 (Islam et al. 2013)

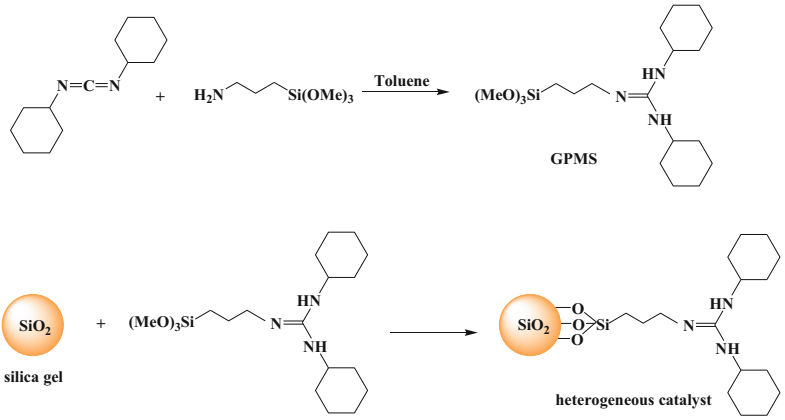

Fig. 23.10 Synthesis of the silica gel/GPMS catalyst

Balbino et al. (2011) synthesized a novel silylating agent through the reaction between 3-aminopropyltrimethoxysilane and dicyclohexylcarbodiimide, as shown in Fig. 23.10. This new organosilane (GPMS) was covalently linked to silica gel and used as a heterogeneous catalyst (silica gel/GPMS) in the transesterification of soybean oil at 80 °C for 3 h, achieving 99% yield of biodiesel. This catalytic system was transposed to a semi-pilot scale in a continuous flow reactor. The best results were found at 126 °C with 20:1 molar ratio of methanol/oil, resulting in 44% conversion to biodiesel. Through calculations of the energy of activation, the author suggested that the reaction is likely under mass transfer limitations.

1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) was anchored on the surface of MCM-41 by the post-synthesis method, as shown in Fig. 23.11. The post-synthesis method favors the functionalization on the external surface and pore entrance of the support. These materials were tested in the transesterification of soybean oil, yielding 99% of biodiesel after 3 h of reaction under reflux, using a 9:1 molar ratio of methanol/oil and 0.1 g of catalyst (de Lima et al. 2014).

The reuse indicated a gradual loss of catalytic activity with no conversion after five consecutive cycles. Elemental analysis of the catalyst after the cycles showed loss of 20% of the nitrogen atoms, indicating a partial leaching of the active phase.

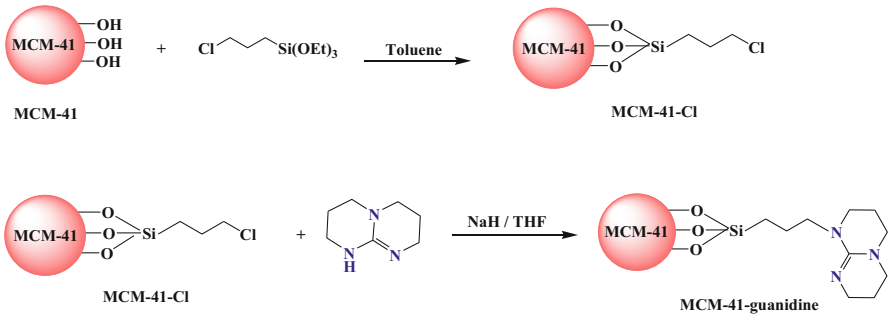

Fig. 23.11 MCM-41 functionalized with TBD

Notwithstanding, the loss of catalytic activity cannot be completely assigned to leaching. The neutralization of the basic sites by the free fatty acids present in the oil plays a considerable role, and it was assumed to be the main cause of the catalyst deactivation (de Lima et al. 2014).

In a later work, the same catalyst was synthesized by the co-condensation method, where the TBD firstly reacted with (3-chloropropyl)triethoxysilane and the resulting compound was included in the synthesis medium of the MCM-41 silica material. Co-condensation leads to a better dispersion of the base on mesoporous silica, with functionalization at the inner and outer surface. The catalyst was tested in the transesterification of soybean oil with methanol at 70 °C, 1:9 oil to methanol molar ratio, and 0.15 g of catalyst loading, achieving 99% yield of biodiesel within 2 h (de Lima et al. 2017).

The reuse of the catalyst was carried out without any pre-treatment between the cycles. A gradual loss of activity was observed after each run culminating to a biodiesel yield of 31% after the fifth cycle. Chemical analysis indicated a loss of 18.5% of nitrogen atoms after five catalytic cycles, similar to what was found when the catalyst was prepared by post-synthetic method, indicating some leaching of the active sites. Again, neutralization of the active sites by the free fatty acids of the oil may be the main cause of deactivation. However, the loss of activity was lower compared to that observed with the catalyst prepared by post-synthesis, possibly because of the better dispersion of the amine on the surface of the mesoporous silica (de Lima et al. 2017).

Xie et al. (2015) synthesized a heterogeneous basic catalyst functionalizing SBA-15 with 1,3-dicyclohexyl-2-octylguanidine (DCOD) by the post-synthesis method. This material was tested on the methyl ester transesterification of soybean oil at 65 °C for 15 h, using 8 wt% catalyst loading and 15:1 of methanol to oil molar ratio, obtaining a maximum conversion of 92.6%. Nevertheless, the activity of the catalyst also decreased upon reuse.

23.7 Conclusions

The main commercial technology to produce biodiesel from plant oils and fats uses transesterification with methanol in the presence of a homogeneous basic catalyst. Reaction conditions are relatively mild, and the biodiesel yield is above 96.5%, within a couple of hours. Nevertheless, this technology has some shortcomings, which are (i) a moderate level of glycerol purity; (ii) the need of extensive washing steps in the biodiesel in the transesterification process, resulting in huge amounts of waste; and (iii) the impossibility to reuse the catalyst, which is lost in the glycerol phase. Acid catalysis is significantly slower and requires more drastic conditions. In addition, only moderate conversion rates are achieved, which yield to a product out of specification. Enzymatic catalysis is still expensive and time-consuming.

The future of biodiesel technology relies on the development of heterogeneous catalysts, especially of basic nature, capable of working on continuous flow. However, before this technology could be commercially available, development of more stable catalysts, resistant to deactivation and leaching, is of prime importance and must be the focus of future researches.

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Part VII
Socio-Economic Sustainability

Chapter 24
Economic Feasibility and Sustainability of Jatropha as a Crop

George Francis

Abstract *Jatropha curcas* L. has the potential to be a suitable source of biofuel feedstock because of its favourable physical and chemical properties. All reports till date show that jatropha oil can reduce greenhouse gas emissions of up to 82% as opposed to fossil fuel indicating the ecological value of the jatropha system. Jatropha cropping has also been shown to be energy efficient, i.e. it results in production of more energy than it uses. Its cultivation has, however, not gained momentum in the last years due to plantation productivity concerns and the general lack of new investments in the biofuels sector. This chapter attempts to determine the intrinsic value of the various jatropha products relative to other comparable products now in the market. Based on this estimated intrinsic value of jatropha seeds, it could be deduced that a seed productivity of between 2000 and 4300 kg per ha per year from mature jatropha plantations would be required to reach crop profitability depending on framework conditions existing at the cultivation site. The main factors that influence the required range of yields for profitability are the selling price of oil at farm gate, the variety of jatropha (edible or toxic) cultivated and labour costs. Jatropha seeds improved by selection and breeding are required for reaching this range of seed productivity on the type of lands usually available for jatropha cultivation.

Keywords Biofuel · By-products · Profitability · Value addition

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24.1 Jatropha in the Context of Biofuel Market Development

Biofuels in general has been passing through a difficult phase over the last years. Following the spike in petroleum prices during 2006–2007, there was high interest in biofuels from the fuel market. This along with promotion by governments through public policy resulted in a surge of interest and investments during that phase. The rise in world food prices in 2007–2008, however, led to criticism that the increased use of grains and plant oil for the production of bioethanol and biodiesel is causing a shortage of these products in the world market leading to a rise in prices. This situation resulted in decreasing political support for biofuels, which together with the decline in crude oil prices resulted in decreased market attractiveness for biofuel alternatives. This market-oriented approach to biofuel might change as there is an increasing agreement on the non-sustainability of fossil fuel in the long term.

There is unanimity of opinion that maintaining enough food stocks and crop production areas at a national and global level to ensure nutritional security for the world population is the top-most priority. However, the production data from the last 10 years show that fears of increased biofuel production compromising world food security was highly exaggerated. According to the Renewables Global Status Report 2017 from the UNEP sponsored REN21 (2017), the world production of transport biofuels increased threefold from 45 million litres in 2006 to 135 million litres in 2016, making up 2.6% of the total transport fuel use in that year. UFOP (2018) estimated that biofuel production used about 11% of the total world food crop production in 2016 as raw material, mainly in the form of sugar cane, maize, wheat, sugar beet, palm oil, soy oil and rapeseed oil in that order of importance. And yet, the world market prices for agricultural commodities have either stagnated or declined over the last 10 years despite the larger demand they experienced from the biofuel sector. This is reflected in the commodity food price index (derived from various commodity food prices on international mercantile exchanges), which declined from 100.3 in December 2007 to 91.0 in December 2017 and the broader commodity agricultural raw materials index, which declined from 83.1 to 81.4 during the same period (Indexmundi.com 2018). It is being recognized that the demand from the biofuels sector in fact kept prices of select commodities (e.g. soybeans, palm and maize oils) from falling even further, in the face of overproduction relative to the stagnating demand from the food sector. The emergence of such data will have the effect of putting the food versus fuel debate on a more factual basis, which will benefit the biofuels sector in general.

24.2 Positive Characteristics That Would Promote Jatropha

Jatropha can play a positive role in this emerging situation as it has the potential to amplify the various positive aspects of biofuel production and use. The basis of the interest in jatropha has been due to its versatility as a multipurpose plant. Several studies have shown its adaptability to suboptimal soil and climatic conditions (see Basili and Fontini 2012; Lama et al. 2018 and cross references therein). Jatropha cultivation has also been shown to contribute to the reclamation of degraded land over time by preventing erosion and increasing the organic matter content of soils (Basili and Fontini 2012; Wani et al. 2012; Srivastava et al. 2014; Baumert et al. 2016). It is thus a potential candidate for cultivating marginal lands that are likely to be available and should be preferably used for biofuel production, namely, those that are not usually preferred for food crop production. Another advantage of jatropha is that it is not capital intensive. Its seeds can be easily stored and processed to produce oil. Jatropha oil has proven to be a high-quality fuel feedstock for the production of biodiesel and hydrotreated plant oil (used as renewable diesel or as bio-aviation spirit). Pure jatropha oil has also been successfully used as a fuel for operating automobiles and stationary engines with no or slight modification.

The greenhouse gas (GHG) and net energy balance of a fuel are important criteria for ascertaining its environmental sustainability. Especially the GHG balance has been made a condition for acceptance of a biofuel in many important markets (e.g. European Union, Switzerland). In these markets, the pricing of a biofuel is also sometimes coupled with its GHG saving potential compared to the petroleum-derived fuel that it replaces. There are several reports that describe the greenhouse gas balance of jatropha biofuel (Basili and Fontini 2012). Baumert (2014) found that all investigated jatropha production pathways in Burkina Faso showed GHG emission reductions and energy savings of up to 82% and 85%, respectively, as opposed to fossil fuel. Kgathi et al. (2017) reviewed several studies and found high-energy return on investment and high GHG savings when jatropha was cultivated on abandoned agricultural fields in some parts of West Africa. Even under more intensive cultivation conditions in Yucatan, Mexico, involving higher use of chemical fertilizers and pesticides, Rivero et al. (2016) predicted large savings of greenhouse gas emissions of >50% compared to fossil diesel using life cycle analysis. Navarro-Pineda et al. (2016) calculated the net energy ratio (NER) of jatropha biodiesel production to be 2.88 in Mexico, indicating the high net energy production from the jatropha system.

24.3 Economic Feasibility of Jatropha Plantations

Despite the positive properties of jatropha, its cropping has been attractive only where the framework conditions have been extremely positive. This has been mainly the case in certain areas of African countries (Madagascar, Mozambique, Mali, Burkina Faso, Kenya, etc.) that (i) possess large tracts of unused and deforested land, (ii) have low labour wages and (iii) are fully dependent on imported crude oil. These features combined with inadequate infrastructure result in bottlenecks for fuel availability and, consequently, high prices for end-customers, especially in rural areas.

Many jatropha initiatives that started in response to favourable market conditions for biofuels during the first decade of this century were abandoned due to several reasons. All of these plantations were established using wild-collected seeds since standardized seeds were not available at that time. Other important reasons for the discontinuation and failure of some plantations were unrealistic expectations about the adaptability of jatropha to adverse conditions and their low and non-uniform seed yields under the prevalent crop conditions as well as improper consideration of the gestation period, lack of knowledge on best practices of jatropha agriculture, etc.

Since that time, several companies (including Jatropower AG) have initiated genetic improvement programmes with the purpose of selecting and breeding accessions for the production of elite jatropha seeds. Seeds that are capable of developing into plants with several times the average yield of wild jatropha germplasm have been developed. Research and development have also resulted in increasing the yield of naturally occurring edible accessions of jatropha, which has high potential as a multipurpose crop yielding both oil and animal feed (seed kernel meal).

An objective evaluation of the economic viability of jatropha based on results obtained under routine plantation conditions is not possible at this stage because of gaps in primary data on performance in terms of seed yields (especially with improved jatropha cultivars and varieties presently available on the market), maintenance costs, etc. under such conditions. The published evaluations have therefore relied on assumptions of seed productivity (mostly with wild-collected seeds) or on results of trials under controlled conditions in research farms. This report proposes an analysis based on seed yields needed to reach the break-even of jatropha plantations where the relationship between seed productivity and profitability starts to be positive.

24.3.1 Cultivation Costs

The land available for jatropha plantations are usually those where other crops usually cultivated in the area have profitability issues. To present a standard cost of cultivation would not be correct as it will depend on several factors that will vary

Table 24.1 Factors affecting jatropha cultivation costs

No.	Variable affecting cultivation costs	Measures/comments	Indicative cost or cost range, €
1	Land has bush growth	Bush clearing required	250–500/ha
2	Highly compacted soil	Deep ploughing required	100/ha
3	Pit size	30–60 cm^3	–
4	Number of pits per ha (assuming 60 cm^3 dug with excavator)	625–2500 pits	100–400/ha
5	Seed cost	Cultivars or hybrids	30–75/ha
6	Direct seeding or nursery raised seedlings	Nursery costs/seedling	0.05–0.1/seedling
7	Labour cost	Highly variable according to locations	2–10/persons/day

from site to site as can be seen from the following description (Table 24.1). For instance, whether the land to be used has bush vegetation on it or not (land clearing would be required in the former case) or whether the soil is compacted (as is usual in sites where the land has been left fallow for long, e.g. large tracts in Madagascar) is important to start with. If the soil is compacted, a general deep plough may be required to loosen the soil to enable root penetration. In areas with looser soil or where other agriculture has been practised prior to jatropha cultivation, pits need to be dug after weed clearing. Experience values for size of the pits range from 30 cm^3 for sites with loose soil to 60 cm^3 for sites with compacted soil. The number of plants per ha will also vary from site to site (depending on the jatropha cultivar selected, topography and rainfall distribution from 625 to 2500 mm).

The establishment costs per ha can vary from about 350 €/ha to over 1000 €/ha. If we assume typical jatropha cultivation areas, an average experience value could be in the range of 500 €/ha for small to medium scale plantation (<100 ha).

Once jatropha is planted, growth and yield depend very much on the care provided to the plantation. Depending on climate conditions, activities will range from weed clearance, plant fertilization, disease management, pruning and harvesting. These costs also vary according to labour and chemical costs in the region. As a working value, we may consider a figure of 100 € for routine maintenance per ha per year and an average harvesting cost of 100–150 €/ha in a mature plantation (assuming full manual harvesting of 60 to above 80 kg of dry seeds per worker and per working day).

The site- and country-related influences are also noticeable on the speed at which the plantations mature and start to produce fruits. In regions with reasonable soil and well-spread rainfall in the range of 800–1000 mm per year, jatropha will start yielding commercial harvests already within year 2 and reach full yield by year 4. Under more stressful circumstances, this figure may be delayed by 1–2 years with the first harvest in year 3 and full yield by year 6, respectively.

The above description shows clearly that plantation-related costs (establishment and maintenance) vary from site to site and from country to country. Also variable is the speed of maturity of the plants, maintenance costs and seed harvests. Under such

conditions, it is hazardous to generalize the costs of cultivation. If one considers the above-mentioned indicative average values, a planter would spend 700–1000 € until he starts getting a commercial seed yield and would have continued costs of 250–300 €/ha/year.

24.3.2 Jatropha Varieties

Another factor that can influence jatropha crop profitability is the variety planted. Conventional jatropha plant parts and seeds contain toxic phorbol esters in addition to other antinutrient secondary compounds, which makes the oil and the seed cake toxic to humans and animals (Devappa et al. 2012). An edible variety of jatropha occurs naturally in Mexico. Its seeds have a similar chemical composition to the toxic varieties except for the fact that it lacks toxic phorbol esters (Francis et al. 2013).

The seed kernels of the non-toxic genotype are traditionally used in Veracruz, Puebla and Hidalgo States of México for preparing a variety of traditional dishes for human consumption. Observations have shown that the seeds produced by non-toxic jatropha plants are always non-toxic and edible, even if their male parent is toxic. The daughter plants developed from such non-toxic seeds produced by cross pollination would, however, be toxic as this character is dominant.

The non-toxic variety is supposed to have evolved as the result of a mutation at a single genetic locus of conventional jatropha. The seeds of both varieties are indistinguishable, but the plants show some differences. The most obvious ones are (i) the slightly upwardly curved borders of leaves, (ii) the long fruit stalks of fruits and (iii) the oblong fruit shape of the non-toxic accession (Fig. 24.1) compared to the short pedicels and spherical fruits of conventional toxic jatropha accessions.

Fig. 24.1 Young fruit clusters of non-toxic jatropha plants showing their long fruit peduncles. (Source: Jatropower AG)

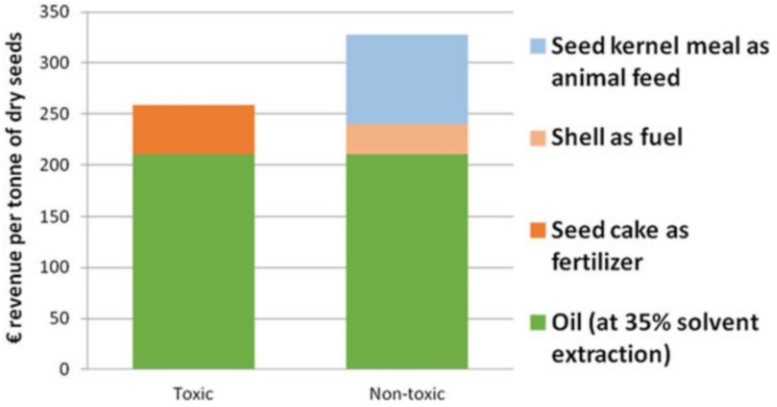

Fig. 24.2 Comparison of non-toxic and toxic jatropha in terms of revenues per tonne of seeds

According to the published data and our own analysis, the oil content in seeds, the nutrient content in kernel meal and the amino acid content of the crude protein are all similar in the non-toxic and toxic varieties of jatropha. As for fatty acids in oil, non-toxic jatropha has higher content of unsaturated fatty acids (C:18-2) compared to its toxic counterpart (Francis et al. 2013).

Compared to toxic jatropha, where there are two major products, viz. fuel oil and seed cake (this often has a mostly regional market as fertilizer only), the non-toxic variety has three products: vegetable oil well suited as fuel feedstock, seed kernel meal that is an ideal animal feed ingredient with higher nutritional quality compared to soybean meal and seed shell pellets as solid fuel (Francis et al. 2013). The non-toxic jatropha kernel meal has been shown to be suited as an animal feed ingredient (Richter 2012). Because of its higher value and the availability of a ready market for its products, non-toxic jatropha yields potentially more revenues compared to the conventional toxic one per tonne of seeds (Fig. 24.2).

For non-toxic jatropha to be successful, good quality seeds are to be made available on the market (jatropha breeding companies including Jatropower AG are involved in non-toxic jatropha improvement). Alongside development of productive seeds, small-scale seed processing machinery is also being developed to help deshell the seeds and extract oil from kernels resulting in the three products cited above that can be marketed even at a small-scale, local level.

24.3.3 Jatropha Products and Possible Processing Methods

The primary product of jatropha plantations are the fruits that are harvested. These fruits are dehusked either when they are fresh or after drying. The seeds thus obtained still have a shell covering the white kernel inside. The various fractions

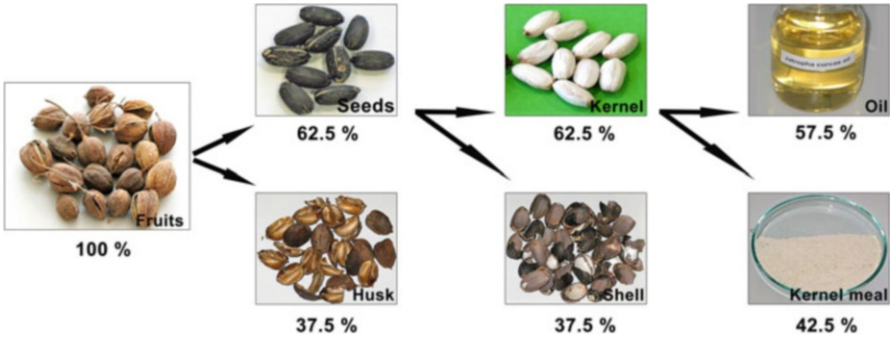

Fig. 24.3 Proportion of various fractions in jatropha fruits. (Source: Jatropower AG)

and their average relative weight percentages in the dry condition are depicted in Fig. 24.3.

In most of the present jatropha production systems, the seeds are crushed with their shells in a screw press to obtain oil. In this process, the two main products will be oil (approximately 28% by weight for most commercial screw presses) and oil cake (72% including about 7% residual oil). In large-scale plantations in the future, it is expected that seeds will be deshelled before oil extraction resulting into further separation into shells and kernels. Kernels are then subjected to pressing or solvent treatment to extract oil. In the latter case, substantial quantities of seed shells (37.5% by weight of seeds) and kernel meal will be generated as by-products. This latter type of processing would be necessary for the seeds of the non-toxic jatropha varieties in order to maximize the value of its by-products. A comparison of the two possible jatropha seed processing systems is presented in Fig. 24.4.

24.3.4 Value of Jatropha Products and By-products

The profitable edible seed oil crops either have markets for oil and their by-products (soybean, rapeseed, sunflower, etc.) or have very high oil productivity (oil palm). Current jatropha plantations are all raised with conventional toxic varieties, and whole seeds are crushed to extract oil mechanically, which is the worse economical case. During the process of mechanical extraction, only a range of 17–18% of the total dry fruit weight results in oil, which is the only product from the jatropha system that is commercially available on the market. The future processing model of jatropha seed deshelling and processing will result in a high percentage of solid residue (husk and shell amounting to 61% by weight of the dry fruit biomass). The pruned twigs also result in a substantial biomass fraction that is currently not commercially exploited.

The energetic evaluation (Navarro-Pineda et al. 2016), nutritional value (Francis et al. 2013) and added values (Steinbruck et al. 2018) of by-products of jatropha fruit

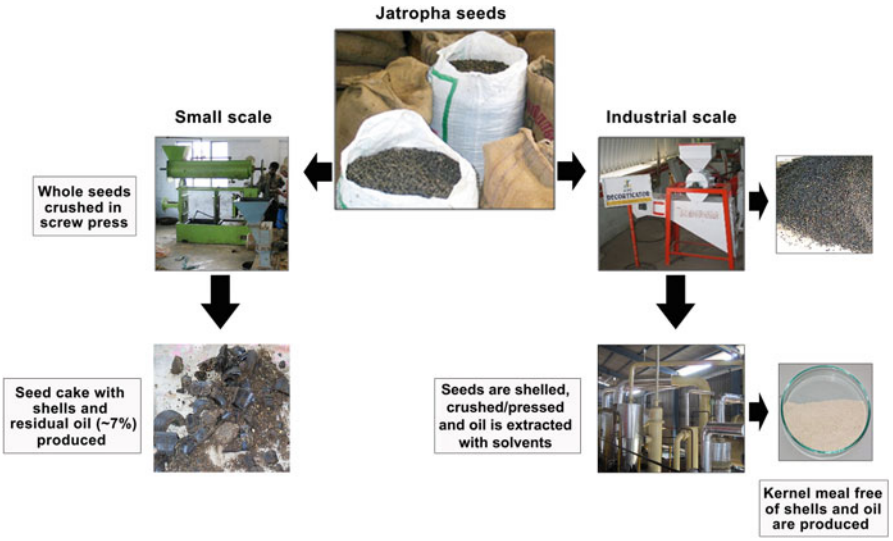

Fig. 24.4 The two different pathways of jatropha seed processing

processing and residues from plantation management were reported earlier and will not be detailed again here. An attempt is made at Table 24.2 to associate a value to the products and by-products of jatropha plantations. We are conscious that it is hazardous to apply market prices at global level for items where no global markets currently exist. However, it is still possible to propose some ranges of prices based on personal experience given the intrinsic value of the respective products.

Table 24.3 provides an overview of the jatropha product and by-product intrinsic values broken down based on fractional weights per kg of seed. The costs of processing seeds to produce by-products are not deducted from their value. When these processing costs are included, they reduce the real market return of the respective components compared to theoretical values (net values) as estimated in Table 24.3.

Considering that the net value corresponds to 70% of the sum of intrinsic values associated to each potential seed components (the remaining 30% being an estimate of processing costs), a market price range of 0.19–0.27 €/kg for conventional toxic jatropha seeds and 0.28–0.41 €/kg for edible jatropha seeds, one may evaluate the profitability of jatropha cropping (see below).

24.3.5 Seed Yields Required to Reach Profitability

The indicative cost of jatropha was estimated to be (i) 700–1000 €/ha for seeding/plantation, establishment and maintenance until maturity and (ii) 250–300 €/ha for annual maintenance and harvesting under Sect. 24.3.1. At these costs, a return of at

Table 24.2 Estimated price ranges of jatropha products

No.	Item	Price range €/tonne	Comment
1	Jatropha oil	650–1000	A 10% premium to crude palm oil (CPO) price is taken as the bottom of the range, given the superior quality of jatropha oil as a fuel feedstock compared to CPO. European biodiesel manufacturers buy sustainability certified vegetable feedstock at a 20% premium to CPO. Locally in many African countries where the *transport and poor infrastructure* premium results in diesel prices around 1 € at the fuelling station, jatropha oil can be sold at the same price as diesel at the farm gate (e.g. Madagascar, Mali, Burkina Faso, etc.)
2	Jatropha seed kernel meal	400–500	This is the price range offered by animal feed manufacturers for edible jatropha kernel meal with no phorbol esters and having a crude protein content of around 58%
3	Jatropha seed cake with shell	60–80	The cake has only fertilizer value slightly better than high-quality chicken manure
4	Jatropha seed shell pellets	70–100	Having high burning value of around 18 MJ/kg, it will fetch prices comparable to wood fuel where an international market exists
5	Jatropha fruit husk	60–80	Can be mixed with the whole seed cake to increase its fertilizer value and therefore will fetch the same price
6	Pruned twigs	–	No price can be derived without converting it into marketable products such as bio-coal

Table 24.3 Estimated intrinsic value of products and by-products per kg of jatropha seeds

Sl No.	Jatropha product and by-product	Intrinsic value in €/kg	
		Screw press	Extraction process involving deshelling of jatropha seeds
1	Jatropha oil	0.18–0.28	0.23–0.35
2	Jatropha seed kernel meal (only for non-toxic variety of jatropha)	–	0.11–0.14
3	Jatropha seed cake with shells	0.04.0.06	–
4	Jatropha seed shell pellets	–	0.03.0.04
5	Jatropha fruit husk	0.04–0.05	0.04–0.05

least 800 €/ha per year at maturity would be necessary for making the crop viable for the planters having access to suboptimal lands. At the indicative seed prices given above, this would mean that the seed productivity would have to be 2900–4300 kg/ha/year for conventional toxic jatropha and 2000–2800 kg/ha/year for non-toxic jatropha at plantation maturity (the range depending mainly on the price at which jatropha oil can be sold, the variety of jatropha cultivated and labour costs) for the crop to be financially viable for planters. As mentioned before, literature references relative to the break-even of jatropha plantations are scarce, but Lama et al. (2018) mentioned an economic yield over 2500 kg/ha/year after reviewing information published recently. Models of profitability based on real costs of jatropha plantations

in Mexico (apparently with more inputs in terms of chemicals and labour costs) indicated that the break-even would be reached for a seed yield of 3244 kg/ha/year for conventional toxic jatropha (Navarro-Pineda et al. 2017). This study also mentions that the yields obtained in existing plantations in Yucatan, Mexico, presumed to be established using non-improved, wild-collected seeds, were 1495 kg/ha/year at maturity. It appears obvious that the seed yields required to reach the break-even of jatropha cannot be obtained using non-improved seeds. It is imperative that planters use jatropha seed material that has been improved and standardised through plant breeding techniques in order to attain profitable seed yields on the type of soils that would be available for jatropha plantations in a given region.

24.4 Conclusions and Future Perspective

Despite several positive aspects, jatropha cultivation has not picked up in the last years due to plantation productivity concerns and the general lack of new investments in the biofuel sector. Based on the estimated price of the relative intrinsic value of jatropha products and by-products, a market price in the range of 0.19–0.27 €/kg for conventional toxic jatropha seeds and 0.28–0.41 €/kg for the edible jatropha seeds was estimated. At these prices, seed yields between 2000 and 4300 kg/ha/year from mature jatropha plantations would be required to reach the point that warrants crop profitability. The main factors that will influence the yield necessary to reach profitability are the selling price of oil at farm gate, the variety of jatropha (edible or toxic) cultivated and the labour costs in the cultivated area. Jatropha accessions improved for yield and agronomical features by breeding are required for reaching this range of seed productivity on the type of lands usually available for jatropha cultivation.

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Chapter 25
Experience with Farming Models, Socio-economic Issues and Agronomic Performance of *Jatropha curcas* L. in Sub-Saharan Africa

Raphael Muzondiwa Jingura, Reckson Kamusoko, and Abel Chemura

Abstract Sub-Saharan Africa (SSA) is a geographical region consisting of 49 countries, out of which at least 39 countries have experience with the cultivation of *Jatropha curcas* L. Since the year 2000, jatropha production escalated in SSA and peaked around 2007/2008. The major drivers of this trend were initial claims made about jatropha including its ability to grow on marginal lands, high seed and oil yields and being drought tolerant, amongst other attributes. This chapter describes the experience in SSA with jatropha cultivation. The objective is to outline how jatropha has performed in the region with a view to promote development work on the crop. It is now clear that the initial claims about jatropha have not been realized. Evidence in SSA has shown the major challenges as low seed yields, vulnerability to pests and diseases and low economic viability. This has been due to lack of elite planting materials, poor agronomic practices and inadequate crop management systems. These hindrances have to be addressed in order to transform jatropha into a viable commercial crop.

Keywords Agronomic performance · Crop management · jatropha production · Seed yield

25.1 Introduction

Jatropha curcas L. is a multipurpose crop that was promoted extensively in sub-Saharan Africa (SSA) in the early 2000s. Despite being undomesticated it was promoted as an energy crop whose main economic significance was production of biofuels. Prior to the year 2000, jatropha was rarely considered as a feedstock for biofuels in SSA. The interest in jatropha in SSA escalated in the mid-2000s and

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peaked around 2007/2008. This was driven mainly by international development agencies. During the period 2007–2009, investment in jatropha projects accounted for 31% of total area acquired for large-scale farmland investment in SSA (Schoneveld 2014). According to Schoneveld (2014), the largest areas acquired for jatropha were in Madagascar (979,610 ha), Zambia (707,476 ha) and Ghana (671,951 ha).

The promotion of jatropha was premised on multiple factors. Most notable was its promotion for pro-poor development (Brittaine and Lutaladio 2010). SSA is a region with substantial levels of poverty amongst its populace. As an example, the jatropha project which was started in 2005 in Zimbabwe was promoted under the mantra of rural development (Mubonderi 2012). The logic was that jatropha could be integrated into mixed crop-livestock farming systems in rural areas and would enable to diversify revenues for smallholder farmers. Throughout SSA, countries that embarked on jatropha production bet on its potential to contribute to economic development. It is also worth noting that there was strong political support and private investment into jatropha production in the early 2000s.

SSA is a geographical region that covers the area of the African continent that lies south of the Sahara desert. The region is considered to be suitable for jatropha cultivation as it lies in the so-called jatropha belt which is delimited by the tropics of Cancer and Capricorn (Basili and Fontini 2012). There are 49 countries in the region, of which at least 32 have experience with jatropha production. Parsons (2005) postulated that prime land suitable for cultivation of jatropha in SSA stretches for over 10.8 million km^2 or 1 billion ha. As of 2008, 119,000 ha were put under jatropha in the region (Global Exchange for Social Investment (GEXSI) 2008a). However, there are regional disparities in jatropha production across the five regions of Africa. There is minimal production in Northern Africa mainly due to its aridity, although there were several projects in Egypt that used sewage water for irrigation (GEXSI 2008b). Figure 25.1 is a map showing countries in SSA with substantial jatropha activities.

Most of the planted areas were in Madagascar, Mali, Zambia, Tanzania and Mozambique. Many other countries in SSA established jatropha plantations, and expectations in 2008 were that the plantations would reach 2 million ha by 2015 (GEXSI 2008b). Ghana and Madagascar together were projected to be the largest producers in Africa with an area of 1.1 million ha by 2015 (Brittaine and Lutaladio 2010). The driver of this trajectory was the forecast that jatropha should feed the liquid biofuels industry in SSA, particularly through processing of jatropha oil into biodiesel. Many countries in SSA import diesel and this has huge financial implications.

It is important to point out that prior to the surge of jatropha in 2000, the crop had a history in SSA. It was grown as a fence to prevent animals from entering reserved areas. It was used to control erosion or to reclaim degraded land, for soap making, as a bio-fertilizer and for medicinal and pesticidal applications. As such, jatropha was not a new crop in SSA. However, its status dramatically changed with the emergence of biofuels as alternatives to fossil fuels in an era of climate change. jatropha was branded as the ‘miracle crop’ or ‘wonder crop’ (von Maltitz et al. 2014). According

Fig. 25.1 Countries in sub-Saharan Africa growing jatropha

to von Maltitz et al. (2014), these terms are used to describe crops that are promoted due to their perceived disproportionately high benefits or, on the contrary, appear to be overpromoted on the basis of unrealistic claims.

In this chapter, we revisit the claims made about jatropha prior to its popularization in SSA with the concern of reporting on the reality as it results from many years of varied experience with the crop. We also look at models that have been used to grow jatropha in the region. We further consider the agronomic performance of jatropha in SSA with a view to highlight challenges and opportunities. The socio-economic dimensions of jatropha production are also analysed. The objective is to use the lessons that have been learnt for further development of jatropha production in SSA.

25.2 Jatropha in the SSA Context

The motivation to establish jatropha plantations in SSA can be ascribed to multiple considerations. It was claimed that jatropha could solve some of the region's multifaceted challenges. The major drivers for jatropha production in SSA were fivefold:

(a) Promotion of economic development
(b) Protection of the environment and mitigating climate change
(c) Mean to ensure sustainable energy supply
(d) Desire to reduce dependency on external energy sources (energy security)
(e) Promotion of rural development

Literature abounds with numerous claims originally made about jatropha as a 'miraculous' energy crop. The claims include high seed yields, drought tolerance, low nutrient requirements, low water and management requirements and adaptation to grow on marginal lands (Achten et al. 2008; Sale and Dewe 2009). It was claimed to provide employment opportunities and offer good economic returns (Borman 2011; von Maltitz et al. 2014; Mmopelwa et al. 2017). It was also claimed to be a major tool for rural development (Baker 2018). In fact, out of the myriad reasons for promoting jatropha, rural development was third after climate change and energy security (Brittaine and Lutaladio 2010). It is no surprise that a study in Kenya (Mogaka et al. 2014) showed that smallholder farmers were adopting jatropha on expectations of high income and ability for on-farm energy supply.

The acclaimed attributes of jatropha were well elucidated as the crop entered cropping systems in the SSA (Openshaw 2000; Brittaine and Lutaladio 2010). It can be seen that the claims resonated very well with some of the challenges in SSA, and the jatropha system offered possible solutions. One of the major challenges in rural SSA is poverty. The link between poverty alleviation and energy supply made jatropha attractive (Brittaine and Lutaladio 2010). Any meaningful discourse on the performance of jatropha in SSA needs to be premised on the claims that have been made about the crop. This will enable a reality check using empirical evidence and provide for means to address the shortcomings. A typology of the claims made up of agronomic, environmental, economic and social factors is presented in Table 25.1. The impact of the claims shown in Table 25.1 was a massive planting programme where thousands of farmers were encouraged to plant jatropha in many countries in the SSA.

Climate adaptation is an important factor which should be taken into account when characterizing crops for their suitability to be grown in any area. Climatic conditions are an important factor for crop production. It is well known that crop productivity depends on physical environmental conditions, which influence the availability of basic resources such as light, heat and water. Several studies

Table 25.1 A typology of claims about jatropha and their implications for SSA

Factor	Claims	Implications
Agronomic	High seed yield resulting in high oil yield	Easy to grow and will require low inputs
	Grows on marginal or degraded land	High seed yields to be obtained in poor conditions
	Grows in arid conditions (drought tolerant)	Low level of care
	Resistant to pests and diseases	
Environmental	Reclaims degraded land	Reclamation of large tracts of marginal lands in SSA
	Climate change mitigation	Balance of greenhouse gas emissions, erosion control
Economic	High return on investiment matches with both high income flows and profit margins, and income from clean development mechanisms and carbon credits	High income flows and profit margins
	High potential to create jobs matches with employment creation	Income from clean development mechanism and carbon credits
	Low management requirements	Employment creation
Social	Promotes rural development	Poverty alleviation and rural development
	Pro-women development	Economic empowerment of women
	Does not threaten food security	

have examined the correlation between jatropha production and agroclimatic conditions, but there are no universally accepted criteria to guide jatropha plantations (Wu et al. 2009). However, there is a consensus in literature that temperature, annual precipitation, soil quality and land slope are the key factors (Wu et al. 2009).

As indicated earlier, the 'jatropha belt' is defined to be stretching 30°N and 35°S (Jongschaap et al. 2007). This can be defined as the area with suitable conditions for the growth of jatropha. SSA largely lies in this 'belt'. Agroecological zones (AEZ) in SSA are varied. However, the region has suitable AEZ for production of jatropha (Parsons 2005). The dominant AEZ in the SSA are warm arid and semiarid tropics.

When describing SSA in terms of AEZ, temperature and moisture are the main factors that determine the types of crops that can be grown in a specific area. Temperature zones in SSA are either tropical or sub-tropical. Tropics are areas where all months have monthly average temperature, corrected to sea level, above 18 °C and sub-tropics below 18 °C (Food and Agricultural Organisation (FAO) 1994). Average annual rainfall in the greater part of SSA exceeds 800 mm, and the minimum temperature of the coldest month is greater than 2 °C (Parsons 2005). Smaller areas in the region have average annual rainfall around 300 mm (Parsons 2005). Thus, the climatic conditions of SSA are suitable for jatropha production.

25.3 Jatropha Farming Models in SSA

In SSA, land tenure plays a significant role in determining the types of farming models that are used (Borman 2011). It is also a truism that land tenure in Africa is complicated, and this affects farming models, more so in rural areas. However, several production models have been used for jatropha throughout SSA. jatropha cultivation in SSA has been deployed as large-scale stand-alone plantations as well as outgrower model schemes and a mixture of both in some situations (Osei et al. 2016). The production systems in SSA can be divided into five categories as follows:

(a) Plantations
(b) Plantation plus outgrower schemes
(c) Outgrower schemes
(d) Smallholder production
(e) Hedges

The most prevalent farming model is large-scale plantations (Wahl et al. 2012). Plantations are large schemes which can be under public or private ownership and are usually larger than 5 ha (GEXSI 2008b). Large plantations in Africa mainly range between 100 and 10,000 ha (Borman 2011; Wahl et al. 2012). Some of these plantations are on wastelands or marginal lands. Most private investment in jatropha in SSA targeted large-scale plantations (Schoneveld 2014). GEXSI (2008b) reported that plantations represented 31% of all jatropha projects in Africa. Plantation projects were projected to grow faster in the future (Wahl et al. 2012). Plantation schemes were reported to be more efficient not only in production but also in building rural infrastructure and providing employment opportunities for communities (German et al. 2011; Osei et al. 2016). One of the main challenges with large-scale plantations is that they can lead to complications in land issues with communities, mainly in rural SSA.

In the model of 'plantation plus outgrower', the outgrowers are linked to a commercial plantation, which provides support to the farmers in the form of planting material, inputs and technical advice (Brittaine and Lutaladio 2010). Outgrowers are mainly smallholder farmers. In the pure outgrower model, the farmers are not contractually linked to a commercial plantation but to a central organisation. For example, in Zimbabwe the National Oil Company of Zimbabwe once contracted smallholder farmers to grow jatropha, and in turn the farmers were to sell the seeds to the company. It must be pointed out that in SSA about two thirds of jatropha projects in 2008 integrated smallholder farmers either as outgrowers or stand-alone farmers (GEXSI 2008a). In Tanzania, for example, more than 10,000 smallholder farmers had established jatropha plantations (Wahl et al. 2009). The stand-alone smallholder production model involves smallholder farmers who have no contractual obligations with anyone. These farmers grow jatropha on their own will and choose their buyers.

The outgrower model is not very common in SSA (Wahl et al. 2012). According to Wahl et al. (2012), the outgrower model poses at least three major challenges: identifying farmers and organizing them effectively; achieving continuous yields and constant seed quality at reasonable costs; and creating added value for farmers.

This requires the contractor to work well with communities and establish communication channels and a robust farmer support system.

In 2008, the total length of jatropha hedges in Africa was estimated at 75,000 km (Muok and Kallback 2008). jatropha is not palatable to livestock and therefore can serve as live fence (Henning 2004). A number of rural households in SSA use jatropha as a hedge to protect their crops or to mark homestead boundaries. jatropha hedges are also used to reduce wind erosion by lessening wind velocity and binding the soil with their surface roots (Henning 2004). It was reported that the only profitable business case for jatropha in rural areas is where farmers were planting the crop as hedges (Romjin et al. 2014; FACT Foundation 2010).

25.4 Cropping Systems

Cropping systems are the frameworks used for managing crop production. There are basically two types of cropping systems for jatropha that are found in SSA (Gesellschaft für Technische Zusammenarbeit (GTZ) 2009). These are:

(a) Monoculture
(b) Intercropping

Monoculture plantations are pure plantations. These are common for most commercial crops grown on a large-scale. Monocultures are considered efficient for plantations (Brittaine and Lutaladio 2010). However, monoculture plantations have challenges such as disease and pest build-up and thus have more risk in the long run (Wahl et al. 2012). As jatropha sheds off leaves in the dry season, the anti-erosion effects are limited, and this is the period when wind erosion is highest. In addition there will be no canopy to protect the soil when first rains fall (Brittaine and Lutaladio 2010). In terms of ease of operations, monoculture prevents interference with operations from other crops.

It is generally accepted that both mono- and intercropping strategies can be practised according to farmer preference (Bogdanski et al. 2011). Intercropped jatropha plantations incorporate other crops, such as maize, beans, elephant grass, lablab and others. jatropha has little negative allelopathic effect on other plants (Wiesenhütter 2003). Intercropping with food crops is interesting in that it creates a food-energy agro-system. Plant spacing for jatropha is generally 2 × 2 m, 2.5 × 2.5 m or 3 × 3 m, and this provides intra-row space which can be occupied by other crops. Generally, intercropping is feasible in the first 2 or so years of growth when the plant has not yet fully grown. When fully grown, the canopy might interfere with the intercrops. Empirical evidence exists in SSA on the productivity of both monoculture and intercrop plantations. Wahl et al. (2009) in Tanzania concluded that intercropping with annual crops was only cost-effective in the first 2 years of growth. The GEXSI report (2008a) showed that 70% of jatropha schemes on a global basis practised intercropping. The advantage of intercropping is that where jatropha has not done well, farmers are compensated by the intercrop. This is a

strategy that can mitigate the challenges that have been experienced with jatropha in SSA.

25.5 SSA Experience with Jatropha Cultivation

It is more than a decade now since the jatropha surge in SSA. It is important to remember that the surge was based on unsubstantiated data or substantiated claims extrapolated from outside SSA. These claims were not adequately corroborated by local systematic research in SSA. However, substantial experience now abounds in SSA in terms of commercial cultivation of jatropha as an energy crop. Reality on the ground has shown that the scenario that had been projected for 2015 (GEXSI 2008a report) did not happen. By 2012, the results were clearly demonstrating the weak business case for jatropha (Romijn et al. 2014; Kgathi et al. 2017).

It has been a while now since doubts have been raised over the viability and profitability of jatropha cultivation (Valdés-Rodríguez et al. 2014). Over the years there has been a lull in the implementation of new jatropha projects across the world, and some established projects have even ceased their activities (van Eijck et al. 2014; Ahmed et al. 2017). Put rather candidly by von Maltitz et al. (2014), it is now clear that jatropha projects were based on incorrect information, misinterpretation of available data and poor knowledge of jatropha and its agronomy. There is also evidence outside SSA that jatropha has been abandoned. For example, in Mexico, despite its assumed economic, environmental and social benefits, many farmers have abandoned jatropha cultivation (Soto et al. 2018). jatropha has been abandoned due to many factors militating against the continuation of its cultivation.

It is now a truism that the interest in jatropha has subsided in the last few years in SSA. Reasons for the decline can be discerned from the experiences with jatropha cultivation accrued over years in the region. The experience that has accrued in SSA with the commercial cultivation of jatropha is described below under four domains (Table 25.1) which are:

(a) Agronomic performance
(b) Economic viability
(c) Environmental effects
(d) Social impact

25.6 Agronomic Performance

There are several parameters that are covered under agronomic performance ranging from planting to seed yield.

25.6.1 Establishment of Plantations

One of the claims attributed to jatropha was its ease of establishment (Brittaine and Lutaladio 2010). There are generally three methods used in establishment of jatropha plantations. These are vegetative propagation, direct seeding and transplantation of seedlings from nurseries. Propagation methods varied across SSA. jatropha has been propagated both sexually and asexually in SSA. Most of asexual establishment was done using cuttings obtained mainly from existing jatropha hedge rows (Jingura and Kamusoko 2014). Where jatropha was propagated from seeds, both direct seeding and transplanting from nurseries were practised (GEXSI 2008b). Eighty-five percent of the projects in the SSA reviewed by GEXSI were established from transplanted seedlings (Renner et al. 2008).

Evidence from Zimbabwe showed that jatropha established through pre-cultivated seedlings outperformed non-rooted cuttings and direct-seeded plants in terms of survival (Jimu et al. 2009). This could be due to the ability of seedlings to produce a taproot. Heller (1996) considered the ability of seedlings to develop taproots to be important for plant survival, especially under water-limiting situations. One advantage of cuttings is their genetic uniformity, rapid establishment and early yield (Brittaine and Lutaladio 2010). A major disadvantage is that they do not produce a taproot. The performance of plants generally appears to depend on crop management practices, other than means of propagation. Two important parameters are survivability and seed yield, which have been observed to be very variable in SSA.

There was very little or no certified jatropha planting material in SSA in the period of the jatropha surge in 2008 (Justiça Ambiental (JA) and União Nacional de Camponeses (UNAC) 2009). Most of the plantations were established using uncertified planting material. This is basically not a good practice in crop production. Figure 25.2 is a picture of women planting jatropha in Rwanda (ProjectRwanda.org 2010).

jatropha plantations appear to have been fairly easy to establish in SSA. The main problem has been the survival of the plants in the early post-establishment phase (Jingura and Kamusoko 2015a). There were observations of poor early survival of plants in some plantations in SSA. For example, it was observed in Mozambique that jatropha required a lot of care in the 1st year or so of growth (JA and UNAC 2009). Farmers in Mozambique had to provide 5–7 litres/day water in order to ensure early survival of the plants (JA and UNAC 2009). Thus, plant survivability has been an issue in SSA. Experience in Mexico has shown that poor seed germination and plant survival rate are determinants that caused abandonment of jatropha cultivation (Soto et al. 2018).

Fig. 25.2 Women planting jatropha in Rwanda. (Source: ProjectRwanda.org)

25.6.2 Hardiness

Plant hardiness generally refers to a plant's ability to survive under adverse conditions (Perry 2003). Hardiness is a genetic factor and is also a function of location. A number of countries in SSA lie in warm arid and semiarid tropics. With climate change, trends in SSA show a warming trend, with frequent occurrence of extreme heat events, increasing aridity and changes in rainfall patterns with a pronounced decline in Southern Africa and an increase in East Africa (Serdeczny et al. 2017). These climatic conditions present adverse growing conditions for many crops.

One of the claimed attributes of jatropha was its reported ability to grow under harsh conditions, which include marginal lands, dry conditions and tolerance to pests and diseases (Sale and Dewe 2009; Achten et al. 2010). This ability would have been a good feature to thrive in the climatic conditions in SSA. Farmers in SSA ventured into jatropha cultivation expecting a crop that would have low water requirements (JA and UNAC 2009). Most of the plantations in SSA were established under rainfed conditions (GEXSI 2008b). Annual precipitation range suitable for jatropha cultivation is 250–1500 mm, with an optimum range of 900–1500 mm (Benge 2006; Trabucco et al. 2010). This amount of precipitation hardly occurs in SSA. Reality on the ground has shown that jatropha does not perform well under water-limiting situations. For example, in Mozambique farmers had to apply 5–7 litres of water per day per plant to supplement rainfall in the early phases of growth of jatropha in order to achieve good stand establishments (JA and UNAC 2009). Data

from Kenya also showed that at least 40% of jatropha farmers practised irrigation (GTZ 2009).

As pointed out earlier, jatropha was acclaimed to thrive on marginal lands. Marginal lands are characterized by low soil fertility and high fragility. Limitations of soil fertility hamper crop development (Jongschaap et al. 2007). Where jatropha has been grown on marginal lands without application of fertilizers, seed yields have been as low as 0.4 t/ha (Openshaw 2000). The use of fertilizers in jatropha production, especially organic types, became a common practice in SSA. In Kenya, 50% of farmers used organic fertilizers in jatropha production (GTZ 2009). In Tanzania, no farmers were observed to apply fertilizers or any other inputs (Brittaine and Lutaladio 2010). What has been observed is that jatropha seems to respond to both organic and inorganic fertilizer applications. For example, in India, treatment of jatropha plantations with 3 t/ha of jatropha seedcake increased seed yield by 120% and 93% at different plant densities of 833 (4 × 3 m) and 1667 (3 × 2 m) plants/ha, respectively (Ghosh et al. 2007). In Burkina Faso, addition of organic matter to jatropha on degraded soils significantly enhanced the growth rate of seedlings (Kagamèbga et al. 2011). It is not really correct that jatropha does not need fertilizer application as it has been shown to respond well to fertilizer application.

Resistance to pests and diseases is a desirable crop trait. This has been one of the major claims attributed to jatropha. Pests and diseases cause adverse impact on jatropha production. jatropha has been shown to be susceptible to many diseases and pests, which include more than 35 fungal species, 4 viral and 4 bacterial pathogens, 6 nematodes and about 60 insect species (Anitha and Varaprasad 2012). In SSA, observations in countries like Zimbabwe, Kenya, Mali and Tanzania provide evidence of the vulnerability of jatropha to pests and diseases (Jingura and Kamusoko 2015a). Diseases such as collar and root rot and pests such as golden flea beetle and stem borer have been reported to cause extensive damage in jatropha plantations (FACT Foundation 2006; Wahl et al. 2009). The bottom line is that jatropha is a plant that is susceptible to pests and diseases and this is a fact that cannot be overlooked. As such appropriate crop protection practices are essential in jatropha production.

25.6.3 Seed and Oil Yield

Seed yield is one of the most important economic traits in commercial production of jatropha. jatropha was claimed to be capable of producing 12 t/ha of seed (Achten et al. 2008). According to Achten et al. (2008), this expectation of 12 t/ha of dry seeds was based on faulty extrapolation. Most jatropha plantations in SSA did not realize such levels of seed yield. Most plantations that were established in the late 1980s to the 1990s were abandoned due to low productivity and/or high labour costs (Jongschaap et al. 2007). As early as 2008, jatropha cultivation was being discouraged in SSA. Reasons given by JA and UNAC (2009) in Mozambique and Wahl et al. (2009) in Tanzania were all centred on poor seed yields and poor viability. Seed

Table 25.2 Reasons for low seed yields of jatropha in sub-Saharan Africa

Parameter	Description
Planting material	Absence of certified planting material
	Use of wild-type germplasm
Land type	Use of marginal lands
	Low fertility and fragile soils
Climatic conditions	Arid and semiarid conditions
	Variable rainfall patterns and prevalent droughts
	Extreme temperatures
Agronomic practices	Little or no use of fertilizers
	Reliance on rain-fed plantations
	Absence of production information
Pests and diseases	Prevalence of pests and diseases
Management	Low management levels

yields reported include 1.65 t/ha in Tanzania (Brittaine and Lutaladio 2010), less than 1 kg per tree in Mozambique (JA and UNAC 2009), 0.63 t/ha in Mali (FACT Foundation 2006) and up to 0.86 kg per tree in Kenya (GTZ 2009).

The seed yields that have been obtained in SSA have been far removed from projections that had been given. However, the yields are consistent with estimates of 0–2.2 t/ha given for marginal lands (Ouwens et al. 2007). The low seed yields affect oil yields as well. The conversion ratio of seed to oil is about 3:1 for high-efficiency systems (Pohl 2010). Using this ratio (33% oil), oil yields from 2.2 t/ha seeds were about 0.7 t. This is very low and means that seed yields of 5–7 t/ha would be necessary to reach 1.7–2.3 t/ha oil, which is the target quantity needed for profitable jatropha plantations. Several factors can be ascribed to the poor seed yields of jatropha in SSA. These are shown in Table 25.2.

It is known that when grown on marginal lands under arid conditions jatropha does not perform well (Maes et al. 2009; Trabucco et al. 2010). The seed yields that have been obtained in SSA and the production environment (Table 25.2) provide such evidence. Kant and Wu (2011) have argued that the productive efficiency of jatropha is dependent on soil fertility, available moisture and temperature and these factors affect seed yield. Given this perspective, it can be argued that the performance of jatropha in SSA has been an epigenetic response to the varied environmental features that it has encountered (Kant and Wu 2011). Thus, the performance of jatropha in SSA needs to be viewed in the context of the interaction between seed yield and local edapho-climatic environmental conditions.

25.7 Economic Viability

The overarching claim of jatropha being able to promote rural development through labour production gained a lot of traction in SSA around 2008. One of the major drivers of jatropha production in SSA was the claim for pro-poor development leading to poverty alleviation (Jingura and Kamusoko 2015a). It was postulated that the jatropha system would promote multiple revenue sources. The economic dimensions of *jatropha* cultivation included diversifying income opportunities for farmers; creating opportunities arising from the value chain (production, transportation and processing); as well as generating incomes from clean development mechanism (CDM) and carbon credits. Economic viability of jatropha production is determined by several factors including seed yield, seed price, oil price and the cost of production inputs. Opportunity costs resulting from land used for jatropha production can be very significant and need to be considered in economic analysis, especially when the same land could be used for growing food crops or used for cultural or social activities (Gasparatos et al. 2015).

As already stated, there have been concerns with financial viability of jatropha production. The low seed yield, low seed prices, limited valorization of by-products (van Eijck et al. 2014; Jingura and Kamusoko 2017) and the underestimated labour and maintenance costs of fields (von Maltitz et al. 2014) have caused great concerns. The intention here is not to provide a detailed economic analysis but to describe how jatropha has fared in SSA from an economic perspective. In a review of jatropha performance in Southern Africa, Mmopelwa et al. (2017) concluded that most projects, especially commercial plantations, were not economically attractive to the extent that they had been abandoned in some countries in the region.

Studies in Tanzania (Wahl et al. 2009) and Kenya (GTZ 2009) showed that jatropha cultivation was not a viable enterprise for the farmers. Seeds are the major saleable product from jatropha plantations for farmers who do not perform value addition. In Tanzania, a research study showed that the net present value of a 5-year investment in jatropha plantation was negative with a loss of US$65 per ha on lands with yields of 2 t/ha (Wahl et al. 2009). In Zimbabwe, and as early as 1992, a jatropha project which was initiated by the Plant Oil Producers Association was abandoned after it was realized that the profit margins were not as big as originally expected (Henning 2003).

In Ghana, Osei et al. (2016) showed that outgrower schemes where oil is exported and presscake utilized for compost and outgrower schemes where oil and by-products are used were not profitable for the farmers. The major determinant of profitability was the seed price (Osei et al. 2016). In a study in Mozambique, Mali and Tanzania, jatropha plantations were found to be unviable (Romijn et al. 2014). This was ascribed to colossal upfront capital requirements, slow and unreliable crop maturation, inefficient oil pressing owing to a lack of scale and experience, inadequate utilization of by-products and competitively priced fossil diesel and palm oil (Romijn et al. 2014). Taking all the observations in SSA, the reality has been that jatropha production has not been a viable business.

25.8 Social and Environmental Impact

At the surge of jatropha in SSA in 2008, it was claimed that jatropha could widen the incomes to rural farmers. An argument that has been proffered in literature is that for jatropha to contribute to sustainable rural development, its cultivation must be small-scale, inclusive and community-based (Brittaine and Lutaladio 2010). This realization led to a number of outgrower schemes and stand-alone farmers. A strong case was made for jatropha-based entrepreneurship driven by the multiple products from jatropha. jatropha has multiple uses, and technologies exist that can be used to convert it into various products creating a technology and entrepreneurship interface for pro-poor development (Jingura and Kamusoko 2015b). Possible social impacts of jatropha can be summarized as follows:

(a) High potential to create jobs along its value chain
(b) Women empowerment mainly through projects such as soap making
(c) Poverty alleviation
(d) Reclamation of degraded lands
(e) Environmental protection

High potential to create jobs is one of the claims made about jatropha. This is a pro-poor development strategy and would tally well with reduction of unemployment levels in SSA. Large jatropha plantations in Mozambique created jobs ranging from 0.03 to 1.03 per hectare (Mmopelwa et al. 2017). The jobs in Mozambique were mainly contract-based, whilst in Tanzania and Mali, the jobs were mostly seasonal, which were offered during the harvest season (Mmopelwa et al. 2017). There are other studies that have also highlighted the link between jatropha development and employment creation where jatropha was found to provide employment in rural areas (Grass and Zeller 2011). It can be concluded that jatropha projects in SSA generated employment mostly on a temporary nature basis. However, as the value chain of biofuel production from jatropha has not flourished, neither have the employment opportunities. It is for this reason that the high potential for job creation has not been achieved.

One of the components of the jatropha value chain was women empowerment via projects such as soap making in rural areas. This was touted as a means to alleviate poverty. Soap making is a simple and affordable process, which Benge (2006) described as village or cottage industry technology. The attraction of jatropha soap is that it has medicinal properties and can be priced higher than ordinary soap. Some rural women in SSA ventured into jatropha soap production, but there have been very few success stories.

It was reported in Zambia that the following happened in rural areas due to jatropha production (Kalinda et al. 2015):

(a) Farmers lost out on time to carry out other activities.
(b) Income from sale of edible non-wood forest products was lost.
(c) Farmers experienced reduction in maize (*Zea mays*) and bean (*Phaseolus vulgaris*) production.
(d) All these negative factors worsened household economic conditions.

The ability to grow on marginal lands meant that jatropha could be grown on wastelands. This would limit competition with food crops for arable lands. Growing jatropha on land used for food and fibre production would entail huge opportunity costs (Mmopelwa et al. 2017). However, evidence in the SSA show that few plantations in the region were established on wastelands. The reality is that most of them were established on arable lands (Gasparatos et al. 2015). In such cases, jatropha competes with food crops for production inputs. This would be at variance with the original claim of no impact on food security and household labour. In addition, the ability to reclaim wasteland in SSA seems not to have been a major focus.

One of the 12 principles of the Roundtable on Sustainable Biofuels is that biofuel production shall avoid negative impacts on biodiversity, ecosystems and other conservation value areas (Ismail and Rossi 2010). jatropha has been used in rehabilitation of degraded soils, erosion control and soil improvement. When planted as hedge rows, jatropha reduces wind erosion. The plant improves infiltration when planted in lines to form contour bunds. Thus, jatropha has some benefits to conservation issues. However, the cumulative impact of jatropha on the environment in SSA remains to be seen in the long run (Jingura and Kamusoko 2015a).

25.9 Conclusion

jatropha is a crop that has gone through its first phase of development in SSA. The challenges that have been experienced with the crop need to be dealt with accordingly. It is now widely accepted that jatropha is a crop under development. Its major limitation in SSA has been very low seed yields. Perhaps it is reasonable to acknowledge that the low seed yields are consistent with a plant still under domestication and will improve over time. The need to optimize the performance of jatropha as a multipurpose crop is imperative.

It is now very clear that jatropha is a crop that requires sound agronomic practices like all commercial crops. Early claims about jatropha seem not to have stood the test of time and the plant cannot be relegated to wasteland status. The experience with jatropha cultivation that has accrued in SSA provides a reality check that helps set an agenda for enhancing the status of jatropha as a viable energy crop. It is worth noting that despite a lull in its production, research and development work have not stopped. The major issue is to improve seed yields. There are six critical areas that need attention. These are:

(a) Improvement of quality of planting materials through breeding programmes
(b) Establishment of good crop management practices with appropriate fertilizer regimes and water requirements
(c) Enhancement of seed yield
(d) Development of competitive markets for jatropha seeds

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