Handbook of Plant Breeding

Von Mark V. Cruz
David A. Dierig *Editors*

Industrial Crops

Breeding for BioEnergy and Bioproducts

Industrial Crops - Breeding for BioEnergy and Bioproducts

HANDBOOK OF PLANT BREEDING

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Breeding for BioEnergy and Bioproducts

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Foreword

The last decade of plant breeding truly has been transformational in creating a unified global effort in several key areas, with bio-energy close to the top of that list. Finding alternative energy sources has become a priority for economies facing urgent energy policy decisions. These chapters are very well timed to highlight some of the key breeding challenges. Feedstock development for both fuel and bio-based products is at the forefront of this discussion, which of course comes with the challenge of prioritizing to meet projected needs over the next two decades.

There are numerous political and social discussions surrounding the development of bio-products and bioenergy economies, and whether or not adequate answers emerge for these, the scientific components are important for successful economic progress. Many of the challenges on the plant breeding and crop production side of developing a bio-economy intersects with food production challenges, and it is not the intention of these chapters to make judgments about where those lines intersect, but to focus on a scientific solution. Entering into the discussion also is the need to reduce the carbon footprints, which can be partially solved by developing plants less dependent on energy-intensive fertilizers and fungicides to produce higher outputs of cellulose. Plant breeding strategies will depend on type of feedstock, as well as size of markets, desired availability and strategic placement of processing and distribution facilities for best accessibility. Breeding issues for cellulosic ethanol will be different from biodiesel needs, and smaller local energy demands will bring different breeding challenges to the table. These issues will determine the types of feed stocks to be developed.

I believe a certain level of harmony exists in the food vs. fuel debate, if the discussion leans more towards effective breeding and development of integrated crop production systems. In this regard, research must take into account the critical emerging themes that are now dictating the need for more integrated systems approaches, including environmental friendliness, sustainability and regulatory requirements for specific needs of the production system. There is a real need to reduce agricultural inputs, but this must be done with more sustainable methods; plant breeding is well positioned to lead this charge. The next decade will see the

private sector working in better harmony with the public sector to shift more rapidly towards systems approaches to enhance the assisting technologies driving them to higher yields. In concert with advances in breeding, we will see integrated farming systems combined with new biological products from RNAi technology, biotechnology, and novel agricultural chemicals to produce innovative advances and sustainable yield gains.

Government assistance is an important requirement for successful development and implementation of new initiatives. The US Federal government is actively promoting the development of ethanol from cellulosic feedstocks. The US Department of Energy (DOE) supports research to develop better cellulose hydrolysis enzymes and ethanol-fermenting organisms, as well as ethanol production from cellulosic biomass. The 2008 farm bill allowed for the commercialization of advanced biofuels, including cellulosic ethanol. The Food, Conservation, and Energy Act of 2008 provided grants covering up to 30 % of the cost of developing and building demonstration-scale bio-refineries for producing "advanced biofuels," which includes all fuels that are not produced from corn kernel starch. It provided loan guarantees of up to $250 million for building commercial-scale biorefineries to produce advanced biofuels.

The Renewable Fuels Standard, which is a part of the 2007 Energy Independence and Security Act, stipulated an increase in biofuels production to 36 billion US gallons a year by 2022. In January 2011, the USDA approved $405 million in loan guarantees through the 2008 Farm Bill to support the commercialization of cellulosic ethanol at three different facilities to develop a combined 73 million US gallons per year production capacity. The USDA also allocated payments to expand the production of advanced biofuels. In July 2011, DOE granted $105 million in loan guarantees for a commercial-scale plant to be built in Iowa.

Contributions of plant breeding to the renewable energy strategy should be aimed at improving energy efficiency and provide economic growth for as many rural communities as possible. In this discussion, we need to recognize the importance of total cost of production (from developing and growing feed stocks all the way to market costs), reduction of greenhouse emissions, and conservation of natural resources. The crops highlighted here in each of the categories (biodiesel, sugar, starch, cellulosic crops) are sensible targets to develop a national strategy which accommodates many rural communities. For biodiesel, adequate availability of feedstocks is an important issue, as is high sugar, starch, and cellulose production for the "non-oil" crops. Sustainable yield and efficient digestibility are important for the native grasses to maintain consistent biomass conversion. Sugarcane varieties must have high yield and high sugar content, but at the same time need to have cold tolerance and adequate disease packages to maintain stability across wider geographical ranges. Sugar beet has seen its share of challenges with emerging and endemic diseases and, although current breeding programs have saved and earned the industry billions of dollars, must be improved for sugar content and processing quality to sustain both sugar and bioenergy industries.

There are many skeptics regarding the potential success for a bio-energy and bioproduct economy. Many countries, however, currently either have fully

operational, or "soon to be on line," biofuels plants. GraalBio in Brazil for example built a facility estimated to produce 82 million liters of cellulosic ethanol per year. Another success story comes from Denmark, where Inbicon's bioethanol plant, with a capacity of 1.4 million gallons annually, has been operating since 2009. An E85 blend of 95 % gasoline and 5 % cellulosic ethanol from wheat straw has been available since 2010 at many filling stations across Denmark.

In the USA, there has been an increasing effort to commercialize cellulosic ethanol during the last 5 years, concentrating on conversion of cellulose into fuel. About a dozen cellulosic ethanol plants in different states are currently either operating or soon to open. Companies, such as Iogen, Poet, and Abengoa, are building, or completed, refineries to process biomass into ethanol. Other companies, such as Diversa, Novozymes, and Dyadic, are producing enzymes to enable cellulosic ethanol conversion. These options will enable shifting from using food crops feedstocks to waste residues, native grasses, and other non-food plants. The first commercial-scale plants to produce cellulosic biofuels began operating in 2013. Among these, multiple pathways for the conversion of different biofuel feedstocks are being used. These refineries are currently expensive to operate, but in the next 5 years the cost of the conversion technologies at commercial scale will predictably become lower.

It is important that the plant breeding research be coordinated and linked with the policy, education and outreach efforts for effective communication with farmers, processors and other renewable energy efforts in the rural community that are involved in feedstock production and value-chain logistics. These efforts must be in sync with feedstock conversion and commercialization strategies. As we go forth in this process, we must acknowledge the role that renewable energy from plant biomass will play in this grand challenge. Integration with other sources in the renewable electricity arena, such as solar, geothermal, wind, and anaerobic digestion will be essential to a sustainable system.

For a plant breeding strategy to be effective, improvement or development of new industrial crops must take into account the challenges of climate change as it relates to the entire agricultural system. Changing temperatures, precipitation and carbon dioxide concentrations generally are thought of as the most major concerns, but equally important are the interactions of new varieties with other inhabitants of the ecosystem, such as insects, weeds, and pathogens that may cause diseases and have significant soil and plant impacts. Breeding crops resilient to these components, while at the same time maintaining the quality components necessary for bioenergy and bioproduct components, though challenging, are essential now and in the future.

It is well known that temperature ranges for optimal biomass production and effects of CO_2 concentrations on crop growth vary with species, especially based on photosynthetic pathways. The greater sensitivity of C3 plants to increased CO_2 levels and effects on water-use efficiency, though not well studied, is not unknown. Effects of ozone fluctuations may impact effects of CO_2 concentrations and must be considered, as breeders develop new selection tools. Field-based phenotyping of new varieties will be essential, and the use of accurate crop models will be

important to assist effective genetic manipulations. The well-known strategy of breeding new plant varieties resilient to changing agricultural systems that is evident in these chapters is a sustainable way to adapt the breeding component of production agriculture to climate change. This strategy serves to temper the negative economic implications of displacing a potentially profitable crop from its original production system. In addition, new varieties usually have the advantage of higher productivity.

The bottom line is that breeders of crops for bioenergy and bioproducts must be even more mindful of the entire agricultural system than ever before, and must collaborate with other components, since it takes a strong adaptive capacity at all levels to highlight the plant breeding benefits. If we consider that the entire agricultural system must be made resilient to climate change, it is then evident that breeders will continue to be held responsible for dealing with the eminent evolution of resistance of pests to genetically modified crops and new chemistries used to maintain economic stability. Breeders must be involved in managing the newly created biodiversity at both field and landscape levels through breeding to address environmental, pest and pathogen issues.

Since feedstock development is of major importance in a successful future for bioenergy, and since bioproducts are deeply engrained in this system, all participants of the system are essential partners, including farmers, ranchers, landowners, crushers, fuel producers, etc. Law-makers and policy makers worldwide are key to successful implementation. Providers of energy and consumers at various levels must engage and communicate effectively to develop and maintain a successful bioenergy and bioproduct future. The top players at all levels should not forget the huge role that plant breeding has in maintaining a viable bio-economy.

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Preface

The scope of the definition of industrial crops undoubtedly has changed. The traditional distinction between food crops and industrial crops have blurred with the emerging opportunities and additional uses of food crop species. Among these uses include being as source of raw materials for non-food products such as fibers, energy, industrial lubricants and starches, resins, plastics, cosmetics, and many other important compounds that are used for manufacturing. This handbook presents advancements in research and breeding for non-food applications and associated commercialization efforts in a selected set of crop species.

The idea of this book volume was initially brought to us by Hannah Smith of Springer Media, and it is a timely suggestion due to rapid advancements in plant science technologies that are important in accelerating developments in crop improvement and the changing pace of agricultural materials being tapped as source of industrial raw materials. Among these technologies also include advances in screening methodologies to look at genotypic and phenotypic variation and the greater inclusion of molecular markers and biotechnology applications in industrial crop breeding programs. Some crops presented in this volume may also have additional information in other handbook volumes in this Springer series and we encourage the readers to consult them.

As part of *The Handbook of Plant Breeding* series, we hope that the collection of papers in this volume will be useful to plant breeders, biologists, students, and other stakeholders of these important species and promising new crops. We attempted to gather developments in these species globally and we have organized this volume by categorizing crops according to their primary non-food use, whether for biodiesel, bioenergy, or bioproduct. A separate section was also assembled to present current issues and emerging technologies in bioenergy and biofuels, providing a situation overview of advances in biofuel technologies, economic feasibility, and the perceived effects of public policy mechanisms at the time this volume was written.

We sincerely thank Springer Science for making the production of this handbook possible and are grateful for the valuable help of their staff especially Michael Sova, Hannah Smith, Melissa Higgs, Brian Halm, and Kenneth Teng. We greatly acknowledge the contributors and all authors in this volume for taking time to share their expertise and specialized knowledge on the crop species and the respective topics.

Fort Collins, CO, USA

David A. Dierig
Von Mark V. Cruz

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Chapter 1
Sweet Sorghum: Breeding and Bioproducts

P. Srinivasa Rao, C. Ganesh Kumar, R.S. Prakasham, A. Uma Rao, and Belum V.S. Reddy

Abstract Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most important cereal crop and is the dietary staple of more than 500 million people in over 90 countries, primarily in the developing world. However, sweet sorghum which is similar to grain sorghum except for accumulation of stalk sugars, is considered as a potential energy crop without impacting the food security of millions. Further, the sorghum stover is considered to be a potential lignocellulosic biofuel feedstock. Being a C4 plant, it has high photosynthetic rate, and several mechanisms are known to confer resilience that help produce higher yield in varied environmental conditions. This chapter not only discusses different breeding methodologies for improving candidate sugar and biomass traits but also the possible utilization of this smart feedstock for diverse biochemicals (lactic acid, xylitol, glycerol, etc.) and bioproducts (nanomaterials, anticancer and microbial compounds, adhesives, polymers, antidiabetic compounds, etc.) development.

Keywords Sweet sorghum • Biofuel • Stalk sugar • Genetic variability • Biochemicals • Bioproducts

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Introduction

The current scenario of declining fossil fuel reserves along with increased concerns on environment pollution and climate change is fundamentally responsible for greater interest in renewable energy sources globally. Sustainable availability of raw material for any economic and constant product production is one of the essential requirements. This has become more appropriate for the constant consumption products like biofuels, as the entire world economy is dependent on the availability of fuel resources. Interest in sweet sorghum (*Sorghum bicolor* (L.) Moench) in semiarid and rain-fed environments is increasing because of the multiple uses of this novel feedstock either for production of biofuels from stalk juice or for power generation from bagasse or for utilization in dairy industry as nutrient rich and easily digestible fodder [1, 2]. Additionally, sweet sorghum biomass is used for fiber, paper, syrup, and biopolymers. Sweet sorghum being a C4 crop has wide environmental adaptation, rapid growth, high grain and biomass productivity, suitability for marginal soils, and high concentrations of the easily fermentable sugars like sucrose, glucose, and fructose [3]. Drought and salinity are widely prevalent abiotic stresses that significantly lower the yields of various crops, and their frequency of occurrence is expected to increase due to climate change. Sweet sorghum grows in marginal areas because of its high tolerance to saline and drought conditions. Sweet sorghum has higher water-use efficiency than other summer crops under both well-watered and water-stressed conditions [4–6]. From the agronomic point of view, sweet sorghum is more environmentally friendly than maize because of its relatively low nitrogen needs and water requirements. It was reported that sorghum requires 310 kg of water to produce 1 kg of biomass, while maize consumes 23 % more water, i.e., 370 kg to produce same quantity of biomass [7]. Besides biofuel production from sweet sorghum, a plethora of food products such as beverage, cookies, syrup, sweets, chocolates [8], and bioproducts like biopolymer resin can be produced [9]. However, the commercialization of this smart feedstock primarily hinges on the national biofuel policy of respective countries besides identification of productive cultivars adapted to the targeted region owing to significant genotype × environment interaction [10].

This chapter will focus on genetic enhancement of sweet sorghum through conventional plant breeding and the production of various bioproducts based on this novel feedstock.

Food: Fuel Trade Off

It is often stated that sweet sorghum cultivars do not produce grain yield or the grain yield is very less vis-a-vis that of grain sorghum. Studies at the International Crops Research Institute for the Semiarid Tropics (ICRISAT) showed that sweet sorghum hybrids had higher stem sugar yield (11 %) and higher grain yield (5 %) compared

to grain sorghum types, while sweet sorghum varieties had 54 % higher sugar yield and 9 % lower grain yield compared to non-sweet stalk varieties in the rainy season. On the other hand, both sweet sorghum hybrids and varieties had higher stalk sugar yields (50 and 89 %) and lower grain yields (25 and 2 %) in the post-rainy season. Thus, there is little trade-off between grain and stalk sugar yields in the sweet sorghum hybrids in the rainy season, while the trade-off is less in varieties in the post-rainy season [2, 3].

This is further corroborated by other published work [11] showing that there is significant soluble sugar content in the stems (79–94 %) during post-anthesis period, with the hybrids exhibiting significantly high soluble sugar content over varieties with same maturity period and effects of year, harvest time, and genotype on calculated ethanol yield (CEY) are highly significant. The experimental data on the relationship between stalk sugar traits and grain yield shows that the regression coefficient of stalk sugar yield on grain yield is not significant, thereby indicating that the grain yield is not affected when selection is done for stalk sugar yield. Hence, selection programs can aim to improve both the traits simultaneously.

Climate Change

Global warming due to climate change will affect grain and stover yields in crops, more so in tropical Africa and Asia where sorghum is a major food crop. Most climate change models predict rise in air and soil temperatures and sea levels and increased frequencies of extreme weather events leading to unprecedented changes in agricultural production in the years to come. In the Intergovernmental Panel on Climate Change (IPCC), climate models predict an increase in global average surface temperature of between 1.4 and 5.8 °C from 2001 to 2100, the range depending largely on the scale of fossil fuel burning between now and then and on the different models used. At the lower range of temperature rise (1–3 °C), global food production might actually increase, but above this range, it would probably decrease [12]. However, broad trends will be overshadowed by local differences, as the impacts of climate change are likely to be highly spatially variable. In general, the sorghum maturity period of current varieties decreases with increased temperatures. Climate change effects in terms of high temperatures and erratic rainfall may drastically reduce sorghum yields in South Asia, Southern Africa, and West Africa [13]. Climate change will cause changes in the length of the growing period (LGP) in some regions. Cooper et al. [13] showed that the extent of global semiarid tropical (SAT) areas will be changed through (1) SAT areas being "lost" from their driest margins and become arid zones due to LGPs becoming too short or (2) SAT areas being "gained" on their wetter margins from

subhumid regions through the reduction in the current LGPs in those zones. It means sorghum could be grown in new areas of the currently humid tropics where sorghum is not grown at present. Therefore, development of crop cultivars with a maturity duration that suits the prevailing LGP will be one of the best options to cope with changes in LGP. ICRISAT and Indian National Agricultural Research System (NARS) have developed a wide variety of sweet sorghum female parental lines and restorers besides varieties with altered LGP that can play pivotal role in achieving the above said option.

Taxonomy

Sorghum was first described by Linnaeus in 1753 under the name *Holcus*. In 1974, Moench distinguished the genus *Sorghum* from genus *Holcus* [14, 15]. Subsequently, several authors have discussed the systematics, origin, and evolution of sorghum since Linnaeus [16–19]. Sorghum is classified under the family *Poaceae*, tribe *Andropogoneae*, subtribe *Sorghinae*, and genus *Sorghum*. The genus was further divided [20] into five subgenera: *Sorghum*, *Chaetosorghum, Heterosorghum, Parasorghum*, and *Stiposorghum*. Variation within these five subgenera except the subgenera *Sorghum* has been described [14]. *Sorghum bicolor* subsp. *bicolor* contains all of the cultivated sorghums. Harlan and deWet [20] have developed a simplified classification of cultivated sorghum which proved to be of real practical utility for sorghum researchers. They classified *Sorghum bicolor* (L.) Moench, subsp. *bicolor* into five basic and ten hybrid races as depicted in Table 1.1. The 15 races of cultivated sorghum can be identified by mature spikelets alone, although head type is sometimes helpful. The Biodiversity International [formerly International Plant Genetic Resources Institute (IPGRI)] advisory committee on sorghum and millet germplasm has accepted and recommended this classification to be used in describing sorghum accessions.

Table 1.1 Classification of *Sorghum bicolor* (L.) Moench. subsp. *bicolor*

Basic races	Intermediate/hybrid races
(1) Race bicolor (B)	(6) Race *guinea-bicolor* (GB)
(2) Race guinea (G)	(7) Race *caudatum-bicolor* (CB)
(3) Race caudatum (C)	(8) Race *kafir-bicolor* (KB)
(4) Race kafir (K)	(9) Race *durra-bicolor* (DB)
(5) Race durra (D)	(10) Race *guinea-caudatum* (GC)
	(11) Race *guinea-kafir* (GK)
	(12) Race *guinea-durra* (GD)
	(13) Race *kafir-caudatum* (KC)
	(14) Race *durra-caudatum* (DC)
	(15) Race *kafir-durra* (KD)

Sweet Sorghum Distribution and Climatic Conditions

In simple terms, wherever sorghum is currently grown, sweet sorghum can also be cultivated commercially. Thousands of hectares are grown with sweet sorghum for biofuels production in Brazil, China, and the USA, while in the Philippines, it is grown for vinegar synthesis, and considerable areas in India, the USA, Indonesia, West Asia, and North Africa go for fodder production. In Western and Southern Africa, it is widely used for chewing purposes and local beverage production. This feedstock is well adapted to the SAT and is one of the most efficient dryland crops in converting atmospheric CO_2 into sugar [3]. The crop can be grown in a wide range of climatic conditions as given below.

Latitude

Sweet sorghum can be grown between 40°N and 40°S latitude on either side of the equator.

Altitude

Sorghum can be found at elevations between sea level and 1,500 m asl. Most East African sorghum is grown between the altitudes of 900–1,500 m, and cold-tolerant varieties are grown between 1,600 and 2,500 m in Mexico.

Environmental Conditions

Sweet sorghum can be grown in the temperature range of 12–37 °C. The optimum temperatures for growth and photosynthesis are 32–34 °C, day length is 10–14 h, optimum rainfall 550–800 mm, and relative humidity between 15 and 50 %. However, the lower the diurnal and nocturnal temperature differential, the less stalk sugar accumulation observed is in tropical sweet sorghums.

Soil Conditions

Alfisols (red) or vertisols (black clay loamy) with pH 6.5–7.5, organic matter >0.6 %, soil depth >80 cm, soil bulk density <1.4 gcc, water holding capacity

>50 % field capacity, N $\geq$ 260 kg ha^{-1} (available), P $\geq$ 12 kg ha^{-1} (available), and K $\geq$ 120 kg ha^{-1} (available) are optimal soil conditions for sorghum growth.

Water

While sorghum will survive with a supply of less than 300 mm over the season of 100 days, sweet sorghum responds favorably with additional rainfall or irrigation water. Typically, sweet sorghum needs between 500 and 1,000 mm of water (rain and/or irrigation) to achieve good yields, i.e., 50–100 t ha^{-1} total aboveground biomass (fresh weight). The great advantage of this feedstock is that it can become dormant, especially in the vegetative phase, under adverse conditions and can resume growth after relatively severe drought. Early drought stops growth before panicle initiation and the plant remains vegetative; it will resume leaf production and flowering when conditions become favorable for growth again. Mid-season drought stops leaf development. Although this crop is susceptible to sustained flooding particularly at early vegetative phase, it tolerates water logging better than maize and sugarbeet [2].

Radiation

Being a C4 plant, sweet sorghum has high radiation use efficiency (RUE) (about 1.3–1.7 g MJ^{-1}). It has been shown that taller sorghum types possess higher RUE, because of a better light penetration in the leaf canopy.

Photoperiodism

Most hybrids of sweet sorghum are relatively less photoperiod-sensitive vis-a-vis purelines. Traditional farmers, particularly in West Africa, use photoperiod-sensitive varieties. With photoperiod-sensitive types, flowering and grain maturity occurs almost during the same calendar days regardless of planting date, so that even with delayed sowing, plants mature before soil moisture is depleted at the end of rainy season.

Reproductive Biology

Breeding procedures that are used with a particular crop species are determined by its mode of reproduction. Understanding the details of phenology, i.e., floral biology, pollination, fertilization, and seed development in a crop, makes it possible to develop orderly and efficient breeding procedures.

Panicle Initiation

Sorghum blooming is hastened by short days and long nights. However, varieties differ in their photoperiod sensitivity [21]. Tropical sweet sorghum varieties initiate the reproductive stage when day lengths return to 12 h. Usually, the floral initial is 15–30 cm above the ground when the plants are about 50–75 cm tall [22]. Floral initiation marks the end of the vegetative growth due to meristematic activity. The time required for transformation from the vegetative apex to reproductive apex is largely influenced by genetic characteristics and the environment (photoperiod and temperature). The grand period of growth in sorghum follows the formation of a floral bud and consists largely of cell enlargement. Hybrids take less time to reach panicle initiation and are relatively less influenced by photoperiod and temperature [2, 3].

Panicle Emergence

During the period of rapid cell elongation, floral initials develop into an inflorescence. About 6–10 days before flowering, the boot will form as a bulge in the sheath of the flag leaf. This will occur, in a variety that flowers in 60–65 days, about 55 days from germination. Sorghum usually flowers in 55 to more than 70 days in warm climates, but flowering may range from 30 to more than 100 days. These observations are valid for tropical sweet sorghums, while temperate sorghums that mature in 5 months take 20–30 days longer for panicle emergence [2, 3].

Panicle Structure

The inflorescence is a raceme, which consists of one or several spikelets. It may be short, compact, loose, or open and composed of a central axis that bears whorls of primary branches on every node. The spikelet usually occurs in pairs, one being sessile and the second borne on a short pedicel, except the terminal sessile spikelet, which is accompanied by two pediceled spikelets. The first and second glumes of

every spikelet enclose two florets: the lower one is sterile and is represented by a lemma and the upper fertile floret has a lemma and palea. Two lodicules are placed on either side of the ovary at its base. Androecium consists of one whorl of three stamens. The anthers are attached at the base of the ovule by a very fine filament and are versatile and yellowish. Gynoecium is centrally placed and consists of two pistils with one ovule from which two feathery stigmas protrude. The sessile spikelet contains a perfect flower. It varies in shape from lanceolate to almost rotund and ovate and is sometimes depressed in the middle. The pediceled spikelets, usually lanceolate in shape and possess only anthers, occasionally have a rudimentary ovary and empty glumes [9].

Anthesis and Pollination

Anthesis starts after panicle emergence from the boot leaf. Flowers begin to open 2 days after full emergence of the panicle. Floret opening or anthesis is achieved by swelling of the lodicules and is followed by the exertion of anthers on long filaments and of stigmas between the lemma and palea. Sorghum head begins to flower at its tip and flowers successively downward over a 4- or 5-day period. Flowering takes place first in the sessile spikelets from top to bottom of the inflorescence. It takes about 6 days for completion of anthesis in the panicle with maximum flowering at 3 or 4 days after anthesis begins. Flowering proceeds downwards to the base in a horizontal plane on the panicle. When flowering of the sessile spikelets is halfway down the panicle, pedicellate spikelets start to open at the top of the panicle and proceed downwards [22]. Anthesis takes place during the morning hours and frequently occurs just before or just after sunrise, but may be delayed on cloudy damp mornings. It normally starts around midnight and proceeds up to 10:00 AM depending on the cultivar, location, and weather. Maximum flowering is observed between 6:00 and 8:00 AM. The anthers dehisce when they are dry and pollen is blown into air. The pollen remains viable several hours after shedding. The flowers remain open for 30–90 min. Dehiscence of the anthers for pollen diffusion takes place through the apical pore. The pollen drifts to the stigma, where it germinates; the pollen tube, with two nuclei, grows down the style, to fertilize the egg and form a *2n* nucleus [2, 3, 19].

Cytoplasmic male sterility has been found in sorghum (A_1-A_4 systems) and has made possible the development of a hybrid seed industry. A good male-sterile plant will not develop anthers, but in some instances dark-colored shriveled anthers with no viable pollen will appear. Partially fertile heads are also observed, and although the anthers frequently have viable pollen, the quantity is less than in normal plants. There are two types of male sterility, viz., (a) genetic male sterility (GMS) and (b) cytoplasmic nuclear male sterility (CMS), both widely used in sorghum improvement programs [4].

Table 1.2 Genetic male sterility genes, their designated symbols, and mechanism of sterility

Gene symbol	Mechanism	Reference
ms_1	Normal pollen is dominant over aborted or empty pollen cells	[25]
ms_2	-do-	[26]
ms_3	-do-	[27]
ms_4	Empty pollen cells	[28]
ms_5	Aborted pollen	[29]
ms_6	Micro anthers without pollen	[29]
ms_7	Empty pollen cells	[30]
al	Antherless stamens	[31]

Genetic Male Sterility

Genetic male sterility is expressed in sorghum in many ways. Several sources of male sterility are identified. In all the cases, it was shown that a recessive allele in homozygous condition designated with a series of alleles, ms_1, ms_2, ms_3, ms_4, ms_5, ms_6, ms_7, and *al*, confers male sterility [19, 23, 24]. The genetic male sterility genes are represented in Table 1.2.

Cytoplasmic Nuclear Male sterility

The discovery of the male sterility resulting from the interaction of cytoplasmic and nuclear genes [32] laid the foundation and revolutionized the development of hybrid cultivar and hybrid seed production technology. The milo cytoplasm was from *durra* race, which induced male sterility in the nuclear background of *kafir* race, and this is designated as A_1 cytoplasm. Since then, several sources and types of male-sterile-inducing cytoplasms have been discovered and reported. In all these cytoplasms, recessive genes in the nucleus and sterile cytoplasm induce male sterility. These male-sterile cytoplasms have been differentiated based on the inheritance patterns of their fertility restoration. The inheritance of fertility restoration is not clear, as it is dependent on the specific cytoplasm and nuclear combinations. Fertility restoration is controlled by single gene in some combinations (e.g., A_1) but is controlled by two or more genes when the same nuclear genotype interacts with a different cytoplasm [33]. Although diverse male-sterile cytoplasms have been identified, by far, only the milo cytoplasmic male sterility system is widely used because the hybrids based on this cytoplasm produce sufficient heterosis (20–30 %) over the best available pure lines in sweet sorghum. In spite of A_2 cytoplasm being as good as A_1 cytoplasm for mean performance as well as heterosis for economic traits such as stalk yield, juice yield, grain yield, days to 50 % flowering, and plant height, it is not popular as the anthers in A_2 male steriles, unlike the A_1 male steriles, mimic the fertile or maintainer lines and lead to

difficulties in monitoring the purity of hybrid seed production, and also the restoration frequency is low. ICSSH 58 (ICSA 738 × ICSV 93046) is the first A_2-based sweet sorghum hybrid in the world bred at ICRISAT and reached the farmers' fields. Other alternate sources like A_3, A_4, A_{4M}, A_{4VZM}, A_{4G1}, A_5, A_6, 9E, and KS are not useful primarily because (1) restorer frequencies are low (restorer frequency: $A_1 > A_2 > A_4 > A_3$) and (2) male steriles cannot be readily distinguished from male fertiles. There is a need to search for more useful form of male sterility yet different from milo (A_1). Milo restorers need to be diversified in guinea background to further enhance the yield advantage in hybrid development. Restorer frequency is very low on non-milo cytoplasms. So, there is a need to identify and breed for high-yielding non-milo cytoplasm restorers [2, 34]. The high Brix% possessing (>14 %) female hybrid parents are not available in plenty on sweet sorghum breeding programs across the globe to exploit the potential heterosis for stalk yield and juice yield [2].

Breeding Sweet Sorghum

Breeding Behavior

Sorghum is basically a self-pollinating crop, but natural cross-pollination varies from 0.6 to 6 % depending on the cultivar. Sorghum has the advantage of possessing complete self-pollination due to its floral biology, cleistogamy, and genetic and cytoplasmic genetic male sterility. Breeding methods relevant to self as well as cross-pollinated crops are, therefore, applied to breed pure line varieties, hybrids, and populations in sorghum. Hand pollination should begin around 9:30 or 10:00 AM and can be extended up to 11:30 AM to 12:30 PM on a foggy morning [22].

Candidate Traits and Variability

The major characteristics which a sweet sorghum cultivar should possess are:

1. High biomass productivity (75–100 t ha^{-1})
2. High Brix% (20–23 %)
3. Thick stems and juicy internodes
4. Photo- and thermo-insensitivity aids to fit into diversified cropping systems
5. Tolerance to shoot pests and diseases
6. Good digestibility of residues when used as forage
7. Tolerance to mid-season and terminal drought
8. Salinity and heat tolerance
9. High water, nitrogen, and radiation use efficiencies
10. Juice quality and quantity sustenance during post-harvesting
11. Grain yield (4.0–7.0 t ha^{-1})

Ayyangar [35] suggested that a single dominant gene confers the non-sweet character. Later, it was reported that stalk sugar is under the control of recessive genes with additive and dominance effects [36]. On the contrary, subsequent studies provided support for the existence of multiple genes with additive effects. Continuous variation in the amount of extractable juice was observed in juicy genotypes and inbred progeny of juicy × dry lines, suggesting multiple genes may be involved in controlling the trait [8, 37, 38]. There was also a report suggesting the involvement of several genes affecting the biofuel traits in sweet sorghum background. The evaluation of four promising sweet sorghum lines [Keller, BJ 248, Wray, and NSSH 104 (CSH 22SS) along with the check SSV 84] indicated substantial genotypic differences for extractable juice, total sugar content, fermentation efficiency, and alcohol production [39]. An analysis of 53 ICRISAT-bred elite hybrids in both the rainy and post-rainy seasons showed that the correlation and regression coefficients are significantly high for all the component traits of sugar yield (Brix%, stalk yield, juice weight, and juice volume) [2]. Knowing general (GCA) and specific (SCA) combining ability effects of genetic materials is of practical value in breeding programs. GCA effects represent the fixable component of genetic variance and are important to develop superior genotypes. SCA represents the non-fixable component of genetic variation, and it is important to provide information on hybrid performance. The line × tester analysis of 171 hybrids along with their parents in both rainy and post-rainy seasons showed that the magnitude of SCA variance was higher suggesting the importance of nonadditive gene action in inheritance of sugar yield-related traits though both additive and dominant genes controlled overall sugar yield during both rainy and post-rainy seasons in tropical sweet sorghums. Hence, selection in early generations would be ineffective and recurrent selection with periodic intercrossing is advocated. However, breeding for good combining restorer parents can produce high sugar yields in post-rainy season. There is an indication of existence of transgressive segregation for sugar yield that can be exploited [39]. The heritability for traits such as stem juice content, stem sugar concentration, total stem sugars, juice glucose, juice fructose, and juice sucrose was low [40, 41]. The predominant role of nonadditive gene action for plant height, stem girth, total soluble solids, millable stalk yield, and extractable juice yield and substantial magnitude of standard heterosis for candidate sugar traits (stem girth: up to 5.3 %, total soluble solids%: up to 7.4 %, millable stalk yield: up to 1.5 %, and extractable juice yield: up to 122.6 %) indicate the importance of heterosis breeding for improving ethanol productivity of cultivars [42]. The significant positive correlation of general combining ability (GCA) effects with per se performance of parents in sweet sorghum facilitates quicker identification and development of sugar rich, high biomass yielding hybrid parents [2, 43]. The generation mean analysis of two crosses has shown predominantly additive gene action for traits like sucrose% and Brix% of juice. However, for cane and juice yield, dominance gene action and dominance × dominance gene interaction were of higher magnitude in both the crosses. Since the traits important for high sugar content have

dominance and overdominance inheritance, utilization of hybrid vigor by developing sweet sorghum hybrids is an attractive option. Also one of the parents with high sucrose content will suffice in getting good hybrids with high sugar and juice yield [44].

From these studies, it is quite evident that significant diversity exists in traits important for biofuel production and this opens up excellent opportunities for sweet sorghum improvement. Biofuel traits are governed by multiple genes and both additive and dominant components of gene action have to be exploited while breeding for high stalk sugar and juice-yielding genotypes. It was demonstrated that the improved hybrids top ranking for grain and sugar yields in rainy season are not top ranking in the post-rainy season and vice versa. It is important to breed for rainy and post-rainy seasons separately [2–4]. The selections for post-rainy season adaptation should be made in post-rainy season only, and for rainy season adaptation, selections can be made in both rainy and post-rainy seasons.

Breeding Objectives

In general, the sweet sorghum breeding programs aim to develop parents and hybrids which can address both first and second generation (lignocellulosic feedstock development) biofuel production issues. The breeding objectives are:

1. To develop sweet sorghum female parents with high stalk sugar and grain yield
2. To develop restorer lines/varieties with high sugar content and resistance to stem borer and shoot fly
3. To develop and identify sorghum hybrids (amenable for mechanical harvesting) with high biomass suitable for use in bioethanol and bioenergy production

Breeding Methods

The most commonly used programs in sweet sorghum improvement are short-term programs (pedigree method and backcross) and long-term programs (population improvement methods). The most common approach in sweet sorghum breeding has been elite × elite crosses followed by pedigree selection. Breeding new female lines, B and R lines have increasingly become dependent on crossing elite by elite lines, B × B and in some cases such as improving for resistance B × R lines. In case of male lines (R lines) improvement, it is R × R crosses. This process progressively narrows the genetic base of breeding programs and requires new traits, especially resistances, to be brought in by pre-breeding and often backcrossing. The success of a backcrossing program depends on the precision with which the desired trait can be identified and thus introgressed into the recurrent parent through backcrossing.

Fig. 1.1 Comparison of grain sorghum (front) and sweet sorghum crop (rear) at flowering

Pedigree Method

Pedigree breeding method is the most commonly used method of breeding in sorghum where the selection begins in the F_2 generation targeting superior plants which are expected to produce the best progenies. Hybrids between diverse parents segregate for a large number of genes, and every F_2 individual is genetically different from other individuals. The population size becomes crucial for the success of recovering desirable genotypes, when several genes are involved. In this method (Fig. 1.1), superior individual plants are selected in successive segregating generations from the selected families, and a complete record of parent progeny relationship is maintained. Identifying a potentially good cross is essential since the best F_1 plants produce better yielding F_4 progenies. The selection in segregating generations should be based on (1) performance of the families of the selected cross on the whole and (2) the individual plants performance within the selected family. Selection for many of the per se selection criteria encompassing various traits like tallness, stem thickness, and juice yield can be rapidly applied in the first two or three segregating generations since crosses between elite lines produce a high proportion of progeny with desirable per se values. Once the promising lines have been identified, they can be test crossed onto male-sterile lines for checking fertility restoration and may be classified as B or R lines. Lines with high biomass yield and other desirable agronomic characters can be released as varieties. The pedigree method has been utilized to create new recombinants, transfer of few to many genes governing resistances to various insects, diseases, cold tolerance, etc. in sorghum. In India, the important sweet sorghum genotypes released through pedigree method of selection are SSV 74, SSV 84, CSV 19 SS, and CSV 24SS [45].

Backcross Method

This method does not offer an opportunity to provide new recombinants as hybrids are crossed back to either of the parents and thus they cannot be fixed. However, it can be utilized to incorporate brown-midrib (*bmr*) or specific defense-related alleles (e.g., stem borer resistance) or improve other traits like seed size, seed shape, and cold tolerance through repeated backcrosses. The backcross method has also been successfully employed in the Indian and ICRISAT breeding programs for transfer of BMR genes and genes which confer high digestibility into elite dual-purpose varieties. Several *bmr* lines in sweet sorghum background, stacked *bmr* mutants, stem borer tolerant lines, etc. have been developed through this method. Several stay-green QTLs (*stgB, stg2*, *and stg3*) are being introgressed into elite sweet sorghum cultivars by deploying this method.

Population Improvement

This method provides long-term breeding strategy to derive diverse and broad genetic-based superior varieties/hybrid parents. Therefore, a comprehensive crop improvement strategy has to combine both short- and long-term progress for continuous improvement of economic traits. The population improvement procedure involves selection of component parents with high GCA, incorporation of genetic male sterility, intercrossing and random mating among parents, and applying appropriate recurrent selection schemes. At ICRISAT-Patancheru, 24 sorghum populations encompassing characters like grain mold, good grain, photo-insensitive, and early dual purpose were developed and maintained. Recently, ICRISAT has started developing sweet sorghum population with ms_3 gene for applying recurrent selection. While population improvement programs are not the most common in sweet sorghum breeding, they are an important source of genetic variation and improved traits.

Genomics

The availability of genomic sequence for sorghum has made it possible to carry out genome-wide analyses. Whereas earlier studies on simple sequence repeat (SSR) marker development primarily utilized anonymous DNA fragments containing SSRs isolated from genomic libraries, more recent studies have used computational methods to detect SSRs in sequence data generated from genomic sequences projects. In the sorghum genome, a total of 109,039 tandem repeats were detected, of which 15,194 were microsatellite (SSR) markers [46]. In a recent studies, several major QTLs for grain and stem sugar composition and yield and their results indicated that overall energy yields could be increased by concurrent improvement for both sorghum grain and sugar traits [37, 40, 41]. Elucidating the genetic basis of stem sugar and stem juice accumulation, modifying cell wall composition so that sorghum biomass can be processed more efficiently, maximizing biomass yield for

a given geographic area and production system, and understanding the different mechanisms underlying drought tolerance are the main focus areas among sorghum researchers who target bioenergy traits.

Transgenic approaches to improve stem sugar accumulation have not been attempted in sweet sorghum. However, differential expression of some genes related to sucrose metabolism has been observed between sweet sorghum and grain sorghum [47]. Further, mature internodes of sweet sorghum showed a lower expression of sucrose transporters suggesting that sucrose accumulation may result from lower transport of sucrose from sink tissues. These genes could serve as important candidate genes for transforming sorghum to achieve better stem sugar yields. However, genetic manipulation of some key enzymes involved in sucrose metabolism did not bring about greater sucrose accumulation in the mature internodes of sugarcane, suggesting their inadequacy in overcoming the osmotic limits of the sugar-storing vacuoles [48]. A microRNA miR169 was recently shown to be involved in regulating sugar levels in sweet sorghum stems suggesting epigenetic regulation of sucrose accumulation [49]. Similarly, a wide hybridization is another useful approach to transfer biotic and abiotic stress tolerance conferring gene transfer from tertiary gene pool *sps* to sweet sorghum cultivars exploiting *iap* (*i*nhibition of *a*lien *p*ollen) lines like T × 3361, Nr481 [50].

Bioproducts of Sweet Sorghum

A profile of different biomass and grain-based bioproducts derived from sweet sorghum is represented in Fig. 1.2. The following section details these bioproducts.

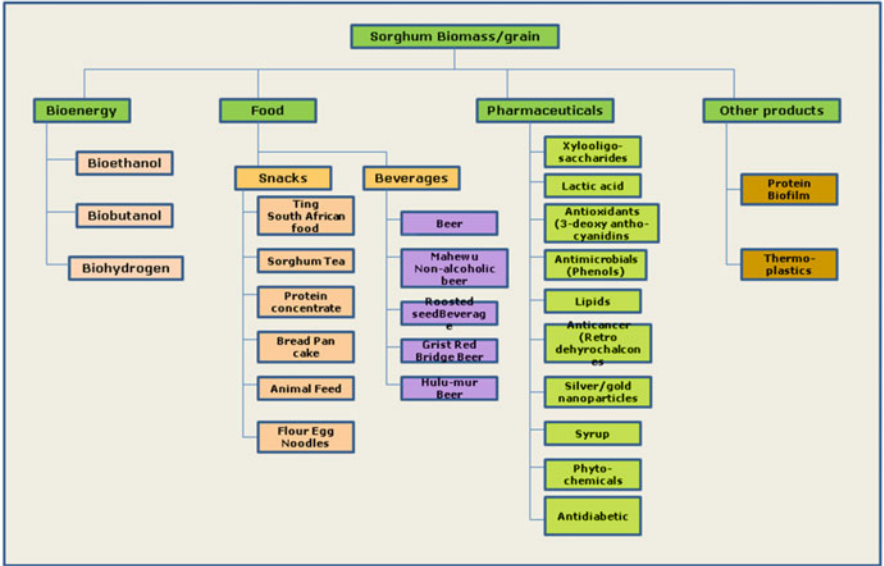

Fig. 1.2 Sorghum-based bioproducts profile

Beverages

Sorghum grain-based beverages are consumed in Africa [51] and known by different names across Africa, including *burukuto* (Nigeria), *pombe* (East Africa), *bjala* (Northern Sotho), and *bil-bil* (Cameroon). African sorghum grain-based beer is produced by lactic acid as well as alcoholic fermentation to achieve distinct sour taste. The souring process is initiated using yogurt, sour dough starter cultures, or by spontaneous fermentation. Opaque sorghum beers are also popular alcoholic sorghum-based beverages in Africa which is known by *tchoukoutou* (Benin) in West Africa, *dolo* (Burkina-Faso), *pito* (Ghana), and *burukuto* or *otika* (Nigeria) [52]. These lager beers are characterized by sour taste with relatively high dry matter content (5–13 g 100 ml^{-1}) and low alcohol content (2–3 ml 100 ml^{-1}) [53]. These beers are mostly prepared with Guinea corn (*Sorghum bicolor*) along with other cereals such as millet or maize as adjuncts or substitutes [52]. The manufacturing process consists of malting, brewing, and fermentation steps. Depending on the geographical location, variations have been observed in the production process [54].

In China, sorghum is fermented to produce distilled beverages, like *baijiu* (sorghum white wine), *maotai* (sorghum liquor), and *kaoliang* (sorghum wine). In USA, two sorghum-based beer products “New Grist” and “Redbridge” have been marketed since 2006, which are gluten-free and hence preferably consumed by people suffering with celiac disease and also popularized among health-conscious drinkers due to its low-carbohydrate content [54]. The nonalcoholic fermented African sorghum beverages like *kunun-zaki* (Nigeria), *hulu-mur* from sorghum malt and flour (Sudan), and *motoho-oa-mabele* from sorghum meal (South Africa) involve some form of lactic acid fermentation [55]. *Kunun-zaki* is a highly perishable product and has a short shelf life (24 h) under tropical ambient conditions; however, the shelf life may be extended under refrigerated conditions [56] or by using 0.1 % sodium benzoate or sodium metabisulfite in combination with pasteurization at 60 °C for 1 h by more than 3 weeks [57]. *Hulu-mur* is a traditional Sudanese nonalcoholic beverage made from a fermented mixture of unmalted flour of sorghum (*Sorghum bicolor*) and malt [58, 59].

Foods

Nearly 30 different fermented sorghum/sweet sorghum-based food products are consumed in Sudan. *Injera* is a leavened, spongy, and sour thin flat, round, staple fermented Ethiopian traditional bread prepared with flour from either of different cereals, water, and starter (*ersho*, a liquid saved from the previously fermented dough). Injera prepared using tef [*Eragrostis tef* (*Zucc*) Trotter], a tiny millet-like grain, is the most popular and preferred cereal ingredient, although other different types of cereals, including sorghum, tef, maize, wheat, finger millet, and barley, are used [60]. The white tannin-free sorghums are preferred due to the light *injera* color

or because of relative brittleness and dryness of sorghum injera after storage [60]. Kisra (aseeda or aceda) is a traditional bread for Arabian Gulf, Sudan, and Iraq which is similar to injera. It is made from the fermented dough of sorghum (*Sorghum bicolor*) or pearl millet (*Pennisetum typhodium*) grains. The fermented dough is baked into thin sheets and consumed along with stew prepared from vegetables and meat. *Lactobacillus* sp., *Acetobacter* sp., and *S. cerevisiae* were the main microflora isolated from kisra and responsible for the fermentation process [61]. Studies on kisra preparation indicated that the fermentation of kisra enhanced riboflavin and significantly decreased thiamine, without any change in the mineral contents [62]. Sweet sorghum porridges produced by hard grain and cysteine addition to wheat flour accelerated the stress and structural relaxation [63] and reduced the mixing time by facilitating the breakdown of the wheat proteins by splitting the disulfide bonds rapidly thus aiding faster dough development [64]. Addition of L-cysteine hydrochloride (0.1 %) increased water absorption, but decreased dough development time and dough stability [65]. The Pampanga Agricultural College, Philippines, has pioneered in this work and published a compendium that enlisted a huge array of products such as cakes, cookies, biscuits, rice, porridges and beverages.

Bioethanol

Sorghum-based ethanol production is a very recent area (two decades old) which involves preprocessing steps like harvest approaches [66], juice processing techniques [67], as well as fermentation and depends on yeast strain used and yield ranges from 78 to 90 % [68, 69]. Biomass-based solid-phase fermentation and juice-based liquid batch fermentation [70] and fed-batch fermentation [71] were also been investigated. Application of immobilized yeast in a fluidized bed reactor [72], gelatin bead-packed bed reactor [73], stirred tank, and tubular bioreactors [74] as well as application of statistical approaches [72] and use of very high gravity (VHG) [75] shortened the fermentation time significantly and increased the conversion efficiency. Higher ethanol yields were reported by fermenting 30 % sulfuric acid-treated sorghum [76] and from sorghum fibers pretreated with dilute ammonia followed by enzymatic hydrolysis and fermentation [77]. Specifically, the energy yield from ethanol obtained from the above-referenced studies ranged between 6,500–8,900 kJ/kg dry and 1,400–2,700 kJ/kg fresh sorghum biomass, respectively (assuming that the energy yield from ethanol is 26,500 kJ/kg). To date, ethanol and methane are the well-known microbial-derived products from sweet sorghum [78]. The data indicates that from a metric ton of sweet sorghum having 18 % Brix, 350–450 L of juice can be realized and up on fermentation 45–55 L of transport grade ethanol can be realized [2–4]. The utilization of bagasse has a most promising future for its conversion to ethanol or butanol, while the residual solids (mainly lignin) can be incinerated to cogenerate heat and power [2]. Other byproducts of sweet sorghum ethanol value chain are vinasse and furnace oil. Vinasse can be converted into a valuable fertilizer.

Biohydrogen

Among different biofuels, hydrogen is widely acknowledged as an attractive candidate for replacement of fossil fuels as it is a clean and renewable energy carrier with high energy yield as compared to other biofuels and emits water as an end product upon consumption. Several approaches such as thermochemical (gasification, pyrolysis, supercritical conversion, etc.) and biotechnological (photo-fermentation, water–gas shift reaction or uptake hydrogenase, dark fermentation, direct and indirect biophotolysis, etc.) have been evaluated [79–82] for hydrogen production. Evaluation of different resources and available processes suggests the use of renewable energy material with high carbohydrate content which would offer better solution. Rich fermentable carbohydrate containing energy crops such as *Miscanthus*, sugarcane, and sweet sorghum is advantageous over other biomass resources. Thermodynamically, simple sugars are advantageous over complex carbohydrate substrates for microbial metabolism and also known to influence the biohydrogen yields. Sweet sorghum biomass has high concentration of soluble sugars 18–22 % on dry weight basis [83] predominantly sucrose with variable levels of glucose, starch, and fructose depending on genotype and has an edge over other biomasses [84]. The fermentation-based production of biofuel is mainly regulated by the structural complexity of substrate material, microbial nature, and other physiological factors [82, 85]. A significant negative correlation between lignin content and fermentative biohydrogen production was reported by Prakasham et al. [80] while working with low lignin containing brown-midrib sorghum mutants.

Multi-substrate utilizing microbial strains would offer edge over single carbohydrate metabolizing strains as conversion yields improve substantially. Rumen bacteria has such potential, and it was reported that when grown on different subparts of sweet sorghum like sorghum stalks and sorghum water extract, the biohydrogen yields were comparable [83]. The biohydrogen production was from xylose, cellobiose, arabinose, formic acid, etc. besides glucose. In a mixture of cellobiose, arabinose, xylose, and glucose, glucose was most preferred and arabinose was least for microbial metabolism and differed with microbial genetics as the initial enzymatic conversion of these carbohydrates to intermediates of glycolysis played a significant role. Glucose gets metabolized via EMP, while xylose enters only after the conversion to xylulose and subsequently to xylulose-5-phosphate by the sequential catalysis of xylose isomerase and xylulose kinase [86]. Irrespective of microbial strains and biomass material, all biohydrogen processes are regulated by hydrogen-producing enzymes [79, 87, 88] and associated with CO_2 production as well as may be combined with other gases like methane and hydrogen sulfide depending on the biological source and substrate. In fact, studies on hydrogen production inhibition indicated that higher hydrogen gas concentration shifts microbial metabolic pathways to produce more lactate, ethanol, acetone, butanol, or alanine [89]. In addition, biohydrogen yield is regulated by different bioreactor conditions such as pH, microbial consortia, structural complexity of biomass,

Table 1.3 Comparison of biohydrogen yield with different plant biomasses

Biomass type (treatment conditions)	H_2 yield (ml H_2/g TVS)	Reference
Corn stover (220 °C for 3 min)	49.00	[90]
Corn husk	62.30	[82]
Corn straw	9.00	[91]
Corn stalk	3.00	[92]
Corn stalk waste	149.69	[92]
Corn cobs (1 % HCl + 100 °C for 30 min)	107.9	[93]
Maize leaves	18.00	[94]
Rice husk	40.38	[82]
Wheat straw	6.40	[95]
Wheat powder	281.00	[96]
Sugarcane bagasse (130 °C for 30 min)	19.70	[94, 97]
Groundnut shell	44.12	[82]
Sweet sorghum plant (130 °C for 30 min)	32.40	[94]
Whorled Rosinweed leaves	10.30	[94]
Switchgrass	27.10	[98]

hydraulic retention time (HRT), and hydrogen gas partial pressure during anaerobic fermentation [79, 80] irrespective of the biomass used. A comparative account of biohydrogen yields using different plant biomasses is shown in Table 1.3.

Lipids

Sweet sorghum extract was also evaluated for production of lipid using *Chlorella protothecoides*. This microalga exhibited dry cell yield and lipid content of 5.1 g L^{-1} and 52.5 %, respectively, when sweet sorghum extract was used as carbon source. However, when the sorghum extract was supplemented with yeast extract, the dry cell yield and lipid productivity of the microalga reached to 1.2 g L^{-1} day^{-1} and 586.8 mg L^{-1} day^{-1}, respectively [99]. Similarly, another heterotrophic thraustochytrid, *Schizochytrium limacinum* SR21, was explored for lipid production using sweet sorghum juice [100]. Semi-solid-state fermentation of crushed sweet sorghum has been reported to produce single cell oils (SCO) using the oleaginous fungus, *Mortierella isabellina*. The sugars and nitrogen present in sweet sorghum were used by the fungus for oil accumulation, and the maximum oil efficiency of 11 g/100 g dry weight of substrate was observed [101].

Nanomaterials

Sweet sorghum syrup-based facile, easy, reproducible, stable, spherical, and rapid synthesis of stable gold and silver glyconanoparticles was demonstrated at room temperature without the use any surfactants [102, 103]. Glucose and fructose present in the syrup were responsible as capping ligands along with sucrose

resulting in the formation and stabilization of nanoparticles with unique H-bonding capabilities for building smart nanomaterials which find application in biomedicine as probes of carbohydrate–carbohydrate interactions and carbohydrate–protein interactions, anti-adhesive therapy, biolabels, bioamplification strategies, antimicrobial agents, and in material science for microstructure manipulation, quantum dots, and magnetic bioconjugation [102, 104].

Xylooligosaccharides

Xylooligosaccharides (XOS) have a great prebiotic prospective and their production on an industrial scale is carried out from lignocellulosic materials (LCMs) rich in xylan by chemical and enzymatic methods, and the latter is preferred in food industry because of lack of undesirable side reactions [105]. XOS seems to exert their nutritional benefits in relation to human health exhibiting excellent physiological properties including improvement in decreasing cholesterol, bowel function, calcium absorption, and lipid metabolism [106]. Furthermore, they can promote a favorable intestinal environment by selectively enhancing the growth of colonic microbiota such as *Bifidobacterium* and *Lactobacillus* [107]. In the recent years, enzymatic production of xylooligosaccharides has attracted more industries in order to make the conversion process economical and also for the effective utilization of renewable plant-based biomasses [108]. In view of this fact, sorghum grain or sorghum bagasse after pretreatment can be used as excellent sources for XOS production as its hemicellulose content varies based on the genotype of cultivar. Four types of oligosaccharides were reported from alkali-extracted sorghum glucurono-arabinoxylan by digestion with a combination of (1- > 4)-β-D-arabinoxylan arabinofurano-hydrolase (AXH) and endo-(1- > 4)-β-D-xylanase (Xyl I), both from *Aspergillus awamori* and were purified by size exclusion chromatography followed by preparative high-performance anion-exchange chromatography [109].

Antidiabetic Compounds

The extracts of sorghum contain various phytochemicals like tannins, phenolic acids, phytosterols, and policosanols. Phenolic extracts of some varieties of sorghum exhibited antidiabetic effects by increasing serum insulin in diabetic rats, and the effect was comparable with glibenclamide, a powerful antidiabetic drug [110]. Sorghum tea made from roasted grains is rich in procyanidins which exhibited stronger α-glucosidase and α-amylase inhibitory activities [111]. Commonly, acarbose, a commercially available drug, is used as an alpha-glucosidase inhibitor which reversibly and competitively inhibits the digestion of oligo- and disaccharides at the brush border of the small intestine and helps to keep blood sugar levels within a target range. This effect controls diabetes and also the development of obesity [112]. In this view, efforts can be made to validate the clinical role of procyanidins for the treatment of alpha-glucosidase inhibition.

Antioxidant Compounds

Antioxidant activity of various foods is significantly correlated with total phenols and tannins and based on this feature; sorghum foods were shown to possess antioxidant activity [113], hence prevents a plethora of physiological complications like cancer, early aging, diabetes, and cardiovascular diseases [114]. The quantity of antioxidant activity is based on the processing of sorghum samples and decorticated sorghum; cooking based on extrusion was shown to reduce the phenol content and accordingly the antioxidant activity [115]. To obtain the whole health benefits of sorghum, it is better to select a process which retains its total phenolic contents. Pigmented testa contains condensed tannins composed of flavan-3-ols which are excellent antioxidants [116]. The tannin sorghum contains high dietary fiber content which slows the hydrolysis of food in the GI tract and the calorific availability which may be responsible for reduced weight gain (antiobesity effect) in animals. Pigmented sweet sorghums have high concentration of 3-deoxyanthocyanins (luteolinidin and apigenidin) [117].

Antimicrobial Compounds

Studies conducted on the antimicrobial properties of sorghum extracts showed strong inhibitory activity against *Escherichia coli* [118]. The antimicrobial property of sweet sorghum against a specific microorganism is based on the type of cultivar as the antimicrobial property of a plant extract is based not only on the phenolic content but also on the presence of various secondary metabolites [119].

Cytotoxicity Against Cancer Cell Lines

Sorghum grain contains retrodihydrochalcones, 3-(2,4,6-trihydroxyphenyl)-1-(4-hydroxyphenyl)-propan-1-one, and 3-(2,6-dihydrox-4-methoxyphenyl)-1-(4-hydroxyphenyl)-propan-1-one which are cytotoxic in nature against various human cancer cell lines [116]. However, future studies are required to evaluate the cytotoxic effects of retrodihydrochalcones from sweet sorghum.

Polylactic Acid

Polylactic acid (PLA) is a biodegradable thermoplastic resin that can be substituted for petroleum-based thermoplastics, reducing environmental pollution and other problems associated with petroleum-based plastics [120]. Lactic acid can be produced either through chemical synthesis or through a fermentation process [121]. Agro-based materials such as cereal grains like corn, sorghum, and sweet sorghum bagasse are major potential sources to produce lactic acid through

fermentation [122]. There are reports on the production of lactic acid monomer from different varieties of sorghum wherein the whole ground sorghum grain was liquefied and fermented to lactic acid using *Rhizopus oryzae* NRRL 395 and the efficiency of saccharification was dependent on the native glucoamylase [123]. Sweet sorghum bagasse residue after alcohol fermentation can also be used for the preparation of biodegradable PLA with a tensile strength of 49.5 M and a flexible strength of 65 MPa [124].

Protein-Based Films and Adhesives

Sorghum grain has an average protein content of 11 % and its proteins are classified as prolamins (kafirins) and non-prolamin proteins. Kafirins constitute 77–82 % of the endosperm proteins and are involved in intermolecular cross-linking. Kafirin was reported to have potential in biofilm-forming applications. Its mechanical, water-vapor barrier and color properties of free-standing films from laboratory-extracted kafirin were comparable to those of zein films of commercial importance [125]. Sorghum flour as such can be used as protein extender in phenol-formaldehyde-based plywood adhesive for sprayline coaters or foam extrusion. The sorghum-based plywood glue had a viscosity of 1,104 cP and adhesion strength of 1.37 MPa which was comparable with the industry standard glue [126].

Summary

Sweet sorghum is the only first generation feedstock that provides both food and fuel besides fodder with relatively high RUE, WUE, and NUE with greater adaptation to semiarid regions [127]. Though it has gained importance as a stable food and fodder crop, recently it is increasingly viewed as a viable feedstock for the production of various bioproducts ranging from biofuels, beverages, food, pharmaceuticals, antioxidants, antimicrobial, and antidiabetics. Hence, focused research on its production and processing is required for efficient exploitation of polymeric carbohydrates, fermentable sugars, and biomass for varied needs of the society.

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Chapter 2
Breeding of Sugarcane

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Abstract Sugarcane is the main source for sugar production and the most important crop for energy production, as well as for byproducts like ethanol and fibers in the world. With a complex genome, the plant has its species from crosses between species of the genus *Saccharum*, which were the basis for sugarcane breeding programs worldwide. The production of sugarcane has increased worldwide due to breeding programs that have developed more productive clones for specific uses and adapted to different climatic conditions. The future objective of breeding programs is to develop sugarcane with high productivity, high sucrose content, drought tolerance, and high production of ethanol and biomass, i.e., plants with high fiber content and with cell walls easily broken to favor the production of ethanol from bagasse, efficient plants with low nitrogen fertilizer use, and others, and consequently to reduce environmental impacts. Currently, the demand for products derived from sugarcane is consistently increasing; the ethanol byproduct has been pointed out as one of the important sources to feed the demand for renewable energy in fossil and nonrenewable fuel substitution programs in different countries around the world. This chapter describes the genetic improvement of sugarcane and its current goals.

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Introduction

More than 1 billion tons of sugarcane are harvested each year. This exceeds the production level of the main food crops of the world, corn, wheat, and rice. Each of these has an annual production of about 600 million tons per year of plant biomass. Sugarcane is the world's biggest source of sugar surpassing sugar beet and is without doubt the most important crop for the production of energy and bio-products such as fibers (paper, cardboard, plastic) [1, 2]. Sugarcane has an average yield of 40–70 t per hectare depending on the country. Some specific varieties are able to yield up to 150 t per hectare under experimental conditions. The production of cane has been increasing and will likely continue to expand in tropical and subtropical environments, if the use of cane as an energy source continues to grow.

Crop improvement programs have been successful, because in the last 50 years, the cane has had an increase of about 40 % in its productivity. Crop improvement however takes about 8–12 years to achieve a new variety. Biotechnological approaches can become crucial to overcome the limitations of classical breeding. Besides the length of time needed to obtain new material, sugarcane is a complex organism because of its high ploidy levels (aneuploidy and polyploidy). Sugarcane's genome structure creates challenges for the development of transgenic plants that may foster the development for new market segments, such as with high saccharose content (richness), drought tolerance, and high yield of ethanol and biomass for biofuels. This chapter describes the genetic improvement of sugarcane and general breeding objectives.

Taxonomy and Domestication

Sugarcane is a semiperennial plant belonging to the Poaceae family, Panicoideae subfamily, Andropogoneae tribe, and genus *Saccharum* L. [3]. Actually the varieties cultivated in Brazil and the world are hybrids from the genus *Saccharum* which includes six species: two wild species, *S. spontaneum* L. ($2n=40$–128) and *S. robustum* Brandes and Jeswit ex Grassl. ($2n=60$–205), and four cultivated forms, *S. officinarum* L. ($2n=80$), *S. barberi* Jeswit ($2n=81$–124), *S. sinensis* Roxb. ($2n=111$–120), and *S. edule* Hassk. ($2n=60$–80). The species *S. officinarum* and *S. spontaneum* are the biggest contributors to the genome of the modern varieties. The species *S. officinarum* or "noble cane" is able to accumulate high saccharose levels in the stem but offer low resistance to diseases. It originated from hybrids between *S. spontaneum*, *S. miscanthus*, and *Erianthus*

arundinaceus and constituted the base of cane improvement programs around the world [4]. Continuous cultivation and the susceptibility to diseases made cane-producing countries start improvement programs aiming to cross the species *S. officinarum* with other species rich in saccharose, but resistant to the present diseases. So, the canes planted nowadays are referred to as *Saccharum* spp. and not only *S. officinarum* [5].

The centers of the origin of these species are hypothesized to be the islands of the Polynesian archipelago, New Guinea and India. It has been proposed that the sugarcane may be a native from Southwest Asia. The centers where the major diversity of some species is found include New Guinea for *S. officinarum* and *S. robustum*, China for *S. sinense*, and Northern India for *S. barberi* [4].

The domestication process of the cane is believed to have begun with the Muslim expansion introducing the crop in areas where it has not been previously cultivated. People originating from regions in Syria and Iraq, in their travels and conquests when they occupied the South and East Mediterranean, brought their culture including animals and plants; the cane was cultivated in the gardens of their palaces. In the ancient times, sugar was extremely rare and expensive. It was a product from pharmacies and consumed exclusively by kings and nobles. The Genoese merchants were the source of the sugar supply since the peak of the dominion of the Roman Empire over the Orient and held the monopoly of commercial relations. With the commercial expansion of merchandise coming from the Orient, Europe came to know the sugarcane. In the European continent, sugarcane was cultivated in Spain and then later brought to the Americas during the maritime expansion and was cultivated in countries such as Brazil, Cuba, Mexico, Peru, Ecuador, Colombia, and Venezuela [6].

In Brazil, the first cane seedlings were brought from the Madeira Island by Martin Afonso de Souza in 1532, and three sugar mills were built by 1534. The crop's cultivation area expanded and Brazil started to lead the worldwide sugarcane production in 1650 but later lost its top position to India in the seventeenth century [7]. In the twentieth century, Brazil recovered its leadership and currently being considered as the world's largest sugar producer.

The first utilization of sugarcane was for food, later on as fuel for ethanol production and pharmaceutical industries producing anhydrous and hydrous alcohol. Right now, besides these products, cane is seen as source of raw materials for waxes, insulation materials, pure alcohol, paper, medium-density fiberboard panels, vegetable hormones, and plastics, as well as being used to generate electric power [8].

Areas of Production

Sugarcane is the main crop for sugar production, covering 22 million hectares worldwide. Brazil and India are the main producers which account for 60 % of the world's cane production. According to the Food and Agriculture Organization

of the United Nations (FAO), Brazil is the world leader with a cane production of 719 million tons, followed by India (227 million tons), China (111 million tons), and Thailand (70 million tons). Countries like Mexico, Pakistan, Australia, Argentina, Philippines, Indonesia, the United States, Colombia, Guatemala, South Africa, Vietnam, Egypt, Cuba, Peru, Venezuela, and Myanmar are among the 20 countries with the highest production, ranging from 68 million tons by Mexico to 95 million tons by Venezuela [9].

In 2012, the area cultivated with sugarcane in Brazil covered 8.6 million hectares. The State of São Paulo had the largest area with 51.66 % of the planted area, followed by Minas Gerais (8.97 %), Goiás (8.54 %), Paraná (7.17 %), Mato Grosso do Sul (6.31 %), Alagoas (5.35 %), and Pernambuco (3.48 %). The production was 602,178.8 million tons with an average productivity of 70 t per hectare. From the total production, 50.19 % was used for producing ethanol and 49.81 % for the production of sugar [10].

In India, sugarcane is considered as one of the most important industrial crops occupying around five million hectares [11]. The main producing states in India include Maharashtra, Andhra Pradesh, Tamil Nadu, and Gujarat. The productivity in these states ranges from 70 to 100 t per hectare, whereas in other regions that include Uttar Pradesh, Bihar, Punjab, and Haryana, the yield ranges from 40 to 70 t per hectare.

China is considered the world's third largest producer with a production of 111 million tons in 2010, on an area of 1.70 million hectares and an average productivity of 65.75 t per hectare. According to Li [12], Guangxi is the Chinese province considered the biggest sugarcane producer in the country, followed by the provinces of Yanon and Guangdong.

Genetic Resources

There are many collections and germplasm gene banks of sugarcane and related genera worldwide. The United States and India have centers that serve as the world's main repositories of germplasm, being recognized by the International Society of Sugarcane Technologists (ISSCT) as the holders of the worldwide collection of materials of the *Saccharum* complex. The collections were assembled based on dozens of collecting activities of wild materials made from 1892 to 1985. The Sugarcane Breeding Institute (SBI) with headquarters in Coimbatore in the South of India is one of the pioneer institutions in breeding research on the crop having started its activities in 1912.

The SBI has more than 2,600 accessions of diverse species such as *Saccharum officinarum, S. barberi, S. sinense, S. robustum, S. edule, S. spontaneum, Erianthus spp*. and related genera, as well as more than 2,000 accessions of improved genetic material (interspecific hybrids, hybrids from the local breeding program and from other institutions, and improved clones of species such as *S. spontaneum* and *S. barberi*) [13].

In the United States, the USDA Repository located in Miami, Florida, maintains more than 2,400 sugarcane accessions representative of the various species of the complex *Saccharum* and related genera, mainly *S. arundinaceum* (124 accessions), *S. officinarum* (748), *S. spontaneum* (635), *S. barberi* (57), *S. sinense* (61), and *Saccharum* hybrids (383). The information on these materials are publicly available in the Germplasm Resources Information Network (GRIN) database maintained by the National Plant Germplasm System (NPGS) of the United States Department of Agriculture (USDA). GRIN has detailed information about the conserved accessions including responses to different diseases and data related to growth, morphology, and phenology [14].

In Brazil, four sugarcane improvement programs are currently in progress, those of the CTC (Sugarcane Technology Center), IAC (Agronomic Institute – Center for Cana), Monsanto (that includes Canavialis), and RIDESA (University Network for the Development of Sugar and Alcohol). The RIDESA is responsible for more than 50 % of the sugarcane varieties planted in the country. The germplasm program has more than 2,700 accessions. Majority are hybrids from RIDESA and other institutions, as well as species of *Saccharum* and related genera. The RIDESA is an agreement between ten public universities in Brazil [Federal University of Paraná (UFPR), Federal University of São Carlos (UFSCar), Federal University of Viçosa (UFV), Federal University of Rio de Janeiro (UFRRJ), Federal University of Alagoas (UFAL), Federal University of Pernambuco (UFRPE), Federal University of Sergipe (UFS), Federal University of Goiás (UFG), Federal University of Mato Grosso (UFMT) and Federal University of Piauí (UFPI)]. Significant part of the sugar and alcohol sector research and development activities are conducted at the experimental research stations located in the States where the crop is being cultivated in addition to the research activities in the campuses of the ten federal universities.

The conservation of the sugarcane germplasm is generally made asexually through clonal propagation, made by cutting stalks and replanting. There is also in vitro conservation where the germplasm is maintained in the laboratory by culturing plant parts under controlled conditions and preservation of seeds. Clonal propagation is preferred over the preservation of seeds because it maintains the genotype while the seeds are preserved as a sample of gametes produced by the clone.

Over the years, cane sugarcane improvement programs have generated clones that are increasingly productive and specific for the different edaphoclimatic conditions. The sugarcane clones are set apart in few generations from their wild ancestors, indicating the importance of the knowledge about the available genetic resources and the high potential of use in the expansion of the genetic variability and generation of more productive varieties and with other attributes in the improvement programs.

Major Breeding Achievements

Currently, the breeding of sugarcane varieties aims to obtain, through the selection of clones that have high productivity and high sucrose per hectare, more rustic, as drought tolerance, resistance to pests and diseases and better adaptation to mechanical harvesting [15].

Within the programs for genetic improvement of sugarcane, conventional breeding today is still the main route for obtaining improved varieties. Over the years of cultivation of sugarcane, problems with diseases were solved with the introduction of resistant varieties. Sugarcane clones are tested to establish reaction to the main diseases such as smut, mosaic, leaf scald, rust, and ratoon stunting. Currently, ratoon stunting can be controlled with thermal treatment and by disinfecting instruments used in the cutting of the sugarcane.

One of the great advancements of conventional improvement was the development of clones resistant to ratoon stunting and smut. At present, 216 diseases were identified in sugarcane. Among these, ten can be considered to have great economic importance. The most important diseases are controlled with the use of resistant varieties; through crossings (hybridization), breeders incorporate genetic resistance in the new varieties of developed canes. It is worthwhile to mention that currently no control measure similar to other crops, like regular application of fungicides and bactericides, is made on sugarcane.

The production of sugarcane has been increasing globally due to the development of improved varieties adapted to their regions of cultivation. The genetic improvement programs are fundamental, because they accumulate alleles of agronomic interest within the set of genes submitted to successive selection processes. In the last 50 years, cane improvement was highly influenced by the improvement of machines and equipment, technological progress, precision agriculture, and new market requirements that influence selection of new varieties showing that the present cane is an integrated package that offers benefits to the sugar and alcohol industries. Over the last 30 years, breakthroughs in genetics, especially in molecular genetics, also happened.

Over the last two decades, the improvement programs have dedicated part of their studies and investments in the area of biotechnology [16]. The utilization of a series of biotechnological tools for genetic analysis had the main objective to expand the existing knowledge as well as clarify the structure and the complex behavior of the cane genome, one example of these studies was SUCEST (the Sugarcane EST Project) [17] which identified 43,000 genes. Starting from the project, the researchers aimed to exchange information with researchers involved in saccharose metabolism. Seven genes were identified responsible for transporting sugars and are more active in stem sections closer to the root where more sugar accumulates. The advancement of biotechnology for sugarcane consists in creating transgenic plants with the identified genes.

Future breeding objectives include the development of highly productive sugarcane, with high saccharose content, drought tolerance, and high production of

ethanol and biomass. Ideally, breeders want a plant with high fiber content and a cell wall that are easy to be breached to obtain ethanol, and an efficient plant requires only a small use of fertilizers and low environmental impact.

Target Traits and Current Breeding Goals

Many of the quantitative characteristics which constitute one of the main goals of the sugarcane improvement are very complex, with variation in dozens if not hundreds of underlying genes [18].

Therefore, quantitative characters are difficult to control mainly because they have multiple genes involved in the expression of a specific characteristic and the major part of the variations being due to the environment. Among the main quantitative characteristics in the sugarcane include productivity (yield) and resistance to diseases. Qualitative characteristics, different from the quantitative, are controlled by few genes, which therefore suffer less environmental influence. The main example of a qualitative characteristic in sugarcane is saccharose content.

The main characteristics which the genetic improvement programs of sugarcane aim to introduce in new varieties are:

1. *Yield*: Present estimates of heritability in the broad sense are of low magnitude, around 0.50. Therefore, of the observed phenotype, only 50 % expressed the genetic value of the cultivar [19].
2. *Resistance to diseases and pests*: Diseases like orange rust, brown rust, smut, leaf scald, and mosaic among others are genetically controlled. It would be desirable that new varieties exhibit tolerance to pests so that they could be used in integrated pest management systems [20].
3. *Richness*: One of the main characteristics for the selection of genotypes is high saccharose accumulation for the beginning, middle, and/or end of the harvest [21]. Estimates of broad sense heritability are of magnitude around 0.92 [19].
4. *Diameter of the stem*: Desired genotypes have uniform stems and medium or large diameters that do not break easily in order to increase yield and reduce waste on mechanized harvesting.
5. *Growth speed and tillering*: The varieties should have fast initial development, good tillering, and proper canopy closure of intervals between plants, minimizing the competition with infesting weeds [20].
6. *Fiber content*: Fiber has been important for the generation of electric power to address the needs of power plants and distilleries, as well as for the sale of surplus energy. The ideal quantity of fibers ranges between 12 and 13 %. However, it is certain that in the future, the demand for bioenergy will influence the selection of clones for fiber quantity and quality [22].
7. *Absence of flowering*: Excessive flowering can cause losses in the quality of the raw material due to the isoporization of the stems, increase of fiber content and lateral bud germination, reduction of the juice extracted by the mills, and

paralyzation of the development of the flowered stems, causing reduction of productivity.

8. *Erect growing habit*: This trait is important for mechanization as well as the manual harvest.
9. *Easy or natural straw down*: This will help the harvester in the stem cleaning operation and generate less vegetal impurities.
10. *Sprouting*: The yield of the stalks of sugarcane decreases each cut. In breeding programs and also commercial areas, it is desirable that cultivars show high ability to sprout after cutting.

Besides these characteristics, others include adaptability and stability, stem height (directly related to productivity), and tolerance to non-biotic stresses like cold and heat. The selection and commercialization process of a new variety is long, on the average about 10–13 years. In this time span, new technologies can be used to maximize the efficiency of the breeders work and reduce the time to develop a new sugarcane variety with favorable characteristics.

Breeding Strategies and Integration of New Biotechnologies

Gene discovery and genomics are essential tools for the future of sugarcane improvement. Sequencing of sugarcane expressed sequenced tags (ESTs) greatly contributed to gene discovery process, e.g., Sugarcane EST Project (SUCEST) initiative [23]. Currently, the information from the SUCEST, the Sugarcane Gene Index (SGI), gene expression data, and records of the agronomic, physiological, and biochemical characteristics of sugarcane cultivars are all integrated in SUCEST-FUN database (http://sucest-fun.org) [17]. Many large-scale array-based studies of gene expression have been performed in sugarcane in the past decade. Gene expression studies have been conducted using a variety of platform array technologies including cDNA macroarrays using nylon membranes, cDNA microarrays spotted onto glass slides, and oligonucleotide microarrays either spotted or synthesized in situ. In some instances, gene expression profiling using arrays has been used to identify genes specific to a tissue (e.g., stems, leaves, roots) related to various traits such as sucrose content, cell wall synthesis, and cold and drought response [24, 25].

The transcriptome projects have contributed to advances in the understanding of gene regulation system of sugarcane. However, there are still gaps in key information as variation among different copies of the same gene in the same individual and discovery of promoter sequences. The genome of commercial sugarcane is estimated to be approximately 10Gb [26], but the polyploidy nature of sugarcane complicates genome sequence assembly into contiguous hom(oe)ologous chromosomal sequences [27]. Thus, obtaining the reference assembled monoploidy genome for sugarcane is a critical step to solve such issues. Importantly, an international consortium, SUGESI (http://sugarcanegenome.org), has been formed

to tackle the challenging task of sequencing the sugarcane genome [25]. Other efforts that are underway include BAC-by-BAC and whole genome shotgun sequencing (WGS). Sequencing of R570 using the BAC library is being pursued by groups in Australia, France, South Africa, USA, and Brazil (http://sugarcanegenome.org). Furthermore, there is an ongoing sequencing effort for SP80-3280, the Brazilian cultivar that contributed most of the available ESTs [17].

In sugarcane improvement, the choice of parents for crossing requires careful characterization and evaluation of the germplasm as well as good knowledge and breeding skills to make the right decisions. Molecular markers can be a tool to help guide this route. This technology has been used in genetic diversity studies, cultivar identification, and genetic mapping. In addition, it is also used in molecular diagnostic tests to detect various sugarcane pathogens in different laboratories worldwide.

Several types of molecular markers have been used in genetic studies sugarcane, e.g., restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), and target region amplification polymorphism (TRAP). With transcriptome studies, the abundance of microsatellite sequence ESTs allowed the emergence of EST-derived SSRs (EST-SSRs). New techniques using a high-throughput microarray platform, like DART (Diversity Arrays Technology), were implemented in sugarcane and can generate a final array comprising 5,000–7,000 polymorphic markers [28]. More recently, next-generation sequencing (NGS) technologies have been used for whole-genome sequencing to discover large numbers of single nucleotide polymorphisms (SNPs). The markers in sugarcane SNPs may be useful for genome saturation, estimates of allelic dosages, and genome-wide association studies (GWAS).

Investigation of genetic diversity within sugarcane cultivars has shown that modern sugarcane cultivars are highly heterozygous with many distinct alleles at a locus. Some authors also investigated the association between genetic similarity (AFLP data) and pedigree data from improved genotypes and species [29]. Moreover, molecular profiles of sugarcane varieties can also be used as additional information in Plant Breeding Rights applications.

Genetic mapping is a basic tool of genomic research. Molecular linkage maps provide information about the organization of the genome and may be used for genetic studies and breeding applications. Unfortunately, genetic linkage maps are inherently difficult to construct in sugarcane for several reasons: (a) elevated ploidy levels (presence of simplex and multiplex alleles), (b) irregular chromosome numbers in various homo(eo)logy groups, and (c) a wide array of genotypes is expected in segregating population (due to heterozygosis and ploidy) [30]. Over the past two decades of early studies of sugarcane genetic maps, there have been 19 linkage maps constructed from 13 pedigrees. Although there are huge efforts of researchers to incorporation molecular markers in genetic maps of sugarcane, they are still incomplete [17]. Currently, the genetic maps are constructed from 1,500 to 2,500 markers, and there are no saturated genetic maps covering all sugarcane chromosomes [31]. In addition, current mapping methods are restricted to the use of single-dose markers (1:1 and 3:1). Thus, the use of few dosages

represents only a subset of genetic information from existing genome of sugarcane. The presence of markers with higher allele doses, as well as combinations of markers with different doses, makes it imperative that additional segregation patterns be considered.

The application of molecular markers for either trait or genotype selection in the breeding of sugarcane has lagged behind other crops despite a substantial research effort in the past decade on sugarcane molecular genetics. Most important traits in sugarcane are explained by multiple quantitative trait loci, each only contributing a small proportion of the overall phenotypic effect. The percentages of phenotypic variation explained by QTLs were in general low, 4–26 %. The most studied traits can be yield components (POL, tons of cane per hectare, fiber content) and disease resistance (brown rust, leaf scald, Fiji leaf gall, Pachymetra root rot) [32, 33]

There have been no reports of effective use of Marker-Assisted Selection (MAS) in sugarcane; however candidate markers are described for durable rust resistance gene Bru-1. Bru1 PCR diagnostic markers should be useful to identify cultivars with potentially alternative sources of resistance to diversify the basis of brown rust resistance in breeding programs [34]. However, the efficiency of this marker has not been demonstrated on different germplasms.

In recent years, association mapping strategy has been used in sugarcane. This methodology consists of evaluating marker–trait associations attributed to the strength of linkage disequilibrium between markers and functional polymorphisms across a set of diverse germplasm. The development of modern sugarcane cultivars was based on a strong genetic bottleneck, followed by a small number of cycles of intercrossing (small number of meiotic divisions) and vegetative propagation, suggesting that linkage disequilibrium should be extensive. However, because only low-density markers are available and statistical methods have not been refined, association studies are at the initial stages [17, 35].

Transgenes can be used to introduce genes from other species and have the potential to incorporate new characteristics to elite genotypes. In the case of sugarcane, there have been transgenes for tolerance for herbicides tolerance, pest resistance (*cry* genes), diseases resistance (mosaic and leaf scalding), resistance to abiotic stresses (higher accumulation of proline and trehalose), higher accumulation of saccharose, suppression of flowering, and other characteristics [36].

The main difficulty found in the research with transgenic cane is the gene silencing, probably caused by the high complexity of the genome (polyploidy and aneuploidy). To achieve transgenic events with a stable expression of the transgene, some groups have been studying the influence of diverse promoters. Besides gene silencing, another difficulty is the impossibility of backcrossing to a species. This means that each genotype of transgenic cane for the same gene must be transformed separately, which depends on the regeneration capacity and also makes the process more expensive.

Institutions of several countries (Argentina, Australia, Brazil, Colombia, the United States, South Africa, India, China, and Indonesia) already obtained transgenic cane in laboratory. Field tests with transgenic events also have been made in several countries, but no commercial release was made. Because of the growing

interest for the production and use of biofuels, several multinational companies that have commercial transgenic events in other crops (soy and corn for example) started to invest in research with transgenic cane, which increases the chance of a commercial release in the next years.

Seed Production

Sugarcane is an allogamous species following a sexual mode of reproduction; but, when cultivated commercially, it is multiplied asexually by vegetative propagation [37]. In cane production, the seeds are important in genetic improvement programs and those with high genetic quality are sought [15].

For the production of seeds, sugarcane needs specific environmental conditions so that flowering can be induced. The flowering of cane is a complex physiological process formed by several stages of development. Each stage has its own environmental and physiological needs. Factors that influence the induction of cane flowering include photoperiod (12–012.5 h of light), temperature (18–32 °C), thermal amplitude (<13 °C), latitude (between 10°N and 10°S), and humidity (>60 %), among others [38]. Outside of these conditions, or under adverse conditions, flowering occurs, but often synchrony between male and female lines remains a challenge; therefore, strategies such as sowing at different dates and photoinductive treatments are needed to aid crossbreeding.

In sugarcane breeding programs, the production of seeds is done using basically two types of crossings: biparental and multiple. In biparental crosses, hybridization is done between two genotypes of interest, while in the multiple crosses, only the identity of the mother plant is of interest, the pollen coming freely from diverse individuals [39]. The production of seeds with high physiological potential represents the basis for a good genetic improvement program. However, once a crop variety is selected and released, production of seeds is not ideal, because flowering is suppressed to avoid reducing the productive potential of the cane [40].

Market Challenges

In spite of the successive economic crises that the world is going through, the demand for products of sugarcane is rising and consistent. The world demand for energy is projected to grow by 4.5 % per year and ethanol has been identified as an important source for meeting this demand. Attached to this, two economic phenomena pose potential challenges – the insertion into the market of billions of new consumers in developing countries and the implementation of programs that seek substitution of fossil fuels by renewable fuels in different countries of the world. To exemplify, the United States in the next 10 years intends to increase the use of ethanol mixed with gasoline. Put into practice, this will result in the United States

consuming six times more ethanol than what Brazil consumes today which is about 32 billion liters.

Countries with emerging national economies that compose the BRICS association (Brazil, Russia, India, China, and South Africa) with an average economic growth above 5 % will add a mass of new consumers of over one billion people. This population will access new industrialized products such as soft drinks, juices, cookies, and food products that are rich in sugar.

Considering other possibilities such as energy production from bagasse and straw, lignocellulosic alcohol, biodegradable plastics, drinks, sweeteners, citric acid, and animal food among others, the sugarcane expands even more its future market perspectives.

New investments are forecasted for the next years, and industrial groups who until then were characterized by a family administration are being incorporated by big multinationals in the food and fuel sector. This phenomenon can contribute in the improvement of the trading rules of these products in the international market. Today, the small transparency of the application of the rules of the Technical Barriers to Commerce (TBT) has allowed convenient interpretations which prohibit that ethanol reach the status of a worldwide commodity. Europe and the United States are the two biggest markets and also are the two groups that use this stratagem to overtax and block the entry of these products. The imposed commercial trade barriers are today the main challenge of the worldwide sugar and alcohol market.

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Chapter 3
Miscanthus

Toshihiko Yamada

Abstract *Miscanthus* is a perennial rhizomatous warm-season C_4 grass species and is native throughout Eastern Asia and Pacific islands, ranging from tropical Polynesia to southern Siberia. Conventionally, the genus showed some attractive features for domestic uses such as livestock feed, as green manure, as well as roof materials for traditional Asian houses. In recent years, the genus has received considerable attention as a feedstock source of biorefineries such as biofuel production for sustainable renewable energy in cold and temperate environments. *Miscanthus* × *giganteus*, which is a triploid hybrid between *M. sinensis* and *M. sacchariflorus*, exhibits promise as a biomass crop because it has high biomass productivity under cold and temperate environments, low fertilizer requirements, and high ability of carbon stock in soil. However, presently only one genotype of *M.* × *giganteus* is widely cultivated. This came from a germplasm introduced to Europe from Japan in 1935, resulting to an increased risk of widespread plant mortality due to diseases or pests. Therefore, the collection of genetic resources of *Miscanthus* spp. and genetic improvement of *Miscanthus* spp. through hybridization and selection methods is essential for future increase in feedstock production. Molecular breeding will offer good opportunities, especially for value-added traits such as enhanced biomass, abiotic stress tolerance, and saccharification efficiency. This chapter describes the genetics and breeding of *Miscanthus* spp., their characteristics and their taxonomy, and progress in genetic improvement of *Miscanthus* spp. through conventional and molecular breeding including current research activities of the author's group.

Keywords *Miscanthus* spp. • *M.* × *giganteus* • *M. sinensis* • *M. sacchariflorus* • Feedstock source • Biorefineries • Interspecific hybridization • Renewable energy • Lignocellulosic energy crop • Warm-season C_4 grass

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Introduction

Global climate change and energy security have accelerated the interest in production and increased availability of alternative energy sources. Considerable research has been accomplished in developing maize (*Zea mays*) grain as ethanol for fuel consumption in the USA [1]. However, large energy and economic inputs are required to maintain the high productivity of maize [2]. Lignocellulosic biomass is an important feedstock source for biorefineries as biofuel production, is able to mitigate greenhouse gas emissions [3–5], and reduces dependency on fossil oil [6, 7]. The second generation of bioenergy crops has targeted nonedible plants species [8–10] owing to their advantages in response to land utilization and avoiding conflict with food security [11–13] and to establish an efficient production system at low cost. All these factors if well combined are believed to contribute in the scaling up of biofuel production. Currently, increase of bioenergy is the main priority to meet predicted energy demand [14, 15]. However, feedstock supply is still limited because of the few number of suitable energy crops and their low productivity due to inadequate management system and poor performance under various environmental stresses [16].

The genus *Miscanthus* is among the promising candidate lignocellulosic energy crops [16–18]. It is a rhizomatous and perennial warm-season C_4 grass species. *Miscanthus* spp. is native to East Asian tropical and subtropical regions and is endemic in high-latitude areas up to 45°N, where the climate is cool [19, 20]. In recent years, the genus has received considerable attention as a potential bioenergy crop in Europe and the USA [21, 22]. To date, *Miscanthus* is considered with reference to the single clone, *Miscanthus* × *giganteus*, a sterile interspecific hybrid between *M. sinensis* and *M. sacchariflorus* (originally collected from Japan) (Fig. 3.1). Field trials showed that *M.* × *giganteus* is easy to grow and resistant to diseases. Very few insect and other invertebrate pests have been found to infest *M.* × *giganteus* [23], and, to date, no report of yield reduction has been cited. It has high biomass production even under low temperature, which is an efficient physiological function for carbon fixation [24]. Genetic uniformity, however, has increased *M.* × *giganteus* vulnerability to diseases, pests, and environmental stresses [25]. Furthermore, *M.* × *giganteus* sterility prevents development of new varieties of *M.* × *giganteus* [25]. Improving *M.* × *giganteus* has been attempted by restoring fertility through polyploidization [26–28]. A genetic transformation system has been successfully established in *Miscanthus* [29]. Efforts at artificial crossing between *M. sinensis* and *M. sacchariflorus* have also been documented [30, 31]. Recently, Nishiwaki et al. [32] investigated sympatric populations of *M. sinensis* and *M. sacchariflorus* to locate natural hybrids between *M. sinensis* and *M. sacchariflorus*. Three natural hybrids were successfully identified and subsequently verified by morphological analysis and sequencing of ribosomal DNA internal transcribed spacer regions [33].

Fig. 3.1 *Miscanthus* × *giganteus*

This chapter describes the breeding of *Miscanthus* species, their characteristics and their taxonomy relationship, genetic resources, and progress of genetic improvement through conventional and molecular breeding.

Taxonomy and Domestication

The taxonomy of the genus *Miscanthus* was studied initially in 1855 by Andersson [34], thereafter followed by the works of Honda [35], Adati [36], Hirayoshi et al. [37–40], and Adati and Shiotani [41] in Japan. In general, the taxonomy of the genus is complex and confusing because of the high level of diversity. However, a wide range of species, hybrids, and cultivars have been identified. The cytogenetic and phylogenetic studies combined distinguished a group of 14–20 species recognized by most horticultural organizations such as the Royal Horticultural Society and members of the International Botanical Congress [42]. On the basis of the description of Clayton and Renvoize [43] and Hodkinson et al. [44], *Miscanthus* was classified to the Poaceae family, in a subfamily of Panicoideae placed in the tribe of Andropogoneae [45, 46].

The genus *Miscanthus* is divided into three sections: *Eumiscanthus* Honda, *Triarrhena* (Maxim.) Honda, and *Kariyasua* Ohwi (Table 3.1). Each section contains several species found in Japan [36, 41].

Traditionally, the genus *Miscanthus* showed attractive features for domestic use such as livestock feed, green manure, as well as roof materials for traditional houses in Japan (Fig. 3.2). Stewart et al. [47] reviewed the ecology of *M. sinensis* in Japan. Natural and seminatural grasslands in Japan, which historically comprised 10 % of

Table 3.1 Classification of the *Miscanthus* species in Japan

Section	Species (scientific name)	Japanese name
Eumiscanthus Honda	*M. sinensis* Andersson	Susuki
	M. sinensis Andersson form. *gracillimus* (Hitchcock) Ohwi	Ito-susuki
	M. sinensis Andersson form. *zebrinus* (Nicholson) Nakai	Takanoha-susuki
	M. sinensis Andersson form. *variegatus* Nakai	Shima-susuki
	M. sinensis Andersson var. *condensatus*	Hachijyo-susuki
	M. floridulus (Labill.) Warburg	Tokiwa-susuki
Triarrhena (Maxim.) Honda	*M. sacchariflorus* (Maxim.) Bentham	Ogi
	M. sacchariflorus (Maxim.) Bentham var. *brevibarbis* (Honda) Adati	Ogi-susuki
	M. sacchariflorus (Maxim.) Bentham var. *glaber* Adati	
Kariyasua Ohwi	*M. tinctorius* (Steudel) Hackel	Kariyasu
	M. oligostachyus Staff	Kariyasumodoki
	M. intermedius (Honda) Honda	Oohigenagakari-yasumodoki

Fig. 3.2 A traditional style of Japanese architecture with a thatched roof

the land area of Japan in the early 1900s [48] but in recent years only have constituted 4 % of the country [49], are comprised of several graminoid and forb species, including *M. sinensis* and *M. sacchariflorus*. Between the two, *M. sinensis* dominates most of these highly diverse grasslands. *M. sinensis* grasslands in many locations have been managed for hundreds of years in Japan by annual harvesting and/or burning [50, 51]. In the Aso mountain region of southern Japan, seminatural grasslands have been maintained by annual harvests and/or burning for more than 1,000 years. Grassland ecosystems can contribute to carbon (C) mitigation through

biomass feedstock production (substituting fossil fuels) and through C sequestration in the soil. The mean total C stock of six sites of seminatural grasslands in Mt. Aso, Japan, which have been managed for more than 7,000 years, was 232 Mg C ha^{-1} (28–417 Mg C ha^{-1}). This equates to a soil C sequestration rate of 32 kg C ha^{-1} year^{-1} over 7,300 years. *Miscanthus* being the predominant C_4 plant species in the grassland exhibits tremendous potential as a stable C sink [52]. The seminatural grasslands in Aso potentially acts as an important C sink in Japan because of their ability to sequester large amounts of atmospheric C. The coupled natural and human systems of the seminatural grassland in Aso act as a model in terms of demonstrating the sustainable use of grassland for animal and renewable bioenergy production as they relate to C accumulation in soil [52, 53].

Miscanthus is still a new bioenergy crop. Scientists, primarily those in Europe [20, 54–56] and the USA [13, 57, 58] have evaluated the potential of several members of the *Miscanthus* genus as bioenergy crops, particularly high-yielding taxa such as *M. sinensis*, *M. sacchariflorus*, and their hybrids [21, 55]. Owing to its C_4 photosynthesis [59], low-nutrient requirement [60], high water-use efficiency [61], capability of C mitigation [21], and high yields in various climates and environments [25], *M.* × *giganteus* has been determined as a very promising bioenergy crop [13, 17, 62, 63].

It has been reported that in the USA 11.8 million hectares (ha) of *M.* × *giganteus* would be required to produce 35 billion gallons of ethanol per year. In comparison, it would require 18.7 million ha of corn (grain plus stover) or 33.7 million ha of switchgrass to produce the same volume of ethanol [13].

Recent cultivation of *Miscanthus* as energy crop has gained momentum, and hectarage has increased mostly in Europe and somewhat in the USA. But much remains of further expansion to other geographical areas of suitable climate. The adoption of *Miscanthus* as biofuel feedstock on industrial scale is still being awaited.

M. × *giganteus* clones that are now available in the market all seem to have been derived from a single plant introduced by a Danish plant collector, Aksel Olsen, into Europe from Yokohama, Japan, in 1935 [64]. *M.* × *giganteus* ($2n = 3x = 57$) is a natural triploid hybrid between diploid *M. sinensis* ($2n = 2x = 38$) and tetraploid *M. sacchariflorus* ($2n = 4x = 76$) [30, 65, 66]. *M.* × *giganteus* has been also known as *M. sinensis* "Giganteus," *M. ogiformis*, and *M. sacchariflorus* var. *brevibarbis*. Recent classification work at the Royal Botanic Gardens at Kew, England, has designated it as *M.* × *giganteus* Greef & Deuter ex Hodkinson & Renvoize [67], a hybrid of *M. sinensis* Anderss. and *M. sacchariflorus* (Maxim.) Bentham. [65, 68]. *M.* × *giganteus* is a sterile triploid, so it can be propagated only asexually by its rhizomes [30, 65]. Molecular marker analysis on several *M.* × *giganteus* clones showed that there is very little genetic variation between clones [30, 65]. This apparent genetic uniformity increases *M.* ×*giganteus* vulnerability to diseases, pests, and environmental stresses [25]. Furthermore, *M.* × *giganteus* sterility prevents development of new varieties of *M.* × *giganteus* [25].

The area planted to *M.* × *giganteus* was about 12,700 ha in the UK and 4,000 ha in Poland in 2009 [16]. In the USA (Arkansas, Missouri, Ohio, and Pennsylvania), *M.* × *giganteus* was planted in about 40,000 ha (Long pers. comm.).

Genetic Resources

Miscanthus is broadly distributed throughout Eastern Asia and the Pacific islands, ranging from southern Siberia to tropical Polynesia, with a current center of diversity in temperate northern latitudes. Thus, adaptation to temperate climates is a feature of many *Miscanthus* populations, and this makes the genus especially attractive for development of a perennial biomass crop adapted to North America and Europe [69].

Section: Eumiscanthus

Miscanthus sinensis

Miscanthus sinensis is one of the major species of the genus *Miscanthus* and is diploid with a chromosome number of 38 (Fig. 3.3). The species has plants that have short and stout rhizomes, scabrous margins on leaves, no hairs or sparse hairs on leaf sheaths, no branching of the culms, high stem density, and tufted structure [41]. *M. sinensis* shows wide phenotypic variation. According to Adati [36], the width of leave blades varied from 1.0 to 2.7 cm, the length varied from 47 to 98 cm, and the culm length varied from 81 to 250 cm. *M. sinensis* has been reported to have 5–7-mm spikelets with awns and callus hairs with the same length as the spikelets [41].

Seeds are generally wind dispersed, which could be considered a factor in its potential invasiveness if fertile varieties are widely cultivated [70–72]. Although the pollen fertility of *M. sinensis* is more than 86 %, the self-pollination rate is very low [37], indicating self-incompatibility.

M. sinensis has the broadest distribution among the *Miscanthus* species. Its native range includes eastern Russia, eastern China, Korea, Japan, Taiwan, and

Fig. 3.3 *Miscanthus sinensis*

Southeast Asia [73–75]. *M. sinensis* is found in a wide range of habitats, from mountain slopes to coastal areas [76]. It is able to establish on varying soil types, with preferences for exposed, well-drained habitats [47, 76]. Conspecific plants naturally occur on soils with pH values ranging between 3.5 and 7.5, although most plants were found growing in soils with pH between 4.0 and 6.0 (summarized in Kayama [77]). However, An et al. [78] reported that *M. sinensis* colonized soils with pH values ranging from 2.7 to 5.4 in Rankoshi, Hokkaido, Japan. *M. sinensis* is also tolerant to high aluminum, chromium, and zinc [77, 79]. Its tolerance to heavy metals can be utilized to develop a bioenergy crop that is also suitable for remediating polluted soils [80].

M. sinensis from high-latitude flower earlier than those from low-altitude areas [36]. The difference in flowering time can be up to two months. For example, an accession of *M. sinensis* collected from Hokkaido, northern region of Japan, flowered in the middle of August and one from Kyushu, western region of Japan, flowered in October in the evaluation test at Sapporo, Japan. In general, *M. sinensis* has lower lignin content compared to *M.* × *giganteus* [81, 82]. This trait is desirable for bioethanol production, because high lignin content will inhibit cellulase activity in breaking down cellulose [81, 82]. In addition, sulfur, phosphorus, potassium, chlorine, and calcium content variations were observed among *M. sinensis* genotypes [83, 84]. High levels of minerals can lead to unacceptable emissions of dioxins during combustion process.

Miscanthus sinensis var. *condensatus*

M. sinensis var. *condensatus* is generally diploid, with a chromosome number of 38 [41], and has more condensed panicles, broader leaves with white spots, and more secretion of wax on the leaf sheaths compared to *M. sinensis* [36, 41, 84] (Fig. 3.4). It is mainly distributed in coastal areas of Japan, Korea, Taiwan, and the

Fig. 3.4 *Miscanthus sinensis* var. *condensatus*

Philippines [85] and is salt tolerant. Hybridization between *M. sinensis* and *M. sinensis* var. *condensatus* resulted in fertile F_1 progenies with wide variations in their morphologies [41].

Although *Miscanthus* exhibits a primarily self-incompatible mating system, *M. sinensis* var. *condensatus* appears to be self-compatible or at least can reproduce apomictically [86]. This partial self-compatibility is considered as the result of strong selection from high-salinity habitat that limits diversity [86]. Understanding the genetic regulation of the self-compatibility character may provide insight on how to break it or utilize it in other *Miscanthus* species.

One other particular trait of *M. sinensis* var. *condensatus* is that it does not senesce in winter. Adati and Mitsuishi [85] reported that in the same habitat on Hachijo Island, *M. sinensis* senesced before winter, while *M. sinensis* var. *condensatus* leaves stayed green. However, *M. sinensis* var. *condensatus* exhibits poor winter survival in cool areas such as Sapporo, Japan.

Miscanthus floridulus

M. floridulus is diploid, with a chromosome number of 38 [41] (Fig. 3.5). The width of leaf blades of *M. floridulus* ranged between 2.0 and 2.6 cm, and the length ranged between 75 and 90 cm. This species is classified as having the largest leaves in the *Eumiscanthus* section. The panicles are about 50 cm long and the axis is generally elongated [36]. Plants of this species can reach a height of 2.5 m in Japan, while in Taiwan plants with about 3 m height were reported [36]. However, the spikelets are smaller than *M. sinensis*, with lengths ranging from 3 to 3.5 mm [36].

M. floridulus is distributed in tropical and subtropical regions, particularly the Pacific side of Japan (except for Hokkaido), Taiwan, Southeast Asia, and Polynesia [31, 36]. Although most *M. floridulus* generally populates coastal regions, it also

Fig. 3.5 *Miscanthus floridulus*

has been found in high-altitude areas [87]. However, Chou et al. [88] found that the *M. floridulus* that are dominant in Taiwan lowlands could not grow well at 2,600 m.

M. floridulus flowers around July, earlier than other *Miscanthus* spp. such as *M. sinensis* [36]. Similar to *M. sinensis* var. *condensatus*, *M. floridulus* does not senesce in winter [36] and is adaptable in warm areas. Because it is distributed mainly in tropical areas, Deuter [31] suggested that *M. floridulus* could be used as a parent stock for biomass crop breeding in the tropics or areas with warm, moist climates.

Section: Triarrhena

Miscanthus sacchariflorus

Another major species of the genus *Miscanthus* is *M. sacchariflorus* (Fig. 3.6). Lafferty and Lelley [89] reported that there are two types of *M. sacchariflorus*, one with 38 chromosomes and another with 76 chromosomes. Hirayoshi et al. [38] found that *M. sacchariflorus* in Japan were tetraploids ($2n = 4x = 76$), while those found in China were diploids ($2n = 2x = 38$) [31, 69].

The tetraploid *M. sacchariflorus* has larger and hardier stems with higher lignin content; taller and branching culms, which can reach more than 3 m; lower culm number; and creeping, stout rhizomes than the diploid type. Its leaf sheaths are densely covered with bristles when young [36, 41]. Also, it has culm nodes from which aerial branches and roots develop [41]. *M. sacchariflorus* develops hollow stems to adapt to soils with high moisture [69]. It has awnless spikelets with callus hairs that are about 2–4 times longer than the spikelets [41].

Fig. 3.6 *Miscanthus sacchariflorus*

M. *sacchariflorus* is distributed from southern Siberia, China, Korea, and Japan [17, 42]. It prefers exposed, fertile, and moist places such as flood plains, river-banks, and lakes [76]. It is also more sensitive to frost than *M*. *sinensis* [90].

Jensen et al. [91] reported that *M*. *sacchariflorus* accessions from different regions of Japan, China, and Korea started flowering from mid-July to late November [91]. *M*. *sacchariflorus*, irrespective of habitat, is considered a quantitative short-day species [92]. In contrast to *M*. *sinensis* that forms new tillers during vegetation period, *M*. *sacchariflorus* forms about 80 % of its tillers in spring. This may be the reason why *M*. *sinensis* has a relatively long flowering period, whereas *M*. *sacchariflorus* has a shorter but more concentrated flowering time [31]. *M*. *sacchariflorus* from more northern locations have been reported to go dormant in autumn even when grown under greenhouse conditions [31].

M. *sacchariflorus* has a high lignin-to-cellulose ratio similar to that of *M*. × *giganteus* [81]. *M*. *sacchariflorus* loses its leaf sheaths early [93] relative to *M*. ×*giganteus*, which retains its leaf sheaths during the winter. Leaf sheaths attached to the culms improve plant resistance to lodging [93]. On the other hand, given that leaves generally have the highest mineral content in a plant [4], selecting for accessions that readily senesce their leaves may be needed to improve combustion quality of the crop.

Section: Kariyasua

Miscanthus tinctorius

M. *tinctorius* is a diploid with 38 chromosomes [36] (Fig. 3.7). The name "Kariyasu" means "easy to cut" in Japanese, and it reflects the fact that *M*. *tinctorius* has been long utilized as fodder. The leaves are broader and thinner

Fig. 3.7 *Miscanthus tinctorius*

than *M. sinensis* or *M. sacchariflorus*, which makes it less likely to inflict damage to skin when harvesting. Traditionally, it was also used for yellow dye ("tinctorius" came from "tinct" = color) [69]. *M. tinctorius* has a small stature (1–1.8 m), sparse pubescence on the outer surface of leaves, smooth inner leaf surfaces, short rhizomes, awnless spikelets, and short callus hairs [41]. The spikelets of *M. tinctorius* have short callus hairs, usually only half of the spikelet length [41, 94]. Short callus hairs prevent wind dispersal of seeds, and the trait could be introgressed into *M. sinensis* to limit seed dispersal [31]. *M. tinctorius* is mainly distributed in the mountainous region of central Honshu, Japan [36]. The flowering time of *M. tinctorius* is between August and October [36].

Miscanthus oligostachyus

M. oligostachyus is a diploid species ($2n = 2x = 38$). *M. oligostachyus* has short and slender rhizomes, and, on average, reaches between 0.6 and 0.8 m in height. The outer leaf surface is smooth, but there is pubescence on the inner leaf surface. The spikelets have awns, and the callus hairs are 2–5 mm long [41]. *M. oligostachyus* is distributed in the mountainous region of Kyushu to southeastern Tohoku in Japan [36, 41]. The flowering time of *M. oligostachyus* is from August to October [36].

Miscanthus intermedius

M. intermedius is a hexaploid ($2n = 6x = 114$). *M. intermedius* has thick rhizomes and 1–2 m culm height, and its leaves are smooth on the outer surface but hairy on the back surface. Its spikelets are 6–8 mm long, with 2–4 mm awns [41]. The spikelets also have 5–7-mm-long callus hairs, which are longer than those of *M. tinctorius* or *M. oligostachyus* [41]. The distribution of the species is restricted to the northwestern part of Tohoku, Japan [41]. *M. intermedius* flowers from August to October [36]. Based on morphological characteristics and cytological analysis, it is considered an amphipolyploid that originated from a cross between *M. tinctorius* and *M. oligostachyus* [95].

Major Breeding Achievements

Until recently, some *Miscanthus* species have been mainly used as ornamental garden plants and have still a gardening market in Europe and North America, and in some cases for thatching and as animal feed in Asia. Currently, the main potential economic use of *Miscanthus* is for feedstock of biofuel and biorefineries due to its high biomass potential [22]. Clifton-Brown et al. [42] reported that *Miscanthus* breeding programs at the Aberystwyth University in the UK and Plant Research International in the Netherlands were initiated in 2004 and are focusing on improvement of *M. sinensis* and using selections to develop improved versions of *M.* ×

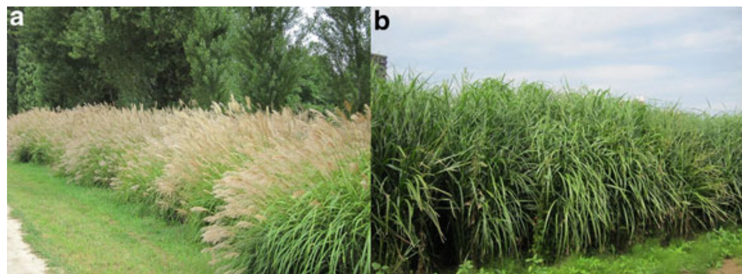

Fig. 3.8 The comparison of new cultivar of *M. sinensis* selected from breeding materials derived from lower latitude and native material of Hokkaido. (**a**) Native in Hokkaido, early flowering time. (**b**) New cultivar, late flowering time (Photos: 31 Aug 2012)

giganteus. A German breeding program led by M. Deuter at Tinplant (http://www.tinplant-gmbh.de/) was established in 1992 and already released two cultivars of *M.* × *giganteus* in 2006, "Amuri" and "Nagara."

M. sinensis can be used as a breeding material given its fertility and abundant genetic diversity [47]. It is also known that selections of *M. sinensis* have biomass yields comparable to that of *M.* × *giganteus* in some experimental sites, such as those in Northern Europe [96]. It is also capable of being established by seeds. Hokkaido University initiated a breeding program on *M. sinensis* in 2006. *M. sinensis* selected from lower-latitude region with high elevations of Japan had high biomass productivity compared with native populations in Hokkaido, indicating delayed flowering time in Sapporo (Fig. 3.8). "Shiozuka" from Shikoku Island and "Akeno" from central of Main Island of Japan were recently released cultivars of *Miscanthus* that are adapted for cool regions such as Hokkaido (Yamada unpublished).

Target Traits and Current Breeding Goals

Because *Miscanthus* spp. is still an undeveloped crop, it is important to identify the trait selection criteria. Karp and Shield [15] listed possible traits for sustainable yield and quality improvement in bioenergy crops. In the context of sustainable production systems in bioenergy crops, they concluded that there are three main challenges facing yield improvement, which are interlinked: how to change thermal time sensitivity to extend the growing season; how to increase aboveground biomass without depleting belowground biomass, so that sufficient reserves are still available for next year's growth (and thus without increasing the requirement for nutrient applications); and how to increase aboveground biomass and not be limited by water.

Increasing biomass yield is the main objective of breeding programs. Delaying or eliminating flowering, increasing plant height, tiller number and density, and stem thickness are also breeding challenges that will enable maximization of biomass yield [97]. In addition, improving the tolerance to biotic and abiotic

stresses can guarantee good biomass production. Cold stress tolerance is important for the cultivation in cooler regions. No reports of yield reduction by disease and pests have been cited at the present time. However, disease and pest problems may happen if the cultivation areas increase in the future. One of the targets for improving *Miscanthus* is to change the content and optimize the ratio of lignin, cellulose, and hemicelluloses in cell walls [98]. This is one of the most popular approaches for improvement of saccharification which is important for a bioenergy crop because high saccharification benefits fermentation of sugars into ethanol [98, 99].

Breeding Strategies and Integration of New Biotechnologies

Germplasm Collection and Characterization

The most important factor for breeding programs is a thorough knowledge of the genotypic and phenotypic variation available in the genetic resources of *Miscanthus* spp. There are wide ranges of phenotypic variation resulting from environmental pressures (e.g., cold temperature, acid soil, salinity) and from genetic isolation leading to specific ecotypes. Genetic resources from Japan [36, 47, 100], China [75, 101, 102], and Taiwan [24, 103] are available for breeding of *Miscanthus* spp. With an increase of interest in *Miscanthus* as a sustainable renewable energy crop, germplasm collection of *Miscanthus* spp. has been intensively carried out in native vegetation areas of Asia [17]. In our group, a total of 1,000 accessions of *Miscanthus* spp. have been collected throughout Japan recently. Germplasm can be collected in the form of seeds or rhizomes, but seeds may be preferable over rhizomes because they can be stored for longer periods of time. The Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth, UK, and the University of Illinois have collected many germplasm of *Miscanthus* spp. from the countries growing *Miscanthus* (Clifton-Brown, Sacks personal communication).

Flowering time is one of the several factors to be considered in selecting high biomass *Miscanthus* [25]. *Miscanthus* plants that are late flowering or nonflowering tend to yield higher than those that are early flowering [25]. Jensen et al. [91] observed the flowering time of various *M. sinensis* for 3 years at Aberystwyth, UK. The plants were collected from Japan, China, Korea, and Russia (latitude range 32.2–43.6°N). The onset of flowering time varied from mid-June to late November. Accessions from more northern areas and higher altitudes tended to flower earlier than accessions from southern areas or lower altitudes. Our evaluation of *M. sinensis* collected throughout Japan also revealed that accessions from northern areas, such as Hokkaido, generally showed an earlier flowering time and ripening than those from southern areas. *M. sinensis* accessions from high-altitude regions of central Main and Shikoku Islands show that late flowering was tolerant to Hokkaido's cold environment and showed high biomass potential [104].

M. × *giganteus* is a promising biomass crop in temperate and cool regions, since the yield is still high at low temperature compared to C_3 plants or C_4 plants such as

maize [62]. However, *M.* × *giganteus* cannot survive winter conditions at some North European areas, particularly in the first season when started from tissue-cultured plantlets [17]. Clifton-Brown and Lewandowski [105] compared cold tolerance in *Miscanthus* spp. using a test for freezing tolerance, finding out that the lethal temperature at which 50 % of rhizomes killed (LT_{50}) for *M.* × *giganteus* and *M. sacchariflorus* genotypes was −3.4 °C, while LT_{50} for *M. sinensis* genotype Sin-H6 was −6.3 °C. Low temperatures during spring delay the timing for shoot emergence, subsequently reduce growth time, and reduce *Miscanthus* yields in autumn [90]. The onset of frost after shoot emergence kills shoots and reduces shoot number. Some genotypes of *M. sinensis* are able to grow shoots at lower temperatures than *M.* × *giganteus* [90].

Characterization of the lignin, cellulose, and mineral content is useful for breeding programs for biorefinery feedstock development. For biorefineries, processing *Miscanthus* with low lignin content is preferred because high lignin content decreases the efficiency of scarification process and increases the cost of pretreatment of feedstock [81]. On the other hand, high lignin concentration gives higher heating value that is preferable for combustion [106]. *M. sacchariflorus* and *M.* × *giganteus* constantly showed higher lignin content and lower hemicellulose content than *M. sinensis*, and variation of lignin content was observed among different genotypes [81]. Our evaluation of lignin content also showed substantial variation among genetic resources from different collection sites (unpublished).

For biomass combustion process, good quality depends largely on minimizing moisture, ash, potassium (K), chloride, nitrogen (N), and sulfur (S). Delaying the harvest of *Miscanthus* spp. improved the combustion quality by reducing ash, chloride, nitrogen, and moisture [60]. Silicon (Si) is important for grass species to increase the plant resistance to lodging and drought; improve disease, insect, and nematode resistance; improve soil nutrient availability; and improve reproductive fertility [107]. However, silicon reacts with aluminum, chlorine, potassium, and other alkalis to form slag during the combustion process. Woli et al. [107] found that Si concentration in *M.* × *giganteus* plants from several locations in the USA ranged from 0.72 to 1.62 %, indicating that soil type influences Si uptake. Si concentration in *M. sinensis* ranged between 0.81 and 3.56 %.

Interspecific Hybridization

The currently propagated natural triploid hybrid *M.* × *giganteus* clone came from a plant that was introduced to Denmark in 1935 from Yokohama, Japan, by a Danish plant collector, Aksel Olsen [108].

Being the center of origin of *Miscanthus* species, there are many sympatric populations of different *Miscanthus* species in Japan. In those overlapping populations, natural hybrids between *Miscanthus* species can be found [36, 38, 109]. Recently, three triploid hybrids were found in seeds collected from *M. sacchariflorus* that grew in Kushima, southern Japan [32]. The plants were confirmed to have 57 chromosomes. Through internal transcribed spacer (ITS)

sequence and chloroplast DNA analysis, the plants were confirmed as hybrids between *M. sinensis* and *M. sacchariflorus* [33]. One of these three hybrids showed the good biomass potential in Sapporo (unpublished). Finding new natural hybrids is important to help broaden the genetic resources of *M.* × *giganteus*.

In parallel to finding natural triploids, artificial hybridizations have been attempted. Flowering time synchronization, parent compatibility, pollen amount, and morphological structure are important factors in determining seed-setting rate in hybridization [31, 37]. Two cultivars of *M.* × *giganteus* "Amuri" and "Nagara" were released by M. Deuter at Tinplant 2006. "Amuri" was derived from a cross between North Asian *M. sacchariflorus* and *M. sinensis*, while "Nagara" was derived from a Japanese *M.* × *giganteus* (described as *M. sacchariflorus*) crossed with *M. sinensis* [69].

Besides *M. sinensis* and *M. sacchariflorus*, hybrids have been created between other *Miscanthus* species. These hybrids may be useful for cultivation in specific locations, such as sodic soils and tropical regions. Hybridization between *M. sinensis* and *M. sinensis* var. *condensatus*, *M. sinensis*, and *M. tinctorius*, resulted in F_1 hybrids that were self-incompatible but could produce fertile pollen [39]. A triploid and unexpected tetraploid were obtained from crossing between *M. sacchariflorus* and *M. sinensis* var. *condensatus* [40]. The culm length, leaf length, leaf width, and ear size of both polyploids exceeded those of the parents. However, the triploid was sterile, and the hair length of spikelets, silky lustre, width of lemma, length of awn, grass type, and evergreeness resembled *M. sacchariflorus*. Meanwhile, the tetraploid resembled more *M. sinensis* var. *condensatus* in those characteristics and the pollens were fertile. Matumura et al. [110] investigated the rhizome structure of triploid, tetraploid, and their parents and found that triploid resembled *M. sacchariflorus* more while the tetraploid resembled *M. sinensis* var. *condensatus*. Adati and Shiotani [41] also reported hybridization between *M. floridulus* and *M. sinensis*, producing F_1 hybrids with regular meiotic division showing 19 bivalents at first metaphase.

Polyploidization

Chromosome doubling is a valuable breeding approach for increasing vegetative biomass production. Głowacka et al. [26] applied colchicine treatment to induce polyploids from *M. sinensis* and *M.* × *giganteus*. Higher colchicine concentrations reduced the survival rate and tillering rate of the plants. Plant genotypes influenced the polyploidization rate in *M. sinensis* genotypes. Generating hexaploid *M.* × *giganteus* to restore fertility may be a way to improve *M.* × *giganteus* through conventional breeding. Yu et al. [27] generated hexaploid plants from *M.* × *giganteus*. The team induced calli growth from immature inflorescence tissue and treated the calli with colchicine or oryzalin in various concentrations and exposure time. The rate of calli survival was generally higher in calli treated with colchicine, but more hexaploids were generated from calli treated with oryzalin at the concentrations tested. The hexaploid plants had slightly broader stems and larger stomatal

size compared to the triploid plants. The pollens from hexaploid plants were found viable by fluorescein diacetate staining and field trials are currently underway [69].

Genetic Transformation

Another approach to improve *M.* × *giganteus* as feedstock materials is genetic modification through biotechnology. The genetic transformation method for *Miscanthus* is still difficult, because at present, there is only one detailed report of success in *Miscanthus* [29]. Particle bombardment-mediated transformation systems were used to transform *M. sinensis* with perennial ryegrass fructosyltransferase (*prft*) genes enabling transgenic plants to produce fructans. Fructans were detected in transgenic plants expressing *prft4* gene, encoding sucrose-sucrose 1-fructosyltransferase (1-SST). The transgenic plants showed minor chlorophyll loss and fewer leaves wilting under cold temperature conditions. This indicated that the transgenic plants are expected to produce higher photosynthetic biomass under low temperature (unpublished data).

Linkage Map and QTL Analysis of Traits

Genetic map construction is useful for the identification and characterization of genes regulating traits related to biomass production. The first genetic map for *Miscanthus* was developed based on random amplified polymorphic DNA (RAPD) markers because of the limited information about the *Miscanthus* genome [111]. Simple sequence repeats (SSR) and restriction fragment length polymorphisms (RFLP) markers were also used in genetic map construction [111]. Atienza et al. [112, 113] analyzed quantitative trait loci (QTLs) related to yield (based on stem diameter, total height, flag-leaf height, total yield, stem yield, tops yield, and leaves yield) using constructed linkage map. QTLs related to chlorine (Cl), potassium (K), calcium (Ca), sulfur (S), and phosphorus (P) have been also analyzed by Atienza et al. [83, 84].

After the preliminary linkage map, a more substantial genetic map of *Miscanthus* has been constructed. Several cDNA-derived SSR markers were mapped using a two-way pseudo-testcross between *Miscanthus sacchariflorus* Robustus and *M. sinensis* [114]. A total of 261 loci were mapped in *M. sacchariflorus*, spanning 40 linkage groups and 1,998.8 cM, covering an estimated 72.7 % of the genome. For *M. sinensis*, a total of 303 loci were mapped, spanning 23 linkage groups and 2,238.3 cM, covering 84.9 % of the genome. The use of the cDNA-derived SSR loci permitted alignment of the *Miscanthus* linkage groups to the sorghum chromosomes, revealing a whole genome duplication affecting the *Miscanthus* lineage after the divergence of subtribes Sorghinae and Saccharinae, as well as traces of the pan-cereal whole genome duplication.

Swaminathan et al. [115] used deep transcriptome sequencing (RNAseq) from two *M. sinensis* accessions to define 1,536 single nucleotide variants (SNVs) for a GoldenGate™ genotyping array and found that simple sequence repeat (SSR) markers defined in sugarcane are often informative in *M. sinensis*. A total of 658 SNP and 210 SSR markers were validated via segregation in a full sibling F_1 mapping population. Using 221 progeny from this mapping population, we constructed a genetic map for *M. sinensis* that has 19 linkage groups, the haploid chromosome number expected based on cytological evidence. Comparative genomic analysis documents a genome wide duplication in *Miscanthus* relative to *Sorghum bicolor*, with subsequent insertional fusion of a pair of chromosomes. The genus *Miscanthus* experienced an ancestral tetraploidy and chromosome fusion prior to its diversification, but after its divergence from the closely related sugarcane clade. A high-resolution linkage map of *Miscanthus sinensis* was also created using genotyping by sequencing (GBS), identifying all 19 linkage groups [116]. Comparative genomics analyses of the *M. sinensis* composite linkage map to the genomes of sorghum, maize, rice, and *Brachypodium distachyon* indicate that sorghum has the closest syntenic relationship to *Miscanthus* compared to other species. The comparative results revealed that each pair of the 19 *M. sinensis* linkages aligned to one sorghum chromosome, except for LG8, which mapped to two sorghum chromosomes (4 and 7), presumably due to a chromosome fusion event after genome duplication. The data also revealed several other chromosome rearrangements relative to sorghum, including two telomere-centromere inversions of the sorghum syntenic chromosome 7 in LG8 of *M. sinensis* and two paracentric inversions of sorghum syntenic chromosome 4 in LG7 and LG8 of *M. sinensis*.

Seed Production

To date, plant establishment is based on vegetative propagation of *M.* × *giganteus* owing to its sterility. Propagation by rhizomes poses problems of scaling up industrial planting because large numbers of plants are needed to produce the number of rhizomes required. Excavating and splitting the rhizomes to generate separate plants and replanting are complex and costly operations and present a bottleneck for *Miscanthus* commercialization.

Propagation of *Miscanthus* by seeds as in their wild environments is done at some research centers for research purposes. In general, direct seed method for field establishment is unreliable because seeds are too small to support sufficient seed carbohydrate reserve and ensure good germination and healthy seedlings [42]. As a result, seedling mortality is high. Consequently, *Miscanthus* establishment in the field using direct seeding is not practiced. The propagation via rhizomes, on the other hand, provides healthy seedlings and establishes plants easily. In Europe, many plant propagation centers exist. Comparisons of propagation methods, however, revealed that vegetative propagation provides propagules at a higher cost compared to seeds. In theory, seed-based cultivars could considerably reduce

establishment costs, probably to around $700/ha [17]. Finding ways to improve germination of seeds and tillage methods to support small seed sprouting and early growth or seed germination in a nursery environment and subsequent field transplanting may reduce start-up cost and ensure availability of adequate quantities of planting material. Tissue-cultured plants if economically produced and transplanted may provide another option.

Two approaches for *M.* × *giganteus* in vitro propagation – direct and indirect – have been optimized and can be used as effective alternatives to asexual propagation from rhizomes. The direct multiplication using stem segments containing axillary buds combined with in vitro tillering phase turns out to be 50–60 times more effective than the conventional ex vitro rhizome-based approach [117].

Market Challenges/Barriers to Commercialization/ Opportunities

In vitro propagation systems can accelerate the cultivation of *M.* × *giganteus*. Most breeding programs on *Miscanthus* are still at the initial phases. The perennial habit and complexity of the genome are limiting factors in elucidating the genetic basis of *Miscanthus* agronomical traits and quality traits. But the recent advances in "-omics" technologies may accelerate the progress of whole genome sequencing, genetic marker development, and elucidation of physiological process in *Miscanthus*.

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Chapter 4
Breeding Willow for Short Rotation Coppice Energy Cropping

Ian Shield, William Macalpine, Steve Hanley, and Angela Karp

Abstract Willow (*Salix*) is a diverse and adaptable genus that has served human beings well for many thousands of years. The Roman scholar Pliny the Elder (AD 23–AD 79) advised on willow planting in the Roman Empire. However, it has only recently been subjected to controlled breeding (twentieth century). Willow breeding has been able to benefit from the knowledge and technologies developed by plant breeders across the globe. The breeding exploits the tremendous genetic diversity and specifically the rapid growth rates observed in response to coppicing on a 2–4 year cycle. Willow breeding cycles are short and commercial exploitation rapid via vegetative propagation of the F_1 progeny. The latest molecular genetics techniques are being deployed in Europe and North America to advance and accelerate crop improvement. Willow is now being rapidly improved and deployed for production of woody biomass, much of it for energy, but also for pulp, potentially specific high value extracts, and applications associated with the multifunctionality of the crop such as bioremediation. Most northern temperate latitude areas have an interest in willow cropping.

Keywords Willow • *Salix* • Short rotation coppice • Breeding • Molecular genetics • Biomass • Bioenergy

Introduction

The willow tree has been associated with and utilized by humans for many thousands of years. Initially (and for several millennia), willow stems were used for baskets on account of their flexibility and to some extent lightweight characteristics. Perhaps implicit within this use was also a recognition that many species are fast growing and could be easily cultivated. Long ago it was no doubt recognized that after a willow had been cut back for useful wood, it quickly regrew from the cut base again. However, it was not until the mid-twentieth century that this ability for regrowth and the subsequent fast growth rate (compared to other trees)

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were widely exploited, first during a shortage of pulp for paper, card and packaging and then as a wood fuel.

The propensity for fast growth of willows is now exploited in the shrub species (primarily from subgenus *Vetrix*) by growing them as coppiced stools. Commercial short rotation coppice (SRC) willow plantations for biomass typically consist of a mixture of specially developed elite varieties, grown in dense plantations of 15,000–17,000 plants per hectare. Planting mixtures of varieties enhances and extends plant breeding efforts by exploiting genetic diversity to limit pest and disease development. Pesticides are virtually excluded from production systems by the physical size of the crop and the impracticality of application. SRC willow plantations are harvested on a 2–4 year cycle and will normally remain in place for more than 20 years. Coppicing reinvigorates the growth of a plantation and only minimal fertilizer inputs are needed as the willows redistribute nutrients during their perennial cycle

Willow can grow well in environments where the alternative land uses are limited, such as cooler and wetter areas of northwest Europe and North America and the high clay content soils of those and other areas of the temperate latitudes. In these environments, because alternative land uses are limited, the production of SRC willow is financially very competitive. Here, we summarize the main activities that have led to improved willow varieties, including brief coverage of the germplasm available and the main target traits for breeding. We finish by discussing prospects for further crop improvement and expansion of SRC.

Taxonomy and Domestication

The family Salicaceae is commonly considered to consist of only three genera; *Salix*, *Populus*, and *Chosenia*, although more recently the Angiosperm Phylogeny Group have added a further 52 genera. *Chosenia* is an unusual genus, containing only one species, *Chosenia arbutifolia*, sometimes considered as *Salix arbutifolia* Pall. *Populus* is a well-known and widely studied genus and of similar interest for bioenergy as *Salix* across much of the world.

The authoritative taxonomy of *Salix* has been conducted by representatives from the major centers of biodiversity, G. W. Argus in North America [1, 2], A. K. Skvortsov in Russia [3], and Zhenfu, Shidong, and Skvortsov in China [4]. Argus and Skvortsov agree upon the division of the genus into four subgenera; *Salix*, *Longifoliae* Andersson, *Vetrix* Dum, and *Chamaetia* Nasarow (Table 4.1). The authorities disagree upon a total species number, placing the sum between 350 and 500.

Subgenus *Longifoliae* comprises only a few New World species with specific morphological traits such as *S. exigua* with stomata on the lower and upper leaf surface. The subgenus containing the greatest number of species and of greatest interest in commercial breeding is *Vetrix*; they are the species best adapted to rapid growth rates and coppicing in cultivation. A few species of subgenus *Salix* have

Table 4.1 The subgenera of *Salix*, the willows, and their uses

Subgenus	Common name	Typical species	Uses
Chamaetia Nasarow	Alpine and Arctic willows		Ornamentals
Longifoliae Andersson	New World willows	*Salix exigua*	
Salix	Tree willows	*S. alba, S. babylonica, S. daphnoides*	Ornamentals, frost protection, and cricket bats. Occasionally biomass (pulp and bioenergy)
Vetrix Dum	Shrub willows	Sallows, *S. aurita*, *S. caprea*, *S. cinerea;* and osiers, *S. purpurea* and *S. viminalis*	Basketry, hurdles, biomass (pulp and bioenergy)

also been used in breeding, *S. alba* and *S. daphnoides* in particular, but for many species there appear to be incompatibilities and/or crossing barriers between members of *Salix* and *Vetrix*. *Salix triandra* has historically been classified as subgenus *Salix* until recently when AFLP analysis of 154 accessions from the UK National Willow Collection placed it between *Salix* and *Vetrix* [5]. *S. triandra* has been a useful parent in European breeding programs and freely hybridizes with *S. viminalis* in nature (creating *S.* x *mollisima*). Within *Vetrix,* interspecific hybridization is widespread and routinely exploited in breeding. The extent of natural interspecific hybridization within subgenera has traditionally made willow classification very difficult. These uncertainties in *Salix* taxonomy account for how variable the estimates of numbers of species within the genus are.

Willow ploidy ranges from diploid to the atypical dodecaploid. *Circa* 40 % of the species in the genus *Salix* are polyploid [6]. Breeding programs commonly exploit a range of ploidy levels from the common diploid up to hexaploid. There are several cultivars that are sterile triploids, the result of diploid x tetraploid crosses.

Despite the historic association of humans with willow, it has not been necessary to subject willow to domestication until relatively recently. The enormous diversity of wild forms has served the past needs of human beings well. Prehistoric human populations in northern Europe built simple boats (coracles), and the Romans made fine, delicate woven willow baskets; Pliny the Elder (AD 23–AD 79) advised on willow planting in his extensive “Natural History.” Those first willow plantations were probably made up from the selections from the wild for characters such as multiple branch-free stems, flexibility of stems, and potential to regrow after cutting. As willow constructions, particularly at the scale of domestic containers (baskets), became more popular, bark color and ease of stripping bark would have also been considered.

Mechanical harvesting did not become widespread until the later part of the twentieth century by which time agronomic practice was being used to render growth habits more suited to the machines available. Specifically, practices such

as planting in distinct rows at high density ensured that the stems were upright and branch-free to be easily processed mechanically. Today large-scale harvesting machines can cut and chip stems with diameters at the base of 120 mm, the maximum encountered on a 3 or 4 year coppicing cycle, while on the move. Lower-cost, lower-capacity machines are restricted to 80 mm diameter stems. For combustion, larger stems are preferred as the wood-to-bark ratio is increased (see section "Quality traits" below).

Deliberate breeding of willows for commercial purposes began at a similar time to mechanization, during the late 1970s, at which time some more classical domestication was undertaken. Biomass for pulp or bioenergy was considered best produced on a 3-year cutting cycle rather than the annual cycle that many had traditionally used. This was best achieved by planting at lower density and allowing stem diameter to develop over the period in between harvests. Growth in larger commercial plantings also necessitated greater pest and disease resistance and the selection of growth forms that inherently produced multiple upright, branch-free stems.

The most recent phase of domestication has involved selection for characters suitable for the different energy conversion processes including minimizing the inorganic component of the wood (ash), and especially the alkali metals, for thermochemical conversion and maximizing sugar release from the cell wall (lignocellulosic) fraction for biological conversion.

Areas of Production

Willow is a temperate genus, and the use of willow as a crop is confined to the temperate areas of the Northern Hemisphere. Most northern European countries have an interest in the crop, but further south in Europe, other species take precedence, particularly poplar. In North America, the greatest interest in willow cropping is in the Northeastern United States and across southern Canada. China, Japan, and Russia and other parts of more northern Asia hold important genetic resources in the genus (*S. sachalinensis, S. miyabeana, S. rehderiana*), and many of these areas have increasing interest in growing willow.

In the UK, the calculation of area planted is complicated by the differing systems employed by the devolved administrations. England represents the greater area of farmed land in the UK, but Northern Ireland probably contains the greatest density of willow plantings per unit of farmland. Eason et al. [7] summarized the data available in 2009 as 7,400 ha willow in the UK. This may have fallen in more recent years as the improvement in agricultural commodity prices and the associated removal of a compulsory set-aside requirement on EU farms favored more arable cropping. Many earlier plantings had exploited set-aside land where food crops could not be grown as a supply control measure.

A positive policy framework in Sweden between 1991 and 1996 resulted in rapid increases in crop area. Planting grants worked in conjunction with environmental

taxes based upon the polluting potential of fossil fuels to make the crop financially attractive. Joining the European Union in 1996 temporarily slowed the rate of expansion, but policy was able to maintain incentives and 16,000 ha were planted by 2000. More recently there has been turnover of crop area but no great expansion [8, 9].

Other European countries are known to have up to several thousand hectares of willow in production: France, Belgium, Denmark, Germany, and Poland. Further east, Czech Republic, Romania, Ukraine, and the Baltic States all have an emerging interest in willow cropping for biomass. The majority of those countries already have an established basketry industry.

In the Northeastern United States, there were more than 400 ha of willow planted by 2008. Plantings in Canada are thought to be of similar area. As in Europe recent increases in returns from arable farming have slowed the rate of increase, but an additional major factor in North America has been the decrease in gas prices as greater quantities of shale gas are exploited. This has impacted upon the price for biomass for energy.

Genetic Resources

The UK National Willow Collection is one of the largest genetic resources of *Salix* spp. known. At the present time there are approximately 1,500 accessions grown as coppiced stool beds at Rothamsted Research. Collecting began at the former Long Ashton Research Station in 1922, and the germplasm collection increased to approximately 1,000 accessions before the closure of the site and transfer of the collection to Rothamsted in 2002. The composition of the collection reflects the history. Collecting began in response to the rapid decline in the willow basketry and hurdle industry. Many of the early additions were of naturally occurring willows that had been taken into cultivation by people engaged in these craft industries (the equivalent of landraces). Many willows had been moved around the world and were often given different names, so origins were not always clear. Therefore, not all willows collected could be described as native. Recently, AFLP and microsatellite analyses of the germplasm collection have identified several duplicates.

The collection has always been associated with basketry, hurdles, and biomass (whether for pulp or bioenergy), and the application has primarily been to exploit the rapid growth characteristic that follows coppicing. Therefore, the collection shows a domination of species from the shrub willows, subgenus *Vetrix*. Work in the 1960s on using willow and poplar for frost protection of fruit orchard trees brought the tree willows, subgenus *Salix*, greater prominence. Finally, recent scientific investigation of the phenomenal morphological diversity observed in the genus has encouraged collection of many new genotypes from across the world, regardless of their immediate application. Apart from Europe, the collection now contains representative accessions from North America, North Africa, the former Soviet Union, China, Japan, and other parts of the Far East.

In Sweden, collaboration between Svalöf Weibull and SLU has resulted in a collection dominated by northern European and Siberian willows. It is used for commercial breeding by Svalöf Weibull and for underpinning science by SLU. In the Northeastern United States, the willow breeding program at State University of New York (SUNY) had 730 accessions in a germplasm collection as of 2005 [10]. The collection was dominated by native *S. eriocephela*, *S. discolor*, and *S. bebbiana*. It also included naturalized imports from Europe such as *S. viminalis* and *S. purpurea* and imports from Asia. Much of the collection originated from the Canadian program of the 1970s and 1980s based in Toronto. The collection was used to breed for bioenergy and phytoremediation as well as other environmental projects. The breeding work moved to Cornell University in 2009, but work on the application of willows to environmental problems continues at SUNY.

Major Breeding Achievements

Karp et al. [11] presented a graph of willow yield increases based upon the year of first appearance of improved genotypes in yield trials (Fig. 4.1). The early genotypes were little more than landraces, selected from the wild, but during the 1980s improved cultivars began to appear. The data indicates a rate of increase in yield of 100 kg ha^{-1} $year^{-1}$ resulting from selection and breeding. This represents an impressive rate of gain when compared to other temperate crops; however, it must be remembered that willow is a new crop that has been subjected to little

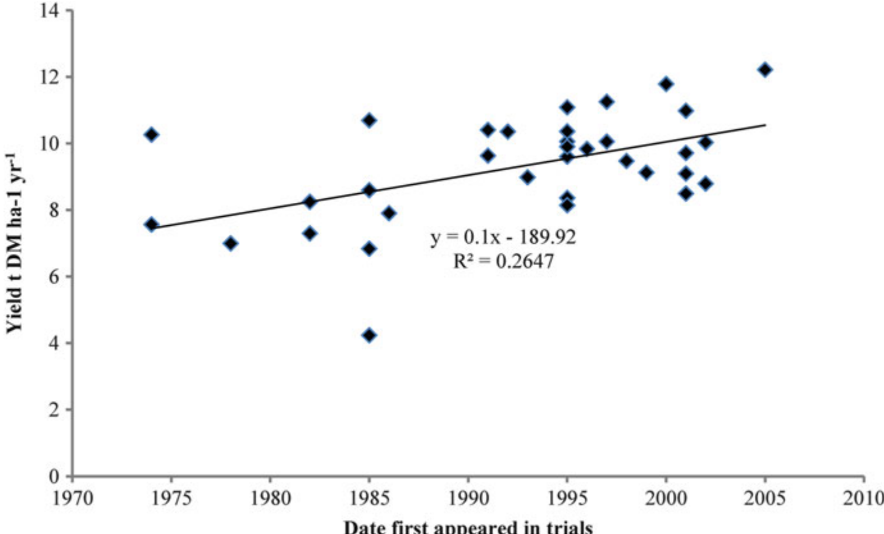

Fig. 4.1 Changes in yield over time, based upon the date of appearance in trials

breeding effort so far. The challenge will be to maintain this rate of gain. Modern molecular breeding techniques offer such an opportunity.

Disease control has also been a major breeding objective. Leaf rust (*Melampsora* spp.) resistance has been an ever-present challenge and a primary target. Leaf rust can severely defoliate willows and reduce yields by up to 40 %. Early resistant cultivars soon saw their resistance mechanism overcome by the fungal pathogen. Most recently the high-yielding hybrid Ashton Stott has suffered complete breakdown in resistance. However, successful breeding of resistance derived from *S. schwerinii* (or possibly an *S. schwerinii* x *S. viminalis* hybrid) collected in Siberia (L79069) has persisted largely unbroken for more than 20 years (see later).

Other major advances have been the identification of QTLs affecting important agronomic traits, particularly yield and component traits (stem diameters and heights, shoot numbers). Many yield traits have been found to collocate to the same regions of the chromosomes [12]. Markers for these QTLs are already being used in marker-assisted selection at Rothamsted.

Target Traits and Current Breeding Goals

Breeding objectives fall into two categories. The first is the creation of high potential yield and the ability to achieve that yield under stress from biotic and abiotic challenges. This incorporates many of the traits that breeders of a diverse range of crops would seek to improve such as pest and disease resistance and drought tolerance. Secondly, there are quality traits that are specific to the bioenergy sector. Minimizing inorganic components of the harvested biomass is important when thermochemical conversion is the target market, while when biological conversion technologies are targeted, the organic composition of the biomass and the ease with which biological processes may access the energy contained therein take precedence.

Yield and the Protection of Yield Potential

Early willow breeding work was relatively simple. Willows are dioecious and are heterozygous. Crosses give rise to highly variable F_1 progeny from which it was possible to select the highest yielders and propagate them vegetatively for trialling and selection. Often early selections were based on visual assessments; subsequently, when sufficient planting material was generated to carry out designed experiments, yield could be directly measured. However, willows are highly variable, and previous studies suggest that more than one growth strategy may result in high biomass. Stott [13] noted that some high-yielding willows had large numbers of thin stems, while others had fewer stems with large diameters. Recent research in the United States on 32 different willows concluded that there are at least two

functional groups with alternative growth strategies for high biomass production. The first had a large number of stems (typically 11 per stool), relatively low leaf area index (LAI) and specific leaf area (SLA), but high foliar N concentrations and wood-specific gravity. The second group had a small number of large diameter stems (typically 6 per stool), high LAI and SLA, but low foliar concentrations of N and low wood-specific gravity [14]. These findings are reflected in the current European varieties, with the majority (e.g., Tora) falling into the former class, and some varieties exhibiting a "wall of stems" (e.g., Terra Nova). From these studies it would seem that that multiple ideotypes may need to be incorporated into willow breeding and selection efforts.

It rapidly became evident that leaf-infecting rust caused by *Melampsora* spp. was a major threat to achieving the yield potential. Research effort was placed on the causal fungus and on the genetics of resistance, particularly as some early sources of resistance proved durable only in the short term.

The most durable source of resistance so far in western Europe has come via the accession L79069 used in the Svalöf Weibull breeding program in Sweden (described earlier). The first bred products were the cultivars Tora and Bjorn. Subsequently, Bjorn was used as a parent to create a mapping population segregating for the rust resistance in order to identify the underlying genetic basis. A single locus with a major effect was identified as responsible for conferring resistance, although several more loci with minor effects were also identified. The study also revealed that the vast majority of cultivars on the market were reliant upon the major locus based resistance mechanism derived from L79069. This resistance has proved durable so far, but planting a perennial crop with a life expectancy of 20 or more years that is reliant upon one source of resistance can clearly be considered high risk. Therefore, identifying and incorporating new, unique sources of resistance into the breeding pool and stacking multiple sources of resistance into cultivars released to the market are a breeding priority.

Melampsora rust has infected willows in the Northeast United States also. Damage has been less severe than in parts of northern Europe. Insect pests are a greater threat to willow cultivation in the Northeastern United States. *S. viminalis* is particularly sensitive to potato leaf hopper *Empoasca fabae* (Harris) damage, but because it conveys many useful traits for biomass production, it is used in interspecific hybrids such as *S. viminalis* x *S. miyabeana*. Further north, in Canada, leaf hopper damage is less of a concern. In continental areas, where the winters are particularly cold, an early and reliable (in time) autumn senescence is sought. Native *S. eriocephela* routinely survive these conditions and certain *S. viminalis* genotypes from Europe are proving promising potential to lift the yield potential under such conditions.

Quality Traits

When breeding willow for biomass production for bioenergy, there are two broad categories with distinct quality specifications based upon the energy conversion process. It has been difficult to identify useful variation in gross energy value of the biomass generated by different breeding strategies, but thermochemical energy conversion requires that inorganic composition of the biomass is minimized. In particular the elements N, K, Na, Cl, and Si can cause problems as they influence volatile behavior, ash melting point (thereby making the ash difficult to use), and the corrosive potential of the gases within the boiler and heat exchangers. The proportion of the harvested biomass that is bark can strongly influence the concentration of these elements [15] and is related to the ideotype chosen for yield (above). Biological energy conversion processes focus on traits that maximize the accessible proportion of the gross energy (although the residues may be subjected to thermochemical conversion). The carbohydrates within the biomass must be accessible to the biological system employed, which often requires lower lignin contents and greater cellulose [16, 17].

Breeding Strategies and Integration of New Biotechnologies

Utilization of natural genetic variation by traditional breeding methods is very relevant to willow as the naturally occurring genetic variation is vast and largely unexploited. In addition, the F_1 generation is exploited by vegetative reproduction, so fixing alleles by inbreeding is avoided. The phenotype of the progeny is sufficient for rapid selection to be made. An additional advantage of willow is that the majority will flower very early in their life, when grown from seed rarely in year 1 but routinely in year 2, and when grown from a cutting more likely in year 1. This allows the breeding program to move quickly, either backcrossing or making recurrent selections for yield as soon as good phenotype (and genotype) data becomes available. This is uncommon amongst tree species, even the closely related *Populus* requiring 7 plus years before flowering.

However, such traditional methods rapidly hit their limitations. Some phenotypes are difficult to ascertain, whether it be due to financial costs or time constraints. Traits such as disease resistance may be most powerfully deployed by gene stacking, whereby multiple resistance sources are combined in one cultivar for maximum durability against an evolving pathogen population. This is particularly valuable in a perennial crop planted for a 20-year life and physically very difficult to treat with agrochemicals. Following recombination the phenotype recorded by the breeder may be resistant to the pathogen due to only one of the sources. Confirmation of the incorporation of multiple sources requires a molecular genetics approach.

Modern commercial scale willow breeding has developed very much in line with the latest biotechnology in support of plant breeding. Molecular genetics studies of willow began in the 1990s in Sweden, the UK, and the United States. The first European mapping family (K3) was created in 1999 by Svalöf Weibull. Subsequently, the larger K1 and K8 families, which comprise around 1,000 progeny from full sibling crosses in a *S. viminalis* and *S. schwerinii* background, were developed. Initially created to study the inheritance of rust resistance, the K8 family, in particular, has been the source of many QTLs acting as markers for a number of valuable traits. Rothamsted Research has continued the work started at Long Ashton Research Station (closed in 2002), and there are now three copies of K8 planted in differing environments plus a further 12 families of 240–550 progeny created since at Rothamsted [12].

The Swedes also studied families of their own creation, and in 2009 Svalöf Weibull, SLU at Uppsala, and Rothamsted Research joined forces to create an Association Mapping Population consisting of *S. viminalis* accessions from across Europe. This population avoids some of the limitation associated with biparental linkage mapping and thus gives the power to seek to identify QTL through two primary routes.

Using these resources, QTLs have been mapped for a large number of traits in willow including rust resistance [18–20]; insect resistance [21]; shoot height, stem diameter, and stem number [12, 22]; frost tolerance; phenology [19, 23]; water-use efficiency and drought tolerance [24, 25]; and saccharification potential [26].

Initially, molecular genetics in willow relied upon transfer of information from the closely related genus *Populus*, for which a full genome sequence has been available since 2006. This was facilitated by direct alignment of the K8 genetic map [27] to the poplar genome [28] and provided an efficient route to identifying the genes residing in the QTL identified from the willow mapping families. More recently Rothamsted Research has begun sequencing an accession of *S. viminalis*. In the United States, a similar effort is underway using a *Salix purpurea* of interest to their breeding program, and in China a genome sequence has been developed for *S. sachalinensis*.

At the time of writing, there is no working transformation system for willow. Research effort continues at Rothamsted and other institutions. Some confirmation of genes identified in the willow genetics research has been achieved by using willow sequences to rescue *Arabidopsis* mutants [29]. Transformation systems for poplar are routinely deployed and may be used for willow genetics research in the near future.

Seed Production

Willow is dioecious and therefore an obligate outcrossing genus. Uniformity of cultivars is achieved by vegetative reproduction of the F_1 generation. The majority of species readily produce roots from lenticels on the stem when placed in dark

moist conditions. Shoots are produced from dormant vegetative buds. Therefore, the commercial crop is produced by planting winter dormant, woody stem cuttings, usually of about 20 cm length and 15 mm diameter.

Willow seed does not contain endosperm, an individual seed weighs only a few milligrams. This makes them difficult to store in a viable condition. Seeds are collected at capsule dehiscence, this typically this occurs 4–6 weeks post pollination [28, 30]. Although seed storage protocols exist [31, 32], following crossing the resultant seeds are germinated almost immediately. Seedlings are reared in a glass house before being transferred to an outdoor irrigated nursery in June. The progeny seedlings produce a single stem of 2 m or more in height in their first growing season. All further reproduction is vegetative from stem cuttings and there is no further use of seeds in the multiplication of willow. The F_1 generation is subjected to selection and ultimately multiplication for sale.

Once selected as a cultivar, multiplication beds are planted specifically to produce stems for planting new areas. These beds are planted at greater density than a commercial wood production field. Often 40,000 cuttings will be planted per hectare on a 0.5×0.5 m grid compared to 15–17,000 cuttings per hectare on a double row system in wood production. The high planting density ensures that low diameter, straight, branch-free stems which are optimal for machine planting are produced. After grading and trimming to 2 m long, such beds may produce up to 600,000 new cuttings (at 20 cm length) which represents a multiplication ratio of 15:1 on a cutting basis, but 40:1 on an area basis when planting new crop at 15,000 cuttings per hectare.

Some commercial nurseries now also utilize micro-propagation techniques to multiply new genetic material more rapidly. This has a financial cost but may be justified by early market entry. Such micro-propagation methods may also be valuable to overcome phytosanitary restrictions on movements of stem material between territories.

Market Challenges/Barriers to Commercialization/ Opportunities

Recently, SRC willow production has sat awkwardly between agriculture and forestry. Surplus grain production in Europe, low commodity prices, and land set-aside schemes provided a place for energy crops on arable farms. The recent changes in arable farming fortunes have totally reversed the situation. Fortunately willow is an immensely flexible crop now finding a more suitable niche in traditionally non-arable areas. The one potential drawback of this change has been the difficulties experienced with winter harvesting on wet land in northwestern Europe. This is much less of a problem in the cold winters of more continental climates where harvests can take place on frozen soils.

SRC willow produces wood chip as a basic fuel. However, many consider it second rate when compared to wood chip from high forest systems. Clearly the greater proportion of bark in a multiple, low diameter (120 mm) stem coppice system increases the inorganic components of detriment to thermochemical conversion technologies relative to larger diameter tree branches. As the demand for wood fuel continues to grow, this is placing tremendous pressure on forestry resources in many areas of the world. Planting new forests has a long lead time, and planting newly bred SRC willow cultivars (with low inorganic components) on non-arable land offers great potential to fuel thermochemical energy conversion facilities. Forest trees have low adaptability; breeding is extremely limited, and the long lifetime of a plantation leaves them vulnerable to rapid environmental change. Classical breeding, backed up by modern biotechnology, in willows offers much greater adaptability.

The multifunctionality of SRC willow plantations are already being utilized and have the potential to be exploited further. They provide biodiversity benefits, when compared to conventional arable cropping rotations, and can be used for bioremediation, establishing riparian buffer zones to prevent erosion and agrochemical runoff and potentially carbon sequestration.

Fermentation of willow (to liquid fuels) is less well developed commercially than the thermochemical conversion technologies. Initially there is potential to ferment the available sugars and direct the residue to thermochemical conversion facilities. Even greater promise is offered by more advanced bio-refining where energy is only one product from the crop [33]. Rothamsted Research have begun a metabolomics profiling program, in part to identify novel and higher value products from the willow crop but also to support the crop improvement per se.

Conclusion

Willow offers tremendous natural genetic variation identified in the world's germplasm collections and potentially much more to be discovered. The breeding system is simple and breeding timescales short. Therefore, there is vast potential for bioenergy today and for rapid progress into the future. Unlike major food crop species where breeding effort must be concentrated on arable land, the willow breeding effort may be concentrated upon land types offering fewer options and greater challenges with, it may be expected, greater gains on such land.

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Chapter 5
Sugar Beet, Energy Beet, and Industrial Beet

J. Mitchell McGrath and Belinda J. Townsend

Abstract Sugar beet (*Beta vulgaris*) is a temperate root crop grown primarily as a source of sucrose for human diets. Breeding has focused on sucrose yield, which is simply the product of total root yield times the proportion of sucrose in the harvested roots, minus loss of sucrose in molasses due to impurities. Molasses is a source of betaine, which is recovered as a feed supplement. The non-sucrose dry matter (marc), mostly cell wall material, is used primarily for fodder. Beet juice, molasses, or sucrose solutions are easily fermented into ethanol, while whole beets or pulp is being used for biogas production. Beets have potential as a bio-resource for additional industrial and chemical feedstocks. Sugar beet vinasse is rich in glutamate that may be economically converted and substituted for some high-value petrochemicals. Cell wall material is low in lignin and thus is readily saccharified and fermented and may also serve as an economical resource for monosaccharides for which novel polymers may be developed. Procedures for sugar beet breeding are directly applicable to breed beets for alternative and novel uses.

Keywords Sucrose • Ethanol • Biogas • Glutamate • Cell wall • Betaine • Beet • Betalain • Marc • Molasses • Vinasse

Introduction

Beet (*Beta vulgaris*) is a root crop grown in temperate climates primarily for the production of the disaccharide sucrose as a sweetener in human diets. Recent reviews detail sugar beet breeding, agronomy, and processing for sucrose production and also detail breeding for crop protection needs that are specific to beet production [1–4]. These issues are germane to cultivation of beet for any purpose,

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including as a biofuel feedstock or for production of other industrial chemicals [5–7]. Indeed, much of the recent historical work of beet breeding has been toward improving crop protection traits. Varieties continue to improve incrementally for sucrose yield and content, and projected sucrose needs may be satisfied for the foreseeable future with the same number, or fewer, hectares of sugar beets as are currently grown.

Sucrose is perhaps the most abundant, chemically pure, renewable resource available, and numerous chemical transformations of sucrose have been described [8, 9]. Conversion of sucrose to ethanol is an ancient and simple biochemical transformation (via fermentation with yeast), and chemical transformations of ethanol also lead to useful industrial compounds. Modification of sucrose itself has non-caloric sweetener value (the artificial sweetener sucralose is an example), and its fatty acid derivatives can be used for coatings and polymers of various types. The breeding targets to increase the yields of these specialty products remain the same as for increasing sucrose yield per hectare. The sugar industry continues to look into alternative products, such as energy beet and beets for other industrial uses, for their long-term sustainability. Sugar beet germplasm and breeding methods will have a primary role in creating new beet crops to satisfy industry needs.

Beets solely intended for energy production were conceived during the first US oil shortage in the 1970s when sugar beet breeders recognized the potential for converting sucrose to ethanol for use as a liquid transportation fuel [10, 11]. These research activities declined when oil prices stabilized. Over the past 10 years, interest in beets for energy production has rebounded, with increasing petroleum prices and the restructuring of the EU sugar industry converging to improve the economic climate for biofuels and other industrial feedstocks derived from beets [12, 13]. Real and potential uses for sugar beets are quite diverse, in addition to sugar beet's primary use as a source of sweetener in human diets. Coproducts such as pulp (the insoluble root tissue after sucrose extraction) and molasses (the liquid remaining after sucrose refining) are used as animal feed or as feedstocks for the manufacture of specialty chemicals.

Taxonomy and Domestication

Beets (*Beta vulgaris* spp. *vulgaris* L.) are dicots in the family Amaranthaceae (formerly Chenopodiaceae) in the order Caryophyllales. Beets are classified by crop type (sugar, fodder, leaf, or table). The wild sea beet (*Beta vulgaris* spp. *maritima*), often found within a few meters of mean sea level, is considered the ancestor of the crop types [14, 15]. All types are outcrossing, wind pollinated, and cross compatible. Wild beets are indigenous to the Mediterranean coastal area, from the Cape Verde Islands in the west and south along Moroccan coast, east through the Middle East to India, and north along the Atlantic coast to the UK and Scandinavia [14]. As a group, Caryophyllales are often found in marginal and stressful environments.

Humans have used beet as early as the late Mesolithic, and it was probably first domesticated for their leaves for food and medicinal uses [14]. Selection transformed the annual habit into the more or less biennial habit characteristic of current crop types. The origin of the swollen root is not clear, but by the eighteenth century, large swollen roots were widely used for food and fodder. The sugar beet was selected from fodder beets from the late 1700s, and the first dedicated sugar beet varieties were available by the 1860s [15]. Sucrose production from sugar beets is an industrial process and requires dedicated factories for processing roots and refining sucrose. Improvements in processing have been continuous over the past 150 years, and breeding over this time has increased sucrose content of early varieties from <10 % (fresh weight) to a US industry average >15 % [16].

Beet morphological variation is impressive. In table, fodder, and sugar beets, the shape and morphology of the enlarged and (ideally) unbranched taproot is completely different from wild types, whose roots are thin and highly branched. In leaf beet, only the foliar apparatus has been modified in size and shape, often with thick, wide, and long petioles in a wide array of appealing colors. Color in beet is taxonomically diagnostic, consisting of the alkaloid betalain pigments that replace anthocyanins but serve similar functions as anthocyanins in most angiosperms [17, 18].

Common beet pathogens do not discriminate between crop types, so breeding for disease resistance is a common feature of all beet improvement programs. Often, resistances identified in sugar beet have been transferred to other crop types, and vice versa, although breeding efforts for vegetable (e.g., leaf and table beets) and fodder beets are not as extensive as for sugar beet. Wild beets will continue to be used as a resource to improve the current cultivated germplasm [14, 15].

Areas of Production

Sugar beet is grown on every continent except Antarctica but yields best in temperate climates, with major areas of production found across Northern Europe and North America. Total production of sugar beet is relatively stable worldwide. Yield of sugar beet per unit area has doubled since the early 1960s, and thus the total area of beets in cultivation has decreased worldwide (Figs. 5.1 and 5.2). In drier climates, irrigation is necessary. In warmer climates, sugar beet is often grown as a winter crop, notably in North Africa and the Imperial Valley of California (USA). Some of the excess factory production capacity caused by EU regulation changes in Northern Europe was diverted to energy production, particularly in France, Germany, and Scandinavia, and the short-term trend for beets as an energy crop, for both bioethanol and biogas (methane), will likely continue to develop in the North Atlantic and Baltic areas [19–21]. Beets grown strictly as an energy crop are not currently planted in the USA or UK, although a factory is being built in Mendota California for year-round beet ethanol production [113] and energy beets are being considered as cool season crops in the Mid-Atlantic and Mid-South regions of the USA [22] where it is anticipated that sweet sorghum and other

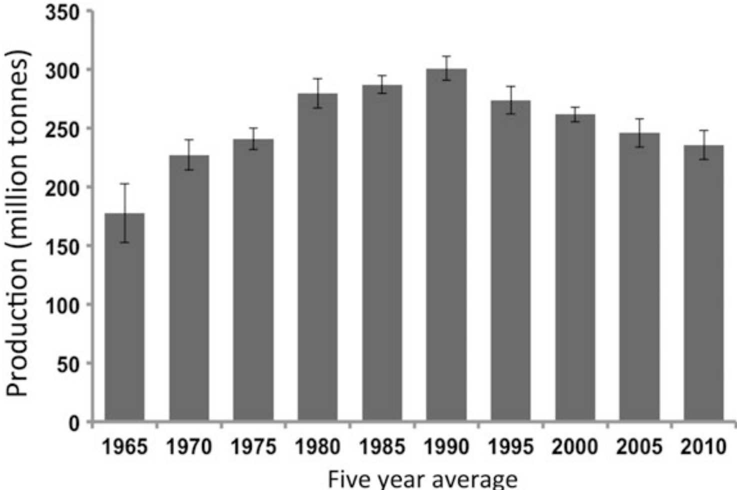

Fig. 5.1 World production of sugar beet in metric tons since 1961 averaged across five-year periods. *Error bars* are standard deviation (Based on data from FAOSTAT. http://faostat.fao.org/site/567/default.aspx#ancor. Last Accessed on 23 Jan 2013)

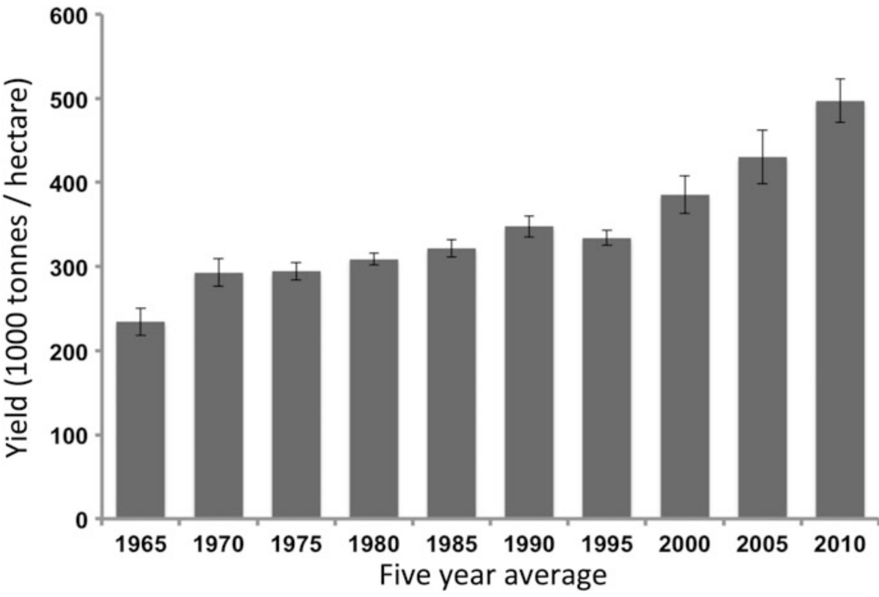

Fig. 5.2 World yield (fresh weight) of sugar beet in metric tons per hectare since 1961 averaged across 5-year periods. *Error bars* are standard deviation (Based on data from FAOSTAT. http://faostat.fao.org/site/567/default.aspx#ancor. Last Accessed on 23 Jan 2013)

warm season bioenergy crops would be grown during the summer, supplemented with energy beet during the winter. Sugar beets have been grown in all states in the USA and could be grown profitably with proper varieties (with particular attention to disease resistance) and husbandry. While these regions do experience freezing temperatures, progress is being made on winter hardiness and frost tolerance (aka winter beets) [23]. Also, beets are being considered for energy production in the major US sugar beet growing regions as a distinct crop, where the processing stream of sugar beet for energy would differ from that for sucrose in a few subtle ways, such as grinding and pressing pulp for juice extraction rather than diffusion from sliced beets for sucrose recovery [24].

Genetic Resources

Extensive germplasm resources exist for *Beta vulgaris* and related species. The USDA-ARS National Plant Germplasm System (NPGS) collection has seed available through the Germplasm Resources Information Network (www.ars-grin.gov/npgs/), with seed stocks maintained at the Western Regional Plant Introduction Station in Pullman, WA, and duplicates held in long-term backup storage at the USDA-ARS National Center for Genetic Resources Preservation in Fort Collins, CO. Approximately 1,900 accessions are currently available for distribution, including official USDA-ARS germplasm releases, with another 25 % unavailable for reasons such as reduced viability and low seed quantities, for which seed increases occur yearly depending on available space. Another large, well-maintained collection is the Dutch-German *Beta* collection with >2,000 accessions housed at the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. An International Database for *Beta* is maintained by the Julius Kühn-Institut, Quedlinburg, Germany (http://idbb.jki.bund.de/idbb) as an international repository of global network of *Beta* germplasm holdings. Each of these holdings represents a diverse collection of wild species, crop types, landraces, and USDA-ARS released germplasm. Efforts to characterize this germplasm have been ongoing for a decade or more, mostly for disease resistance traits [25]. Core collections have been defined in collaboration between US and European gene banks.

Beets are typically outcrossing, enforced by a complex system of self-incompatibility, and genetic diversity is high within *B. vulgaris* [26, 27]. Allelic diversity among cultivated species may be ~25 % of that found in wild germplasm [28, 29]. This underutilized diversity is relevant for germplasm enhancement [30]. Breeding is typically through population improvement approaches, and selection has reduced the level of heterozygosity in USDA-ARS germplasm releases over time [31]. Relatively few morphological mutants have been described in beets [32, 33], and, unfortunately, genetic stocks for most of these are no longer available. Newer approaches involving mutagenesis [34], TILLING [35], and recombinant inbred lines [36] are being used to uncover and tag genetic variation at individual

genetic loci. Linkage group nomenclature is based on the Butterfass trisomic series [37]. Genetic maps of (mostly) sugar beet have been created based on morphological, isozyme, and molecular markers [27, 38]. Results suggest that single nucleotide polymorphisms occur at 1 per 50–130 bp within genic regions [39, 40].

Major Breeding Achievements

Breeding achievements for sugar beet are notable, beyond ancient selection diversifying root and leaf crop types. Perhaps the first character subject to modern progeny test breeding methods was for increased sucrose content during the latter 1800s, followed by a rapid improvement in technologies for measurement and selection of fodder beet genotypes with increasing sucrose levels, from ~4–8 % to 12–15 % (fresh weight) over the next 100 years [41], and continuing at a less rapid pace today where modern sugar beet hybrids routinely achieve 18 % sucrose in optimal growing regions. Today, private companies produce sugar beet seed for commercial planting. Each of these companies has an extensive variety development program, and most of the worldwide yield gains over the past 25 years are directly due to their efforts (Fig. 5.2).

Initial varieties were open-pollinated populations and mass selected for increased root weight (e.g., tons/ha) as well as increased proportion of sucrose in the root. These values determine the yield of sucrose, minus what is unrecovered during processing. Two major innovations were developed in the 1940 and 1950s, and each of these is a staple in sugar beet breeding programs today. The first was discovery and deployment of the monogerm seed character, a single recessive gene (*m*) that conditions one seed per seedball (the ancestral state is a multigerm seedball which is a fused group of 2–8 woody flower tissue with one seed per flower, botanically a utricle). The second major development was cytoplasmic male sterility (CMS) as well as development of fertility restoration genotypes, which are conditioned by a pair of recessive nuclear genetic loci (*x* and *z*) that prevent pollen fertility in CMS mitochondrial genotypes but restore fertility in normal cytoplasmic genetic backgrounds (e.g., maintainer lines).

There are many diseases and pests affecting beets from the seedling stage through flowering [42]. Predominant seedling diseases include *Pythium*, *Aphanomyces*, and *Rhizoctonia*, for which some chemical treatments are effective and genetic resistance (or tolerance) is available, but additional genetic controls are continuously sought for sustainable long-term disease control. Major fungal root diseases include *Rhizoctonia*, *Aphanomyces*, and *Fusarium*, and genetic resistance or tolerance is the only effective control for these diseases. In warmer climates, should energy beets find a niche, *Sclerotium rolfsii* could be a yield-limiting disease, and genetic resistance is not currently available. Rhizomania, "crazy root," caused by beet necrotic yellow vein virus (BNYVV) which is transmitted by the soil organism *Polymyxa betae*, is perhaps the major root disease of beet worldwide. At least two genes (*Rz1* and *Rz2*) are deployed singly or in combination in the majority of sugar beet hybrids; however, these are not effective against

resistance-breaking strains of the virus, and transgenic approaches are being tested [43]. Also very important is resistance to the foliar *Cercospora* leaf spot fungus (caused by *Cercospora beticola*), which has the potential to defoliate the crop if uncontrolled. Genetic resistance is controlled by at least two genes, to as many as eight, and, along with sucrose content, is one of the few traits in beets with demonstrated quantitative inheritance. Other diseases can be yield limiting in certain areas, such as curly top (Geminiviridae, *Curtovirus*) in the Western USA and powdery mildew (caused by *Erysiphe betae*) in the UK. Sugar beet cyst nematode (*Heterodera schachtii*) is the major pest of beets worldwide and is yield limiting. Major achievements for beet breeding relate to developing germplasm resistant or tolerant to each of these diseases and pests [2, 25]. However, combining these into a common genetic background is still problematic, primarily because their precise genetic control is generally not well defined and many of the relevant genes have not yet been identified; thus marker-assisted technologies for trait stacking are still in their infancy [44].

Target Traits and Current Breeding Goals

The sugar beet taproot consists of water (~75 %), soluble solids (~20 %; ~75 % as sucrose), and insoluble solids (~5 %) [4, 45–47]. Values range by genotype and environment, and genotype x environment interactions for sucrose and marc (non-sucrose dry matter) are rarely significant [48, 49]. Sucrose biosynthesis occurs as in other plants, although the mechanism of sucrose accumulation is uncertain [50–52]. Sucrose and betalain pigments accumulate in root parenchyma vacuoles. Sucrose, but not betalain, is concentrated within the innermost five of 12–15 concentric cortical rings around the point of maximum root girth. These features begin developing within 3 weeks after germination and may be discriminated as significantly different between varieties by 10 weeks of age and continue to increase through the growing season [53, 54]. Varieties result from repeatedly selecting high sucrose segregants in heterogeneous breeding populations. Sucrose percent is quantitatively controlled with high heritability. Schneider et al. [55] detected five quantitative trait loci (QTLs) associated with sucrose content in multilocation replicated field trials using a molecularly mapped population. Interestingly, only two QTLs influenced root yield (mass), which is generally considered a nonadditive trait.

Sucrose yield is determined by total harvest weight multiplied by their proportion of sucrose, minus loss during storage and processing. Root sucrose content is expressed as a percent of fresh weight primarily because of the ease of specific gravity and refractometric measures and later polarimetry [41]. Nearly unanimous consent exists for a strong negative correlation between sucrose content and root yield [56]. Few factors in beets could simultaneously influence both yield (e.g., mass/area) and physiological (e.g., proportion of total mass) components, and of these, water exerts the greatest effect on both yield and percent sucrose.

Incorporation of water content measures demonstrated genetic variability for water content, and that commercial hybrids tested have a slightly, but statistically significant, lower water content than USDA-ARS germplasm (McGrath unpublished). Interestingly, the majority of putative QTLs for both sucrose content and sucrose yield appear to co-segregate with QTLs for water content and/or water yield [57]. By definition, the proportion of sucrose reflected in dry matter (e.g., dry matter = biomass = 1 − water content) is also heritable, and breeding for dry matter content will be a primary consideration for energy beets. Fortunately, sucrose content and total dry matter are highly correlated in sugar beet [46].

Sucrose and betalains accumulate in vacuoles of parenchyma cells located in between concentric cortical vascular rings that are a unique and distinguishing feature of beets [58, 59]. Accumulation and storage of biochemicals to economic levels is currently limited to sucrose, glycine betaine, and betalain pigments, but sucrose esters, fructans, specialty lipids, ascorbate, vanillin, and others theoretically could be produced in beets [8, 60, 61]. Glycine betaine is an osmoprotectant and feed additive. It is recovered from molasses during the removal of residual sucrose [62, 63]. Fresh beet products are good dietary sources of potassium and folic acid, betalain pigments are considered antioxidants [64, 65], and betaine has benefits to human health mostly due to its role as an osmolyte and methyl donor [66]. Betalains are used commercially as food colorings [67, 68], and breeding for increased dye concentrations shows that the quantity of betalain synthesized in the beet root is under genetic control [69]. Protein concentrations are generally low in storage roots, and assuming an adequate protein expression system in beets could be developed, recovery of high-value bio-ceuticals could be facilitated.

Disease management is critical for beets, and recent reviews reinforce the need for adequate levels of genetic resistance or tolerance to a range of biotic and abiotic stressors [1, 3, 30]. As well, early season growth (e.g., the first 8–10 weeks) is a critical phase for obtaining good field stands as well as for developing metabolic capacity for biomass accumulation. A phase change from embryonic/juvenile growth to adult vegetative growth coincides with an increased growth rate, accompanied by warming temperatures [54, 70]. Sugar beets are normally planted into cool (10–15 °C, optimal germination is 20–25 °C) soils to reduce the impact of seedling diseases. Acquisition of disease tolerance from acute seedling diseases to chronic root rot symptoms also occurs concomitantly with the juvenile to adult root growth phase change.

Breeding Strategies and Integration of New Biotechnologies

Interest in sugar beet for biofuel production has renewed in recent years [5, 16, 71]. Briefly, the state of the art is the deployment of the highest quality seed made from hybrids with highest demonstrated performance in each particular market. Hybrids have evolved from 3- and 4-way combinations from the 1960 and 1970s, through triploid hybrids from the 1970 to 1990s that exhibited performance gains

over early diploid hybrids, to a majority of diploid hybrids in the current market. Breeding at the diploid level gives breeding companies greater flexibility in creating hybrids tailored for particular niches, for example, in markets where rhizomania is a serious problem and where homozygous genetic resistance is essential in both seed and pollen parents for effective control. In these cases, marker-assisted selection for rhizomania resistance is routine (so far, perhaps the only trait in beet where this is true). Dosage of *Rz1* and *Rz2* genes can be more easily ascertained at the diploid level, and inbreeding within each of the parental lines can be used to fix such monogenic traits relatively quickly.

Genetic modification for sugar beet improvement offers the potential for the introduction of new traits that are not possible to introduce by traditional breeding approaches. One transgene event (H7-1) for resistance to the glyphosate herbicide was deregulated in the USA in July 2012. Other traits such as virus (rhizomania) and fungal (*Cercospora*) resistances are targeted but are not yet being evaluated for regulatory approval. Transgenic approaches for sugar beet improvement are recently reviewed [60].

Beta vulgaris has a haploid genome size of ~750 Mbp and a base chromosome number of $n = x = 9$. Approximately 60 % of the genome consists of moderately to highly repetitive elements. Many molecular marker maps have been constructed [27]; however, their resolution and their ability to discriminate QTLs (quantitative trait loci) have been still somewhat limited, and progress has been deferred in anticipation of higher-density mapping approaches and whole genome sequences. The beet genome is in the process of being assembled and annotated [72, 73, 114], and this will help immeasurably with SNP discovery and perhaps with genotyping-by-sequencing approaches. Part of the lack of progress in applying markers to beet breeding is that the majority of breeding materials are self-incompatible and thus population improvement approaches are most appropriate for new variety development. Relatively few inbred lines are available, particularly in the public sector; thus genetic dissection of traits of interest via conventional Mendelian transmission genetic approaches requires molecular analyses of sibmated lines or of entire populations, and these approaches have been prohibitively expensive to date. Association mapping has been suggested as one means to circumvent this limitation and is quite promising [74], but marker density is still too low for regular discovery of trait genes via such fine mapping approaches.

Also lacking is knowledge of the genes that control agronomic trait expression in different environments and during development, although significant progress has been made via transcript profiling approaches in recent years [70, 75, 76]. Plant development occurs under favorable conditions of water, light, and moderate temperatures, irrespective of most response-to-environment influences, such as temperature extremes, nutrient and soil (moisture) stress, and pathogen infection. Response-to-environment genes affect development, but developmental paradigms should be canonical for crop type and vary mostly in the timing of their expression, at least as a first approximation. Consequently, transcription profiling methods have been more successful in defining beet developmental genes and pathways than they have for response-to-environment genes. Developmental changes are quite

extensive during early season growth [54], and inferring gene functions and important biochemical pathways (including signal perception and transduction) among differentially expressed genes allowed better estimates of the magnitude of these changes. For example, an unusual involvement of hydrogen peroxide in stimulating germination appeared to activate stored lipid metabolism during germination, but only under stress conditions [77], and in essence defined a component of seedling vigor, which is an otherwise nebulous concept because it is difficult to measure empirically.

For energy beets, breeding progress and approaches are still in their infancy, and as with sugar beet variety development, private companies such as KWS (Einbeck, Germany) and its US subsidiary Betaseed Inc. (Shakopee, MN) are taking the lead in variety development. Interest in fodder beet as a source of germplasm for creating energy beets was rekindled due to the high root yields of fodder beet relative to sugar beet. However, most if not all of this yield boost is due to increased water content and thus is not relevant for biomass energy production [78]. Thus, well-adapted, high sucrose content sugar beets will likely be the best choice for founder populations of energy beets. Breeding dedicated energy beets has some advantages over breeding sugar beets. A major consideration for sugar beets is the loss of sucrose to molasses, which is caused in part by anionic sucrose molecules in solution charge balancing with cationic sodium and potassium ions as well as amino acids. Breeding against these loss-to-molasses (e.g., melassinogenic) substances can be problematic [79] but is a goal of most sugar beet breeding programs. The level of impurities is not a known issue for energy beet, so there is more freedom in the ability to breed for high dry matter varieties without selection against high impurity levels, although mechanical properties of the beet root such as density, elasticity, and slicing or crushing resistance will still be important [4]. Commercial energy beet varieties are being marketed in Europe and are not suitable for sugar processing due to excessive impurities but are instead being targeted for biogas production. Dedicated energy and industrial beets could be bred with pigments (either superficial epidermal pigments or wholly pigmented root tissue) to distinguish them from other crop types, as many fodder beet varieties have pigmented root epidermis but colorless internal tissues.

High biomass, defined as the yield of dry matter, is desirable for an energy crop. Relationships between water and dry matter, and dry matter and sucrose content, have a genetic and physiological basis. The literature has addressed some of these, but a clear picture is yet to emerge. An inverse correlation between sucrose content and root yield may largely be explained by differences in water content [80]. Between extreme high and low sucrose germplasm, consistent differences in the proportion of dry matter were evident [81]. Water content decreases early during development but appears to remain constant thereafter through the growing season [48, 82, 83]. Sucrose is readily fermentable, so varieties with a high dry matter content as well as a high proportion of sucrose to dry matter may be the best germplasm resources for energy beet breeding. The easy fermentation of sucrose would provide a direct boost to bioenergy yields and the low non-sucrose dry matter

content would create less processing load on the conversion of complex cell wall carbohydrates into readily fermentable energy resources.

The demonstrated ability of beets to accumulate high-value compounds such as sucrose, betaine, and betalain suggests that other chemicals could be accumulated in a similar industrial fashion. Which chemicals could be manufactured profitably depends on economics; however, it is likely that current and additional coproducts from beet sugar manufacture could be developed into specific industries on their own, and thus breeding would need to address the needs of such industrial beets. A US Dept. of Energy study [84] listed a dozen high-potential value-added chemicals from biomass, for which beet coproducts might yield economic benefit in the current bio-economy, namely, glutamic acid and sugar alcohols of xylose and arabinose. Glutamic acid comprises roughly 8 % of amino-N compounds (excluding betaine) and glutamine 31 % in sugar beet roots, which is synthesized from glutamate and ammonia enzymatically via glutamine synthetase, whose activity is correlated with glutamine levels in high- and low-amino-N varieties of sugar beet [85]. Inhibition of this enzyme in beet roots may result in larger pools of glutamic acid, and it is suggested that total amino-N and specific ratios of amino acids in the beet root are at least partially heritable but also markedly affected by environment, including N fertilization [49, 86]. Glutamic acid comprises >50 % of sugar beet vinasse (liquid remaining after ethanol fermentation) [87], and it appears that some glutamate-derived products can be made economically on par with their petrochemical equivalents [61].

Sugar beet cell walls are unusual when compared to nearly all other crops. Pulp that remains after sucrose is extracted is mostly plant cell wall material. Sugar beet has a highly atypical cell wall in that it has very low levels of xyloglucan and high levels of pectin [88, 89]. Sugar beet pectin is rich in neutral sugar side chains (arabinan) and highly acetylated pectic homogalacturonan [90, 91]. The neutral sugars appear to directly link together pectin and cellulose [92, 93]. These unusual properties directly influence the properties of sugar beet pectin as a food additive [94].

Sugar beet and other members of the Caryophyllales are unique in that ferulic acid is esterified to pectic arabinosyl and galactosyl residues of the pectic side chains [95–97]. Ferulic acid cross-linking is thought to influence the properties of the cell wall such as extensibility, control of growth, intercellular adhesion, microbial digestion, protein binding, and lignification [98–100]. Phenolic cross-linking also occurs in grasses, although it occurs on arabinoxylans, in addition to lignin-mediated reinforcement of secondary cell walls [101, 102]. In contrast, sugar beet storage roots contain mostly primary cell walls, so there is negligible lignin (~1.5 %) [4, 103]. The dry matter content of pulp is usually only 18–23 %, making it a "wet" feedstock which, in combination to the low lignin content, makes it unsuitable for combustion to produce heat and power [104]. Therefore, the challenges to decomposition of the cell walls are different to those of lignocellulosic biomass crops such as corn stover or sugarcane bagasse. Deconstruction of the cell wall involves the hydrolysis of polysaccharides in a process termed saccharification. The products of saccharification can then be used for fermentation or as

industrial precursors. It has been suggested that phenolic cross-linking interferes with efficient saccharification and fermentation by reducing access of hydrolytic enzymes and inhibiting microbes [105].

Seed Production

Beets are long-day plants and normally flower in the spring and early summer, after vernalization for biennial types. Vernalization can be completed on plants at practically any stage of growth. Typically, temperatures of 4–6 °C for 10–16 weeks are sufficient for vernalization, after which the plants should be moved to 12–15 °C with higher-intensity lighting for a period of a few weeks to ensure the flowering response is committed. Beets will de-vernalize if temperatures are too high for too long (>20–25 °C for >1 week). The de-vernalization process is not very well understood but is often more a problem in greenhouse seed production than field environments. The requirement of a period of cold to induce bolting and flowering is governed largely by the bolting locus, *B* [76, 106]. Annual beet generally has the dominant allele *B*, and commercial beets are homozygous recessive (*bb*) at the flowering locus. Understanding genetic pathways controlling vernalization and bolting is fundamental for controlling weed beet contamination, synchronizing flowering for seed production, and enabling earlier spring or fall sowing without yield reductions caused by premature flowering [107].

Cytoplasmic male sterility (CMS) makes hybrid sugar beet production practical. Beets are normally allogamous, governed by a complex gametophytic self-incompatibility system, which prevents self-pollination but allows almost any two plants to cross-pollinate. Commercial seed production [108] relies heavily on stecklings, which are young field-grown roots of 8–12 weeks of age, or otherwise kept small by dense planting, which are sown in one environment, harvested and vernalized en masse, and transplanted into a hybrid seed production field. A few specialized areas support the full life cycle for seed production, such as those found in the Willamette Valley of Oregon, USA, or areas closer to the northern shore of the Mediterranean Sea (e.g., southern France, northern Italy). In Mediterranean areas, care must be exercised that wild and ruderal beets are not flowering in the same area, or the hybrid seed crop will be contaminated with annual beets. Typically, seed production fields are isolated from other pollinators by a distance of 2–4 km. Commercial sugar beet hybrid seed is typically harvested from 8 to 12 rows of a CMS parent, which is alternated with 4–6 rows of a suitable pollinator parent. Often the CMS parent is directly sown in late August and early September and vernalized in situ, while pollinator stecklings are vernalized elsewhere and transplanted into the seed production field the following spring as temperature and day length increase. Seed yield and quality are often quite variable, and a great deal of processing is devoted to removing immature seed, removing the corky fruit tissue surrounding the seed (decortication), sizing fruits, and, in many instances,

priming and pelleting seed for commercial distribution. Stand establishment can be dramatically improved by such extensive processing.

Market Challenges/Barriers to Commercialization/ Opportunities

The market for energy and industrial beets is in its infancy. A number of studies have been conducted regarding feasibility, and in some situations, there is benefit to using beets as an energy feedstock [20, 16, 109]. The "right" mix of technologies, inputs, as well as the path to development depends on the local growing region, availability of complementary bioenergy feedstocks, familiarity with growing and processing beets, and availability of seed. Whether used as a sole feedstock or in mixed biofuel systems, a steady supply of fresh or processed beets needs to be available throughout the year. This is unlikely in growing areas exposed to prolonged freezing conditions, although growers in milder climates may benefit from improvements to frost tolerance of sugar beet that could allow autumn sowing and spring harvesting. Post-harvest loss of sucrose due to increased respiration is significant in warmer climates, and direct delivery and processing of roots at the factory are common in areas such as Imperial Valley of California and Egypt [110]. Post-harvest decay is also a significant risk to beets during storage. Dehydrating beets on a commercial scale is possible and may solve long-term storage issues and reduce transportation costs, but at additional energy inputs and expense to factories. Pressed, cooked, and filtered juice could be stored during the off-season as well. However, the potential for energy beets may be greater as a biomass input when other crops are unavailable, such as where cool and warm season crops can be grown in the same growing region at different times of the year. In factories where white sugar is already processed from beets, the non-sucrose materials may be used for alcohol or biogas production. Of the molasses (liquid) and pulp (solid) coproducts, molasses has the greatest energy conversion content, but supplies are generally insufficient for biofuel production year-round [111, 112]. Plus, these uses would compete with high-value sugar beet coproducts like glycine betaine and perhaps other nitrogenous compounds which are currently extracted from molasses, although perhaps their isolation from vinasse would complement existing uses while providing ethanol as well. A comprehensive assessment of the economic value and environmental impacts of inputs and products of sugar beet production will be required in each situation to determine the most favorable scenario. The potential of sugar beet as an energy and industrial crop has improved greatly in recent years. Breeding for specific energy and industrial components beyond that of sucrose production will likely succeed and perhaps lead to new beet crops with new and specific germplasm improvement goals. We can expect steady yield gains of energy and industrial feedstocks from beets.

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Chapter 6
Native Grasses for Biomass Production at High Elevations

Calvin H. Pearson, Steven R. Larson, Catherine M.H. Keske, and Kevin B. Jensen

Abstract Herbaceous perennial grasses as lignocellulosic resources are a preferred feedstock source for biofuels because they have a neutral carbon budget, require few agronomic inputs, can be readily managed to be environmentally friendly, and have the potential to be grown on a variety of lands, soils, and crop production situations. The Mountain West at elevations of 1,200 m, and higher, typically have unique and variable conditions typified by dry climates, cold-season precipitation, cold winter temperatures, hot summers with cool nights, large areas of public land, long distances to markets, large variations in soil types, variable soil quality such as salinity, changing field topography, and other factors. Large regions of the Mountain West are dominated by cool-season grasses that could be a desirable source for biofuel production. Tall-statured, cool-season perennial grasses including basin wildrye, creeping x basin wildrye hybrids, intermediate wheatgrass, and tall wheatgrass are viable candidates for lignocellulosic biomass production in this region. Developing a locally grown biomass and biofuel products could provide economic diversification to rural communities in the Mountain West. Establishing a regional supply chain for biofuel production could diversify fuel sources and provide a degree of energy security. Cool-season biomass grasses are not currently cost-competitive with other biomass feedstocks or other Mountain West energy sources. Policies that encourage market development, energy diversification and security could jump-start the market for cool-season biomass grasses, although long-term market viability hinges on their production at competitive costs. Furthermore, commercial production of cool-season perennial grass species will require considerable genetic improvement to develop these plant species for suitable biomass production.

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Introduction

Considerable interest has focused on producing plant biomass for conversion into biofuels for the USA. Many biomass crop specie candidates and biomass resources have been proposed in recent years. Herbaceous perennial grasses as lignocellulosic resources are a preferred feedstock source for biofuels because they have a neutral carbon budget, require few agronomic inputs, can be readily managed to be environmentally friendly, and have the potential to be grown on a variety of lands, soils, and crop production situations. Plant species and their associated crop production systems used for sustainable biomass crop production have a number of requisites that should be taken into account when considering crop selection for commercial biomass production (Table 6.1).

Much of the popular and scientific attention on plant biomass has been centered on warm-season grasses with their production being located primarily in the Great Plains, midwest, and areas of the east and southeast USA where these warm-season grasses are adapted. Accordingly, a considerable amount of breeding and genetic research on the development of dedicated energy crops in the USA has been directed on warm-season perennial grasses, such as switchgrass (*Panicum virgatum*) and *Miscanthus* sp.

Large regions of the Mountain West in the USA are dominated by cool-season grasses. In a study conducted in Utah comparing the performance of warm- and cool-season grasses for biomass production, cool-season grasses were found to be the most productive for total annual biomass production [1]. Elevations of 1,200 m,

Table 6.1 Prerequisites of plant species used for biomass production for biofuel

High yields in comparison to inputs	Low-input production requirements
Does not compete with established food/feed systems or for established food/feed cropland	Drought tolerant with high water-use efficiency/low water-use requirements
Should be carbon neutral and preferably carbon negative	Facilitate mechanical harvesting and processing
Have desirable sociological aspects	Perennial growth and long-lived
Little allocation of dry matter to reproduction	Not weedy
Low moisture content at harvest	Minimum plant-to-plant competition
Good competition against weeds	Must be profitable to agriculture and others
Resistant against diseases and insects	Inexpensive and easy to plant and establish
Have positive environmental characteristics	

or higher, in the Mountain West typically have unique and variable conditions typified by dry climates, cold-season precipitation, cold winter temperatures, hot summers with cool nights, large areas of public land, long distances to markets, large variations in soil types, variable soil quality such as salinity, changing field topography, and other factors. Although the average annual precipitation is relatively low, seasonal climate patterns often provide adequate soil moisture and temperatures for cool-season perennial grasses in the spring and early summer. Specific areas of this highly varied region provide ideal growing conditions for tall-statured cool-season grasses.

The higher-elevation environments in many areas of the Mountain West are limited by the number and type of crops they can produce. Nevertheless, crops and cropping systems needed to produce low-input herbaceous perennial crops to support a bioenergy economy in the Mountain West are essentially unknown, especially for large-scale production [2].

Recent policies such as the Energy Independence Security Act of 2007 (EISA) and the second US Renewable Fuel Standards (RFS) have targeted biofuel production and domestic energy independence. In contrast to most of the country, the Mountain West has attracted few biorefineries (http://www.ethanolrfa.org/bio-refinery-locations/) and is not expected to provide abundant biofuel development [3]. From a cost standpoint, this is not unexpected, due to high-input costs associated with irrigation water for production and transportation costs of biomass [4, 5]. Overcoming transport distances and water requirements are considered to be especially challenging for Mountain West biomass production [6]. The arid western states are not expected to provide abundant biofuels due in large part to the difficulty of identifying biomass crops that can sustain success in an arid setting. However, despite the low EISA projections for the region, energy disruptions and high energy prices would also presumably affect the Mountain West. Biomass crops that facilitate energy security on farms in the Mountain West benefit the region as a whole and make the RFS goals more attainable. Irrespective of the EISA biofuel mandates and policy targets, if bioenergy crops (grasses or oilseeds) are shown to be economically feasible for agricultural producers, their commercial production, and subsequent market development will result.

This chapter is intended to contribute towards developing biomass crop production and biofuel markets in the Mountain West with a focus on the potential of cool-season perennial grass species for biomass production and on modeling the profitability of agronomic production of perennial grasses. Tall-statured, cool-season perennial grasses including basin wildrye, creeping x basin wildrye hybrids, intermediate wheatgrass, and tall wheatgrass are viable candidates for lignocellulosic biomass production in this region. While this chapter is charged to focus on native grasses, we have arguably included grass species that are not native but have historically been grown in the USA and have been widely used in many applications. Based on their historic use over a sustained period of time, they are considered to be naturalized. These naturalized species along with native grass species have merit for consideration in biomass/bioenergy applications.

Basin Wildrye

Taxonomy and Domestication

Basin wildrye (*Leymus cinereus* (Scribn. & Merr.) À. Löve), also known as Great Basin wildrye, includes grasses previously treated as *Elymus cinereus* Scribn. and Merr. Creeping wildrye (*Leymus triticoides* (Buckley) Pilg.), also known as beardless wildrye, includes grasses previously treated as *Elymus triticoides* Buckl. Basin and creeping wildrye are closely related species of the tribe Triticeae genus *Leymus*, which encompasses about 50 perennial grass species from temperate regions of North America, Europe, and Asia [7]. Of the 17 *Leymus* taxa in North America, four species are introduced, two are naturally occurring hybrid taxa, and 11 species including basin wildrye and creeping wildrye are native [7, 8]. Both basin and creeping wildrye are highly self-sterile ([9]. The most obvious differences between these two species is that basin wildrye is generally taller (up to 270 cm) strictly caespitose with few if any short rhizomes, whereas creeping wildrye is typically shorter (usually less than 125 cm) and strongly rhizomatous [7, 10]. Basin wildrye also has relatively large spikes, up to 29 cm long, with 2–7 spikelets per node, whereas creeping wildrye also has smaller spikes with 1–3 spikelets per node. Hybrids between creeping and basin wildryes occur naturally in regions of overlapping distribution, but have not been formally named [7]. Basin and creeping wildrye are the only native *Leymus* wildrye species cultivated for seed or forage in the western USA. Cultivars and germplasms of basin and creeping wildrye have been developed from natural collections [11–14]). Seeds from at least several *Leymus* wildrye species, including basin and creeping wildryes, have been utilized as food grains by Native Americans of California and the Great Basin, Vikings, and other human societies [14–16]. There has been some effort to domesticate mammoth wildrye (*Leymus racemosus* (Lam.) Tzvelev) and beach wildrye (*Leymus arenarius* (L.) Hochst.) as perennial grain crops [15]. However, neither basin nor creeping wildrye can be considered fully domesticated.

Areas of Production

Basin and creeping wildryes are both native to western North America. Basin wildrye is widespread throughout this region including Alberta, northern Arizona, British Columbia, the California Sierra-Nevada, Colorado, Nevada, Montana, Washington, and Wyoming [7, 16]. Like most *Leymus* species, basin and creeping wildryes are well adapted to alkaline soils and cold-growing environments of this region. Although basin wildrye can be found throughout a wide variety of desert and mountain habitats, large native stands are typically restricted to specific areas where soil and water accumulate including roadsides and irrigation borders. Very few native species are so equally well adapted to both saline soils of the desert

basins and nonsaline soils of the upland sagebrush/grassland environments [17]. Basin wildrye was presumably once abundant on more productive soils of the intermountain valleys and floodplains that are now cultivated croplands and pastures [16]. Basin wildrye supplements tall wheatgrass in saline pastures, where it is grazed in the spring and fall [18–20], and provides valuable forage for winter grazing across western rangelands [16, 21, 22]. Basin wildrye is primarily used for large-scale rangeland rehabilitation, erosion control, and other conservation uses throughout western North America [23]. It is recommended for areas receiving 250–400-mm precipitation [21], but is often found growing in drier saline desert basins where surface or subsurface moisture may accumulate [24–26]. Basin wildrye has demonstrated tolerance to phytotoxic soils contaminated by heavy metals and is one of the more useful native grasses for mine reclamation in the western USA [27–30]. Although crops and forages grown on contaminated soils may pose health risks to humans and livestock, these areas may be suitable for biofuel production if the contaminants can be properly managed.

Creeping wildrye is commonly found on harsh alkaline sites in California, Nevada, Utah, and southeast Oregon [7, 21]. Creeping wildrye may have been one of the dominant species in the prairies and lowland oak woodlands of the California Central Valley [31]. Creeping wildrye is primarily used for soil stabilization, especially along channel or river banks, and for wildlife habitat in wetland and riparian plantings [14]. It is also recommended for use as forage and for reclamation of croplands and pasturelands contaminated by saline irrigation water [14]. The salt tolerance of creeping wildrye approaches that of tall wheatgrass and both species are being evaluated for forage and biomass production using saline irrigation water in the San Joaquin Valley of central California [32–34].

Genetic Resources

Next to *Thinopyrum* (wheatgrass), species in the genus *Leymus* have been of greatest interest to Triticeae grain breeders since the early 1940s when N. V. Tsitsin initiated hybridizations between *Leymus* and the Triticeae cereal genera *Triticum*, *Hordeum*, and *Secale* [15, 35]. Several *Leymus* species have been successfully hybridized with wheat, and some of the resulting introgression lines display potentially useful traits including biological nitrification inhibition [36], resistance to *Fusarium* head blight [37–39], and salt tolerance [40]. The genus *Leymus* are comprised of allopolyploid member species that contain the **Ns** genome of *Psathyrostachys* (Russian wildrye) and the **J** genome of *Thinopyrum*, based on chromosome pairing of interspecific hybrids [35]. However, early cytogenetic experiments raised doubt on the putative genome relationship between *Leymus* and *Thinopyrum*, which led to the currently accepted **NsXm** subgenome designations where **Xm** is from an unknown diploid ancestor [41, 42]. In any case, it is should be relatively easy to hybridize species and transfer chromosomes or genes between congeneric *Leymus* species, but introgression between genomically

defined Triticeae genera requires more sophisticated breeding and cytogenetic techniques [35, 43]. Most *Leymus* species are allotetraploid ($2n = 4x = 28$); however, octoploid ($2n = 8x = 56$) and duodecaploid ($2n = 12x = 84$) species and races exist. About 40 % of the surveyed basin wildrye accessions are tetraploid as are all creeping wildrye accessions [44]. However, the majority of basin wildrye accessions are octoploid.

Genetic markers and maps have been specifically developed for gene discovery and breeding research using hybrids of basin and creeping wildryes. Nearly 1,800 simple sequence repeat (SSR) markers were designed from 11,281 expressed gene sequence tags (ESTs) from creeping x basin wildrye hybrids [45]. Most of the 12,000 *Leymus* ESTs have been aligned to *Brachypodium*, and other grass genome reference sequences on the biofuel feedstock genomics resource from Michigan State University (http://bfgr.plantbiology.msu.edu/) and GrainGenes [46]. Three full-sib genetic mapping populations comprised of 586 progenies from reciprocal backcrosses of creeping x basin wildrye hybrids to creeping and basin wildrye testers were developed to map genes and markers associated with functionally important trait differences between these species [46–48]. Molecular genetic maps were constructed by genotyping these highly polymorphic mapping populations using nearly 2,000 DNA markers including 435 *Leymus* EST and 28 marker loci for nine of the ten known lignin biosynthesis genes. A large-insert bacterial artificial chromosome (BAC) libraries were developed from a creeping x basin wildrye hybrid including 405,888 clones with an estimated average length of 150.5 kb per insert, which represents 6.1 haploid genome equivalents of these allotetraploid *Leymus* wildryes [49]. These experimental plant materials and DNA libraries provide valuable tools for gene discovery research and plant breeding.

The USDA National Plant Germplasm System (NPGS) currently holds 242 accessions of basin wildrye and 20 creeping wildrye accessions. Three geographically significant landraces of basin wildrye were identified by DNA analysis of the NPGS accessions [44]. The Columbia race extends from British Columbia in the north, south through the Columbia River Plateau of Washington and Oregon, and further south into the Sierra Steppe of southeastern Oregon and northern California. About 91 % of the accessions classified in the Columbia race were octoploid. The Rocky Mountain race extends from the Rocky Mountain Piedmont of Alberta and Montana in the north; south through Wyoming, Utah, and Colorado; and west across the Snake River Plateau of Idaho and the Intermountain region of Nevada and Utah. About 82 % of the accessions classified in the Rocky Mountain race were tetraploid. The Great Basin race is interspersed with the Rocky Mountain accessions, but it is restricted to the Great Basin region of southwestern Idaho, Nevada, and western Utah. The Great Basin race is genetically more similar to the Rocky Mountain race, but like most of the accessions from the Columbia race, 73 % of the Mountain race accessions are octoploid.

Major Breeding Achievements

The octoploid ($2n = 56$) basin wildrye cultivar "Magnar," released in 1979 [11], is believed to have originated from southeastern British Columbia. The tetraploid ($2n = 28$) basin wildrye cultivar "Trailhead," originally collected near Roundup, Montana, was released in 1991 [12]. Cultivars Magnar and Trailhead represent the two most widespread and important genetic races, the Columbia race and Rocky Mountain race, respectively [44]. Magnar and Trailhead can be visually distinguished by the presence or absence of glaucous cuticle wax, which appears to be controlled by a single dominant gene orthologous to the wheat *Inhibitor wax* (*Iw*) gene [46]. Both Magnar and Trailhead have been widely used in seed mixtures with other grass species on public and privately owned rangelands of the western USA. "Continental" is a cultivar [50]) derived from a chromosome-doubled Trailhead pollinated by the natural octoploid, Magnar, which shows increased seed mass and seedling vigor compared to the parental cultivars [50]. The cultivar Continental segregates for the glaucous trait [50] and presumably segregates for other genes that distinguish its Columbia and Rocky Mountain parental races. The basin wildrye cultivar "Washoe" was collected from a natural population growing on phytotoxic soils near the now defunct Washoe smelter stack in western Montana, which is contaminated with arsenic, cadmium, copper, lead, and zinc [13].

The only creeping wildrye cultivar Rio, released in 1991, was originally collected in Kings Valley, California, and is used for soil stabilization in riparian areas, forage production, and reclamation of saline, irrigated croplands and pasturelands [14, 32]. Another cultivar, "Shoshone," was originally released as creeping wildrye, but morphological characters [21] and chloroplast DNA sequences [51] of Shoshone are similar to Eurasian *Leymus multicaulis* [14].

Breeding Strategies, Traits, and Goals

Growing up to 3 m tall [7, 10], basin wildrye has relatively high biomass accumulation potential, with up to 13,300 kg ha^{-1} observed with no irrigation or fertilizer in Cache Co., UT. Basin wildrye has a deep and extensive root system [52, 53]; high photosynthetic capacity, nitrogen-use efficiency, and intrinsic water-use efficiency [54]; and salt tolerance [24–26] that enable basin wildrye to maintain growth and physiological activity during dry summer periods when many other perennial grasses are dormant [54]. Basin wildrye tends to begin spring growth early, flower later, and stay green longer than other cool-season native perennial grasses, which extends the vegetative growth of this species [54, 55]. Biomass production can be enhanced from low levels of fertilization and irrigation, but once established it is a low-maintenance plant requiring little additional treatment or care [23]. These traits of basin wildrye can be useful for low-input biomass production in high-elevation environments of the Mountain West that are often favored by winter-precipitation

patterns. However, the high growing point of basin wildrye is susceptible to clipping and grazing [23, 55, 56], and it is difficult to establish good stands due to poor seedling vigor [21]. The release of Continental basin wildrye demonstrates that there is sufficient genetic variation within the species to improve seedling establishment [50]. Moreover, interspecific hybridization is being used to introgress rhizome genes into basin wildrye [10], which is expected to improve its grazing tolerance. Basin wildrye is susceptible to black grass bugs, including *Irbisia pacifica* and *Labops hesperius*, which can decimate grass monocultures [57].

Poor seed fill, low germination, and weak seedling vigor are the major limitations of basin wildrye [21]. Although the relatively large spikes of basin wildrye can produce thousands of seeds, these seeds readily disarticulate from the spikelet rachilla [7] and are prone to seed shattering [58]. Basin wildrye seed production fields require close scrutiny to prevent seed losses and ensure complete physiological development of the caryopsis [23, 58]. Moreover, the timing of seed harvest may be complicated by the fact that individual plants are genetically variable and may show variation in the timing of flowering and seed development [10, 58]. Thus, it has been speculated that seed performance problems associated with basin wildrye may be partly attributed to the temptation to harvest seed before it is physiologically mature [58].

The potential of creeping wildrye as a forage or biomass crop is derived from its adaptation to moist saline-alkaline soils [34, 59]. The cultivar Rio produced between 10,000 and 13,800 kg ha^{-1} in fields with soil salinities of 12.9–21.0 dS/m EC_e [34]. Although creeping wildrye is a relatively poor seed producer and has dormant recalcitrant seeds and weak seedling vigor, this species is not prone to seed shattering [14, 58, 59]. Once established, the aggressive rhizomes of creeping wildrye rapidly spread to produce better coverage, provide exceptional resiliency to clipping and mowing, and typically survive for many years [14]. However, this species may lack the biomass accumulation potential of taller statured species such as tall wheatgrass or basin wildrye [48, 60]. Moreover, seed and forage production typically declines when stands are left to become sod bound.

Hybrids between creeping and basin wildryes are partially fertile, and it has been suggested that it may be possible to introgress simply inherited traits from one species to another [43]. In particular, Dewey [43] suggested that the seed germination of creeping wildrye could be improved by introgression of genes from basin wildrye and the growth habit of caespitose basin wildrye could be improved by introgression of rhizome genes from creeping wildrye. Likewise, Larson and Kellogg [58] suggested that introgression of a recessive gene variant that abolishes seed abscission from creeping to basin wildrye could be used to improve ripening and development of basin wildrye seeds. Moreover, the F_1 hybrids of creeping wildrye and basin wildrye hybrids display increased plant biomass, with up to 14,100 kg ha^{-1} observed with no irrigation or fertilizer in Cache Co., UT. The creeping and basin wildrye parents of this hybrid produced 4,600 and 9,600 kg ha^{-1} in the same experiment. Progeny of these hybrids display transgressive segregation for biomass, forage quality, and many other traits including rhizomes and seed retention [10, 60, 61]. These observations suggest possibilities of improving the

biomass yield and composition; however, some of these traits may be multigenic or recessive making introgression by phenotypic selection difficult. Thus, genetic markers associated with seed retention [58] other important traits [10, 60, 61] can be used to introgress functionally important genes between these species with documented precision not possible by phenotypic selection alone. This approach of marker-assisted gene introgression is fundamentally based on conventional breeding techniques and natural processes. For example, some natural basin wildrye populations contain DNA alleles and traits, such as short rhizomes, which may result from introgression from natural hybrids between basin and creeping wildryes [7, 44]. However, the molecular markers enable selection of recessive genes, complementary genotypes, and other cryptic factors that are difficult to detect by conventional breeding procedures.

Seed Production

Methods of seed production for basin and creeping wildryes are well established, and rhizome sprigs can also be used to establish creeping wildrye in areas that are inundated by water or where rapid cover is needed [14, 23]. However, methods of seed production for creeping x basin wildrye hybrids have not been firmly established. The hybrids are partially fertile and synthetic populations derived from creeping x basin wildrye hybrids are being developed and tested at the tetraploid level [47] and colchicine-doubled octoploid level [62]. However, it is not clear if hybrid heterosis and fertility can be stabilized and maintained in synthetic populations that may be segregating for cryptic chromosome differences, which may cause problems with meiosis in later generations. Novel methods of producing F_1 hybrid seed of creeping and basin wildryes may be possible by taking advantage of the highly rhizomatous nature and strictly self-incompatible mode of pollination in creeping wildrye [9]. Creeping wildrye can be clonally propagated by rhizomes [10, 14], and it has been observed that some clones readily hybridize with basin wildrye especially if no other pollen source is available [47]. Thus, it may be possible to select and propagate a single creeping wildrye genotype, clonally, for use as a hybrid seed parent that would be pollinated by basin wildrye cultivars or populations.

Intermediate Wheatgrass

Taxonomy and Domestication

Under the present taxonomic treatment [63], intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth and D. R. Dewey) includes grasses previously treated as *Agropyron intermedium* (Host) Beaus., *A. trichophorum* (Link) K. Richt.

(pubescent wheatgrass), and *A. pulcherrimum* Grossh. Barkworth et al. [63] recognize two subspecies within intermediate wheatgrass subsp. *intermedium*, which is glabrous and subsp. *barbulatum* (Schur) Barkworth and D. R. Dewey, which is pubescent (syn. pubescent wheatgrass). Intermediate wheatgrass was first described from a collection in Yugoslavia in 1805 as *Triticum intermedium* by Host [64]. Intermediate wheatgrass spikes are borne on erect stalks and seeds are easily threshed, lending itself as a possible perennial grain crop on hilly or otherwise marginal land, thus reducing the farmers economic costs (i.e., labor and fuel) along with soil erosion (i.e., low-impact sustainable agriculture) [65]. Intermediate wheatgrass is tolerant of some saline soils, used as a fall and early winter forage [66], as well as providing an immense genetic reservoir to select from for disease and insect resistance in the cereals [67]. Intermediate wheatgrass is generally considered to be highly self-sterile, although self-fertile plants occasionally occur [9]. The first introduction (PI 20639) came into the USA from Trans Ural, Siberia, in 1907 [68].

Areas of Adaptation and Production

Intermediate wheatgrasses' natural distribution is found in steppes, on open stony and aleurite slopes among shrubs up to the lower mountain belts of southern Europe through the Middle East and southern USSR to western Pakistan [64, 69]. Dewey [68] reported that no intermediate wheatgrass collections have been recorded south of 30° north lat. and the more southerly collections were made only at higher elevations. Most collections within Iran were between 1,200 and 2,100 m.

In North America, intermediate wheatgrass is used for hay and pasture on sites receiving at least 35-cm annual precipitation at altitudes up to 3,000 m. It is widely distributed in the Intermountain Region and northern Great Plains of the USA and Canada where it grows best on well-drained, fertile soils that receive 30–46 cm of annual precipitation. It is recommended for sagebrush sites and high mountain areas up to 2,700 m. It is moderately tolerant of shade and alkalinity. As a general rule, intermediate wheatgrass is adapted to sites currently occupied by smooth bromegrass (*Bromus inermis* Leyss.). Yields and stand persistence can be increased in intermediate wheatgrass if grown with a legume. In drier areas (less than 38 cm of precipitation), intermediate wheatgrass yields more than smooth brome and crested wheatgrass [*Agropyron desertorum* (Fisch. ex Link) Schult]; however, after several years of harvesting, intermediate wheatgrass yields decline. The pubescent form is considered to be better adapted to the more southern limits of the species adaptive range in Asia [70] and the USA [71]. It appears to be better suited to droughty, infertile soils and saline sites that receive 30–35 cm of annual precipitation than typical intermediate wheatgrass [66].

Intermediate wheatgrass will outyield bromegrass and reed canary grass when grown on fertile well-drained irrigated land and will equal crested wheatgrass and outyield bromegrass under drought conditions on dryland. Under favorable conditions, intermediate wheatgrass will outyield both crested and bromegrass under

moist years on dryland cites [72]. Intermediate is less competitive with alfalfa (*Medicago sativa* L.) than bromegrass or crested wheatgrass and maintains a more desirable grass-alfalfa balance. Smart et al. [73] reported that May to June forage of smooth bromegrass outyielded intermediate wheatgrass by 750 kg ha^{-1} during the first harvest season, but only by 275 kg ha^{-1} at the second harvest season. Across three locations in Nebraska, intermediate wheatgrass averaged 5,301 kg ha^{-1} and ranged from 3,801 to 6,401 kg ha^{-1} [74]. At Mead, NE, between 1986 and 1987, Manska intermediate wheatgrass averaged 7,201 kg ha^{-1} compared to 6,800 kg ha^{-1} for cultivars Oahe and Slate; however, by 1989, the overall biomass was twice that with differences between cultivars reported [75]. Black and Reitz [76] reported that with increased row spacing width from 76- to 152-cm biomass went from about 3,500 kg ha^{-1} to 3,100 kg ha^{-1} under fertilization (67 kg ha^{-1} N and 22 kg ha^{-1} P); however, under no fertilization biomass production remained at around 2,000 kg ha^{-1} regardless of row spacing width. Dry matter yields averaged over four test sites, and multiple years in North Dakota were 4,226, 4,228, and 4,509 kg ha^{-1}, respectively, for intermediate wheatgrass cultivars Manska, Oahe, and Reliant [77]. Rush intermediate wheatgrass cultivar ranked among the four highest entries for overall biomass yield across five irrigation levels, averaging 23,700 kg ha^{-1}, and was the single best entry at low-irrigation levels, 19,100 kg ha^{-1}, in a comparison of 21 warm-season and six cool-season grasses [1].

Intermediate wheatgrass has increased the productivity of marginal land where bromegrass and orchard grass (*Dactylis glomerata* L.) are not well adapted. Its water requirement is between smooth bromegrass and crested wheatgrass, and it flowers from 1 to 2 weeks later than these grasses. Because of its relatively late maturity and quality retention after frost, intermediate wheatgrass has been effectively used for grazing during the fall and early winter in the Intermountain Region.

Although intermediate wheatgrass is noted for its productivity, it is sensitive to mismanagement or intense defoliation. Early cultivars failed to persist more than 4–5 years and were not good seed producers, prompting many to prefer either smooth bromegrass or crested wheatgrass. However, these problems have been overcome through the development of improved cultivars [78]. The forage quality of intermediate wheatgrass also declines at advanced stages of maturity. Intermediate wheatgrass is sensitive to mismanagement at the time of harvesting in the shooting stage [79].

Genetic Resources

Species in the genus *Thinopyrum* [35] have been of the greatest interest to wheat breeders since the early 1930s when N. V. Tsitsin first demonstrated that *T. ponticum* (Podp.) Barkworth and D. R. Dewey, intermediate wheatgrass, and *T. junceum* (L.) Á. Löve hybridized readily with various species of *Triticum* [80]. Chromosome numbers in intermediate wheatgrass range from $2n = 42$ to 52 with the aneuploids arising from unequal chromosome disjunction or unreduced

gametes. Its stable chromosome number is $2n = 42$ [81]. Based on chromosome pairing and C-banding patterns in intermediate wheatgrass, Liu and Wang [82] proposed that it is described as a segmental autoallohexaploid with the genomic formula of $\mathbf{E^eE^eE^eE^eStSt}$. Subsequently, Xu and Conner [83] described it as an allohexaploid $\mathbf{E^bE^bE^eE^eStSt}$ with its origin resulting from hybrids between diploid *T. elongatum* (Host) D. R. Dewey ($\mathbf{E^eE^e}$) or *T. bessarabicum* (Savul & Rayass) Á. Löve ($\mathbf{E^bE^b}$) and one of several tetraploid species *Elytrigia caespitosa* (C. Koch) Nevski ($\mathbf{E^eE^eStSt}$), *Elytrigia nodsa* (Nevski) Nevski ($\mathbf{E^eE^eStSt}$), and *Pseudoroegneria geniculata* (Trin.) Á. Löve ssp. *scythica* (Nevski) Á. Löve ($\mathbf{E^eE^eStSt}$).

A total of 1083 EST-SSR markers were developed from 16,128 Sanger DNA sequencing reads, with 6,450 contigs and 2,330 unmatched reads, of *Pseudoroegneria spicata* (**St St**) [45]. A total of 1,379,000 pyrosequencing reads, with an average length of 427 bp, were obtained from cDNA of hexaploid *Thinopyrum intermedium* ($\mathbf{E^eE^eE^bE^bStSt}$) and two diploid *Thinopyrum* species, *T. bessarabicum* ($\mathbf{E^b\ E^b}$) and *T. elongatum* ($\mathbf{E^e\ E^e}$), using next-generation techniques [84]. These short-read EST sequences were assembled into 71,300 contigs (667-bp average length), with 123,200 unmatched reads, containing an abundance of putative single-nucleotide polymorphisms (SNPs) and other possible DNA polymorphisms [84]. These *Pseudoroegneria* and *Thinopyrum* ESTs are being used to test and develop genome-specific EST-SSR markers and high-throughput SNP genotyping assays for intermediate wheatgrass.

The Montana-1 male sterile intermediate wheatgrass cultivar was derived from amphiploid hybrid *Triticum turgidum* L. var. *durum* x intermediate wheatgrass [85]. In 1986, the Montana Agricultural Experiment Station released Montana-2 perennial X *Agrotriticum intermediodurum* Khizhnyak resulting from a cross between durum wheat and intermediate wheatgrass. Seed of this hybrid is nearly three times as heavy as that of typical intermediate wheatgrass. It is proposed as a potential perennial grain crop in areas where soil erosion and production costs are limiting factors. The germplasm has potential as a genetic donor for disease resistance, winter hardiness, drought resistance, and semi-dwarfness in wheat breeding programs [86]. Subsequently, Jones et al. [87] concluded lines derived from Montana-2 contained individuals that could be used to improve biomass production if the population could be stabilized with improved seed production.

Within the National Plant Germplasm System (NPGS), there are 161 active collections of intermediate wheatgrass. The collections cover the following countries Afghanistan (5 accessions), Austria (1), Canada (1), former Soviet Union (17), Iran (83), Kazakhstan (21), Portugal (1), Russian Federation (8), Turkey (10), Turkmenistan (2), Ukraine (2), the USA (9), and Uzbekistan (1).

Breeding Strategies, Traits, and Goals

Accessions initially introduced into the USA failed to create significant interest for intermediate wheatgrass [78]. However, the introduction of PI 98568 from Maikop, USSR, in 1932 was the foundation for cultivar development in establishing intermediate wheatgrass as a forage grass in the USA. This PI was released as the cultivar “Ree” by the South Dakota Experiment Station and has contributed parental germplasm for the cultivars “Chief,” “Greenar,” Nebraska 50, “Oahe,” and “Slate.” Additional cultivars “Luna” (pubescent form) and Mandan 759 (pubescent form) were selected from PIs 106831 and 116252, respectively [21].

In 1966, the Canada Agricultural Research Station at Lethbridge released a 12-line synthetic cultivar “Greenleaf”, a pubescent wheatgrass type selected for increased seedling vigor, earliness of spring growth, forage yield, winter hardiness, predominance of bright green foliage, and pubescence of spikelets [88]. In 1980, the Research Station at Swift Current, Saskatchewan, released the cultivar “Clarke,” a 20-clone synthetic with breeding emphasis on drought tolerance, winter hardiness, good seed quality, and productivity of forage and seed [89].

The cultivars “Reliant” and “Manska” were released in 1991 and 1992, respectively. Reliant combines traits from 24 different hexaploid intermediate wheatgrass cultivars and experimental lines selected for improved persistence, forage quality, and forage and seed yields [90]. Manska was derived from Mandan 759 pubescent wheatgrass. It is particularly noted for its high nutritive value, based on in vitro dry matter digestibility (IVDMD) and animal performance [77].

In 1994, 2003, and 2003 cultivars “Rush,” “Haymaker,” and “Beefmaker” were released by Aberdeen Plant Materials Center (PMC) and the University of Nebraska, respectively. Rush was selected directly out of PI 281863 from Germany with emphasis on increased seedling emergence and plant vigor [91]. Haymaker originated from PIs 440015, 440008, and 440011 from the former USSR, and the cultivar Slate. Haymaker was selected for increased forage yields and in vitro dry matter digestibility [92]. Beefmaker intermediate wheatgrass is a broadly adapted cultivar that produces forage with high IVDMD and high protein concentration in the tallgrass, midgrass, and shortgrass ecoregions of the central Great Plains, USA. It was developed by intercrossing six plant introductions (PI 345586, PI 273733, PI 273732, PI 315353, PI 315067, and PI 3155355) that were identified as having superior agronomic performance in the central Great Plains in a germplasm evaluation [93]. The most recent cultivar release was “Manifest” in 2007 by ARS, NRCS – Bismarck, ND (PMC), and the North Dakota Agricultural Experiment Station. Manifest originated from ten collections near Stavropol and Svetlograd, in the Caucasian region of Russia collected by the late Douglas R. Dewey. It was selected for forage yield, seed yield, spring recovery, and resistance to leaf spot. Its higher tiller density results in improved persistence and stand longevity [94].

Seed Production

Intermediate wheatgrass spikes are borne on erect stalks, and seeds are easily threshed [95]. Because of its relatively high yield of large seed and vegetative characteristics, it has been proposed as a possible perennial grain crop in a low-impact sustainable agricultural system [96]. For optimum seed production, row spacing of 60 cm under irrigation and 90 cm under dryland conditions are recommended at a seeding rate of 9.2 kg ha^{-1} (irrigated) and 6.9 kg ha^{-1} (dryland). Seed fields should be planted in late summer by mid-August with adequate soil moisture or supplemental irrigation or early in the spring. If fall moisture and/or spring moisture is not reliable, then a fall-dormant seeding just prior to the soil freezing is recommended. Under irrigation, seed yield will range from 728 to 1,176 kg ha^{-1} averaging 952 kg ha^{-1}. When grown as a dryland crop, seed yields average 392 kg ha^{-1} and range from 224 to 560 kg ha^{-1}. Seed production fields remain productive between 5 and 10 years [97].

Tall Wheatgrass

Taxonomy and Domestication

Tall wheatgrass [*Thinopyrum ponticum* (Podp.) Liu and Wang] was previously treated as *Agropyron elongatum* (Host) Beauv., *A. elongatum* ssp. *ruthenicum* Beldie in North America, and as *Elytrigia pontica* (Podp.) Holub by Asian botanists [35]. The true *A. elongatum*, now excluded from *Agropyron* sensu stricta, is a diploid ($2n = 14$), while the robust grass known as tall wheatgrass in North America is a decaploid ($2n = 70$) [35].

Tall wheatgrass is indigenous to southern Europe and Asia Minor and was originally introduced into North America from Turkey in 1909 [98]. In its native habitat, it is often associated with saline or alkaline soils in meadows, salt marshes, and seashores [99]. It is a long-lived, coarse, vigorous, perennial bunchgrass with leaves that are long and erect. It is the latest maturing of the grasses adapted to the temperate rangelands of the west.

Areas of Adaptation and Production

Tall wheatgrass is one of the most saline or alkali-tolerant cultivated grasses and is particularly noted for its capacity to produce forage and persist in areas that are to alkaline or saline for other productive crops. On less favorable sites, e.g., saline and low moisture, it is short-lived unless there is a water table below the dry surface.

It can tolerate up to 1 % soluble soil salts. Tall wheatgrass increases production yields in soils with salinity levels of 6,000–18,000 ppm and persists in soils with electrical conductivity (EC) up to 26 mmhos/cm [100].

Tall wheatgrass is adapted to semiarid range sites receiving a minimum 35–40 cm of precipitation annually, or on irrigated or subirrigated soils at elevations from 1,300 to 1,850 m. In North America, it is widely used throughout the Mountain West and the northern Great Plains in salty areas in association with greasewood and salt grass. In the Columbia River drainage and the Great Basin, it competes well with native species such as basin wildrye on saline soils [101]. Tall wheatgrass has large seed that is easy to harvest and plant. It has good seedling vigor, and established plants have an exceptionally deep root system, which contributes to its resistance to drought [98, 102]. Under favorable conditions, it establishes as a dominant and may form a monoculture, thereby reducing diversity [101].

Tall wheatgrass remains green 3–6 weeks later than most other range grasses and is often valued as a source of forage during late summer, fall, and early winter [66]. It also has been used successfully as a silage crop. Because of its late maturity, it is usually recommended that tall wheatgrass be seeded alone. Leaving 20-cm stubble is recommended at year's end to prevent animals from grazing too close the following year. Grazing should not be initiated until at least 25 cm of new growth has accumulated above last year's stubble [21]. To ensure a successful seeding, it is recommended that one growing season be required for establishing tall wheatgrass on irrigated land and two growing seasons under dryland conditions. The major limitation in establishing tall wheatgrass stands are that young seedlings are slow to establish. Due to its late maturity, competitive ability, and tendency to become coarse during the growing season, it is recommended that tall wheatgrass be seeded alone rather than in a mixture with other grasses [66]. Although it tends to become coarse at advanced stages of maturity, when managed properly, tall wheatgrass has relatively good palatability and nutritional value. It is usually recommended for cattle; however, it has proven to be a good source of grazing for sheep [98].

Based on Vogel and Moore [103], sufficient variation exists in NPGS collections, particularly, PIs 98526, 264770, 283163, and 401006 to improve biomass production through selection in tall wheatgrass. On saline soils that ranged from 1.7 to 21.7 mmhos under dryland conditions, tall wheatgrass (cv. Alkar) averaged 4,331 kg ha^{-1} over a three-year period compared to 4,405 kg ha^{-1} for intermediate wheatgrass (cv. Greenar) and 4,107 kg ha^{-1} for the RS hybrid (cv. NewHy) in NRCS plant materials salinity trials in Roosevelt, UT [104]. In an irrigated trial near Elmo, UT, on saline soils that ranged in EC values from 5.7 to 20, tall wheatgrass (cv. Alkar) averaged 3,319 kg ha^{-1} over a four-year period compared to 4,624 kg ha^{-1} for tall fescue (cv. Festorina) and 2,376 kg ha^{-1} for the RS hybrid (cv. NewHy) [104]. On an upland site near Hays, Kansas, tall wheatgrass cultivars Alkar and "Jose" averaged 5,600 and 4,26 kg ha^{-1} over 3 years, respectively, compared to 3,696 and 2,800 kg ha^{-1} in intermediate wheatgrass cultivars Oahe and Slate, respectively [105]. Alkar tall wheatgrass ranked among the four highest

entries for overall biomass yield, averaging 22,800 kg ha^{-1} per year over five cuttings, in a comparison of 21 warm-season and six cool-season grasses [1].

Because of its tall stature and caespitose growth habit, tall wheatgrass provides excellent nesting and cover for upland game birds. Its seeds remain on the plant relatively well, providing feed for birds during periods of deep snow cover. The species has shown to have value in plantings as a barrier against wind and drifting snow [102].

Genetic Resources

Tall wheatgrass is genomically related to the intermediate wheatgrass complex. Intensive cytogenetic studies have established that it is essentially an autodecaploid, comprising five sets of genomes, designated $\mathbf{E^e}$ or $\mathbf{E^b}$ [106]. The $\mathbf{E^e}$ genome originated from the diploid *T. elongatum* and the $\mathbf{E^b}$ genome originated from *T. bessarabicum*. Tall wheatgrass has proven to be valuable in wide hybridization programs to transfer genes conditioning resistance to salinity, drought, and disease to wheat [35, 107]. Molecular genetic markers developed from *T. intermedium* ($\mathbf{E^eE^eE^bE^bStSt}$), *T. bessarabicum* ($\mathbf{E^bE^b}$), and *T. elongatum* ($\mathbf{E^eE^e}$) should also be useful for tall wheatgrass [84].

Breeding Strategies/and Traits

The gene base of tall wheatgrass included in North American breeding programs is relatively narrow, with most cultivars tracing to one or two plant introductions. "Largo" the first cultivar to be released was derived from PI 109452, an accession collected by the Westover-Enlow expedition in Turkey. It was originally increased at the USDA-SCS nursery at Albuquerque, New Mexico, and the Utah Agricultural Experiment Station at Logan, Utah, cooperatively with USDA-ARS and released in 1937 [91].

Alkar, which is the widest used cultivar, was selected at the USDA-SCS Plant Materials Center at Pullman, Washington, and released in 1951 [91]. Its parental germplasm was derived from PI 98526, an accession obtained from the USSR via the N. I. Vavilov Institute of Plant Industry in 1932. Alkar is widely used in the Pacific Northwest and the Intermountain Region for pastures in wet, alkaline conditions [102, 108].

The cultivar "Jose" was released in 1965 and has been used for pasture and hay in irrigated areas of New Mexico and Colorado at elevations up to 2,300 m, as well as on range sites where alkali and salinity prohibit the use of other productive grasses. It is reported to be more acceptable to grazing animals than cultivars such as Alkar and Largo [109].

"Orbit" is the first cultivar of tall wheatgrass to be licensed for sale in Canada and was released in 1966 by Agriculture Canada at Swift Current, Saskatchewan. It is a composite of nine open-pollinated lines and one three-clone synthetic that were derived from PI 98526 and locally selected strains. The breeding population benefited from natural selection for winter hardiness. It is also characterized by relatively good seed and forage yield.

"Tyrrell" was registered in 1981 by the Victorian Department of Agriculture, Australia. It was selected from Largo, and subsequent evaluation trials have established that it is distinctly different from Largo. Its main assets are high salt tolerance and ability to grow and persist on highly alkaline soil sand salt seepage areas. It is particularly well adapted to salt-affected land typical of northwestern Victoria in Australia [110].

The cultivar "Platte" was released by the USDA-ARS and Nebraska Agricultural Experiment Station. Its parentage consists of selections from Nebraska 98526 and another breeding line. The cultivar is noted for its winter hardiness and improved forage and seed production. It is particularly well adapted to alkaline sites in lower valleys of Platte River drainage [111].

Seed Production

Tall wheatgrass grown for seed production should be planted in 71–91-cm rows and cultivated or 30–36-cm rows uncultivated. It typically produces 336 kg ha^{-1} under dryland conditions and 672 kg ha^{-1} under irrigated conditions [97].

Market Development and Macroeconomic Considerations

At this writing, cool-season biomass grasses are not cost-competitive with other biomass feedstocks or other Mountain West energy sources like natural gas. The agronomic cost analysis presented in this chapter provides a step towards cultivating the market for cool-season biomass grasses. With a better understanding of agronomic costs, the biomass industry may eventually become cost-competitive with other energy sources, especially when environmental benefits are calculated. A biomass market in the Mountain West could also contribute to regional economic prosperity if the market diversifies the region's energy portfolio. The first part of this chapter provides a review of macroeconomic, supply chain, and policy considerations that could be used to establish markets and policies for cool-season biomass grasses. This is followed by an agronomic cost analysis and discussion about barriers to commercialization.

Stable, low energy prices are directly linked to economic prosperity. Sharp commodity price increases, like abrupt energy price increases, can create an economic domino effect. For example, disruptions in crude oil supplies result in

rising diesel prices. This subsequently increases transportation and agricultural production costs, eventually leading to inflation. Policymakers take considerable measures to diversify energy sources for transportation, electricity generation, and aviation so that energy costs remain stable. Adequate diversification and domestic production of fuel sources is defined by many policymakers as "energy security" [112]. An energy portfolio diversified with biomass-based energy could improve the Mountain West's energy security. Policies that encourage market development, energy diversification, and energy security could jump-start the market for cool-season biomass grasses, although long-term market viability hinges upon whether crops can be produced at competitive costs.

Ideally, profitability calculations should include net environmental costs. Laws and regulations require policymakers to balance energy security with environmental targets that consider greenhouse gas emissions, soil reclamation and remediation, and nutrient management. As established earlier in the chapter, cool-season biomass grasses may provide several of these environmental benefits. If a full environmental cost accounting of environmental impacts is conducted, then biomass-based energy cost-competitiveness may improve. Energy security and environmental policy goals provide both opportunities and challenges for biofuel production in the Mountain West.

On a national level, biofuels have been promoted as a means for increasing domestic energy production while meeting environmental regulations, although the effectiveness to which biofuels fulfill these objectives is continually under discussion. As previously stated, EISA and the RFS policies have attempted to increase domestic biofuel energy production. These policies are linked with considerable US biodiesel production increases from 87 million L in 2004 to 3,107 million L in 2011. The RFS calls for 136 billion L of biofuels to be created annually by the year 2020.

Based on current examples, biofuel policies have arguably been successful in establishing markets for US biofuels. Recent studies demonstrate that biofuel production is a commercially viable enterprise in some regions of the USA. For example, in the Midwest, there is sufficient supply and demand for corn ethanol, and the market in this region is now considered economically viable. At this writing the corn ethanol market can function without the support of many US subsidies [113]. Results from life cycle analysis that evaluate environmental impacts from "cradle to grave" note varying degrees of environmental benefits. There are notable concerns as to whether biofuel feedstocks displace food and whether US biofuel policies could contribute to higher domestic and international agricultural prices along with food prices. Some authors have demonstrated a strong correlation between biofuel production and rising global food prices, although other economists have noted that periods of high global food prices have resulted from a complex set of issues and that only select US biofuel policies have a minimal effect on food prices [114]. This debate will likely continue into the future as additional data become available. Nevertheless, what is relevant is that biofuel crops produced in the Mountain West states should be produced at low-input costs and ideally should not compete with crops intended for established uses such as food and feed.

If biofuel production can be established in an economically feasible way in the Mountain West, then this may be a market-based, environmentally desirable solution to energy security.

Recent studies show that the farm costs of producing switchgrass for cellulosic biofuel are estimated to be $40–60 per metric ton. A key component to profitable biomass production is maintaining consistently low-input costs [115]. A central criterion for the production of biomass crops are high yields with low production costs. The high price of production is driven, in part, by the high cost of inputs, such as water, as well as high transportation costs [4, 5]. The requirement of a low-cost delivered feedstock may be challenging to producers.

In theory, perennial grasses could be a desirable source for biofuel production because they can be grown on marginal lands with low water and fertilizer requirements and do not otherwise compete with other food/feed crops. Preliminary data suggest that perennial grasses could also improve soil carbon and nitrogen balances, indicating that this could be an environmentally desirable source of energy biofeedstock. The challenge is producing grasses in sufficient quantities to establish a regional market. Agronomic and biorefining costs must be low enough so that the prices are competitive with energy sources such as natural gas. Likewise, prices must be high enough so that agricultural producers will be willing and able to supply a consistent amount of biofuel feedstocks for profitable biorefinery production and to consistently fulfill fuel delivery contracts, ensuring that there are no fuel shortages. In other words, perennial grass biofeedstocks must be cost-competitive and reliably available to maintain stable consumer fuel prices while providing the supply chain with enough incentive to reliably produce the biofuel feedstock.

Identifying the incentives for producers in the Mountain West to grow biomass for biofuel is likely to be challenging. Biorefinery owners need a known, available, and constant supply of biomass to maintain an uninterrupted operation of their biorefinery, and it would seem appropriate for them to secure multiyear contracts with biomass producers. Will producers be willing to enter into multiyear contracts that will set contract conditions over multiyears? If biorefinery owners choose to lease fields from producers and produce their own biomass, will land owners be willing to enter into multiyear leases that result in their land being committed to a single production system over a long period of time? If these two options are not feasible, can biorefineries capitalize themselves sufficiently and secure the needed human resources and expertise to create the entire supply chain and own and operate a large enough land area to produce sufficient biomass to operate their biorefinery?

Not surprisingly, many steps along the supply chain must be cultivated for perennial grasses to be a viable source of bioenergy feedstock. Calculating and improving agricultural production costs at the farm level is a good way to begin supply chain development. Fore et al. [115], for example, calculated niche biofuel and feedstock costs for small, on-farm production. In their example, they find that neither soybeans nor canola was cost-competitive with petroleum diesel when feedstocks were valued at market price, but that under certain scenarios, the economic feasibility of straight-vegetable-oil (SVO)-based fuels and diesel could

be similar. In a Colorado-specific example, the economic feasibility of growing the oilseed crop *Camelina sativa* (“camelina”) in the Mountain West was modeled to produce value-added protein feed supplement, SVO-based biofuel, and farm energy independence [116]. Results from stochastic crop rotation budget showed that producers have a 50 % likelihood of breaking even when diesel prices reach $\$1.14\ L^{-1}$, although an experienced producer could achieve profitability 90 % of the time (using expected values of input prices) when diesel is at $\$0.81\ L^{-1}$.

Using a specific location as a test case allows an agronomic-economic model to be populated with examples, so that variables can be isolated and replicated elsewhere. This approach is supported by the literature, as others have noted that feedstocks must be tailored to be region specific in order to feasibly grow biofuels throughout the USA [117]. In other words, if economic profitability can be achieved at the farm level for niche biofuel markets, like those presented above, others may be encouraged to replicate the results. This momentum may lead to improvements in cost-efficiency and expanded production of perennial grasses as bioenergy feedstocks in the Mountain West. Ensuring the availability of a consistent number of producers/suppliers is a positive step towards attracting investment in a regional biorefinery and establishing a supply chain. Some people argue that the production of grass species will provide growers with dual market opportunities. Farmers can sell harvested grasses into traditional feed markets, and when prices are favorable, they can sell harvested grasses into the biomass/biofuel market. This approach does not provide for a reliable supply chain to meet the needs of a biorefinery.

Economic Feasibility of Perennial Grass Production in Western Colorado

Using the case study approach, this section presents an agronomic-economic model and a crop enterprise budgeting tool for growing perennial grasses in the Mountain West, with the intention that the model can eventually be replicated elsewhere and a regional market for perennial grasses can develop.

The cost of producing biofuel feedstocks is a major hurdle for growers [118], as production has been on too small of a scale to ensure consistent profitability [119]. One key component to profitable commodity production is maintaining consistently low-input costs [115]. Agronomic-economic data were collected to develop a crop enterprise budget tool [120] for herbaceous plant species in western Colorado. Field performances of four herbaceous biomass entries (factor 1) and four fertility input levels (factor 2) are currently being evaluated to assess their effect on biomass production over a long-term testing period at three locations in western Colorado. A more elaborate description of the agronomic parameters is outlined in Pearson et al. [121].

The objective of the budget tool was to model the impact of agronomic changes on production costs. The enterprise budgeting tool was developed in an Excel spreadsheet that is user-friendly for a variety of audiences, including producers,

crop consultants, extension agents, and others. Parameters can be adjusted to reflect variations in location, crop management, best/worst case scenarios, or optimizing a specific input. For the purposes of this paper, the parameters of the crop enterprise budget have been adjusted to reflect specific agronomic scenarios. Naturally, these scenarios, and the corresponding results, can vary according to the agronomic model.

As demonstrated in the crop enterprise budget scenarios presented in Table 6.2, of the four species, the introduced grass species demonstrates the lowest per hectare break-even price ($51.59) when grown using efficient agronomic management. In contrast, the native grass mix demonstrates a relatively lower yield and a substantially higher break-even price, at $315.35 per hectare, even with efficient agronomic practices. Increases in two key costs, diesel fuel, and irrigation water, not unexpectedly, directly affect production costs. Regardless of the scenario, producers with capital equipment constraints (e.g., no disking) incur approximately 20 % higher break-even prices due to reduced yields. The crop enterprise budget tool quantitatively shows how changing different input parameters affects potential profitability.

As additional agronomic data becomes available, comparisons can be made about the expected break-even costs in the years following crop establishment. At the moment, it appears that establishment costs for the native grass mix and switchgrass are higher than the introduced grass mix, because yields are not as high during the first 2 years of production. Preliminary data from Pearson et al. [121] demonstrates considerable increases in switchgrass yields in its third year of production. Thus, while there could be a higher opportunity cost at least

Table 6.2 Enterprise budget scenarios for biomass species trials in Fruita, Colorado

	Switchgrass	Tall fescue	Introduced mix	Native mix
Biomass production costs	Assumes 2 cuttings with the average yield per species, per cut, expressed in tons/acre for average of 2011 and 2012 (early establishment years' yields)			
Efficient management				
Cost per acre	$182.70	$152.10	$154.78	$157.67
Break-even price per acre	$121.80	$152.10	$51.59	$315.35
Inefficient management				
Cost per acre	$229.97	$191.72	$195.08	$198.69
Break-even price per acre	$153.31	$191.72	$65.03	$397.38

[a]Inefficient management is defined as a scenario in which the agricultural production is the capital equipment constrained so that production is conducted at 80 % of the efficiency of an optimal producer. The following input parameters were assumed for both management scenarios: $30/acre cost for deficit irrigation in a typical non-drought year, $10/h labor costs, 3 % operating loan, $4.00/gallon diesel fuel prices, and two cuttings. Yields of 1.5, 1.0, 3.0, and 0.5 t/acre, respectively, were used for switchgrass, tall fescue, and introduced and native species. These reflected the approximate 2-year average yields for early establishment years at the Fruita site (2011 and 2012)

initially for switchgrass, production costs could decrease if yields increase considerably in nonestablishment years. At this writing, switchgrass and the introduced grass mix could show promise as economically feasible crops on marginal lands.

In summary, this crop rotation budget exercise should be viewed in context as a first step towards isolating the agronomic and economic variables that influence the profitability of agronomic perennial grass biomass production targeted for the Mountain West. Additional field trial data will provide necessary information to agricultural producers who must decide whether or not to grow the crop. The crop budget and profitability estimates are steps in building a perennial grass market that could ultimately lead to a critical mass of agricultural producers who are willing and able to cultivate a crop that can be used as a biofuel in the arid Mountain West.

Barriers to Commercialization

At present, there are several supply chain barriers to commercialization. Agronomic production of perennial grasses has only been at the pilot scale. Before investing in commercial scale development, biorefineries must ensure that there will be both adequate biomass supply and demand for the finished product. Ultimately, biofuels must be reliably available and offered at a price point that competes with other fuel sources. However, investment in the biofuel supply chain could help overcome production and cost barriers and improve the overall economic structure of remote, agriculturally based Mountain West communities.

Developing a locally grown biofuel product could provide economic diversification to rural communities in the Mountain West. Establishing a regional supply chain for biofuel production could diversify fuel sources, thereby providing a degree of energy security against price increases or shortages. With strategic biorefinery locations, transportation costs could be minimized, so that the biofuel products could be competitively priced. If the perennial grasses are grown on marginal lands, it could provide agricultural producers a diversified agricultural product mix and an additional revenue stream from land that may not otherwise be in production.

The identification of sufficient land area within a cost-effective distance to support year-round operation of a biorefinery is a significant barrier to commercialization in many areas of the country including the Mountain West. The production of ethanol that uses corn for conversion has been economically feasible to locate biorefineries close to by-product users rather than only near the resource production sites. Corn grain can be railed across state lines to ethanol conversion facilities locations where by-products from a biorefinery are sold to cattle feeders.

Biomass from perennial grasses must be grown on land that does not compete with land that is currently being used for food/feed production. A variety of potential types of land that could be used for biomass production are marginal land, abandoned land, degraded land, idle land, underutilized land, wasteland, reclaimed land, and inefficient land. Identifying and quantifying such land that is

suitable and available for biomass production is challenging. Determining the production and production stability potential of these lands for biomass production to meet the demands of a particular biorefinery is equally challenging.

As previously discussed, in order to establish the agricultural production segment of the supply chain, the perennial grass field trials should be replicated at multiple sites. Great care should be taken to measure cost, input, and yield data to ensure that agricultural producers set proper expectations for field performance. Cost and yield trends will help growers estimate feedstock quantity and contract price parameters for biorefinery contracts, and a critical mass of agricultural producers will be necessary in order to attract capital investment for a biorefinery.

Likewise, biorefineries will need to ramp up production levels to a point where they are able to provide predictable quantities of biofuel to fulfill fuel contracts and to ensure that there are no fuel supply disruptions. As previously noted, in order to establish a commercially viable market, it is important to control production costs, from growing and biorefining the feedstock, so that per gallon biofuel price is competitive with other commercially available fuels. It is also expected that the agricultural producers and biorefinery will earn a reasonable rate of return on their production – otherwise, there would not be an incentive for them to continue the biofuel supply chain. A fuel supply contract, possibly for a city or county service vehicle fleet, could provide assurance to producers that there will be a demand for the products that they produce. In small, rural economies like those in the Mountain West, there is a potential for the regionally grown, processed, and supply chain to develop and provide a cost-competitive product.

From a practical perspective, recent technological advances have improved the economic feasibility for developing non-conventional natural gas plays (defined as shale gas, coal bed methane, and tight gas sands) that can yield reliable natural gas production with high immediate payback on investment and competitive consumer prices. Many of these natural gas resources are located in the rural Mountain West communities that have been the subject of this chapter. Hence, natural gas development, rather than biofuel development, may actually serve as the low-cost energy resource that drives economic development in these regions. On the downside, while the benefit of low energy prices have been well established, unlike agricultural-based economies that create diversified economic sectors, oil and natural gas development leads to notoriously undiversified regional economies often leading to boom and bust economic cycles [122, 123]. While the USA is projected to be a net energy exporter during the next 30 years, this enthusiasm should be put in perspective with the perceived natural gas shortage from just a decade earlier. There is considerable economic benefit projected for natural gas development in the Mountain West; diversification of energy resources should always be an important goal to manage risk and to facilitate energy security.

At the present time, Mountain West communities are poised to benefit from the anticipated boom in natural gas production that is projected to displace coal as an electricity generation resource and eventually displace gasoline and diesel as a source for heavy fleet vehicles. There is little disagreement that natural gas reduces net greenhouse gas emissions compared to coal-based electricity generation

[124–126], although there are concerns about fugitive methane emissions, in part due to shale disturbance. Despite technological advances and improvements in environmental assessment and accountability for hydraulic fracturing, there is much to be learned about the non-conventional natural gas development process and accompanying environmental impacts. As recent, controversial, community meetings have shown decisions as to whether or not to proceed with natural gas development should be made on a community-specific basis with an attempt to include multiple stakeholders. Agriculturally based communities could make an informed decision about the mix of locally based energy production that best suits their community values, and this mix will likely address locally produced natural gas.

Another plausible scenario is that biomass could be coproduced on lands that primarily serve to meet soil protection and wildlife conservation goals [127]. Herbaceous perennial grasses provide benefits for land cover that improves soil and water protection, nutrient management, and wildlife habitat. The marginal lands that qualify for agricultural policies like the Conservation Reserve Program could serve dual policy goals of providing wildlife habitat and biomass production to establish a supply chain to sustain a regional economy. Considerable agronomic and economic work is necessary to make this economically desirable proposition for biomass supply chain in the Mountain West a sustainable reality.

Opportunities

For biomass production in the Mountain West, a goal of 6.7 dry ton ha^{-1} and a biofuel yield of 330 L ton^{-1} of biomass would produce 2,211 L ha^{-1} of biofuel. Compared to much of the rest of the country, the Mountain West has a large acreage of idle cropland, has a majority of the land in grassland pasture and range, and has one of the highest rates of crop failure. Using sustainable cropping practices for biomass production, well-adapted, dedicated perennial biomass crops would reduce the incidence of crop failure [128]. If 4 million hectares of the 142 million hectares of cropland, grassland, pasture, and range could be used for biomass, this land has the potential to produce 9.1 billion liters of biofuel annually, thus creating a significant economic opportunity. Production of this quantity of biobutanol in the Mountain West would make a significant contribution towards meeting the US Energy Independence and Security Act of 2007. The realization of these targets in the Mountain West will not happen in the short term. Certainly, such a successful enterprise in the Mountain West would create new business and thousands of new jobs.

Biofuel crops may require a small amount of supplemental irrigation to ensure their economic viability [129]. Nevertheless, some dedicated herbaceous energy crop species, such as native and naturalized grasses, may have higher water-use efficiencies and be more heat and drought tolerant than annual row crops. Furthermore, in some cases, the use of municipal, industrial, or gray water may be

available for irrigating biofuel crops and would not compete with freshwater sources.

Production of perennial grasses for biomass would create opportunities that are environmentally beneficial. Dedicated biofuel crops are not likely to have adverse impacts on water quality because the use of pesticides and fertilizers is limited. It is possible that the production of dedicated biofuel crops could actually improve water quality under the proper crop management production system. For example, in western Colorado, the production of low-input biomass would reduce irrigation applications and thus reduce salt and selenium loading into the Colorado River and could improve water quality for downstream users in California and other western states.

Because of their deep systems and year-round cover, herbaceous perennial energy crops have the potential to reduce soil erosion rates, sequester and enhance soil organic carbon, and increase soil fertility over time compared to annual corn grain production. For example, soil erosion when growing switchgrass was approximately 30 times lower during the first year, and in the second and third years, soil erosion was 600 times lower compared to soil erosion that typically occurs in annual crops [130].

Conclusion

Herbaceous perennial grasses as lignocellulosic resources are a preferred feedstock source for biofuels because they have a neutral carbon budget, require few agronomic inputs, can be readily managed to be environmentally friendly, and have the potential to be grown on a variety of lands, soils, and crop production situations. Large regions of the Mountain West are dominated by cool-season grasses. These cool-season perennial grasses could be a desirable source for biofuel production because they can be grown on marginal lands with low water and fertilizer requirements and on such land that does not otherwise compete with food/feed crops. Basin wildrye, basin x creeping wildrye hybrids, intermediate wheatgrass, and tall wheatgrass are considered to be viable candidates for lignocellulosic biomass production.

Agronomic production of perennial grasses for biomass to date has largely been at the pilot scale in many areas of the country. Crops and cropping systems needed to produce low-input herbaceous perennial crops to support a bioenergy economy in the Mountain West are essentially unknown. Identifying sufficient land area within cost-effective distances to support year-round operation of a biorefinery is a significant barrier to commercialization in many areas of the country including the Mountain West. A variety of potential types of land that could be used for biomass production are possible, but identifying and quantifying such land that is suitable and available for biomass production will be challenging.

Stable energy prices are a critical component for maintaining a stable macroeconomy, which presents both challenges and opportunities for developing

new energy sources. A viable market requires both product demand and a reliable supply chain. Steps towards achieving this goal include quantifying biomass production costs and developing approaches to improve these agronomic costs.

Policymakers should consider instituting policies that encourage supply side contracts for locally produced energy sources, in order to encourage local economic development and to diversify energy resources. Policies are already in place that target biofuel production, which provides a critical link between agriculture and energy. Agricultural production is the economic and cultural lifeblood of many western rural communities [131]. In the event of a fuel supply disruption, it would be important to these rural communities and the agricultural supply chain to ensure that agricultural production continues. It is once again essential to emphasize that successful integration of perennial grasses would only eliminate a small fraction of the need for energy sources. Small fractions can quickly add up to significant sums if other biofuels options are implemented elsewhere.

Developing a locally grown biomass and biofuel products could provide economic diversification to rural communities in the Mountain West. Establishing a regional supply chain for biofuel production could diversify fuel sources, thereby providing a degree of energy security against price increases or shortages. The commercial production of cool-season perennial grass species as found in basin wildrye, basin x creeping wildrye hybrids, intermediate wheatgrass, and tall wheatgrass for lignocellulosic biomass production in the Mountain West will require considerable genetic improvement to develop these plant species for suitable biomass production. Since the 1990s, there has been a constant decline in range grass breeding programs in the USA due to reduced budgets and other program changes. Current range grass breeding programs have emphasized forage quality over yield in more recent intermediate wheatgrass cultivars as well as emphasis on developing plant materials that establish and are more persistent on dry, harsh disturbed rangelands capable of competing against invasive annual grasses, thus reducing the frequency and magnitude of wildfires and maintaining our natural resources. Numerous other aspects of the supply chain and conversion processes appropriate for the Mountain West will also require research and development efforts.

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Chapter 7
Canola, Rapeseed, and Mustard: For Biofuels and Bioproducts

Peter B.E. McVetty and Robert W. Duncan

Abstract *Brassica* species have been used as sources for edible and nonedible oil for thousands of years. These species include *Brassica carinata*, *B. juncea*, *B. napus*, *B. nigra*, and *B. rapa*. Currently, canola, rapeseed, and mustard oilseed species are the third largest source of vegetable oil globally. Production of *B. juncea* and *B. rapa* occurs in warmer semitropical regions of the world, while *B. napus* is produced in cooler temperate regions. Most of the canola/rapeseed/mustard breeding achievements have been associated with oil and/or meal quality, including the development of low erucic acid rapeseed *B. napus*, high erucic acid rapeseed in *B. napus*, and high oleic, low linolenic *B. napus*. A reduction in glucosinolates in *B. napus* and *B. rapa* occurred almost simultaneously to the reduction of erucic acid to create double low rapeseed, renamed canola in Canada. Significant breeding effort is directed to increasing seed yield; enhancing yield stability; improving seed quality, oil quality, and meal quality; as well as herbicide tolerance and resistance to abiotic and biotic stress. Breeding strategies now focus on doubled haploid line development and hybrid development because of significant heterosis for seed yield. Pollination control systems for hybrid development include cytoplasmic male sterility, genetic male sterility, nuclear male sterility, and self-incompatibility. Genomic strategies and marker-assisted selection have been successfully incorporated into *Brassica* breeding. *Brassica* oils compete with fossil oils in the biofuel and bioproduct markets, and further *Brassica* breeding advancements are required to meet the quantities and quality necessary for successful biofuel/bioproduct production.

Keywords Canola • Rapeseed • Mustard • *Brassica napus* • *Brassica rapa* • *Brassica juncea* • Oil quality • Erucic acid • Hybrid

Abbreviations

CGIAR	Consultative Group on International Agricultural Research
CMS	Cytoplasmic male sterility

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GMS	Genetic male sterility
HEAR	High erucic acid rapeseed
HOLL	High oleic, low linolenic
IPGRI	International Plant Genetic Resources Institute
LEAR	Low erucic acid rapeseed
MSL	Male sterile Lembke
NMS	Nuclear male sterility
QTL	Quantitative trait loci
SHEAR	Super high erucic acid rapeseed
SI	Self-incompatibility
SRAP	Sequence-related amplified polymorphisms

Introduction

Oilseed crops have been grown for thousands of years as sources of edible and nonedible (industrial) oils for a wide range of end uses including fuels and bioproducts. Rapeseed was used in India as a source of vegetable oil for edible and nonedible oil purposes as early as 4000 BC [1]. Rapeseed oils were initially used for lighting in oil burning lamps, religious ceremonies, medicinal purposes, cooking and frying foods, and food seasoning [2]. Interest in *Brassica* oilseed crop species for edible and industrial oils has been due to their high oil content and high-protein meal left over after oil extraction [3], because these oilseed species are adapted for production in temperate climatic zones and able to germinate and grow at low temperatures [4, 5]. Rapeseed/mustard species have moderate to high erucic acid ($22{:}1^{cis\Delta 13}$) content in the oil that provides oil characteristics that make it an excellent lubricant for steam and water-washed surfaces [3]. However, there were numerous reports from the 1960s that rapeseed/mustard oil depressed growth in rats [6] and a report in 1970 that the erucic acid content in rapeseed/mustard oil caused heart health problems in rats [7]. Rapeseed/mustard meal naturally contains high levels of glucosinolates, plant defense-related secondary metabolic compounds [8]. Glucosinolates provide the sharp tasting effect when rapeseed/mustard seed/meal is eaten. The breakdown products of glucosinolates adversely affect iodine uptake by the thyroid gland, which can reduce weight gain in animals fed such meal [9].

To improve oil and meal quality and enhance the value of the crop, double low rapeseed (low erucic acid content in the oil and low glucosinolate content in the meal) varieties in *B. napus* oilseed rape and *B. rapa* turnip rape were developed in Canada in the 1970s [10]. These double low rapeseed varieties were renamed "canola" circa 1980. More recently, double low *B. juncea* mustard varieties have been developed in Canada [11]. These new double low mustard varieties are known as canola *juncea* varieties in Canada.

Many different fatty acid profiles in canola/rapeseed/mustard for particular end uses have been developed or are under development [12]. Canola oil has been used

for biofuel production and for dielectric fluids in electrical transformers, while high oleic acid canola oil is being developed for use in hydraulic equipment. Rapeseed oil has been used as a lubricant, and high erucic acid rapeseed oil has been used as a lubricant and for select chemical feedstock applications [13]. Recently, medium erucic acid mustard oil and canola *juncea* oil have been deemed suitable for biofuel production [14, 15].

Canola/rapeseed/mustard oilseed species are currently the third largest source of vegetable oil globally after palm and soybean [16]. Considerable research and development effort throughout the world to develop new cultivars of canola/rapeseed/mustard for biofuel and bioproducts is occurring currently. The research and development focus in canola/rapeseed/mustard is on hybrid development, agronomic performance, seed quality, disease resistance, and novel traits to provide advantages for these oilseed crops in an intensely competitive global oilseed market.

Taxonomy and Domestication

The *Brassica* plant genus belongs to the Brassicaceae family (formerly known as the Crucifer family), which is a large family of substantial economic importance throughout the world [17]. The Brassicaceae family contains 338 genera and over 3,700 cultivated or wild species [18]. This family is characterized by plants that have conduplicate cotyledons and/or two-segmented siliques which contain seeds in one or both chambers and only have simple hairs, if present [19].

Several species within the *Brassica* genus including *B. carinata* (Abyssinian mustard), *B. juncea* (Indian mustard) and *B. napus* (oilseed rape), *B. nigra* (black mustard), and *B. rapa* (turnip rape) are grown as oilseed crops. *Brassica napus* and *B. rapa* species are classified as "rapeseed," while *B. carinata*, *B. juncea*, and *B. nigra* are classified as "mustard." While *B. nigra* was grown as an oilseed in the past, it is currently grown exclusively as a condiment crop [20]. A closely related species, *B. oleracea* (cabbage, kale, and cauliflower) is primarily a biennial plant grown as a vegetable, not as an oilseed. All of these *Brassica* species naturally produce long-chain fatty acids in their seed oil and defense-related secondary metabolites called glucosinolates found throughout the entire plant [2].

Of these six *Brassica* species, three are diploids (*B. nigra*, *B. oleracea*, and *B. rapa*), and three are amphidiploids (*B. carinata*, *B. juncea* and *B. napus*), which combine the chromosome sets of the diploid species. The relationship among these six *Brassica* species was first outlined by U in 1935 [21, 22] (Fig. 7.1). U's triangle describing the genomic relationship of these six *Brassica* species has greatly facilitated interspecific breeding activities within the *Brassica* genus [23].

The three diploid *Brassica* species *B. nigra*, *B. rapa*, and *B. oleracea* are thought to have arisen approximately 4–8 million years ago, while the three amphidiploid species are very young in evolutionary terms, arising spontaneously in the last

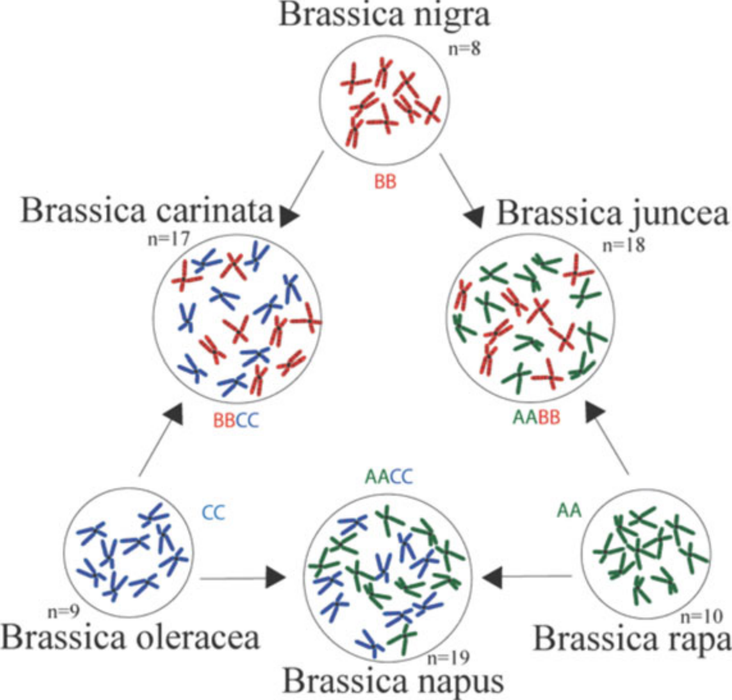

Fig. 7.1 Triangle of U (1935) (Reprinted from Triangle of U Simple1. Wikipedia. http://en.wikipedia.org/wiki/File:Triangle_of_U_Simple1.PNG. Last accessed on February 26, 2013. With permission from Creative Commons License)

10,000 years or less in areas where the natural range of distribution for the parent species overlapped in the wild or agricultural settings [24, 25].

Studies of cytoplasmic genetic diversity indicate that the amphidiploid cultivated species originated several times from independent interspecific crosses [26, 27]. Further, the cytoplasmic diversity studies suggest that the interspecific hybridization events giving rise to the amphidiploids occurred mostly unidirectionally [27]. *Brassica juncea* has the cytoplasm of *B. rapa*, while *B. carinata* has the cytoplasm of *B. nigra* [28]. *Brassica napus* has a cytoplasm which is most similar to that found in *B. oleracea* ssp. r*obertiana*, a wild species of *B. oleracea* found in the Mediterranean region [27].

The evolutionary origins of *Brassica* species are complex and uncertain. Some cytological studies of *Brassica* species suggest a common ancestor with a base chromosome number of six once existed [29, 30]. Other cytological and/or molecular studies of *Brassica* species suggest an ancestor with a base chromosome number of seven or eight [31, 32]. More recent molecular studies suggest that the *Brassica* species may share a common ancestor with the weedy species *Arabidopsis thaliana* (L.) Heynh. ($n = 5$) and that several chromosome rearrangements and ploidy level changes have occurred during the evolution of the *Brassica* species

[23, 33–35]. There is tremendous variability in *Brassica* genomes, and some plant scientists have speculated that this made *Brassica* unusually responsive to selection, a reason for their widespread adoption and success as oilseed and vegetable crops [25].

Plants of the Brassicaceae family were among the first to be domesticated by man, with initial domestication occurring several thousand years ago. The three diploid *Brassica* species *B. nigra*, *B. rapa*, and *B. oleracea* were the first to be domesticated, and these species have been cultivated for a very long time. The amphidiploid species were domesticated later but probably still very early in mankind's agrarian evolution [36]. Rapeseed (likely *B. rapa*) was cultivated in India in 4000 BC [37, 38]; in China, Japan, Greece, and Italy 2,000–2,500 years ago [16, 23, 39]; in Europe 800 years ago [23]; and in North America 60 years ago [37].

Centers of origin provide useful information regarding probable areas of initial domestication. *Brassica rapa* is thought to have a primary center of origin in the Indian subcontinent with secondary centers of origin in Europe, the Mediterranean area, and in Asia [36]. *Brassica oleracea* and *B. napus* are presumed to have originated in the Mediterranean area [36]. *Brassica nigra* and *B. juncea* species are thought to have originated in the Middle East [40], while *B. carinata* is presumed to have originated in northeast Africa [41]. All of the *Brassica* species of the U triangle have spring habit forms. *Brassica oleracea* is primarily a biennial, but *B. oleracea* var. *alboglabra* is a spring habit variant in this species. In addition, *B. napus* and *B. rapa* species have winter-annual habit types [42]. The winter habit types of *B. napus* and *B. rapa* species are more productive than the spring forms; however, they are less winter hardy than winter cereals [41].

Brassica rapa appears to be the most widely distributed oilseed *Brassica* species. At least 2,000 years ago, it was distributed from the Atlantic islands in the west to the eastern shore of China and Korea and from northern Norway to the Sahara and northern India [36].

Canola/Rapeseed/Mustard Production

Different *Brassica* oilseed species are adapted to, and grown in, different regions of the world. *Brassica juncea* and *B. rapa* are the predominate species in the warmer semitropical regions of the world, while in cooler temperate regions, *B. napus* and *B. rapa* predominate. *Brassica carinata* is limited to Ethiopia and northeast Africa [43], while *B. nigra* is grown in Europe and Asia [44]. *Brassica oleracea* is exclusively a vegetable crop produced globally [20]. *Brassica juncea* is an important oilseed species in India [45, 46], Pakistan, and China [43] and is an important condiment crop in Canada [43], which has recently been converted to a new Canadian double low edible oilseed crop called canola *juncea* [11]. *Brassica napus* is the most important oilseed species in Australia, Europe, Canada, and China, while *B. rapa* is the predominate species in India and northwest China [16, 38]. Winter habit *B. napus* types are grown in southern Europe and China,

while spring habit *B. napus* and *B. rapa* types are grown in northern Europe, Australia, Canada, India, and northwest China [16]. *Brassica carinata* is an important oilseed in Ethiopia and east Africa [47, 48].

Canola and high erucic acid, low glucosinolate rapeseed (HEAR) cultivars are grown in both Europe and Canada. Spring habit *B. rapa* canola/rapeseed cultivars previously grown in Canada have progressively been replaced by *B. napus* cultivars in the last two decades [43]. Spring habit *B. napus* canola/HEAR cultivars are grown in the Canadian Prairie provinces and northern Europe, while winter habit *B. napus* canola/HEAR cultivars are grown primarily in Europe [41]. Canada grows *B. juncea* as a condiment crop, while canola *juncea* (canola-quality mustard) has been developed and is being grown as an oilseed crop in Canada [11]. Australia also grows spring *B. napus* canola cultivars over their fall and winter period [49]. Spring and semi-winter habit *B. napus* canola/rapeseed is grown in China. Spring habit *B. rapa* canola/rapeseed and *B. juncea* mustard as an oilseed crop are grown on the Indian subcontinent. *Brassica carinata* mustard as an oilseed crop is grown on the highlands of Ethiopia and is starting to be grown in Canada. *Brassica napus* canola/rapeseed is the predominant *Brassica* oilseed species globally, and it produces the highest seed yields of all the species. Seed yields for spring habit *B. napus* canola/rapeseed types are approximately 1.0–1.5 tons/ha, while seed yields for winter habit *B. napus* canola/rapeseed types are approximately 2.5–3.0 tons/ha [3].

Canola/rapeseed/mustard is the world's third largest source of vegetable oil (22.7 M tons), behind only palm (43.6 M tons) and soybean (39.8 M tons) [50]. The top ten canola/rapeseed/mustard seed-producing countries in 2011 (production in millions of metric tons: mmt) were: Canada (14.1 mmt), China (13.4 mmt), India (8.1 mmt), France (5.3 mmt), Germany (3.8 mmt), the United Kingdom (2.7 mmt), Australia (2.3 mmt), Poland (1.8 mmt), Ukraine (1.4 mmt), and the Czech Republic (1.0 mmt) [50]. The ranking of the top five canola-/rapeseed-/mustard-producing countries has remained similar for the last 30 years, with Canada overtaking China in 2011 [50]. There has been change in the rank for the next five canola-/rapeseed-/mustard-producing countries over the last 30 years with Australia joining the top ten circa 1995. The United States joined the top ten in 2010 but dropped to twelfth in 2011. Total world production of canola/rapeseed/mustard has increased steadily over the last 30 years, with production of: 10.7 mmt in 1980, 19.3 mmt in 1985, 24.4 mmt in 1990, 34.2 mmt in 1995, 39.5 mmt in 2000, 49.9 mmt in 2005, and 59.0 mmt in 2010 [50]. This sixfold increase in global canola/rapeseed/mustard production in the last 30 years has been in response to increased market demand for canola/rapeseed/mustard oils for edible, industrial, and biofuel uses. Further increases in global canola/rapeseed production are anticipated as demand for all vegetable oils is steadily increasing.

Genetic Resources

The genetic diversity in the Brassicaceae family is very large. Crosses between a large number of wild and cultivated *Brassica* species are possible despite large differences in chromosome numbers. A comprehensive listing of the numerous crosses possible within the Brassicaceae has been reported by [51]. Interspecific crosses in the Brassicaceae are most frequently made among species in the U triangle (Fig. 7.1). Crosses between each amphidiploid species and their two respective diploid parents as well as between amphidiploids having a parental genome in common are readily possible [41]. In addition, intergeneric crosses between *Diplotaxis*, *Eruca*, *Raphanus*, and *Sinapis* genera to one or more of the diploid *Brassica* species in the U triangle are possible [41]. As a general rule, the interspecific crosses are more successful if the amphidiploid parent in the cross is used as the female parent [46]. Crosses between the diploid species of the U triangle are possible, but these have a low success rate [52]. Crosses of the diploid species which originally created the amphidiploid species have been successfully repeated by researchers to create resynthesized hybrids for *B. carinata*, *B. napus*, and *B. juncea* [41]. These resynthesized hybrids display poor agronomic performance, poor seed quality, moderate to high erucic acid content, and high glucosinolate content, rendering them of little direct use; however, they have been used as bridging species for transferring desirable genes or traits from the diploid parent species into adapted amphidiploid cultivars [53]. Resynthesis of the amphidiploid species to enhance the genetic variability of *B. napus*, *B. carinata*, and *B. juncea* also has been and continues to be a frequently used approach in amphidiploid species breeding [43, 46].

While a huge number of interspecific crosses within *Brassica* can be successfully undertaken, few are actually attempted. Some *Brassica* researchers have indicated that the available natural variation in *Brassica* oilseeds has not yet been fully assessed or exploited with few exceptions and suggest that systematic searches will identify the needed traits within the six *Brassica* species in the U triangle [46]. Regardless of the reasons, most canola/rapeseed/mustard breeding programs tend to stay within an even more restricted gene pool of adapted *Brassica* material as genetic resources [54, 55].

There are large public gene bank collections of *Brassica* species cultivars/lines located in the United Kingdom, Germany, the United States, India, and China [16, 56]. Bioversity International, formerly International Plant Genetic Resources Institute (IPGRI), a member of the Consultative Group on International Agricultural Research (CGIAR) consortium, collects and preserves *Brassica* species accessions and maintains a Germplasm Database website (ecpgr.cgiar.org/germplasm_databases.html) [57] that provides information on accessions of *Brassica* species cultivars/lines stored in gene bank collections globally. There are Brassicaceae collections held at the Universidad Politécnica de Madrid, Spain [58]; the Tohoku University, Japan; the Nordic Gene Bank in Sweden; and the Australian Temperate Field Crops Collection in Australia [16].

Major Breeding Achievements

There have been numerous major breeding achievements in canola/rapeseed/mustard in the last 40 years. Canadian canola/rapeseed/mustard breeders have been the originators for many of these major breeding achievements. Canadian *Brassica* breeders develop short-season spring habit canola/rapeseed/mustard crops that can complete three complete growth cycles per year in the greenhouse. Canadian canola/rapeseed/mustard breeders initially focused their attention on quality improvements in *Brassica* crops to create high-quality, high-value crops for export into competitive world markets. Many of the canola/rapeseed/mustard major breeding achievements have been associated with oil and/or meal quality [43]. Major breeding achievements in oil quality improvement include the development of low erucic acid rapeseed (LEAR) *B. napus* and *B. rapa* varieties [10]. Low erucic acid mustard (*B. juncea*) has also been developed in Australia [59]. Other oil quality improvements include high oleic, low linolenic (HOLL) *B. napus* and high erucic acid rapeseed in *B. napus* [43]. Meal quality improvement through reduction of glucosinolates in *B. napus* and *B. rapa* occurred nearly simultaneously to the reduction in erucic acid in the oil to create double low rapeseed [10]. Low erucic acid, low glucosinolate rapeseed (*B. napus* and *B. rapa*), initially called double low rapeseed, was renamed canola in Canada circa 1980 to distinguish it from rapeseed [10]. *B. napus* rapeseed was the first species to be converted to canola (1974) followed closely by *B. rapa* (1978) [10] and much later *B. juncea* (2002) [11]. These oil and meal quality improvements created high-quality edible oil and good quality meal for feed rations and are the major breeding achievements in *Brassica* oilseeds that set this crop on its path of tremendous success in the last 40 years [10].

Brassica napus canola cultivars provided the foundation for oil profile modifications to better meet end-user requirements. A major breeding achievement has been the development of high oleic acid, low linolenic acid content (HOLL) oil profiles to create high-stability oil specialty canola in 1987 [60]. Major breeding achievements in *B. napus* HEAR have also occurred where HEAR lines with 62–64 % erucic acid content have been developed [61] and where the world's first transgenic SHEAR line with 72 % erucic acid in the seed oil has been developed [62].

While canola cultivars provided a source of edible vegetable oil and an industrial source of oil for biofuels and bioproducts, a substantial market provided by rapeseed (*B. napus* and *B. rapa*) continued unabated. Major breeding achievements involved increasing erucic acid content to create high erucic acid rapeseed in *B. rapa* (1975) [10] and then combining reduced glucosinolate content in the meal with high erucic acid rapeseed in *B. napus* to create high erucic acid, low glucosinolate *B. napus* rapeseed (HEAR) in 1982 [10].

The development of herbicide tolerance systems in canola/rapeseed/mustard in *B. napus* and *B. juncea* has been a major breeding achievement beginning in the 1980s [63]. Several different herbicide tolerance systems in *B. napus* canola/

rapeseed including triazine tolerance, glufosinate tolerance, glyphosate tolerance, and imidazolinone tolerance in *B. napus* canola/rapeseed as well as imidazolinone tolerance in *B. juncea* canola have been developed and commercialized in Canada [63]. Triazine-tolerant and glyphosate-tolerant *B. napus* canola cultivars have also been developed and commercialized in Australia [64]. Australia also developed and grows imidazolinone-tolerant canola and, more recently, Roundup Ready canola in some Australian states [65]. Biotechnology created herbicide-tolerant canola occupied 26 % of global canola production area (8.2 m ha of 31 m ha total) in 2011 [66].

The development of synthetics, varietal associations, and hybrids in *B. napus* using genetic male sterility systems, nuclear male sterility systems, and cytoplasmic male sterility systems is a more recent major breeding achievement which significantly increased seed yield in *B. napus* canola/rapeseed grown in Canada starting in 1989 and elsewhere in the world shortly thereafter [1, 43]. The development of hybrids in *B. juncea* has also been successful in India [67].

Target Traits and Current Breeding Goals

There are several common target traits and current breeding goals applicable to all *Brassica* oilseeds and all areas of world production. These include increased seed yield; enhanced yield stability; adaptation to local environments; improved seed quality, oil quality, and meal quality; herbicide tolerance; as well as improved abiotic and biotic stress (disease and insect) resistance [1, 16, 43, 46]. Because of variation in the current state of development of different *Brassica* oilseed species, different growing conditions throughout the world, different weed spectrums, varying abiotic and ever-changing biotic threats to production, constantly evolving market requirements and opportunities, and differing resources available to different *Brassica* breeding programs, the relative importance of these breeding targets is oilseed species, location, and time specific [43]. Increasing seed yield in *B. juncea* and *B. rapa* was a primary breeding target in India; however, there was shift in the early 1990s to equal emphasis on improving seed yield and seed quality [46, 68]. In contrast, in the western world, breeding for seed, oil, and meal quality were primary target traits in *B. juncea*, *B. napus*, and *B. rapa* starting in the 1970s [16, 69]. The emphasis in breeding targets in the western world has now shifted to improving seed yield and yield stability while maintaining seed quality [43]. For *B. carinata*, canola quality is a target trait for crop researchers in Spain, while increasing erucic acid content is a target for Canadian researchers [43]. While breeding for biotic stress resistance such as disease resistance is a common target, the important diseases vary in different parts of the world, with blackleg a major disease in Australia, Canada, and Europe, while sclerotinia stem rot is the major disease in China [46]. Similarly, breeding for abiotic stress tolerance is a common target; however, important stresses range from frost in Canada, northern Europe, and the highlands of China to drought, heat, and salt in India [46].

Increased seed yield in *Brassica* oilseeds through the exploitation of heterosis has become a major breeding target globally in recent years since reports of heterosis for seed yield in *B. rapa*, *B. juncea*, *B. napus*, and *B. carinata* have appeared in the literature [43]. *Brassica* breeders are attempting to reach the yield stability target through the development of hybrid canola/rapeseed/mustard cultivars [43] because hybrid cultivars are thought to contribute to yield stability since they are usually more abiotic and biotic stress tolerant [70].

Brassica breeders have focused on early maturity in spring habit canola/rapeseed/mustard and have focused on winter hardiness in winter habit *B. napus* canola [1, 46] to achieve their adaptation to local environments. In short-season growing areas such as Canada or northern Europe, early maturity is required to avoid frost damage, while in longer-season growing areas, early maturity is required to permit annual multi-cropping [46].

Improved seed quality as a breeding target globally involves increasing oil content and protein content while reducing fiber content [43]. Oil quality improvement targets involve modifying oil profile for specific end-use markets. Reduced erucic acid content for edible oil, biofuels, and bioproducts; increased erucic acid content for industrial oil; and reduced linolenic acid and increased oleic acid content for improved oil stability are common breeding targets throughout the world [1, 43, 69]. High lauric acid canola was developed as a substitute for palm oil [71]. The development of very high oleic acid canola (80–90 % oleic acid) is a target since high oleic acid levels are required for industrial applications because oleic acid is a chemical feedstock for further chemical synthesis [72]. The development of super high erucic acid *B. napus* rapeseed with erucic acid levels over 66 % has been a breeding target for 20 years [1]. Oil profile modifications are a target in *B. juncea* where several fatty acid profile modifications have been developed including reduced linolenic acid types as well as mid and high oleic types [73].

Meal quality improvement targets include increasing protein content and reducing fiber, sinapine, and phytic acid in *B. napus*, *B. rapa*, and *B. juncea* [41]. The development of yellow-seeded *B. napus* cultivars (which would increase protein content and reduce fiber content in the meal simultaneously) is a major seed quality improvement target globally [1].

The development of herbicide-tolerant canola *B. juncea* and *B. napus* has been a major breeding target in Canada and Australia since the 1980s [63]. Canada was the first canola-/rapeseed-producing country to begin commercial production of herbicide-tolerant canola/rapeseed/mustard [63].

Resistance to diseases and pests in canola/rapeseed/mustard is an important breeding target globally, with different diseases and pests of importance in different production areas of the world [1, 46].

Abiotic stress resistance is also an important breeding target that involves improving the frost, heat, drought, and salt tolerance of *Brassica* oilseeds [16].

Breeding Strategies and Integration of New Biotechnologies

Breeding strategies depend on the pollination behavior of the crop under development. The diploid species in the triangle of U *Brassica* species are generally self-incompatible obligate outcrossing species although there are exceptions such as *B. rapa* yellow sarson and *B. oleracea alboglabra* [41]. In contrast, the amphidiploid species of the U triangle are self-compatible species. For the self-incompatible *B. rapa* types, mass selection and recurrent selection are the main breeding strategies [10, 46]. For the facultatively outcrossing but predominately self-pollinating *B. rapa* types, *B. carinata*, *B. juncea*, and *B. napus*, a wide range of breeding techniques appropriate to self-pollinating crops have been successfully used including single plant selection, self-pollination and pedigree selection, backcross breeding, doubled haploid line development, and more recently the development of hybrids [1, 46].

The breeding strategy for oil quality improvement in *B. napus* and *B. rapa* involved identification of naturally occurring mutations for low erucic acid in the oil of the forage *B. napus* cultivar "Liho" followed by crosses to *B. napus* and *B. rapa* rapeseed to produce low erucic acid rapeseed in the 1960s [10].

Meal quality improvement in *B. napus* and *B. rapa* involved identification of naturally occurring mutations for low glucosinolates in the meal of the *B. napus* cultivar "Bronowski" followed by crosses to *B. napus* and *B. rapa* rapeseed to produce low glucosinolate rapeseed in the 1970s [10].

The breeding strategy to develop low erucic acid, low glucosinolate rapeseed used crosses involving Liho and Bronowski with adapted double high rapeseed cultivars produced the world's first *B. napus* canola cultivar in 1974 [10]. Crosses between *B. rapa* and *B. napus* were used to produce the world's first canola *B. rapa* cultivar in 1978 [41].

Specialty canola cultivars in *B. napus*, with reduced levels of linolenic acid and increased levels of oleic acid, the so-called high-stability oil canola cultivars, were developed using crosses and backcrosses of Regent with the M11 low linolenic acid mutant line [74] with the world's first high-stability oil canola cultivar released in 1987 [10].

The breeding strategy to develop high erucic acid, low glucosinolate *B. napus* HEAR cultivars used a cross of a high erucic acid Swedish rapeseed strain to low glucosinolate progeny from the Bronowski x Target cross [10].

To develop super high erucic acid rapeseed, SHEAR, with erucic acid content over 66 %, two breeding strategies are being used. In the first, microspore mutagenesis of resynthesized *B. napus* lines to create doubled haploid lines with homozygous mutations for fatty acid profile variations, including possible SHEAR genotypes, is being used [61]. In the second, transformation technology is being used to introduce transgenes which control erucic acid pool size and triglyceride formation into HEAR and resynthesized HEAR lines [61, 75]. Recently, development of the world's first transgenic SHEAR line with 72 % erucic acid in

the seed oil has been reported by [62]. This indicates that SHEAR development from HEAR is possible using transgenic technologies.

The breeding strategy to develop canola-quality *B. juncea* (canola-quality mustard) has involved identification of natural mutant zero erucic acid mustard lines [59] and interspecific cross-derived low glucosinolate mustard lines [76]. Crosses of the low erucic acid lines with the low glucosinolate lines were used to develop canola *B. juncea* [11].

Disease resistance especially to blackleg was greatly improved in Canadian canola/rapeseed cultivars using European and Australian sources of blackleg resistance within *B. napus* initially, but later using *B. juncea* as a blackleg resistance gene source [77] and wild *B. rapa* subsp. *sylvestris* as a blackleg resistance gene source [78].

Several different breeding strategies have been used to develop herbicide-tolerant canola/rapeseed/mustard in Canada. Triazine tolerance, the first herbicide tolerance to be developed in Canada, was transferred from naturally occurring mutant *B. rapa* plants to *B. napus* using a backcross approach to insert the *B. napus* nucleus into the *B. rapa* cytoplasm [79].

Glufosinate-tolerant *B. napus* was created using transformation technology to add a gene for an enzyme from a soil actinomycete which detoxified glufosinate to *B. napus* canola [80]. Glufosinate tolerance is also part of the genetically engineered nuclear male sterility (NMS) system created by Bayer CropScience [81], which is used to produce InVigor hybrid canola cultivars [43].

Glyphosate-tolerant *B. napus* was created using transformation technology to add two genes, one gene coding for a glyphosate-insensitive target enzyme and another to detoxify glyphosate to *B. napus* canola [82]. Glyphosate tolerance is currently based on the RT73 transgenic construct; however, new canola cultivars with a new transgenic construct, MON88302, conferring enhanced tolerance to glyphosate are currently under development [83].

Imidazolinone-tolerant *B. napus* was created using mutagenic treatment of two genes involved in amino acid biosynthesis to produce enzymes that are no longer targets for imidazolinone herbicides [84]. The use of microspore mutagenesis combined with doubled haploid line development greatly improved the efficiency of development of imidazolinone-tolerant *B. napus* [85]. Herbicide resistance to imidazolinone herbicides in *B. juncea* has also been developed [84] and patented [86].

The breeding strategy to develop pod shattering-resistant *B. napus* canola/rapeseed uses interspecific crosses of *B. rapa* or *B. juncea* to *B. napus* since both *B. rapa* and *B. juncea* are pod shatter-tolerant species [43, 87].

Breeding strategies to improve yield by up to 3.6 % per year (equivalent to a doubling of seed yield in 20 years) and yield stability have been species specific. For *B. rapa*, mass selection and recurrent selection have been used to produce improved population cultivars of self-incompatible *B. rapa* [10]. For *B. napus* canola/rapeseed cultivars, breeding for improved seed yield has involved successively selection within open-pollinated population landraces and crosses of open-pollinated populations and pedigree selection of derived families, the development

and selection of pure breeding doubled haploid lines, and the development of synthetics, varietal associations, and finally hybrids [1].

For *B. juncea* canola/mustard oilseed cultivars, breeding for improved seed yield has involved successively selection within open-pollinated populations and crosses of open-pollinated populations and pedigree selection of derived families and the development and selection of pure breeding doubled haploid lines and more recently hybrids [43, 46, 67].

For *B. carinata* mustard cultivars, breeding for improved seed yield has involved successively selection within open-pollinated population landraces and crosses of open-pollinated populations and pedigree selection of derived families [88].

A breeding strategy focusing on hybrids has developed in four *Brassica* oilseed species because of reports of significant heterosis for seed yield. This strategy has been coupled with a pollination control system development strategy to facilitate the production of large quantities of hybrid seed for commercial use.

Reports of heterosis for seed yield in *B. rapa* [89–92] created interest in the development of hybrid *B. rapa* cultivars using either self-incompatibility [46] or cytoplasmic male sterility [16] pollination control systems.

Reports of heterosis for seed yield in *B. napus* [93–97] created interest in the development of hybrid *B. napus* cultivars using genetic male sterility, self -incompatibility, or cytoplasmic male sterility [10]. Pollination control system development in *B. napus* including cytoplasmic male sterility (CMS) systems [98], genetic male sterility (GMS) systems [99], and self-incompatibility (SI) systems [100] has been conducted for several decades. The *nap* CMS [101], *pol* CMS [102], *mur* CMS [103], *ogu* CMS [104], and *ogu* INRA CMS [105] systems have been developed for use in hybrid canola/HEAR seed production. Initially, absence of a restorer gene for the *ogu* INRA CMS system resulted in the development of non-restored hybrid canola cultivars (known as varietal associations) in Europe [2]. Varietal associations had widely varying seed yields correlated to the extent of insect cross-pollination occurring in the field [2]. The development of restorer lines for the *ogu* INRA CMS system produced a fully functional CMS system [106–108]. Genetic male sterility (GMS) systems have also been developed and evaluated for use in hybrid canola/HEAR cultivar development [99]. The male sterile Lembke (MSL) GMS pollination control system is widely used to produce hybrid canola/HEAR cultivars globally. The development of a genetic male sterility system based on recombinant DNA technology created a successful pollination control system in *B. napus* based on barstar and barnase genes, both linked to broad-spectrum novel herbicide tolerance [81]. This nuclear male sterility (NMS) pollination control system is being used to produce very successful hybrid canola cultivars globally. *B. napus* canola hybrids are being developed in Canada and Europe using male sterile Lembke (MSL) GMS, Bayer CropScience nuclear male sterility (NMS), and *ogu* INRA CMS pollination control systems [81, 98]. A current breeding objective in hybrid *B. napus* breeding is the expansion of the genetic variability in the spring canola gene pool to maximize heterosis for yield in hybrids [109]. In addition to the standard crosses using spring habit *B. napus*, spring habit-

by-winter habit canola crosses are also being used to expand genetic variability [43].

Reports of heterosis for seed yield in *B. juncea* [110–113] created interest in the development of hybrid *B. juncea* cultivars using cytoplasmic male sterility [16]. Several cytoplasmic male sterility (CMS) pollination control systems in *B. juncea* have been under development for several years [16, 67]. Most of these CMS systems lack functional restorer lines so are not useable for hybrid *B. juncea* cultivar production. However, Sodhi et al. [114] reported that a new CMS system in *B. juncea*, the 126-1 CMS system, has been developed and successfully used to produce a mustard hybrid in India.

A report of heterosis for seed yield in *B. carinata* [115] provides *Brassica* breeders with the development of hybrids as an obvious seed yield improvement breeding strategy. However, there are no reports of pollination control system development in *B. carinata* in the literature currently.

Breeding strategies also depend on the availability of tissue culture techniques for DH line development, for microspore mutagenesis, and for plant transformation technologies. Doubled haploid line development techniques have been established for *B. rapa* [116–119], *B. napus* [116, 118–120], *B. carinata* [118, 119, 121], and *B. juncea* [122]. With the successful adoption of microspore-based doubled haploid line development techniques [123, 124], a number of canola cultivars in Canada were based on DH lines. Doubled haploid lines now provide the parents for *B. napus* hybrids. Transformation technologies provided the tools needed to develop glufosinate and glyphosate herbicide-tolerant canola cultivars. Finally, transformation technologies applied to fatty acid biosynthesis in *B. napus* led to the development of high lauric acid specialty oil profile canola cultivars [1].

Genomics is making contributions to *Brassica* oilseed development through the development of genetic maps, the identification of quantitative trait loci (QTL) for polygenic traits, and the identification of molecular markers for Mendelian traits and through map-based gene cloning. Recently developed high throughput genome sequencing may provide additional tools for *Brassica* breeders to assess whole genome genetic variability.

Numerous maps of the *Brassica* oilseed species have been developed in *B. rapa* [125], *B. napus* [126, 127], *B. juncea* [128], and *B. carinata* [129]. These maps provide a foundation for all subsequent genomic research.

Molecular markers are being developed and widely used in canola/HEAR breeding programs. A number of genetic maps have been developed in *B. napus* starting with an RFLP map [130]. Recently, an ultradense sequence-related amplified polymorphism (SRAP) [131] genetic recombination map for *B. napus* has been developed [127]. This map has great potential for map-based cloning and within-gene molecular marker development. The high-density genetic maps developed for each *Brassica* oilseed species facilitate positional cloning of selected genes, for example, *Rfp*, the nuclear restorer gene for the *pol* CMS system in *B. napus* [132] or a blackleg resistance gene [133]. In addition, a review of *B. napus* mapping populations, marker systems, maps, and traits studied has been published

[134]. QTL identification for numerous traits in *Brassica* oilseeds is common, for example, QTL for oil content in *B. napus* [135].

Marker-assisted selection is being successfully used in *Brassica* oilseed breeding programs. For example, the development of genome-specific erucic acid gene molecular markers for the two erucic acid controlling genes in *B. napus* [136] has permitted the selection of homozygous canola and high erucic acid genotypes in segregating generations and backcross generation progeny of canola x HEAR crosses, greatly improving breeding efficiency for such programs.

Brassica oilseeds are highly amenable to all of the procedures required to transform plants. The disarmed *Agrobacterium tumefaciens* vector has been used routinely to move cloned genes into the chromosomes of *Brassica* oilseed species [137]. Many of the herbicide tolerance systems and the NMS pollination control system have been developed using *B. napus* transformation technology.

More recently, very-high-throughput sequencing is providing genome sequencing, genome resequencing, and targeted resequencing capabilities for all plant genomes including *Brassica* oilseeds. This technology will facilitate the complete assessment of individual *Brassica* genotypes by sequencing, an advanced form of genomics-based assisted selection.

Seed Production

Flower type, pollination type, and crop type have considerable impact on seed production procedures used in different crops. The vast majority of the *Brassica* species are perfect flowered types [18]. Further, as a general rule, for the six *Brassica* species of the U triangle, the diploid species (*B. nigra*, *B. oleracea*, and *B. rapa*) are self-incompatible obligate outcrossing species, while the amphidiploid species (*B. carinata*, *B. juncea*, and *B. napus*) are self-compatible self-pollinating species [54]. However, variations exist among plant types for pollination mode within species [138]. For example, *B. rapa* var. *sarson* (brown and yellow) and *B. oleracea* var. *alboglabra* are exceptions, since they are self-compatible diploids. Pollen transfer between plants can occur by physical contact or by insects for the self-compatible species and by physical contact, by wind, or by insects for the self-incompatible species.

There are numerous crop types in the *Brassica* oilseed species. Cultivars for the diploid species are usually genetically diverse outcross populations, while cultivars for the amphidiploid species can be pure inbred or DH lines, open-pollinated populations, synthetics, or hybrids. *B. napus* canola/rapeseed hybrid cultivars are rapidly becoming the predominant type in this species grown throughout the world.

Brassica species seed multiplication rates are very high, from 300 to 1,000: 1 [41], so a limited number of generations of seed multiplication are needed to produce the required quantities of commercial planting seed [139] for all cultivar types. The canola/rapeseed/mustard pedigreed seed generational cascade in Canada involves breeder seed, foundation seed, and certified seed [140].

Brassica species seed remains viable in the soil for many years, creating a volunteer problem for pedigreed seed production [139]. For canola/rapeseed/mustard pedigreed seed production in Canada, the use of land not previously sown to *Brassica* species for a minimum of a 3–5-year break between a previous *Brassica* crop and the pedigreed seed production year is required [140]. Isolation distances vary from 100 m for different varieties of canola/rapeseed for open-pollinated population cultivar pedigreed seed production to 800 m for different varieties of canola/rapeseed for hybrid cultivar pedigreed seed production [140]. Similarly, isolation distances from distances vary from 200 m for different varieties of mustard for open-pollinated population cultivar pedigreed seed production to 800 m for different varieties of mustard for hybrid cultivar pedigreed seed production [140]. The canola/rapeseed/mustard pedigreed seed plots must be free of primary noxious weeds and must have not more than 1 in 10,000 plants of harmful contaminant species which include *B. napus*, *B. rapa*, *B. juncea*, *R. raphanistrum*, and *S. alba*. Canadian canola/rapeseed/mustard pedigreed seed production standards are similar to the canola/rapeseed/mustard pedigreed seed production standards used by most Organization for Economic Cooperation and Development (OECD) member countries. This facilitates the movement of pedigreed canola/rapeseed/mustard seed globally.

Pure inbred line, pure DH line, and open-pollinated population rapeseed/mustard/canola cultivar pedigreed seed lots are sown in plots that meet the above land requirements and are grown subject to all of the above requirements. The pedigreed seed production plots are bulk harvested and the pedigreed seed produced assessed for purity, germination percentage, and seed quality [140]. Hybrid rapeseed/mustard/canola cultivar pedigreed seed lots are sown in plots that meet the above land requirements and are grown subject to all of the above requirements. The management of hybrid cultivar component pedigreed seed production plots depends on the type of pollination control systems used.

If a genetic male sterility (GMS) pollination control system [99] is used to produce the hybrid seed components, the female line is homozygous recessive (ms/ms) for male sterility and the maintainer line is heterozygous for male fertility/male sterility (Ms/ms). 1:1 to 4:1 strips of the female line and maintainer line are grown with honeybees used to assist in the transfer of pollen from the maintainer to the female line. The maintainer line is removed from the plot post-flowering and the seed on the female line bulk harvested. The maintainer line and the pollen parent for hybrid seed production are male fertile and are grown in isolated fields and bulk harvested [141]. The hybrid seed is produced using strips of varying ratios (from ~1:1 to 6:1) of the female line and the pollen parent for hybrid seed production. The GMS female line segregates 1:1 for male sterility and male fertility and must be rogued prior to flowering to ensure pure hybrid seed production. Honeybee hives at ~2–4 hives per ha are used to ensure adequate pollen transfer [141]. The pollen parent for hybrid seed production is removed post-flowering. The female line is bulk harvested and the pedigreed hybrid seed produced assessed for purity, germination percentage, hybridity, and seed quality [140].

The nuclear male sterility (NMS) system [81] is a genetically modified genetic male sterility system that uses herbicide tolerance/susceptibility to purify the female line in the hybrid seed production plots. With the exception of the use of a herbicide to purify the female line in hybrid seed production plots, production of the NMS pollination control system components and hybrid seed would use similar procedures to those for GMS pollination control systems.

If a cytoplasmic male sterility (CMS) pollination control system [98] is used to produce the hybrid seed components, the female line is homozygous recessive (rf/rf) for male fertility restorer gene(s) and present in male sterility-inducing cytoplasm, the maintainer line is homozygous recessive for male fertility restoring gene(s) and present in a male fertile cytoplasm, while the restorer line is homozygous dominant for male fertility restorer gene(s) and present in a male sterile or male fertile cytoplasm. Production of the CMS pollination control system components and hybrid seed would use similar procedures to those listed above for GMS pollination control system.

Market Challenges/Barriers to Commercialization/ Opportunities

Market challenges exist because *Brassica* oils for biofuels and bioproducts compete with fossil oils, the traditional source of oils for transportation fuels and chemical feedstocks. *Brassica* oils, while acceptable for biodiesel production currently, may not be suitable in the future as evolving clean diesel engine technology may require shorter chain length components in biodiesel fuel than currently available in *Brassica* oils. Another market challenge is the food versus fuel/bioproduct debate, which continues with no simple resolution. The development and production of nonfood Brassicaceae species on marginal farmland has been proposed as a solution. Finally, a significant volume of *Brassica* oil is required to meet even a small portion of the biofuel/bioproducts market needs. For example, one million tons of *Brassica* oil will be needed annually to meet Canada's 2 % biodiesel in diesel fuel mandate. This will require 530,000 ha of canola production in Canada at the current average yield of 1.89 t/ha. This additional production can be accommodated in Canada but may lead to shorter rotation times between plantings of canola, which may increase disease pressure and volunteer canola issues.

Barriers to commercialization include the high costs of *Brassica* oilseed-related research and development, especially when it involves advanced biotechnologies. The high costs of obtaining and maintaining all of the regulatory approvals for commercial production of new transgenic traits limit the commercialization of such transgenic plants to a very few organizations. Another current but perhaps declining barrier to commercialization of transgenic plants is the lack of acceptance of transgenic plants by certain areas of the world.

The opportunities for *Brassica* oils for biofuels and bioproducts are substantial. The emerging emphasis on renewable energy, chemical feedstocks, industrial oils, and novel uses of vegetable oils and the steadily growing bio-economy will provide significant growth opportunities for *Brassica* oils as biofuel and bioproduct sources. The reduction of greenhouse gas emissions from diesel-powered transportation (through the use of biodiesel) and the replacement of nonrenewable fossil oil feedstocks with renewable *Brassica* oil-based chemical feedstocks should be drivers of the shift from fossil fuels to *Brassica* oil. Canola, modified fatty acid profile canola, rapeseed (HEAR), and possibly SHEAR cultivars will be developed and grown to meet emerging and enlarging markets. Double-digit annual growth in demand for *Brassica* oils for biofuels and bioproducts is anticipated [1].

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Chapter 8
Camelina sativa: For Biofuels and Bioproducts

Freeborn G. Jewett

Abstract Oilseed crops have the potential to increase the stability and sustainability of American agriculture by replacing a portion of the fossil fuels consumed by this sector. There are several candidate oilseed species that have been identified as compatible with a dryland winter wheat-fallow rotation. Of these species, *Camelina sativa* has been previously identified as being a promising species for drought-prone areas of the American High Plains. This is due to its short growing season, drought tolerance, cold tolerance, and resistance to many of the insect and pest species that cause yield reductions in other *Brassica* oilseed species. Camelina seed oil has high concentrations (30–40 %) of linolenic fatty acid (C18:3), which is a valuable product and also improves the cold-flow properties of the feedstock oil. Camelina is a native of Europe, and breeding efforts have so far focused on optimizing the varieties to produce high yields in agricultural regions of the United States and Europe. Breeding and research efforts have created linkage maps and identified QTL for yield, agronomic characteristics, and oil characteristics. Researchers have also found success in creating transgenic varieties of camelina, which could greatly facilitate the optimization of the oil profile for use as a feedstock for industrial oils and as a biofuel.

Keywords Oilseed crops • *Camelina sativa* • Fatty acid • Linoleic fatty acid • Cultivar development • Agronomy

Introduction

Camelina sativa, or "gold of pleasure," belongs to the Brassicaceae family and has been cultivated in Europe as an oilseed since the Bronze Age, which began around 4000 B.C. [1]. Numerous archeological studies have shown that camelina, flax, and other assorted cereals constituted a significant portion of the human diet in Europe and Scandinavia during the Bronze Age [1]. Cultivation of camelina waned until recent interest in low-input biofuels resulted in a reexamination of its value as an oilseed crop and as a potential source of omega-3 fatty acids for human and animal

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consumption [1, 2]. Interest in camelina as a biofuel feedstock stems from its drought tolerance and compatibility with existing cropping systems.

In the United States, camelina can be incorporated into a dryland winter wheat-based cropping system where it can be treated as a summer annual or a fall-seeded annual [3]. Camelina is a small-seeded crop and can be broadcast or direct seeded using existing wheat or canola planting equipment at a shallow depth of no more than 12 mm, with 6.3 mm being optimal [3]. The optimal seeding rate has been found to be 5.6–7.8 kg ha^{-1} depending on planting conditions such as seed bed quality, soil humidity, and pressure from weed competition [4]. No-till conditions are appropriate for camelina planting, although there are some weed-control issues that arise from this method of planting due to the lack of herbicide-resistant varieties [5].

There are two types of camelina varieties: winter varieties that are planted in the fall and allowed to overwinter in the rosette stage (fall-seeded) and those that do not have a vernalization requirement (spring-seeded) [12]. Fall-seeded varieties have a growth cycle similar to wheat in that they establish a stand and overwinter in a dormant stage. Of course, this is dependent on the presence of fall rains. Spring-seeded camelina does best when planted early [3, 6]. If camelina is planted in early March before weed emergence, it will have enough time to allow it to compete more vigorously with spring weeds [3, 5]. Camelina is a short-season crop, requiring roughly 80 days to reach maturity. Early spring planting or late winter planting will allow camelina to mature before high summer temperatures cause heat stress and lower yields [7]. The required cumulative growing degree days (GDD) for camelina are estimated to be 1,300 [8]. The optimal temperature for germination is 3.3 °C and delay of planting from March until April results in yield reductions of up to 25 % due to heat stress [6]. Dryland trials of camelina in Colorado have demonstrated superior yields compared to other oilseed crops such as canola [9, 10]. The seed oil content of camelina ranges from 30 to 45 % [1, 10, 11]. The protein content ranges from 39.2 to 47.4 %/DM, while the fiber content varies from 12.5 to 16.8 % *f.f.* DM [11].

Although camelina is a low-input new oilseed crop, it responds well to fertilization [12]. A general rule of thumb is that camelina needs 2–2.7 kg of N to produce 45 kg of seeds [13]. This can be applied during the growing season, or if residual nitrogen is available from previous crops, this can be utilized by the plant as well [13].

The flowers of camelina are generally autogamous and are between 5 and 7 mm in diameter [1]. These flowers become silicles which vary in number between 126 and 283 [14]. Each silicle can contain between 10 and 15 seeds [1, 14].

During growth, camelina is not susceptible to insect pressure from flea beetles (Chrysomelidae) that have been shown to negatively affect yields of canola and *Brassica juncea* [1]. The resistance to flea beetles is thought to be the result of defense compounds present in the leaves of camelina. A class of compounds known as quercetin glycosides has been identified as contributing to its resistance to damage from the crucifer flea beetle [15]. The presence of additional leaf compounds means that camelina is naturally resistant to some fungal infections, which

is important in irrigated situations [16]. Camelina has also shown allelopathic relationships with flax, *Linum usitatissimum,* under controlled conditions [17, 18].

Camelina is well suited to growth in low-moisture environments. The minimum water requirement for camelina to reach its maximum yield potential has been calculated to be 333 to 422 mm in Arizona [7]. The required minimum irrigation varies with climatic conditions and evapotranspiration rate. Below this minimum, yields are negatively affected. Irrigating above the minimum does not show any positive effect on seed yields and has been shown to only raise evapotranspiration of the plant [19]. The root zone of camelina is relatively shallow compared to wheat, reaching a maximum depth of 1.4 m [19, 20].

Taxonomy and Domestication

The name camelina is derived from the Greek words *chamai* (dwarf) and *linion* (flax) [21]. The center of origin of camelina is thought to be Europe. Ghamkahar et al. [22] used amplified length fragment polymorphism (AFLP) to assess genetic diversity among accessions collected in different geographic locations. They found that Russia and Ukraine are likely a center of origin of the species due to the higher level of diversity among accessions from these areas.

The species *Camelina sativa* (L.) Crantz and its wild relatives, which include *C. microcarpa* and *C. linicola,* have been reported by humans since the Bronze Age (1500–400 B.C.) [1]. Wild camelina species are present throughout North America and likely arrived as contaminants of flax and other agricultural products from Europe [23]. Wild accessions of *Camelina microcarpa* have been found that are resistant to ALS herbicides [24]. In Montana, there are over 120 varieties found in the wild [25].

Genetic Resources

Camelina ploidy varies among accessions. Camelina has been observed to have a chromosome count of $2n = 12$ to $2n = 40$ [23]. The most common count has been observed to be $2n = 40$ [26]. Analysis of the activity of desaturation and elongation genes has revealed *Camelina sativa* to be an allohexaploid [27].

The genetic diversity of existing camelina germplasm is relatively low. Vollmann et al. [28] analyzed a subset of 41 accessions of camelina using randomly amplified polymorphic DNA (RAPD) markers. They found that these accessions were also classified into four main groups based on the seed weight, oil content, and protein content, which suggested a low level of diversity among these accessions. Diversity of available camelina germplasm can be supplemented with accessions recently made available from Eastern European collections formerly inaccessible to Western researchers. Analysis of camelina accessions from Russia and Ukraine

show higher levels of diversity among these accessions [22]. There is the possibility of utilizing the numerous wild relatives of camelina; however, between *C. sativa* and *C. microcarpa,* there is a barrier to cross-pollination, limiting *C. microcarpa*'s value as a source of new traits [21].

Major Breeding Achievements

Breeding efforts of camelina have so far succeeded in producing several widely available varieties that show high yields. Yields of trials in Western Nebraska in 2005 and 2006 were between 556 and 1,456 kg ha^{-1} depending on the date of planting [29]. Yields of winter camelina varieties in Minnesota from 2007 to 2008 were also reported to be within the range of 311–625 kg ha^{-1} [30]. Camelina in Arizona under irrigation yielded over 1,500 kg ha^{-1} in 2009 and 2010 [8]. Camelina yields in Chile have also been reported to vary between 420 and 2,314 kg ha^{-1} for 2008 and 2009 [14]. Mean yields across several environments in Germany ranged from 1,460 to 1,715 kg ha^{-1} [31]. Camelina is able to produce adequate yields under dryland conditions, but exposure to excessive heat during flowering negatively affects its ability to produce higher yields and affects the oil profile [32].

Public and private breeding programs for spring camelina development exist in the American Midwest, Montana, and Western Europe. In the American Midwest, the Yellow Stone variety was developed by Great Plains Oil in Ohio. High Plains Crop Development, LLC, is currently active in Torrington, WY, and is producing varieties for the High Plains Region. Blue Sun Biodiesel is currently active and has previously developed the varieties BSX G22, BSX G24, and Cheyenne. The varieties Suneson and Blaine Creek were developed in Montana at Montana State University by Dr. Duane Johnson. Dr. Johnson currently works under Clear Skies Inc. out of Big Fork, MT, developing camelina varieties. The varieties Ligena and Celine were both developed in Europe and have demonstrated high yields in Europe and in the United States under a variety of conditions. The variety Celine was developed by Limagrain and has been observed to have a lower content of glucosinolates than other varieties but is shatter-prone [33]. Sustainable Oils of Global Clean Energy Holdings, Inc. is close to releasing their proprietary high yielding varieties SO-40, SO-50, and SO-60. Due to the low rate of outcrossing in camelina, major breeding programs have developed their varieties through open-pollinated stands, using either pedigree selection or recurrent selection. Inbred camelina lines used for the production of genetic maps have been derived through single-seed descent to the F6 generation [31]. Some testing has been done to determine the combining abilities of camelina lines for use in the creation of hybrid varieties, but so far, no male sterilization methods have been commercialized [34].

Protoplast fusion has been used to create a somatic hybrid between *Camelina sativa* and *Brassica oleracea*, with the intention to transfer camelina's resistance to the black spot leaf disease, although this was met with limited success [35].

Mutagenesis has been used as a technique to introduce new and novel traits into existing camelina germplasm. Ethyl methanesulfonate (EMS) has been used by researchers interested in developing camelina lines with resistance to residual herbicides. Walsh [36] used EMS mutagenesis to develop two camelina genotypes that demonstrated tolerance to acetolactate synthase (ALS) inhibitor herbicides. These mutant camelina lines showed increased resistance to imazethapyr and sulfosulfuron herbicides. Limited quantities of these mutant lines are available from Washington State University.

A mapping population was developed in Germany (Deutsche Saatveredelung, Lippstadt, Germany) and has been used to create linkage maps and identify QTL for favorable agronomic traits in several studies. This mapping population was derived from a cross between the European varieties "Lindo" and "Licalla." Gehringer et al. [31] created a linkage map of camelina with 157 amplified fragment length polymorphism (AFLP) markers and 3 *Brassica* SSR markers and identified quantitative trait loci (QTL) for seed yield, oil content, plant height, thousand-seed weight, and fatty acid composition. Using this same mapping population, Enjalbert [32] identified 29 significant QTLs for yield, drought tolerance, and oil quality characteristics. Of these, six were found to be in common with Gehringer et al. [31].

Target Traits and Current Breeding Goals

Yield

Yield is the subject of most improvement programs. Camelina seed yield has shown a high degree of heritability at 86.5 % [37]. Oil content and thousand-seed weight also demonstrate high degrees of heritability at 95.6 % and 97.6 %, respectively [38]. Thousand-seed weight could be a characteristic of interest for breeders interested in increasing yield, as this character is easier to select than yield and has a higher heritability than yield [31]. There is evidence that higher thousand-seed weight comes at the expense of oil content and the number of seeds per plant [39].

Breeding for yield stability over environments will become more important in the future as climate change and global warming affect both absolute environmental conditions and variability of environmental conditions. Optimizing camelina growth habits to better avoid summer heat can potentially minimize environmental effects on yield. Flowering time may be a useful characteristic as a target for selection, as earlier flowering varieties can better escape heat stress and earlier flowering is positively correlated with seed yield and linolenic acid content [32].

Oil Characteristics

The four most important components of the fatty acid profile of camelina are C18:1, C18:2, C18:3, and C20:1, as they comprise the majority of the fatty acid profile of camelina, and environmental conditions affect the concentrations of these fatty acids [10, 32, 40]. The most economically important fatty acid, linolenic fatty acid (C18:3), has been shown to vary in concentration between 30 and 40 % [14, 31, 32]. Breeding efforts might focus on increasing the percentage of linolenic fatty acid to optimize the value of camelina press cake as an animal feed and its value as a source of polyunsaturated fatty acids. Enjalbert [31] determined the heritability of linolenic (C18:3) fatty acid to be between 0.40 and 0.83 and the heritability of oil content to vary from 0.42 to 0.87. An increase in the amount of linolenic fatty acid has been shown to improve heat and drought tolerance in some *Brassica* species such as canola and *Arabidopsis* [41–45]. Camelina seeds are composed of 30–40 % oil [1].

The high concentration of polyunsaturated fatty acids (~50 %) and protein present in the press cake is a valuable addition to feed but must be added in modest proportions. It is recommended that camelina meal comprise no more than 10 % of the feed weight due to the concentration of toxic glucosinolates that can negatively affect growth of livestock and poultry [46, 47]. Camelina meal can replace up to 5 % of broiler chicken feed without negatively impacting the quality of the meat. The incorporation of this feed increases the intramuscular concentration of omega-3 fatty acid [48]. The protein content of the press cake left over from the hexane solvent extraction process is suitable for animal consumption. It is lower in fat due to the increased efficiency of the extraction technique, but it has protein content similar to that of soybean meal. Any harmful compounds such as glucosinolates and erucic acid present in the seeds before pressing can be extracted by subsequent solvent treatment of the seed meal [49]. Vegetable consumers can recognize glucosinolates from the pungent odor that is released when cooking those in the *Brassica* family such as cabbages and Brussels sprouts. The leftover camelina seed meal can be heated to reduce the glucosinolate content prior to consumption, similar to these vegetables [50].

The most common, easiest, and least expensive method of oil extraction uses a mechanical oilseed crusher. This machine heats and crushes the seeds, which causes the separation of the oil from the seed meal [51]. The resulting press cake contains approximately 10 % oil by weight and is considered to have an extraction efficiency of 75 % [52]. The leftover seed meal from camelina pressing contains 40–45 % crude protein and 10 % fiber, which is lower than soybeans but comparable to rapeseed press cake [48]. With the residual fatty acids and absence of erucic acid (C22:1), the molecular profile of the leftover seed meal indicates that it could be a potentially valuable coproduct as animal feed. Future improvements and plant breeding research will need to focus on optimizing the biofuel oil profile to raise the percentage of oleic acid (18:1) and decrease concentrations of linolenic acid (18:3) [53]. This would reduce the iodine value (a measure of the degree of unsaturation of

the oil) to a value that is below the acceptable limit of 120 as established by the European biodiesel standard [53].

Recent analysis of Eastern European camelina germplasm collections has identified an accession with desirable oil profile of greater than 30 % linolenic fatty acid, less than 3 % erucic acid, less than 10 % saturated fatty acids, and a ratio of linolenic to linoleic acid greater than one [22].

Breeding Strategies and Integration of New Biotechnologies

Analysis of the agronomic characteristics of recombinant inbred lines (RIL) formed from a cross between the varieties Lindo and Licalla showed that 25 % of the offspring outperformed both parents, meaning that camelina shows transgressive segregation [31]. Of these RILs, Gehringer et al. [31] identified five promising varieties as candidates for possible release. Two of these were also identified at Colorado State University as containing QTL for yield and drought tolerance and demonstrating significantly higher yields [32]. These are currently undergoing further yield evaluations with the possibility for commercial release [32]. The difference in climate between Germany and Colorado suggest that these varieties are widely adapted and show high yields in a variety of environments.

Gamma ray irradiation was used by Vollmann et al. [37] to induce mutations in camelina germplasm for improving linolenic acid content. Lines were isolated that contained higher concentrations of the fatty acid (40.8 %). In addition, some mutants were identified that contained lower concentration of erucic acid (less than 2 %).

Camelina is a suitable candidate species for transgenesis. It is widely considered a primarily self-pollinating species with a low rate of outcrossing [23]. Field experiments have estimated the outcrossing rate in camelina to vary between 0.01 and 0.28 % [54]. This is nearly equal to soybean, which is approximately 0.30 % [55]. The fact that camelina is a facultative outcrossing species means that it can be bred through recurrent selection or through the creation of hybrids by creating male sterile lines for crossing. A methodology for developing doubled haploid camelina would also greatly reduce the number of generations necessary for new lines of camelina to reach homozygosity [39].

Research looking to characterize the genome of camelina has been ongoing for some time. There have been notable efforts to identify several target genes with respect to their potential for genetic manipulation. Hutcheon et al. [27] targeted genes regulating fatty acid synthesis in *Arabidopsis* that were hypothesized to be homologous in camelina. These genes include fatty acid desaturase 2 (FAD2), which converts oleic acid (C18:1) to linoleic acid, and fatty acid elongase 1 (FAE1), which adds two carbons to an 18-carbon chain. It is presumed that downregulation of these genes could lead to increased production of oleic acid, a favorable fatty acid for biodiesel feedstocks. RNA sequencing techniques revealed that unlike in *Arabidopsis*, where only one copy of each gene is found, the camelina

genome contains at least three functional copies of FAD2 and FAE1. This suggests at least three full genome duplication events occurred at some point in the evolution of the camelina genome. The polyploid nature of the camelina genome was proposed to be conducive to reverse genetic (TILLING) manipulations using protocols developed for wheat, another allohexaploid. The use of TILLING technologies would make it possible to identify individuals with mutations in the FAD1 and FAE2 genes.

Another attempt to modify the oil profile of camelina through the use of transgenics was carried out by Lu and Kang [56]. Using a seed-specific phaseolin promoter and the floral dip method, the authors were able to carry out a successful plant transformation where they inserted a castor oil gene FAHI2, which codes for a novel fatty acid (C18:1OH). The oil profiles of the resulting transgenic plants had elevated levels of oleic acids from 14.4 to 21.6 % and a resulting decrease in the polyunsaturated fatty acid percentage from 37.8 to 13.3 %.

Seed Production

Camelina can be directly harvested using existing wheat harvesting equipment with a screen of 3.6 mm installed over the lower sieves of the harvester [3, 5]. Harvesting efficiency can be improved if future varieties are selected to reduce shattering. If weeds are a problem, camelina can be swathed when the pod color is about 65 % yellow [5].

Today, camelina is being produced as a biofuel feedstock crop. Commercial camelina production in the United States centers on the Pacific Northwest region and Montana, and the majority of this production is sold to the US Air Force for its green fuel program. According to the USDA National Agricultural Statistics Service [57], about 19,500 acres of camelina were harvested in 2009 and 9,400 acres were harvested in 2010 in the United States. Camelina is currently being produced exclusively under contract; there is no open market for the crop.

Although the production areas of camelina include Montana, Oregon, and Washington, camelina testing and varietal evaluation is being conducted in several states including Colorado, Wyoming, California, Kentucky, Iowa, Florida, and Arizona. Photos of camelina stands grown in Craig, Colorado, are shown in Figs. 8.1 and 8.2.

Camelina contract price varies depending on the location and year, but in 2010, the average price was $0.16/lb [58]. Assuming the cost of meal is $0, which is not the case, the value of the camelina biodiesel would be $5.00/gallon [58]. Keske et al. [59] estimated that producing camelina for on-farm use of straight vegetable oil would have the highest probability of return when conventional diesel prices reach and exceed $1.31/L. The cost of production for camelina based on models developed at Montana State University is estimated to be $80.27/acre at 1,350 lbs/acre yields [34, 58].

Fig. 8.1 Camelina stands in the high-altitude location of Craig, Colorado, 60 days after planting. July 11, 2011

Fig. 8.2 Camelina stands ready for harvest in Craig, Colorado. August 30, 2011

Winter Varieties

Limited development of winter camelina lines exists today. High seed yield from fall-seeded winter varieties remains to be proven [30, 60]. The advantage of these varieties is to allow the camelina seedlings to be in the soil when weather conditions are optimal for emergence. Phenotypically, these varieties differ from spring varieties in leaf shape and overwintering ability.

In the case of fall-seeded, the plant establishes itself in the fall and overwinters as a rosette. The following spring, when temperatures reach 3.3 °C, growth is initiated and the plant emerges from the rosette and resumes growth [6]. The vernalization requirement for winter camelina has not been well characterized. Previous experiments have found that fall-seeded camelina has enough winter hardiness to survive the harsh winters of Minnesota, where average winter air temperatures are far lower than those found in Colorado [30]. As the plant is already established, it reaches maturity far earlier than spring-seeded camelina. Earlier maturity means that the plants are not exposed to as much of the heat and drought stress that occurs during the warmest months of summer.

In addition to the potential for increasing yields, earlier harvest allows more time for moisture recharge in the field during the summer. This could result in higher yields for wheat that is planted after fall-seeded camelina than spring-seeded camelina. This may vary based on spring temperatures and moisture conditions. Another advantage of winter camelina is that fall planting is generally drier and the seeds are already planted when spring rainfall arrives. Winter seeding of camelina would be particularly advantageous in southeastern regions of the United States, where the winters are warmer and the spring arrives earlier. In colder climates, overwintering ability is increased with snow cover [61, 62]. Aase and Siddoway [61] determined that 7 cm of snow cover is sufficient to buffer wheat seedlings from temperatures as low as −40 °C. With the increased stubble as a result of the implementation of no-till agricultural systems, there is a greater amount of snow capture on fields.

Winter camelina trials in Akron, Colorado, have encountered failures related to the presence of *Ceutorhynchus cyanipennis* and *Ceutorhynchus americanus* ([60, B. Kondratieff and G.L. Hein (2011), personal communication). These insect pests appear frequently on plant species in the *Brassicaceae* family, which includes camelina [63]. These insects most strongly affect winter camelina that is planted earlier in the fall, especially in August, as this is the time adult insects lay eggs [63]. Later fall planting dates have been shown to reduce the impact of these pests. Studies of winter camelina in Ireland have shown that earlier planting dates are prone to high rates of lodging [64]. This also may be due to the damage from these insects, as the larvae feed on stems at or below the soil line (G.L. Hein (2011), personal communication).

Market Challenges/Barriers to Commercialization/ Opportunities

Barriers to the wide-scale adoption of camelina as a biofuel feedstock come from a variety of factors. Camelina sativa is related to weedy species that are recognizable to farmers. This might contribute to their reluctance towards planting large swaths of land with camelina. This can be overcome through education and awareness, as camelina is not very competitive as a weed species and volunteer camelina can be easily controlled with available herbicides.

The lack of an open market for camelina may dissuade some potential producers. Farmers operating outside of certain areas where contract camelina growing operations exist may have trouble selling excess product, as it is not certified for human consumption.

The main opportunity for camelina production exists in the on-farm production of biofuels. This eliminates the need for an open market, as local consumption is not subject to certification in the same way as biodiesel producers looking to sell to large blenders. Local camelina production is an opportunity for farmers to offset their costs in two ways. The first is through the utilization of the camelina oil as a diesel substitute that will offset the annual consumption of diesel fuel. The second is by utilization of leftover seed meal, or press cake, to offset consumption of animal feed.

Camelina-derived diesel fuel can be utilized directly without any fuel processing. This is known as straight vegetable oil (SVO). The SVO can be directly burned in an engine that has been modified with tank heaters to increase the viscosity of the fuel, or else, it can be blended with diesel fuel and used in an unmodified engine.

Local production of camelina is dependent on collaboration between producers. A possible model for the production of camelina-based fuel involves a community-funded crushing facility that is shared between several producers. The meal and camelina oil can be distributed among the producers along with the costs. Currently, there are locally sourced crushing facilities in three locations in Colorado. These are in Rocky Ford, Burlington, and Costilla County, Colorado [32].

More advanced producers will take advantage of the specialized oil profile of camelina and produce with the intention of selling the components of the oil such as linolenic and linoleic fatty acids, which has a higher value than vegetable oil for use as diesel fuel alternatives. Selling the components of the camelina oil is a way to increase production when fuel prices are low and ensure profits during low yielding years.

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Chapter 9
Maize Starch for Industrial Applications

Brad M. Ostrander

Abstract Starch is used in many industrial applications as viscosifiers, emulsifiers, defoaming agents, for encapsulation, and as sizing agents. Starches are valued for their ability to impart textural characteristics and provide gelling or film formation. Much of the starch used for industrial purposes must be chemically or physically modified to improve performance or provide functional persistence. Increasingly, however, as the genetics behind starch biosynthesis are better understood, native starches can be selected to allow chemical or physical modification protocols to be optimized or to be more fully utilized as non-modified starches. This review discusses the types of starch commonly used in industry, the development and availability of specialty corn types, breeding methods used, and the challenges and potentials for new approaches.

Keywords Specialty starches • Amylose • Amylopectin • Waxy • High amylose • Wet milling • Papermaking • Bioplastics

Introduction

Starch provides the maize plant with the energy resource to fuel seedling emergence. Seedling growth and leaf development rapidly increase the plant's photosynthetic capability to fix carbon as glucose. The glucose is polymerized by the plant to form starch, initially in relatively small quantities in the leaves. As the plant matures and enters the reproductive stage of the life cycle, an increasingly higher proportion of glucose is sequestered in the endosperm as starch, in the newly formed, next-generation kernel.

In the endosperm of the maize kernel, starch granule development occurs by successive layering of starch in crystalline and amorphous configuration (Fig. 9.1). Maize starch is classified as either amylopectin or amylose. Amylopectin and amylose are distinguished by degree of branching and molecule size. Amylopectin maize is larger than amylose and is composed of 2,000–20,0000 α-(1 → 4)-linked glucose units, with branching α-(1- > 6) linkages occurring every 24–36 units.

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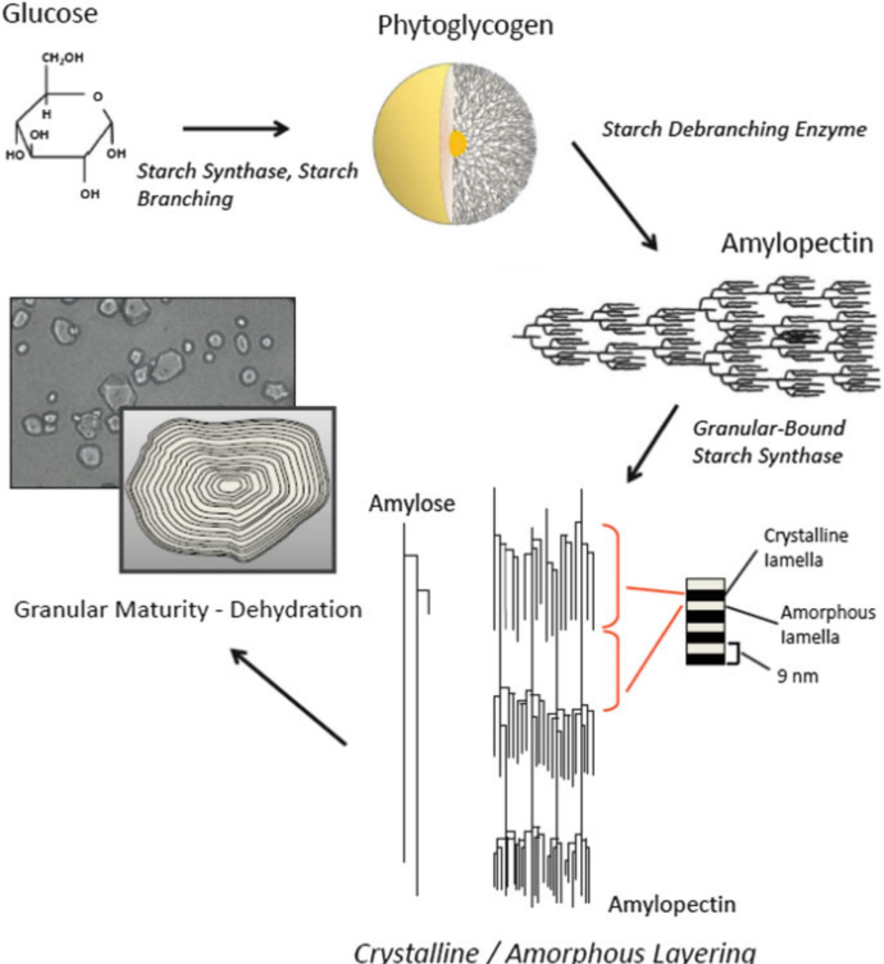

Fig. 9.1 Starch production and granule organization

Amylose typically is composed of only 300–3,000 α-(1 → 4)-linked glucose subunits, and it forms very few α-(1 → 6) branches and typically maintains a helical configuration.

On extraction from the kernel, the relative proportion of amylopectin to amylose in the granule is the major determinant of the starch functional properties in food and industrial use. Commercial maize starch types include regular starch, waxy starch, and high-amylose starch. Regular starch, or simply cornstarch, is approximately 75 % amylopectin and 25 % amylose. Waxy cornstarch is nearly 100 % amylopectin starch. The high-amylose cornstarch most commonly used in the industry is 25 % amylopectin and 75 % amylose, the inverse of the regular starch

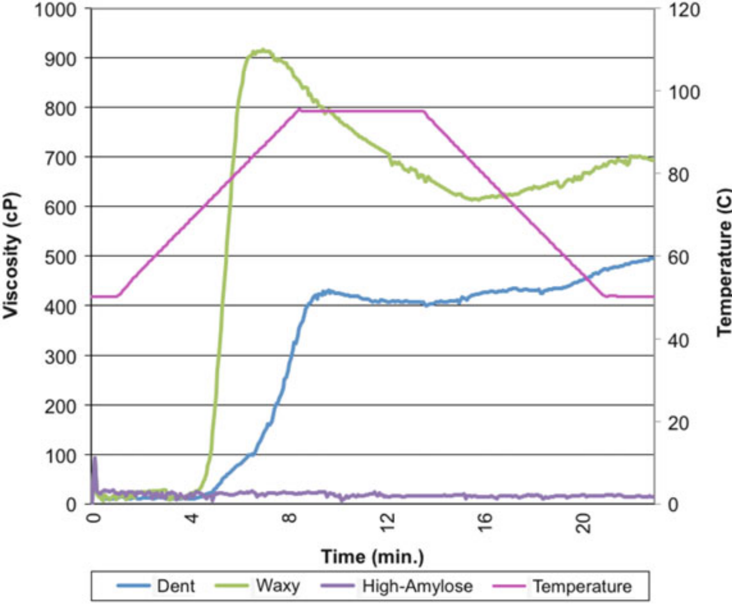

Fig. 9.2 Viscosity profile of dent, waxy, and high-amylose starch in response to heat

ratio. Additionally there is also a limited commercial market for high-amylose starch that has 55 % amylose and 45 % amylopectin.

Regular starch, made from yellow dent corn, is most readily abundant and can be most economically produced. However, because of limited functionality, it often must be chemically or physically modified to provide improved utility in food and nonfood uses.

Starch functionality can refer to the properties of the granule, or may refer to the starch matrix that associates the following disruption of granule structure. In response to heat, pressure, or chemical input, the amylopectin and amylose molecules are released from the granule structure and reassociate to form a gel or film.

The granule will maintain itself to a variable degree dependent on amylose-to-amylopectin content, degree of amylopectin branching, branch length, and intermolecular bonding that occurs. The swelling and collapse of the granule structure is described as gelatinization, and regular, waxy, and amylose starches display characteristic profiles in response to heat and moisture (Fig. 9.2).

Chemical or physical modification is often performed after the initiation of gelatinization but prior to granule breakdown. Typical industrial applications of native and modified regular, waxy, and amylose starches are listed in Table 9.1.

Industry wide approximately 40 % of wet-milled starch is used for nonfood applications. The most predominant industrial uses are papermaking, corrugating, laundry, gypsum wallboard construction, and adhesives.

High-amylose starches, valued for their use in corrugating, are of growing importance in the bioplastic industry. The long straight chains of the amylose

Table 9.1 Properties and industrial applications of specialty starches

Starch	Gelatinization properties of native starches[a]		Industrial use of starch/modified starch base
	To	Tp	
Dent			
Native	78	95	Corrugating, face and talcum powder
Modified	Variable		Papermaking, laundry starch, textiles, bioplastics, glass-fiber sizing
Waxy			
Native	70	83	Foods, adhesives
Modified	Variable		Papermaking, pharmaceuticals, adhesives, bookbinder, construction, mining, home, and fabric care products
Amylose 5			
Native	>95	>95	Corrugating, glass-fiber sizing
Modified	Variable		Corrugating, textiles, papermaking, glass-fiber sizing
Amylose 7			
Native	>95	>95	Films, bioplastics, textiles
Modified	Variable		Corrugating, textiles, papermaking, bioplastics, glass-fiber sizing

[a]*To* gelatinization onset temperature, *Tp* gelatinization peak temperature

polymer can form either biodegradable or nonbiodegradable plastics. Bioplastics from starch can be moisture permeable and so have increased utility as food wraps or packaging.

The starch amylose and amylopectin molecules have distinctive characteristics that provide a wide array of properties. This functional variety makes starch a unique, valuable, and adaptable industrial resource.

Starch Production and Development of Specialty Starch Hybrids

One of the first commercial corn wet-milling operations in the United States was the Oswego Starch Works, founded by Thomas Kingsford in Oswego, New York, in 1848. The plant milled about 850,000 bushels and produced more than 8.25 t of starch annually. Oswego, in the mid to late nineteenth century, was one of the major great lakes shipping ports, linking the eastern seaboard and Chicago grain trade [1].

Initially, all corn wet milled was regular corn. The waxy corn type was not discovered until 1909 in samples categorized from Chinese landraces [2]. Waxy is most prominent in SW China with 18–22 % of the landraces from the provenances of Yunnan, Guizhou, and Guangxi reported as waxy-type corn [3, 4].

The ability to visually distinguish the waxy kernel phenotype made it very useful in early genetic studies of recombination frequency and gene distance chromosome

mapping [5, 6]. The functional properties of waxy were not documented until 1922, when it was found that waxy corn "contains but one kind of carbohydrate" and that it is "soluble in hot water, the solution or viscous suspension giving the characteristic red reaction with iodine" [7].

Tapioca starch (from cassava) was commonly used in foods and glues in the United States, with approximately 230,000 tons of it being imported in 1937 [8]. Disruption of shipping lines from the Dutch East Indies during World War II gave leverage to the US starch industry's desire to increase domestic starch production and to promote waxy maize as a viable substitute.

The waxy breeding programs of Iowa State in Ames, Iowa, and Purdue University in Lafayette, Indiana, provided breeding resources for early waxy development. Waxy corn was initially marketed as Tapicorn® and became established as demand for canned and packaged foods increased in the 1950s and 1960s [9].

As the value of waxy corn became recognized, and the nature of amylopectin and amylose starch types better understood, the search was begun for high-amylose corn. The high-amylose corn type was discovered in 1948 when kernels with a slight color or "tarnish" were observed to be segregating out of research populations at the Bear Hybrid Corn seed company [10, 11].

The new corn type was named "amylomaize" [11]. The Bear Hybrid Corn Company, already in the business of developing and marketed waxy corn, worked toward the development of commercial high-amylose corn. In 1948 National Starch teamed with American Maize Products to support the Bear Hybrid Corn breeding effort to produce hybrids with 55 % amylose starch [12]. This was done in cooperation with the USDA regional laboratory in Peoria, Illinois. The 10-year breeding program culminated with an experimental milling of amylose in 1958 and the marketing of Amylon® starch in 1959 [12].

Areas of Production

To reduce transport cost and processing margins, specialty maize has been grown local to milling facilities whenever possible. Waxy and amylose grains are typically grown under production contracts. Since the 1970s, there has been sufficient market demand for growers to produce additionally waxy grain for the speculative market. Annual production acres for waxy are estimated at 600–700 k acres, depending on demand, with some years spiking to over 900 k production acres [13]. Waxy production areas for the United States are shown in Fig. 9.3.

The amylose acres grown in the United States is less well documented but is estimated at 50 k acres annually. Nearly all production is grown under contract. Because both waxy and amylose are recessive traits, a minimal separation distance is required to prevent cross-pollination with dent.

Waxy production in Europe is mostly in southwest France, with approximately 30 k hectares grown annually and is significantly less than 1 % of the total French corn production [14, 15].

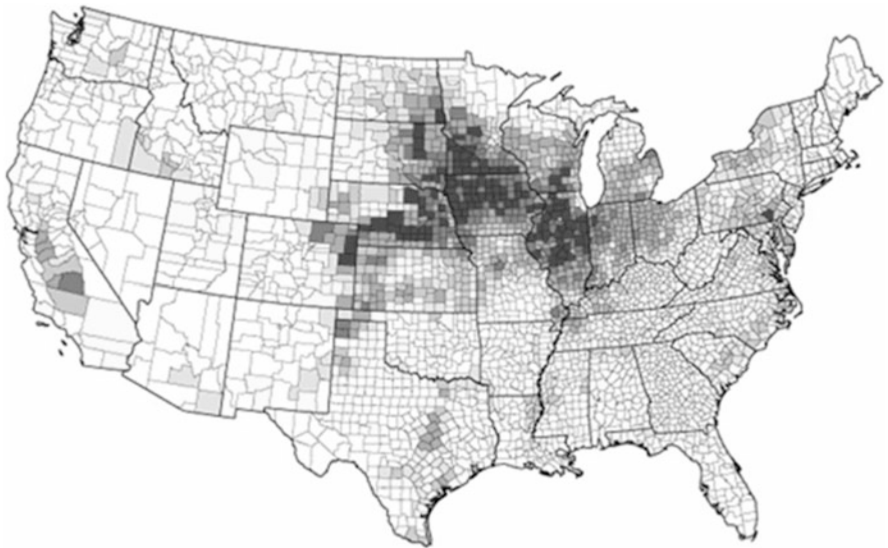

Fig. 9.3 Waxy production areas in the United States (Based on data from Ref. [13])

Australia grinds more than 60 k tons of corn annually, utilizing more than 15 % of the total corn production [16]. Waxy grain production in Asia is increasingly being used for food, beverage, and industrial use. A significant portion of the waxy grown in China and other areas, such as Thailand, is grown as fresh market "sticky corn." Waxy grown for the fresh market may be white, yellow, blue, or black and is harvested in a similar manner as sweet corn and often sold on the ear.

Specialty Starch Hybrid Genetic Resources

The controlling genetic components of the waxy and high-amylose specialty grain types are starch biosynthetic pathway enzyme mutations. The waxy gene is a recessive, null mutation of the pathway enzyme granule-bound starch synthase (GBSS) [17, 18]. The *amylose-extender* gene, responsible for the amylose phenotype, is the starch branching enzyme IIb (SBEIIb) [19].

The *waxy-1* gene has been independently discovered numerous times, and many of the allelic seed stocks are conserved at the Maize Genetics Cooperation Stock Center (Urbana, Illinois) or the Germplasm Resources Information Network (Ames, Iowa). The Germplasm Enhancement of Maize program (GEM), begun in 1995 at Iowa State University, has available diverse public domain breeding materials including a limited number of waxy breeding lines [20].

Several of the alleles found to have arisen spontaneously out of breeding populations were shown to be the result of the action of transposable elements [21]. Waxy alleles have also been recovered out of induced mutation populations using ethyl methanesulfonate or gamma radiation induction.

The availability of several independent waxy alleles provided a unique resource for early research into the organization of the gene. Using 22 of the waxy alleles, a structural gene map was constructed and provided seminal insight into the nature of molecular change at a level of detail not previously possible in an agricultural crop [22].

The amylose mutation has been rediscovered less frequently than waxy; however several independently derived alleles are listed with the Maize Genetics Cooperative Stock Center. Mark Campbell, of Truman State University, has released a public domain amylose breeding line, GEMS-0067 [23]. In addition to *amylose-extender*, GEMS-0067 has been shown to possess a mutation of the gene for starch branching enzyme I (SBEI) [24]. The mutant SBEI gene serves as a significant amylose modifier, elevating grain amylose content in the evaluation population to above 70 %. Amylopectin branching has also been shown to be impacted by SBEI and to alter starch granule structure [25].

Specialty Starch Hybrid Breeding Advancements

Breeding for specialty starches routinely involves backcross conversion of waxy or amylose genetics into elite performing dent or flinty breeding lines. The line to be converted, in addition to yield potential, should be considered for its ability to confer agronomic stability, kernel starch content, ear disease resistance, good fall dry down, kernel size, and kernel test-weight characteristics. In contrast to dent breeding with its focus on capturing top-end yield, with specialties, performance longevity and environmental stability are often of superior premium.

Comparisons of hybrids produced using waxy and dent isolines reported an average yield reduction of 3.5 % [26]. A portion of the waxy yield drag may be an artifact of linkage drag resulting from the use of less productive donor materials. Improved waxy donor germplasm that is as closely related to the elite recurrent as possible should be used for conversions. Marker-assisted selection, highly useful in transgenic inbred conversions also can be effective in minimizing linkage drag around the specialty genes of interest.

A biological or biochemical causal effect for waxy yield drag is not fully apparent although waxy breeding lines have, in some evaluations, been observed to have lower starch content in comparison to dent [27]. Waxy ears have also been found to have slower dry down in the fall, a characteristic that could contribute in some growing conditions to reduced yield and increased susceptibility to fungal infection [28, 29].

Genetically engineered lines with transgenes for protection against herbicide and insect damage have been available in waxy hybrid seed since 2007 [30]. Engineered

traits have been available in regular field corn since 1996 and have simplified production, decreased costs, and increased yield [31–33]. The potential to capture similar gains has motivated the promotion of genetically modified waxy hybrids.

Amylose specialty hybrids, in contrast to waxy, have a yield reduction of 20–25 % in comparison to dent isogenic materials [26]. Amylose starch granules are smaller and irregularly formed, suggesting that the rate of starch polymerization or the configuration of the granular crystallized lamella is disrupted [34]. The enzyme complex formed in high-amylose corn is distinct and does not follow the same activity cycle as compared to the dent wild type [35, 36]. Effectively the plant is unable to assemble the most efficient protein configuration and defaults to a less efficient aggregate [36].

As with waxy, slower grain dry down and higher grain moisture later in the season may contribute to yield loses and disease susceptibility. High-amylose hybrids have been commercialized on a much smaller scale than waxy hybrids, and fewer breeding programs pursue their research and development. The additional requirement of amylose modifier genes also contributes to limit development of high-amylose donor material, and linkage drag effects impact yield to greater degree than in waxy conversions.

Transgenic conversions to control expression of the amylose-extender gene are a strategy that has been pursued not only for corn but also for several other agronomic crops including rice, wheat, barley, potato, and sweet potato [37–42]. Transgenic conversions provide valuable insight into starch functionality but have not been widely used for any commercial applications.

Not all wet-mill facilities accept genetically modified waxy grain, and especially those that serve food industry will often require grain channeling, identity preservation, and handling documentation.

Starch Pathway Genetics

In high-moisture food applications such as sauces, spreads, fillings, and soups, starches can affect and improve sensory aspects and confer creaminess, flowability, and smoothness. In lower moisture foods like bakery, snacks, cookies, crackers, and cereals, starches contribute to products perceived softness, crispness, stickiness, or crunch.

The impact of native starch on food functionality is the result of several factors including amylopectin and amylose content, amylopectin chain length and branching properties, and starch interactions with lipids and proteins. Because of the very specific nature of taste and food perception, small and subtle changes in the starch can greatly impact the food experience.

With industrial use, the starch is often required to perform as a permanent application with performance characteristics that must be maintained for a much longer time frame. Chemical modification can be applied at a higher rate and produce starches that are much more persistent. Industrial starches also do not

command the same price premium and may not be as quickly adaptable to new or small production specialty grain types that may find profitable niche application with food.

The genetics behind starch biosynthesis has been extensively researched in maize for food applications and is well defined by numerous evaluations of null mutations (Table 9.2) [19, 43–49]. In addition, many gene combinations have been discussed and reviewed [35, 45, 50–55].

Waxy and amylose-extender are genetics on which commercial businesses have been built; improvement on these starch types provides immediate application. Unique gene combinations that could expand on these applications are highly desirable and would provide the industry with new commercial grain types.

Disruption of the initial steps of the pathway results in very little or no starch being produced. Both *shrunken-2* and *brittle-2*, code for subunits of the tetrameric ADP-glucose pyrophosphorylase, prevent starch production, and *shrunken-2* is used in commercially released sweet corn hybrids. The ADP translocator enzyme coded by the *brittle-1* gene prevents transport of glucose-6-phosphate across the amyloplast organelle membrane.

The *sugary-1* mutation, no longer greatly used in the sweet corn industry, codes for starch debranching enzyme and results in phytoglycogen as the major endosperm carbohydrate rather than starch. Phytoglycogen is more highly branched than amylopectin; it does not form granules but is retained in the dried kernel where it is 35 % of the dry-matter weight [56].

Phytoglycogen molecules, with a diameter of 40–50 nm, are much smaller than starch granules (15 μm). While not a starch, phytoglycogen is a very interesting hydrocarbon and has been shown to have many useful food and industrial applications [56, 57].

Substrate availability, enzyme availability, and enzymatic activity are the predominant regulators of biosynthesis. While much of the current breeding for specialty corn is built on null mutations, and thereby enzyme availability, the starch pathway in maize appears also to include some more complicated aspects. The observations and descriptions of ADP-glucose pyrophosphorylase (*shrunken-2*) provide an interesting example and demonstrate the potential of starch genetics to contribute to improved yield and agricultural performance [58–60].

Soluble starch synthase (*dull-1*) gene has been shown to reduce SSII and SBEIIb [61]. The formation of multi-subunit protein complexes containing SSIIa, SSIII, SBEIIa, and SBEIIb has been well described and outlines a higher-order structural component within the pathway [45].

Simple starch pathway genetic combinations that are most prominent in the literature are *waxy:sugary2* and *waxy:amylose-extender*. *Sugary-2* has been shown to have a dosage effect on amylopectin chain length and produces a change in the onset of gelatinization temperature [62]. The shorter amylopectin chain lengths have been cited as contributing to reduced starch retrogradation in *waxy:sugary2* starch [55]. The *waxy:amylose-extender* combination changes the amylopectin chain length in the opposite way, extending chain length [55].

Table 9.2 Major starch pathway enzymes and respective genes

Starch biosynthetic enzyme	Protein abbreviation	Maize gene	Chromosome	Comm. specialty	Starch effect
Sucrose synthase					
	Sus	*shrunken-1*	9S	Not used	Little or no starch produced
ADP-glucose pyrophosphorylase – heterotetramer					
Large subunit	AGPase LS	*shrunken-2*	3L	Sweet corn	Little or no starch produced
Small subunit	AGPase SS	*brittle-2*	4S	Not used	Little or no starch produced
ADP-glucose translocator	ADPGluc transporter	*brittle-1*	5L	Not used	Little or no starch produced.
Starch debranching enzymes	ISAI or DBE	*sugary-1*	4S	Sweet corn	Phytoglycogen predominantly produced with very little starch
Soluble starch synthases					
	SSIIa	*sugary-2*	6L	Waxy	Causes a reduction in amylopectin and results in higher amylose. Reduces amylopectin chain length in combination with GBSS
	SSIII	*dull-1*	10L	Not used	Reduced activity of SBEIIb
Granule-bound starch synthase	GBSSI	*waxy-1*	9S	Waxy	Endosperm starch content is approximately 100 % amylopectin
Starch branching enzymes					
	SBEIIb	*amylose-extender*	5L	Amylose	Increased amylose content to 45–55 %
	SBEI	*sbe-1*	5S	Amylose	Increases amylopectin branching, in combination with SBEIIb elevates amylose content

The *waxy:sugary-2* and *waxy:amylose-extender* gene combinations have interesting food applications and potential to reduce reliance on chemical modification for functionality. Neither gene combinations seem to have an immediately apparent large-scale industrial application. Petrochemicals in the United States and many other countries continue to provide the least expensive source of hydrocarbons for industry; however the sustainable production of naturally sourced starch can be a very attractive resource for some markets.

High-amylose starch can be heated and electrostatically aligned to form strong films. These films differ from oil-derived films in that they have greater permeability and are biodegradable [63] . These attributes have the potential to make them preferable in use for food packaging and other applications that require short-term, rather than indefinite or long-term, persistence.

Amylose starches have been extruded to form packing peanuts and into sheets and shapes for packaging. For forms and films, greater amylose content confers greater strength, and amylose modifier discovery, such as SBE1, will facilitate amylose hybrid development and work to reduce the high-amylose yield drag.

Because the majority of high-amylose starches are used in food applications, most if not all, high-amylose hybrids used for industrial starches are also used for food applications. The development of a high-amylose hybrid specifically for an industrial production target relies on the establishment of a market of sufficient scale and size.

The ethanol industry provides a large market, and while profit calculations revolve around grain yield, total starch content, and ethanol/acre, some very interesting approaches have been taken. The Enogen® trait is transgenic bacterial α-amylase that becomes active during the fermentation process [64]. A similar approach that affects amylose or amylopectin structure for industrial use would be very interesting.

Breeding Strategies for the Development of Specialty Hybrids and Integration of New Approaches

The genetics of the waxy and amylose specialties accommodate different breeding approaches. For the development of waxy inbred lines, backcross conversion and line verification are commonly used. With amylose, the quantitative nature of the trait is more amenable to population development and pedigree line selection.

Both waxy and amylose must, at some point, use self-pollination to identify and fix the homozygous recessive specialty gene. Testcrosses or markers to verify the presence of the heterozygote are effective and save generational time but require extra cost.

A transgenic waxy or amylose specialty trait would have several advantages, dominant expression of specialty starch genetics would allow visualization of the trait in each generation, combination with a herbicide selection marker would allow

elimination of trait-negative plants at the seedling stage, and less contamination at the grain production stage would be expected.

Neither transgenic waxy nor amylose have yet been commercialized in corn. Waxy has been shown to be an improved source material for ethanol production [27]. It is ethanol or other large-scale industrial markets that would most likely provide the demand for a genetically modified waxy or amylose. Aside from approaches to use genetic transformation, and with attention to the dual food/ industrial use required of specialty hybrids, several interesting breeding strategies have been and continue to be applied.

Advantages of Marker-Assisted Selection

Marker-assisted selection (MAS) is a very valuable breeding tool to accelerate backcrossing (Table 9.3). The most rapid conversions to the elite are captured in the BC1-BC3 generations. Use of the marker-assisted backcross is somewhat complicated by recessive genetics, as the recessive gene, if not fixed prior to marker analysis, will reduce the number of useful selections by half, and two recessive genes will reduce selections to 25 %.

Greenhouse or continuous nursery production, especially in combination with MAS, can fully convert an inbred in less than 2 years. Seed increase, yield testing, and functionality qualification however add significant time to specialty inbred release schedules.

The major advantage for the use of MAS for waxy is that it can be used to reduce breeding generations and field pollinations. The waxy trait is a phenotype that is easy to distinguish visually and is a binary phenotype, producing either 100 % amylopectin or wild type. Traditional backcrossing is an effective process to convert elite regular corn to the waxy specialty, and using MAS the process can be accelerated.

The use of MAS for the development of amylose inbred lines is less straightforward. There is at least one major modifier gene required to produce inbreds with greater than 70 % amylose [66]. Additional genetics require additional costs when either traditional or MAS breeding methods are employed. The breeding dynamics

Table 9.3 Comparison of backcrossing efficiency using marker-assisted selection

<table>
<tr><th>Breeding generation</th><th>Average elite contribution (%)</th><th>Potential elite contribution using markers [65]</th></tr>
<tr><td>F1</td><td>50</td><td>Markers not useful</td></tr>
<tr><td>BC1</td><td>75</td><td>83.8 %</td></tr>
<tr><td>BC2</td><td>87.5</td><td>91.2 %</td></tr>
<tr><td>BC3</td><td>93.75</td><td>97.3 %</td></tr>
<tr><td>BC4</td><td>96.88</td><td rowspan="2">Markers effective but small differences</td></tr>
<tr><td>BC5</td><td>98.44</td></tr>
</table>

and trade-offs for simultaneous selection for multiple genes require consideration of specific systems and resource availabilities; however the potential to eliminate environmental effects on amylose content and remove laboratory sampling error provides compelling reasons to employee MAS.

As with all marker projects, economics of cost must be considered, and row costs, quantitative laboratory costs, and marker costs must be balanced. The continuing evolution of reduced laboratory costs, not only in genomics but in spectrophotometry, proteomics, and data analysis, continues to change the equilibrium of breeder formulae and facilitates new avenues of germplasm evaluation and advancement.

Use of Haploids

Haploid breeding, prominently used in dent corn breeding, can be effectively implemented with specialties. The relative cost to produce inbreds by use of doubled haploids decreases as row costs and specialty trait evaluation and identification costs increase (Table 9.4).

The use of doubled haploids to facilitate single event backcross conversions is not effective unless trait identification costs are very high. Waxy breeding is probably best served by a marker-assisted selection program. Amylose, however, where the recessive amylose-extender gene is expressed in combination with potentially one to three modifiers, suggests a situation where a doubled haploid approach would hold benefit. If there are five genes needed for top-end amylose inbred development, using the F2 approach would require the evaluation of over 1,000 plants (40 nursery rows) and could potentially cost many more times as much as using doubled haploids.

Many traditional breeding approaches for amylose inbred line development have been used. Recurrent population development and pedigree line development are likely much more effective approaches than F2 line selection; however, the major advantages of haploid breeding are still apparent. Homozygous lines are obtained quickly, genetic recombination is maximized, environmental error is reduced, and a smaller number of individuals are needed for gene pyramiding. The major difficulty in using doubled haploids is the requirement of specialized labor and knowledge to perform the technique and limited access to inducer genetics. Outsourcing doubled haploid line generation can provide a convenient and economical solution for many specialty breeding programs.

Table 9.4 Cost comparisons of maize inbred production using F2 and doubled haploids

	Selfing to F2 generation		Use of doubled haploids		Costs – $47/DH, $5/trait analysis		Costs – $47/DH, $10/trait analysis	
Genes	Probability of homozygosity	Number needed	Probability of homozygosity	Number needed	F2 cost ($)	DH cost ($)	F2 cost ($)	DH cost ($)
1	0.2500	4	0.5	2	20	104	40	114
2	0.0625	16	0.25	4	80	208	160	228
3	0.0160	64	0.125	8	320	416	640	456
4	0.0040	256	0.0625	16	1,280	832	2,560	912
5	0.0010	1,024	0.03125	32	5,120	1,664	10,240	1,824
6	0.00025	4,096	0.015625	64	20,480	3,328	40,960	3,648
7	0.00006	16,384	0.007813	128	81,920	6,656	163,840	7,296
8	0.00002	65,536	0.003906	256	327,680	13,312	655,360	14,592
9	0.000004	262,144	0.001953	512	1,310,720	26,624	2,621,440	29,184
10	0.000001	1,048,576	0.000977	1,024	5,242,880	53,248	10,485,760	58,368

Genetic Engineering

Genetic engineering has potential to expand the functionality of starch dramatically. As mentioned previously making waxy or amylose-extender dominant would greatly facilitate breeding efforts, it simplifies backcrossing, reducing testcrossing and trait verification self-pollination steps.

A transgenic amylose-extender that includes an enhancing modifier construct would reduce breeding difficulties in dealing with independently segregating modifiers. Resequencing the promoter code of starch biosynthetic genes to upregulate expression can effectively increase starch content [66]. Another highly interesting use is gene sequencing and genomic comparisons, genes from different species can be used for comparison purposes, and potentially maize genes could be altered to be more like those of cassava or potato.

Cassava differs greatly from corn in functionality and granule structure, and the genetics behind these differences may be transferable. Some aspects of the root and tuber starches are a result of starch development in the subsoil environment. It would be very interesting to better understand the associated genetic contributions of characteristics like the increased phosphate content observed in potato starches.

The Enogen® transgenic event and other similar approaches have a high potential for reward but also a significant risk component. The ethanol industry is a multibillion dollar industry; capturing even a portion of that market could be very lucrative. The risks, however, include the possibility that the event escapes grain channels and enters the food or other non-approved markets. The StarLink™ corn recall is a stark example of that risk and has been estimated to have cost the Aventis company and US corn producers hundreds of millions of dollars [67].

Given that risk can be managed, it would be very interesting to consider the possibilities of including branching enzymes or other proteins that could be activated postharvest in the starch manufacturing stage. The proteins could be embedded in the granule and activated during wet milling, starch drying, or through a specialized manufacturing step. Starch chains could be broken and reformed to provide cross-linking, improving granule integrity and resistance to sheer, heat, or other stresses. Such an approach could greatly impact starch chemical modification and potentially provide safe and sustainable materials for many unique industrial starches.

Commercial and Foundation Seed Production

Seed production for specialty maize is much like that used in production of other commercial corn seed; however, because of the very specific end use of waxy and amylose grain, control and reduction of seed contamination are priorities. Commercial dent seed production requirements include an isolation distance of not less than 660 ft from any other corn, greater than 99 % of seed parents must be

detasseled prior to 5 % silk emergence, and less than 0.1 % of the pollen-parent plant can be offtype at pollen release.

Sold seed must meet tag requirements stating that at least 98 % of the seed is the hybrid listed. Purity determinations can be made using genetic markers or field grow-outs of seed lots and graded samples. Waxy and amylose seed conform to these requirements, but given the value-added nature of the grain, efforts are often made to exceed 98 %.

Beginning with breeder seed, contamination evaluation must be as thorough and as complete as is possible. Isolation distances far greater than 660 ft are commonly used especially for the production of foundation seed, or if the pollen parent is not a strong shedding inbred. Numerous field visits during the production are required to monitor and execute rouging and removal of offtype materials.

Evaluation of ears prior to shelling provides a critical opportunity to use visual phenotypic selection to remove any dent or obviously offtype ears. While ear inspection is not feasible for large acreage productions, it can be a very important step in breeder seed and small foundation increases. Pollen and seed parent synchronicity is necessary to reduce blow-in contamination. Delayed pollen will result in higher contamination at the base of the ear, whereas delayed silking can increase contamination at the ear tip.

Kernels at the base of the ear tend to be larger rounds or flats and at the tip are typically smaller rounds. A thorough evaluation of seed conditioning lots can provide a very descriptive analysis of the production, and problematic seed lots can be discarded. Because waxy and amylose seed productions are often smaller than dent productions, attention must also be paid to postharvest contamination sources. These include equipment cleanout and drier and bin-storage cleanouts to reduce potential for contamination.

Market Challenges for Specialty Starch Maize

The major market challenge for the development of specialty starch hybrids is to reduce production and grain premium costs. Premiums for waxy and amylose grain are calculated based on formulations that take into account grain availability, local costing basis, deliver costs, seed cost, increased cost for water, fungal or insecticidal applications, specialty drying, costs of identity preservation, grain channeling, and quality controls, but more than all of these things, the premium is paid based on the yield of the specialty hybrid. All efforts have to be made to ensure the specialty hybrid performance matches pace with yield advances in regular field corn.

Adaptation of the newest technologies provides new opportunities in breeding for industrial maize starches, and those that are most profitable need to be captured and capitalized. Importantly, markets need to be identified where industrial starches are the go-to choice, either because of functionality, sustainability, or resource availability. Specialty grain production is a growing market with many exciting future possibilities.

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Chapter 10
Cotton Breeding for Fiber Quality Improvement

Greg Constable, Danny Llewellyn, Sally Ann Walford, and Jenny D. Clement

Abstract Cotton (*Gossypium hirsutum* L.) is the world's leading fiber crop, grown or processed in many countries, providing a major contribution to their economies. Yield is economically most important to a producer which drives cultivar development and adoption; however, fiber quality is the primary focus for spinning mills. Cotton fiber quality must improve to remain competitive with synthetics due to increased demands for lightweight casual garments which require longer, stronger, and finer fibers. Improved cotton yields and fiber quality have continued to be realized through science-based plant breeding, particularly in countries and production systems with suitable climate and appropriate management inputs to maximize those improvements. The most significant challenge for cotton breeders has been to combine high yield with improved fiber quality, due to negative associations between yield and quality attributes in *G. hirsutum*. This chapter highlights practices to enable simultaneous improvement of yield and fiber quality during conventional breeding. There are adequate genetic resources available for innovative cotton breeders to make more progress, but new tools being offered by modern molecular technologies will achieve those gains more efficiently. Advances in fiber quality science have been made in cotton biotechnology – by improving our understanding of fiber development phases that contribute to fiber quality through gene discovery, genome mapping, and identification of linked molecular markers. Novel biotechnology traits have the potential to improve fiber yield and quality by altering the developmental phase associated with fibers per seed, fiber length, strength, and fineness. Biotechnology tools to facilitate improved conventional breeding through marker-assisted selection are also under development, particularly high-throughput techniques based on single nucleotide polymorphisms derived from next-generation sequencing. There are clearly great opportunities for better integration of conventional breeding and molecular biology, and as new GM traits are developed, a future challenge will be to combine multiple GM traits into elite cultivars. This could be assisted by the judicious use of molecular markers to herald a new age in cotton improvement. Cotton is one of the pioneer crops for

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the introduction of genetically modified (GM) insect and herbicide resistance, with about 80 % of global cotton being GM by 2012. That experience of research and deployment of these first-generation GM traits provides the foundation for development and exploitation of GM novel fiber property traits in the future.

Keywords Cotton • Fiber quality • Plant breeding • Genetic resources • Genetic diversity • Fiber development • Genetic engineering • Transgene • Fiber biotechnology • Transcription factor • Quantitative trait loci • Marker-assisted selection • Marker-assisted backcrossing • Single nucleotide polymorphism • Next-generation sequencing • Genome sequencing

Abbreviations

AFIS	Advanced Fiber Information System
AFLP	Amplified fragment length polymorphisms
AOSCA	Association of Official Seed Certifying Agencies
BAC	Bacterial Artificial Chromosome
CesA	Cellulose synthase A
CS-B	Chromosome substitution
CSIRO	Commonwealth Scientific and Industrial Research Organisation (Australia)
DNA	Deoxyribonucleic acid
dpa	Days post anthesis
eQTL	Expression quantitative trait loci
FMT	Fineness and maturity tester
GBS	Genotype-by-sequencing
GM	Genetically modified
GS	Genomic selection
HD	Homeodomain
HVI	High volume instrumentation
IAA	Indole-3-acetic acid
ISTA	International Seed Testing Association
KAP61R	Keratin-associated protein
KASPar	KBioscience competitive allele-specific polymerase chain reaction assay
MABC	Marker-assisted backcrossing
MAS	Marker-assisted selection
MYB	V-Myb avian myeloblastosis viral oncogene homolog
NGS	Next-generation sequencing
NIL	Near-isogenic line
OECD	Organisation for Economic and Co-operation and Development
PCR	Polymerase chain reaction
PHB	Polyhydroxybutyrate
QTL	Quantitative trait loci

RAD-Seq	Restriction site-associated DNA sequencing
RAPD	Restriction fragment length polymorphism
RIL	Recombinant inbred line
RNAi	RNA (ribonucleic acid) interference
SCW	Secondary cell wall
SNP	Single nucleotide polymorphisms
SSCA	Southern Seed Certification Association
SSR	Simple sequence repeats
SusA1	Sucrose synthase A1
TILLING	Targeting induced local lesions in genomes
TM-1	Texas marker-1
XTH	Xyloglucan endotransglycosylase

Introduction

Cotton is the generic term for a small number of indeterminate perennials of the *Gossypium* genus that produce long fibers on its seed coat, which develops in a fruit or boll. Cotton is used in about 35 % of global textiles because yarn made from cotton fiber is soft, absorbent, cool, comfortable, and durable. It is grown as an annual crop, important economically in agriculture and industry, and grown in up to 100 countries.

Cotton fibers are comprised of nearly pure cellulose and represent the main economic value or primary yield, due to its use in yarn production. Two types of fibers can develop on cotton seeds, 20–30 mm long, easily detached fibers called lint and a much shorter linter or fuzz fibers only a few millimeters long that develop later and interspersed between the lint fibers in most species (some species/cultivars have a naked seed that lacks linters).

Physical and chemical attributes (fiber quality) vary by species, cultivar, region, or farm and affect their potential usage and hence value in the textile market. There are many measures of fiber quality used in marketing to indicate the fiber value for spinning into yarn. Cotton breeders use these measures in developing new cultivars with improved fiber properties. The linters contribute little to yield, but have value as fillers in cosmetics and paper products or as chemical feedstocks for acetate production. The oil- and protein-rich seeds have value as animal feed (mainly for ruminant animals, as these seeds also contain toxic secondary chemicals such as gossypol) and for their extractable oil commonly used as frying oil. Cotton is inadvertently one of the world's largest oilseed crops, although mainly grown for its seed fiber.

By 2013, much of the world's cotton was genetically modified (GM) with novel traits for better insect and weed control. These include the insecticidal genes from *Bacillus thuringiensis* commercialized as Bollgard II® and the herbicide tolerance genes from *Agrobacterium* sp. (Roundup Ready Flex® trait) or *Streptomyces* sp. (LibertyLink® trait) conferring tolerance to glyphosate and glufosinate,

respectively. These traits have revolutionized cotton pest and weed control and made life considerably simpler for cotton growers. As of yet, there are no GM traits for fiber quality that have been commercialized.

In this chapter, we will focus on the background, methods, and future opportunities for simultaneously improving yield and fiber quality in *Gossypium hirsutum* ("upland") cotton. We will particularly highlight opportunities and potential for integrating conventional breeding with future biotechnology traits and molecular tools.

Taxonomy, Early Domestication, and Selection in Cotton

Cotton taxonomy has been widely studied since the mid-nineteenth century because of its industrial importance with 50 or more *Gossypium* species believed to have appeared 10–20 million years ago in three centers of diversity: Australia, Africa-Arabia and, Central America [1, 2]. Recent comprehensive reviews on history and taxonomy of *Gossypium* species include Fryxell [3, 4], Brubaker et al. [5], and Percival et al. [6]. These reviews are recommended for more detail than presented below.

Cotton is divided into eight diploid genome groups with $2n = 26$ chromosomes plus five tetraploid species ($2n = 52$) (Table 10.1). The A genome diverged from genomes B, E, and F, 4–9 million years ago in Africa-Arabia [2], and the two important A genome species with spinnable fibers, *G. arboreum* and *G. herbaceum* appeared over 1 million years ago [8]. The tetraploid species appeared in Mexico-Guatemala, 1–2 million years ago from a chance hybridization and chromosome doubling between A and D genome ancestors of *G. arboreum* and *G. raimondii*, respectively [9], and subsequently radiated into the five tetraploid species, *G. hirsutum*, *G. barbadense*, *G. mustelinum*, *G. darwinii*, and *G. tomentosum* of which only the first two are used in agriculture.

There are therefore only four species grown commercially because they produce fibers long enough to be spun into yarn for textiles, and these are *G. hirsutum* (upland cotton), *G. barbadense* (Pima, Egyptian or Sea Island cotton), *G. arboreum* (desi cotton), and *G. herbaceum* (Levant or Arabian cotton). *G. hirsutum*, because of its higher fiber yields and greater adaptability, is by far the most widely grown with over 90 % of world production from this species, followed by *G. barbadense* (6 %) and *G. arboreum* and *G. herbaceum* (less than 2 % each).

The cultivated diploid and tetraploid species differ significantly in their morphology and agronomic performance, but also in their fibers, although that distinction is narrowing with focused genetic improvement of all commercial species. Table 10.2 shows the typical fiber properties of the two cultivated tetraploid species compared with fiber from the diploid *G. arboreum*. Although there is variation between cultivars within each species, the shorter, weaker, and coarser fiber of *G. arboreum* compared with the long fine fibers of *G. barbadense* dictates different

Table 10.1 List of species identified in the Genus *Gossypium*

Subgenus	Species	Genome	Origin
Sturtia	*G. sturtianum* J. H. Willis	C_1	Australia
	G. robinsonii F. Mueller	C_2	Australia
	G. costulatum Todaro	K_1	Australia
	G. populifolium (Bentham) F. Mueller ex Todaro	K_2	Australia
	G. cunninghamii Todaro	K_3	Australia
	G. pulchellum (C. A. Gardner) Fryxell	K_4	Australia
	G. pilosum Fryxell	K_5	Australia
	G. anapoides Stewart Wendell, and Craven	K_6	Australia
	G. enthyle Fryxell, Craven, and Stewart	K_7	Australia
	G. exiguum Fryxell, Craven, and Stewart	K_8	Australia
	G. londonderriense Fryxell, Craven, and Stewart	K_9	Australia
	G. marchantii Fryxell, Craven, and Stewart	K_{10}	Australia
	G. nobile Fryxell, Craven, and Stewart	K_{11}	Australia
	G. rotundifolium Fryxell, Craven, and Stewart	K_{12}	Australia
	G. bickii Prokhanov	G_1	Australia
	G. australe F. Mueller	G_2	Australia
	G. nelsonii Fryxell	G_3	Australia
Houzingenia	*G. thurberi* Todaro	D_1	Mexico
	G. armourianum Kearney	D_{2-1}	Mexico
	G. harknessii Brandegee	D_{2-2}	Mexico
	G. davidsonii Kellogg	$D_{3\text{-}d}$	Mexico
	G. klotzschianum Andersson	$D_{3\text{-}k}$	Galapagos Islands
	G. trilobum (DC) Skovsted	D_8	Mexico
	G. turneri Fryxell	D_{10}	Mexico
	G. aridum (Rose and Stanley ex Rose) Skovsted	D_4	Mexico
	G. raimondii Ulbrich	D_5	Mexico
	G. gossypoides (Ulbrich) Standley	D_6	Mexico
	G. lobatum H. Gentry	D_7	Mexico
	G. laxum Phillips	D_9	Mexico
	G. schwendimanii Fryxell and S. Koch	D_{11}	Mexico

(continued)

Table 10.1 (continued)

Subgenus	Species	Genome	Origin
Gossypium	*G. herbaceum* L.	A_1	Africa, Arabia
	G. arboreum L.	A_2	Africa, Arabia
	G. anomalum Wawra and Peyritsch	B_1	Africa
	G. triphyllum (Harvey and Sonder) Hochreutiner	B_2	Africa
	G. capitis-viridis Mauer	B_3	Cape Verde Islands
	G. longicalyx B. Hutchinson and Lee	F_1	Arabia
	G. benadirense Mattei	E	Africa, Arabia
	G. bricchettii (Ulbrich) Vollesen	E	Arabia
	G. vollesenii Fryxell	E	Arabia
	G. stocksii Masters in Hooker	E_1	Arabia
	G. somalense (Gurke) J.B. Hutchinson	E_2	Arabia
	G. areysianum Deflers	E_3	Arabia
	G. incanum (Schwartz) Hillcoat	E_4	Arabia
	G. trifurcatum Vollesen	B	Somalia
Karpas	*G. hirsutum* L.	$(AD)_1$	Mexico
	G. barbadense L.	$(AD)_2$	Peru, Ecuador
	G. tomentosum Nuttall ex Seemann	$(AD)_3$	Hawaii
	G. mustelinum Miers ex Watt	$(AD)_4$	Brazil
	G. darwinii Watt	$(AD)_5$	Galapagos Islands

Based on data from Refs. [4, 6, 7]

Table 10.2 Typical fiber quality of three cotton species

Species	Fiber length (mm)	Fiber strength (g/tex)	Fiber linear density (μg/m)	Micronaire
G. barbadense	36	45	150	3.8
G. hirsutum	29	30	170	4.2
G. arboreum	24	20	187	6.0

Based on data from Ref. [10] (*G. arboreum*)

end use and quality of yarn produced after spinning and hence has different economic value.

Man has been successful in domesticating many crops and animals. That capability defines human endeavor because otherwise our species would not be so numerous. It is understandable that food crops (wheat, barley, maize, potatoes) were domesticated before cotton [11], but the domestication process has been similar for all crops through a process of conscious or unconscious selection for those characteristics that made it easier to grow, collect, and utilize the useful products of the plant. By 5,000–7,000 years ago, man had already heavily influenced the utility of the fiber of *Gossypium* species for the production of yarn to be used to make fabric, particularly in India and the Central Americas [5, 12, 13].

After European colonization of the Americas, *G. hirsutum* and *G. barbadense* were further selected for their ability to be cultivated commercially, and modern hybridization and breeding were eventually used to create the current range of cultivars. An account of the early history of cotton in the southern USA gives an insight into more recent selective breeding of cotton [14]. Between 1800 and 1850 in Mississippi, there was an increase in productivity of *G. hirsutum* cotton as a result of developments in "breeds." This innovation was stimulated by a demand for cotton as a result of industrialization, so better yield and fiber quality (price) were required. The main types grown up to 1800 were "Georgia Green Seed" and "Creole Black Seed," both imported from other environments. Around 1805, Burling collected seed from Indians in Central Mexico that had better plant type, improved fiber quality, and resistance to boll rot. This seed ("Burlings Mexican") was shared with neighbors and was used into the 1830s. However, with no pure seed scheme, all "cultivars" became mixed physically and by natural cross-pollination. In 1824, Price selected a line ("Prices Mexican") that was further selected in 1833 by Nutt, who carefully chose planting seed by its appearance and developed the "Petit Gulf" cultivar. This was also used in Georgia. In 1845, Vick selected planting seed (by mass selection) from the best Petit Gulf plants in the field to produce the "Hundred Seed" cultivar. Many cultivars were subsequently developed from this line [15]. By this time, the cotton seed business was already a profitable industry in itself. In 1851, Phillips proposed that new cotton cultivars could be produced by direct transfer of pollen from one cotton plant to the stigma of another and heralded the birth of modern cotton breeding.

Thus, in little over 50 years, the cotton industry in Mississippi had progressed from simply sowing imported non-adapted seed to a vibrant business utilizing new cultivar development regimes. The same principles of discovery of local plant types, testing them for suitability in growing and spinning, then sharing with family and neighbors would have applied in India and Central America over the previous 5,000 years. However, the speed of this process was more rapid in the 1800s, with better communication in letters, magazines, and newspapers, and also the commercial incentive was greater in marketing of both the fiber and seed of newly developed cultivars, many of them eventually moving across international borders to dominate global cotton production.

Areas of Production

The cotton production system covers a wide range of practices across the world. There are many small-scale production systems that are hand sown, relatively low in inputs, and hand harvested. At the other extreme are large-scale row crop production systems, fully mechanized, and with high input of pesticide, water, and fertilizer. For mechanical harvest, the crop requires prior chemical defoliation to remove leaves and ensure low trash content. Harvested seed cotton is ginned to separate seed from the lint. The lint is pressed into bales, ranging in size from 100 to

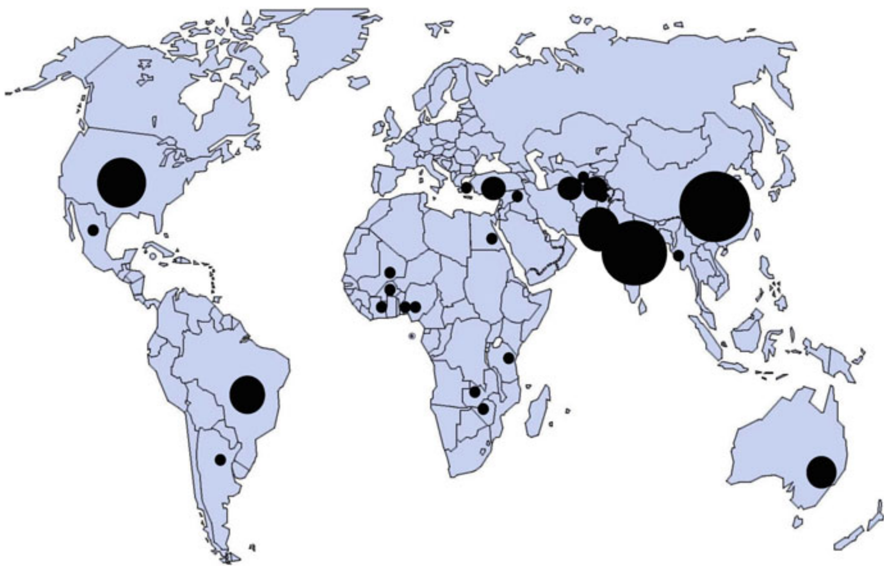

Fig. 10.1 Major cotton-producing countries. Area of each circle is proportional to total cotton produced in 2011 (Based on data from International Cotton Advisory Committee)

227 kg (depending on country), for transport to spinning mills where the bale is opened and the lint processed through spinning. Fuzzy seed from ginning is crushed for oil or used as a stock animal feed.

Although originating from more tropical environments, human selection, breeding, and changes in agronomic management have allowed cotton to expand into more temperate regions (outside its original area of evolution), and now the bulk of production is well removed from the equator (see Fig. 10.1). World output of cotton was over 27 million tonnes in 2011 representing about 35 % of global textile fiber. It was produced on less than 36 million hectares spread across up to 100 producing countries [16]. The top ten producers, however, generate over 90 % of the worlds crop, with China and India together amounting to half of all the cotton harvested. The other major producers in decreasing order of output were the USA, Pakistan, Brazil, Australia, Uzbekistan, and Turkey (Table 10.3). India has by far the largest area under cotton cultivation (over 12 million ha), but its relatively low yields relegate it to only the second largest producer.

The importance of yield in modern cotton production systems cannot be overstated, particularly in an era with fierce competition from synthetic textiles. High yield ensures cheaper cost of production per unit of fiber, and yield-gross margin will keep the crop profitable to encourage the choice of cotton as a crop by growers. There is a wide range in national yields across the major producers (Table 10.3), and this is a reflection of the differing levels of fragmentation, mechanization, irrigation, and chemical inputs and varying agronomic and environmental challenges in those different countries. Around half of the world crop is irrigated, and the rest is either rain-grown or only partially irrigated [17]. Figure 10.2

Table 10.3 World production of cotton 2011/2012

Country	Harvest area (10^3 ha)	Lint yield (kg/ha)	Production (10^3 tonne)	Net export (10^3 tonne)	Net import (10^3 tonne)	Mill use (10^3 tonne)
World	**35,726**	**749**	**27,884**			**24,957**
China	5,500	1,326	7,605		3,638	10,033
India	12,199	482	6,129	1,260		4,427
The USA	3,945	865	3,558	2,512		772
Pakistan	3,200	680	2,270		182	2,361
Brazil	1,400	1,400	2,043	863		976
Australia	600	1,815	1,135	908		9
Uzbekistan	1,340	683	953	624		284
Turkey	480	1,407	704		528	1,203
West Africa	1,710	371	662	530		43
Mexico	190	1,375	272		182	386
Bangladesh	36	399	15		715	749
Indonesia	9	605	6		427	431
Vietnam	10	501	5		341	352

Based on data from Ref. [16]

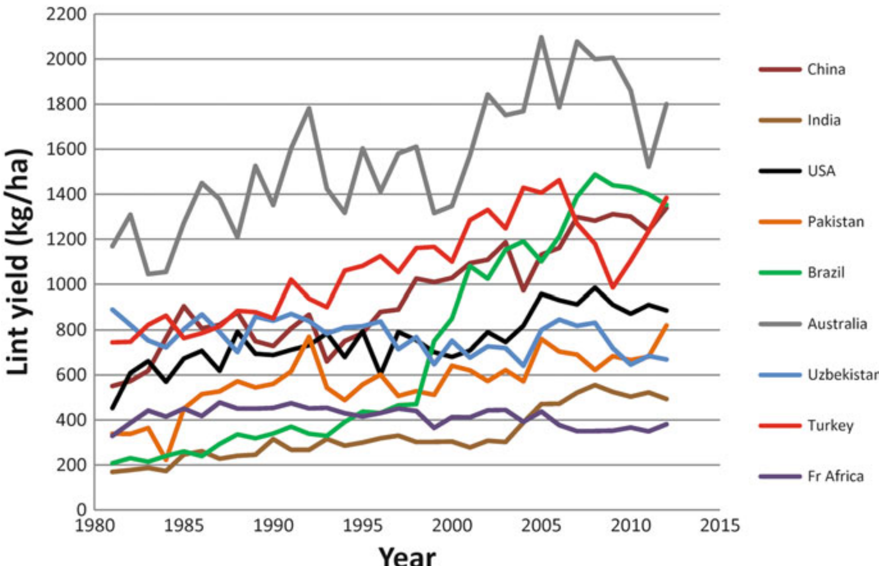

Fig. 10.2 Yield of cotton from 1980 to 2011 from a number of countries. French Africa includes Benin, Burkina Faso, Cameroon, Chad, Ivory Coast, Mali, Senegal, and Togo (Based on data from International Cotton Advisory Committee)

shows the last 30 years of yield records for nine countries to illustrate a range of yields and historical yield levels. It is notable that countries with favorable climate and/or irrigation such as Brazil, China, Australia, and Turkey have relatively high yield and have increased in yield by more than 20 kg lint/ha/year over a 30-year period. French Africa and Uzbekistan are trending to yield reduction over the same period, while India, Pakistan, and the USA are intermediate, in some cases, because of a high proportion of rain-fed production.

Genetic Resources Available for Cotton Improvement

In breeding, there is a central role to be played from coordinated interactions between cotton germplasm collection, maintenance and utilization, targeted improvement programs, and the modern science of genomics [18]. The genetic resources available for cotton have been growing rapidly over the last 10 years and have been extensively reviewed [6, 7, 19–22]. These resources include: a number of significant collections of *Gossypium* species and cultivars totaling over 50,000 accessions (the bulk being cultivars), novel cytogenetic stocks with targeted additions or deletions of specific chromosomes or chromosome segments from different species, large mapping families, and genetic populations including immortalized recombinant inbred lines (RILs) and near-isogenic lines (NILs). There are also high-density genetic maps and DNA marker databases, physical resources of large insert DNA libraries such as bacterial artificial chromosomes (BACs) from several *Gossypium* species, extensive expressed sequence tag (EST) collections from a relatively limited number of species, and more recently whole assembled *Gossypium* genomes.

The International Cotton Genome Initiative was formed in 2001 to coordinate cotton genomics research, and as a result, more comprehensive genetic maps for the diploid and allotetraploid cotton genomes were published [23] or available through accessible Internet resources [24]. Public marker discovery is also gaining momentum from these new genomic resources, and large consortia are being formed to pool data across academia and other publicly funded research programs toward some broadly useful tools for use in genomic-assisted plant breeding. This coordination of efforts has increased the number of markers available for QTL analysis and positional cloning and provided a solid basis for the more complete sequencing of the cotton genome starting with the smaller, less complex D genome of *G. raimondii* [25, 26].

Is the Genetic Diversity of Current Cotton Germplasm Limiting Breeding Progress?

Despite the considerable achievements of the past 160 years of science-based cotton breeding, concerns are beginning to be raised of a leveling off in yield progress (note yield progress of different counties in Fig. 10.2). There have clearly been a number of bottlenecks in the ancient and modern history of the development of current cotton cultivars, starting with the original hybridization and chromosome doubling more than a million years ago to produce the original tetraploid cotton, followed by domestication through selection by ancient tribes in the last 7,000 years, then selection by Europeans over the last 200 years, including "genetic cleanup" of late maturing cultivars after the boll weevil crisis in the USA in the 1930s [27]. Because of these bottlenecks, there is a perception of low diversity and a narrow genetic base for most of today's cottons.

There are two schools of thought on the extent and limitations of the genetic diversity in cotton. One view, based on the low rate of DNA polymorphism found in *G. hirsutum* [28–30], is that genetic diversity is low relative to other crops and that this leaves commercial cotton crops in danger of a yield plateau and susceptibility to pest and disease outbreaks [31]. There is now growing evidence in the USA, where yield progress is slowing down, that it is critical new sources of variation be injected into those programs [32].

The other view is that DNA sequence diversity does not fully measure genetic diversity. Many conventional breeders still note and exploit the ability to make substantial changes to plant phenotype and performance by hybridization and targeted selection. Meredith [33], for example, commented that "the occurrence of unusual positive genetic deviates has puzzled cotton breeders for a long time." Furthermore, Bowman et al. [34] studied the genetic base of US germplasm in 1970–1990 and even though up to 95 % of modern cultivars had descended in part from a small number of the mid-nineteenth century cultivars, such as Petit Gulf. They concluded that the genetic base of 1990 cultivars was not narrow based on the broad range of agronomic performance characteristics being generated in more recent cultivars and predicted further significant performance improvements would continue to be made in cotton breeding.

This incongruity suggests that there may be another level of variability. Epigenetic regulation occurs in plants through changes in the methylation status of the cytosine residues of the DNA or changes in chromatin structure through chemical alterations to the histones around which the DNA is packaged [35]. These epigenetic changes could be contributing to variation in plant phenotypes, not accounted for by any sequence variation between individuals. There has been considerable interest in how DNA methylation might act within the different genomes when a polyploid, like tetraploid cotton, has been formed and how it might affect the expression of homoeologous genes [36], but the potential for such epigenetic regulation to contribute to agronomic performance remains largely unstudied. As next-generation sequencing (NGS) technologies can be used to study patterns of

DNA methylation, it is likely more information about the cotton epigenome will be uncovered over the next decade, and ways of exploiting this in breeding or even management will be explored.

While there are questions about whether the overall genetic diversity of modern cotton cultivars is narrowing and limiting genetic gain, most would accept that breeding programs should ensure they maximize genetic diversity within their material. Maintaining access to germplasm resources will therefore be important in cotton breeding. Breeders will need to actively introgress interesting and important traits from non-adapted germplasm including wild cotton relatives, as well as access lines or cultivars from other breeding programs that may have similar goals.

Utilization of Germplasm Resources in Modern Cotton Improvement

Traditionally, and in increasing order of complexity and effort, there are three gene pools, primary, secondary, and tertiary, that can be accessed for genetic improvement of a crop like cotton. Given the dominance of *G. hirsutum* in the world production, most of the global breeding effort is toward that species. The primary gene pool is other tetraploid cottons with the same A and D genome complement that are sexually compatible with *G. hirsutum*. For example, considerable time and effort has gone into interbreeding *G. hirsutum* and *G. barbadense* cultivars to move agronomic traits in both directions improving productivity and/or quality of cultivars in both species [8, 19, 20], although those efforts appear to have mostly run their course. Race cottons, often domesticated but largely unimproved *G. hirsutum* or *G. barbadense* accessions, also offer a potential source of new variability for the cultivated species, but their use has been hampered by a number of factors including their photoperiodicity that makes breeding difficult in more temperate regions away from the equator. This has been partly alleviated by converting them to a day-neutral habit [37], although they are still poorly utilized because of severe yield drag. It remains to be seen whether the promise of marker-assisted selection (MAS) (see sections "Molecular approaches to yield and quality enhancement" and "Breeding strategies and integration of new biotechnology") will improve upon the current level of trait introgression between and within these different species. Most breeders are reluctant to sacrifice the high level of linkage drag, aberrant phenotypes, and often fertility issues associated with genomic breakdown when crossing these two species (even between elite cultivars) or the loss in agronomic performance when crossing elite cultivars with wild or unimproved material. There is justification for ongoing research on wide introgression. The wild tetraploid cottons (*G. tomentosum*, *G. mustelinum*, and *G. darwinii*) have also been relatively underutilized in cotton breeding because of these same issues, but there is considerable scope for using them in developing intermediate breeding stocks to produce material with enhanced pest, disease, or stress tolerance not available from existing

cultivars or closer relatives. The nectariless trait, for example, has been introgressed from *G. tomentosum* [38].

The secondary gene pool for *G. hirsutum* cotton is represented by a number of diploids with compatible A, D, B, or F genomes, and there are ongoing efforts in a number of public and private programs to introgress unique pest or disease resistance from these species via the synthetic tetraploid route or via synthetic hexaploid bridging species [6]. Such approaches require substantial investment in time and resources (often 10–15 years) to achieve a usable outcome, but there are a number of successful examples, including cytoplasmic male sterility from *G. harknessii* [39] and reniform nematode resistance from *G. longicalyx* [40]. Beasley sourced improvements in cotton fiber strength (reportedly from the D genome contributor), with his triple hybrid ((*G. thurberi* × *G. arboreum)* × *G. hirsutum)* that has made a contribution to the high fiber strength of modern cultivars. The use of molecular markers to accelerate the introgression of traits from these more distant sources is clearly warranted.

The tertiary gene pool consists of those other diploid species with a completely different genome type such as C, E, G, or K that show relatively poor or no recombination with the A or D genome. These include a number of the Australian endemic species some of which have unique traits such as glandless seed-glanded plant that have been the focus of introgression from the C-genome species *G. sturtianum* [41], although without much success and suggest that, in the long run, GM approaches may be more useful for transferring such traits into the cultivated species.

Finally, modern biotechnology, sometimes dubbed the quaternary gene pool, allows the transfer of genes from any organism, for example, Bt cotton expressing the insecticidal genes from the bacterium *Bacillus thuringiensis*. Such techniques can also be used to transfer any trait identified in any plant, including other sexually or nonsexually compatible *Gossypium* species (especially tertiary but also secondary gene pools), into the cultivated forms of cotton, regardless of any ability to generate a viable hybrid between those species. This process is still considered recombinant DNA by GM regulators, so it would still carry a large cost for registration of the traits in global markets, but perhaps lower than for genes from non-plant sources. To this time biotechnology traits have, however, been limited to high-value trans-kingdom transgenes, such as pest and herbicide tolerance, so the likelihood of plant to plant gene transfers being commercialized will depend on the ability of the biotech developers to recapture their investments through trait licenses to seed companies and cotton growers.

Mutants have played a significant part in increasing our understanding of gene action and in dissecting biochemical and developmental pathways in model plants such as *Arabidopsis* and rice and are also being used in cotton. Natural mutations in fiber development, for example, have been critical to the discovery of the key regulatory genes in fiber initiation through genome scale gene expression comparisons between fiberless mutants and wild-type cotton seeds [42]. While induced mutation in breeding was a fad of the nuclear age and did deliver some variation to breeding programs, it was never widely successful. However, with the new

genomics age, it is being revisited as a potentially valuable resource in functional genomics and may have applied uses in breeding when combined with next-generation sequencing (NGS) technologies. Chemical and radiation mutation can be used to saturate the genome, screened for mutations in specific target genes and can be isolated and phenotyped. Such TILLING (Targeting Induced Local Lesions in Genomes) [43] or TILLING by sequencing approaches, particularly in polyploids like cotton, should allow the development of specific gene knockout plants with agronomic potential because of their modified metabolism or biochemistry from loss of that particular gene function. As this is non-GM, it may overcome some of the cost and regulatory hurdles faced by a GM approach such as using gene silencing of the same pathways.

Major Breeding Achievements to Date

The process of domestication and conversion of cotton to an annual were obviously the first steps in enhancing lint yield by man. It was a major step in changing a tropical-subtropical perennial shrub with relatively poor fiber and low productivity into a temperate annual crop with fiber suited for textile production. There have been many significant achievements in cotton breeding since the first deliberate attempts to apply crossing and selection to improve performance. Tolerance to pests, diseases, and adverse environmental conditions all contribute to protecting yield and quality but are not covered here in detail as they do not directly enhance yield potential or fiber quality.

Fiber Yield

Being morphologically indeterminate, cotton does not stop growing vegetatively to start reproductive growth and thus requires a relatively long growing season to maximize yield. This indeterminacy, while it allows the crop to compensate for damage or fruit loss throughout the season by producing more fruits, can also extend the growing season and increase production costs. Cotton breeders have addressed this by targeting crop earliness. Early or short-season genotypes are considered more determinate when compared with late-season (indeterminate) genotypes; they flower rapidly, and the length of time to boll opening is reduced. This is advantageous for milder climates and has been the major aid in breeding for regional adaptation. To a degree, earliness can reduce loss from insects and diseases and minimize inputs such as fertilizer or irrigation. However, utilizing the fullest growing season maximizes yield potential in cotton, so optimizing season length, productivity, and costs is a challenge for agronomy and breeding.

Yield is a complex trait and can be broken down into a number of components: the weight of fibers per seed, the number of seeds per boll, and the number of bolls

per plant or unit planted area [44]. The proportion of lint removed from seed cotton is the lint fraction and is positively correlated with yield. It has provided an indirect and cost-effective way to breed for improved yield potential [45, 46], with increases of approximately 10 % over the past 60 years [47]. However, lint fraction can also be negatively correlated with seed size, potentially reducing seedling vigor [48, 49].

There can also be an interaction between breeding and management, with modern cultivars being more responsive to improved agronomy than older cultivars [50]. Yield improvement in national datasets with lint yield >1,000 kg/ha are more likely to have larger contributions of breeding to yield improvement than low yielding systems, but a common result is for breeding to contribute approximately 50 % of yield improvement, with management contributing the rest [51]. Disease resistance has also been important in protecting yield [52].

As noted in the Introduction, cotton has been one of the pioneer crops introducing transgenic or GM traits for insect and herbicide resistance, reducing cotton's environmental footprint [53]. Since the original introduction of Monsanto's Bollgard® insect resistant trait in 1995, the area has grown rapidly, and approximately 80 % of global cotton area in 2012 was transgenic [54]. In Australia, GM insect resistance has been shown to give an 80 % reduction in insecticide use compared with conventional cotton [55]. Likewise, a glyphosate resistance trait can reduce the need for residual herbicides by 50 % [55] and makes weed control relatively reliable and simple for growers.

Fiber Quality

In the last 50 years, giant strides have been made in plant breeding to improve fiber quality. In fact, the definitions of preferred levels of fiber quality properties are constantly moving as spinners demand improvements to facilitate faster spinning and weaving (reflected in base quality, discussed in section "Current trait targets and breeding goals"). Figure 10.3 shows progress in the decade up to 2010 with fiber length, strength, and micronaire in the USA, China, India, and Australia. Note values for each fiber property are averages of a wide range of cotton with different management, climate, and cultivar (China reached up to 579 cultivars in 2008 [56]). For example, for an average national micronaire of 4.3, there will typically be about 10 % of the cotton with micronaire less than 3.5 and 10 % greater than 5. In all these countries, there has been a conscious decision by the cotton industry and breeders to improve fiber quality to meet market demands.

Although there was little progress and even some decreases in fiber length and strength in US cotton from the 1940s to 1980s [57], individual breeding programs have demonstrated improvements in fiber length and strength through time (e.g., Bassett and Hyer [58] in California; Zhang et al. [59] in New Mexico). Since that time, there have been gradual improvements in length of 0.08 mm per year and strength of 0.1 g/tex per year [60].

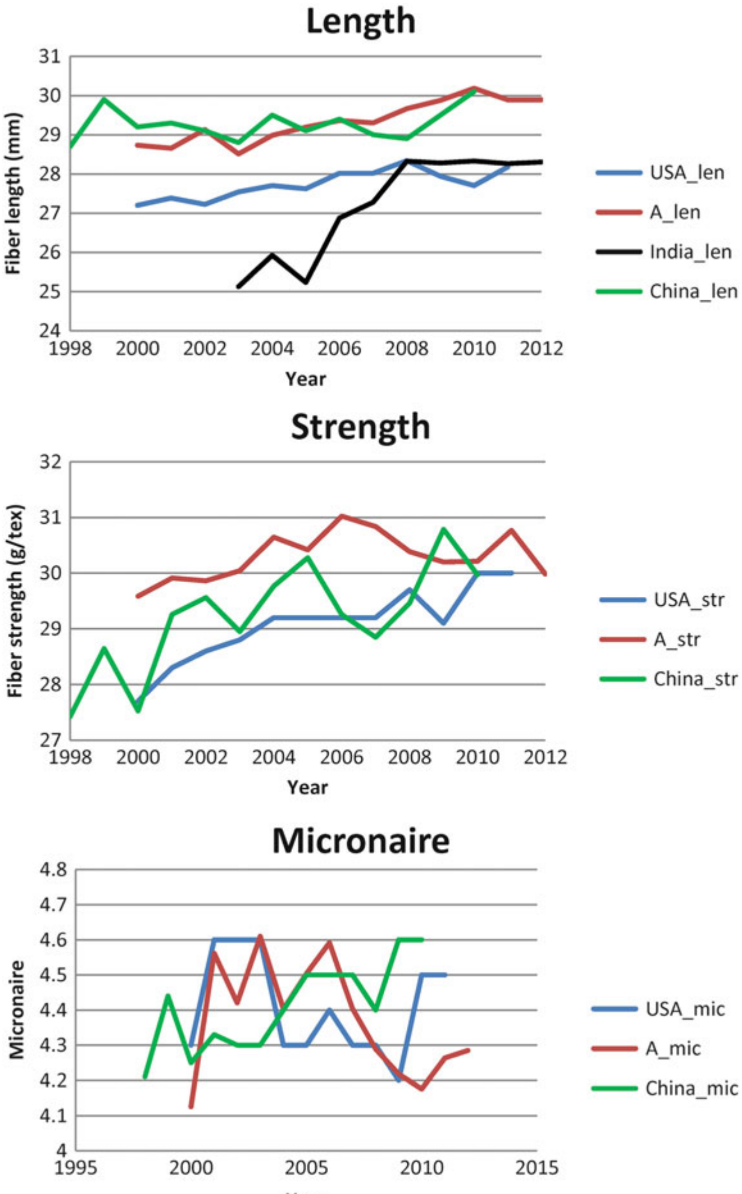

Fig. 10.3 Fiber quality improvement of cotton from 2000 to 2012 for the USA, China, India, and Australia (Based on data from USA data Refs. [60, 64, 65]; China data Refs. [56, 61, 62]; India data Ref. [63]; Australia data Ref. [66])

In Australia, in the 10 years up to 2012, there was a substantial increase in fiber length (0.16 mm per year), but fiber strength was constant at about 30.5 g/tex through the same time and a trend for reduction in micronaire to more favored values between 4.1 and 4.3 (Fig. 10.3). The length and micronaire improvements were the direct result of intensive breeding efforts over the previous 10 years [51]. China reached 30 mm fiber length, while India has reached 28 mm fiber length after great improvements since 2002 to reach the same levels as the USA. China has improved fiber strength over a 10-year period to reach 30 g/tex by 2010. Micronaire is usually influenced more by climate and management than cultivar choice, so micronaire trends through time were variable in all countries.

Current Trait Targets and Breeding Goals

As a breeder, fiber quality improvement is seen as a way to maintain market demand and price for cotton producers and a way for cotton to compete with synthetic fibers in textile mills. Depending on the market, price premiums may be applied for cotton fiber with higher values of length and strength, intermediate micronaire values, and clean grade (as defined below), while conversely, price discounts may apply to cotton fiber that is below industry-defined base levels for key quality parameters. These parameters affect the efficiency of spinning, weaving, and dying which ultimately influence the profitability of the textile industry and in turn the profitability of cotton production.

After ginning, cotton fiber samples are tested for quality by standardized instruments such as a High Volume Instrument (HVI) [67]. The HVI measures the length by creating a "beard." The beard is a clamped section of fiber that is combed to remove any loose fibers; it is scanned from the clamp to the tip of the longest fiber. The upper half mean length is determined by the average of the longest half of fibers [67]. Fiber bundle strength is determined from this beard by using another clamp to apply force. The amount of fibers in the beard and the force required to break them determine the fiber strength.

Micronaire is a measure of fiber resistance to airflow, which is an indirect way to estimate fiber fineness (linear density) and maturity. High micronaire vales (>5.0) indicate a coarse fiber (less fine), while low values (<3.5) indicate immature fibers, both undesirable for spinning. Assessment of fiber economic value based on maturity and linear density separately are slowly being adopted as new instrumentation is developed to measure them at a realistic speed to fit into a standard HVI quality testing stream [68].

Grade currently encompasses color and trash content. Cotton is naturally white and most desirable but due to environmental variables can turn yellow or even gray in extreme situations. Leaf, dust, and other foreign material make up the trash content. Excessive leaf hair is prone to adhere fragments to the cotton lint after defoliation and during harvesting thus increasing trash content. Trash contaminates the cotton and directly impacts the quality of the yarn. Environment and

management predominantly determines grade, but breeding for reduced leaf hair, for example, minimizes trash content.

Cotton fiber is spun into yarn by tightly twisting the fibers together to form a cohesive structure. Yarn quality and overall mill productivity are key concerns for spinners, which make length, strength, and fineness the most attractive traits to buyers. Longer fibers were first recognized by spinners for their efficiency in processing and contribution to yarn strength by providing more surface area contact during twisting. Fiber strength is directly correlated with yarn strength [69] and is valued because stronger fibers can withstand the vigorous processes of spinning. Finer fibers promote strength by allowing more fibers per cross section of a yarn thread.

There are two primary types of spinning, rotor or open-end ring spinning. Rotor spinning is rapid and spins the fiber into yarn without needing a spindle. It is generally used with coarser fibers [70] and, being a more aggressive procedure, generates a larger diameter, weaker yarn compared to ring spinning. Coarse fibers are usually brittle and decrease the total number of fibers in the cross section of a yarn resulting in thick and thin places that are weaker and slow down spinning when the yarn breaks. Rotor spinning has an economic advantage over ring spinning due to its higher output and lower costs; however, it is incapable of producing fine yarns. Ring spinning draws out the fibers between rollers, and then they are spun and wound around a rotating spindle, which in turn is contained within an independently rotating ring flyer. The majority of *G. hirsutum* cotton fiber from the major exporters is destined for ring spinning, so breeding targets need to be adapted to that end use. Ring spinning requires longer and more uniform fibers and produces a finer and stronger yarn [70]. Uniform fiber creates a consistent product with few thin or thick places, preventing breakage or fabric defects later in the production line. Cotton fiber can vary in length due to its location on the seed, creating nonuniform fibers even in a premium quality genotype. This renders cotton less attractive compared to the uniform synthetic fibers, at least for spinning.

Other fiber traits, short fiber index (percent by weight of fibers shorter than 13 mm), elongation (extension before breakage), and maturity, are important for different reasons. Short fiber represents a loss in being combed out in spinning and contributes to a lack of uniformity. Elongation is the amount of elasticity in the fiber, and some elongation is necessary in preventing breakage as a yarn is stretched during spinning or weaving. There is an inverse relationship between strength and elongation, but elongation is important in that a very strong fiber can become brittle and will break if stretched. Fiber elongation is significant in spinning for measuring work-to-break of the yarn [71]. Fiber maturity is the amount of cell wall thickening inside the fiber, and a mature fiber is more advantageous because it contributes to fiber strength. It can also determine how readily dye is absorbed; consistency in maturity is important to guarantee a uniform fabric color. Neps, fiber tangles due to immature fibers, slow productivity and do not absorb dye thus producing light flecks in the woven fabric.

Commercial *G. hirsutum* cultivars already have a range of inherent fiber quality properties, most of which are also affected by environment and management. So, to

Table 10.4 Comparison of the 2012 base HVI fiber quality properties for marketing in Australia and the USA

Target trait	Base USA	Base AUS	Breeding goal	Comments
Fiber length (mm)	26.9	29.0	>29.0	Also high uniformity and low short fiber index. Premium length is 32 mm
Fiber strength (g/tex)	26.0	27.0	>28.0	Also high elongation. Premium strength is 34 g/tex
Micronaire	3.5–4.9	3.5–4.9	3.8–4.5	Linear density 155–180 μg/m; fiber maturity ratio 0.85–1.00; premium micronaire range is 3.8–4.2

ensure that they will be fit for purpose for spinning and textile manufacture, minimum acceptable fiber parameters (base levels) are set, below which crops can attract a penalty or discount on the standard price when marketed. Table 10.4 shows the base levels for three main fiber quality traits in the USA and Australia for *G. hirsutum* cotton. While most commercial breeding is currently directed at enhancing yield and at least consistently meeting base fiber quality, there is a case to be made to continually push that target to embrace significantly higher quality and develop premium fiber cultivars that attract higher prices.

There are price premiums available to growers who produce fiber strength and length in excess of the base level and with intermediate micronaire, but premiums are not always enough to compensate for yield loss. Breeding for improved fiber quality is, therefore, only beneficial if yield is maintained or simultaneously improved and most commercial breeding programs adopt this strategy of placing fiber quality behind yield, but try to advance both.

Molecular Approaches to Yield and Quality Enhancement

Biotechnology has clearly provided powerful tools for the improvement of cotton as evidenced by the rapid and widespread adoption of insect- and herbicide-tolerant GM cultivars over the last two decades. Several fiber characteristics such as yield, fineness, strength, and length are also objectives for improvement, and there are a number of genes and gene networks that have been suggested as potential targets for genetic modification. Cotton fibers are single hyper-elongated cells arising from the epidermal cells of the outer integument layer of the seed coat. The synchronous growth of thousands of terminally differentiated single-celled fibers per ovule [72] is characterized by four overlapping developmental stages, namely, fiber initiation, primary elongation, secondary cell wall (SCW) synthesis, and maturation [73]. Fiber initiation begins on the day before anthesis, although fertilization must occur for elongation to begin in earnest [74]. This initiation phase determines the number of ovule epidermal cells that will differentiate into fiber cells and

ultimately the number of fibers per seed, a yield component. Once the fiber cell protrudes from the epidermal layer, it elongates rapidly beginning primary elongation. The rate and duration of this phase determines many important fiber traits, including the length, shape, structure, and composition of the fiber cell [75]. Fiber elongation occurs largely by diffuse growth that coordinates cell turgor (the driving force of cell expansion) and cell wall loosening [76]. During the latter stage of elongation, ~16 and 21 days past anthesis (dpa), there is a transition from primary elongation to SCW synthesis [77] distinguished by the realignment of parallel microtubules and cellulose microfibrils in a steeply pitched manner [78]. Though once considered mutually exclusive, there is a period of overlap between phases [79, 80], since elongation can continue up to 45 dpa in longer genotypes [81] or cease around 25 dpa in shorter genotypes [80]. These differences are due to genetic variation between cotton species and cultivars [82]. During SCW synthesis, cotton fibers increase in dry mass due to cellulose deposition which continues until the boll sutures open. Fiber strength is directly influenced by the rate of cellulose deposition during SCW synthesis [83], while the amount of cellulose partially determines yield [84]. A greater understanding of the molecular physiological processes that regulate which cells become fibers and control each of the stages of their development could enhance the ability to either breed for or to engineer cotton plants with a higher density of fibers which are longer, stronger, and finer, hence higher yielding.

Discovery of Genes Involved in Fiber Development and Their Manipulation Through Genetic Engineering

The recent advances in functional genomics, genetic, and analytical tools, especially comprehensive gene expression profiling of cotton fiber cells, together with the availability of a sequenced genome, have provided new opportunities to improve cotton fiber traits through genetic modification. Many fiber-specific genes involved in fiber cell initiation, fiber elongation, or cell wall biogenesis have been identified as candidates for genetic manipulation to improve fiber yield and/or quality (Fig. 10.4). For example, two MYB genes, *GhMYB25* and *GhMYB25*-like, which are related to a petal epidermal cell patterning MIXTA-MYB from *Antirrhinum majus*, and a homeodomain transcription factor (*GhHD-1*) were identified from microarray comparisons between fiberless mutants and wild-type cotton [42]. Silencing these genes in tetraploid cotton affects either the initiation or timing of expansion of fiber initials and their overexpression under a constitutive or seed coat-specific promoter results in an increased number of fiber initials on the surface of the ovule [85–87]. Whether this increased fiber initiation translates into an increase in lint percentage or yield remains to be tested in the field. Transcript profiling and ovule culture experiments both indicate that several phytohormones, including auxin, gibberellic acid, and brassinosteroids mediate cotton fiber initiation and early growth [88–90]. Seed-specific expression of the *iaaM* gene

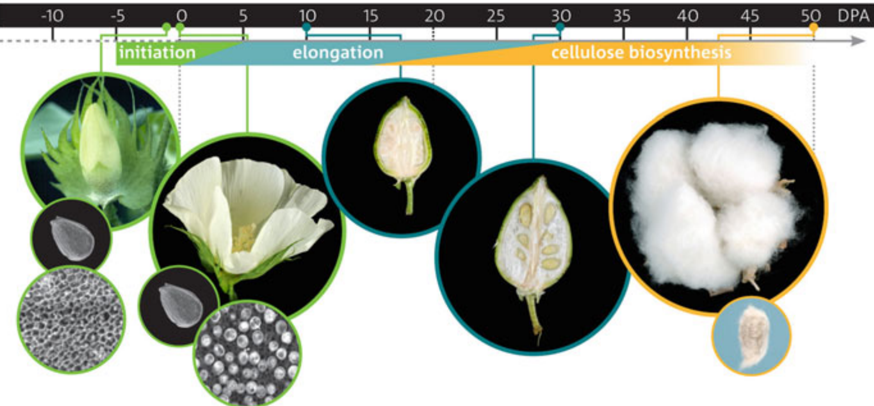

Fig. 10.4 Fiber development of cotton from flower opening to boll opening showing targets for fiber quality improvements. Fiber growth occurs over about 60 days, passing through the three overlapping stages of fiber cell initiation, elongation, and secondary cell wall (*SCW*) thickening when large amounts of cellulose are laid down inside the fiber. These different stages determine final fiber yield and quality. The number of fiber initials formed on the surface of the ovule (high-magnification scanning electron microscopy inserts) predetermines the number of fibers on the mature cotton seed, an important component of yield. Transcription factors, such as GhMyb25, GhMyb25-like, and GhHD-1, have been shown to be important regulators of fiber initiation. Auxin promotes fiber initiation and the early stages of elongation. The higher rates of expression of genes involved in auxin signaling and pectin modification are linked with longer fibers. Downregulation of the abscisic acid (*ABA*) and ethylene signaling pathways and secondary cell wall synthesis may contribute to longer periods of fiber elongation. Rapid cell elongation requires buildup of turgor pressure and cellulose and sucrose synthase provides the UDP-glucose and fructose that increase osmotic pressure and provide substrates for cellulose synthesis. Differences in cellulose content may contribute to changes in fiber fineness and/or strength. All these processes and genes have been targets for manipulation in genetically modified cotton

(a gene involved in auxin indole-3-acetic acid synthesis), for example, increased the number of fiber initials, mature lint fibers, and cotton yield with no deleterious effects on fiber fineness, strength, or maturity [90]. Manipulating the other hormones or hormone response pathways may offer alternate targets.

During fiber elongation, cell wall extensibility is essential to allow the rapid expansion and elongation of these single fiber cells. Xyloglucan endotransglycosylases (XTHs) cleave cell wall xyloglucans and reconnect them to other xyloglucan molecules [91], allowing movement of cellulose microfibrils relative to each other for rapid cell expansion. Overexpression of *GhXTH1* in cotton resulted in longer fibers than their controls, without adversely affecting other

fiber characters. Fiber elongation also relies on the cleavage of sucrose into UDP-glucose and fructose to increase osmotic pressure. These compounds also provide the substrate for cellulose synthesis during later SCW thickening. Sucrose synthase is a key enzyme in this reaction and is abundant in fiber initials [92, 93]. A novel cotton sucrose synthase gene, *GhSusA1*, was identified from *G. hirsutum*. Silencing of *GhSusA1* reduced fiber length and yield, whereas overexpression of this gene increased fiber length and strength [94]. Additionally, overexpression of a potato sucrose synthase in transgenic cotton enhanced leaf expansion and improved early seed development, thereby enhancing seed set and promoted fiber elongation [95]. Both of these studies suggest that sucrose synthase is an important regulator of sink strength in cotton that is tightly associated with productivity. It is therefore a promising candidate gene that can be developed to increase cotton fiber yield and quality – possibly by improving seed development as a whole, rather than solely focusing on manipulating fiber growth [95]. Cellulose synthesis is a key biochemical event during SCW formation, and at least five cellulose synthase (*CesA*) genes have been shown to increase in expression during this stage [96], so increasing cellulose production is an obvious target for improving fiber quality. The fibers from transgenic cotton expressing two cellulose synthase genes (*acsA* and *acsB*), from the bacterium *Acetobacter xylinum*, were approximately 15 % longer and 17 % stronger than wild type [97], but it is unclear how the bacterial cellulose affects the structure and composition of the fiber SCW to change these properties.

Genetic Modification for Novel Fiber Traits

Along with genetic improvements to yield and conventional quality traits, attempts have been made to genetically alter other fiber traits such as color and thermal properties. The dyeability of cotton fibers is an important trait in the textile industry. The process of dyeing cotton fibers is expensive and creates large volumes of toxic waste, and, as a result, there has been an increased focus on naturally colored cotton (mocha, brown, red, and green) by the organic cotton industry and environment-minded consumers alike. Although only grown on a small-scale, colored cotton represents a niche market. Genetic engineering of cotton to produce a greater variety of colored fibers has received some attention in recent decades with a primary focus on the two main colors used for mass-produced blue and black denim. Genes responsible for melanin and indigo production were inserted into cotton resulting in some color formation in the fibers [98]. While the color intensity was not sufficient for commercial use, these attempts suggest that there is potential for producing novel fibers through genetic modification.

The synthetic textile industry has produced many innovative fiber products, including bicomponent fibers that contain a core polymer surrounded by a sheath polymer that combines the properties of the two polymers in one fiber. Attempts at replicating this innovation in cotton fiber have included the introduction of bacterial genes for the production of an aliphatic polyester compound, polyhydroxybutyrate

(PHB) [99], a natural biodegradable thermoplastic with physical and chemical properties similar to polypropylene. The fibers of the transgenic plants showed slower rates of heat uptake and cooling compared with fibers from wild-type plants and, although the effects were small, provide some promise for this approach. There have been attempts at expressing this biopolymer in fiber plants such as flax and poplar [100, 101], but nothing further in cotton. Zhang et al. [102] produced transgenic cotton expressing rabbit keratin-associated protein (KAP61R) genes in the fiber from the fiber-specific E6 promoter. The fibers from the transgenic plants were reported to have improved strength and thermal properties and were 60 % longer than the wild-type controls. However, this GM trait has not appeared in commercial use, presumably because its reported unique properties were not inherited. In a similar vein, Huang et al. [103] have expressed a spider silk protein in cotton fibers, but it had no statistically significant effect on fiber quality. Fiber engineering is clearly complex, but this has been recognized for many years in many different systems where biotechnologists have attempted to alter plant metabolism.

Identification of QTLs Linked with Fiber Quality and Yield

Many traits of agronomic interest are monogenic, meaning they are controlled by a single gene, but most important targets for crop improvement, such as yield and quality, are invariably polygenic. The genes that contribute to a multigenic trait are referred to as quantitative trait loci or QTLs. A QTL can be statistically associated with a trait and tracked using linked molecular markers to enable identification of superior cotton lines. The DNA markers in cotton have included a range of types such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), simple sequence repeats (SSRs), amplified fragment length polymorphisms (AFLPs) (reviewed in Mei et al. [104]), and, increasingly, single nucleotide polymorphisms (SNPs) [105]. The advantages of DNA markers over conventional phenotypic selection are that they are independent of environment, age of the plant, or the presence of a pathogen or pest. Therefore, it is possible to select for the progeny of a cross carrying the genomic regions which contain the desired alleles that contribute to a trait, even at a stage (ie., as seeds or seedlings) before the trait is expected to be expressed, using the marker instead of the trait phenotype.

QTLs associated with fiber quality and other traits have been identified in a number of studies involving both inter- and intraspecific crosses between the tetraploid species (see reviews [106–108]). Hybridization of *G. hirsutum* × *G. barbadense* through conventional breeding programs to improve fiber traits of commercial cultivars has been difficult because of their genome incompatibilities. QTLs and genes linked to high-quality fiber traits from *G. barbadense* have been introgressed into *G. hirsutum* [46, 109] allowing the identification of large numbers of fiber quality-related QTLs [110–113] and the construction of many high-

resolution genetic maps [23, 114]. This includes the *qFL-chr1* locus, which provides a valuable source of fiber length [115] and a number of QTLs for fiber strength [116, 117].

Stelly and colleagues [118] released a set of 17 disomic alien chromosome substitution lines (CS-B lines), produced through hypoaneuploid-based backcrossing in a near-isogenic genetic background of the *G. hirsutum* genetic standard Texas Marker-1 (TM-1) line of *G. barbadense* chromosomes. They also generated a set of chromosome-specific RIL populations from these substitution lines which provide a novel source of germplasm for fine mapping of fiber and other agronomic traits, with potential for introgressing those QTLs present on those chromosomes or chromosome segments into elite *G. hirsutum* germplasm. Most CS-B lines had different fiber quality properties to TM-1 so should be a better source of well-characterized and additive fiber quality QTLs than using the *G. barbadense* parent directly. Similar approaches are being explored with unadapted *G. tomentosum* and *G. mustelinum* accessions. A total of 28 QTLs for fiber elongation, length, fineness, fiber uniformity, boll weight, and boll number [119] have been identified in *G. tomentosum*. Considered to be an unfavorable parent to contribute to commercial targets, a number of QTLs contributed by this species have resulted in the improvement of several *G. hirsutum* fiber characteristics and potentially offers novel and valuable genetic diversity and needs to be further exploited.

The classical genetic approach of QTL analysis has been combined with a genomic approach. The expression levels of tens of thousands of genes or gene clusters are analyzed within a segregating population, expression quantitative trait loci (eQTL) are mapped like conventional QTLs, and their locations compared with fiber quality QTLs from the same populations [120, 121]. This genetic genomics approach provides a novel way to close the gap between (structural) genetics and (functional) genomics to discover chromosomal regions and eventually genes important for fiber quality. This will ultimately facilitate the breeding of superior genotypes through marker-assisted selection (MAS) or biotechnology [122].

Many mapping and genomic studies done to date have been in isolation from operational breeding programs, and when associations between genomic regions and fiber traits were identified, they were rarely picked up by breeders. Fiber traits are complex; the QTL region detected are large (10–20 cM) and may contain hundreds of genes, so identifying the underlying genes is not usually possible. Other QTL studies have used too small a population, are cultivar-specific, and the QTLs are too loosely defined to be of value in selection, particularly when there are many of small effect [120]. Where QTLs are cultivar-specific, they are not applicable beyond the populations originally studied.

Most public marker discovery and mapping efforts in cotton have involved using SSR markers (e.g., the Cotton Marker Database at http://www.cottonmarker.org/, also available through http://www.cottongen.org/), but SSR technology is tedious and labor-intensive, and larger breeding programs have moved away from SSR to SNP markers. Relatively few studies have been done on QTL mapping between different *G. hirsutum* cultivars mainly because of the low levels of marker

polymorphism within this species, but the advent of better genome sequence data has helped with the discovery of an abundance of SNP markers between cultivars. This has cleared the way for more in-depth analysis of commercially important agronomic traits within breeding populations.

Once SNPs are discovered, various technologies are available for large-scale genotyping each depending on the number of SNPs needing testing and the number of DNA samples to be assayed, as well as the budget of the program. These include the Fluidigm, KASPar, GoldenGate, and Infinium systems [123]. The use of SNPs in breeding and research will be supplanted by NGS technologies over the coming decade. As the cost of this type of short read, high coverage analysis becomes cheaper and the depth of sequencing increases, it will become possible to pool more samples in a single run and analyze many individual plants. Combined with some genome complexity reduction techniques such as restriction site-associated sequencing (RAD-Seq) or genotype-by-sequencing (GBS) [124] to simplify SNP discovery, it will be possible to discover SNPs "on the run" in the populations or collections being studied [123] and without a reference genome sequence.

Bioinformatic challenges can be high, particularly in polyploid species, but they are not insurmountable. GBS will democratize the application of genome-wide analysis methods into non-model plants and, by simplifying SNP discovery, will move cotton breeders toward using genomic selection (GS) techniques in their breeding. GS is a form of MAS [125] that selects for the best individuals based on genomic estimated breeding values determined from marker-trait associations. They are derived from a training subpopulation and then applied to predict the phenotypes of the breeding population based on their individual genotypes, but require the assessment of large numbers of markers. Such GS strategies have been successfully used in animals and should have value in plant breeding programs such as cotton as the methodologies are refined and applied to our crop plants.

Integration of Biotechnology and Conventional Breeding

The increasing knowledge about the cotton genome at the structural, gene expression, and epigenetic levels, as well as the collation of the QTLs linked with fiber quality, are contributing to fledgling "breeding by design" programs for cotton fiber improvement based on existing SSR and some SNP markers [126–128]. Their exploitation is still not yet widespread in mainstream commercial breeding programs and certainly not among public cotton breeders. To be successfully integrated in breeding programs, there needs to be both a step change in the reliability and relevance of particular trait-marker associations and a willingness to adopt marker systems by breeders. This will only be achieved when there are closer ties between the people carrying out genetic studies and the breeders who will be applying them.

MAS in many crop species has only been used in a small number of situations where traits are determined by a relatively few major genes and have a high value

[129]. In cotton, markers are reportedly only being used for introgression of existing GM insect and herbicide tolerance traits or novel sources of nematode resistance and mostly in private companies. Recent advances in high-throughput genotyping and high-throughput sequencing are shifting the pendulum back in favor of using DNA markers in breeding. A number of the larger maize and soybean breeding companies have already started down this route and have adopted a nondestructive seed-based screening strategy to apply markers. “Seed chippers” [130] help automate the process of removing a small portion of seed, extracting DNA and running panels of SNP markers in a high-throughput manner. Cotton is likely to be more difficult as the seeds contain secondary chemicals that inhibit common molecular biology reactions. Seed-based screening will be essential in any large-scale GS program as it would be impractical to plant extremely large populations just to find the small numbers of plants to be kept that may contain the required large number of favorable alleles. If the correct genotypes can be identified from dried seed, then only small numbers of individuals would need to be planted out for phenotypic evaluation.

The availability of the new SNP genotyping technologies should begin to address the issues of the low numbers of markers and high costs associated with MAS in cotton genetics and breeding. Public SNP discovery efforts in cotton are well under way [131], and a public release of a high-density SNP chip was launched at the end of 2013 by the Illumina Company. The availability of hundreds of thousands of SNPs on these chip platforms should allow the use of association mapping on big collections of genotypes or breeding lines representing a broad genetic base to discover trait-marker associations quicker and at much higher resolution than has been possible in traditional biparental populations and SSR markers. The rapid and widespread adoption of GM traits for insect and herbicide tolerance in cotton has, out of necessity, begun to change breeder attitudes about the value and the practicability of DNA markers as plant breeding tools. GM insect and herbicide traits are mostly single traits that need to be combined in various combinations depending on market demands. Each trait segregates independently, but must be stacked together in the best available genetic backgrounds. They are generally first introduced in poorly adapted backgrounds that must be backcrossed into elite material suited to each region where they are to be deployed, or even later when they are already in elite material, they must be continually reincorporated into any new conventional germplasm as it is developed. A new cultivar with better fiber quality or disease tolerance, for example, is not going to achieve widespread adoption unless it also incorporates the current advantages of existing GM cultivars on the market. DNA diagnostic markers (both trait-specific and event-specific) have been developed for all released GM traits because of their utility in breeding and as a regulatory requirement for being able to detect any traits that might contaminate non-GM cotton seed or products (reviewed in [132]). These diagnostics are in essence a “perfect marker” for the genomic region containing the GM trait and are treated just like a marker would for a QTL. Breeding GM traits are similar in many ways to breeding with a small number of QTLs using MAS or marker-assisted backcrossing (MABC). If DNA has already been prepared to screen

populations for GM traits, then why not combine that with a screen for another useful trait for which there are available markers? In MABC, cotton breeders can use markers distributed across the genome of a parent to provide an additional level of selection against, or for, the donor or recipient in their backcrosses to accelerate the recovery of the recipient genotype carrying the GM traits. So with the increasing number of transgenes being deployed internationally, cotton breeding programs are facing resource challenges maintaining breeding progress as more effort is absorbed into backcrossing GM traits into their elite conventional germplasm. To maintain flexibility and contain regulatory costs, GM trait providers prefer to offer each of their traits as an independently segregating transgenes, and many of the next-generation GM cotton products may contain four to seven different transgenes. The population sizes required to recover parental performance in backcrossing are beginning to strain both glasshouse and land requirements as well as the ability to screen that material for the presence and zygosity of multiple transgenes. This may drive faster adoption of high-throughput genotyping and the development of robust seed genotyping technologies.

Breeding Strategies and Integration of New Biotechnology

The driving force in cotton breeding is to produce a commercial cultivar with improved performance; however, not every cross is destined for release. A cultivar must have competitive yield before release, so introducing an exotic or unadapted genotype into a breeding program usually requires several crosses with elite material before the desired trait is integrated with appropriate yield. The integration is necessary to maintain variability, but the unadapted material normally has undesired traits which takes longer to breed out. Once a breeding line possesses both the trait and yield required, it is then ready to be used within a breeding program. This process is termed germplasm enhancement, but in many instances, the desired trait cannot be successfully integrated.

Breeding populations are formed by first crossing parents and then applying a breeding method to advance the lines until they are homozygous. Breeding lines or individual plants are selected and advanced based on the mean of the desired trait. The selected lines are then evaluated in replicated tests to confirm the value of the selection, and superior lines are released commercially or distributed to the general public. This cyclic process is repeated by adding new germplasm. Breeding methods (mass selection, bulk selection, recurrent selection, and pedigree selection) for cotton have been well established over many years, and reviews by Lee [133] and Calhoun [134] demonstrate their procedures and utility. A breeder has to be flexible in applying or choosing a breeding method due to particular breeding goals and available resources. There is hybrid vigor in cotton with up to 200 kg lint/ha yield heterosis, but limited heterosis for fiber properties [135]. Large proportions of India and China use hybrid cultivars, but costs of hybrid seed production limit their use in most other counties.

As yield and fiber quality traits are quantitatively inherited, breeding strategies will require approaches involving careful selection of parents, effective plant selection, and large breeding populations. Parental selection can be done at random or based on performance data from a previous season, its pedigree, or more quantitative data such as breeding value or combining ability, gained from prior mating studies. Once selection has been made, careful evaluation is necessary before advancing. Experimental designs for field trials are based on reducing the amount of variation within the trial; the more replications either through years, locations, or within locations, strengthen a dataset. Larger breeding populations, whether through the number of crosses or selections, provide a better chance of identifying and confirming the desired trait.

In the USA, there are both public and private breeders with defined roles. Public breeders are primarily focused on germplasm enhancement. Improved breeding lines are then released and available for private breeders to use in generating commercial cultivars. Bowman [136] in a survey of breeding practices in the USA showed the pedigree method was used in up to 82 % of private programs, and backcross methods were used in up to 28 % of public programs. The average cotton breeding program had about 100 crosses annually, creating up to 3,700 nursery plots and 7,500 plant selections. Yield testing began at F_4 stage, but this stage had considerable range across programs. Detailed evaluation of performance of elite lines was done at about six sites in private companies. Other than having many more plant selections, similar practices are used in Australia.

Breeding for improved fiber quality is only beneficial if yield is maintained or simultaneously improved. Long-standing negative associations between yield and fiber traits, due to linkage, pleiotropy, or physiological factors [31, 137, 138], slow the development of cultivars with premium fiber quality which are competitive in yield with existing cultivars. In a detailed analysis of 6–11 years of cotton breeding data from Australia and the USA, Clement et al. [138] reported the magnitude of these associations was greatest for fiber strength, and despite differences in yield and fiber strength between countries and seasons, there was a consistent and strong negative association (Fig. 10.5). For Australian data, a strength improvement from 32 to 34 g/tex was associated with a mean yield reduction of 1,000 kg lint/ha.

Fortunately, there were outliers in that association, albeit at low frequency (1.4 % in Australian data), that have allowed some progress to be made in improving fiber quality without loss of yield. It must be noted that in the last 30 years, the definition of high or premium fiber quality has shifted to much greater values of fiber length and strength. For example, Culp and Harrell [139] and Scholl and Miller [140] reported strength values of approximately 22.4 and 21.3 g/tex, respectively, in their strongest lines which are well below current base grade values (Table 10.4). The triple hybrid line, TH 131–5, was 26 g/tex with 601 kg/ha lint yield [137]; this shows the strongest genotype at the time was barely at the current base grade, while the low yield displays the negative association. Figure 10.3 also shows that fiber properties considered above average 30 years ago would now be considered below average. Thus the task of achieving high yield and premium fiber quality has also become more challenging. Breeding practices for improving fiber

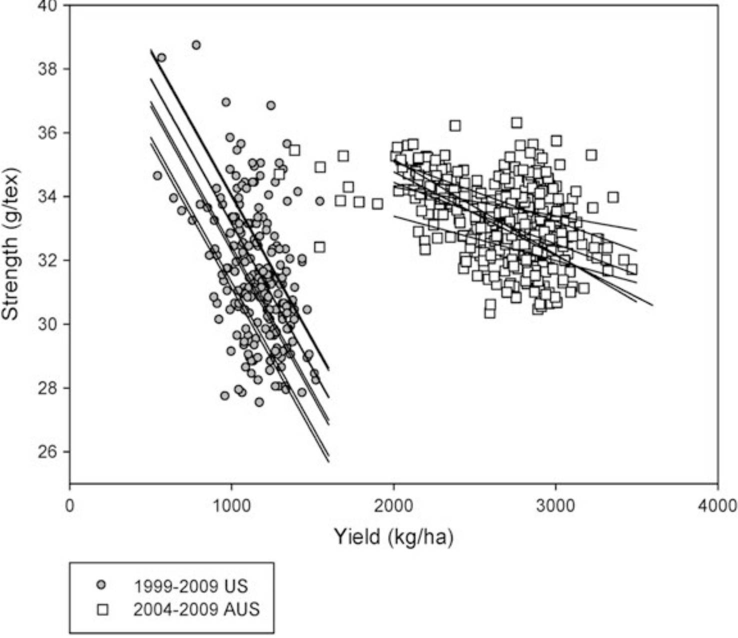

Fig. 10.5 The negative association between lint yield and fiber strength in 6–11 years of data from Australia and the USA. R^2 values for multiple regressions were 0.35 ($P < 0.001$) and 0.32 ($P < 0.05$) for Australian and USA data, respectively (Reprinted from Clement et al. [138]. With permission from Elsevier)

quality from relatively poor values to base values or from base to premium values would be similar, although different parents would be used.

Broad sense heritability estimates for fiber quality are considered moderate to highly heritable (>0.50) [27]. This is desirable for breeding, indicating the potential inheritance of the trait and predicting gain from selection. Genetic variability for the trait of interest is an essential component for gains to be made, and there is ample evidence for fiber quality improvement. Western US Acala cottons, eastern US PeeDee cottons, and transgressive segregants have been shown to be sources of improved fiber length and strength [59, 141, 142]. Interestingly, both Acala and PeeDee pedigrees involve introgressions of both *G. barbadense* and diploids [33]. Smith et al. [143] reported *G. hirsutum* breeding lines with substantially improved fiber length (Upland Extra Long Staple) with little or no recent introgression from *G. barbadense*. New cotton lines have also been generated with the aid of mutagenesis which had 8–9 % greater fiber length than its original cultivar [144].

Meredith and Bridge [45] suggest that any method that allows hybridization and recombination should assist in breaking the negative association between yield and quality, provided that populations are large enough, but this is a significant constraint in a number of programs. Clement et al. [138] concluded that recurrent

selection should be used as part of the breeding strategy to more effectively assemble desired alleles for yield and fiber quality and to weaken and/or break their negative relationship. Outliers such as in Fig. 10.5 with higher yield and fiber strength need to be used in further intermating, with increased population sizes during evaluation in subsequent generations. Bowman and Gutierrez [59] and May [27] also recommended larger breeding population sizes in seeking improved fiber quality and identifying transgressive segregants. Culp et al. [145] showed desirable strength at a frequency of only 0.3 % in breeding populations, but they found the frequency of desirable combinations increased in subsequent intermating.

Diallel studies have identified sources of improvement for fiber quality and yield based on the combining ability of select breeding lines [146, 147]. This breeding analysis tool aids in determining the usefulness and variability of a program's breeding material by qualifying and quantifying the trait of interest and its gene action. Additive gene action has predominately been found more than nonadditive for fiber quality especially when contrasting genotypes are used [148–150]; however, there have been reports of greater nonadditive gene action for fiber length [151–153]. A review of gene action studies for lint yield and fiber quality are reported in Meredith [154] and May [27]. As indicated in section "Molecular Approaches to Yield and Quality Enhancement," most fiber properties have been found to have many QTL, confirming multigenic control and quantitative inheritance which present challenges for using molecular markers.

Backcrossing may be used to transfer one or a limited number of desirable traits from one parent (possibly not adapted) to an elite or adapted parent. Meredith [141] proved fiber strength improvement was achievable when utilizing backcrossing; however, it is commonly used at the early stages of transgenic trait development. Stiller et al. [155] in a study of breeding methods with transgenic traits highlighted that at least three backcrosses were required, but even then, there was considerable variation in yield, fiber quality, and disease resistance in the progeny. They concluded that a standard pedigree method was required to even recover the recurrent parent characteristics. The aim of backcrossing should be to equal, if not exceed, the recurrent parent performance.

In order to improve fiber quality for end users, breeders must know how particular properties relate to spinning or spun yarn quality. In this sense, yarn quality provides the fullest description of fiber quality. However, spinning yarn from breeding populations is a high-cost exercise particularly if proper control of spinning parameters (yarn count, twist, and production speed) is applied. Therefore breeding programs use fiber testing instruments such as HVI, AFIS, and others as a means for predicting yarn quality [156–161]. May and Green [152] reported that selecting solely for fiber strength was not effective for improving yarn strength indicating that there are more properties than fiber strength affecting yarn strength. The value of assessing or even predicting yarn is that it provides information on the *integration* of all fiber properties and their interactions in supporting yarn structure.

Breeders should be carefully measuring routinely selecting for improvement in fiber properties while also selecting for yield and other key attributes. An HVI is adequate for measuring fiber length and strength in breeding, despite being more

rapid but less accurate than single instruments such as fibrograph and stelometer [162]. However, relying on HVI micronaire to predict spinning performance can be misleading because there are different combinations of linear density and maturity for the same micronaire value [163]. When selecting simultaneously for high yield and intermediate micronaire, the likely long-term breeding outcome will be coarse, immature fiber, the exact combination *not* wanted by spinners. This is a result of the strong positive correlations between yield and linear density [138]. Therefore, to meet desired combinations of fiber linear density and maturity (Table 10.4), other instruments are required to measure these properties during breeding and selection such as the Advanced Fiber Information System (AFIS), Cottonscope [164], and Shirley Fineness Maturity Tester (FMT). These instruments were designed to quantify fiber traits not measured by HVI [165]. Target fiber properties for breeding are listed in Table 10.4.

Commercial Seed Production

Having invested considerable effort in the production of a high-performing cultivar, ensuring that it stays true to the breeder's original selection and maintains good germination and seedling vigor, requires quality control and seed certification processes. Certification limits physical contamination, cross-pollination, and natural selection from altering the valuable characteristics of a cultivar that has gone into commercial use. As more cotton-producing countries shift to adopting GM traits in their regionally adapted cultivars, this adds additional constraints on seed producers to ensure that those traits remain pure. Certification in those regions also requires a process of quality assurance to detect and quantify GM traits especially at the point of hand over of seed from the breeder to the seed producer, but also at all stages during seed increase prior to sale. It is clearly important to have purity of GM traits so that producers can capture the high costs paid to access those technologies. The ability to track those traits in general commerce is also critical as countries with zero tolerance for those traits in seed of conventional cultivars that may be crossing international borders, and this adds a high level of responsibility (and liability) on seed producers and breeders alike to ensure good seed and trait stewardship.

The International Seed Testing Association (ISTA) and the Organisation for Economic and Co-operation and Development (OECD) are two international organizations that standardize and accredit seed certification methods and agencies for numerous agricultural crops and ornamental germplasm. These groups work together with numerous other regional organizations such as the Association of Official Seed Certifying Agencies (AOSCA) used in Canada and the USA.

AOSCA, for example, defines four classes in certifying seed. *Breeder seed* is seed directly controlled by the breeder; it is used to develop and maintain a cultivar and is not available to the public. This seed is used for the production of *Foundation seed* that is grown to maintain specific genetic identity as described by the breeder in its registration. It can only be produced by or under supervision of a licensed

plant breeder. *Registered seed* is grown from foundation seed that has passed certification for genetic purity, while *certified seed* can be produced from foundation or registered seed that has passed certification. In producing these different types of seed, standard growing and testing practices must be adopted to obtain certification, and this includes specifications on isolation distances and standardized equipment clean-downs.

Isolation avoids physical contamination and along with buffers of the same cultivar, especially reduces outcrossing by pollinators such as bees [166]. Seed crops should be grown in ideal locations to avoid poor growing conditions affecting seed quality. Seed production of new (regulated) traits may require special conditions of greater buffer sizes and isolation distances. Certified cotton must be isolated by 5 m or have a barrier that prevents mechanical mixture. It must be isolated by 800 m from other cotton species and at least 30 m from any other cultivars that differ in morphological features. During each generation, before field inspection, the plants are rouged for off types, and weeds are removed where weed seed could contaminate the certifiable seed. Off types are allowed 2.5 plant/ha for foundation seed, 5 plants/ha for registered seed, and 25 plants/ha for certified seed [167].

It is imperative that all planting, harvesting and ginning equipment and storage containers be cleaned properly and inspected to avoid contamination. Once harvested, seed must be placed in appropriate storage that prevents high moisture or humidity from affecting germination. Samples are tested for germination which must be >70 % (preferably >90 %, especially for cool conditions) and must contain <2 % inert material for all certification classes.

For GM cotton, quality assurance is performed at each stage of certification by immunoassay tests, ELISA plates, and lateral flow strips, which determine if the GM protein is present and how much is present. This can be done on leaves or seeds. Herbicide bioassays can be used on seeds to detect genetic purity for herbicide traits. Polymerase chain reaction (PCR) detects and identifies DNA specific to the biotech trait. In most countries, quality assurance must demonstrate up to 99 % GM event purity for commercial sales, but this can vary depending on company or country regulations. For conventional cotton, approved traits must not exceed 1 % contamination. In countries that are GM-free, there is a zero tolerance for GM contamination of conventional cultivars, but in practice, this must be set at a level consistent with the ability to detect contamination which is often below 0.02 %.

Good seed production and stewardship procedures have been the key to the successful adoption of the outputs of breeding programs, and this will need to be maintained as new high-performing cultivars are delivered using the combination of conventional- and genomics-assisted breeding in to the next decade.

Market Challenges, Opportunities, and Commercialization Barriers

All industries face challenges into the future, and cotton is no exception. Competition between cotton and synthetics has shown cotton to have a steady decline in market share. However, with population increase, the total consumption of cotton has remained relatively constant. Of course competition is healthy, and there will continue to be blends between cotton and other fibers, but to compete with synthetics, cotton will need to improve quality without being more expensive. The economics around the use of cotton for textiles also creates challenges for breeders striving for enhanced fiber quality, as the incentive to advance quality is constrained by the lack of economic rewards for producers who achieve any higher quality fiber. Such a moving target is common in breeding but does add complexity to the process.

The anticipated availability of genome sequences of all the cultivated cotton species will open up many new opportunities to integrate conventional and marker-assisted selection and backcrossing or genomic selection. The higher marker density and tighter associations possible in association mapping will more accurately identify multiple regions contributing to traits. This will allow the identification of the underlying genes and open up new approaches to manipulating those traits either through selection or GM.

The push toward incorporation of high-value GM traits such as insect and herbicide tolerance can lead to reduced attention on the discovery of new conventional breeding material and a concomitant drop in public breeding efforts [168]. Adequate resourcing of breeding programs is therefore required as well as attention to traits that may be of specific regional value that may be easily swamped by the push to adopting the latest “global” GM traits.

Many of the novel GM fiber traits attempted to date still remain academic exercises, and while they do provide useful biological information about the roles of various genes in fiber development or the types of novel traits that could be supported at a cellular or metabolic level, there is considerable research required before they could be deployed commercially. Firstly, most have been introduced into nonelite cultivars and would need to be introgressed into elite backgrounds for proper assessment and deployment. Secondly, it is unclear whether they would be additive, as they might improve the properties of an originally poor quality, but transformable cultivar, but would they provide the same enhancement to an elite cultivar. Many of the changed properties of GM fibers are still small and, particularly where metabolic pathways are being manipulated, would require further optimization. Finally, the cost of registration of a GM trait is still incredibly high, so can only be justified when it provides a high-value outcome. Many potentially useful GM traits, such as those for better fiber quality, would struggle to return the value of the trait registration and so are unlikely to be taken up unless they can establish a unique niche market or make a substantial contribution to increased yield.

Genetic diversity in breeding is facilitated by exchange of genetic material between breeders or germplasm collections. However, restrictions imposed by patents on cultivars (GM or not), as well as a desire to protect regional intellectual property, have made it more difficult to share germplasm between regional programs or even countries. There is a good case to ensure some exchange between different programs is possible or for creating partnerships to facilitate this diversity aim. Access to Australian commercial germplasm from 1998, for example, has brought in enhanced fiber quality to the USA, particularly in Texas where environmental conditions are similar to those in Australia.

In this chapter, we have highlighted a number of opportunities to ensure continued improvement in yield and fiber quality of cotton. We have proposed breeding strategies to limit the negative association between yield and future desirable fiber properties, and we also demonstrate that molecular approaches to directly altering fiber properties are possible. Different GM yield and quality traits may need to be stacked together to achieve a commercial novel fiber, but stacking multiple transgenes is already becoming a limiting activity in breeding with the existing herbicide and insect tolerance GM traits. Future breeding will therefore require novel breeding strategies and/or application of high-throughput marker technologies to integrate breeding for the presence of multiple transgenes and multiple conventional agronomic traits.

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Chapter 11
Flax and Linseed

Martin Pavelek, Eva Tejklová, and Marie Bjelková

Abstract Flax has a long history of utilization dating back to ancient times. This dual-purpose crop is believed to have originated somewhere in Central Asia, Near East, or Mediterranean region with oil flax (linseed) predating fiber use (fiber flax). Current breeding work on the crop focuses on several characteristics of the oil and fiber to make the crop more competitive for use in the food industry and as source of bioproducts. For both types, increasing yield and improving resistance to lodging, pests, and fungal diseases are important goals. Genetic resources collections of flax are abundant, and breeding programs have been integrating new biotechnology applications into crop research and improvement activities. Overall, global crop production of flax is decreasing, though the trend in opposite in linseed has slight increase in Canada, Egypt, and some member countries of the European Union (EU). The policy environment significantly affects linseed production in the EU and has substantial influence on the marketing and commercialization strategies of the crop.

Keywords *Linum usitatissimum* • Linseed production areas • Breeding aims • Breeding methods • Genetic resources • Fatty acids • Lignans • Cyanogenic glycosides • Crop utilization

Introduction

Flax (*Linum usitatissimum* L.) was grown 6,000–8,000 years ago in Egypt and Samaria and belongs (together with barley and wheat) to the oldest of cultivated plants [1]. The dual purpose of flax, as source of fiber (fiber flax) and oil (linseed or oil flax), was already known in ancient times. In ancient Egypt, linen (derived from the fiber) was used for wrapping the royal mummies, and linseed oil was used to embalm the bodies of deceased Pharaohs [2]. For a long time, flax has been

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cultivated as a dual-purpose crop, but nowadays fiber flax and linseed represent different gene pools. Recently, a 30,000-year-old processed and colored flax fiber was found, indicating that early humans made fabric or threads from the flax [3].

There has been a decrease in the number of flax-producing areas all over the world in recent years. In traditional western European countries like France, Belgium, and the Netherlands, flax production areas have been decreasing since 2006 [4, 5]. In Egypt, however, there has been a small increase in production areas since 2007. The first production reports from China in 2006 confirmed a flax area of approximately 130,000 ha of which only 78,000 ha were processed. The world production areas as of 2009 covered approximately 400,000 ha of which about 25 % were located in France, Belgium, and the Netherlands [4]. Fiber production from western European countries represents approximately 60 % of the world market. The available statistical data show that worldwide fiber production is mainly concentrated in western European countries between the Escaut and Seine rivers [4, 5]. At the beginning of the 1990s, the production areas in EU countries reached 50–80,000 ha, but decreased massively between 1991 and 1992 to 44,000 ha during the peak of the flax crisis. Because of the endowment support from the EU, flax areas stabilized and interest in flax production rose. Flax areas again increased and reached more than 100,000 ha in 1995. However, the main reason for this unexpected increase was speculative flax growing in nontraditional flax-growing countries with the aim of receiving subsidies. That is to say, the financial support was provided to these countries without any attention to the crops' subsequent processing and utilization. Since 2000, rules for providing subsidies have been changed, and many nontraditional flax-growing countries like Great Britain, Spain, and Portugal lost interest in flax growing. For this reason the flax areas in the EU again decreased from almost 214,000 ha in 1999 to 87,000 in 2002. The admittance of new countries to the EU (i.e., Latvia, Lithuania, Poland, the Czech Republic) influenced the increase in flax areas in 2004, but in the long term, a continuous decrease in flax areas has been observed [4, 5]. The decline of cultivation areas in traditional western European flax countries has been carried out by the CELC (the European Confederation of Linen and Hemp) recommendation to balance supply and demand for long fiber due to previous large stocks which were not liquidated even at minimum fixed price of 1.5 EUR per kg. In the other flax-producing countries like the Czech Republic, Lithuania, Latvia, and Germany, the decrease of flax areas was not caused be the CELC regulation but under the influence of unfavorable economic conditions which were not improved by the EU subsidy policy in the individual countries. The world linseed areas and production data are described in detailed in section "Areas of production" of this chapter and presented in Tables 11.1 and 11.2 and Figs. 11.1 and 11.2.

Linseed oil is primarily used for medical and food purposes and then for industrial purposes, such as the production of paints and oil-based coverings and the manufacture of linoleum flooring [6]. The seeds are also used in some food products, e.g., as an ingredient in bread. Linseed oil is high in linolenic fatty acid content (45–60 %), making it a very effective drying agent. Linseed oil also offers important nutritional benefits because of the high levels of omega-3 fatty acids,

Table 11.1 Linseed areas (ha) in the world, by country

	2005	2006	2007	2008	2009	2010	2011
Russia	61,410	84,000	74,000	57,380	80,700	226,500	472,700
China	490,000	485,000	339,900	337,800	336,930	340,000	350,000
India	448,700	436,800	426,000	468,000	407,900	342,000	338,810
Canada	732,600	785,200	524,000	625,200	623,300	353,300	273,200
Kazakhstan	1,100	5,100	4,500	12,800	58,400	225,200	90,000
France	78,644	74,641	76,200	67,904	66,178	73,285	77,292
Ethiopia	215,107	215,106	174,108	152,129	180,873	140,801	73,688
USA	386,480	310,397	141,237	137,595	127,070	169,160	70,010
Ukraine	25,000	51,400	24,100	19,100	46,800	56,300	58,700
Belarus	72,132	65,807	65,476	74,181	48,086	46,762	49,981
UK	48,000	36,000	12,500	16,078	28,000	44,000	36,000
Argentina	37,180	46,690	28,400	9,450	17,370	37,960	25,600
Nepal	13,087	13,500	13,244	12,982	13,062	14,272	15,593
Sweden	9,854	8,690	4,321	3,500	9,900	19,100	14,660
Brazil	21,914	18,679	16,223	12,245	13,037	16,584	11,190
Bangladesh	4,769	13,585	14,075	8,823	11,176	10,542	10,251
Belgium	19,288	16,168	14,297	11,986	11,227	14,000	10,160
Australia	10,000	10,000	8,000	8,000	8,000	8,000	8,000
Spain	6,000	6,201	6,250	6,426	7,547	6,320	6,724
Uruguay	3,135	3,130	3,148	2,700	3,445	4,743	4,700
Germany	14,000	13,900	6,300	4,200	4,100	7,100	4,600
Pakistan	5,448	5,816	5,241	4,647	5,432	4,207	3,946
Afghanistan	16,588	1,840	2,000	3,160	1,780	1,788	3,943
Egypt	15,000	6,560	8,748	8,443	5,369	3,339	3,389
Italy	3,000	3,000	3,000	3,000	3,000	3,000	3,000
Uzbekistan	3,000	3,000	3,000	3,000	3,000	3,000	3,000
Mexico	1,400	8,500	10,300	7,300	2,600	3,600	2,600
Czech Republic	7,335	7,869	2,640	1,171	2,631	4,094	2,475
Chile	1,250	1,280	1,150	1,571	2,185	2,326	2,442
Poland	1,016	1,391	1,759	1,341	1,624	3,441	2,162
Netherlands	4,600	4,400	3,500	2,618	2,163	1,422	2,156
Slovakia	2,850	4,474	1,726	880	1,444	2,114	2,116
Romania	65	290	473	313	838	1,608	1,447
Eritrea	330	893	788	258	1,612	1,255	1,355
Latvia	1,600	600	500	200	200	800	1,330
New Zealand	1,500	1,250	1,000	1,198	1,261	1,276	1,276
Iran	1,218	1,105	1,143	1,181	1,124	1,013	1,069
Peru	1,010	1,500	1,600	1,133	1,342	1,174	1,052
Kenya	875	877	710	619	961	849	935
Tunisia	3,419	3,237	2,867	2,200	1,399	870	883
Austria	5,367	4,804	2,012	677	535	669	669
Hungary	2,009	1,865	1,713	673	910	354	527

(continued)

Table 11.1 (continued)

	2005	2006	2007	2008	2009	2010	2011
Lithuania	4,300	1,400	500	200	200	400	500
Iraq	537	400	365	309	296	351	324
Bulgaria	120	170	146	179	180	280	291
Ecuador	100	111	130	129	165	173	177
Estonia	163	207	136	200	236	173	115
Denmark	0	120	100	96	91	93	84
Kyrgyzstan	521	285	246	2	25	30	33
Turkey	176	146	81	67	20	10	15

Based on data from FAO stat data 2013 (http://faostat.fao.org)

Table 11.2 Linseed production (mil·t) in the world, by country

	2005	2006	2007	2008	2009	2010	2011
Canada	990,600	988,800	633,500	861,100	930,100	423,000	368,300
China	475,000	480,000	268,301	349,655	318,135	340,000	350,000
Russia	55,890	78,982	79,573	92,930	102,620	178,210	230,000
India	169,700	172,500	167,900	163,000	169,200	153,700	147,000
UK	89,000	49,000	23,000	29,298	54,000	72,000	71,000
USA	500,273	279,894	149,764	145,192	188,550	230,030	70,890
Ethiopia	125,907	125,907	108,222	169,855	156,079	150,629	65,420
Kazakhstan	1,000	5,390	5,220	10,300	47,650	94,610	64,000
Ukraine	28,200	61,500	11,400	20,800	37,300	46,800	51,100
Argentina	36,100	53,780	34,065	9,564	19,505	52,075	32,170
Sweden	15,700	11,900	6,700	5,400	18,800	23,900	23,200
France	59,236	43,155	33,801	14,600	21,500	20,400	16,000
Belarus	19,460	11,099	14,497	19,468	10,033	10,399	13,143
Brazil	15,819	13,442	14,722	11,333	9,873	16,159	11,046
Spain	7,000	6,800	6,800	6,746	7,000	8,083	8,649
Nepal	6,500	6,400	6,251	6,194	6,255	6,988	8,070
Australia	10,000	8,000	7,000	8,000	8,000	8,000	8,000
Bangladesh	2,970	8,485	8,180	7,810	7,050	6,870	6,705
Uruguay	3,821	4,442	5,078	3,320	4,326	5,840	6,146
Germany	25,370	22,400	6,000	4,000	4,000	7,000	6,000
Belgium	10,270	10,578	10,031	8,800	7,393	8,600	5,689
Egypt	28,000	9,602	12,226	11,879	7,888	4,552	4,536
Czech Republic	8,851	7,990	1,742	1,405	4,291	3,928	3,433
Pakistan	2,558	2,781	3,631	3,105	3,056	2,714	2,776
Romania	55	321	394	221	1,099	1,817	2,626
Poland	1,685	1,348	2,039	1,564	2,082	3,379	2,611
Tunisia	3,516	3,624	3,071	3,347	2,381	2,232	2,568
New Zealand	2,250	1,900	1,663	1,901	2,291	2,351	2,351
Slovakia	2,674	4,548	1,337	1,237	1,876	1,672	2,331
Italy	2,000	2,000	2,000	2,000	2,000	2,000	2,000

(continued)

Table 11.2 (continued)

	2005	2006	2007	2008	2009	2010	2011
Mexico	960	6,000	7,200	5,100	1,800	2,500	1,800
Afghanistan	10,000	1,100	1,200	1,300	1,200	1,400	1,500
Latvia	600	300	100	200	100	800	1,500
Chile	1,125	1,150	1,026	1,040	859	1,160	1,221
Netherlands	3,641	3,400	2,400	2,162	2,499	1,422	1,195
Uzbekistan	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Kenya	921	916	1,017	1,141	945	912	926
Austria	6,441	5,765	2,415	812	642	852	906
Iran	1,013	892	978	983	958	861	879
Peru	552	844	900	867	1,173	970	784
Eritrea	11	380	306	13	610	589	598
Hungary	2,818	1,896	2,155	610	948	339	547
Lithuania	2,000	700	300	200	200	200	400
Switzerland					325	358	398
Bulgaria	87	160	140	171	150	201	189
Iraq	168	150	161	144	145	189	183
Estonia	209	140	159	192	175	157	101
Kyrgyzstan	372	173	186	4	50	55	65
Ecuador	24	40	48	51	40	56	57
Denmark		45	40	47	44	40	35
Turkey	86	84	48	40	10	3	3

Based on data from FAO stat data 2013 (http://faostat.fao.org)

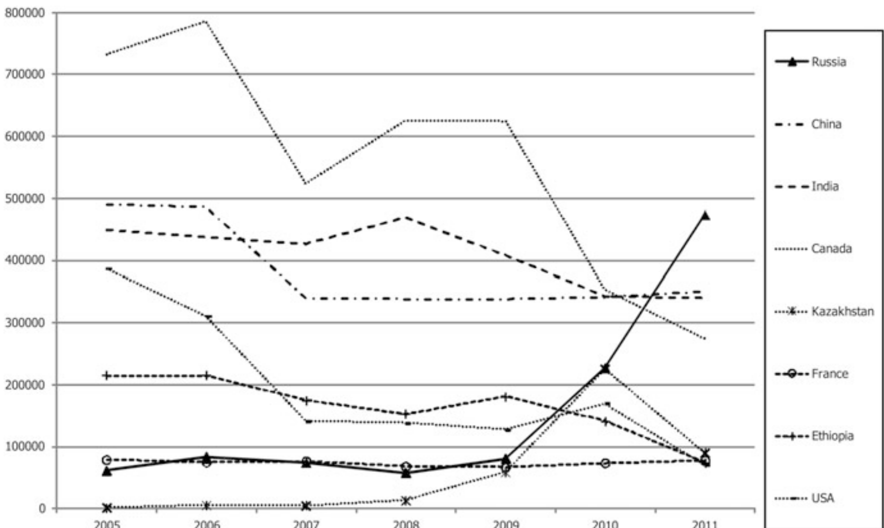

Fig. 11.1 Linseed areas (ha) in the eight most important countries (Based on data from FAO stat data 2013 (http://faostat.fao.org))

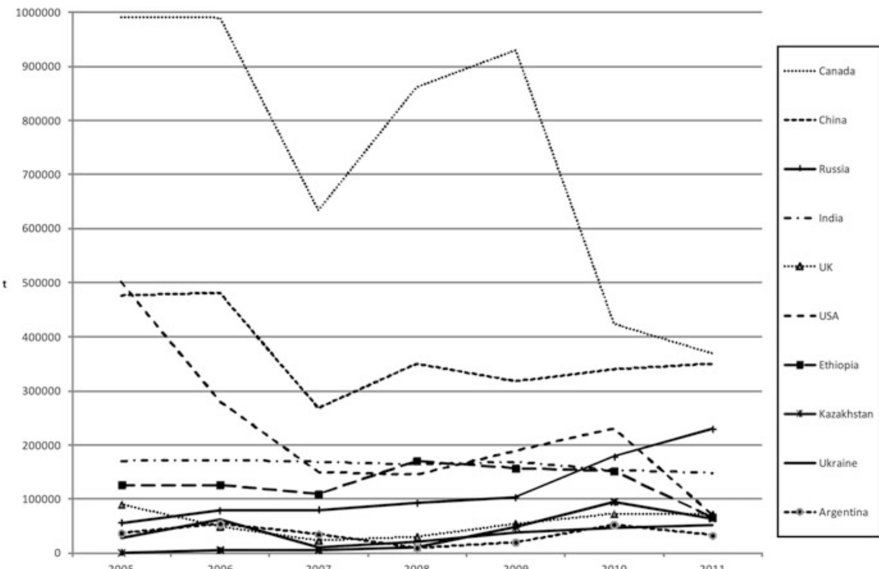

Fig. 11.2 Linseed production (mil·t) in the ten biggest producers (Based on data from FAO stat data 2013 (http://faostat.fao.org))

specifically alpha-linolenic acid (ALA). There is an interest to increase its production because it is considered as functional food [7, 8]. Animal experiments and clinical studies indicate that omega-3 fatty acids have anti-inflammatory properties and, therefore, might be useful in the management of inflammatory and autoimmune diseases, including coronary heart disease, major depression, aging, rheumatoid arthritis, Crohn's disease, and cancer [9]. Studies on animals have confirmed that flaxseeds also reduce LDL (low-density lipoprotein) and total cholesterol levels [10, 11]. Several clinical studies using flaxseeds (whole flaxseeds, flax oil, or lignans) to determine their effect on different cardiometabolic risk factors, particularly blood lipids, have been conducted [12–15]. Many studies have shown that whole flaxseed can reduce total cholesterol in both healthy [16] and hyperlipidemic people [17]. Linoleic acid (C18, n-6 fatty acid) and alpha-linolenic acid (C18, n-3 fatty acid) can be desaturated and elongated to arachidonic acid (AA, C20, n-6 fatty acid) or eicosapentaenoic acid (EPA, C20, n-3 fatty acid). Both these fatty acids are present in cell membranes and can be further metabolized to the eicosanoids (leukotrienes, prostaglandins, and thromboxanes), different group of fatty acid metabolites that are linked to homeostatic functions [18]. Increased cellular concentration of EPA can prevent cardiovascular diseases, hypertension, and inflammatory diseases [19]. The EPA concentration in tissues increases when food is supplemented with flax oil which has a high alpha-linolenic acid content, a precursor of EPA. Several meta-analysis-type studies of flaxseed effect on blood lipids have also been done [20–25]. There have been 28 studies conducted between January 1990 and October 2008 that focused on this area. The results of total

Table 11.3 Classification of linseed varieties according to the content of fatty acids

	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
			ω 9	ω 6	ω 3
Low content LA, **traditional content ALA**	4.9	1.8	16.6	**17.6**	**59.1**
High content LA, **low content ALA**	5.7	1.4	13.3	**73.3**	**6.3**
Medium content LA, **medium content ALA**	5.3	1.6	15.5	**41.2**	**36.4**

Comment: *LA* linoleic acid (omega 6), *ALA* linolenic acid (omega-3)

cholesterol were assessed in 36 comparisons from these studies having 1,548 participants, while LDL cholesterol level in blood was observed in 35 comparisons from 27 studies with 1,471 participants. Flaxseed was found to reduce total cholesterol by 0.10 mmol·l^{-1} and LDL cholesterol by 0.08 mmol·l^{-1}. A significant reduction in total and LDL cholesterol levels was observed when using whole flaxseeds (−0.21 and 0.16 mmol·l^{-1}, respectively) and lignans (−0.28 and −0.16 mmol·l^{-1}, respectively), but not with flax oil. Cholesterol decrease was more significant in women (especially those at postmenopausal stage) and individuals with high initial concentration of cholesterol. No significant changes were observed in HDL (high-density lipoprotein) cholesterol and triglycerides.

Flaxseed (Table 11.3) contains on average 36–41 % of fat of which approximately 70 % are polyunsaturated fatty acids (PUFA) and more than half of them is created by alpha-linolenic acid (ALA, C18, n-3/omega-3/fatty acid) with 50–62 % from linseed oil. Linoleic acid [LA, C18, n-6 (omega-6)] represents approximately 17 % of all fatty acids. This characteristic corresponds to the linseed varieties with traditional fatty acid composition of alpha-linolenic acid content [26]. Flaxseed with high content (approximately 73 %) of linoleic acid (LA, omega-6) is separately categorized from those with very low content of alpha-linolenic acid (ALA, omega-3) (Table 11.3). There is also a completely new type with medium content of both fatty acids (LA and ALA) as shown in Table 11.3.

Taxonomy and Domestication

The exact geographic origin of flax or linseed (*L. usitatissimum*) is unknown [27]. Among the eight independent centers of origin of the world's most important cultivated plants [28], *Linum* species were reported to have probably originated in four: the Central Asiatic, the Near Eastern, the Mediterranean, and the Abyssinian Center. Gill [29] and Rakousky et al. [30] have also discussed these four probable centers of flax origin. Alternatively, other researchers believe that Egypt could be a center of origin [31] as well as an area east of the Mediterranean toward India because a diverse form of flax is found in the area [31, 32].

The progenitor of cultivated flax *L. usitatissimum* ($2n = 30$) is also uncertain [29]. Many authors reported that cultivated flax was derived from two or more ancestral forms [31, 33, 34]. The species cultivated by ancient Egyptians were believed to be different from those indigenous to Russia and Siberia. Alternatively, it was suggested that cultivated flax originated from a single wild species *L. angustifolium* [33, 35–39]. This hypothesis is supported by morphological [40, 41] and cytological studies [38, 39, 42, 43]. Some authors consider *L. bienne* ($2n = 30$) as the progenitor of small seeded flax, originating from Kurdistan and Iran, whereas others consider *L. angustifolium* ($2n = 30$) containing high oil content and seed weight, as progenitor, originating from the Mediterranean region [32, 44]. Other authors suggest that *L. bienne* and *L. angustifolium* are the same species, and both are widely distributed over western Europe, the Mediterranean basin, North Africa, the Near East, Iran, and Caucasus [45, 46]. Recently, a study using molecular markers suggested that the three species originated from one common ancestor, with *L. angustifolium* being the most ancient [47]. While *L. usitatissimum* is an annual crop species, the wild forms can also be biennial or perennial. All species are predominantly self-pollinated [46]. However, cross-pollination may occur via honeybees [48] or by artificial means. A RAPD marker analysis of seven *Linum* species revealed that *L. angustifolium* and *L. usitatissimum* have high genetic similarity and these two species consistently clustered in the same group [49, 50]. A different AFLP study indicated that *L. bienne* is the sister species to *L. usitatissimum* [51] although some consider *L. angustifolium* and *L. bienne* to be the same species [45, 46]. However, genome comparisons using molecular markers of these three species confirmed that they are very closely related genetically and *L. bienne* can be considered as a subspecies of *L. usitatissimum,* rather than a separate species [47].

The distribution of flax from the Near East into Europe is well documented [46]. It is thought that flax cultivation in western Europe (i.e., the Netherlands, northern France, Belgium, and Switzerland) started about 5000–3000 BC when seminomads from the Middle East settled in Flanders and introduced flax cultivation [2]. Since the domestication of flax, there has been a preference for growing flax either for its fiber or oil. In the western region of Eurasia, flax is mainly grown for its fiber, whereas in the eastern region of Eurasia, it is grown for its oil [29]. Fiber flax has a long unbranched growth habit, whereas linseed (oil flax) is much shorter and highly branched. Throughout this chapter, the distinction between fiber flax and linseed is made. Based on molecular studies concerning the conversion of stearoyl ACP to oleoyl ACP [52], it was estimated that flax was domesticated first for oil [53]. This analysis supported the antiquity of oil flax over fiber use. On the other hand, from ancient times until the twentieth century, flax was the important source of fiber for the textile industry in temperate regions, rather than cultivated for oil use [54]

Areas of Production

According to FAO Statistics, the world production of linseed has been recorded decreasing trend since 2005 accompanied with decreasing harvesting areas (Tables 11.1 and 11.2, Figs. 11.1 and 11.2). Until 2010, the largest areas were in Canada, China, and India. In 2011 the largest area was sown in Russia. EU areas increased twice in 2010 (23.27 %) compared to the period 2000–2009 (13–14.38 %) and world production [55]. In 2011, flax and linseed sowing areas reached 36.56 % of world areas [55]. EU flax and linseed production areas were associated with increasing trend of harvesting areas when the share on total world production was 12.31 % in 2005–2009, 20.64 % in 2010, and more than 27 % in 2011.

Genetic Resources

Various research studies on the genetic resources of flax/linseed have already been considered as the basis for successful breeding work. The first attempt to make an inventory of flax/linseed European gene pool was made at the ad hoc meeting of flax germplasm collection holders within newly established Industrial Crops and Potato Network during the ESCORENA Network meeting by the European Cooperative Programme for Crop Genetic Resources Networks (ECP/GR) held in Prague, on December 7–8, 2001 [56]. Based on this inventory, the total European gene pool is estimated at approximately 27,437 accessions, maintained at 16 genebanks. The majority of these accessions are maintained in Russia, Romania, Germany, the Czech Republic, and France. In EURISCO there are approximately 17,175 *Linum* ssp. accessions recorded in the holdings of 31 institutes from 21 countries [57], while another estimate indicated that there are about 18,950 records [58]. The detailed analysis of flax/linseed national inventories covering most of the European countries has been previously described by Pavelek et al. [59]. The structure of these national collections according to the origin and type of use as well as from the number of accessions maintained was presented by the respective authors and national collection curators (Bulgarian collection [60, 61], Czech collection [62, 63], French collection [64], German collection [65], Hungarian collection [66], Dutch collection [67], Polish collection [68], Romanian collection [69, 70], Russian collection [71], Ukrainian collection [72], Italian collection [73], Latvian collection [74], Portugal collection [75], Slovak collection [76], Lithuanian collection [77].)

The traditional methods of evaluation based on passport and special descriptors analysis are often augmented with molecular methods in order to characterize collections, to create core collections, as well as to identify and to distinguish the respective accessions. Various DNA markers have been widely used for diversity analysis in plants, including random amplification of polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism

(AFLP), and simple sequence repeat (SSR). All these marker systems have also been applied to study flax germplasm diversity [37, 41, 49, 50, 78–82]. One of the latest molecular methods is inter-retrotransposon amplified polymorphisms (IRAP) which is now often used in germplasm collection analysis [82–85].

Central crop databases play very important roles in the distribution and inventory of plant genetic resources. These central crop databases are accessible via the European Internet Search Catalogue (EURISCO, http://www.ecpgr.cgiar.org/epgris/index.htm) [56]. The EURISCO web catalogue automatically receives data updates from the National Inventories (NI), and the database effectively provides access to all ex situ PGR information in Europe and facilitates locating and accessing specific PGR accessions. EURISCO is hosted and maintained by the International Plant Genetic Resources Institute (IPGRI) on behalf of the Secretariat of the European Cooperative Programme for Crop Genetic Resources Networks (ECP/GR). EURISCO and the list of FAO/IPGRI *Multicrop Passport Descriptors* (MCPDs) agreed for data exchange by the European database managers in 1996 [86] and have served as a starting point for the International Flax Database (IFDB) development. The IFDB has been managed and coordinated by the Agritec Ltd. since 1993 within the framework of FAO ESCORENA Flax and Other Bast Plants Network (FAO FOBPN) [87–91] and then subsequently since 1999 under the framework of IPGRI Coordination Group Network for Sugar, Starch and Fibre Crops (CGN-SSFC), now known as the Sugar, Starch, Fibre Crops & Aromatic Plants Network (CGN SSFC&APN) at Bioversity International. The IFDB presently includes passport data of 17,152 accessions of 21 collections from 15 countries in an EXCEL IFDB structure [87]. From available data, the structure of IFDB is shown in Figs. 11.3 and 11.4. Based on geographical origin, the majority of accessions are of European origin (Fig. 11.3). Asian accessions are kept as part of the NI Vavilov Institute of Plant Industry (VIR) collection, and accessions with "unknown" origin are kept in several genebanks such as IPK Gatersleben – Germany, CGN Wageningen – the Netherlands. Based on germplasm origin, the biggest proportion of accessions in most European genebanks is comprised of

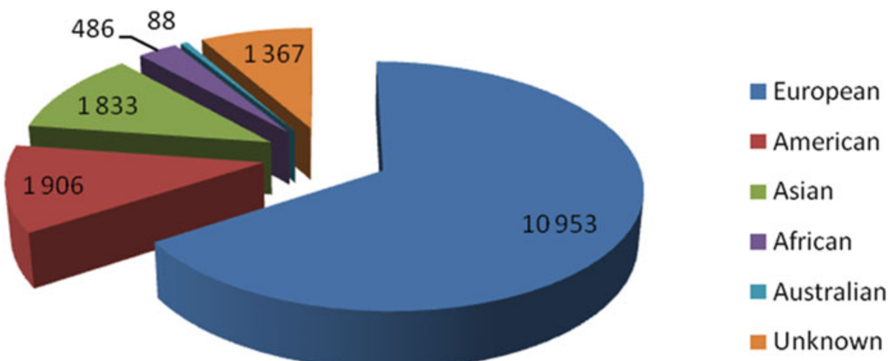

Fig. 11.3 Structure of the flax database (IFDB) according to the geographical origin

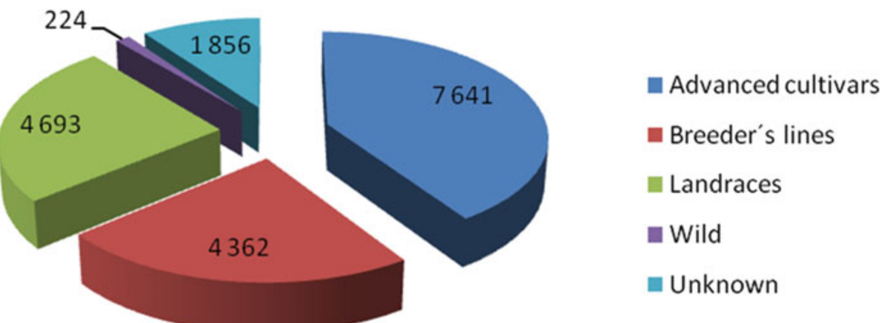

Fig. 11.4 Structure of the flax database (IFDB) according to the origin

advanced cultivars, followed by the breeding lines. This trend is evident in the genebanks which are often located in the research or breeding institutes especially interested in breeding activities and the development of local varieties. Accessions categorized as "landraces" are maintained in the genebanks with long history like in VIR, Russia; Sadovo, Bulgaria; and Sumperk, Czech Republic, where the base collection of historical flax and linseed materials are found. Accessions categorized as "breeder's lines" and "landraces" in IFDB are practically comparable. Those categorized as "wild material" are registered in individual genebanks such as in Sadovo, Bulgaria; IPK, Germany; and IBF, Poland. The decision of the respective genebank managers will determine if accessions in this last category will become the part of *L. usitatissimum* collection or not. Accessions categorized with "unknown" origin comprise nearly 57 % in ISCI Bologna, Italy; 37 % in IPK, Germany; and 30 % in IBC, Ukraine.

Major Breeding Achievements

Contemporary trends, breeding aims, and methods of flax/linseed breeding have been recently described in the *Handbook of Natural Fibres* [59]. The publication presented the latest up-to-date methods of linseed breeding specifically focused on increasing its utilization in the food industry, for bioproducts, and in medicinal and pharmaceutical applications due to its nutrition value and demonstrable benefit for human health.

Flax/linseed is very important crop with regard to its content of essential unsaturated fatty acids (EFAs) in its seed oil and phytoestrogens (plant lignans, SECO – secoisolariciresinol), proteins, and dietary fibers in seed meal. The seeds also contain less amount of carotenoid lutein and considerable amounts of antinutritional substances cyanogenic glycosides (CG) as well.

Target Traits and Current Breeding Goals

Breeding work in flax/linseed is being carried out in agreement with the breeding objectives. For flax and linseed, these are presented in Table 11.4 [59]. These general objectives are practically common in all countries conducting breeding activities and research. Nearly all of Europe was focused on breeding of flax in the recent past (such as France, Belgium, the Netherlands, the Czech Republic, Poland, Russia, Ukraine), while countries like Canada, the USA, and also India were predominantly interested in linseed breeding. Nevertheless, the characters affecting yield are common for both types of use, like resistance to lodging and resistance to fungal diseases and other pests [29].

The current breeding work in Canada is focused on dual-purpose flax *L. usitatissimum* for seed/oil and straw/fiber [92]. The goal is to find a compromise proportion between straw and seed yield. Opinions vary on the best way to breed dual-purpose *Linum* crops. The breeding work that has been carried out at the University of Saskatchewan pointed out that linseed can yield only about 33 % of the fiber yield of flax, whereas flax can yield 60 % of the seed of linseed, so selection for dual-purpose varieties should be based on flax varieties [93]. However, others have recognized that the seed component has been the most important economically and, considering that fiber length and fineness is less important for many modern applications, have opted to use linseed x linseed or linseed x flax as parents for dual varieties [94–98]. Recombinant inbred lines (RILs) from crosses of linseed x linseed or linseed x flax lines showed positive genotypic correlations for both high seed/oil and straw/fiber yields [99]. Foster et al. [95] also suggested that there is lower genetic variation for important traits in flax than in linseed. The main problem in breeding dual-purpose varieties is the different rate of maturation of the seed and fiber, with the fiber typically reaching optimum maturity around 3 weeks before maximal seed yield is reached [94]. Attempts have been made to address the synchronization of stem and seed maturity through breeding [100, 101]. However, attempts to find viable dual-purpose varieties have been met with only limited success [99, 102].

Table 11.4 Breeding aims for flax and linseed

Flax	Linseed
High resistance to lodging	High resistance to lodging
High resistance to pathogen complex	High resistance to pathogen complex
Middle vegetation period	Middle vegetation period
Average unretted stem yield	Low stem yield
High content of fiber in the stem	High seed yield
High yield of fiber per hectare	High content of fat in the seeds
New quality: low linolenic acid content, high content of lignans (SECO), low content of cyanogenic glycosides	High fat yield per hectare New quality: low linolenic acid content, high content of lignans (SECO), low content of cyanogenic glycosides

Low-linolenic acid linseed breeding is the second main objective of Canadian linseed breeding. During the 1980–1990s, Australian and Canadian breeding programs have developed and used linseed varieties with completely different and unique quality of oil compared to the common profile of *L. usitatissimum*. This work is specific to Agriculture and Agri-Food Canada (AAFC), Crop Development Centre (CDC) in Saskatchewan, and Agricore. As the result of these breeding programs, low linolenic linseed varieties under the common trade Linola have been developed [61, 103–107]. Linola is the trademark name of solin, a mutant strain of flax (*L. usitatissimum*) developed in the early 1990s by the Australian agency, Commonwealth Scientific and Industrial Research Organisation (CSIRO). It was developed and released by Australia in 1992 and first commercially grown in 1994. This variety was developed to provide a source of edible linseed oil with a low alpha-linolenic acid (ALA) content of approximately 2 %, as compared to 50 % in the standard type variety. It was done to improve the storage quality of linseed when used as a bulk livestock feed. Linseed's previous main use had been linseed oil for use as a paint ingredient, with the ALA (omega-3 fatty acid) being a quick-drying component. With the advent of "plastic" water-based paints, the linseed market fell into decline. When marketed as a stock feed, linseed oil's omega-3 content also deteriorated quickly in storage. It also has a correspondingly higher content of the gamma-linolenic acid and omega-6 fatty acid, around 65–75 %. The lower level of ALA increases the oxidative stability of the oilseed, which remains edible when stored. Selection for seed color was also conducted changing the standard type dark brown seed to a light yellow seed, which consequently gives an oil of a lighter color, easily distinguished from the darker linseed oil, and this is the flaxseed oil that we consume today. Linola oil does not contain any useful omega-3 fatty acids and is not likely to be found as a human food/health supplement. Linola can specially help against Neurodermitis. Linola is being produced in Australia, Canada, the UK, and the US states of Washington and Idaho. Linola substitutes for flax in cropping rotations because of lower production costs than canola, but brings prices comparable to canola or other edible oils.

The breeding programs in the USA have been partly reduced. Flax breeding activities in Minnesota have been terminated in 1984 in connection with V. E. Comstock's retirement. In South Dakota, the breeding of flax was combined with other oil crops. In North Dakota State University, the breeding activities have been reduced to Word Flax Collection maintenance and coordination of regional variety testing.

The US breeding activities are now focused mainly on the below objectives:

– To develop flax cultivars with desirable agronomic characteristics, seed yielding ability, quantity and quality of oil
– To obtain varieties with tolerance to wilt and pasmo diseases and resistance to known North American races of rust

Table 11.5 Target traits and parameter levels in flax and linseed

Flax ideotype	Linseed ideotype
Resistance to lodging: 9	Resistance to lodging: 9 b
Resistance to pathogen complex: 8	Resistance to pathogen complex: 8 b
Middle vegetation period	Middle vegetation period
Yielding potential of unretted stem reached in trials: 7–8 t ha^{-1}	Seed yielding potential reached in trials: 2,30–2,40 t ha^{-1}
Yielding potential of the seeds reached in trials: 1,10–1,30 t ha^{-1}	Fat content potential reached in trials: 42–46 %
Long fiber content potential reached in trials: 22–25 %	Fat yielding potential reached in trials: 980–1,000 kg ha^{-1}
Total fiber content potential reached in trials: 39–41 %	Low linolenic acid content: less than 5 %
Long fiber yielding potential reached in trials: 1,25–1,40 t ha^{-1}	High content of lignans (SECO)
Total fiber yielding potential reached in trials: 2,50–3,0 t ha^{-1}	Low content of cyanogenic glycosides
Low linolenic acid content: less than 5 %	Increasing absorption and accumulation of Cd and Pb, increased tolerance to Cd and Pb
High content of lignans (SECO)	Resistance to herbicide Basta
Low content of cyanogenic glycosides	
Increasing absorption and accumulation of Cd and Pb, increased tolerance to Cd and Pb	
Resistance to herbicide Basta	

Flax breeding is focused on population improvement. A secondary objective is to develop and maintain populations with useful genetic variability necessary to support the development of improved cultivars. Several target parameters (Table 11.5) were also established in the Czech breeding program [59] resulting in the registration of new linseed varieties Amon [108] and Raciol [109]. The current breeding targets of the Czech linseed breeding program are: (1) high-linolenic type: C18:3 content 55–65 %; (2) low-linolenic type: C18:3 content <10 %; (3) intermediate type: C18:3 content 30-40% of both linoleic and alpha-linolenic acid. These three types are bred both for brown and yellow colour of seeds. The same traits are being developed with increased content of secoisolariciresinol (SECO) for high and low C18:3 and with decreased cyanogenic glycosides content [110].

Breeding Strategies and Integration of New Biotechnologies

Crop improvement is based on increasing genetic variability followed by the selection of suitable phenotypes with desired combination of features and then with reduction to eliminate genetic variability to meet the conditions of distinctness, uniformity, and stability in newly developed varieties. Next to the traditional breeding (intra or intergeneric hybridization, pedigree selection, bulk breeding),

new biotechnological methods are increasingly used resulting to the stabilization of genotypes via the process of homozygosity – haploid, double haploid production, or increasing genetic variability of genotypes using other DNA molecular methods resulting in GMO flax/linseed development.

Using double haploids is a very effective method since completely homozygous genotypes, non-segregating in any trait, are obtained in one generation. The oldest method of haploid production is polyembryonic method [111]. Some genotypes are able to produce double embryos in one seed, from which mostly one is diploid and one is haploid, but mostly in a very low frequency. Moreover, induction of polyembryos into breeding materials is very lengthy. There are several methods to produce double haploids in flax/linseed which include microspore culture [112, 113], ovule culture [114–120], and anther culture [121–123]. The details of these methods have been previously described by Pavelek et al. [59].

In order to evaluate the level of genetic variability of genotypes, breeding lines, or developed varieties, molecular methods play a very important role, and compared to the traditional methods of evaluation based on morphological traits, characterization is considered more effective and accurate. The abundance of DNA markers, their environmental insensitivity, and non-tissue-specific characteristic are some of their advantages. Markers are useful for varietal identification and evaluation of genetic variation. Among the different marker systems include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), or simple sequence repeat (SSR). Previous applications of these methods in the crop have been described [79, 80, 124].

To incorporate various genes into the flax/linseed genome, different genetic transformation methods have been developed. Flax, like most dicotyledonous crop species, is amenable to gene transfer via *Agrobacterium* [125]. Flax cells are easily transformed with *Agrobacterium tumefaciens*, and these can be easily grown but require an elaborate inoculation/selection/regeneration procedure [126]. The success of these methods is very strongly influenced by the chosen part of the plants. It has been reported that the hypocotyl is the most regenerable part of flax/ linseed plant [127, 128]. To enhance transformation efficiency, an improved procedure for the production of flax plant was developed [129].

Next to *Agrobacterium* transformation, other methods are developed like particle bombardment. Wijayanto and McHughen [130] documented a successful biolistic process for producing transgenic linseed flax. Successful plant regeneration is closely influenced by the respective chosen selection medium (kanamycin, hygromycin B, spectinomycin) [131, 132] and protocols [30].

For monitoring gene expression in transgenic tissues, markers such as β-glucuronidase (GUS), luciferase (LUC), or β-galactosidase (LazC) are used. The GUS assay is the most useful assay in flax transformation [129] but destructive. As an alternative, green fluorescent protein (GFP) is used as a visual marker during the establishment, evaluation, and improvement of transformation procedures for flax plants. GFP allows nondestructive evaluation and enables plant growth and development without damage to transgenic tissues [133].

Linum was among the first crop species to benefit from a herbicide resistant construct, as glyphosate (Roundup) resistance. Sulfonylurea and glufosinate resistance all were quickly introduced and field tested in commercial linseed flax genotypes. Flax has been transformed with the aim to improve resistance to *Fusarium* [134]. The idea was that the increase in the flavonoid content in transgenic flax plants might be the reason for observed, enhanced antioxidant capacity of those plants. The increased antioxidative properties of transgenic plants may lead to improve resistance to *Fusarium*. Successful transformation of *L. usitatissimum* plants with bacterial genes involved in polyhydroxybutyrate synthesis has also been reported [135]. This offers new perspectives for environmentally safe production of basic components for modern biodegradable composites. Flax as an industrial crop can be utilized for phytoremediation as well. Flax transformation with heavy metal-binding proteins has been reported [136]. However, to date, no transgenic linseed/flax is permitted to grow for commercial utilization. Only one transgenic linseed has reached registered cultivar status, "CDC Triffid," but authorization of the variety was rescinded in Canada in 2001 and commercial cultivation of CDC Triffid flax has since been banned.

GM flax is being developed primarily for agronomic traits. Herbicide tolerance is very important for weed control, as well as fungal resistance, insect resistance, and stress tolerance to adapt to changing climate and local factors. In addition, quality traits of flax that are of interest include modified oil composition in particular enrichment for health-promoting ingredients, such as flavonoids (antioxidants) and omega-3 fatty acids. The shift to renewable resources also influenced the development of flax with modified fiber composition (modified elasticity and thermoplastic characteristics of the flaxseed fiber for the synthesis of biological degradable synthetic material), production of pharmaceutical agents (molecular pharming usage of GM flaxseed as a system to produce pharmaceuticals, which to date is only experimental), and land reclamation (specifically phytoremediation of heavy metal-contaminated soil where the plants are modified so they are able to grow within this soil and extract heavy metals and accumulate them within the plant biomass) [136–138].

Field trials of GM flaxseed have been conducted in the EU under three applications in three countries (Sweden, Poland, the Czech Republic) during 2005–2007. The traits evaluated in these experiments were oil composition, flavonoid content, elasticity (bioplastics), herbicide tolerance, insect resistance, insect and fungal resistance, and heavy metal absorption. Also, there have been 198 applications for transgenic field trials in Canada during 1988–2002, mostly for evaluating herbicide tolerance.

No genetically modified flax is currently commercially available. A herbicide-resistant GM flax was introduced in 2001, but was soon taken off the market because European importers refused to buy it. In September 2009, it was reported that Canadian flax exports had been contaminated by the unapproved, illegal, genetically modified (GM) variety Triffid. Since linseed derived from GM flax has not been authorized in Europe, products containing even minimal amounts cannot be made commercially available. Transgene contamination of flaxseed has

been found in the food industry. On September 10 2009, the European Union (EU) Rapid Alert System for Food and Feed (RASFF) reported finding an unapproved genetically modified (GM) flax/linseed event in cereal and bakery products in Germany. Traces of GM linseed have been found in at least seven states. Baking ingredients contaminated with the GM linseed had been distributed to 15 German states and also exported to other countries by a German company based in Hessen. Linseed is an ingredient in baked goods and muesli. Consumption of products containing minute traces of GM linseed however does not present a health risk, but the GM variety FP967 (CDC Triffid) is not authorized for food or feed use in the EU. The variety has tolerance to soil residues of sulfonylurea-based herbicides and was developed by the Crop Development Centre (CDC) at the University of Saskatchewan in Canada. As previously mentioned, the authorization of the variety was rescinded in Canada in 2001 and the cultivation of CDC Triffid flax has since been banned. Canada supplies approximately 70 % of the total flax/linseed in the EU annually. Because GM flax FP967 is not authorized in the European Union, there is zero tolerance for the variety. That means any raw material or flax/linseed derivative analyzed to be positive for FP967 is illegal and not marketable in the EU. The Canadian Grain Commission is investigating how the admixture of the GM flax in linseed products could have occurred.

Seed Production

The physiology of yield in flax/linseed is influenced by a lot of internal as well as external factors (plant morphology, sowing density, plant density, date of harvest, type of harvest process, climatic conditions, and others). While there have been several studies investigating the effects of plant density on yield and/or quality in flax [93, 139–142], there have been only few studies investigating the detailed physiology of yield per se in either flax or linseed. All of the below mentioned studies tried to find and investigate the relationships among different outside factors influencing the ratio of stem, fiber to seed, and oil and their mutual relations resulting to the respective quality of both stem-fiber and seed-oil. For example, Casa et al. [140] found significantly higher influence of planting year and soil conditions compared to plant density. However, total biomass production can be greater in flax than in linseed [93] possibly due to higher height of plants and the greater energy requirement associated with the higher oil seed yield in linseed. Differentiation of biomass into stem and fiber in flax compared to branch, capsule, and seeds in linseed is predominantly influenced by plant density. Low plant density positively affects greater branching and higher seed yield but limits stem and fiber yield, whereas less branching in flax and high plant densities give greater stem yields. Flax has more rapid and sustained fiber growth, whereas linseed has more rapid and sustained seed growth [93]. However, the harvest index for seed in flax and linseed can be very variable [143]. Use of high-quality seeds for sowing is recommended – these include seeds treated with fungicides. The optimum sowing

quantity of fiber flax for industrial production is 110–130 kg ha^{-1} (2,000–2,400 seeds per m^2), while for seed production is 50–70 kg ha^{-1} (1,000–1,100 seeds per m^2) [144]. Nevertheless, higher sowing quantities are published reaching up to 140 kg ha^{-1} [145] or even 170–180 kg ha^{-1} [146] corresponding to a plant density of 3,000 plants per m^2. In practice however the optimal stand at harvest time reaches 1,600–1,800 plants per m^2 [147].

With regard to the direction of cultivation (stem, fiber/seed, oil), also the respective type of harvest is used. The harvesting process for stem and fiber includes several operations – stem pulling, deseeding, stretching on the field, retting, turning, and collecting the stem. Sometimes biphasic harvest can be used, when the stem is pulled and stretched on the field with the bolls. Just after several days when the stem is turned and colored, the bolls are harvested. Specific machines are used for the abovementioned operations. On the other hand, the seed harvest requires a combine harvester. Linseed harvesting by combine has important impacts in energy, exploitation, and economical parameters [148]. The four major producers of linseed are Canada, China, the USA, and the European Union (Table 11.6). Casa et al. [140] also mentioned Argentina and India. Linseed production is still insufficient to meet the demand and the EU is a major importer [149].

For seed production, there should be established rules and requirements in every country regulating statutory distance from neighboring sources of pollen which may result in undesirable cross-pollination. These rules include species and category of the seeds (level of multiplication), varietal purity, and analytical purity. Table 11.7 shows the evaluation system used in the UK [150]. A similar evaluation system for seed production is used in the Czech Republic (Table 11.8) [151]. As far as the varietal purity is concerned, one plant per of other species 30 m^2 from the variety is acceptable for basic seed production and one plant per 10 m^2 for certified seeds C1, C2, C3 [146]. In the Czech system, there are three plants per 100 m^2 in categories SE and E and 10 plants per 100 m^2 in category C [151].

According to Casa et al. [140] and Diepenbrock and Iwersen [152], the sowing dose (density) is not a limiting factor of seed yield. Based on their results, the seed yield is mainly influenced by soil and climatic conditions compared to sowing dose which in their case was 200–800 plants per m^{-2} because the plants with lower plant number have a tendency to increase the number of capsules per plant. However, different levels of sowing doses were studied [153–156]. Currently European growers use the optimal sowing dose of 800 plants per m^{-2} (unpublished results).

Table 11.6 The world's top 20 linseed-producing countries

Rank	Country	Production (mill tons)	Proportion of world production (%)
1	Canada	633,500	34.1
2	China	480,000	25.9
3	India	167,900	9.0
4	USA	149,963	8.1
5	Ethiopia	108,222	5.8
6	Russian Federation	79,573	4.3
7	Bangladesh	50,000	2.7
8	France	33,801	1.8
9	Argentina	34,065	1.8
10	Egypt	12,226	0.7
11	United Kingdom	23,000	1.2
12	Brazil	14,722	0.8
13	Afghanistan	13,500	0.7
14	Ukraine	11,400	0.6
15	Belgium	10,031	0.5
16	Mexico	7,200	0.4
17	Spain	7,300	0.4
18	Australia	7,000	0.4
19	Germany	6,000	0.3
20	Sweden	6,700	0.4

Based on data from FAO stat data 2007 (http://faostat.fao.org)

Table 11.7 UK varietal and analytical purity standards for flax and linseed

Species and category	Varietal purity (%)	Analytical purity (%)
Flax		
Basic	99.7	99.0
C1	98.0	99.0
C2	97.5	99.0
C3	97.5	99.0
Linseed		
C1	99.7	99.0
C2	98.0	99.0

Based on data from Guide to The Oil and Fibre Plant Seed (England) Regulations 2002. Department for Environmental Food and Rural Affairs, Plant Varieties and Seeds Division. Cambridge, UK; 2004. http://www.fera.defra.gov.uk/plants/seeds/seedCertification/documents/guideOilFibrePlantSeeds02.pdf. Last Accessed on April 23rd, 2014

Table 11.8 Moisture, germinability, and varietal purity standards for flax and linseed in the Czech Republic

Species and category	Moisture (%)	Germinability (%)	Varietal purity (%)
Flax			
SE	13	92	99
E	13	92	99
C	13	92	99
Linseed			
SE	13	85	99
E	13	85	99
C	13	85	99

Based on data from Ref. [151]

Market Challenges/Barriers to Commercialization/ Opportunities

The current situation is not favorable for flax but more favorable for linseed. There is a continuous decrease in flax areas all over the world during the last several years including in traditional western European countries like France, Belgium, and the Netherlands. In these three countries, flax areas have been decreasing since 2006 [4, 5]. On the opposite side, in Egypt there was a small and insignificant increase in flax areas. There were accurate reports that China in 2006 had a confirmed flax area about 130,000 ha, but only production from 78,000 ha was processed. These data are related to the planted areas where they were collected. Area harvested therefore excludes planted areas which were damaged or destroyed due to outside unfavorable factors [157].

The world flax areas total approximately 400,000 ha which about 25 % are located in France, Belgium, and the Netherlands. Fiber production of western European countries represents approximately 60 % of the world market. Based on these statistical data, it is obvious that world long fiber production is mainly concentrated in western European countries between the Escaut and Seine rivers [4].

At the beginning of the nineteenth century, flax production areas in EU countries reached 50,000–80,000 ha. It decreased to 44,000 ha in 1991/1992 during the peak of the flax crisis. Because of the new endowment support from the EU, flax areas stabilized and the interest in growing flax has increased. Flax areas increased and reached more than 100,000 ha in 1995. However the main reason of this unexpected increase was the speculative flax growing in untraditional countries – those that do not grow flax regularly but did so to receive subsidies. The financial support was provided without any view to the following processing and utilization. Flax-growing areas and their development during last 10 years are presented in Table 11.9.

Since 2000, the new rules for providing subsidies have been adopted, and a lot of untraditional countries (e.g., Great Britain, Spain, Portugal) lost their interest in

Table 11.9 Trend in flax-growing areas in EU countries

Country	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
France	49,129	53,680	66,561	66,772	74,439	78,281	81,843	76,278	74,500	67,688
Belgium	12,176	13,320	16,860	15,315	19,306	19,823	18,761	15,919	14,740	12,230
Netherl.	3,570	4,016	4,415	4,062	4,615	4,517	4,691	4,366	3,500	2,525
Total	64,875	71,016	87,836	86,149	98,360	102,621	105,295	96,563	92,740	82,443
G. Britain	15,000	12,089	4,860	177	1,976	1,820	21	0	0	0
Finland	863	1,067	365	202	97	67	57	0	0	0
Germany	569	402	200	200	224	180	38	30	51	42
Spain	126,226	13,895	457	55	0	0	0	0	0	0
Austria	336	450	132	171	142	110	133	129	0	0
Sweden	1,327	21	0	25	0	30	0	0	34	
Denmark	11	45	19	2	0	0	0		0	0
Italy			0	6	20	80	18		0	0
Ireland									0	0
Portug.	4,678	3,810	0	0						
Latvia						2,400	2,072	1,057	0	356
Lithuania						5,600	3,599	1,420	424	247
Poland						5,745	1,507	788	1,044	779
Czech R.						5,499	4,311	2,736	824	156
Total EU	213,885	102,795	93,869	86,987	100,819	104,908	105,562	96,722	92,825	82,485

Based on data from DG VI – C4 European Commission, Vlas Berichten 8/02, 12/03, 22/03, 22/04, 23/05, Czech Flax Union

growing flax. Due to this reason, the flax areas decreased from practically 214,000 ha in 1999 to 87,000 in 2002. After addition of new countries to EU (i.e., Latvia, Lithuania, Poland, the Czech Republic), flax areas again increased in 2004, but since this year, long-lasting decrease of flax areas has been again observed [4]. For example in 2010, the processing industry collapsed in the Czech Republic. All machinery was mostly sold to foreign countries and flax-growing and breeding activities have been stopped. The situation with flax is not so serious in western European countries although the areas also declined. Breeding activities have been stopped in the UK, while in France (Terre de Lin) or in the Netherlands (CEBECO, Van de Bilt), these are still carried out [158].

Also linseed production showed a slightly decreasing trend, while linseed areas increased in the EU (Table 11.10). The average yield in the EU fluctuated between 0.65 and 1.17 $t{\cdot}ha^{-1}$ [55]. In 2008, Canada increased linseed areas in approx. 100,000 ha followed by Belorussia and the Russian Federation. The share of EU linseed production on the total world production reached 5.33 % in 2007 and only 3.66 % in 2008. In the Czech Republic, there has been a decreasing trend since 2005 with the share on the world production down from 1.7 to 0.06 % compared to EU [159]. Seed of linseed varieties is imported into the Czech Republic, but the volume is decreasing. In 2009, the import was reduced by 36 % equivalent to 1,854 tons compared to 2008. The biggest importers of seeds for technical applications are Ukraine (404 tons), the Russian Federation (324 tons), Belgium (238 tons), Germany (187 tons), Slovakia (185 tons), and Poland (124 tons). Exports of linseed from the Czech Republic increased to 3,040 tons in 2009 and focused mainly to Austria (1,344 tons), Poland (702 tons), Slovakia (297 tons), France (192 tons), and Italy (123 tons) [130].

The main barrier for finding a balanced proportion between flax and linseed market is the lack of a common EU policy for all members. Subsidy rules should be based on compromise approach between flax and linseed using clearly defined evaluation criteria for both types. An example is given using the Alice variety in the National Listing of Varieties in the United Kingdom is a typical example of these barriers. It was not possible historically to grow linseed-type varieties and claim the processing subsidy for its fiber production because only true flax (i.e., linen type) varieties were allowed on the EU Approved List. This resulted in a real problem in developing multifunctional flax varieties for the UK: A notable example of this was the Alice variety, which was entered on the National Listing in the UK where it passed the DUS tests and VCU criteria. Subsequently, it was accepted as a flax variety, but was only allowed entry on the EU Common Catalogue with the footnote: "Not clearly classifiable as a linseed or flax variety in the absence of established definitions for those common names and not uniformly classified by Member States" [158]. The position was challenged with the commission, but the above classification was upheld. This position meant that the variety was excluded from the EU Approved List and was not eligible for processing aid as an eligible flax variety and effectively could not be marketed as a fiber variety. While this apparent barrier to development of dual-purpose varieties would no longer be present if processing subsidies were removed (as there would no longer be a need

Table 11.10 Linseed areas and production in the European Union, 2005–2011

EU	2005	2006	2007	2008	2009	2010	2011
Area (ha)	208,211	186,789	138,073	121,642	141,004	182,253	166,308
Production ($t \cdot ha^{-1}$)	237,650	164,446	101,553	79,665	128,799	156,790	148,412
Average yield ($t \cdot ha^{-1}$)	1.14	0.88	0.74	0.65	0.91	0.86	0.89

Based on data from FAO stat data 2011 (http://faostat.fao.org)

for an EU Approved List for flax), there may still be difficulties in getting dual-purpose varieties approved on the Common Catalogue if they do not meet VCU criteria for “flax” types. Therefore, some consideration should be given at national level to develop simple descriptive lists for *Linum* which had rates or grades for their main characteristics. The development of protocols for testing such diverse types would require some thought and discussion, because, for instance, flax and linseed types are sown at different seed rates. In the UK, no VCU criteria exist for hemp or flax for fiber, as no varieties have been registered. However information on the criteria in relation to flax quality was available from the Netherlands. UK’s Department for Environment, Food and Rural Affairs (Defra) should give consideration to the VCU criteria and the possibility of developing simple descriptive National Lists. If processing subsidies and an approved list continue to exist for flax, then Defra should give attention to making sure that UK growers are not disadvantaged through exclusion of varieties which do not conform to the type of variety preferred in France and Belgium, but would otherwise be suitable for UK production of flax fiber [158].

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Chapter 12
Abaca (*Musa textilis* Nee) Breeding in the Philippines

Antonio G. Lalusin and Maria Lea H. Villavicencio

Abstract Abaca (*Musa textilis* Nee.), a plant native to the Philippines, is the source of fiber known internationally as Manila hemp. It is indigenous to the Philippines whose favorable climatic condition and volcanic soils are suited to its cultivation. It is often used as raw material for cordage, clothing, and various handicrafts. Furthermore, the fibers can be manufactured into specialty papers such as currency notes, filter papers, stencil papers, and tea bags, among others. The abaca industry is a major dollar earner and an important export crop of the country. Due to the current concern for biodegradable products and forest conservation, it is expected that the abaca industry will continue to flourish in both domestic and international markets. With the advent of new uses of abaca, the crop will be extensively utilized for more industrial applications because it is a natural and superior material. The Philippine abaca industry continues to make a stronghold in both international and domestic markets generating US$80 M annually from 1996 to 2000. Being an export-oriented commodity, the country's abaca industry has maintained its status as the world's largest producer accounting for 97 % share of world imports. However, the abaca industry is still relying solely on traditional varieties, and due to limited attention devoted to sustained varietal improvement, the old abaca varieties had outlived their usefulness and now become easy prey for disease devastation.

Different plant breeding techniques are employed to develop abaca varieties possessing desirable traits like high fiber yield, good fiber quality, and high degree of resistance to major diseases of abaca. With conventional breeding method coupled with the recent advances in molecular biology and biotechnology, a more directed solution to the disease problem of the industry can now be identified. It is possible to isolate resistance genes from abaca varieties or in wild relatives. With basic knowledge on mechanisms of abaca-pathogen interactions, similar approaches can be applied to abaca breeding to produce durable resistance at a much faster pace. These improved abaca varieties can either be used directly for commercial planting or as genetic stocks to develop high-yielding varieties resistant to various diseases. The availability of these improved resistant high-yielding varieties backed by appropriate marketing strategies and employed with sound

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resistance management schemes brings forth a package of technology that promises to make abaca one of the top foreign exchange earners of the country.

Keywords *Musa textilis* Nee • Breeding • Abaca industry • Genetic conservation

Introduction

Abaca (*Musa textilis* Nee.), a plant native to the Philippines, is the source of fiber known internationally as Manila hemp. It is indigenous to the Philippines whose favorable climatic condition and volcanic soils are suited to its cultivation. It is often used as raw material for cordage, clothing, and various handicrafts. In addition, the fibers are manufactured into specialty papers such as currency notes, filter papers, stencil papers, and tea bags. The abaca industry is a major industry in the Philippines. Due to the current interest for biodegradable products and forest conservation, it is expected that the abaca industry will continue to flourish in both domestic and international markets. With the advent of new uses of abaca, the crop will be extensively utilized for more industrial applications because it is recognized as a natural and superior material.

The Philippine abaca industry continues to make a stronghold in both international and domestic markets generating US$80 M annually from 2001 to 2010 [1]. Being an export-oriented commodity, the abaca industry has maintained its status as the world's largest producer accounting for 85 % share of world imports. Domestic consumption is increasing with a 5.7 % growth rate. The economic and social impact of abaca is further indicated by the fact that the biggest sector of the industry consists of farmers with small landholdings, averaging close to only 2 ha. Of the country's total land area planted to abaca, it is the major crop in the Eastern Visayas and the Bicol Region of the country, accounting for 36 % and 30 %, respectively.

Philippine production of abaca fiber for the past decade has been relatively stable, averaging 65,701 metric tons (MT) per year. Three regions in the Philippines (Bicol Region, Eastern Visayas, and Davao Region) account for 79 % of the total national abaca production during the 10-year period. Among the provinces, Catanduanes continues to be the top producer of the crop with 18,971 MT of abaca produced in 2010, equivalent to 33 % of the total regional production (Fig. 12.1). The provinces of Leyte (12 %) and Northern Samar (7 %) rank second and third, respectively. The volume of abaca production in the Bicol Region has been fluctuating for the past 10 years due to the prevalence of typhoons that hit the region, particularly the province of Catanduanes where the bulk of production is found [1].

In general, the Philippine abaca industry still relies on traditional varieties. However, through the years and due to lack of germplasm diversity, the old and traditional varieties are becoming susceptible to various diseases resulting to genetic erosion. Extensive rehabilitation programs in abaca-growing regions are

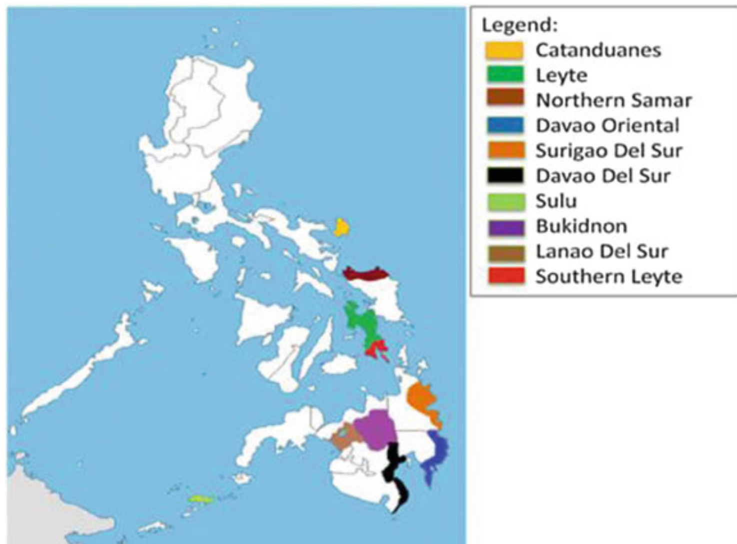


Fig. 12.1 Top ten abaca-producing provinces in the Philippines, 2010

currently being done as a concerted effort in addressing the primary problems of the industry. Breeding programs are being revitalized. In addition, a more flexible breeding methodology is being adapted to anticipate any shift in demand by the abaca industry for fibers.

Different plant breeding techniques are employed to develop abaca varieties possessing desirable traits like high fiber yield, good fiber quality, and high degree of resistance to major diseases of abaca. Conventional breeding methods are coupled with recent advances in molecular biology and biotechnology to come up with a more directed solution to address the disease problem of the industry. Biotechnologies make it possible to isolate resistance genes from abaca varieties or wild relatives. With basic knowledge on mechanisms of plant-pathogen interaction, progress in abaca breeding to produce durable resistances is expected to proceed at a much faster pace. Improved abaca varieties can either be directly used for commercial planting or as germplasm materials to develop other high-yielding varieties resistant to various diseases.

Importance of Abaca in the Philippine Economy

Abaca has been grown in the Philippines for centuries and was known to Filipinos before the Spanish occupation. The crop has been cultivated, processed, and traded, and abaca products have been used for tax payments. When the Spaniards arrived in the island of Cebu in 1521, they noted that the natives were wearing clothes made

from the fiber of the abaca plant and the weaving of abaca fiber was already widespread in the island.

It was, however, only much later that the commercial or export importance of abaca was recognized. According to historical accounts, an American lieutenant of the US Navy brought abaca fiber to the United States in 1820, and 5 years later, the first exportation of the fiber was made. Since then, abaca became well known as one of the strongest materials for marine cordage due to its superior tensile strength and proven durability in saltwater applications. Attempts to introduce the crop in 1822 to India, Borneo, German East Africa, West Indies, and Florida were either unsuccessful or found not commercially viable [2]. During the twentieth century, abaca fiber became the premier export commodity of the country. The US government introduced the crop in many countries with a climate similar to the Philippines to ensure supply of marine cordage for the US Navy [3]. The US government introduced abaca in 1923 in several Latin American countries with tropical, humid, and warm climate similar to the Philippines. These include Panama, Ecuador, Costa Rica, Guatemala, Honduras, Dominican Republic, Brazil, British and French Guiana, Cuba, Jamaica, Puerto Rico, Martinique, Guadeloupe, Trinidad, Mexico, St. Vincent, Bolivia, Peru, Nicaragua, and El Salvador [4]. In 1925, Abaca seed pieces from the Philippines were also used to establish plantations in Sumatra, in British Borneo, and in Malaya. It was also introduced in New Caledonia and Queensland [5]. An abaca plant was noted growing in a garden in New Zealand. The crop was introduced in Vietnam in 1958 with seed pieces from Costa Rica. It was after World War II that a Japanese owner of the abaca plantation in the Philippine province of Davao started a formal field testing and a successful cultivation of abaca in Ecuador. The country now also produces abaca for export, and it supplies approximately 15 % of the world market.

A joint project by the Philippines and Ecuador was conducted from 1998 to 2004 for the improvement of fiber extraction and identification of high-yielding varieties. This was funded by the Common Fund for Commodities (CFC), United Nations Industrial Development Organization (UNIDO), FAO Intergovernmental Group on Hard Fibers (FIGHF), and Philippine Fiber Industry Development Authority (FIDA). The lead collaborating agency in Ecuador was the Ecuadorian Abaca Corporation (CADE). Fiber extraction machines and tools were developed, evaluated, and improved by both countries. New varieties were developed, but the planned varietal exchange activities between countries did not materialized. The variety being planted by the CADE was an introduced Philippine variety "Tangongon" [6].

Abaca is currently cultivated in almost all regions in the Philippines except the Ilocos Region, Cagayan Valley, provinces in Region 3, and Cavite and Batangas provinces in Region 4. Small-hectare experimental plantings have been established in Ilocos Norte, Rizal, Sultan Kudarat, and Tawi-Tawi. Abaca grows best in Philippine provinces characterized with the most amount of rainfall, no dry season, a humidity range of 78–88 %, and with average temperature of less than 27 °C.

Today, abaca – known commercially as Manila hemp – has been transformed from its traditional use as a raw material for rope and paper into a source of material

for the fashion industry and composite fibers used in luxury automobile upholstery. Philippine fashion designers sell intricately embroidered “barong” shirts made of abaca “sinamay.” The Japanese also know it as “makiwara” – the rope tied around posts used in martial arts practice. Its superior tensile and folding strength and high porosity also make it especially suitable for currency papers (the Philippine peso bills have 20 % content, while the Japanese yen has 70 % content), furnitures, home décors, textiles, cosmetics, cigarette papers, surgical masks, sausage casings, tea bags, coffee filters, and others.

In 2007, the Philippines produced 60,000 MT of abaca fiber, while Ecuador produced 10,000 MT. Exports from the Philippines, where 85 % of world supply comes from, are mostly pulp rather than raw fiber. The county generates about US $76.8 million a year from exports of raw fibers, pulp, cordage, and fiber crafts. Only the Philippines (where it is a small farmer’s crop) and Ecuador (where it is grown on large estates) supply the world market. Indonesia is a small producer, but it is currently expanding its abaca hectarage.

In 2004, the price of abaca fiber reached an average of US$0.71 (about ₱40) per kilogram of which US$0.39 or 56 % went to farmers. Abaca farmers produce 78,000 MT a year valued at over US$0.10 billion. By 2020 – when farms expand an additional 32,600 ha – abaca fiber production should reach 152,000 MT. Fiber yield is expected to increase from 565 kg per hectare per year to 900 kg per hectare. This requires an investment of US$5.93 million during the first 5 years. The cost of establishing a low-level technology abaca farm is US$524.02 per ha. Abaca, which matures in 18–24 months, can be harvested three times a year. Abaca extraction is 80 % manual and only 1 % of the fiber is recovered. Simple technology innovations like a portable stripping machine that costs about US$806.02 can increase fiber extraction by 3 %.

Abaca fiber ranks ninth among the Philippine major agricultural exports – after coconut oil, banana, pineapple, tuna, shrimps, tobacco, and desiccated coconut. Besides fiber, abaca pulp and cordage are exported to the United Kingdom, Japan, and the United States. For pulp, Germany is the main market followed by Japan and the United Kingdom. Principal buyers of cordage are the United States, Singapore, and Canada. Foreign exchange earnings from the export of abaca had been declining at 2.8 % each year from 1995 to 2004. Except for abaca pulp which had been increasing at 2.6 % per year, the Philippine foreign exchange earnings from the rest – raw fiber, cordage and yarns, and fabrics and fiber crafts – have declined.

Abaca Market Flow

From the producer/farmer/fiber stripper, the abaca fiber was sold at an “all-in” basis and ungraded to the “barangay” (village) dealer. The fiber then goes to the town/city dealers. To some extent the farmers sell directly to exporters/grading and baling establishments (GBEs). In some cases, farmers’ cooperatives/associations have a direct link to domestic processors.

1. *Local Consumption*
   Domestic processors utilize about 66 % of the country's total production of abaca fiber. A steady increase of 3.2 % per year in the consumption of abaca fiber by local processors resulted to more income through production of high value products and employment opportunities in the Philippines. The pulp sector is considered the highest growth area of the abaca industry due to the favorable developments in the world market increasing demand for its end products such as meat casings, tea bags, cigarette papers, and other specialty paper products. The pulp sector accounted for about 57 % of the total local consumption for the past 10 years. Consumption by this sector grew 6.9 % annually. The cordage sector, on the other hand, accounted for about 31 % of the total fiber usage by the domestic manufacturers. During the 10-year period, a decrease of 1.4 % per year in the sector was observed largely due to stiff competition posed by synthetic cordage.
2. *Export*
   Most of the processed products like pulp, cordage, and fiber crafts are exported by the Philippines to various countries and from which, with raw fiber exports included, an average of US$79 million a year are generated (Table 12.1). About 82 % of export earnings or an average of US$65 million came from abaca manufacturers. The rest (18 %) was contributed by raw fiber exports with annual average earnings of US$14 million. Among the abaca manufacturers, pulp contributed the highest export earnings at 39 % of the annual total followed by fiber crafts at 22 %. Exports of cordage and allied products contributed 14 % and yarns and fabrics 0.7 %. The United States remains as the biggest market for Philippine abaca cordage while Japan and Germany for abaca pulp exports during the past 10 years. Increasing quantities of pulp are also being marketed to the United States as well as the European and Asian countries like France, Taiwan, Korea, and China. The major markets for fiber crafts include the United States, Germany, Japan, and Australia. An average of 12,887 MT of raw fibers

Table 12.1 Average exports of abaca fibers and products 1997–2006

Exports	Volume (MT)	Value (in FOB US $)	Destination
Raw fibers	12,887	14,049,398	United Kingdom, Japan, Indonesia
Pulp	17,384	38,391,313	Germany, Japan, United Kingdom, France, United States
Cordage, ropes, and twines	7,725	11,370,481	United States, Singapore, Canada, Malaysia, United Kingdom, United Arab Emirates
Yarns and fabrics		396,910	
Fiber crafts		15,046,555	United States, Japan, Spain, Italy, United Kingdom, France, Australia
Average total earnings		79,254,657	

were exported every year during the past 10 years with 91 % of the total going to the United States, the United Kingdom, and Japan. For the past 10 years, raw fiber export has been declining at the rate of 2.3 %, but this is offset by an increasing trend in the domestic consumption and export of manufactured abaca in the forms of pulp, cordage, and fiber craft.

Sectors of the Abaca Industry

The abaca industry is made up of five major sectors: farming, fiber stripping, trading, fiber exporting, and processing (Table 12.2).

1. *Farming Sector*
   There are more than 77,500 Filipino farmers that grow abaca on about 127,258 ha. An abaca farmer has a small landholding that averages about 2 ha. The national average production is 850 kg/ha, which is relatively low compared to the potential of 2,000 kg/ha.
2. *Fiber Stripping Sector*
   Stripping abaca fibers is done either by hand or mechanical means. About 80 % of the abaca fibers in the country are hand stripped – practiced mainly in Bicol and some parts of Leyte and Samar provinces. The remaining 20 % of the fiber is produced through spindle stripping and is employed principally in Mindanao and the Leyte provinces. Included in the stripping work are harvesting of stalks and tuxying and drying of fibers. The fiber strippers are paid either in cash or by share. Under the sharing system, they receive 50 %, 60 %, or 70 % of the harvest depending on the prevailing practice agreed upon.
3. *Trading Sector*
   Trading is done at different levels depending on the location of the farmers and where the accumulation of fiber is done. Hence, there are traders in the barrio, town, province, city, and region. In each level, the pricing system includes markup attributable to the service provided by the trader. There are a total of 617 traders at various levels of trading.

Table 12.2 Summary of abaca industry sectors

Sectors		Number
Farmers		77,500
Traders (licensed)		617
Traders-exporters (licensed)		31
Fiber exporting	Grading and bailing (licensed)	20
Processing	Cordage firms (licensed)	6
	Pulp manufacturers (licensed)	6
	Fibercraft processors (licensed)	105

4. *Fiber Exporting Sector*
   The fiber exporters, also known as grading and bailing establishments (GBEs), operate in major abaca regions and usually maintain liaison offices in Metro Manila. The establishments employ classifiers who ensure that the fibers are in accordance with government standards. They likewise operate pressing machines for bailing of fibers intended for trading in both domestic and international markets. The standard bale of fiber is equivalent to 125 kg and measures $100 \times 55 \times 60$ cm.
5. *Processing Sector*

   (a) *Pulp Mills*
   There are six abaca pulp companies operating in the Philippines, which have combined rated capacities of 16,180 MT per year. The companies have well-established market networks for their pulp which are principally destined for the international market.

   (b) *Cordage Manufacturers*
   There are six cordage firms operating in Metro Manila, Cebu, and Davao. They use abaca as the principal raw material for rope, cordage, and twine manufacture. Blending with other natural fibers like maguey is done depending on the specifications of the buyers. The combined rated capacities of these companies are approximately 21,350 MT per year.

   (c) *Fiber Craft Manufacturers*
   The fiber craft sector, including handmade papermaking and carpet manufacturing, is primarily characterized as "cottage based." Operating mostly in the countryside, especially in the central Philippines, the sector is a major source of livelihood especially to the women and out-of-school youth. Several of these manufacturers have successfully established their markets abroad, especially through their unique, functional, and creative designs.

   (d) *Textile/Fabrics*
   The textile/fabric sector produces handwoven abaca fabrics which are used as raw material for making novelty and household items, as décor and wrapping material, as well as for fashion wear and accessories. Some abaca weaves are blended with metallic thread or polyester, while others have striped and ethnic designs to suit the varying needs of the market. The industry is confined in Western Visayas, the Bicol Region, and Southern Mindanao where indigenous people are actively engaged in *tinalak* weaving. Production of new product lines for fashion wear and accessories and specialty/novelty items is based in Metro Manila.

Threats and Problems

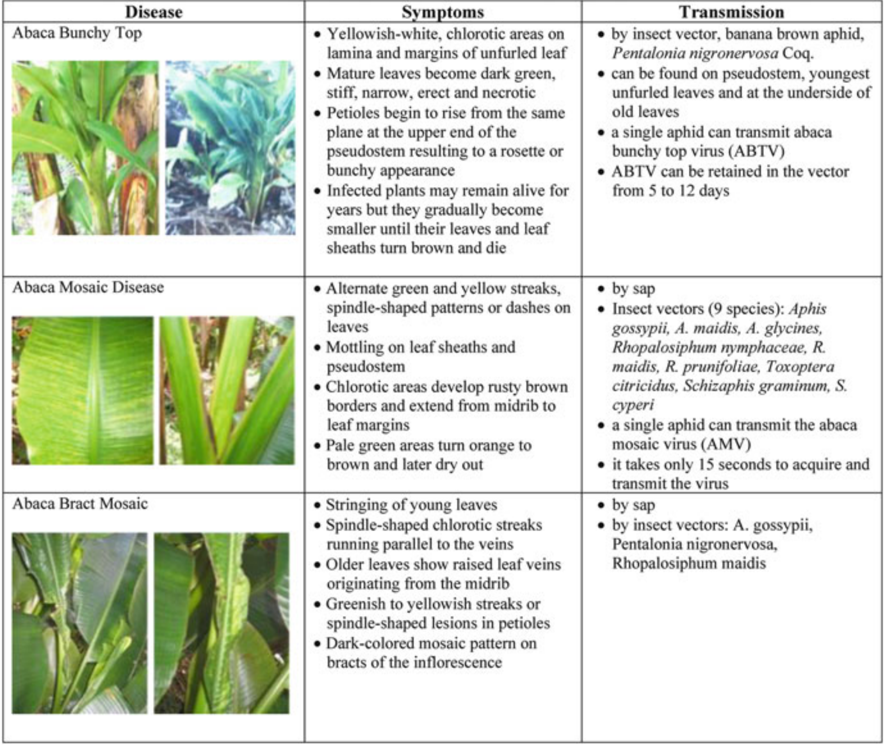
Although abaca has been an established Philippine industry, it is still plagued with problems. Areas that continue to be addressed are (1) farm productivity and (2) fiber quality. Also among the serious challenges in the Philippine abaca industry includes:

1. Aggressive moves by Indonesia to massively produce abaca under the government's reforestation program, increasing market competition
2. Availability of similar materials from China and India and technological advances and breakthroughs which make possible production of cheaper substitutes, whether from natural (e.g., sisal, Ecuadorian abaca) or synthetic-based materials
3. Threat from destructive pests and diseases, natural calamities, use of a few genotypes, overexploitation due to over-harvesting of natural stands, and changing land use brought about by development and population pressure

As early as 1980, there was a rapid decline in abaca production not only due to the unavailability of improved varieties but also due to three major virus diseases – abaca bunchy top (ABT), abaca mosaic (AM), and abaca bract mosaic (BM) (Fig. 12.2). There were resistant lines identified from the abaca germplasm collection. However, these have often fibers of inferior quality. Control of these viruses is difficult even with different disease control strategies. Other diseases remain important as they affect production of abaca. Superior hybrids developed by conventional breeding could be adopted directly by abaca farmers if they possess resistance to diseases caused by viruses and other pathogens. Continuing effort on identifying sources of resistance to these diseases is being done to be able to sustain abaca production in the country.

Abaca bunchy top, abaca mosaic, and bract mosaic virus are economically the most devastating virus diseases in abaca. These often occur in the same growing area. The average incidence of abaca bunchy top and abaca mosaic diseases in Bicol in 1991 was 5.19 % with an estimated fiber yield loss of more than 800,000 kg valued at about ₱18 million [7]. The estimate for the same year in the Eastern Visayas was disease incidence of 8.16 % valued at about ₱8 million. These diseases are known to reduce fiber quality as well.

The symptoms of bunchy top disease were first observed in Albay (Bicol Region) in 1910 and 1911 [8], then in abaca plantations in Silang, Cavite, in 1915. The disease was not a serious threat to abaca cultivation until 1923 [9]. Since that time, increased virulence caused the abandonment of plantations in the provinces of Cavite and Laguna (Paete). Presently, bunchy top which is widespread in abaca-growing areas is considered the most destructive of the abaca diseases because the plants are very much stunted (Fig. 12.2) and are not productive. The most striking characteristic of bunchy top is the crowding of the leaves into more or less a rosette arrangement [8], accompanied by a transparent appearance of the main and secondary veins of the leaves.



Disease	Symptoms	Transmission
Abaca Bunchy Top	• Yellowish-white, chlorotic areas on lamina and margins of unfurled leaf • Mature leaves become dark green, stiff, narrow, erect and necrotic • Petioles begin to rise from the same plane at the upper end of the pseudostem resulting to a rosette or bunchy appearance • Infected plants may remain alive for years but they gradually become smaller until their leaves and leaf sheaths turn brown and die	• by insect vector, banana brown aphid, *Pentalonia nigronervosa* Coq. • can be found on pseudostem, youngest unfurled leaves and at the underside of old leaves • a single aphid can transmit abaca bunchy top virus (ABTV) • ABTV can be retained in the vector from 5 to 12 days
Abaca Mosaic Disease	• Alternate green and yellow streaks, spindle-shaped patterns or dashes on leaves • Mottling on leaf sheaths and pseudostem • Chlorotic areas develop rusty brown borders and extend from midrib to leaf margins • Pale green areas turn orange to brown and later dry out	• by sap • Insect vectors (9 species): *Aphis gossypii, A. maidis, A. glycines, Rhopalosiphum nymphaceae, R. maidis, R. prunifoliae, Toxoptera citricidus, Schizaphis graminum, S. cyperi* • a single aphid can transmit the abaca mosaic virus (AMV) • it takes only 15 seconds to acquire and transmit the virus
Abaca Bract Mosaic	• Stringing of young leaves • Spindle-shaped chlorotic streaks running parallel to the veins • Older leaves show raised leaf veins originating from the midrib • Greenish to yellowish streaks or spindle-shaped lesions in petioles • Dark-colored mosaic pattern on bracts of the inflorescence	• by sap • by insect vectors: A. gossypii, Pentalonia nigronervosa, Rhopalosiphum maidis

Fig. 12.2 Symptoms and methods of transmission of different abaca diseases

The disease itself is caused by a virus very similar to the banana bunchy top virus (BBTV) and transmitted by the aphid *Pentalonia nigronervosa* Coq. [10]. The similarity of the abaca bunchy top virus (ABTV) with the BBTV was observed by comparing the sequences of three genes of the replication initiation protein, the coat protein, and the movement protein. These genes of the abaca bunchy top virus shared 99 % homologies with the BBTV [11].

A mosaic-like disease in abaca has been known to exist since 1925; however, the abaca mosaic disease was first described from an infection observed at the Odell Plantation in Tagum, Davao del Norte, in 1933 [12]. In 1941, the Japanese planters in Davao claimed that 50 % of abaca grown in Eastern Davao was infected because of poor cultural management. The spread of the diseases was rapid for three reasons. First, several aphid species can harbor and transmit the virus. Second, the abandonment of plantations during the World War II left the disease free to spread. Third, after the war, when the price of abaca fiber was good, there was a frenzy to expand abaca production areas. Due to poor information, the planting materials used were diseased. The economic cost of the disease is often estimated from the reduction in fiber yield. There is also loss in income due to poorer fiber quality. Fibers from mosaic-infested plants have higher percentage of stretch which

was attributed to change in chemical constituents. In addition, recent reports confirmed that abaca mosaic not only reduced the tensile strength of abaca fiber but also reduced biomass yield, fiber yield, plant height, stalk diameter, and, more importantly, farmer's income.

Not much is currently known about abaca bract mosaic disease. The first report of natural infection of abaca with banana bract mosaic virus was reported in the Philippines in 2000 [13]. The symptoms of abaca bract mosaic disease are expressed at any growth stage; an infected plant exhibits stringing of young leaves with chlorotic stripes. Leaf lamina symptoms consist of spindle-shaped chlorotic streaks running parallel to the veins which may not be prominent in younger leaves in recent infection. Older leaves also show raised leaf veins originating from the midrib which appear like continuous ripples. Greenish to yellowish streaks or spindle-shaped lesions are present in the petioles but may be absent on petioles of older leaves showing leaf lamina symptoms (Fig. 12.2). When the dead leaf sheaths are pulled away from the pseudostems, distinctive dark-colored mosaic patterns, stripes, or spindle-shaped streaks are visible [11]. The characteristic dark reddish brown mosaic pattern on the bracts of the inflorescence is the distinguishing mark for the disease. In the absence of the bracts, abaca bract mosaic symptoms may be mistaken for abaca mosaic symptoms.

Taxonomy, Domestication, and Genetic Resources of Abaca

Taxonomy

Abaca, (*Musa textilis* Nee) or Manila hemp, is endemic to the Philippines. It belongs to the Musaceae family, a large family that includes majority of the cultivated banana species and cultivars. The genus *Musa* is comprised of 30–50 species and many hybrids, majority of which are triploids. Musaceae is divided in five sections wherein *M. textilis* belongs to the section *Australimusa*. It has been proposed to reduce the sections of this genus to three sections according to the number of chromosomes and amplified fragment length polymorphism (AFLP) analysis.

The general morphological structure of *M. textilis* is similar to that of the edible banana cultivars, but the plant is more slender, the leaves are smaller, and the fruits are seeded (Fig. 12.3). Only 20 of the more than 400 abaca cultivars in the Philippines are of commercial importance. Further taxonomic study of the species is needed.

Spanish friar and botanist Manuel Blanco was the first to classify the Philippine bananas which also included the description of the wild bananas "butuhan" and "saging maching," the abaca, and the "virgin" banana which were all classified under *M. troglodytarum* Linn. Abaca was listed by Fr. Blanco with botanical variety *textoria* [14].

Fig. 12.3 Typical *Musa textilis* plant

A reclassification of Fr. Blanco's Key to *Musa* species in the Philippines was done by Dr. Nicanor G. Teodoro in 1915. Dr. Teodoro used the collections of the College of Agriculture, University of the Philippines Los Baños (UPLB), as working materials for the reclassification. Teodoro's Key to Species and varieties of the Genus *Musa* were extensively used in describing banana cultivars grown in the Philippines, until it was superseded by the taxonomic scheme of Cheesman [15], Simmonds, and Shepherd [16] and lately by the taxonomic classification adapted by the curators of the national banana variety collections of Southeast Asia [14] (Fig. 12.4).

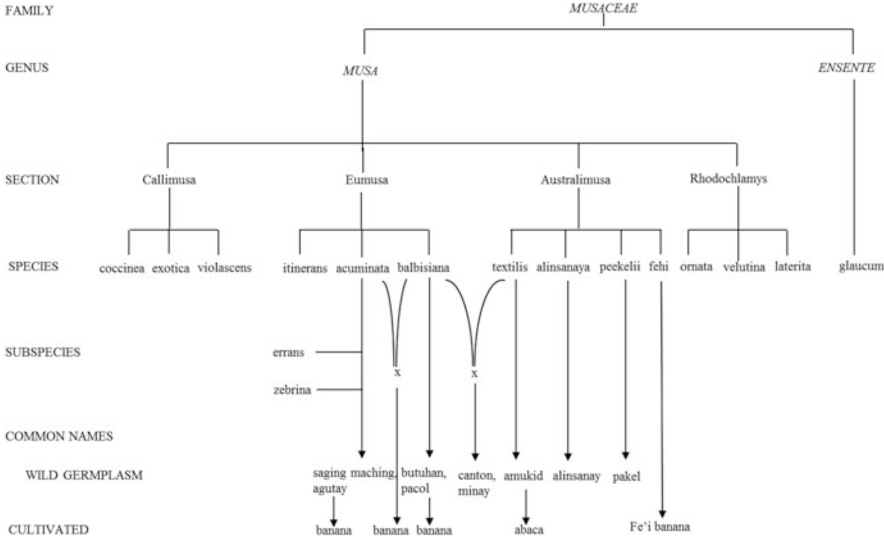


Fig. 12.4 Classification and grouping of Musaceae and its cultivated and wild forms (Reprinted from Valmayor et al. [14] with permission from Ramon V. Valmayor)

Domestication

Abaca is native to the Philippines and northern Indonesia. It was introduced to Sumatra in 1925 [17]. There were unsuccessful attempts to introduce the crop in India in 1822, as well as in German East Africa, West Indies, and Florida, USA. Prior to the 1920s, abaca cultivation is unknown outside of the Philippines, and it is believed that Filipinos are the first to domesticate abaca [3]. Successful cultivation has been reported in other Southeast Asian countries as well as in Central America and Ecuador. At present, it is also grown in Equatorial Guinea and Kenya.

Genetic Resources

Abaca is believed to have the Philippines as its center of origin, from where it then spread southward to Borneo [3, 18]. Based on the Vavilonian crop centers of origin, the abaca is known to be from the Indo-Malayan center. The Philippine archipelago lies within the area of greatest *Musa* diversity. *Musa balbisiana* Colla, *Musa acuminata* Colla, and *Musa textilis* Nee are indigenous to the Philippines (Fig. 12.3). Their natural distribution overlaps, and since they are cross compatible, several interspecific hybrid forms are known to occur in nature, adding to the great wealth of *Musa* germplasm in the country [14].

Historically, the first germplasm collections of *Musa* species in the Philippines started during the Spanish era with the initiative of Spanish friar Manuel Blanco,

author of the illustrated book *Flora de Filipinas*. An extensive banana collection program was started by the Philippine Bureau of Agriculture in 1911–1912, during the American occupation of the Philippines. The initial germplasm assemblage consisted of 276 accessions of 22 species.

The collections from the Bureau of Agriculture, and those of Dr. Teodoro, as well as the descriptions of Fr. Blanco early on, were further studied and compared by Dr. Eduardo Quisumbing, a professor of UPLB, in 1919. Widespread synonymy and duplications were found; thus, there was a reduction of the germplasm collections. During World War II (1941–1945), many of the plant and animal germplasm collections of the country were lost due to war-related atrocities. The wild and cultivated *Musa* accessions of UPLB and the Bureau of Plant Industry then had increased to 298. After the war, the country launched a program for the rehabilitation of the abaca industry. During the time, the abaca plantations in Davao and Bicol regions were severely affected by the abaca mosaic disease. Chemical sprays did not control the malady. The long-term solution was believed to be the use of resistant hybrids, and a breeding program utilizing resistant, wild *Musa* relatives was initiated by UPLB and the Bureau of Plant Industry.

At present, the National Abaca Research Center (NARC), based at Leyte State University in Leyte, Philippines, holds the world's largest collection of *Musa textilis* germplasm, with more than 600 accessions of both cultivated and wild types. Abaca accessions are also conserved in vitro. The collection at NARC has been characterized with respect to fiber morphology, chemical composition, fiber quality, and physical properties. Though *Musa textilis* is a genetic contributor in certain edible seedless hybrid banana varieties, it has not been used in any formal breeding program for edible bananas [19].

A research project that examined the genetic diversity of the Philippine abaca germplasm using microsatellite markers found that the germplasm collection in Luzon island has the highest diversity based on the Shannon diversity index (H) [20].

Also in an attempt to conserve the diversity of Philippine abaca, Villavicencio et al. [21] initiated the establishment of an in situ and on-farm conservation in Lake Sebu, South Cotabato. In situ and on-farm conservation through conservation field schools (CFS) is envisioned to enhance the capacity of farmers and stakeholders on in situ conservation and its sustainable use. With the active participation of local farmers, local government unit (LGU) technicians, and researchers from institutions concerned with conserving the abaca, identification of traditional varieties was conducted in the center of abaca production in the area. Five traditional varieties were identified, namely, Tangonon, Maguindanao, G'nolon, Maguindanao black and Wogu, and wild abaca. These varieties were multiplied and established in the on-farm conservation sites. At the time of this activity, 80 % of the abaca plantations in the Philippines have already been infected by abaca mosaic virus.

Abaca Breeding in the Philippines

History of Abaca Breeding

Musa textilis is cross compatible with several Musa species including *M. acuminata*, *M. balbisiana, M. lolodensis*, and *M. borneensis* [22]. Their hybrids are often partially or highly sterile. Cross fertilization in abaca is facilitated by the stigma remaining receptive for about 2 days. Once fertilized, the flower turns brown and shrivels within 24 h. The heart or panicle containing the male flower may be gathered and kept in the shade for 7 days with the pollen remaining viable and effective for fertilization [5, 23]. Figure 12.5 shows the general method for pollinating abaca to develop hybrids.

The indigenous *Musa* species – *M. acuminata, M. balbisiana*, and *M. textilis* – overlap and natural hybrids among these species exist. One cultivar of *M. balbisiana* known as Pacol produces fiber of low quality that it has been used as an adulterant to abaca. Natural hybrids of Pacol and abaca exist in the Bicol Region and are known as Canton and Minay [24]. The basic chromosome number for the section Eumusa to which the edible bananas belong is $n = 11$, whereas the section Australimusa to which abaca belongs is $n = 10$. The natural hybrid between the diploid banana and abaca called Minay/Minary/Minray has $2n = 21$ [25, 26] and Canton has $2n = 20$ [24].

Canton is highly sterile but Minay occasionally produces seeds. Crosses between *M. balbisiana* and *M. textilis* have produced hybrids with morphological characteristics and chromosomal numbers similar to those of Canton and Minay

Fig. 12.5 Pollination method to develop abaca hybrids

[27]. Hybrids between Minay and abaca, with the latter serving as male parent, have been produced. The hybrids resemble the Minay parent more than the abaca parent [18]. Artificial hybridization proceeds more effectively when the abaca is the male parent.

A triploid and three tetraploid *Musa* which produce low-quality fiber have been studied and used in breeding [27–29]. Two abaca varieties, Inosa and Laguis, were found with chromosome numbers varying from $2n = 17$ to $2n = 23$ and $2n = 16$ to $2n = 24$, respectively [30].

As of 1928, there were already hybrids developed for the varietal improvement of abaca (Table 12.3). Crosses between Libuton and Itom, as well as Canorajan and Lagurhuan, were developed [31]. There were 54 different crosses developed from 1928 to 1931; however, only 29 crosses were successfully planted, 19 in Guinobatan Abaca Experiment Station and 10 in Silang, Cavite [5]. Screening for disease resistance was also carried out in 39 clones. Heterosis was observed in the F_1 abaca hybrids. The F_1 hybrids produced greater number of abaca suckers than either parent. Crosses with Maguindanao were better adapted to different conditions and possess stronger root system.

In 1974, abaca hybrids developed in 1939 were field tested, and they were named after the name of the parental line. The hybrid from Linawaan × Laylay was named as Linlay, Linawaan × Libutanay as Linlib, and Linawaan × Inosa as Linino [32]. Oyardo [33] also field tested and named some abaca hybrids such as from Itom × Lausigon as Itolaus, Itom × Maguindanao as Itomag, and Lausigon × Maguindanao as Lausimag.

The abaca varietal improvement program in UPLB was started in the early 1950s initiated by the university's College of Agriculture (UPCA) and the Bureau of Plant Industry (BPI) with emphasis on varietal collection, classification, evaluation, establishment of disease observation nurseries, clonal selection, and intra- and interspecific hybridization. The cooperative work was centered on the development of resistant abaca varieties, and the most notable achievement was the identification of *Pacol* as a source of resistance. Hybridization was done between *Pacol* and abaca; however, the project was terminated in the 1960s. The abaca collection was then maintained by the UPLB Forestry Abaca Gene Bank and was turned over to the UPLB Experiment Station in 1981.

Diaz [34] generated F_1 hybrids of Mininonga crossed with six varieties of abaca and screened for bunchy top resistance. Of the different crosses developed, only the following crosses produced F_1 seedlings: Malaniceron × Mininonga, Mininonga × Itolaus 39, Mininonga × Layahon, Mininonga × Putumag 22, Mininonga × Tinawagan Puti, and Sogmad Pula × Mininonga. The reaction of these F_1 hybrids to abaca bunchy top varied; Malaniceron × Mininonga, Mininonga × Itolaus 39, and Mininonga × Layahon have resistance to bunchy top virus, while Sogmad Pula × Mininonga hybrids has moderate resistance.

In 1999, six abaca hybrid genetic stocks (Itolaus 39 × Magsarapong 2, Itolaus 39 × Magsarapong 3, Itolaus 39 × Magsarapong 4, Itolaus 39 × Magsarapong 7, Itolaus 39 × Magsarapong 8, and Tetraploid × Ilolaus 39) were identified by the Institute of Plant Breeding. These genetic stocks were developed by conventional

Table 12.3 Review of the breeding works from 1928 to present

Researchers	Year developed	Crosses
Labrador	1928	Libuton × Itom
		Canorajan × Lagurhuan
Torres and Garrido	1939	Bulao × Lausigon
		Bulao × Maguindanao
		Bulao × Tangongon
		Carnajon × Samina
		Inisarog × Maguindanao
		Inisarog × Samina
		Itom × Lausigon
		Itom × Maguindanao
		Itom × Tangongon
		Jolo-lambutin × Putian
		Kinalabao × Putian
		Kinalabao × Sinibuyas
		Lausigon × Bulao
		Lausigon × Maguindanao
		Lausigon × Tangongon
		Libutanay × Tangongon
		Maguindanao × Kinalabao
		Maguindanao × Lausigon
		Maguindanao × Putian
		Maguindanao × Tangongon
		Punukan × Putian
		Putian × Jolo-lambutin
		Putian × Jolo-tigasin
		Putian × Kinalabao
		Putian × Maguindanao
		Putian × Sinibuyas
		Putian × Tangongon
		Puti-tumatagacan × Lausigon
		Puti-tumatagacan × Maguindanao
		Puti-tumatagacan × Libutanay
		Samina × Bulao
		Sinibuyas × Kinalabao
		Sinibuyas × Putian
		Magsarapong × Inisarog
Bernardo and Umali	1956	Putian × Kurisan
		Putian × Magsarapong
		Ugaram × Magsarapong
		Bulaoluna × Magsarapong
Bernardo	1957	Putian × Jolo
Cruz and Balingkit	1974	Linawaan × Laylay (Linlay)
		Linawaan × Libutanay (Linlib)
		Linawaan × Inosa (Linino)

(continued)

Table 12.3 (continued)

Researchers	Year developed	Crosses
Oyardo	1974	Itom × Lausigon (Itolaus 35)
		Itom × Lausigon (Itolaus 39)
		Itom × Lausigon (Itolaus 45)
		Itom × Maguindanao (Itomag 3)
		Itom × Maguindanao (Itomag 16)
		Lausigon × Maguindanao (Lausimag 24)
		Lausigon × Maguindanao (Lausimag 32)
		Lausigon × Maguindanao (Lausimag 35)
Tabora and Carlos	1978	Pacol × CES No. 3
Alcober	1986	Pacol × CES No. 3
Diaz	1997	Malaniceron × Mininonga
		Mininonga × Itolaus 39
		Mininonga × Layahon
		Mininonga × Putumag 22
		Mininonga × Tinawagan Puti
		Sogmad Pula × Mininonga
Engle et al.	1999	Itolaus 39 × Magsarapong 2
		Itolaus 39 × Magsarapong 3
		Itolaus 39 × Magsarapong 4
		Itolaus 39 × Magsarapong 7
		Itolaus 39 × Magsarapong 8
		Tetraploid × Ilolaus 39
Moreno	2001	Canarahon × Korokotohan
		Canarahon × Samoro
		Tangongon × Samoro
Manguiat et al.	2000	BC1 hybrids
Lalusin et al.	2006	BC2 hybrids

crossing Magsarapong, Tetraploid 1, and Itolaus 39, an abaca possessing moderate resistance to mosaic and bunchy top virus. Itolaus 39 is a commercial hybrid between Itom and Lausigon, but the appearance is more of a *Pacol*. All the stocks were moderately resistant to mosaic and resistant to bunchy top under greenhouse and highly resistant under field evaluations, but the fiber quality and recovery were lower than the abaca hybrids. Hybridization between abaca varieties and relatives has been done. Table 12.3 shows the history of breeding works since 1928.

The Institute of Plant Breeding of UPLB started its abaca breeding program in 1981. In 1986 the first six F1 hybrids between *Pacol* and abaca were released by the institute. These hybrids have resistance to bunchy top virus, but the fiber quality is quite poor. The crossing work was ended due to unavailability of funds. It was only in 2006 that the breeding work was continued, although to a limited extent, and

several BC1 crosses were evaluated. A total of 196 BC_2 progenies have been developed and evaluated for bunchy top resistance and fiber qualities. Six promising clones have both the *AbBTV* resistance and good fiber quality.

During the evaluation, 1,300 seedlings from four BC_2 populations were manually inoculated with viruliferous aphids (*Pentalonia nigronervosa*). Each plant was inoculated with 10 aphids that fed for 24 h on abaca leaves infected with the bunchy top disease. Majority of the inoculated seedlings did not show any symptoms of the bunchy top virus after 2 months. One hundred sixty-six inoculated seedlings (12 from BC_1-19 × Abuab; 132 from BC_1-20 × Abuab', and 22 from 'BC_1-19 × Musa tex 51) were selected based on bunchy top virus resistance, plant vigor, and resemblance of morphological characters to true abaca. The seedlings were re-inoculated with the aphids for further confirmation of resistance.

To screen for desirable morphological traits, the BC_2 abaca hybrids were selected and planted in the field with 2.5 × 2.5 m planting distance under coconut trees as partial shade. The abaca hybrids were used for the evaluation of morphological traits, fiber qualities, and field resistance to bunchy top virus disease. These abaca populations were also used to screen the primers and to identify crosses with resistance to abaca bunchy top virus at the same time with good fiber qualities.

Recent Advances in Abaca Breeding

Marker-Assisted Breeding

Plant improvement, either by natural selection or through the efforts of breeders, has always relied upon creating, evaluating, and selecting the right combination of alleles. The manipulation of a large number of genes is often required for improvement of even the simplest of characteristics. With the use of molecular markers, it is now possible to trace valuable alleles in a segregating population and mapping them. These markers once mapped enable dissection of the complex traits into component genetic units more precisely, thus providing breeders with new tools to manage these complex units more efficiently in a breeding program.

Genetic marker systems have numerous applications in *Musa* improvement. These include increasing heritability of difficult to select characters via indirect genotypic selection; complex quantitative traits may be resolved into simple Mendelian loci; gene pyramiding for pest and disease resistance genes can be performed; detailed genetic linkage maps can be constructed [35], and a map-based gene cloning may be performed. Other uses include accurate identification of clones [36, 37]; the determination of evolutionary pathways between clones [38]; the identification of duplications in germplasm banks; and monitoring of somaclonal variation in micropropagated material for commercial use [39]. Identification of PCR markers for detection of A and B genome sequences in *Musa* was also reported [40, 41]. Three 10-mer RAPD primers produced unique banding profiles for the differentiation of *M. acuminata* (A genome) and *M. balbisiana*

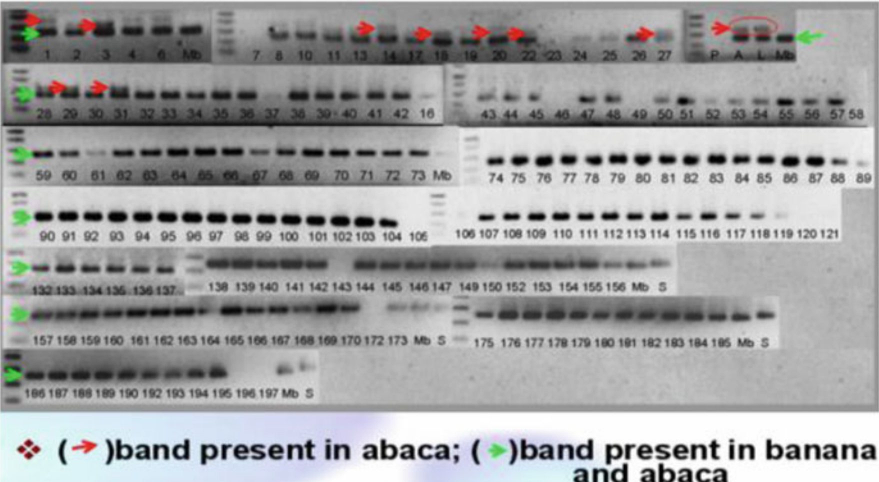


Fig. 12.6 DNA fingerprinting of the 196 BC2 hybrids using mMaCIR45. Abuab (A) and Lausigon (L) (abaca check) were run in parallel with the BC2 samples; *Musa balbisiana* (*Mb*) and "Seniorita" (*S*) were used as positive controls

(B genome). Molecular techniques such as isozymes [42–44], restriction fragment length polymorphisms (RFLP) [36, 45], random amplified polymorphic DNA (RAPD), repetitive elements, diversity of rDNA spacer length (IGS) [46], sequence-tagged site markers (STS), and AFLP [47] have been used to study the origin, relationships, and variability among and within genomic groups in *Musa*.

No studies to date have been done to identify molecular markers that are linked to disease resistance or to high yield of abaca and bananas. At present, the UPLB's Institute of Plant Breeding is developing molecular markers to fast-track the breeding of abaca varieties with high fiber yield and resistance to abaca bunchy top virus. The genetic background and relationships of different abaca accessions are being determined by fingerprinting BC_1 and BC_2 populations using a specific primer set (mMaCIR39, mMaCIR40, and mMaCIR45). This was done to facilitate the screening for resistance to abaca bunchy top virus and the selection of clones with high fiber quality (Fig. 12.6).

Abaca accessions collected from FIDA Bicol, NARC in Leyte, Negros Occidental, and FIDA Davao were also subjected to PCR analysis using the primer set. Fingerprinting analysis was carried out to establish the genetic relationship among the different accessions.

Tissue Culture and Genetic Engineering

The basic protocol for abaca in vitro culture has been published. Production of numerous disease-free abaca varieties through tissue culture has been long realized,

and this technology has been transferred to farmer's level. However, the effectiveness of this technique is limited by the presence of residual and alternative sources of inoculum in abaca plantations, making the absolute eradication of the virus diseases almost impossible. Often, the initially disease-free planting materials will get infected within a few growing seasons. It is therefore of great importance that new abaca cultivars which are resistant to pests and diseases be developed.

Genetic engineering utilizing plant transformation and gene cloning are becoming important tools in plant improvement. However, the success in utilizing the potentials of the technique is largely dependent on the development of efficient and reproducible protocols for the establishment of cell suspension cultures, induction of somatic embryogenesis, and protoplast fusion.

Excellent progress has recently been made in obtaining regenerants from somatic embryoids and recently gene expression in abaca. The technique involves adventitious shoot induction, shoot multiplication in the medium supplemented with BA or 2,4D and coconut water. Addition of 2,4D also induced callus formation. Subsequent root induction in adventitious shoots was also obtained in MS medium supplemented with IAA, NAA, and IBA [48]. Buds and shoots were induced in young floral sections and floral apices in MS medium supplemented with Ki in combination with NAA, IAA, or IBA. The formation of embryogenic (nodular) structures which developed into shoots was first observed in explants grown in MS medium supplemented with 2-ip and Ki. Somatic embryos have been included from cell suspension cultures derived from globules that formed from meristematic buds (scalps) or leaf sheaths in modified liquid MS medium supplemented with 2,4D, zeatin, and L-cysteine. These methods of producing somatic embryos are essential to the process of genetically engineering abaca.

Transient expression of the GUS reporter gene has been observed in transformed meristematic globules of abaca [49]. Researchers at the FIDA and the University of the Philippines Diliman (UPD) and Los Baños (UPLB) campuses are collaborating to genetically engineer abaca bunchy top-resistant and abaca mosaic-resistant abaca.

Opportunities/Prospects and Developments

The Philippine abaca industry is expected to continue making a stronghold in both the domestic and international markets. The growing concern for environmental protection and forest conservation worldwide has further provided demand opportunities to natural raw materials like abaca. Considering its superior fiber qualities over other natural materials, the utilization of abaca for industrial applications is expected to increase.

The total world abaca demand averaged 73,917 MT per year, 85 % of which was supplied by the Philippines. The Food and Agriculture Organization (FAO) of the United Nations projects that global consumption of abaca will increase further to more than 85,000 MT.

Since 1989, the abaca pulp sector registered a growth rate of 6.6 and 7.9 % per annum in terms of export volume and earnings, respectively. This is expected to increase further as the technology and formulations developed and used by the specialty paper manufacturers are becoming principally abaca based. Likewise, demand for specialty papers such as currency notes, tea bags, meat and sausage casings, cigarette papers, and the like will continue to grow as economies of the major consuming countries improve and new markets open up.

Abaca pulp can also be substituted for coniferous pulp in most paper products on the ratio of 4 to 1. Majority of the world's pulp and paper companies use wood pulp with global demand estimated to be at 200 million MT. This is equivalent to about 50 million MT of abaca pulp.

The fiber craft sector is another growth area registering a 4.4 % improvement in earnings per annum. To sustain the increasing demand for fiber crafts, however, functional and innovative designs should continuously be introduced in the market.

Growing awareness and interest on abaca fabrics for décor and wrapping purposes, as well as for fashion, have increased the demand for this product. Since 1989 until the present, the export volume registered a remarkable growth rate of 121.4 % per year. It is expected that demand would be long term due to the growing popularity of environment-friendly materials especially in developed countries.

While the market of abaca cordage, ropes, and twines would not be as promising compared with other sectors of the industry, demand will remain stable as it has specific markets to serve. Abaca cordage is highly preferred in oil dredging/exploration, navies, and merchant shipping as well as in the construction business because of its non-slipping characteristics.

Abaca production is expected to improve in response to the encouraging developments in both the local and world markets.

Direction of Abaca Breeding in UPLB

There are only two agencies in the Philippines doing abaca breeding – the Institute of Plant Breeding Crop Science Cluster, UPLB, and the National Abaca Research Center (NARC), Visayas State University (VSU). Therefore, abaca breeding in UPLB will have a significant contribution in the viability of the Philippine abaca industry. Abaca breeding in UPLB will continue as long as there are researchers, technicians, and laborers who are dedicated to pursue the objective of rehabilitating the Philippine abaca industry. Abaca breeding will concentrate more on the development of high-yielding and virus-resistant varieties using conventional and non-conventional methods and the mass propagation and dissemination of these high-yielding and resistant hybrids to abaca farmers and other interested stakeholders.

Considering that the area devoted to abaca production is more than 144,000 ha, UPLB needs to produce and disseminate about 230,400,000 planting materials in different abaca-growing areas in the country to rehabilitate the abaca industry.

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Chapter 13
Cuphea Production and Management

Marisol T. Berti and Russ W. Gesch

Abstract The genus *Cuphea* (Lythraceae) is quite unique in that most of its 265 different species synthesize and store primarily medium-chain fatty acids (MCFA) in their seeds, and many flourish in temperate climates. Presently, the United States and other developed countries import millions of tons of tropical plant-related oils to provide MCFA for industrial chemical manufacturing. *Cuphea* can serve as an additional source for these fatty acids. Since about the early 1980s, a concerted effort in the United States has been made to domesticate cuphea as a commercial, temperate climate crop source of MCFA for the manufacturing of a myriad of industrial chemicals. The biggest breakthrough came in the 1990s when more agronomically friendly genotypes were developed through the interspecific hybridization of *C. viscosissima* and *C. lanceolata*. Since that time, significant strides have been made in developing best agricultural management practices for the commercial production of cuphea. Currently, small-scale seed production has taken place in the northern Corn Belt region of the United States for high-end value products such as those manufactured by the cosmetic industry. This review primarily focuses on advancements that have been made over the past decade in developing agricultural management for cuphea production.

Keywords *Cuphea* • Seed oil • Medium-chain fatty acids • Agricultural management

Introduction

Cuphea (*Cuphea viscosissima* Jacq. x *C. lanceolata* W.T. Aiton) is being developed in the North Central United States as a new industrial oilseed crop that has oil rich in saturated medium-chain fatty acids (MCFA) 8–14 carbon-chain lengths. These fatty acids, particularly capric (C10:0) and lauric (C12:0), have been traditionally

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important in the manufacturing of soaps and detergents, cosmetics, lubricants, and certain nutraceuticals [1] but more recently have been shown to lend themselves well for replacing petroleum in advanced biofuels and other bioproducts [2]. Except for cuphea, there is currently no domestic crop source of oil rich in MCFA in the United States. All MCFA used in the United States and most other developed nations are derived from plant oils from coconut (*Cocos nucifera* L.) and palm kernel oil palm (*Elaeis guineensis* Jacq.) imported from Southeast Asia and from petrochemicals. The United States alone currently imports about 2.1 million metric tons of palm-related oil at a cost of about \$962 t^{-1}, which has been on the increase since 2005 and is projected to continue to increase.

Considerable effort has been made to domesticate and improve cuphea's agronomic traits to promote commercial production. Important breakthroughs were made in the early 1990s by Knapp and Crane [3–5] toward reducing seed shattering and seed dormancy in cuphea. Interspecific hybrids between *C. viscosissima* x *C. lanceolata*, f. *silenoides* populations were selected for partially non-shattering, nondormant, and autofertile characteristics. From this selection process, the interspecific hybrid line PSR23 (PI 606544) was the latest released in 2000 [3]. The cultivar PSR23 has been used in many agronomic studies to improve production practices in recent years.

Furthermore, over the past decade, substantial progress has been made in developing best management practices for cuphea PSR23 that have led to small-scale production by the specialty seed industry [6]. Cuphea crop establishment has been greatly aided by advances made in cuphea seeding requirements [7–12], weed control [13, 14], fertility [15], and harvesting [16–18]. However, harvest management still remains a challenge largely due to cuphea's indeterminate growth and propensity to shed seed (i.e., seed shattering), which are traits that will undoubtedly require further crop breeding to improvement.

Cuphea Description and Origin

Cuphea genus (Lythraceae) is endemic to the New World with two major centers of diversity located in southeastern Brazil and western Mexico. *Cuphea* species are mostly summer-annual plants with some perennial species that grow from sea level to 3,000 m elevation. In total, there are about 265 different species of the genus *Cuphea* that have been identified, but only six species have their origin in the United States [19]. *Cuphea viscosissima* Jacq. and *C. lanceolata*, which have been used in the development of commercial cuphea lines, are annual herbaceous species. *Cuphea lanceolata* is native to northern and Central Mexico, whereas *C. viscosissima* is native to the United States with its general origin being along the eastern United States inland from the coastal plains in disturbed, relatively wet habitats [19]. *Cuphea wrightii* has been found in the southeastern corner of Arizona, in disturbed, wet habitats, and its native range extends as far south as Costa Rica. The other three species that have been found in the United States (*C. aspera, C. glutinosa,* and *C. carthagenensis*) were introduced from South America [19, 20].

Morphological Description of *Cuphea viscosissima* and *lanceolata*

The leaves of both species are thin, entire-margined, opposites, and diminish in size toward the top of the plant. Trichomes are common in leaf surfaces, the stem, and reproductive structures. Trichomes are glandular and excrete a resinous and sticky exudate. Inflorescences are in the terminal and axillary buds. The flower has an elongated floral tube (hypanthium), dark purple in color, and formed from sepal, staminal, and petal primordial. Two larger petals of dark purple color are attached to the upper part of the end of the floral tube. Four petals, white or pink in color and much smaller than the upper, are in the lower part of the flower tube. Plants with white flowers occur in *C. viscosissima* x *C. lanceolata* very rarely. The fruit is a thin-walled capsule covered by the floral tube. The capsule wall ruptures lengthwise along their dorsal side to shed the seeds attached to the placenta. Several seeds are formed within each capsule ranging between 6 and 20 seeds in each capsule. Seeds are lenticular in shape, smooth, green or brown in color, and 2.5 mm long by 2.2 mm wide. Seed embryo includes two cotyledons and germination is hypogeal [19].

Cuphea Seed Oil and Fatty Acid Composition

Cuphea seed oil is rich in MCFA such as caprylic (C8:0), capric (C10:0), lauric (C12:0), and myristic acid (C14:0) [21]. The oil content varies greatly among the 265 *Cuphea* species. For instance, the oil content of *C. llavea* and *C. wrightii* var. *wrightii* varies between 10.1 and 39.5 % [22]. Cuphea species also vary in their fatty acid composition but typically will emphasize the synthesis and storage of a single medium-chain fatty acid type [23]. For instance, *Cuphea pulcherrima* and *C. paintieri* are very rich in caprylic acid (94 % and 73 %, respectively) [23], while *C. carthagenensis* [24] and *C. wrightii* var. *wrightii* [22] both have high lauric acid, 81 % and 73 %, respectively. *Cuphea lanceolata* has relatively high capric acid content (70 %) [24], while *C. llavea* has the highest level of caproate (92 %) [22]. The interspecific hybrid of *C. viscosissima* x *C. lanceolata*, which the variety PSR23 originated from, has a seed oil content typically in the range of 27.0–31.0 % and whose fatty acid distribution typically includes 70 % of capric, 3 % lauric, 4 % myristic, 6 % palmitic, 9 % oleic, and 5 % linoleic [25]. Cuphea oil has an iodine value of 19.7 and a high oxidative stability of 157 h at 110 °C comparable to that of coconut oil. The content of free fatty acids (4–4.25 %) and chlorophyll (200–260 mg kg^{-1}) in the crude oil is considered high [26].

Fatty Acid Synthesis in Cuphea Seeds

The biosynthesis of MCFA in *Cuphea lanceolata* starts with the carboxylation of acetyl-CoA by the enzyme acetyl-CoA carboxylase to form malonyl-CoA, a pathway common to all plant species. The initial condensation between acetyl-CoA and malonyl-ACP (acyl-carrier protein) is catalyzed by the enzyme KAS III (β-ketoacyl-acyl-ACP synthase III) [27]. In most plants, further chain elongation is catalyzed by acyl-ACP-specific condensing enzymes, KAS I (C6 to C16) and KAS II (C16 to C18). In cuphea, there is a KAS IV enzyme responsible for MCFA synthesis that interacts with specific medium-chain thioesterases that hydrolyze the ACP and release the fatty acid stopping the elongation at C10 or C12 [28]. The increased pool sizes of medium-chain acyl-ACP inhibit the condensation of KAS enzymes downstream after KAS III, then KAS IV is responsible for catalyzing the elongation to C10. The ACPs play an important role in plant fatty acid synthesis since they carry the acyl moieties during fatty acid elongation [29]. Different ACP forms exist in plants with at least three specific ACPs identified in *Cuphea lanceolata* [30]. *C. lanceolata* has a specific ACP isoform (ACP2) compatible with medium-chain fatty acid thioesterase to optimize the synthesis of MCFAs [29].

Cuphea Oil Uses

Medium-chain fatty acids can be used to replace saturated fatty acids and plasticizers in chewing gum. Cuphea oil also works well as a flow carrier and solvent in candy manufacturing and as a defoaming agent and booster in soap and detergent manufacturing [31]. Cuphea oil can be used in high-valued cosmetic products such as lipsticks, lotions and creams, and bath oils [32]. The oil has a high oxidative stability, low- to medium-spreading ability, and low slip value, all of which provide the desirable non-slippery characteristic for use in sunscreens [33].

The properties of cuphea oil make it ideal for advanced biofuels including biodiesel and jet fuel [34]. Because of the already short carbon chain lengths of cuphea seed oil triglycerides, it lends itself well to the manufacture of jet fuel with little chemical modification. The addition of cuphea oil to jet fuel reduces the fuel's freezing point avoiding fuel-gelling flow at temperatures below −20 °C. The long-term use of biodiesel from unmodified vegetable oil from soybean (*Glycine max* (L.) Merr.) and rapeseed (*Brassica napus* L.) can result in the buildup of carbon deposits (coking) on the engine fuel injectors due to incomplete combustion that causes deterioration of engine performance. Therefore, transesterification is needed to break the oil into fatty acid esters and glycerol. Fatty acid esters burn cleanly and efficiently in the engine, but glycerol must be removed and this process is costly. Oils rich in short- and medium-chain fatty acids have a reduced viscosity so they can be used as a diesel fuel substitute without transesterification. Oil from

C. viscosissima VS-320 has a low viscosity, higher than Number 2 diesel fuel but lower than rapeseed oil [35].

Biodiesel from cuphea has also been evaluated as nonselective contact herbicide in turfgrasses. A 2 % cuphea biodiesel caused significant injury to sicklepod (*Senna obtusifolia* (L.) H.S. Irwin & Barneby) and common milkweed (*Asclepias syriaca* L.) and also injury to the perennial rye grass (*Lolium perenne* L.). The low environmental impact, low cost, user friendly, and low risk of over application makes cuphea biodiesel an interesting potential herbicide alternative for turfgrasses [36].

Cuphea oil estolide derivatives have superior physical properties than petroleum-derived estolides and can be used to manufacture biodegradable, vegetable oil-based lubricants, cosmetics, and coatings [37]. Estolides are esters formed from vegetable oils when the carboxylic acid functional group reacts with other fatty acids to form an ester linkage [37]. Secondary linkages in estolides make them more resistant to hydrolysis than triacylglycerol. The oleic-octanoate (caprylic acid) and oleic-decanoate (capric acid) estolide 2-ethylhexyl esters were the best performers of the estolides tested. When compared with commercial lubricants (petroleum oil, synthetic oil, soy-based oil, and hydraulic fluid), the oleic-decanoate estolide 2-ethylhexyl ester had the lowest cloud point (~ −41 °C) indicating excellent performance under cold temperatures [38, 39].

Cuphea pressed cake, a coproduct after oil extraction, can be used for animal feed [40, 41] and as filler in poly(lactic) green composite materials to make the price more competitive [42].

Cuphea's Current and Potential Markets

The world market for lauric acid is 4.5 million metric ton (mt) and the US consumption is at 1.5 million mt or one-third of annual production [43]. Most of the oil rich in lauric acid come from Malaysia and Indonesia. Total coconut, palm, and palm kernel oil imported into the United States reached about 2.1 million mt in 2010 [44] (Table 13.1). The amount and value imported into the United States have about doubled since 2005 (Tables 13.2 and 13.3). Coconut, palm kernel, and palm oil prices fluctuate yearly with a record high value in 2008. Their prices have

Table 13.1 US import volume of coconut, palm, and palm kernel oil from 2005 to 2010

	2005	2006	2007	2008	2009	2010
Oil	(×1,000 mt)					
Coconut	432	493	458	499	484	576
Palm kernel	232	274	279	327	312	578
Palm	416	629	788	997	979	948
Total	1,080	1,396	1,525	1,823	1,775	2,102

Based on data from Ref. [44]

Table 13.2 Average price for imported oils in the United States (coconut, palm kernel, and palm) from 2005 to 2010

Oil	2005	2006	2007	2008	2009	2010	% change 2005–2010
	$ mt^{-1}						
Coconut	666	598	840	1,330	855	984	74
Palm kernel	734	675	791	1,289	817	1,029	70
Palm	458	491	712	1,036	730	872	95
Average	619	588	781	1,218	801	962	

Based on data from Ref. [44]

Table 13.3 US import value of coconut, palm, and palm kernel oil between 2005 and 2010

Oil	2005	2006	2007	2008	2009	2010
	$\$\times10^6$					
Coconut	288	295	385	663	414	567
Palm kernel	170	185	221	327	255	595
Palm	190	309	560	1,032	714	872
Total	648	789	1,166	2,022	1,383	2,034

Based on data from Ref. [44]

increased from 2005 to 2010 to an average value of 962 $ mt^{-1} (Table 13.2) with an imported total value of $2 billion dollars [44] (Table 13.3).

An estimated 14 million hectares of high-lauric cuphea production (based on an average oil yield of 150 kg ha^{-1}) would be required to substitute for current palm, palm kernel, and coconut oil imports. Based on current demand for MCFA, we speculate that cuphea production in the upper Midwest of the United States could feasibly approach that of oilseed sunflower (*Helianthus annuus* L.), which is annually about 810,000 ha. Contracting companies were paying farmers $1.19 kg^{-1} of seed with a gross income of $1,192 ha^{-1} necessary to cover the production costs with a yield of 444 kg ha^{-1} in 2006 and 2007 [6]. The price paid per kg of seed by contractors may decrease as seed yield and oil content increase with the development of new cuphea cultivars and improvements in agronomic management. Currently, the high value paid to the growers for the seed makes the oil much more costly to produce domestically than what it costs to import coconut or palm oil. That is why high-end dollar niche markets, such as cosmetics, will likely be the near-term outlets for cuphea oil until greater yield improvements are made. Current cuphea lines are 10 or 20 times higher in capric acid than coconut (6 %) and palm kernel oils (3 %) [45], and they may have a higher market value because of capric acid's value for cosmetics and other specialty chemicals. This is one of the challenges of bringing a new crop to the market.

Cuphea Genetics, Breeding, and Improvement

In a previous review, Phippen [46] provided an excellent review of research regarding cuphea germplasm sources, genetics, and breeding; so, we will only briefly address it here. PSR23 cuphea is the primary focus of recent breeding work that has been done in Illinois at Western Illinois University and in Minnesota. Also, as cited by Phippen [46], some breeding work continues in India on *C. procumbens* for seed oil production, in South America where *C. carthagenensis* and *C. glutinosa* are being explored for medicinal purposes, and in Japan where *C. leptopoda* is being researched for food uses. *Cuphea* species chromosome number varies from $n = 6$ to $n = 54$ [19]. *Cuphea lanceolata* and C. *viscosissima* have a chromosome number of $n = 6$ which allows interspecific hybrids between these species to be fertile [47, 48]. *Cuphea llavea* and *C. wrightii* var. *wrightii* have a chromosome number of $n = 22$. *Cuphea carthagenensis* and *C. tolucana* have a chromosome number of $n = 8$ and $n = 12$, respectively [19, 47]. Most cuphea germplasm accessions have wild characteristics not suitable for direct utilization and commercial production such as seed dormancy, obligate cross pollination, seed shattering, and indeterminate growth habit prone to enhancing seed shatter and low seed yields. Because of cuphea's indeterminate growth and flowering, seed development can extend over weeks to even months [16].

Morphological variability among accessions of *C. viscosissima* is limited, but differences have been observed in plant mass, height, dormancy, seed shatter, seed yield, oil content, and oil composition [49, 50]. Important breakthroughs toward domestication of cuphea have been accomplished in the last two decades. Several lines with suitable agronomic characteristics such as low seed dormancy self-pollination, partial seed shattering, and high oil content have been released, such as lines LN-183 (*C. lanceolata* Ait.), VL-90 to VL-95, VL160, VL186, and PSR23 (*C. viscosissima* Jacq. x *C. lanceolata* f. *silenoides* W.T. Aiton) [3, 4, 51, 52] (Table 13.4). Also Knapp et al. [53] and Tagliani [54] developed *C. viscosissima* fatty acid mutant germplasm lines with low capric and high caprylic, lauric, and/or myristic acid.

However, seed shattering, self-incompatibility, and indeterminacy are problems that need to be solved to improve cuphea yields and expand commercial production.

Cuphea Physiology

Cuphea Seed Germination and Development

Most of the wild species of cuphea have seed dormancy. Several wild species of cuphea exhibit primary seed dormancy, which leads to poor seedling establishment [55]. Seeds of *C. viscosissima* have been shown to lose most of their dormancy in 4–6 months of cold, moist, storage, or after 4 years of dry storage [56]. Seeds from

Table 13.4 Cuphea accessions released by breeding programs in the United States from 1992 to 2009

Specie	Accession no. (PI)	Line	Release date	Notable characters	Ref.
C. lanceolata	574384	LN-183	1992	Nondormant	[52]
C. viscosissima	574621	VS-6-CPR-1	1993	Low capric and high caprylic and lauric acid	[53, 54]
	574622	VS6-CPR-4			
	574623	VS-6-CPY-1			
	574624	VS-6-MYR-1			
C. viscosissima × *C. lanceolata* f. *silenoides*	574491	VL-90-95	1993	Self-fertile, partially nondormant	[51]
	574492				
	574493				
	574494				
	574495				
C. viscosissima × *C. lanceolata* f. *silenoides*		VL-160	1998	Nondormant and naturally self-pollinated. Seed shattering and stickiness are adverse characteristics yet to be reduced	[4]
C. viscosissima × *C. lanceolata* f. *silenoides*	606543	VL 186	1998	High oil cuphea with an average of 35 % oil	[5]
C. viscosissima × *C. lanceolata* f. *silenoides*	606544	PSR23	1998	Self-compatible, partially non-shattering, and nondormant. Higher seed yield and improved harvest ability. High content of capric acid in the oil	[3]
C. viscosissima × *C. lanceolata* f. *silenoides*		Snow flake, Blizzard	2007	Anthocyanin mutant of PSR23. Inbreeding depression. Diminished seed vigor	[46]

hand-harvested plants have shown higher germination than seed from combine-harvested plants presumably due to mechanical damage [57]. A light requirement of some cuphea species for germination may be one of the factors that limit seedling

emergence from soil depths greater than 19 mm. Light has been reported to be essential for germination in *C. viscosissima* [56].

Seed germination can be affected by storage temperatures prior to planting. *Cuphea carthagenensis* viability, for example, remained high when seeds were stored at 25 °C, but it decreased rapidly when stored at 5 °C. Viability decreased faster in seeds with seed moisture greater than 100 g kg^{-1} than seed at 10 g kg^{-1} moisture. The reduced seed viability is explained by the crystallization of triacylglycerols (TAGs) [58]. Most crop seeds are stored at −18 °C in germplasm banks and germinate without problems after cold storage. Cuphea seeds will not germinate after stored at −18 °C. The low temperature exposure does not kill the seed after storage at −18 °C. Death occurs when the seed is hydrated. Seed imbibition is hindered by crystallized TAGs [59]. Water contact with crystallized TAGs is lethal for the seed by altering the balance between hydrophilic and hydrophobic compounds needed for the transition of the cell to aqueous environment. The crystals cause massive decompartmentalization and interfere with the proper arrangement of oleosin (protein covering oil bodies) in the oil bodies [60]. Fortunately, poor germination of cuphea seed can be avoided by heating seeds from cold storage, such as those of *C. lanceolata*, for 10 min at 45 °C before imbibition [59, 61].

Interspecific cuphea germplasm lines developed from crossing *C. viscosissima* x *C. lanceolata* have been shown to exhibit nondormancy [3, 20]. An extensive amount of work has been devoted to studying seed germination and development of PSR23. Germination, seedling emergence, and vigor of PSR23 cuphea in the field tend to be low and highly variable [8, 10–12, 25, 62]. To a large extent, this is due to the heterogeneity of seed maturity at harvest resulting from cuphea's indeterminate growth. Therefore, any particular seed lot may consist of seed with a range of weights, moisture content, oil content, and composition, which can greatly affect germination potential and vigor.

During a 3-year field study in Fargo, ND, Berti and Johnson [62, 63] studied seed development of PSR23 from anthesis to physiological maturity (PM). They found that an accumulation of 253 growing degree days (using a base of 10 °C and maximum temperature of 30 °C) was required from anthesis to reach PM (i.e., maximum seed dry matter accumulation), which corresponded to approximately 30 days from anthesis. Seed moisture content at PM, however, varied among years ranging from 566 g kg^{-1} the first year to 156 and 52 g kg^{-1} in the second and third years, respectively, while maximum seed weight for PSR23 was found to be about 3.3 mg $seed^{-1}$ in their study. For comparison, Kaliangile [64] studied seed development of *C. wrightii* and *C. lutea* and reported that for these two species, PM occurred at 19 days after anthesis or 219 growing degree days when grown in a greenhouse under 25/18 °C day/night temperatures and a 16 h light cycle. Also, they reported that the seed moisture content for *C. wrightii* and *C. lutea* at PM was 320 and 420 g kg^{-1} and maximum seed weight was 1.89 and 2.62 mg $seed^{-1}$, respectively. When harvested at an optimum time for seed yield [16], seed moisture of PSR23 cuphea generally ranges from 300 to 450 g kg^{-1} [6, 17] and requires further drying before storage.

Several seeds are produced per capsule of cuphea. For PSR23 that depends strongly on cross-pollination by insects [3], any factor affecting pollination, particularly weather conditions, can affect seed set. Berti and Johnson [63] showed that during a wet and mild growing season, the number of seeds per capsule varied from 12 to 16, while in a hot and dry year, the number of seeds per capsule decreased, varying from 8 to 11. A 2-year planting date study in west central Minnesota revealed that the number of capsules per plant can vary from 13 to 121 with a tendency to decline with delayed sowing, when presumably plants are flowering and setting seed under warmer and drier conditions than plants sown earlier [7]. However, in this same study, the number of seeds per capsule only varied from 11 to 13 across all planting dates, which ranged from 15 April to 15 June. In the absence of pollinators, Gesch and Forcella [65] have demonstrated that high temperatures greatly inhibit reproductive growth of cuphea, particularly when daytime temperatures reach or exceed 30 °C.

Cuphea seed germination and vigor are strongly affected by seed maturity. For PSR23 cuphea, which does not have dormancy, the germination rate is greatest at 33 days after anthesis, or just slightly later (i.e., 2–3 days) than PM [62]. As demonstrated by Berti and Johnson [62], both seed germination and vigor sharply declined in younger seed that was sampled prior to PM. Furthermore, seed oil content increases with the maturity of cuphea seed [16] reaching its maximum at or near PM [62], and this may also impact germination as demonstrated for other plant species [66].

An earlier field study performed with PSR23 clearly indicated that soil temperature greatly affected emergence and hence stand establishment. Gesch et al. [7] reported that when the average daily soil temperature at the 5-cm depth was consistently higher than 10 °C, emergence and stand establishment greatly improved. Later, it was shown by Berti and Johnson [10] through a controlled environment study that the optimum temperature range for cuphea seed germination was 18–24 °C with a mean of 21 °C and that germination rate sharply decreased below 18 °C. They also estimated the base temperature to be between 6 and 10 °C and the maximum temperature for germination was between 33 and 38 °C.

Cuphea Plant Growth and Development

The systematic botany including the anatomy, morphology, and some growth characteristics of most, if not all, the wild *Cuphea* species found in North and Central America have been described by Graham [19, 67]. Moreover, Hirsinger and Knowles [68] and Hirsinger [69] have described many of the growth and development characteristics of some of these North American species that are most agronomically promising. Lacking, however, is a compilation of recent findings for advanced agronomic breeding lines of cuphea such as PSR23.

Cuphea generally grows well as a summer annual in the upper Midwest and northern regions of the United States. Interestingly, even several wild *Cuphea* species originating from Mexico and Central America also grow to maturity quite

well under the mild temperate summer climate and relatively fertile soils of the US Corn Belt region. In west central Minnesota, *C. wrightii*, *C. lutea*, *C. calophylla*, *C. carthagenensis*, *C. koehneana*, *C. palustris*, *C. procumbens*, *C. tolucana*, and *C. llavea* have been grown, and they all have flowered and produced seed. Forcella et al. [25] studied the growth, seed yield, and oil characteristics of PSR23 cuphea grown from southwestern Iowa to northwestern Minnesota along a latitudinal transect of 41–49°N. In the absence of drought, vegetative growth of plants did well all along this transect. However, seed yields, oil content, and capric acid content, the major fatty acid of PSR23 oil, were all greater in Minnesota than in Iowa and tended to increase with latitude up to 45°N. Increases in seed yield and oil content were best associated with decreased air temperature, particularly during midsummer to late summer when plants were flowering and setting seed. Similar results were reported by Kim et al. [70] who studied PSR23 and another related cultivar HC-10 at field sites in Iowa, Illinois, Minnesota, and North Dakota. In this study, growth and biomass yield and seed yield and oil content were distinctly greater in Minnesota and North Dakota than Iowa and Illinois for both cultivars. Soil environment was also studied, but the results were generally better associated with climate than soil environment. Three wild species (*C. viscosissima*, *C. lutea*, and *C. wrightii*) that show good potential for domestication were also included in the study and were found to perform equally well across all four locations.

Because field studies indicated that growth of cuphea under high temperatures might be detrimental to its development, Gesch and Forcella [65] studied cuphea's response to temperature under controlled environment conditions. Plants were subjected to day/night temperature regimes of 18/12, 24/18, and 30/24 °C with a 16 h photoperiod. The study confirmed that vegetative growth adapts well to a wide range of temperature. In part, this was due to cuphea's ability to acclimate its photosynthetic machinery to compensate for different growth temperatures. Cuphea's vegetative growth was greatest under the 24/18 °C treatment, and the optimum temperature for leaf photosynthesis was predicted to be 23 °C based on its quadratic response to temperature. Conversely, reproductive growth (i.e., flowering and seed set) was greatest at 18/12 °C and declined linearly with increased growth temperature [65]. The growth rate of reproductive tissues was 18 % less under 24/18 °C than at 18/12 °C, and reduced reproductive growth was mainly due to reduced number of flowers and fertilized seeds per capsule [65]. By comparison, the optimum temperature for cuphea's (PSR23) reproductive growth is much lower than rice (*Oryza sativa* L.) and corn (*Zea mays* L.) and slightly higher than that of spring wheat (*Triticum aestivum* L.) which is 20–25 °C during the day [71]. Other germplasm lines developed from the cross of *C. viscosissima* and *C. lanceolata* are likely to respond to temperature similarly to PSR23, although further research is needed to verify this.

Although PSR23 cuphea has indeterminate growth, about 110–120 days of growth, from the time plants emerge to harvest, are typically required to reach optimum yield maturity in the northern Corn Belt region. When planted in early to mid-May, flowering generally begins in mid-July to late July and peaks in mid-August. After this, cuphea will continue to flower at a minimal rate until killed by a hard frost. Early field studies with PSR23 cuphea indicated that approximately

Fig. 13.1 Cuphea PSR23 growth stages (Reprinted from Berti and Johnson [63]. With permission from Elsevier)

533–578 GDD were required from planting to reach initial flowering [7] and about 1,200–1,350 GDD to reach harvest maturity [16]. Despite cuphea's indeterminate growth, having a descriptive growth staging system, such as that for corn [72], would be beneficial for making agricultural management decisions for its production. Recently, Berti and Johnson [63] developed such a system for PSR23 cuphea from emergence to harvest maturity that associates growth stages with accumulated GDD and includes visual cues for key growth stage events (Fig. 13.1). For instance, they defined initial flowering (R1) to occur between 600 and 800 GDD, physiological maturity occurring around 900–1,000 GDD, and harvest maturity (R5) occurring around 1,000–1,250 GDD [63]. This system greatly aids in the timing of postemergence herbicide application and timing of harvest management.

Cuphea Water Requirements

Soil temperature and water are also critical factors that influence germination and seedling establishment and subsequent plant growth and development. Field studies have indicated that cuphea has low water use efficiency and may be susceptible to drought [25, 73]. A more recent study published by Gesch et al. [74] clearly showed that cuphea seed and biomass production suffer from drought stress when the plant

available water holding capacity of soil decreases to 50 % or less. In this study, under hot dry conditions that prevailed late in the growing season, irrigation led to a 2.7-fold increase in seed yield. Moreover, measurements of photosynthesis, leaf water potential, and seed $\delta^{13}C$ all indicated that nonirrigated plants suffered severe drought stress. The shallow root system and inefficient water use in the interspecific hybrid PSR23 and related varieties perhaps come from the genetic background of *C. viscosissima*, whose native habitat is primarily riparian (relatively wet) areas [67].

The seasonal water use in cuphea varies with sowing date where early sowing allows roots to penetrate deeper into the soil profile. Cuphea's root system is very shallow with most roots (80 % or more) in the upper 20 cm of the soil, but roots may penetrate up to 50 cm if planted early [73]. Water use efficiency of seed production (WUE) ranged from 1.2 to 2.0 kg ha^{-1} mm^{-1} for cuphea grown under dryland condition in west central Minnesota [73] and tended to be highest for early-sown plants (late April to early May). In a separate study conducted on the same soil type, WUE was as high as 2.4 kg ha^{-1} mm^{-1} for irrigated cuphea. Water use efficiency in cuphea appears to be relatively low as compared with other oilseed crops. For instance, WUE of canola and barley (*Hordeum vulgare* L.) is about 10 kg ha^{-1} mm^{-1} [75, 76]. Water use efficiency for sunflower (*Helianthus annuus* L.), soybean, and wheat fluctuates between 5 and 6 kg ha^{-1} mm^{-1} [75]. Flax (*Linum usitatissimum* L.), however, has a WUE very similar to cuphea of about 2.0 kg ha^{-1} mm^{-1} [75]. Based on the relationship of seasonal water use and seed yield of cuphea, Gesch et al. [74] estimated that cuphea requires about 460–490 mm of moisture during the growing season, depending on soil type and climate, to maximize seed yield.

Cuphea Agronomics

Cuphea Seeding Requirements

Seeding depth, soil packing, and seeding rate are also factors that influence plant stand establishment. Roath [57] observed that cuphea seedling emergence was greatly reduced with seeding depths greater than 13 mm. Roath [57] reported that soil packing increased pure live seed emergence by 14 %. This may be due to better seed/soil contact and greater seed hydration for germination. Soil water within the first 20 mm of the soil is likely less than at deeper-profile depths. The recommended seeding depth according to the cuphea grower's guide is 13 mm [77].

In a recent study conducted by Berti et al. [12], soil packing increased pure live seed emergence and plant stand when seeds were broadcasted on the soil surface (Fig. 13.2). When drilled, pure live seed emergence was highest at 13-mm depth compared with 25-mm depth. This confirms the need to seed cuphea to less than

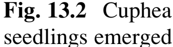

Fig. 13.2 Cuphea seedlings emerged

13-mm in depth or broadcasted on the soil surface; however, lack of soil moisture at the surface may delay emergence in a broadcast seeding.

Optimum growth and yield is achieved by planting in early to mid-May in west central Minnesota. Days from emergence to initial flowering decreased and growth rate increased as seeding date was delayed [7]. Sowing too early when soil temperatures are below 10 °C can lead to poor stand establishment, while sowing at the optimum recommended time lengthens the period for vegetative growth including root development, which typically translates into greater seed and oil yields [7, 73].

Roath [57] showed that as cuphea seeding rate increased from 1.5 to 4.5 kg ha^{-1}, so did the number of plants established. Recommended seeding rates in Minnesota are 9–12 kg ha^{-1} [8, 9]. Plant densities between 118 and 228 plants m^{-2} did not significantly affect seed yield [8]. Also, increasing row spacing between 0.13 and 0.75 m did not have an effect on seed yield. The number of branches and the number of seed capsules increase with less dense stands due to the plasticity of cuphea plants [8].

Cuphea Nitrogen Requirement

There is little reported on the effect of nitrogen fertility on cuphea seed yield, oil content, or oil composition. The cuphea grower's guide, created to give guidelines to farmers in Minnesota, recommends using a band application of fertilizer placed 5 cm to the side and 5 cm below seed placement. For most soils, the recommendation was to apply 45 kg ha^{-1} of potassium sulfate (0–0–20–7) along with 224 kg ha^{-1} of diammonium phosphate (18–46–0) and 112 kg ha^{-1} of urea (46–0–0) [8]. The first commercialization efforts with cuphea started in Morris, MN, in 2004. Producers planted between 2 and 4 ha of cuphea and fertilized their fields

with N, P, and K, prior to planting at 56, 56, and 22 kg ha^{-1} of each nutrient respectively [6] based on soil test results [8].

A recent study conducted in North Dakota and Minnesota by Berti et al. [15], in which fertility treatments (soil + fertilizer) were 44, 60, 80, 100, 150, and 200 kg N ha^{-1}, showed that increased N fertility enhanced N uptake and seed yield in cuphea. According to a regression model of the response, the maximum total N uptake at harvest was 139 kg N ha^{-1} and the maximum seed yield occurred at 185 kg N ha^{-1}. However, the seed yield increase obtained with added fertilizer (134 kg N ha^{-1}) was only 71 kg ha^{-1}. Berti et al. [15] concluded that N fertilizer application, at least for already fertile soils, may not be economic given the minimal yield increase with increased N rate application.

Cuphea Weed Control

Cuphea grows very slowly the first 4 weeks after emergence in the spring. Thus, cuphea is a very weak competitor with early-season weeds. However, once cuphea reaches its reproductive phase (generally around midsummer), it grows vigorous and fills its canopy quickly, thus, competing well against late-season weeds. Soil-applied herbicides are primarily recommended to control broadleaved weeds. Cuphea tolerates preplant incorporated herbicides such as trifluralin (840 g a.i. ha^{-1}) and ethalfluralin (840 g a.i. ha^{-1}) and preemergence-applied herbicides such as isoxaflutole (80 g a.i ha^{-1}). For postemergence broadleaf weed control, mesotrione (105 g a.i. ha^{-1}) and imazethapyr (70 g a.i. ha) can be applied after cuphea plants have at least three pairs of leaves showing [13]. Combinations of soil-applied with postemergence-applied herbicides such as imazethapyr and mesotrione also do not damage cuphea [13]. Two weed species that have become problems in commercial fields of cuphea grown in Minnesota and North Dakota that are not controlled by any combination of these herbicides are Canada thistle (*Cirsium arvense* L.) and biennial wormwood (*Artemisia biennis* Willd.). Recently, however, Forcella et al. [14] showed that cuphea tolerates clopyralid at applied rates of up to 400 g a.i. ha^{-1} without significant plant damage. Therefore, clopyralid at a rate of 200 g ha^{-1}, commonly used for other commercial crops, can safely be applied to cuphea to effectively control Canada thistle and biennial wormwood.

Since cuphea is a dicotyledonous plant, several graminicides can be used to control grass weeds. Sethoxydim (0.3 kg ha^{-1}) is routinely used in cuphea to control grass weeds [6, 77]. If adequate row spacing is used for production, mechanical weed control such as cultivation is a viable option and has been used successfully to control weeds prior to canopy closure.

Cuphea Diseases and Insects

The only disease reported for cuphea is white mold (*Sclerotinia sclerotiorum* (Lib.) De Bary.), which caused patches of dead plants in tests plots at Morris, MN, and Prosper, ND, in 2004. A yield reduction as high as 85 % was found in affected areas compared with that of non-diseased cuphea fields [78].

Seedling blight and damping-off of cuphea was observed in several fields in North Dakota and Minnesota between 2004 and 2006. The efficacy of several fungicide seed treatments was evaluated in several experiments in the field and greenhouse between 2005 and 2007 [11]. According to the results of this study, the superior efficacy of mefenoxam over other fungicides seed treatments indicates that a pathogen from the Oomycetes class, such as *Pythium* or *Phytophthora*, is likely responsible for the seedling blight and damping-off symptoms. Treating cuphea seeds with mefenoxam (0.15 g a.i. kg^{-1} seed) is highly recommended to improve seedling establishment. This is especially important in fields that have had sugar beet (*Beta vulgaris var. saccharifera* L.) grown in the past.

Few insects feed on cuphea plants due to the stickiness of the plant stems, leaves, and flowers. Small insects such as several aphids' species and the insidious flower bug (*Orius insidiosus* (Say)) die after becoming stuck to the plants [79]. A study to determine if cuphea would reduce western corn rootworm (*Diabrotica virgifera virgifera* Le Conte) populations in corn-soybean rotation when cuphea was grown following the corn (*Zea mays* L.) crop indicated that larvae of the western corn rootworm may be able to complete their cycle in cuphea fields by feeding on cuphea roots. Behle and Isbell [79] concluded that it is unlikely that cuphea would greatly reduce corn rootworm populations, thus, reducing damage on the following corn crop, but they also suggested that more research is needed to confirm this. Larvae of corn earworm (*Helicoverpa zea* L.) caused extensive damage in cuphea in Peoria, Illinois, in 2006 (T. Isbell, personal communication). Gall-inducing insects (many species) have been observed in ornamental species of *Cuphea*, *C. appendiculata*, *C. cyanea*, *C. hookeriana*, *C. llavea*, *C. nitidula*, and *C. spectabilis* [80].

Cuphea Harvest

An important factor in determining when to harvest crops is seed moisture. Generally, seed moisture at harvest is high for cuphea because mixtures of seeds at different stages of maturity occur at harvest due to the indeterminate growth habit of the plant [7, 25]. For any crop, as its seed reaches physiological maturity, seed moisture decreases [11, 64]. Seed moisture of cuphea at physiological maturity (30 days post anthesis) fluctuated between 62 and 156 g kg^{-1} in a study conducted in North Dakota [11]. Although in cooler seasons, seed moisture at physiological maturity might be as high as 449 g kg^{-1} [81]. Greatest seed yields were obtained when cuphea was harvested in late September to early October, 139–147 days from

Fig. 13.3 Commercial cuphea harvest

planting date in west central Minnesota and corresponding with a killing frost. Oil content increased as harvest date was delayed from August to late September [16].

Swathing or desiccation can be used in oilseed crops to accelerate moisture reduction in order to facilitate earlier harvest and avoid swathing. Swathing cuphea before combining reduces seed moisture at harvest without significant seed shattering or a decrease in seed yield or oil content [17]. One of the disadvantages of desiccation is seed loss to shattering, which has been reported to occur in cuphea if harvest is delayed. Seed will shatter naturally before the first frost even without the application of a desiccant [7, 16]. The greatest seed yields are obtained when straight combining with a small header [7, 18, 25]; however, this treatment also results in much higher seed moisture than swathing the crop. In a study conducted in North Dakota, seed moisture reduction for the swathed treatment compared with direct combining was 216 g kg^{-1} in the first harvest date in the fall. Harvested seed yield reduction was observed only for the desiccated harvest treatment. Swathing is also acceptable since no significant seed yield reduction was observed. Based on the returns after harvest treatments, the direct harvest may be the most cost-effective method to harvest cuphea; however, it is not the most practical due to clogging of harvesting equipment, which slows down harvest (Fig. 13.3).

Cuphea Drying and Processing

Cuphea seed moisture varies with the final use for the seed. Seed intended for planting is dried to 110–130 g kg^{-1} [59]. If seed is going to be crushed for oil extraction, seed moisture needs to be 30–60 g kg^{-1} before processing. Mechanically harvested cuphea from farmer's fields can contain as much as 450–500 g kg^{-1} of moisture. If swathing or applying a desiccant is used, seed moisture can be lowered to between 150 and 250 g kg^{-1} [18]. Drying is challenging because wet

seeds clump and mold very fast if not dried quickly. Large quantities of seeds can be dried using a Grain Technology 2245XL batch dryer or other commercial dryers adapted to have large air flow [82].

Industrial oil extraction is similar to other oilseeds. Cuphea seeds are cooked then pressed with a mechanical screw. Residual oil in the cake after this process is approximately 8 %. The pressed cake is then extracted once with hexane and then finally is desolventized and toasted. Finished cuphea meal typically has 0.3–0.6 % residual oil, 27.3 % crude protein, 21.8 % crude fiber, 37.8 % carbohydrate, 7.8 % ash, and 5 % moisture [83].

The chlorophyll contained in cuphea seed coat (hulls) is carried into the oil during the extraction process. Oil extracted by screw pressing of cuphea seeds may contain up to 360 mg kg^{-1} of chlorophyll, which can reduce the quality of the oil depending on its use. By dehulling the seed prior to hexane extraction, the oil contains 70 % less chlorophyll than that extracted from the whole seed [84]. Also, supercritical carbon dioxide extraction at low temperature and pressure (i.e., 50 °C and 20.7 MPa) can be used to obtain a very high quality cuphea seed oil [85].

Cuphea in Crop Rotations

Because cuphea is well suited for northern US climates and is mainly being developed for the Corn Belt region, it is vital to understand the effects that it has in rotation with the predominate crops for this region, which are corn, soybean, and wheat. Diversifying crop rotations can add environmental and economic benefits. Adding a crop to rotation can suppress pathogens [86] and increase nutrients [87] and more available water in the soil for the next season's crop [88]. However, sometimes, the previous crop can have a negative impact on the next crop in sequence due to such things as allelopathy [89] or by leaving less available soil water for the next crop [90].

To date, only two studies have addressed the effects of cuphea in rotation with the major crops in the Midwest. Behle and Isbell [79] conducted a 4-year study (the first year being an establishment year) in central Illinois with the primary objective of testing the hypothesis of whether cuphea (PSR23) could help alleviate western corn rootworm by disrupting its life cycle when used in rotation with corn and soybean. Generally, fewer adult corn rootworm beetles were trapped in plots of cuphea as compared with corn and soybean plots in this study. However, results also indicated that larvae may be able to complete their development by feeding on cuphea roots, although this was not conclusive. Planting cuphea in rotation with corn significantly reduced the amount of root feeding by corn rootworms in two out of three evaluation years compared with rotations without cuphea, but some economic damage still occurred. Interestingly, this study also showed that corn growth and subsequent grain yield were greater when following cuphea in rotation compared with continuous corn and tended to be as high as that when corn followed soybean.

In another 4-year rotation study conducted by Gesch et al. [9], 2-year rotational sequences of cuphea with corn, soybean, and spring wheat were evaluated. In this study, seed yields of cuphea were unaffected by the previous crop, and vice-versa, corn, wheat, and soybean yields were unaffected by cuphea. However, wheat stand establishment was consistently 17 % greater and grain crude protein content was significantly higher when wheat followed cuphea in rotation as compared with following corn or soybean. This response was attributed to nitrate N remaining in the soil following cuphea harvest, which in part may be due to the low C-to-N ratio of its plant material causing it to decompose quicker, thus allowing N available early in the growing season for the next crop. When cuphea was the previous crop, it left more moisture in the soil profile for the next crop than soybean and was equivalent to that of wheat. Cuphea was found to slightly negatively affect soybean stands, although this was not reflected in grain yield.

Gesch et al. [9] also evaluated the economics of cuphea in rotation and reported that the cost of cuphea production averaged \$172 ha^{-1} less than corn and \$118 and \$126 ha^{-1} higher than soybean and wheat, respectively. However, since that study was conducted, more has been learned about fertility and harvest management of cuphea, which have greatly reduced the input cost of its production, bringing it more in line with that of wheat. In the Gesch et al. [9] study averaged across years, cuphea was not found to be profitable at a price less than \$1,830 mt^{-1} for its seed. Nevertheless, it did provide rotational benefits, with net returns for corn and soybean following cuphea comparable to other non-monoculture sequences and greater than corn or soybean grown continuously. It was concluded that cuphea fits well in rotation with corn, wheat, and soybean but may fit best when rotated after soybean and before wheat or corn.

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Chapter 14
Germplasm Improvement to Develop Commercially Viable Lines of the New Oilseed Crop Lesquerella

Von Mark V. Cruz and David A. Dierig

Abstract Lesquerella (*Physaria fendleri*) is a new oilseed crop that is being domesticated as an alternative crop for arid regions in the United States. As a member of the Brassica family, the species exhibits a high seed-oil content that can provide a source of hydroxy fatty acids for industrial use as source of bioenergy and by-products that can be utilized in livestock production. Germplasm evaluation and breeding activities on the crop have been done by the University of Arizona and the US Department of Agriculture (USDA). Lesquerella and other *Physaria* and *Paysonia* species have a substantial germplasm collection that is well characterized for phenotypic traits and oil profiles. There are several improved germplasm released by the USDA with higher oil content and seed yield, enhanced oil profile, abiotic stress tolerance, and harvest index which can be directly utilized for commercial production or used for crop improvement. Additional technologies that can aid breeding, such as molecular marker systems and genetic transformation systems, have been established. A few challenges remain to be surmounted for the crop to be commercialized. A continued concerted effort by public and private institutions may hasten the process of bringing this new crop to commercial production.

Keywords Brassicaceae • Bladderpod • New oilseed crops • Estolides • Hydroxy fatty acids (HFA)

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Introduction

New crops are fundamental to agricultural diversification and could help raise farm income and mitigate the potentially adverse environmental effects of intensive crop production [1]. New crops are plant species that has been identified to have potential for commercialization and can be grown off-season with a commodity crop or utilize land that are not suitable for growing current commodity crops [2]. Historically, new crops in the United States are those that were previously new germplasm introductions. For example, soybean was once considered a new crop in the United States during the late 1700s, but then it eventually became a major commodity crop in the 1930s [3–5].

Lesquerella (*Physaria fendleri,* formerly *Lesquerella fendleri*) (Fig. 14.1) is among the latest new industrial oilseed crops being developed by improving native germplasm for commercial cultivation in the United States. Interest in *Lesquerella* species for domestication came in the late 1950s and subsequent research and commercialization efforts are viewed as a model for new crop species [6, 7]. The beginning of lesquerella as a new crop started with the national oilseeds screening program of over 200 plant families growing in native habitats initiated by the New

Fig. 14.1 A lesquerella (*P. fendleri*) plant during a USDA germplasm regeneration activity. The netting material of the isolation cage is seen on the background (Photo: USDA)

Crops Research Branch of the US Department of Agriculture (USDA), Agricultural Research Service (ARS) [8, 9]. The goal of the screening program was to find new or unusual kinds of oils that will not compete with vegetable oils [10]. The unique hydroxy fatty acids (HFA) from lesquerella seeds were identified [10–12] and the crop's potential for high yield was likewise recognized. During the 1960s when no *Lesquerella* species had been cultivated, it is reported that the original plant collectors of *Lesquerella* species used a combine method in deserts of Texas to collect large quantities of seed from wild populations for initial research activities [13]. A series of articles including *The Search for New Industrial Crops* and *The Search for New Industrial Oils* were published in Economic Botany and the Journal of the American Oil Chemists' Society between 1960 and 1984 and described lesquerella as well as other potential new crops. The second article in *The Search for New Industrial Crop* series was the first description of collections of *Lesquerella* made by USDA-ARS [8].

Lesquerella produces about 30 % seed oil, and estimates derived from trial plots and farmers' fields show that improved varieties can yield more than 2,000 kg/ha [14]. Lesquerolic, oleic, and linolenic acids were found to be significant fatty acids in its seed oil. Lesquerolic acid (14-hydroxy- eicosa-11-enoic, 14-OH-20:1) content in the seed oil of *P. fendleri* and other species is between 45 and 55 % of the total HFA [15]. Lesquerolic acid was first isolated and identified in *P. fendleri*'s sister species *P. lasiocarpa* and *P. lindheimeri* [11]. The species *P. lindheimeri* has about 89 % lesquerolic acid content. There was initial interest also with *P. gracilis* as a "botanochemical crop" since it has about 70 % lesquerolic acid content [16]. It is not common to find plants that produce fatty acids with a hydroxy group, and before lesquerella, the only commercial plant source is castor (*Ricinus communis*) [17, 18]. Lesquerolic acid is very similar to ricinoleic acid (12-hydroxy-octadeca-9-enoate, 12-OH-18:1) in castor oil, except it is two carbons longer [19]. There are also minor components in lesquerella seed oil that include epoxy acids, a C-22 homologue of lesquerolic acid, and sterols [20].

The hydroxy oil of lesquerella is suitable for producing triglyceride estolides that has numerous applications in industry [21, 22]. Biodegradable lubricants from lesquerella have been found to have superior low temperature properties, with a pour point of −48 °C, and have outperformed commercial products even without additives [23]. Estolides synthesized from lesquerella fatty acid esters were reported to be the best performing estolides to date at cold temperatures [22]. Lesquerella oil derivatives were found to be suitable as a biodegradable, renewable, and nontoxic additive to ultralow sulfur diesel (ULSD) that can easily substitute for petroleum-derived additives [24]. The US Department of Energy has been evaluating lesquerella oil products as biodiesel additives [25], and recent testing by the USDA indicated that methyl esters from the crop have favorable fuel properties and can be blended with petrodiesel at reasonable amounts [26].

The major component of lesquerella oil, lesquerolic acid, can also be converted for use in the production of nylon and molded plastics [21]. Other applications of lesquerella-derived fatty acids are in the production of cosmetics, lithium greases, drying agents, and pharmaceuticals [27]. Additional products from lesquerella

include its seed gum which can be utilized as viscosity modifier in edible and industrial applications [28] and press cake that can be used as an organic fertilizer [29]. The mucilage from seeds has also been found effective as biological control agent against mosquito larvae [30]. This mucilaginous trait is of particular interest in brassica improvement that targets drought-prone regions in China [31]. Lesquerella seed meal was reported to be a better alternative to soybean meal as animal feed due to very favorable amount of amino acids even exceeding the average amount of lysine in several *Brassica* species [32, 33]. Seed protein and protein from the seed meal can be harnessed for other various food and nonfood applications [34].

The other unique HFA found in the related auriculate-leaved *Physaria* species that have been reclassified to the genus *Paysonia* include densipolic acid (12-hydroxy- octadeca-9,15-dienoic, 12-OH-18:2) and auricolic acid (14-hydroxy-eicosa-ll,17-dienoic, 14-OH-20:2). The densipolic fatty acids are predominant in seed oils of *P. densipila*, *P. lescurii*, *P. lyrata*, *P. stonensis*, and *P. perforata* while auricolic acid in *P. auriculata* [35]. The biosynthetic pathways of the different hydroxylated fatty acids in lesquerella seeds have already been studied using in vivo experiments [19].

Taxonomy

Lesquerella is a member of the Brassicaceae or Crucifer family. It is commonly known as bladderpod, lesquerella, yellowtop, desert mustard, and cloth of gold. The genus was named in honor of Leo Lesquereux, a Swiss-American Botanist [36]. The genus *Lesquerella* includes member species that were previously classified under *Alyssum* and *Vesicaria* [37]. Majority of *Lesquerella* species including *P. fendleri* (Fig. 14.1) were later transferred to the genus *Physaria* [38]. Prior to this, *Physaria* consisted of 22 species distributed throughout the intermontane region of the United States and extending into Canada. *Lesquerella* was established by Watson [36] as a North American genus. Later, Payson [39] recognized 52 species and Rollins and Shaw [35], and Rollins [37] recognized 69 and 83 species with 5 additional species published at a later date. Al-Shehbaz and O'Kane Jr. [38] believe that 4 additional new species exist in South America, including *P. mendocina* first described in 1893. In Rollins [37] review of *Lesquerella* of North America, 83 species were included. Other species have since been discovered including those by Rollins [40–42] and by O'Kane Jr. [43, 44] bringing the total number of North American species to about 90.

Among the species of *Physaria* later described include *P. lesicii* and *P. pulchella* found in mountainous areas of central and western Montana [40]; *P. tuplashensis* found in the Hanford nuclear site, Washington [41]; *P. navajoensis* in northwestern New Mexico [43]; *P. eriocarpa*, *P. pachyphylla*, and *P. pycnantha* in the western United States [45]; and *P. scrotiformis* from southwestern Colorado [44]. Several

member species of the genus have been identified as endangered including *P. pallida*, *P. douglasii* subsp. *tuplashensis*, *P. thamnophila*, and *P. globosa* [46].

There was a previous proposal to conserve the name *Lesquerella* over *Physaria* since the former genus has a larger number of species than the latter [47]. However, the request was not recommended by the Committee for Spermatophyta who ruled that "the name *Physaria* is already well known and the advantages of conservation are not sufficient to justify over-ruling the principle of priority" [48].

Because of the taxonomic revision, most of the *Lesquerella* species were moved into the genus *Physaria*, while others transferred to *Paysonia*. The species that were moved to *Physaria* have the HFA lesquerolic acid (C20:1 OH) as the primary component of the seed-oil profile, as do all of the previously existing *Physaria* species. The previous *Physaria* species are perennials and do not flower until the second year of growth. The newer *Physaria* species are both annuals and perennials.

Five species with auriculated leaves native to the southeastern United States (mostly Tennessee) that were previously classified as *Lesquerella* are now part of *Paysonia* [49]. These species have the HFA densipolic acid (C18:2 OH) as the primary component of the seed-oil profile. Also included in this genus are two other species native to Texas which contain lesquerolic acid as the primary seed-oil component and another species from Oklahoma, *Paysonia auriculata*. This species is the only one containing significant amounts of auricolic acid (C20:2 OH), the third HFA type found in this taxon.

The expansion of *Physaria* by combining *Lesquerella* is based on molecular data using nuclear ribosomal DNA sequences of internal transcribed spacer (ITS) [49, 50]. Based on these results, *Physaria* was found to be different from *Paysonia*. The results from using diversity array technology (DArT) markers and subsequently DArTseq molecular markers supported the taxonomic classification by O'Kane Jr. and Al-Shebaz [51, 52].

Native Populations and Areas of Production

Wild populations of *P. fendleri* are usually associated with moisture availability in mixed, sparse vegetation, and the plants can be easily recognized by their glabrous siliques and fused trichomes which set the species apart from other *Physaria*. *P. fenderi* plants are found in its native environment on calcareous, well-drained soils in the southwestern states of Arizona, New Mexico, and Texas, and these are the areas identified as suitable for lesquerella crop production. There have been collections of *P. fendleri* germplasm from southern Utah and Colorado made by Rollins and Shaw [35], but cropping experiments have not been done on these areas. Plant collections from the states of Coahuila, Chihuahua, Nuevo Leon, Zacatecas, and Durango, Mexico, were also made [35, 53].

In addition to being suitable in the southwest United States, *P. fendleri* has been tested to be a promising crop in Southwest Oregon [54] and Northern Mexico

[55]. The crop is also being considered as a new crop in Argentina and was found to also grow well in the cold and arid environments of Latin America along with its sister species *P. mendocina* [56, 57] and can be grown as a summer annual in Ontario, Canada [58]. Research to test if *P. fendleri* can be grown commercially in Europe is ongoing as part of the European Multilevel Integrated Biorefinery Design for Sustainable Biomass Processing Project (EuroBioRef) [59]. It has been reported that *P. fendleri* can be grown with less water and on poorer soils than castor [60]. The crop can withstand mild water stress prior to flowering and still produce maximum yields [61]. However, experiments that aim to determine optimal cultural management practices outside the previously mentioned areas (Arizona, New Mexico, Oregon, and Northern Mexico) are still lacking. The crop production and management system for lesquerella was noted to be very similar to that of winter wheat [6]. Wang et al. [14] published a primer for lesquerella production in Arizona stating planting recommendations that include a planting rate of 7–11 lbs/acre (8–12 kg/ha) with 400,000 plants/acre (988,420 plants/ha) for optimum seed yield which averages at about 1,500 lbs/acre (1,681 kg/ha). The estimated seed yield in other areas where lesquerella have been tested varies from 496 kg/ha in Southern Oregon when planted in March [54] to 1,100 kg/ha in Northern Mexico when sown in December [55].

Genetic Resources

Seed collection trips to acquire germplasm of targeted species of *Physaria* and *Paysonia* were first made in the 1960s. Currently, there are 240 germplasm accessions available in the US National Plant Germplasm System (NPGS). One hundred and twenty five of these are *P. fendleri*, and majority of these accessions were collected during the period from 1993 until 2002 through trips supported by USDA-ARS [15, 53]. The geographic distribution of these collected germplasm with information on average seed-oil content is shown in Fig. 14.2. Only 17 species and 21 accessions of *P. fendleri* were previously represented in the NPGS prior to the USDA collecting effort [62]. The working seed collection from the former breeding program at the USDA-ARS location in Maricopa, Arizona, still exists and a duplicate germplasm collection maintained by a curator within the NPGS located at the USDA-ARS National Arid Land Plant Genetic Resources Unit at Parlier, California, who also conducts routine phenotypic characterization and germplasm regeneration activities.

There are 413 accessions of 57 *Lesquerella* and 17 *Physaria* species already collected. Phenotypic evaluations of germplasm available in the NPGS for HFA and other fatty acids have been completed [53, 63]. Characterization data on four HFA (lesquerolic, densipolic, auricolic, and ricinoleic) as well as seven other the fatty acids (palmitic, palmitoleic, stearic, oleic, linolenic, linoleic, and arachidic) have been published [63]. It has been reported that the growth environmental conditions affect the fatty acid profile of lesquerella, with other lesquerella species

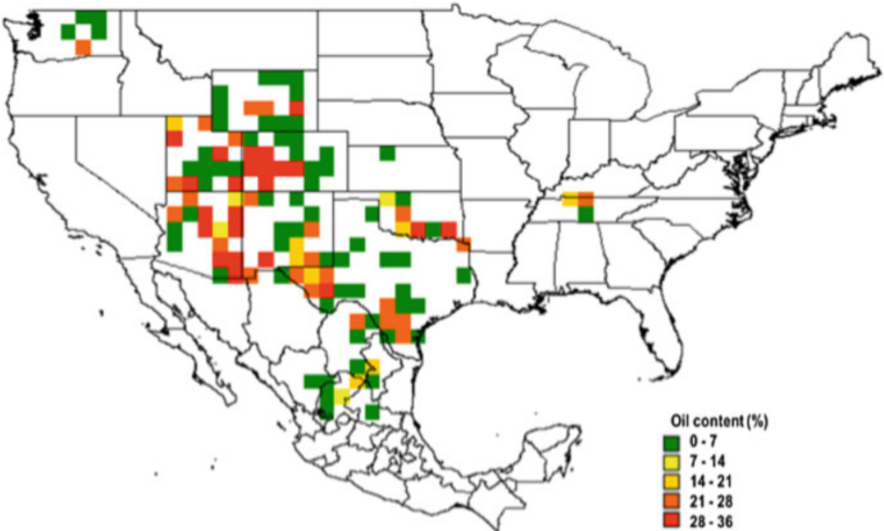

Fig. 14.2 Geographic distribution of lesquerella (*P. fendleri*) and USDA-collected germplasm of related *Physaria* and *Paysonia* species in the United States and Mexico showing variation in seed-oil content

showing higher sensitivity to elevation and temperature. Plant density was also found to affect the content of linolenic and auricolic acids, while late harvesting may cause reduced seed-oil content [64].

Several lesquerella accessions have been described to have potential for ornamental use due to abundance of flowers and the plants' suitability for semiarid and arid landscapes [65]. Likewise, there is a recent interest in the crop as a model species thriving in arid environments. An endeavor to characterize germplasm collections for root traits is ongoing to determine the existing variability in the collection as well as identify associated molecular markers [66].

A few *Physaria* species are on federal or state lists as rare, endangered, or threatened species. Among these species, *P. pallida* has been valuable to the crop's breeding program because of its high lesquerolic HFA content (85–90 %). This species is also autofertile compared to the self-incompatibility and open pollination of *P. fendleri*. No collections have been made of this species since the original collection in the 1800s, until a report by E.S. Nixon and J. Ward in 1981 on its rediscovery [67].

There are several *Physaria* species that have traits of interest for genetic improvement, although none have been found to have the equivalent productivity of *P. fendleri*. The lesquerella database contains over 10,000 evaluation records including various traits such as yellow seeds, non-shattering selections, salt tolerance, male sterility, five-petal plants, multilocule silique selections, and other traits and crosses.

Major Breeding Achievements and Target Traits

The concerted effort to conduct research activities on lesquerella happened several years after the crop's potential as a new crop has been recognized [68]. The history of lesquerella breeding program started at the University of Arizona, and activities were conducted by D.D. Rubis in 1966 which continued until 1978. These initial activities at the University of Arizona were only documented in the annual reports of the university [69]. The USDA-ARS US Water Conservation Laboratory in Arizona took over the crop's research and breeding activities in 1984 until 2010, and the program was continued by A.E. Thompson and D.A. Dierig [69]. The USDA program included materials from the previous breeding program of the University of Arizona.

The domestication and crop improvement activities in *P. fendleri* have resulted to the development of several improved germplasm by the USDA. Eight registered germplasm and one genetic stock have been released and seeds are available through the US NPGS (Table 14.1). These lines came from research activities utilizing a breeding population from a bulk of accessions of one accession from Arizona and nine from Texas established in 1986 [70]. The materials have been selected for seed and oil yield, unique flower color, seed coat color, plant

Table 14.1 Genetic stocks and improved germplasm of lesquerella (*P. fendleri*) developed by USDA

Germplasm	NPGS accession	Trait(s) of interest	Seed yield/ plant (g)	Oil content (%)	Year released	Reference
WCL-LY1	PI 596362	Higher seed oil content than unselected populations	22–27	24.4	1998	[70]
WCL-LO1	PI 596363	Improved oil content	22	26.4	1998	[70, 72, 86]
WCL-LH1	PI 596364	Improved lesquerolic acid content	15	–	1998	[70]
WCL-YS1	PI 610492	Yellow seeded	–	19.2	2000	[71]
WCL-LY2	PI 613131	Improved oil content, lesquerolic acid, and seed yield	24–36	29.4	2001	[72]
WCL-SL1	PI 613132	Salt tolerant	1.5	–	2001	[73]
WCL-CF1	PI 642048	Cream-colored flowers	8	18.0	2006	[74]
WCL-LO3	PI 642047	Higher oil content	19	28.7	2006	[75, 86]
WCL-LO4	PI 666045	Increased harvest index	24	28.8	2013	[86]

architecture, abiotic stress tolerance, and seed-oil characteristics. Diseases and insects have not been in issue in the crop as no major incidence related to these has been observed during limited trials. The agronomic performance of lesquerella was found not to be affected by the predominant insect pest, *Lygus* sp., in the southwest United States, but additional studies are needed to be done when the crop is grown under commercial cultivation [77, 78].

The reestablished lesquerella breeding program has contributed significantly in improving the seed-oil content of the crop. Dierig and Ray [79] reviewed that improved germplasm has an oil yield advantage of 60 l/ha over unimproved germplasm materials. When translated into seed-oil content, this is equivalent to an increase of 8–12 % in the improved germplasm. The latest lesquerella lines that were developed have more than 33 % seed oil and a harvest index of up to 16.5, significantly higher and a tremendous improvement compared to 0.14 harvest index observed in unimproved germplasm [80]. Selection for materials with increased lesquerolic acid has also been successful with the release of WCL-LH1 having about 8.6 % increase in lesquerolic acid content in the seed oil [70]. A salt-tolerant germplasm, WCL-SL1, was later developed using plants that survived high salinity levels (21 and 24 dS m^{-1} electrical conductivities) as parental materials [81].

P. fendleri is a diploid ($2x = 2n = 12$). Other species have different chromosome numbers ranging from 8 to 40 [35]. The feasibility of interspecific hybridization has been demonstrated as possible using *P. fendleri*, *P. pallida*, and *P. lindheimeri* [27]. Interspecific hybridization is being explored as a possible strategy to further improve the HFA content of *P. fendleri* which is relatively low (~50 %) compared to the two other mentioned species (both with >80 %) [63]. Interspecific hybrids were observed to have elevated content of HFA (~70 %) indicating the possibility of increasing the crop's oil content by genetic introgression specifically from the endangered species *P. pallida* [82]. Previous reports on lesquerella species with natural habitats in the western United States however indicated that natural hybridization among them is not common. It is the opposite for species found in the eastern United States [83–85]. Additional research to evaluate interspecific hybrids in *Physaria* is needed.

Breeding Methods and Integration of New Biotechnologies

Breeding Methods

The research activities at USDA included development of genetic stocks with improved traits for direct utilization or for use as base materials to develop new lines. Thompson and Dierig [69] outlined the methodologies used to develop different lesquerella breeding populations. The specific approach included using single-plant selections from the unimproved accessions, bulking selections from half-sib families to develop the lesquerella Texas and Arizona subpopulations

during the 1984–1991 phase of the breeding program. All current genetic stocks can be traced back to these subpopulations assembled from ten wild accessions. Mass selection has been extensively used to improve germplasm from the highly heterogeneous wild populations of lesquerella.

The first three germplasm lines (WCL-LY1, WCL-LO1, WCL-LH1) with improved oil traits were registered in 1998 (Table 14.1). These were developed using three generations of recurrent selection for oil traits using a bulk population developed in 1986 [70]. WCL-LY2 was subsequently developed from WCL-LY1 using mass selection to obtain a higher seed-oil quantity [72]. Mass selection was also used to develop WCL-SL1 from the 1986 bulk population using plants that survived high salinity treatments [73], WCL-LY2 from WCL-LY1 with oil quantity and lesquerolic acid quantity as selection criteria [72], WCL-LO3 from WCL-LY2 selecting for oil concentration [75], and WCL-LO4 from WCL-LO3 with harvest index and oil content as selection criteria [86]. An oil content of 45 % was reported in several individual plants following recurrent selection activities [76].

WCL-YS1 and WCL-CF1 were developed using single-plant selections. For WCL-YS1, two plants with yellow seed coat identified in a field trial in Arizona were selected and subsequent selection activities for progenies with yellow seed coat were done [71]; WCL-CF1 was identified from a single plant during a field regeneration activity [74].

In breeding for oil content, progress in evaluating for oil content using the half seed technique (or half seed method) has been made. The nondestructive technique evaluates individual seed and allows good reproducibility of measurements [87]. It also demonstrated that germplasm selections still possess high natural variability in seed oil which provides an opportunity for further improvement [76]. The previous method used for evaluating oil content is by using bulked seeds.

Mutation Breeding

The use of chemical mutagenesis in lesquerella has been viewed as a strategy to increase HFA variability and obtain other novel traits. Cultured microspores have been attempted to generate mutant lines [79, 88]. Initial efforts however indicated that there was no significant advance in generating additional variability as most traits obtained were subsequently observed to be present in the germplasm collection [79].

Molecular Markers

There are several molecular markers for lesquerella which include protein- and DNA-based systems. Most, however, have not been integrated for use in the crop's

improvement program but have found utility in helping understand its population genetics and taxonomy and assist in germplasm curation.

Allozyme markers were the first to be employed in the study of the species. Five enzymes and seven polymorphic loci were used to genotype early and late germinating *P. fendleri* plants, as well as those grown under two water treatments [89]. Subsequently, random amplified polymorphic DNA (RAPD) marker amplification have been tested to work during optimization of DNA extraction protocols in the species *P. fendleri*, *P. ovalifolia*, *P. rectipes*, and *P. douglasii*. However, only preliminary results from using one RAPD primer, OPL03, were available in the literature, but the initial work presuppose that other RAPD primers can be useful in genotyping work in the species [90].

Microsatellite markers have been developed for *P. fendleri* and were found to successfully cross-amplify with the following species: *P. acutifolia, P. angustifolia*, *P. cinerea*, *P. douglasii* subsp. *tuplashensis*, *P. gordonii*, *P. gracilis*, *P. lindheimeri*, *P. mexicana*, *P. pallida*, and *Paysonia lyrata* [91]. Microsatellite markers are being used to determine genetic diversity in *P. congesta* and *P. obcordata*, both sister species of lesquerella that are under threatened status in Colorado [92].

Intersequence simple repeat (ISSR) markers have been used in a *Physaria* relative, *P. bellii*, to determine whether native populations in the Colorado Front Range are under threat of hybridization with a more common *Physaria* species [93]. Three ISSR primers (UBC890, UBC809, UBC841) were found to work and have enabled the analysis of genetic variability within and among *P. bellii* populations [94]. ISSRs along with internal transcribed sequences (ITS) of nuclear ribosomal DNA have also been used to assess the taxonomic classification of the genus, consequently providing support for reclassification uniting *Lesquerella* with the genus *Paysonia* [38].

Amplified fragment length polymorphism (AFLP) markers were utilized to determine the genetic composition of intertribal sexual hybrids between *P. fendleri* and *B. napus*. A total of 1,271 bands were obtained from 26 primer pairs and were used to analyze the parental materials and their F_1 hybrids [31].

More recently, Diversity Arrays Technology (DArT) marker systems have been developed and used to analyze the genetic diversity of the *Physaria* and *Paysonia* germplasm in the US National Plant Germplasm System. A total of 2,833 marker loci have been analyzed using microarray DArT while 27,748 marker loci using DArTseq [51, 52]. Results of analysis using these two DArT marker systems provided support to the previous taxonomic revision of the genus and gave information on the genetic diversity of the USDA-ARS lesquerella germplasm collection.

Tissue Culture and Genetic Transformation Systems

Biotechnologies may help overcome the reproductive incompatibility issues in lesquerella and allow transfer of desirable traits to related genera in the Brassica family. Tissue culture studies have been done in *P. fendleri* to support genetic

transformation systems as well as aid recovery of interspecific hybrids [95]. Ovule cultures of interspecific hybrids from the following species – *P. auriculata, P. lindheimeri, P. lyrata, P. pallida*, and *P. fendleri* – were obtained and used in breeding activities [96]. Embryo rescue was utilized to obtain intertribal hybrids between *P. fendleri* and *B. napus* after hand pollination [31]. Somatic hybrids between *P. fendleri* and *Brassica napus* have also been developed through polyethylene glycol-induced protoplast fusion. Somatic hybridization between these species had an efficiency of 1.5–7.3 % with the hybrids reaching reproductive stage in the greenhouse and able to produce seeds [97].

P. fendleri has been demonstrated to be amenable to *Agrobacterium*-mediated transformation as well as biolistic methods. Transplastomic *P. fendleri* plants have been obtained from biolistic approach and the resulting plants were found fertile and successfully produced seeds [98]. *Agrobacterium* transformation protocols have also been reported by Wang et al. [99] and substantial progress to refine the system has been made [100–102]. *P. fendleri* calli were noted to be responsive to the *Agrobacterium* transformation system, but chimeric regenerants have been noted. These were later addressed by screening for non-transformed cells in multiple rounds of shoot regeneration to obtain stable transgenic *P. fendleri* lines [101]. Transgenic approaches have helped provide initial information on the mechanisms of HFA synthesis in *P. fendleri* [103].

Seed Production

Lesquerella is a predominantly outcrossing species, but the seed setting ability was determined to be influenced by how much pollen has been transferred on a flower. The seed set per fruit was correlated to the amount of available pollen grains, but the relationship was determined to be very loose and highly dependent on the variability among plants [104].

One area that received considerable focus after the crop was identified as a promising oilseed is how to break seed dormancy in several species [105]. Lesquerella does not exhibit planting density-dependent germination [89], but the persistence of seed dormancy has been identified as a significant barrier to obtain adequate plant densities in the field and a challenge for seed production [105]. Lesquerella seed dormancy has been observed to last up to 5 years after harvest, although this has not been observed in *P. fendleri* but an issue with *P. gordonii* and *P. palmeri* [106]. The positive effect of gibberellic acid (GA) to break seed dormancy has been determined and the effect of applying GA to seeds was found to last up to 72 months at 5 °C and 35 % relative humidity [105]. GA was found to eliminate the light and temperature requirement for lesquerella seed germination in *P. fendleri*, *P. gordonii*, *P. palmeri* [106, 107], as well as *P. argyraea*, *P. gracilis*, *P. rectipes*, *P. recurvata*, *P. sessilis*, and *P. thamnophila* [108]. GA can be applied to seeds by either soaking the seeds for 8–24 h or spraying it with a concentration of 1,000 ppm [106]. Other studies found that immersion to

100–400 ppm GA for 20 h helped seeds of *P. fendleri* and *P. gordonii* germinate at low (5 and 10 °C) and high (35 °C) temperatures [109], and soaking the seeds for 4 h at 100 mg GA l^{-1} or GA with 2 g potassium nitrate (KNO_3) solution is sufficient to break seed dormancy [110]. Pre-sowing seed treatment in lesquerella will allow better and uniform germination, but field testing remains to be conducted. The current improved lesquerella germplasm does not have significant dormancy issues, and adequate germination can be achieved from fresh seed.

Another significant research area in lesquerella is in identifying possible production field sites for the new crop. Dierig et al. [111] have determined that *P. fendleri* grew well in areas with elevation below 700 m. The range of suitable production areas might be expanded by introgressing genetic material from germplasm of species with close affinity to *P. pallida,* since the latter have better plant performance at higher elevations [111]. Possible production in Northern Mexico has been studied with a mid-December sown crop found to outyield those planted in October [55], while a late March seeding date was suggested when growing the crop in Ontario, Canada [58]. However, exact recommendations for agronomic management in Northern Mexico and Canada are yet to be determined.

Lesquerella plants are similar to short canola crop, but since it is being improved from wild populations, pod shattering during harvest has been an issue during the crop's early stages of development [64]. Terminating the crop by desiccation was found suitable and machine harvesting lesquerella seeds result to low seed losses (less than 5 %) [112]. The crop is harvested when the seeds turn brown and have moisture content below 12 %. The plants can be combined directly or windrowed prior to combining [58]. Harvested yields obtained from windrow combining were less than when a direct combine method was used but not statistically significant [113]. The harvested yield can be affected by the termination date of the plants and a 2-week additional growth period was found to result to higher yields [113].

Market Challenges/Barriers to Commercialization/ Opportunities

The development and commercialization of new crops is a highly integrated process starting from germplasm identification and improvement to getting the necessary market and policy environment and involvement of industry partners for commercialization [69, 114]. The success to commercialize new crops and for enabling farmer adoption entails knowledge and positive experience on them being profitable. Widespread skepticism of alternative crops can result to limited public support and may stunt the potential success of development and commercialization efforts [1].

In the case of lesquerella, there is still limited testing of the crop in commercial scale production and consumers are not fully aware of its potential. There are a few remaining challenges for the crop to be commercialized [7]. Recommendations on

agronomic practices for commercial production are still being studied. Among this includes getting suitable herbicides registered for scaling up lesquerella production [7, 14]. Lesquerella grows slowly in the early season and weed control is an important issue [115]. However, progress is being made in getting lesquerella among the crops included in the labels of herbicides. To date, research studies have shown that Prefar™ (46 % bensulide) can be used at a rate of 4–6 qt/acre during planting, and Prowl® H2O (38.7 % pendimethalin) at 3 pt/acre after the plants have reached 4–10 leaves (McCloskey, 2012, The School of Plant Sciences, University of Arizona, "personal communication"). Fine-tuning other agronomic recommendations for the crop remains with the public sector research centers. Paarlberg [5] noted that the involvement of the private sector is very valuable in speeding up the development of new crops, but it depends whether the private sector sees a potential for the crop to be proprietized.

The economic benefits of lesquerella compared to other crops have been studied. Van Dyne [115] concluded that returns from growing lesquerella can supplement those from other crops grown in counties in Arizona, New Mexico, and Texas, where the crop has the best production potential. However, farmers need to be made aware that net returns from growing the crop are higher than other alternatives for them to consider growing it. Updated economic data on lesquerella production are needed since costs have changed significantly since the last reported economic study.

The properties of lesquerella oil have been fully characterized by the USDA-ARS National Center for Agricultural Utilization Research (formerly Northern Regional Research Laboratory) in Peoria, Illinois. The center has confirmed that thermal behavior data of lesquerolic acid is comparable to ricinoleic acid [19] and there are various advantages of lesquerella oil over other vegetable oils [22]. Additional studies have been suggested to further look on seed-oil extrusion methods to obtain optimal oil qualities and yield during processing [116].

Other potential use of lesquerella is in the research efforts to understand lipid biosynthesis and trait manipulation in plants. Genes from lesquerella have been cloned and studied in *Arabidopsis* and was determined to show both hydroxylase and desaturase activities [117]. A gene (*LfKCS45*) that encodes a 3-ketoacyl-CoA synthase that functions in the production of saturated very long chain fatty acids has been characterized and localized to be in the lateral root cap of *P. fendleri* [118]. Follow-up work to harness and integrate the desirable traits of *P. fendleri* to *B. napus* might also be needed. Characterization of genes and studies to determine the HFA biosynthesis pathway in this new oilseed crop might help the development and improvement efforts in other industrial and oilseed crops.

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Chapter 15
Sisal/Agave

Sarah C. Davis and Stephen P. Long

Abstract *Agave* species have recently emerged as potential bioenergy feedstocks that can be grown on marginal semiarid lands, creating an economic opportunity in regions where there are few agricultural commodities. This chapter provides an introduction to *Agave* species that are currently cultivated at a commercial scale for the tequila and fiber industries. It then reviews the opportunities and challenges associated with developing *Agave* feedstocks for biofuel by integrating recent biotechnological advances with traditional knowledge of *Agave* production. Drought tolerance, high yield, CAM physiology, and genetic diversity are among the characteristics that make *Agave* species apparently attractive as feedstocks. Challenges include manual labor costs and the establishment time that is required for the crop. Opportunities for development include the use of land that is otherwise unsuited, or has become unsuitable, for other agriculture in economically depressed rural areas. Despite the additional research that is needed to identify the varieties most fit for biofuel feedstock, current technology exists to support an *Agave*-based biofuel production system.

Keywords Agavaceae • Biofuel • Ethanol • Lignocellulosic • Semiarid agriculture • *A. tequilana* • *A. sisalana* • *A. fourcroydes*

Introduction

Agave species have recently emerged as candidate bioenergy crops because of their relatively high yield in areas of low rainfall, drought tolerance, and low lignin content [1, 2]. Though commercial production of *Agave* spp. for bioenergy has not yet been demonstrated, several species have been cultivated commercially to

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support alcoholic beverage and fiber industries. Archaeological findings and reports of early Spanish explorers suggest that pre-Columbian communities in the southwestern USA and Mexico cultivated *Agave* on a large scale. This practice provided not only beverages and fibers but also an insurance food crop, rich in carbohydrates, during periods of drought when corn and bean crops failed [3]. The *Agave* genus has been cultivated on four different continents, but the geographic range may be even larger than previously realized because species of this genus are tolerant of a wide range of temperatures, precipitation patterns, and soils.

Adapted to semiarid and arid climates, the *Agave* genus has the potential to grow on lands that have been labeled marginal or unsuitable for other crops. Crops grown on semiarid lands are often heavily irrigated and are thus likely to have a greater environmental impact and lower profitability than crops grown in rain-fed agricultural regions. These dryland agricultural systems, such as those in western Texas and other states in the southwestern USA, are less stable agricultural production systems that move in and out of production depending on climate trends and market conditions for different products. A plant like *Agave* requires little or no irrigation and would be less vulnerable to drought and extreme temperatures as well as monsoon events than irrigation-dependent crops used in semidesert areas across the globe.

There are 208 known species of *Agave*, most of them native to Mexico [4], and more research is needed to determine which of these have optimum traits for bioenergy feedstock. More research is also needed to determine the geographic range across which commercial plantations of *Agave* varieties would be viable. Despite the novelty of this crop, there is enough potential as a successful bioenergy feedstock that research focusing on *Agave* varieties has begun at sites that span the globe. This chapter reviews the current state of knowledge and the traits that make *Agave* a hopeful source of biomass for bioenergy.

Taxonomy, Domestication, and Breeding History

The genus *Agave* is classified in the monocotyledon family Agavaceae, but is sometimes included in either the Liliaceae or Amaryllidaceae [4–6]. There are two subgenera [7] and 200–300 species that are all native to the new world, with most originating from México. Ploidy levels vary, even within species, from $2n$ to $8n$ ($n = 30$) and hybridization has occurred frequently in wild cultivars [8–10]. Higher chromosome numbers are often found in species with larger leaves and more dense fiber tissue [8, 10]. Recent molecular analysis of Agavaceae also shows that the genus *Agave* is paraphyletic with respect to three other genera [4]. Little variation in genome size of diploid varieties in cultivation has been observed [9].

All *Agave* species are xerophytes, but they range in size from a few cm to 4 m in height [7, 11]. The plants consist of a basal rosette of stiff, evergreen leaves that are succulent and usually lanceolate in shape with a terminal spine. Most species have leaves with spiny margins. All species in the genus are expected to use Crassulacean Acid Metabolism (CAM) photosynthesis because all *Agave* spp.

analyzed to date use CAM [12–14]. CAM plants are unique in opening their stomata at night to assimilate CO_2 into organic acids, primarily malic acid. During the day, the CO_2 is released, by decarboxylation of the organic acids, into the internal leaf air space. It is then assimilated into carbohydrate via the C_3 photosynthetic pathway while allowing the stomata to remain closed [15]. Evaporative demand is much lower at night in desert and semidesert environments, where nighttime temperatures may be 20–30 °C below daytime temperatures. In these conditions, CAM plants can use a small fraction (0.05–0.1) of the water used by a non-CAM plant for each CO_2 that is assimilated [16]. Water use efficiencies can therefore be 10–20 times higher than for C_3 plants growing in the same environment. Although there is an additional energy cost in assimilating CO_2 first into an organic acid, this is of little consequence in an environment where water is scarce, but sunlight is plentiful. However, when grown in moist environments, CAM plants will be at a theoretical disadvantage.

As perennial xerophytes, *Agave* spp. are adapted to survive in hot dry conditions. The leaves have a waxy epidermis, sunken stomata, and large water storage cells in the mesophyll [17]. The roots are retractile and shrink in response to low soil water potential, leaving an air space between the soil and root surfaces. This, coupled with the thick waxy cuticle covering the shoot, isolates the plant hydraulically from the dry air and dry soil, allowing it to maintain a high water content through long periods of drought.

There is a long history of *Agave* cultivation in Mexico and the southwestern US desert, where the genus has been used for fiber production, sweet nonalcoholic beverages, low-alcohol fermented beverages, distillation into tequila and mescal, and as a food source, typically by baking the stem bases [3, 18]. In their native range, *Agave* plants hybridized naturally with the assistance of pollinators that range from insects to bats. In cultivation, humans selected and cultivated varieties for two separate traits: high sugar and long fibrous leaves. These varieties serve two separate industries with the high sugar varieties supporting fermentation to alcohol and the varieties with long leaves providing fiber for rope making and textiles [5].

Areas of Production

The *Agave* production system that supports the tequila industry parallels the fuel ethanol production systems from corn in the USA and sugarcane in Brazil. It similarly relies on the fermentation of sugars, and polysaccharides (fructans) that are easily degraded to sugars (fructose), that are concentrated in the stem base [19].

Agave tequilana, like most *Agave* spp., is monocarpic [4], i.e., after producing leaves for a period of time, the stem apex becomes reproductive and on the completion of flowering and fruit formation, no further growth occurs and the entire stem dies. During the vegetative stage, storage carbohydrates accumulate in the stem base which, in the case of *A. tequilana*, swells into a large spherical organ, termed a piña (composition summarized in Table 15.1). When the flowering

Table 15.1 Weight-based percentages of compounds in *Agave* leaves

		A. tequilana [32, 36, 49]	*A. fourcroydes* [32, 50]	*A. sisalana* [51]	*A. americana* [52]	*A. angustifolia* [32]	*A. salmiana* [53]
Leaf	Cellulose	65	78	43	68		
	Hemicellulose	5	5–7	32	16		
	Lignin	16	13	15	4.9		
Piña	Glucose	1–3	4			0–2	
	Sucrose	4–6	8			1–10	
	Fructose	7–10	13			2–7	
	Fructans	43–73	49			36–51	
	Total soluble carbohydrates	55–90	55			52–63	
Bagasse	Cellulose	43					47
	Hemicellulose	19					13
	Lignin	15					10
	Total soluble carbohydrates	5					

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stage is initiated, these carbohydrate reserves are mobilized to elongate the stem into an enormous flowering spike. For tequila production, the stem base must be harvested just before spike development. Cultivars of *Agave tequilana* have been selected for early flowering time relative to other *Agave* spp. so it can be harvested in 5-year rotations. Other species will mature after a decade or longer.

Agave spp. that are grown for fiber have long straight leaves from which long fibers can be harvested for rope and fabrics. *Agave sisalana* (sisal) is the most widely cultivated, primarily in Africa and South America, where this crop occupied roughly 420,000 ha in 2010 declining from 890,000 ha in 1961 [20]. Another fiber variety, *A. fourcroydes* (henequen), is mainly grown in Mexico and occupied 43,000 ha in 2010 [20]. These plants also produce soluble carbohydrates (Table 15.1), but would be most suitable as lignocellulosic feedstock crops. The leaves are harvested instead of the whole plant, as in an *A. tequilana* crop, and biomass from the same plants can therefore be harvested repeatedly at a shorter interval than is typical for the tequila crop. The opportunity to repeatedly harvest leaves from sisal and henequen plants offers a potentially continuous supply of feedstock for lignocellulosic fuel production. Currently, most harvesting is manual, which may be an important source of income in the poorest countries. Elsewhere, viability is likely to depend on mechanical harvesting. However, both manual harvesting aids and sisal harvesters have been proposed and tested [21, 22].

Brazil is currently the largest producer of *Agave* for sisal fiber (Fig. 15.1). Other leading producers, including Tanzania, Kenya, Madagascar, and Mexico, suffered a decline in production since the late 1960s, but most dramatically since 2000,

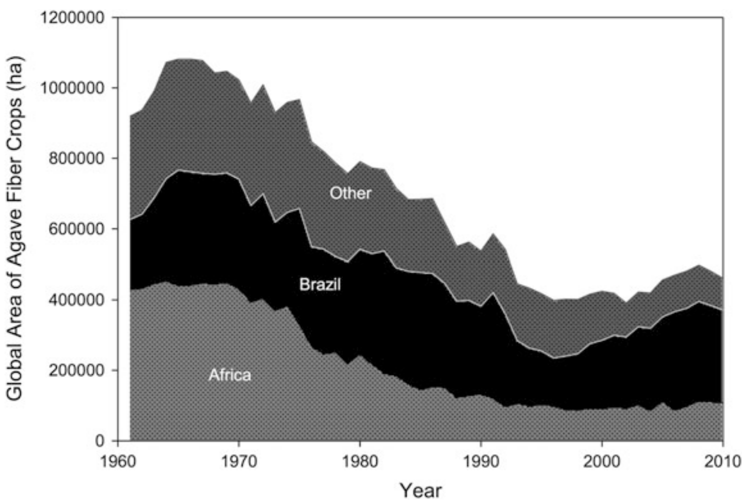

Fig. 15.1 World production of Agave fibers, 92 % of which are sisal, showing the contribution of Africa (primarily Tanzania, Kenya and Madagascar), Brazil, and all other countries (Based on data from Ref. [20])

because natural fibers have largely been replaced by synthetic fibers [2, 20, 23]. Recent increases can be attributed to increased production in Brazil and an increase in interest in natural versus synthetic fibers for select markets (Fig. 15.1). Most of the growing regions for *Agave* are semiarid, but Brazil also produces *Agave* in areas with high rainfall (>1,000 mm) but that have few other present economic opportunities relative to other regions of this resource-rich country.

The demand for natural fibers from sisal peaked in 1964, when > 1 Mha of land around the world was planted in *Agave* [20]. Since 2000, abandoned sisal production, primarily in Africa and Mexico, has led to the abandonment of this agriculture on 0.6 Mha of land globally (Fig. 15.1) [20], and it is estimated that this land alone could support an annual production of 6.1 billion liters of ethanol [2]. As of writing, however, there is no commercial production of *Agave* for biofuel.

Agave crops can be grown in a wide variety of conditions, but the greatest opportunity (economically and geographically) probably exists on semiarid lands that currently do not support other valued crops. Semiarid lands are classified by the United Nations Environment Program as areas where the ratio of actual to potential evapotranspiration is between 0.2 and 0.5 and total precipitation is typically less than 600 mm. Such land represents 17.7 % of the land surface or 2,370 Mha [24].

Genetic Resources

Regardless of the opportunities, there is still a need for research on the yield potentials of different *Agave* varieties for different regions. Yields reported in previous literature reflect a variety of growing conditions and managements (Table 15.2). Large-scale replicated yield trials, and in particular trials comparing varieties, have not been conducted to date, but these will be critical to understand the true potential of Agave as a bioenergy crop [2]. There is a wealth of genetic diversity in the genus *Agave* that has been qualitatively described, but a central depository of defined germplasm for the genus is lacking [7, 25]. Cultivars can be triploid or pentaploid sterile interspecific hybrids, often precluding conventional genetic analysis and further breeding in these lines. However, the rapid growth of sequencing capability and analysis is contributing to a better understanding of inter- and intraspecific variation and genetic resources related to diseases, pest resistance, and environmental resilience [26–29]. This should also provide molecular markers to accelerate breeding. However, targeted breeding efforts have not yet been undertaken to optimize *Agave* as a biofuel feedstock.

Table 15.2 Summary of yields (Mg ha^{-1}) for *Agave* species (As reviewed in Davis et al. [2])

Species	n^a	Mean yield	(±Standard error)
A. deserti	3	6.1	(±0.4)
A. fourcroydes	3	14.0	(±1.5)
A. sisalana	2	14.5	(±2.5)
A. lechuguilla	2	14.7	(±10.9)
A. tequilana	5	23.7	(±0.8)
A. salmiana	7	23.8	(±5.4)
A. mapisaga	4	31.6	(±3.8)

[a] n = the number of studies used to calculate mean yield

Propagation

Commercially produced *Agave* are harvested before the flowering stage, are often sterile hybrids, and even if fertile they are highly heterozygous, and would therefore produce highly variable stands from seed. Therefore, plants are not typically propagated by seed but instead are propagated asexually to provide uniform clonal material. Seeded plants are also disadvantaged because they have a tendency to be more sensitive to drought and sudden temperature changes and require more years to reach harvest maturity than asexual propagules [5, 14, 30]. Asexual propagation can occur by harvesting offset rhizome shoots or bulbils from a plant inflorescence or through micropropagation by tissue culture. These methods provide more robust plantlets and result in lower variation in plants across a field than a seeded crop [5].

The flowering stalk must be allowed to form in the case of propagation by bulbils, but a single stalk can produce thousands of plantlets. Thus, nearly a hectare of *Agave* plants can be planted from one or two mother plants. Sisal plants produce ~2,000 bulbils on a single stalk that can be harvested by simply shaking the plant and collecting the fallen bulbils from the ground. This method will produce more uniform plantlets than propagation from offsets, where only a few plantlets which are typically of variable size can be harvested from a mother plant at a time. The advantage of propagation from offsets is that one need not wait until the plant matures to collect the plantlets. Offsets can be harvested annually, and these are then typically kept in a nursery before planting in the field [31].

Harvest of *Agave* plantations is usually accomplished with manual labor and is therefore an economic challenge for commercially scaled production for biofuel. Nuñez et al. [23] reviewed the economic viability of *Agave* production for biofuel assuming the methods that are currently used for tequila production are transferable to *Agave* bioenergy feedstock production. The economic returns from *Agave* feedstock production cannot compete with those realized by the tequila industry, and manual labor costs incurred during the harvest phase of production limit the economic competitiveness of *Agave* against other biofuel feedstocks [23]. This will of course depend on the labor costs in the region chosen for cultivation.

Integration of New Biotechnologies: *Conversion of Agave Feedstock to Ethanol*

The tequila industry provides one established industrial model from which a biofuel production operation might be envisaged because the plantations and the initial fermentation at a distillery are similar to the steps needed for fuel ethanol production. Tequila Sauza, one of the largest tequila companies in México, processes approximately 400 metric tons of biomass per day into alcohol at a single distillation plant (Del Real Laborde, personal communication, 2010). Approximately one liter of 100 % tequila is produced for every 5.5 kg of dry biomass. At 40 % ethanol by volume of tequila, the ethanol currently produced by this model facility is ~10.6 million l year^{-1}. The piña yields ≥52 % sugar [32] and yields a greater concentration of soluble carbohydrates than maize grain.

If one assumes that the current ethanol conversion efficiency of maize grain [33] can be applied to the sugar-rich piña from *Agave tequilana*, then the potential ethanol production of a facility, the size of a tequila distillery, would be approximately 61 million liters year^{-1}. This does not include conversion of lignocellulosic parts of *Agave* plants (biomass from leaves and bagasse). In tequila production, these are wastes, but with emerging technologies for converting lignocellulose to ethanol, these could in the longer term be used to increase fuel ethanol production [34]. The leaves of *A. tequilana* that are unused in the process of tequila production equate to ~38 % of the total plant biomass [35, 36] and could serve as cellulosic feedstock to produce another 28 million l year^{-1}. In total, the production capacity of an ethanol plant that processes *Agave* from the same amount of land used by a single tequila distillery could be 89 million l year^{-1} of ethanol. Existing technologies for processing and fermenting lignocellulosic biomass from *Agave* to ethanol are described below.

An important practical advantage that biofuel production from *Agave* would have over sugarcane or maize ethanol production is stability of supply. The latter are dependent on yields within a given year, and thus a drought or other disruptive weather events could severely disrupt feedstock supply. *Agave*, with growth cycles of 4–20 years, provide a standing stock so, while annual biomass production may vary from year to year, the net standing stock allows a stable supply in any 1 year and parallels forestry more closely than arable agriculture in its ability to provide a consistent supply of material for processing.

Pretreatment

Lignocellulosic material should be pretreated to reduce the lignin content. This is typically done by steam explosion or with an alkaline or acid solution. In each case, elevated temperature and pressure, ~121 °C with 1.1 kg cm^{-2} for 4 h, are required. Steam explosion would typically be applied prior to acid hydrolysis, but the

alkaline pretreatment, when combined with enzymatic hydrolysis, has been demonstrated recently as an apparently more efficient way to yield reducing sugars from the cellulosic material in *Agave* [37].

Saccharification by Hydrolysis

A recent study that compared acid hydrolysis efficiency with enzymatic hydrolysis found that Viscozyme (Novozymes; Bagsvaerd, Denmark) yields the greatest amount of reducing sugars when compared to acid hydrolysis and other enzyme catalysts [37]. The study evaluated pathways for hydrolysis of bagasse from *A. tequilana* that included raw lignocellulosic material from the leaf bases and outer piña that were trimmed before the piña was crushed to extract sugars (called metzal) as well as the fibers that remain after the piña is processed (called metzontete). Using acid hydrolysis of bagasse in dilute solution of HCl and bagasse at a pH of 5.0, only 4–5 % of the metzal mass was converted to reducing sugars, and 3.5–10 % of the metzontete mass was converted to reducing sugars.

In a recent comparison, the combination of alkaline treatment with enzymatic hydrolysis was more effective in releasing reducing sugars from *Agave* bagasse than from sugarcane bagasse [37]. A 0.005:1 ratio of NaOH and bagasse (15 ml 0.25 M NaOH for 1 g bagasse) was used for the alkaline treatment, and then a solution with 6 % bagasse and 1.33 % enzyme were kept at 55 °C with the pH adjusted to the optimum required by the enzyme catalyst (5.5–7.0). Percent sugar conversions for each enzyme test are shown in Table 15.3 [37], with Viscozyme apparently the most efficient hydrolytic catalyst in this experiment, converting 58 % of metzal to reducing sugars, of which 47 % was glucose and 24 % xylose. Recent advances in yeast engineering would allow complete fermentation of this sugar mix at industrial rates [38, 39]. Metzal is the bagasse component that is comparable to the raw lignocellulosic material in leaves that could serve as bioenergy feedstock. These conversion rates are similar to what can be expected for conversion of *Agave* leaf material.

Table 15.3 Percentage of Agave bagasse converted to sugars by contrasting enzymes after treatment in alkaline solution

	% Conversion efficiency from biomass to reducing sugars	
Enzyme	Metzal (raw)	Metzontete (post piña processing)
Pulpzyme	22	12
Cellubrix	28	14
Novozyme	38	12
Celluclast	43	26
Viscozyme	58	36

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It is likely that separate reaction vessels will be required for conversion of the piña and the cellulosic material from bagasse and leaves to sugars. The piña is primarily comprised of fructans that are more readily converted to reducing sugars than celluloses. However, a different enzyme mix for hydrolysis is required. A recent study found that Fructozyme (Novozymes; Bagsvaerd, Denmark) achieves hydrolytic efficiency of 99.5 % after 3–4 h at 60 °C in a 1:1 biomass and water mixture [40]. The optimum enzyme concentration for hydrolysis of both solid particles and aqueous agave extract is 0.17 % solution. With 100 % hydrolysis, there is a yield of approximately 97.5 g l^{-1} from aqueous extracts and 110 g l^{-1} from agave particles [40]. If a fiber *Agave* such as sisal or henequen was the feedstock, providing a continual supply of leaves rather than a harvest of leaves and piña, then this step would be removed (unless the leaves also prove to have high concentrations of fructans).

There is a loss of efficiency when employing simultaneous extraction and hydrolysis in a continuous diffuser. Because the extraction conditions can deactivate enzymes, the hydrolysis efficiency is 50–62 % or 70–89 g l^{-1} of fructose [40]. This, however, would avoid the downtime that is unavoidable with current batch processing. Further study of the conditions that promote enzyme activity may improve reaction efficiency at the commercial scale.

Fermentation

Agave extracts from the piña are typically stored for 12–24 h before fermentation commences. Fermentation for tequila is initiated with a heat treatment of 100 °C that imparts a desired color and flavor to the final product, and then fermentation continues at a lower temperature of 30–35 °C. Fermentation of piña extracts can be accomplished without heat treatment, but the ethanol production is delayed ~14 h and there is a reduction of yeast cells [40]. Low heat fermentation may be a more energy efficient method for biofuel production, but has not been studied in detail for tequila manufacturing because it negatively affects the flavor and consequently lowers the value in the beverage market. This would not be a concern for the fuel market.

The yeast *Saccharomyces cerevisiae* is used to ferment reducing sugars from *Agave* to ethanol because of its high alcohol tolerance, but low conversion efficiencies are often observed in commercially scaled tequila manufacturing. Fermentation efficiency of piña-derived sugars to ethanol is usually at least 80 % [41, 42], but in a fermentation test of *Agave* bagasse, metzal and metzontete yielded a maximum of only 33 % conversion efficiency of reducing sugars to ethanol [37]. Assuming sufficient nitrogen is available, ethanol yield increases with increasing sugar concentrations, with 94 % efficiency achieved in some cases [19].

Gutiérrez-Loméli et al. [42] tested the effect of overexpressing genes that regulate hexose transport (HXT) and alcohol dehydrogenase (ADH). In solution with 115 g reducing sugar l^{-1} at 33 °C, they discovered that fermentation efficiency increased to 94 % with recombinant *S. cerevisiae* from 89 % in the wild type

[42]. Rapid recent advances in breeding and engineering more efficient and tolerant industrial strains of yeast for other sugar fermentations, enabled by genomics, directed evolution and metabolic engineering suggest that large gains in efficiency are likely in the future [43].

Residue Processing

There are several uses for byproducts from *Agave* conversion to ethanol. The bagasse materials, referenced in the potential hydrolysis and fermentation processes above, are a product of distillation after the processing of the *Agave* piña. The solids that are left after processing the piña could be converted to ethanol using the delignification process previously described or it can be used in combustion for co-powering the conversion facilities. The solid wastes from the cellulosic conversion can then be returned to the agricultural fields as a fertilizer. There are also liquid wastes, called vinasse, that are toxic but as in the case of sugarcane ethanol vinasse can be made useable by wastewater treatment. Such treatment can include methanogenesis to yield methane [44] that could be readily used to provide combined heat and power (CHP) for the processing plant.

Vinasse from the processing of piñas is often returned to fields without treatment, which poses a risk of contamination to groundwater. Vinasse is very acidic (pH of 3.35) with high biological and chemical oxygen demands (BOD and COD), suspended solids, and volatile compounds [45]. Methanogenesis provides an ideal way of recovering energy from this organic waste slurry since as an insoluble gas, it requires no energy loss in dewatering. Simultaneously, it lowers the BOD and COD, which are the main causes of toxicity when the slurry is released to water courses or spread onto the land. However, use of plant residues in general from ethanol production is relatively new and has only recently attracted more intensive scientific analysis. A major limitation is that the methanogens can only produce methane from just two substrates, CO_2 and acetate. They therefore require other microbes to digest the material to these substrates and provide the energy sources to drive reduction to methane. Efficiency of methane production could be improved by understanding how to optimize the community of organisms needed for conversion and by engineering the methanogens so that they may interact with more substrates [46].

A stable digestion system that converts *Agave* vinasse into methane has been demonstrated, although it is not typically used. In a 6 l experimental digester, Mendez-Acosta et al. [45] were able to produce 14 l d^{-1} of biogas comprising 65 % methane under stable reaction conditions. This process is scalable and has low energy inputs because reactor temperature is maintained at 35 °C. Digestion reactor conditions are detailed in Table 15.4 [45]. An acclimation period of 50 days was used to initiate this process, although it is likely that acclimation can be achieved in a shorter amount of time. The acclimation period is followed by a start-up phase with controlled dilutions of vinasse; higher concentrations of vinasse are gradually

Table 15.4 Digestion conditions for a reactor that treats vinasse and produces methane a byproduct

Stage	Conditions	Products
Acclimation	3:7 dilution of vinasse to water	Acclimation achieved when chemical oxygen demand is reduced and > 50 % methane is generated
	pH 7.4	
Dilution tank	pH 6.5–7.0	Mixture of vinasse and water with concentration gradually increased of time for start-up phase
Start-up	40 % volume from acclimated anaerobic sludge	80 % of chemical oxygen demand removed
	3:2 ratio of total suspended solids to volatile suspended solids	Alkalinity achieved
	N additions to achieve anaerobic conditions	Accumulation of volatile fatty acids reduced
	pH 7.4	
	35 °C	
Processing	Conditions in start-up stage stabilized	Alkaline solution
	Raw vinasse inputs	Volatile fatty acids eliminated
		Consistent supply of CH_4

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added until raw vinasse can be added without dilution in water. The result is a constant supply of methane that is produced from an otherwise problematic waste. Following methods used for sugarcane ethanol, treated vinasse could then be returned to the field with a much reduced BOD, yet retaining the nutrients (including N, P and K) that were present in the harvested biomass [47]. Continuous fermentation to methane would in theory be more efficient and require smaller fermentors, but has to operate without contamination by unwanted microbes. However, progress has been made in optimizing semicontinuous fermentation of *Agave* at a pilot scale [48].

Market Challenges/Barriers to Commercialization

A major obstacle to large-scale production of *Agave* spp. for bioenergy is the capital cost of manual labor to manage and harvest the crop [23]. The harvesting of the plants requires a practiced hand to wield a "coa de jima," a sickle-like instrument, cutting individual leaves. Harvesting of offsets, weeding, and pest control also incur substantial manual labor costs. Together with other production costs, the intensive labor requirements amount to a greater cost (per unit volume) to produce ethanol from *Agave* feedstock in Mexico than that of corn ethanol in the USA or sugarcane ethanol in Brazil [23]. For fiber *Agave* crops, a few leaves are removed from each

plant, typically on a 3-month cycle to provide a continuous supply of material for processing [5]. Again, this has been dependent on manual labor, which could benefit rural economies of the poorest potential producer countries. However, elsewhere this is likely to be economically inefficient. As noted above, both mechanical harvesters and mechanical aids to manual harvesting could be developed [21, 22].

Another challenge for widespread adoption of *Agave* for bioenergy is the establishment time. *Agave* plants take at least 5 years to mature. In the case of those grown for fiber, harvesting of the leaves may occur annually (after ~3 years establishment) for up to 15 years before replanting is required. In the case of *Agave* that would be harvested for easily fermented sugars, however, the entire plants are harvested and the field must be replanted following the harvest cycle of 5–7 years.

Opportunities

Despite the novelty and challenges associated with the agricultural production of *Agave* for bioenergy, this genus has many traits desirable for feedstock and there is a rare, perhaps unique, land opportunity associated with *Agave*. With 18 % of the global land surface in semiarid land that does not intersect with prime agricultural lands, and recent abandonment of agriculture for sisal production, there is an opportunity to develop *Agave* feedstocks with less controversy than was experienced for current ethanol feedstocks. Indeed, not only could *Agave* provide biofuel if placed on semiarid abandoned land, without conflict with current food supply, but it could aid in reversing the human-induced degradation of much of this land area, by adding organic matter and stabilizing soil surfaces.

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Chapter 16
100 Years of Breeding Guayule

Terry A. Coffelt, Dennis T. Ray, and David A. Dierig

Abstract Guayule has been known for 100 years as a potential source of natural rubber. Breeding efforts have been sporadic limiting progress in guayule breeding compared to other crops. Even though the genetic base appears to be rather narrow, it has not hindered guayule breeding programs. The most extensively employed breeding approach has been single-plant selections. The primary objective for guayule breeding programs has been increased rubber yield. Limited studies utilizing biotechnology, chemical, molecular, or other new methods of improvement have been conducted in guayule. Most have involved trying to understand the rubber synthesis pathway or to modify the rubber biosynthesis pathway through genetic engineering. While these previous attempts to increase rubber yields have met with little or limited success, the studies have shown that guayule can be successfully transformed. It might be more effective in the short term to use biotechnology to insert genes for other potentially useful traits such as herbicide tolerance and insect resistance. For future progress in guayule breeding to be made, much work remains to be done.

Keywords Guayule • Breeding • Natural rubber • Germplasm • Genetic resources

Introduction

Guayule, *Parthenium argentatum*, (Gray) in the Compositae family has been known for 100 years as a potential source of natural rubber, essentially identical to that from the tropical rubber tree, *Hevea brasiliensis* [1, 2]. The rubber in guayule is located principally in the cortical parenchyma cells of the shrubs, with two-thirds or more in the stem and branches and the remainder in the roots [3]. The use of

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guayule rubber by native populations to make balls for games was first reported by the Spanish in the early 1500s [1]. Use of guayule rubber as a commercial source began in the late 1800s, when it was "rediscovered" by a Mexican Boundary Survey party.

In the last 100 years, breeding efforts for guayule have been sporadic and largely correspond to four major commercialization efforts in North America [4, 5]. These efforts have all centered on the production of guayule as an alternative source of natural rubber. The limited and sporadic nature of these breeding efforts has limited the progress in guayule breeding compared to other crops.

The initial major commercialization attempt started in the early 1900s with harvesting wild guayule stands in Mexico due to the high price of imported rubber from the Amazon region [6]. Production during this effort accounted for up to 24 % of the total rubber imported into the United States by 1910 [6] with 20 extraction plants either operational or under construction in Mexico. Harvesting of wild stands in Mexico came to a halt in 1912 because of the Mexican Revolution. The effort was then moved across the border with plantings in Arizona and California in the United States, [4]. This effort came to a halt in 1929 as a result of the Great Depression [4]. Breeding efforts during this attempt involved mainly the collection and selection of plants and their seed from wild stands.

The second major effort to utilize guayule as a source for natural rubber was the Emergency Rubber Project of World War II. Natural rubber production had moved almost exclusively to large plantations of the Brazilian rubber tree grown in Southeast Asia, and these sources were cut off at the beginning of the war [4]. This second effort was very successful, generating the bulk of our knowledge about the basic biology of guayule, and developed the germplasm upon which the current breeding programs are based [5]. The effort ended with the end of the war, the return of availability of natural rubber from Southeast Asia, and the development of synthetic rubber.

The third major effort to commercialize guayule started in the late 1970s because of the quadrupling of crude oil prices [5]. The fear was if the oil supply could be manipulated, then there might again be a shortage of natural rubber due to either natural disaster or political unrest in Southeast Asia. Two laws were enacted by the US Congress in response to this fear – the Native Latex Commercialization and Economic Development Act of 1978 and the Critical Agricultural Materials Act of 1984 (Laws 95–592 & 98–284). Again, a tremendous amount of work was accomplished, resulting in significant yield increases and the refinement of cultural practices to fit modern mechanized agriculture [4, 5, 7, 8]. This third effort again showed that guayule could be planted, cultivated, harvested, and processed as a source of natural rubber. However, as the political climate changed, this effort was also terminated.

The future for guayule appeared bleak until the report of latex allergy to *Hevea* rubber products in the general population [9]. The development of *Hevea* latex allergy made the development of an alternative, safe source of natural rubber imperative. Guayule proved to be a source of non-allergenic latex for those with latex allergy [10]. Guayule latex was found to contain many fewer proteins than

Hevea latex and in much lower quantities [11–13]. The need for an alternative source of natural rubber latex led to the fourth and current commercialization effort.

Commercialization of non-allergenic guayule latex came closer to reality when Yulex Corporation (www.Yulex.com) was granted the exclusive license to US Patent No. 558094 [11] and to US Patent No. 5717050 [12] on guayule latex processing and products, respectively. Yulex has established a business organization, developed a financial base, increased seed of promising lines, built a latex extraction processing plant, and is in the process of planting large acreages to support the industry. Additionally, another new US company, PanAridus (www.PanAridus.com), has begun a guayule breeding effort to develop improved germplasm and production practices with the hiring of a full-time plant breeder. More recently in 2012, Bridgestone Americas (www.bridgestone.com) has hired a plant breeder and an agronomist to develop new germplasm and production practices for using guayule as a source of natural rubber for tire manufacture and has plans to build a processing facility. All three of the commercial groups described above are putting together plant breeding programs, showing a long-term commitment to guayule. This most recent commercialization effort differs from the previous two in that it is largely being driven by interest in guayule by commercial companies and not by government support. The interest by commercial companies is not only because of the hypoallergenic properties of guayule latex compared to *Hevea* latex but also a predicted increase in demand for natural rubber and an expected decrease in supply of *Hevea* natural rubber, due to plant diseases and political, economic, and social factors in rubber-producing countries [14].

Germplasm Resources

Guayule (*Parthenium argentatum* Gray, Compositae) is a perennial shrub native to the Chihuahuan Desert of North-Central Mexico and the Trans Pecos of Southwest Texas (Stockton Plateau and Big Bend Region) [3]. Native populations are found on semiarid plateaus scattered throughout approximately 300,000 km^2 of rangeland and over a range of climatic conditions [8]. Currently available germplasm can be traced back to the first efforts on guayule breeding.

Parthenium argentatum *Resources*

The first collection of guayule germplasm was made during the first commercialization effort when civil strife and revolution began in northern Mexico. W. B. McCallum employed by the Intercontinental Rubber Company gathered seeds in 1912 from wild stands in order to move his cultural operations into the United States. The germplasm was initially planted at Valley Center, California, and evaluation was subsequently conducted at Continental, Arizona, and Salinas,

California [1]. A selection made by McCallum in Salinas, "593," was the principal germplasm utilized for guayule production in the 1920s, 1930s, and the Emergency Rubber Project [1]. During the Emergency Rubber Project, a major activity was germplasm and cultivar development. The breeding material developed during this time became the basis for the research efforts starting in the 1970s and continuing to the present [1, 5].

There were two main germplasm collection expeditions during the Emergency Rubber Project, the second major commercialization effort. LeRoy Powers, W.B. McCallum, and D.S. Olson collected 66 accessions from 24 locations in Mexico; and Powers and W. Federer collected 368 accessions from 21 locations in Texas. These accessions were then planted and evaluated at Salinas in 1943. In 1948, B.L. Hammond and J. Hinton collected an additional 174 accessions from 93 locations in Mexico [1]. The USDA guayule breeding program at Salinas, California, was terminated in 1959, and 24 germplasm lines, developed by H.M. Tysdal from the Powers, Hammond, and Hinton collections, plus line "593" developed by McCallum, were selected for storage at the USDA National Seed Storage Laboratory (now the National Center for Genetic Resources Preservation) at Fort Collins, Colorado, in 1965. These 25 lines, selected on the basis of their rubber production and plant growth characteristics, were the only ones saved from the hundreds of selections, breeding lines, and accessions stored at Shafter and Salinas, California. These 25 lines plus the line "Bulk Richardson" (from D.D. Rubis; a bulk seed collection from Mexico made by Richardson) became what was commonly called the 26 USDA germplasm lines, from which the breeding programs in the 1970s began [1].

Interestingly, 21 of the 26 USDA lines came from the state of Durango, Mexico. The apparent narrow germplasm base is accentuated by the fact that 15 of the lines descended from the Powers, McCallum, and Olson collection #4265, which was a bulked seed collection from five plants at one location. The original diploid material came from collection #4254, which was also bulked seed from five plants at one location [1].

In 1976, R.C. Rollins made collections from 45 locations in Mexico. In 1977, C.T. Mason collected related *Parthenium* species throughout Mexico, Naqvi and Hanson collected guayule from 50 locations in Mexico (also in 1977), and in 1982 Tipton and Gregg collected seeds from 10 native populations in Texas. An extensive effort was mounted in 1982 by Mexican scientists who collected 3,000 accessions from 310 locations from six states [1]. Unfortunately, it is unclear where most of these accessions are today. This germplasm appears to have been lost during one of the many periods of inactivity when funding for guayule breeding programs was discontinued.

All breeding approaches depend upon the existing genetic variability found in the available germplasm [5]. Even though this genetic base appears to be rather narrow, it has not been a hindrance to guayule breeding programs. This is probably because the highly heterozygous genetic makeup of the plants in the guayule germplasm collection and the facultative nature of apomixis in polyploid guayule continually release new variability with each seed harvest. In fact, with the limited

scale of the present plant improvement programs, this variability is created faster than it can be exploited by breeders [1, 5, 15].

Most guayule germplasm today consists of apomictically reproducing triploid ($3n = 54$) and tetraploid ($4n = 72$) accessions because they received most of the attention in previous breeding programs [1, 5, 15, 16]. When Gore et al. [17] evaluated available germplasm from the GRIN system, they found a natural polyploid series ranging from diploid ($2n = 2x = 36$) to pentaploid ($2n = 5x = 90$), with $4x$ being the predominant ploidy. Interestingly, not all plants sampled from an accession had the same ploidy level (mixed ploidy). This was recently verified by Coffelt et al. [18], when they found similar results with ploidy levels varying from diploid to octoploid in a breeding population derived from diploid crosses and a seed increase nursery of additional plant introductions. Results from these studies [17, 18] confirm the variability available in guayule for breeding improvement. Sexually reproducing, largely self-incompatible diploids ($2n = 36$) have had only limited use in guayule breeding programs.

At present, the USDA-ARS, National Arid Land Plant Genetic Resources Unit in Parlier, California, where guayule is curated has 144 *P. argentatum* accessions and five interspecific hybrids of different *Parthenium* species [5]. Twenty-five of these accessions have PI numbers, with the remainder carrying western regional numbers, but unfortunately as many as 64 accessions may not have viable seed. This is an important problem that has been recognized by the National Plant Germplasm System. A collection trip by T.A. Coffelt, M.A. Foster, and D. Stout (sponsored by the USDA, NPGS, Plant Exchange Office) was made to Texas in 2005 to try to recollect some of the original collections made in Texas. However, guayule could not be found at most of the original collection sites. Where guayule plants were found, little or no seed was present. Other species were found at many of these sites, especially *P. incanum*. It is possible that many of the plants at these original sites were misidentified or that other species have replaced the original guayule populations. One site was found with viable seed of guayule near Bakersfield, Texas. Seed has been collected at this site and added to the USDA collection at Parlier, California. This represents the only wild source of guayule collected since the early collection trip of Tipton and Gregg in 1982. Seeds were also collected and added to the collection from old guayule plantings at the Firestone test track facility near Fort Stockton, Texas. Many of the old USDA lines and materials collected from Mexico were planted at this site from 1940 to 1990, but the plots have not been actively maintained for over 20 years and plot identities have been lost. Seed collected at this site may be the best chance to recover genetic diversity that otherwise may be lost. Studies to determine the best methods for long-term storage of guayule seed to maintain viability have been initiated, but recommendations are not yet available.

Related Species

Sixteen species, in addition to guayule, have been identified in the genus *Parthenium*, but none of these species produce appreciable amounts of rubber. Ploidy level in these species is also variable as seen in guayule. However, they have been identified as potential sources of other useful traits such as disease resistance, increased biomass, cold tolerance, regrowth potential, and drought tolerance [19]. Limited seed of some of the related species is available from the USDA-ARS, National Arid Land Plant Genetic Resources Unit in Parlier, California, through the GRIN system (http://www.ars.grin.gov/npgs/). Reviews of the morphology, anatomy, ultrastructure, and relationships among the species are available [20, 21].

Seed collection of these related species should be a high priority for the near future as native stands of these species are threatened. Seed of three species, *P. incanum, P. hysterophorus, and P. confertum*, were collected by Coffelt, Foster, and Stout during the collection trip in Texas in 2005. Except for *P. incanum*, the stands were very small and seed very limited of these species. Large stands of *P. incanum* were found at several sites. Ploidy analyses indicated that both triploid and tetraploid plants were present at these sites [18].

Breeding Methods

In many instances, the breeding of new and conventional crops is essentially the same [5]. The major differences are that in new crops, (1) the plant breeder starts with a different and frequently unique and exotic germplasm base from which to develop a crop; (2) the breeder is often totally unfamiliar with the species, the germplasm, and potential end products; (3) the traits to be improved frequently have not been identified by researchers, industry, or growers; and (4) there is often a paucity of previous research, including the appropriate technology for evaluating, selecting, and breeding for the commercial production of the products and coproducts sought. New crop breeders must be flexible in their approach to breeding where so much is unknown. The breeder must be innovative and able to change approaches and methodology rapidly to meet the opportunities and constraints as they are encountered.

Guayule yields were first increased by planting larger areas and improving cultivation techniques rather than through breeding. This is standard in many new/minor crops because plant breeding programs take time to initiate and start achieving their desired goals. In addition, many new/minor crops do not have a large industry capable of supporting a plant breeding program. This was true in guayule, as described above, but it also is a more difficult species to work with for some biological reasons: it is a perennial; it is physiologically immature for 1 year (rubber does not produce any appreciable amounts until it has gone through one

winter cycle) before the first harvest at year 2; reproduction is essentially asexual (asexual reproduction by facultative apomixis); and because breeding plots must be maintained for many years to evaluate multiple harvests from regrowth. As described above, at first glance the available germplasm from which selections were made appeared rather narrow, but the first breeding successes were through either mass selection or the selection of individual high-yielding plants [1, 4, 15]. Another limitation to breeding progress is the lack of a nondestructive test to screen for variation in rubber/latex content. Whole plants must be harvested and then ground, subsampled, and analyzed for rubber, latex, and resin content. This severely limits the plant breeder in the number of plant samples to screen from large populations.

The most extensively employed breeding approach in guayule has been single-plant selections from within apomictic polyploid populations. Selection of individual plants is usually the simplest and most rapid method when heritabilities for desired characters are high. If heritabilities are high, increases can be made in a short period of time, but the long-term potential is for only modest gains since new genetic combinations are not being produced. Thus, the degree of success using this method depends first upon the amount of heterogeneity in the population; second, whether or not the differences are genetic; and third, on the number of plants that can be screened [1]. This method increased annual rubber yields from approximately 300–1,000 kg/ha, by selecting for the components of yield described previously, but predominately by selecting simultaneously for increased rubber concentration (%) and dry matter or biomass production [19, 22].

When heritabilities are low, single-plant selection is not as effective as family selection [5]. In family selection, families of progeny, either full sibs or half sibs, are used to evaluate the quality of the parent plants. Thus, parent plants are not selected on their own merits but on those of their progeny. The disadvantage of family selection is that there is a lengthened generation interval. However, because guayule is a perennial plant with almost continuous flowering, many generations of progeny can be obtained from a single plant once it has been selected as a suitable parent.

Mass selection is one of the oldest plant breeding methods, and significant gains can be achieved in a relatively short period of time because only the top yielding plants in a population are selected to become the parents of the next generation. Today, mass selection is used to enhance germplasm and develop cultivars, especially in crops where there are few individuals involved and cross pollination is the major mode of reproduction. Mass selection has been used in sexual diploid populations by Ray et al. [23], in which, after three cycles of selection, a diploid line tolerant to *Verticillium dahlia* was developed.

Mass selection has never been used extensively in polyploid guayule because, to enhance populations using this method, one must be able to screen effectively many plants (hundreds at minimum, to thousands optimally), and cross pollination must be the major mode of reproduction (facultative apomixes will slow the selection process). However, mass selection has been used effectively to develop uniform lines once selections have been made. Mass selection does have potential in

guayule since many of the important characters in guayule appear to be multigenic with low heritability, but as stated above success will be enhanced when a screening procedure is developed that will allow for large numbers of plants to be sampled in a timely manner. Mass selection should be successful since polyploid guayule populations are fairly variable because (1) new genetic combinations are continually being produced, (2) the plants are highly heterozygous due to both self-incompatibility and apomixes, and (3) germplasm lines in which mass selection can be performed have not been selected for uniformity. Unfortunately, many of these germplasm lines are already planted and have been grown for many years, compounding the environmental effects and making selecting for genetic differences more difficult.

Hybridization of apomictic polyploids is a method that has been suggested, but has been used sparingly because of the problems of separating the offspring that arise from sexual reproduction from the apomicts. Plants expressing high levels of sexuality could be identified using the method of Keys et al. [24] and crossed to produce new genetic combinations from which further selections could be made. Seed would be collected from the hybrid plants, planted, and tested for apomictic potential. If the resulting progeny is predominately apomictic, seed from them would be placed in progeny trials and tested for possible release as new lines. If the plants are predominately sexual, they could be backcrossed to enhance certain characteristics, self-pollinated to produce a segregating population from which more selections could be made, or apply standard breeding strategies generally not used in guayule.

Another tool has become available with the successful use of flow cytometry to determine ploidy levels in guayule [17, 18]. Segregating breeding populations can be screened to identify diploid plants for use in crossing programs while at the same time identifying polyploid plants for evaluation in yield trials. This would be especially helpful if large numbers of plants could also be simultaneously screened for rubber and resin content to identify the most promising plants.

Interspecific hybridization has been applied on only a limited scale [5]. None of the other *Parthenium* species produce an appreciable amount of rubber, although they should be considered as potential sources of vigor, increased resin content, increased biomass, disease and insect resistance, regrowth ability after clipping, and cold tolerance. The major disadvantage of interspecific hybrids is that it will take a large number of backcross generations to guayule to increase the rubber content as well as to keep the new desirable trait(s). The University of California-Riverside has released three germplasm lines (Cal-1, Cal-2, and Cal-5) that were developed from interspecific crosses of guayule with three different *Parthenium* species [25, 26]. These three have increased vigor, biomass production, and resistance to Verticillium wilt. AZ-101, a vigorous natural interspecific hybrid, is an open-pollinated cross between guayule and *Parthenium tomentosum* var. *stramonium* [1, 19]. This line was also known as Gila 1 and used extensively during the third commercial effort. The low rubber content made successful rubber extraction more difficult even though the high biomass indicated yields per hectare were higher.

Diploids are potentially useful in guayule breeding because of their sexual (non-apomictic) reproduction and thus the ability to use standard breeding methodologies. While there are problems in using diploids, such as significantly lower yields and increased susceptibility to root diseases, these yield and disease problems have been overcome by using modified recurrent selection schemes to increase yield and mass selection to develop *Verticillium*-tolerant lines [19, 23]. These improved diploid lines can either be crossed to apomictic polyploids or have their chromosome numbers doubled with colchicine. Diploids could also be used to release new genetic combinations by crossing them as the female parent to apomictic polyploids. The resulting apomictic progeny plants might contain new and useful combinations of genes, because meiosis in the microspore mother cells of the apomictic polyploid male plants is normal. Once high-yielding polyploids are identified, they could be crossed onto diploids resulting in populations with enough variation from which to make selections [1, 5, 19].

A potential breeding method that can make the most of limited resources in guayule is the pedigreed natural crossing method [27, 28]. Guayule meets the requirements for use of this method by having natural cross pollination between potential parents (species or diploids or polyploids) and dominant markers to identify hybrids. The advantages of this method are that crossing is not dependent on limited time available for a single scientist or trained assistant to perform the cross; identification, harvesting, and isolation of hybrids can be done by semiskilled workers on land unsuitable for yield trials and other experiments; and it is more economical than making crosses in the greenhouse. The biggest disadvantages are that the pedigree of the hybrids is based on a parental line rather than a single plant and large amounts of land may be needed to identify hybrids. The advantages of this method of producing large numbers of hybrids with little effort should outweigh the disadvantage of individual parent plant identification. The higher outcrossing rate of guayule compared with self-pollinated species should result in a larger number of hybrids being identified with the same amount of land.

Traits of Interest

The primary objective for all guayule breeding programs to date has been to increase rubber yield. Secondary objectives have included improving rubber quality, resin yields, seedling and mature plant vigor, plant architecture, regeneration following harvest by clipping, and tolerance to salinity, drought, diseases, and pests [1, 5, 15, 19]. However, because of the relatively few researchers involved in guayule breeding, the secondary objectives have not received much attention over time.

Selection in guayule has been significantly aided by the description of the components of yield and their relationships to rubber production [29, 30]. In general, rubber content (%) was not positively correlated with rubber yield and in fact was often negatively correlated. Fresh and dry weights, as well as other

characters related to biomass production, were highly and consistently correlated to rubber yield [29, 30]. The characters shown to be the best predictors of rubber content were plant fresh and dry weight and percent dry weight and plant volume, and the best predictive model for rubber yield includes plant height and width and volume and dry weight [29].

Ray et al. [31] tested the relatedness of apomictic parents and their open-pollinated, half-sib progeny families for eight components of yield. Heritability estimates were made by measuring the components of yield in both the parents and progeny. The parent plants were all open-pollinated progeny of a single-plant selection made by D.D. Rubis (University of Arizona), and measurements were made when the parent plants were 3 years old and the progeny plants, 2 years old. For rubber yield, rubber content, resin content, fresh weight, dry weight, percent dry weight, height, and width, none of the parent-progeny regressions were significantly different from zero. For all characters, a large range of phenotypic variation was observed, and the range and standard deviation of the parents were greater than among the progeny. This was probably due to the compounding of environmental effects (the parent plants were a year older than the progeny plants) rather than a difference in genetic variability [32]. Linear correlations were performed to study the relationship between rubber yield and the other seven characters, and fresh and dry weights were highly and positively correlated with rubber yield in all populations. Thompson et al. [30] found significant correlations between rubber content and resin content that were higher than correlations of any other character with rubber content. This high correlation means that breeders should be able to create new lines that are higher in both rubber and resin than older lines. Because both rubber and resin are important characters in determining the value of guayule end products, breeding for simultaneous increases in these traits is important to insure successful commercialization. Evidence that this is possible is found in the release of six new germplasm lines that are higher in rubber and resin the older USDA lines [22].

Biomass appears to be the best predictor of rubber yield (rubber yield = plant biomass × rubber concentration). Thus, plant growth or biomass production can be used as a primary selection index for rubber yield. However, selection for large plant size may be disadvantageous because larger plants may result in mechanical harvesting problems, increased transportation and handling costs, and reduced efficiency of rubber extraction in the processing plant. These are all significant economic factors in the production of rubber from guayule. For this reason, selection of plants with higher rubber concentration in concert with adequate biomass production must receive primary attention. Such selection is difficult because there is often a negative correlation between rubber concentration and biomass [30].

Yield trials have been used successfully to evaluate guayule germplasm lines under various environmental conditions [19, 33]. However, this valuable tool has not been consistently available to breeders due to a lack of continuous funding. More consistent funding is needed to carry these trials to completion and initiate

new ones as new germplasm becomes available. Now that there are significant breeding efforts by industry, these trials should again be implemented.

Another important aspect of yield trials is their use in estimating genotype, environment, and genotype x environment interactions. Coffelt et al. [34] found that location, line, and plant age effects were significant and the interactions not significant for all traits measured in a study at two locations with two plant ages. Environment accounted for over 50 % of the variability observed in all traits, followed by plant age (16 %), and line (10 %). These results point to the tremendous impact that environment has on guayule plant growth, biomass, and latex content. Coffelt et al. [34] could not determine from these tests whether temperature, soil type, moisture, fertility, or a combination of these or other environmental factors were responsible for this response. Some of the nonsignificant interactions may have been significant if a larger or wider germplasm base could have been evaluated. Dierig et al. [32] also observed significant environmental effects even within a single field. Additional studies are needed to determine the environmental factor (s) responsible for the large environmental response observed in these studies.

It is important to breeders that the genotype x environment interaction is not significant since this means selection for superior lines can be done at one location. The superior lines should be superior at other locations where guayule is grown. Antidotal evidence supports this conclusion, since the AZ lines [22] tested by Coffelt et al. [34] have been observed to give similar results when evaluated under diverse environments such as Spain, Australia, South Africa, and China [5].

Progress in selection for rubber/latex traits has been hampered because of the difficulty in determining rubber and latex yield in single plants. The analyses for rubber and latex contents are labor intensive, time consuming, and expensive, greatly limiting the number of samples that can be processed. The amount of leaves, the moisture content of the shrub, and deterioration of the latex during processing all can interfere and must be considered in the analysis of rubber and especially latex [35]. In addition, morphological traits have not been identified that consistently correlate with rubber or latex content. Improvements in these areas could greatly speed the breeding progress.

Research is needed to establish the relationship between latex and rubber concentrations and yields. If rubber and latex concentrations and/or yields are closely related, then previous relationships established between rubber concentration/yield and the various yield components can be expected to be the same as their relationships with latex concentration/yield. However, if rubber concentration is not closely related to latex concentration, then studies will need to be conducted to establish the relationships between latex concentration and traits such as plant biomass, latex yield, rubber concentration and yield, resin concentration and yield, plant height and width, etc. Recent studies [36, 37] have indicated inconsistent relationships between latex and rubber concentrations. In one study [36], latex concentration and yield varied with storage conditions prior to chipping, whereas rubber concentrations and yield did not. In another study [37] of the effects of plant population and planting dates over several harvest dates, rubber and latex concentrations were similar. These studies suggest that more research defining the

relationships between latex concentration and yield and rubber concentration and yield will need to be done before meaningful breeding programs can be started.

In a recent study, Foster et al. [38] evaluated eight guayule lines for cold tolerance and rubber, resin, and biomass yields over a three-year period (2006–2009). Two lines, 11591 and N6-5, had the least cold damage and hold promise for rubber production on the Texas High Plains. Certain production criteria make the Texas High Plains an ideal guayule production site: the long-term annual rainfall averages 460 mm, irrigation water salinity is less than 1 E.C. and is pumped from only 909 m, and center pivots are available for establishing guayule by direct seeding. This study shows that sufficient variability exists within the current germplasm pool to select for cold tolerance to expand the potential production area of guayule.

Guayule still contains many wild characteristics such as indeterminate flowering, seed shattering, natural seed dormancy, and both sexual and asexual reproduction occurring in the same plant, which are desirable to change to facilitate commercialization [5, 39]. To overcome these undesirable characteristics, long-term well-supported breeding efforts will be needed. Hopefully the renewed interest in guayule as a commercial crop will lead to these efforts.

Biotechnology

Limited studies utilizing biotechnology, chemical, molecular, or other new methods of improvement have been conducted in guayule. Most have involved trying to understand the rubber synthesis pathway or to modify the rubber biosynthesis pathway through genetic engineering in guayule. The first chemical studies with isozymes [40] involved the development of isozyme markers to identify genetic differences among diploids, but they are not in use in the current breeding programs since the emphasis is currently on developing polyploid lines.

Rubber synthesis in guayule is temperature dependent, with highest accumulation in the cold winter months [41, 42]. It is important to dissect the effect of cold temperature on this biosynthetic pathway to understand the regulation of rubber biosynthesis in guayule is critical to realize the goal of improving guayule as a domestic rubber crop, by means of breeding or genetic engineering, to achieve high yields of natural rubber.

Ponciano et al. [43] utilized an expressed sequence tag (EST) collection to analyze of the transcriptome of cold-acclimated guayule to search for genes involved in rubber biosynthesis, including the elusive member(s) of the rubber transferase enzyme complex. They found that cold-acclimated, rubber-producing guayule tissue is enriched with allene oxide synthase (AOS) transcript, but not much by other transcripts encoding proteins believed to be associated with rubber particles (e.g., cis-prenyltransferase (CPT) and small rubber particle protein (SRPP)) or the isoprenoid pathway enzymes that make the precursors for rubber biosynthesis (MEV enzymes and farnesyl pyrophosphate synthase (FPPS)).

Moreover, seasonal expression analysis of known and putative rubber biosynthesis-related genes from field-grown guayule shrubs found no cold induction, except perhaps for CPT in which case a sudden increase in ambient temperature at night 10 days before harvest was associated with the highest expression level. Additionally, no positive correlation of expression levels for all genes with rubber transferase activity was found. Ponciano et al. [43] concluded that either [1] gene expression is not controlling the enzymatic activity of the rubber transferase complex, but instead posttranslational modifications are the point of control, or [2] proteins encoding the genes analyzed are not those regulating rubber biosynthesis, thus the critical member(s) of the rubber transferase complex are yet to be identified.

Two studies have been conducted to evaluate the performance of genetically engineered lines. In the first study, Veatch et al. [44] overexpressed allylic pyrophosphate initiators from the isoprenoid pathway, including farnesyl pyrophosphate (FPP) in guayule. They found natural rubber production was not significantly altered, but there was enhanced terpenoid resin production in the lines. Only empty vector controls were used in this study.

In a later study, Dong et al. [45] evaluated transgenic lines involving the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). This study included two types of controls not included in the previous study by Veatch et al. [44], first was a tissue culture control and the second was a non-transformed control derived from seed in addition to the empty vector control. HMGR catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate, a precursor of IPP. This enzyme is considered a key regulatory enzyme of MEV carbon flux in mammals and in microbial systems [46] and possibly in plants as strategic to increased isoprenoid production.

Dong et al. [45] found insertion of the HMGR gene into guayule produced a higher rubber content phenotype in tissue culture for one line of modified plants and produced a dwarf phenotype in the field for that same line. Three other lines did not yield differentiable phenotypes. In order to resolve the discrepancies, gene expression was measured by quantitative real-time PCR analysis for both tissue culture and field-grown plant tissues. Results confirm higher expression of the HGMR gene in modified plants than in control plants. The relative differences in Ct (cycle threshold) values for all tissue culture plants were lower than that of the empty vector control or a non-transformed control in all cases. The reduction in Ct value was even more pronounced for field-grown plants versus the respective controls. However, in no case did the expression vary significantly between lines for which phenotype differences were observed. Field evaluation of plants confirmed transgenic expression but did not validate metabolite accumulation, probably due to the overwhelming influence of the environment over many months in the temperature extremes typically experienced in Arizona. Survival during regrowth of the transformed lines was significantly improved for HMGR overexpressing plants, suggesting enhanced carbon flux to important secondary isoprenoid metabolites, such as growth phytohormones.

While these previous attempts to increase rubber yields have met with little or limited success, the studies have shown that guayule can be successfully transformed. It might be more effective in the short term to use biotechnology to insert genes for other potentially useful traits such as herbicide tolerance, insect resistance, and leaf senescence. Herbicide tolerance would be helpful during the establishment phase of guayule since growth is slow during this period and weed competition is high. Once established guayule has good tolerance to insect pests, but during early stages of establishment and regrowth following harvest, the new tissue is very susceptible to insect pests. These two traits will be even more important to have if guayule production changes from plant establishment by transplanting to a direct seeding system.

Current studies are underway at USDA-ARS to assemble a draft genome sequence of the heterozygous, diploid guayule accession, PI 478663. Preliminary results have indicated an estimated haploid nuclear genome size of ~1,100 Mb [18]. Completion of this study should result in the development of markers that can be used to enhance guayule germplasm.

Summary

Guayule has a long history of use as a source of natural rubber. Although similar in quality to natural rubber from *Hevea brasiliensis*, guayule rubber was not competitive economically until the occurrence of latex allergy in the general population. Continued pressure on worldwide *Hevea* rubber supplies has contributed to renewed interest in the use of guayule rubber in tire applications.

The available germplasm upon first glance appears to be rather narrow, but because of the facultative nature of apomictic reproduction in guayule, genetic variability is continually being released. Three collection trips, one in the early 1900s and two in the early 1940s, account for most of the germplasm used in current breeding programs and what is available in the National Plant Germplasm System today. A more recent collection trip in Texas found guayule was no longer growing at many of the older sites. Collection of guayule germplasm from its natural habitat in Mexico and the United States needs to be a top priority before it is all lost. In conjunction with collection, research needs to be done on properly storing seed for the long term. Much of the previously collected seed is no longer available because it has lost viability.

The major differences in breeding guayule and other new crops compared to traditional crops are that (1) the plant breeder starts with a different and frequently unique and exotic germplasm base from which to develop a crop; (2) the breeder is often totally unfamiliar with the species, the germplasm, and the potential end products; (3) the traits to be improved frequently have not been identified by researchers, industry, and/or growers; and (4) there is often a paucity of previous research, including the appropriate technology for evaluating, selecting, and breeding of the products and coproducts sought. New crop breeders must be flexible in

their approach to breeding where so much is unknown. The breeder must be innovative and able to change approaches and methodology rapidly to meet the opportunities and constraints as they are encountered. Breeding guayule is difficult because of several factors such as its perennial growth, need to overwinter to initiate rubber biosynthesis, facultative apomictic reproduction system, and necessity for evaluating multiple harvests. In spite of these difficulties, there have been successes through guayule plant breeding resulting in significant increases in yield per area. Rubber and resin yields have been increased by 300 % in some lines.

These increases have been accomplished mainly through selection of high-yielding individual plants but also through mass selection. Other breeding methods such as pedigreed natural selection, interspecific and intraspecific hybridization, and family selection can also be used in breeding guayule. Utilizing these methods requires a long-term commitment for the program to be successful. Genetically modifying guayule by transgenesis is another tool that might be used for improving guayule; however, initial experiments using this method have not proved successful as of yet.

Guayule still contains many wild characteristics such as indeterminate flowering, seed shattering, seed dormancy, and both sexual and asexual reproduction occurring in the same plant, which will be desirable to change to facilitate commercialization. To overcome these undesirable characteristics, long-term well-supported breeding efforts will be needed. Hopefully, the renewed interest in guayule as a commercial crop will lead to these efforts. The increased interest in commercialization of guayule should also help breeders as industry identifies those morphological and other traits needed to produce the ideal guayule plant for needed for commercialization.

For future progress in guayule breeding to be made, much work remains to be done. The relationship between solid rubber and latex rubber and the factors affecting this relationship need to be identified and understood. The inheritance of important traits needs to be determined, the genes involved identified, and their location mapped to specific chromosomes. The large environmental effects on resin and rubber also need to be determined.

Guayule research and development priorities during the past 100 years have appropriately focused on variety development, agronomic studies, and latex extraction processes. Rubber remains the primary driver in development. Current commercialization efforts are a culmination of the results obtained from previous breeding efforts as well as agronomic studies. The next priority for complete commercialization of guayule is coproduct development for the bagasse and resin.

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Chapter 17
Algae Crops: Coproduction of Algae Biofuels

Gal Hochman, Michael C. Trachtenberg, and David Zilberman

Abstract The chapter discusses potential uses of algae, and the benefits of coproducing algae biofuels and value-added products such as wastewater treatment and fish. Because of the discussion and the data surrounding algae, and the large technical and economic barriers faced by producers of algae biofuels, we predict that the use of algae to produce energy will likely end up being combined with other value-added products. The combination of energy production and other coproducts (e.g., wastewater treatment) can make large-scale algae biofuel production economically viable. Key, however, to algae biofuel coproduction is the ancillary market's ability to consume large volumes.

The chapter also discusses aquaponics systems, which use algae to filter out water pollution and then recirculate the cleansed water back to the aquaculture production system. This part of the chapter is used to show that the social cost of coproducing algae for biofuel, while employing an aquaponics system is different than the private costs. Thus, an efficient outcome is achieved using public policy—e.g., subsidies, making algae for biofuel production more economical.

Keywords Algae • Microalgae • Macroalgae • Biofuels • Bio-products • Biochemicals • Aquaculture • Aquaponics • Wastewater treatment • Production externality

Introduction

Algae are produced with technology used to farm aquatic organisms such as fish and aquatic plants. It involves the cultivation of freshwater and saltwater populations under controlled conditions. In principle, this technology can eliminate land constraints—a key concern with terrestrial plants used for energy production.

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However, the reality is that the technology is still developing and that poor management introduces many environmental concerns [1].

The industry, in its search for best management practices, has developed approaches that optimize efficiency and create diversification. Conservation, not dilution, is a solution concept much discussed [2]. Best management practices combine aquaculture and hydroponics to integrate fed aquaculture (e.g., fish) with inorganic and organic extractive aquaculture (e.g., seaweed); thus, the waste of one system is used as a resource by the other. This type of production system, termed "aquaponics," results in a sustainable system with very low-input use, especially for water. It is a closed system that, in principle, may produce algae, which, in addition to cleaning the water, can be harvested, sold, and used as an input in production of industrial products at large scales.

Such sustainable systems suggest great potential. The use of algae to capture greenhouse gases has been known for many years [3], as is its potential to sequester carbon and achieve higher energy productivity than land-based crops. However, the discussion and the data surrounding algae, and the large technical and economic barriers faced by producers of algae biofuels, suggest that the use of algae to produce energy will likely end up being combined with other value-added products [4]. The combination of energy production and other coproducts (e.g., wastewater treatment) can make large-scale algae biofuel production economically viable. Key, however, to algae biofuel coproduction is the ancillary market's ability to consume large volumes.

The Economics of Coproduction

The literature discusses the possibilities of coproducing algae biofuel and wastewater [4], as well as other high-value algae products. Lundquist et al. [4] argued that limited demand for many of the high-value products could restrict the benefits of these production processes. They argue that large-scale solutions are needed and conclude that such coproduction systems should be limited to wastewater treatment and algae biofuels. However, aquaponics might offer an economically viable alternative to coproduction of wastewater treatment and algae. Future research will need to show how viable this alternative may be and what are the consequences of algae being the secondary product—the primary being the fish.

Aquaculture production systems result in water pollution. Because aquaculture production relies on artificial feed to grow fish, it faces the quandary of increasing production at the expense of increasing pollution from farm effluent. Because fish do not consume all artificial food, some food reaches the bottom where it is decomposed by microorganisms. These residuals can alter the natural food structure and significantly impact the local environment. Further, fish excretion and fecal wastes combine with nutrients released from the breakdown of excess feed and may raise nutrient levels well above normal to result in anoxic conditions. While farmers can use chemicals to clean the water, the alternative, a sustainable aquaponics

system, can reduce the use of chemicals and bacteria and decrease the energy that would otherwise be needed.

The aquaponics system uses algae to filter out water pollution and then recirculates the cleaned water back to the aquaculture production system. The objective is to create sustainable aquatic production systems, where not only does the aquaculture system produce a product (e.g., fish) but also produces algae; that is, the algae are not only used to filter the water and consume the nutrients but are also harvested and sold to biofuel producers (the economic model abstracts from other options, some of which might be more lucrative than oil production using algae). The idea is to combine aquaculture and hydroponics, such that algae consume the effluents, which accumulate in the water and increase toxicity for the fish, with the cleansed water that has been recirculated back to the aquaculture system. This is a closed system that needs to achieve a delicate balance among various physical and biological factors. Future research will need to show if such systems are sustainable, if they can be scaled up, and if income of coproduction is lucrative. Future research will also need to set up pilot experiments and small-scale operations and use the data collected to evaluate and assess these methods.

Using a stylized model to illustrate the potential benefit of coproduction of algae biofuels, we show how socially optimal policy may impact decisions. Assume an aquaponics production system that is composed of many farmers who want to farm *Tilapia* fish in open ponds. To address the effluents that accumulate in the water, the farmers may consider using a natural filter. They could potentially use algae, which will consume the nutrients in the water—the fish waste will feed the algae. As the algae grow, the farmers could harvest the algae and sell the algae to biorefineries that will extract oil (lipids) from algae biomass and use it to produce biofuels. For simplicity and without loss of generality, assume fish and algae are harvested in batches and the growing period of the two is the same (assuming continuous harvesting does not impact the results). Also, algae would not be used to feed the fish, although such an alternative should be compared with one where the algae are harvested and sold to biorefineries.

The proposed system produces two income streams: income from selling the fish (R_F) and income from harvesting the algae and selling it to biorefineries (R_B). The former is a function of fish biomass (fish weight times number of fish harvested) and price (which may depend on fish age and number of fish sold). The latter is a function of volume of algae harvested, amount of oil (lipids) extracted from algae, and the price. The farmer is also concerned with the production costs (C_P), which are a function of quantity and price of feed, labor, water, electricity, as well as other fixed and variable costs. Farmer i profit function is

$$\pi_i = R_{F,i} + R_{B,i} - C_{P,i}$$

Some farmers may have lower-cost structure than others. The farmer maximizes the profit function (π_i) subject to a time constraint.

The farmer, however, does not internalize the social cost of effluents that accumulate in the water and/or the social benefit from filtering pollutants. The

farmer does not internalize the negative externality from producing fish in open ponds—compared with conventional methods of cleaning the effluents, algae use less chemicals and less electricity, but the aquaponics farmer does not incorporate these costs into the calculations. However, the regulator is aware of the social cost of aquaculture production, and an optimal regime will introduce policy that internalizes these externalities. The policy will impact the farmer's decision process and result in a socially efficient outcome. The regulator sees the social benefit of cleaning the water using algae (R_P) as well as the social cost of growing fish and polluting the water (C_W). The regulator will set policy such that

$$\frac{d(\pi_i + R_P - C_W)}{d(\text{growing period})} = \frac{(\pi_i + R_P - C_W)}{\text{growing period}}$$

where dy/dx denotes the total derivative of y with respect to x.

This suggests that, at the optimum, the marginal effect of the input on the intensive margins $\left(\frac{d(\pi_i + R_P - C_W)}{d(\text{growing period})}\right)$ equals the average benefit from adding a day to the production cycle $\left(\frac{(\pi_i + R_P - C_W)}{\text{growing period}}\right)$. The analysis also suggests that such a system makes algae biofuel production more profitable, because it introduces both a second stream of income as well as government support. In reality, many more considerations impact the optimum social outcome, including cost and efficacy of other methods of wastewater treatment, food, and biofuel production, as well as other alternatives to fossil fuels. However, this work does identify tension between private and social costs of algae production. Future research should try to derive the optimal growing period, for the fish and the algae, and compare it with the various alternatives. When setting the optimal period of growing algae, the farmer's decision will include the optimal amount of oil (lipid) production, which is impacted by many factors including exposure to light and daily average temperature.

While focusing on biofuels, the analysis of the model suggests two streams of revenue: one from selling fish and the other from selling algae to biorefineries. This simple analysis also suggests that the social cost of growing fish is larger than the private cost and that the social benefit of treating the fish effluent using algae is larger than the private benefits. Although the magnitude of these costs and benefits needs to be evaluated using real data, in principle, optimal regulation would increase production cost of fish but lower the cost of cleaning the effluents that accumulate in the water.

Next, we discuss the various types and species of algae, as well as the various products that can be produced using those species. Many high-value products can be produced using algae. However, the volume of the high-value products is usually minimal when compared to the volume of the fuel sector. Wastewater is an exception; aquaponics systems might introduce other exceptions.

Algae

Algae are used to produce numerous products from food to feed to health aids; examples of commercial and industrial use of algae cultivation include production of food ingredients, such as omega-3 fatty acids or natural food colorants and dyes, food, fertilizer, bioplastics, chemical feedstock, pharmaceuticals, and algal fuel, and can also be used as a means of pollution control.

Many of the algae species that are cultivated are microalgae (also known as phytoplankton, microphytes, or planktonic algae), but others are macroalgae. The former are microscopic organisms that form fast-growing populations when supplied with the necessary nutrients, and the latter are commonly known as seaweeds.

In 2008, 15.8 million tonnes of aquatic plants were produced, of which 93.8 % came from aquaculture. Seaweeds dominate the production of aquatic plants in 2008, with 99.6 % by quantity and 99.3 % by value [5]. Commercial production of seaweed occurs mostly in East and Southeast Asia countries [5]. China accounts for 62.8 % of the world's aquaculture production of seaweed by quantity; Indonesia produces 13.7 %; Philippines, 10.6 %; Republic of Korea, 5.9 %; Japan, 2.9 %; and Democratic People's Republic of Korea, 2.8 % [5].

Most of the seaweed species cultured in East Asia are for human consumption, although Japanese kelp is also used as a raw material for the extraction of iodine and alginates. Seaweed farming in Southeast Asia mainly produces raw material for carrageenan extraction [5]. Chile is the most important seaweed-culturing country outside Asia, producing 21,700 t in 2008, and Africa harvested 14,700 t of farmed seaweed during the same year. In contrast, production of algae culture using freshwater was 68,400 t in 2008—where production of *Spirulina* from China contributed 62,300 t, and Chile contributed 6,000 t [5].

The culture of the freshwater alga *Haematococcus pluvialis* is developed in a few countries (e.g., Chile, China, India, Japan, and the USA) for the extraction of *astaxanthin*, a natural pigment and strong antioxidant used in numerous fields including aquaculture feeds. The lipid composition and food quality of freshwater alga cladoceran zooplankters was investigated in [6]. The production and breeding of lipid-rich species of freshwater algae for biofuel production is at its initial stages and is the latest development in freshwater algae culture [5]. Members of the *Scenedesmus genus* have been identified as potential oil-producing species, with both rapid growth and relatively high lipid content [7, 8]. Widjaja et al. [9] measured the effect of lipid production from freshwater microalgae *Chlorella vulgaris* based on the effect of CO_2 concentration, nitrogen depletion, harvesting time, and extraction. Lipids, when fermented, yielded 65 % cellular lipid and 100 g/L biomass. Rodolfi et al. [7] also identified a marine strain that responded to nutrient replete conditions: the *Nannochloropsis sp. F&M-M24*, which in outdoor culture had lipid content varied between 28 and 32 % and lipid productivity between 81 (end of the summer) and 117 (midsummer) mg/L/day.

The American Biomass Program designed several types of large-scale cultivation systems and tested them [10]. Furthermore, a recent study has investigated the

potential of coproducing offshore wind energy in conjunction with the growth of algae [11]. That study expanded upon work done by Buck and Buchholz [12], Chynoweth [10], and Pérez [13].

Macroalgae

Macroalgae (seaweed) are multicellular plants growing in either salt or brackish water. These plants are classified based on their pigmentation:

1. Brown seaweed (Phaeophyceae)
2. Red seaweed (Rhodophyceae)
3. Green seaweed (Chlorophyceae)

These types of seaweed are mainly used for the production of food and for the extraction of hydrocolloids (e.g., agar, alginates, and carageenans) which are used in the pharmaceutical and cosmetic industries. There are several species that are suited for large-scale cultivation. These species require nutrients, salinity, temperature, light depth, and currents [14]. Seaweed is used in production of food, feed, chemicals, cosmetics, and pharmaceutical products, as well as wastewater treatment.

Microalgae

Microalgae are microscopic photosynthetic organisms found in both freshwater and saltwater. In general, these plants are very efficient at converting solar energy into biomass. The most commonly used microalgae are Cyanophyceae (blue-green algae), Chlorophyceae (green algae), Bacillariophyceae (which includes diatoms), and Chrysophyceae (which includes golden algae) [14]. The main species of microalgae used in commercial production include *Isochrysis*, *Chaetoceros*, *Chlorella*, *Arthrospira* (Spirulina), and *Dunaliella* [15]. Currently, the size of the microalgae production is less than 10,000 t a year.

Microalgae are used for food and feed in aquaculture. The therapeutic supplements of microalgae include β-carotene, astaxanthin, polyunsaturated fatty acid such as DHA and EPA, and polysaccharides such as β-glucan [14, 16, 17]. The use of microalgae for the generation of bioenergy, or the combined applications for biofuels production and CO_2-mitigation, such that CO_2 is captured and stored, has been extensively researched [18–29].

Microalgae can be produced using photobioreactors. This technology uses different types of tanks, tubes, bags, or other closed systems in which algae can be cultivated [4]. There is a vast literature that reviews these alternatives (e.g., [30, 31]). Other technologies suggested in the literature, apart from shallow ponds, include growing algae in conventional fermenters instead of photobioreactors

[32]. Instead of light and photosynthesis, heterotrophic algae use carbon sources [33].

The traditional alternative employs open pond systems. The open pond system uses a shallow pond (~1 m deep) in which algae are cultivated. Nutrients are provided through runoff water or by channeling the water from sewage/water treatment plants [34]. Many studies and several commercial plants have demonstrated the profitability of sewage and wastewater treatment [35, 36]. Oxygen production by microalgae for waste oxidation by bacteria in ponds is one example. Others include disinfection, nutrients, and the removal of heavy metals and organic toxins. To this end, Oswald estimates that the saving associated with use of algae instead of electricity for oxygen production in sewage ponds to be between 3,300 and 14,000 US$ per hectare (assuming energy price of 10 cents per kilowatt an hour).

Algae provide oxygen for the bacterial breakdown of the wastes, which in the absence of algae would be achieved using conventional processes of mechanical aeration. The challenge, however, is to harvest the algal biomass; such techniques are only practical using large-scale ponds. Algae are relatively efficient at capturing and removing nutrients, such as N and P. Thus, when wastewater treatment expanded from just oxidizing the organic matter to removing nutrients, it increased interest in employing algae for this purpose.

There is a lot of potential in combining microalgae with waste management as well as other activities [34, 37]. Furthermore, many of the waste management technologies developed using microalgae are appropriate for algal use for other production activities—the case of the Arava, Israel, and the production of β-carotene are just two examples.

Algae Bioenergy

Algae are used to produce bioenergy—i.e., renewable energy made available from materials derived from biology. The use of algae to produce energy has spurred investment by several sectors, some focusing on biodiesel, while other on green gasoline and drop-in fuels.

Recent Trends in Macroalgae Energy Production

Recently, there is rising investment from petrochemical majors, and governments aimed at using seaweed for ethanol, advanced biofuels, drop-in fuels, biochemical, and biopolymers. A recent study (executive summary is available at http://pdf.pr.com/press-release/pr-266157.pdf) suggests macroalgae has potential to become an energy feedstock. That study details emerging projects in macroalgae (Table 17.1).

Table 17.1 Recent trends in macroalgae investments

Project and partners	Products	Description
South Korea National Energy Ministry	Ethanol	Korea—$275 USD million project over 10 years to produce nearly 400 million gallons a year of ethanol by 2020. The project will create an offshore seaweed forest approximately 86,000 acres in size
City of Venice JV with Port Authority and Electric Power Plant	Algae biofuel for electric power	Italy—$200 million Euro project announced in March 2009 by the city of Venice to capture algae seaweed and generate 40 MW of power from algae biofuel. The project will also cultivate microalgae in closed photobioreactors to generate biomass for power generation
Biomara/Scotland's Ministry of Energy	Algal biofuels	Scotland—$8 million USD from Scotland's Energy Ministry and the EU's INTERREG IVA Programme, and Crown Estate in April 2009 to investigate seaweed and microalgae strains for commercial scale production
Chilean Economic Development Corporation (CORFO) and Bio-Architecture Lab (BAL)	Ethanol	Chile—$7 million USD investment in 2010 in a seaweed-based bioethanol project lead by US-based BAL in collaboration with Chilean oil company ENAP and the Universidad de Los Lagos. Project goal is to produce 165 million liters of ethanol
Philippine National Government, Korean Institute for Industrial Technology	Ethanol and biofuels	Philippines—$5 million from the Philippine government to develop a 250-acre, seaweed-based ethanol plant and aquafarm cluster
Statoil and Bio-Architecture Lab (BAL)	Ethanol and coproducts (lipids, proteins, iodine)	Norway—starting in late 2010, Statoil will fund BAL's R&D and demonstrations projects in Norway. BAL will utilize its process technology which will convert seaweed from Statoil's aquafarming operations into ethanol and coproducts

(continued)

Table 17.1 (continued)

Project and partners	Products	Description
Dupont/BAL (Bio-Architecture Lab)	Biobutanol, sugars for advanced and drop-in fuels	USA—$9 million US-based Advanced Research Projects Administration Energy announced in Spring 2010 to fund a DuPont/BAL macroalgae project aimed at supplying biobutanol to be marketed by Butamax, the BP-DuPont JV

Reprinted from Seaweed: A New Wave of Investment in Macro-Algae. BiofuelsDigest. Oct. 4, 2010. http://www.biofuelsdigest.com/bdigest/2010/10/04/seaweed-a-new-wave-of-investment-in-macro-algae/ Last Accessed on January 22nd, 2013. with permission from Biofuels Digest

Microalgae to Energy

Microalgae are composed of three main components: protein, carbohydrates, and oils (lipids). The oil component of the algae would be the base of any future algae oil industry. The first attempt to grow microalgae for oil occurred in Germany during World War II, where researchers noticed that when producing algae with insufficient nitrogen, the algae accumulate oil to levels reaching 70 % of the dry mass [19]. Since then, researchers have attempted mass production of oil (lipids) using microalgae. Although the topic has been neglected during the 1960s and 1970s, in the 1980s renewed interest by US DOE and the initiation of the Aquatic Species Program in 1980 spurred new research [38].

The algae metabolic mechanism is highly complex and efficient, one to which many researchers devoted their life's work. The research has shown that although the theoretical maximum yield observed in laboratories is about 10 %, the actual or simulated unlimited sunlight exposure results in energy conversion rates of only 1–2 % [4, 39]. The major factor limiting the conversion rate (i.e., the photosynthetic process) is the saturation effect, whereby the conversion rate of the reaction center process is much slower than the plant's ability to capture photon energy [4]. Much research has been devoted to achieve sunlight dilution effect, and many innovative ideas have been investigated [40–43].

It should be noted, however, that even these much lower energy conversion rates of 2–3 % are much higher than most other plants. Using the analysis presented in [4], assume that the maximum total solar energy received in the continental USA is just about 7,500 MJ/m^2 a year. Furthermore, assume that algae biomass contains 40 % oil and that the combined biomass low-value heat is 25.6 MJ/kg. Then, assuming the maximum theoretical yield is 290 mt/ha per year of biomass. This is equivalent to 13,500 gal of oil/ha per year. However, introducing unavoidable losses can bring yield to 1.62 %, which then yields an oil production potential of about 2,200 gal/ha per year. Lundquist et al. noted, further, that if the light saturation/photoinhibition effect could be reduced by half from 75 % to a 37.5 % loss, annual productivity will increase to 5,500 gal/ha a year.

Other limiting factors that impact the growth of algae include the supply of nutrients—carbon being the most essential [4]. Temperature is another factor that may limit algae biofuels production. Optimal biofuel algae production occurs between 20 and 35 °C [4]. The diurnal temperature, not the average, is more important because high temperature at night increases metabolism, consuming some daylight production. Also, cold water temperatures in the early morning slow down production substantially. Other factors that impact algae biofuel production include water quality and water use, the physical mixing of the pond, as well as the biotic environment.

Large technical barriers to algae biofuel production prevent it from becoming economically viable. However, coproduction may tip the economics making algae biofuels profitable. Further, as the conceptual framework suggests, coproduction of algae with other value-added products using aquaponics, especially when there is a large gap between the private and social cost of production, may result in algae biofuels becoming economically profitable.

Discussion and Concluding Remarks: Searching for Synergies

Production of algae biofuels is expensive, at least at present and in the near term. Thus, for algae production to become economically viable, additional income streams are required. One example, presented in [4], was to combine algae production with wastewater treatment. Combining algae biofuels and wastewater treatment produced a net cash flow of $0.21/kWh. Other options discussed in the literature consider coproduction of animal feed with algae biofuel. However, [4] argued that the benefits are at most marginal and it might make more sense economically to produce animal feed without the biofuel component. Another option would be to combine biofuel production with high-value coproducts. One example is *Haematococcus pluvialis* for production of astaxanthin.

The concept guiding aquaponics is the creation of sustainable aquatic production systems. The idea is to combine both aquaculture and hydroponics, such that the effluents that accumulate in the water and increase toxicity for the fish can be transferred to a hydroponic system that filters out the pollution from the aquaculture system. The cleansed water is recirculated back to the aquaculture production system. Algae can be used to treat wastewater and consume the nutrients, and this system can support large-scale fish production. Furthermore, such production processes warrant government support because of the pollution they are preventing. Future research will assess such systems and evaluate their sustainability, as well as measure the income flows of coproducing fish and algae for biofuels. This research will derive a threshold price that will determine if algae should go to feed, biofuels, or both—should it be used within a closed system or sold and used in industrial processes. Such analysis will compare the proposed aquatic system with alternative methods as well as alternative fuels.

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Chapter 18
International Policies on Bioenergy and Biofuels

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Abstract This chapter provides an overview of international biofuel polices and their main impacts on food prices and land use. Global biofuel production has experienced a rapid growth by increasing from almost a zero level in 1970 to 29 billion gallons in 2011; the United States, the European Union, and Brazil account for around 90 % of the global biofuel production. Biofuel policies are widely implemented in most developed and many developing countries. Most commonly used biofuel policy instruments are biofuel mandates and consumption subsidies (tax credit and tax exemptions). These policies determine biofuel prices, depending on which instrument is binding. Biofuels may also have unintended effects on other markets. In particular, interlinkages between biofuel and agricultural productions lead to food price responses and land use adjustments.

Keywords Biofuel policies • Price interlinkages • Food prices • Land use changes

Introduction

Bioenergy is renewable energy derived from biological sources to be used for the production of heat, electricity, or fuel. The main input in bioenergy production is biomass. Biomass covers a wide range of plant sources, including those that are used for fuel directly (e.g., firewood) or processed into biofuels (e.g., corn, soy, sugarcane, sugar beet, rapeseed, or wheat). Main biofuel products include ethanol,

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biodiesel, and biogas. Biofuels belong to the most rapidly growing renewable energy technologies and are the main focus of this chapter [1, 2].[1]

There has been a tremendous increase in the production of transportation fuels derived from biomass (i.e., biofuels) in recent years. World biofuel production increased from almost a zero level in 1970 to 29 billion gallons in 2011 [3]. The biggest players in the world biofuel market are the United States, the European Union, and Brazil, accounting for around 90 % of global biofuel production. These three regions practically dominate the sector and determine the development in world biofuel markets.

The rapid growth of the biofuel industry would not have been possible without government intervention. Biofuel policies are widely implemented in most developed and many developing countries. The International Energy Agency (IEA) [2] estimates that in 2009 the combined government support in Brazil, the European Union, the United States, and other countries totaled $20 billion. Governments use many different policy instruments which from an international trade perspective either do or do not discriminate against international trade. The first category includes consumption subsidies (tax credits or tax exemptions) and mandates, while the second group consists of policies such as import tariffs and quotas, production subsidies (for biofuels and feedstocks), and sustainability standards [4]. However, by far most commonly applied instruments are biofuel mandates and consumption subsidies.

There are several seemingly plausible reasons why biofuels are supported by governments.[2] First, biofuels reduce the dependency on imports of crude oil [1, 6–9]. There are concerns that because of limited oil reserves the cost of oil extraction will increase in the future, leading to high relative oil prices. Furthermore, oil price instability stemming from, for example, regional conflicts and political turmoil in the Middle East and other oil-producing regions, has negative consequences for the world economy. Second, increased production of biofuels is expected to improve the environment by reducing the reliance on conventional sources of fuels, thus contributing to the reduction in global greenhouse gas emissions [1, 6–8]. Finally, biofuel support might reduce the cost of agricultural support programs. The biofuel production stimulates higher agricultural prices, thus leading to higher incomes of farmers and contributing to economic growth in rural areas [1, 8]. This interlinkage between biofuel and agricultural markets could possibly allow governments to partially substitute the farm support with the provision of support to the biofuel industry.

[1] The International Energy Agency (IEA) differentiates between “conventional” and “advanced” biofuels. The distinction is based on the maturity of a technology. Conventional biofuel technologies include well-established technologies that are already producing biofuels on a commercial scale. These biofuels are commonly referred to as first-generation biofuels. Typical biomass used for first-generation biofuels includes sugarcane, sugar beets, corn, wheat, rapeseed, soybean, or palm oil. Advanced biofuel technologies are conversion technologies that are still in the research and development, pilot, or demonstration phase, commonly referred to as second- or third-generation technologies. These biofuels are made from lignocellulosic biomass, woody crops, agricultural residues, or waste [2].

[2] de Gorter et al. [5] provide arguments for why these reasons are not justified in reality.

The expansion of biofuels and biofuel policies has sparked a lively debate and controversy about the contribution of biofuels to various issues related to developments in agricultural markets. As shown in recent studies [1, 10–14], biofuels may have far-reaching side effects on agricultural markets due to price interdependencies between the energy, bioenergy, and agricultural markets. For example, they may directly or indirectly increase food prices [10, 13, 15–17], cause negative environmental impacts [14, 18], or induce indirect land use changes [1, 19, 20].

The objectives of this chapter are to provide an overview of biofuel policies implemented around the world and to discuss the implications of biofuels for biofuel markets, food prices, and direct and indirect land use changes.

The rest of the chapter is organized as follows. In the next section, we provide a short overview of global biofuel production and consumption. Then, we discuss feedstocks for biofuel production. The key biofuel policies are overviewed in the fifth section followed by the discussion of the market effects on these policies. The last section provides some concluding remarks.

Biofuel Production and Consumption

Global biofuel (i.e., ethanol and biodiesel) production has experienced a rapid growth over the last two decades; it reached 29 billion gallons in 2011, a 625 % increase from the quantity produced in 1990 [3]. Global ethanol production first started to increase significantly, relative to earlier years, in the period 1975–1978 as a result of the implementation of the Brazilian ethanol mandate originating in the PROALCOOL program in 1975 and the introduction of the U.S. blender's tax credit in 1978 (Fig. 18.1). The second surge in the global ethanol production started

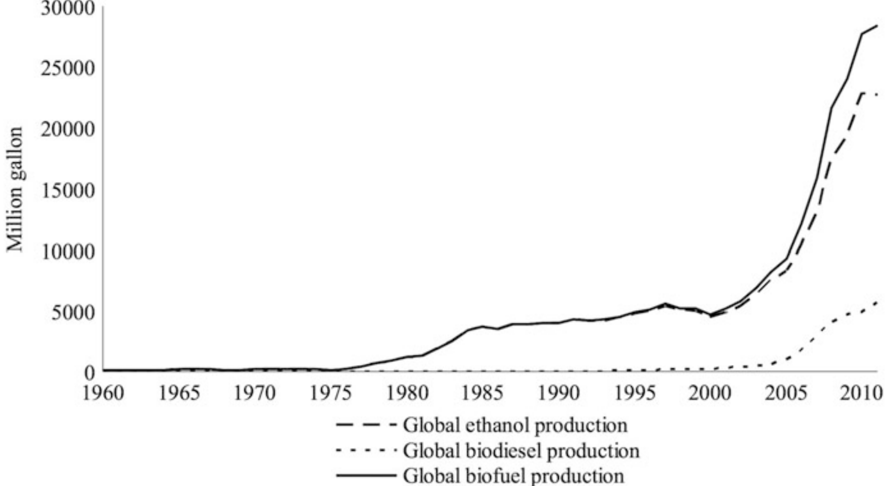

Fig. 18.1 Global biofuel production, 1960–2011 (Based on data from Ref. [3])

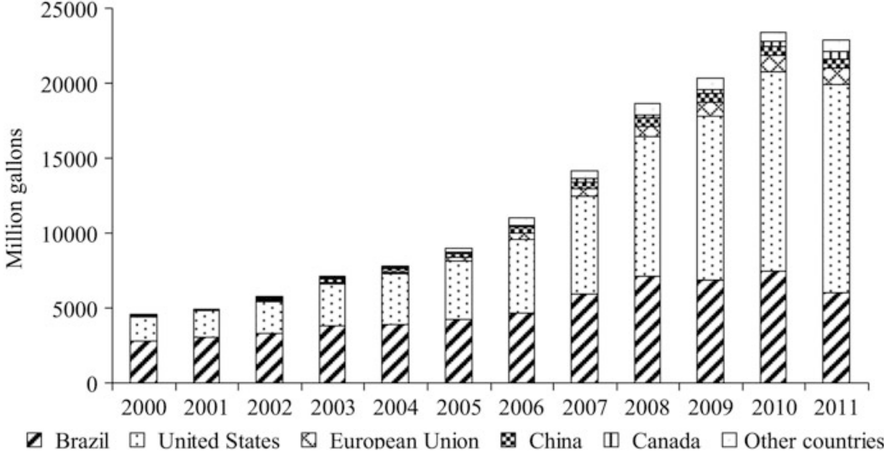

Fig. 18.2 Global ethanol production by country, 2000–2011 (Based on data from Ref. [21])

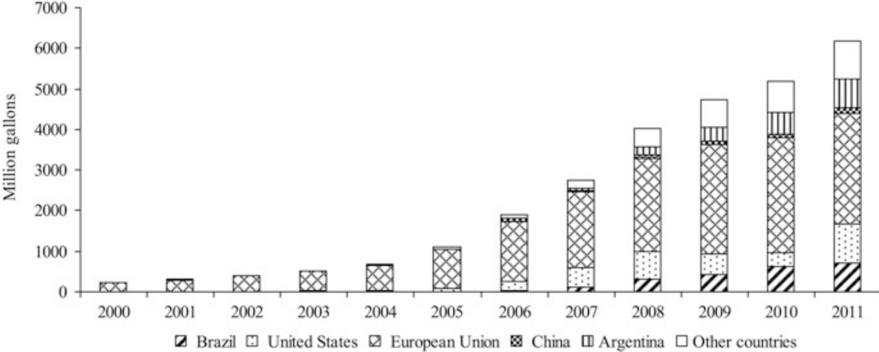

Fig. 18.3 Global biodiesel production by country, 2000–2011 (Based on data from Ref. [21])

in the period 2003–2005 which coincides with the introduction of the U.S. Renewable Fuel Standard Program (first adopted as part of the Energy Policy Act of 2005) and the EU Directive 2003/30 on the promotion of the use of biofuels or other renewable fuels for transport.

Ethanol dominates the global biofuel production; for example, in 2011 its share was 79 % and the rest was biodiesel. The United States and Brazil are the world's largest ethanol producers and consumers. In 2011, the two countries accounted for 87 % of global ethanol production and 74 % of global biofuel production. In contrast, in the same year the world's largest biodiesel producer and consumer, the European Union, produced 44 % of global biodiesel production, which represents 13 % of global biofuel production. Germany and France are the biggest EU biofuel producers, accounting for 48 % (29 % and 19 %, respectively) of the total EU biodiesel production (Figs. 18.2 and 18.3).

Despite the substantial increase in global biofuel production, biofuels' share in global fuel (i.e., biofuel and liquid fossil fuel) consumption is low; worldwide, biofuels represent less than 3% of transportation[3] fuel consumption [2]. The global biofuel consumption is projected to rise to 7 % and 11 % of the world's transportation fuel consumption in 2020 and 2030, respectively [23]. Of course, the share is higher in developed countries where consumption of biofuels is promoted. For example, in 2009 the share of biofuels in total transportation fuel use was 7–8 % in Germany and Spain, 6 % in France, 4 % in Italy, and 3 % in Great Britain [24]. Brazil is an outlier in this cross-country comparison as the volume of biofuels in total transportation fuel use achieved 21 % in 2008 [25].

Biofuel Feedstocks

Currently, ethanol is mostly produced from corn and sugarcane. It can also be produced from wheat, sorghum, sugar beet, or cassava (Table 18.1). These crops are rich in sugar and starch that is fermented into ethanol. Sugarcane is the favorite raw material for ethanol production in Brazil, while corn and cereals are used in the United States, the European Union, and other developed countries with a temperate climate [1, 29–31].

Biodiesel, on the other hand, is mostly produced from oilseed crops like rapeseed, soybeans, sunflower, or palm oil (Table 18.2). Rapeseed is the most popular feedstock for biodiesel production in the European Union and soybean in Brazil and the United States. In some tropical and subtropical countries, palm, and jatropha oils are used for biodiesel production [1].

The second-generation biofuels aim at using lignocellulosic feedstock, including herbaceous lignocellulosic species such as miscanthus, switchgrass, and reed canary grass (perennial crops) and trees such as poplar, willow, and eucalypt (short rotation crops), as well as forestry and agricultural residues. Feedstocks for second-generation biofuels generally produce higher biomass yields than most first-generation feedstocks (sugarcane being an exception). Given their relatively high projected energy conversion efficiency, second-generation feedstocks are projected to have higher overall energy yields (Tables 18.1 and 18.2). However, technologies for second-generation biofuels are still in the research and development stage and are not commercially exploited at a larger scale.

[3] The largest use of ethanol and biodiesel is as a motor fuel and fuel additive. Other uses of ethanol and biodiesel include industrial and residential consumption and represent a small proportion of total production (less than 2 %) [22].

Table 18.1 Yields of biofuels for first-generation feedstocks

Crop	Global/national estimates	Crop yield (t/ha)	Conversion efficiency (l/t)	Biofuel yield (l/ha)
Ethanol crops				
Sugar beet	Global	46.0	110	5,060
Sugarcane	Global	65.0	70	4,550
Sugarcane	Brazil	73.5	75	5,476
Sugarcane	India	60.7	75	4,522
Cassava	Global	12.0	180	2,070
Cassava	Brazil	13.6	137	1,863
Cassava	Nigeria	10.8	137	1,480
Corn	Global	4.9	400	1,960
Corn	United States	9.4	399	3,751
Corn	China	5.0	399	1,995
Rice	Global	4.2	430	1,806
Wheat	Global	2.8	340	952
Sorghum	Global	1.3	380	494
Biodiesel crops				
Oil palm	Malaysia	20.6	230	4,736
Oil palm	Indonesia	17.8	230	4,092
Soybean	United States	2.7	205	552
Soybean	Brazil	2.4	205	491
Rapeseed	European Union	3.1	369	1,140
Jatropha	Global	7.0	250	1,750

Based on data from Refs. [1, 26–28]

Table 18.2 Yields of biofuels for second-generation feedstocks

Feedstock	Current yield (dry t/ha)	Biofuel current yield (l/ha)	Expected yield (dry t/ha)	Biofuel expected yield (l/ha)
Miscanthus	10	1,250–3,000	20	2,500–6,000
Switchgrass	12	1,500–3,600	16	2,000–4,800
Willow	10	1,250–3,000	15	1,875–4,500
Poplar	9	1,125–2,700	13	1,625–3,900

Based on data from Refs. [26, 32]
Note: Yields reported in this table are for test plot scale

Biofuel Policies

There is a plethora of biofuel policies used worldwide. Some of them are used more frequently, such as mandates or consumption subsidies (e.g., tax credits and tax exemptions), and others less so, for example, import quotas. From an international trade perspective, it is important to distinguish between policies that do not discriminate and those that do discriminate against international trade. The former

group includes consumption subsidies (e.g., tax credits or tax exemptions) and mandates, while the latter encompasses production subsidies (for biofuels and feedstocks), import tariffs and quotas, and the zero/one sustainability standards whereby a certain reduction in carbon emissions of biofuels (ethanol or biodiesel) is required relative to fossil fuels (gasoline or diesel) that biofuels are assumed to replace [4]. The choice of particular instrument is a result of comparative advantage in biofuel production, political process, and pressure from interest groups. There is no significant cooperation among countries to harmonize their biofuel policies, although spillover effects due to the learning process among countries cannot be excluded.

Biofuel policies have been shown to be the key driver of biofuel production [11, 12, 16]. This naturally poses a question if there could ever be production of biofuels in the absence of biofuel policies. The answer is positive, but the conditions for that to happen have not historically been met. To illustrate, consider corn ethanol. The ethanol supply curve is given by the difference between the corn supply curve and the non-ethanol demand curve at any corn price. Hence, its intercept – the point representing the minimal ethanol price required for ethanol production to occur – corresponds to the intersection of the corn supply curve with the non-ethanol corn demand curve (i.e., the market price of corn in the absence of ethanol production).

But when no biofuel policy exists, ethanol will only be demanded if the price a consumer pays per mile traveled is the same as for gasoline (ethanol and gasoline are assumed to be perfect substitutes in consumption). This results in a free market ethanol price that is typically much lower than the intercept of the ethanol supply curve; hence, no ethanol production without a government policy (e.g., a high enough blender's tax credit or a mandate) will likely exist.

The most common instruments used to support biofuel consumption/production are biofuel mandates, followed by consumer subsidies (tax credits/tax exemptions), subsidies to feedstock production (e.g., corn production subsidies), and tariffs and quotas. The rest of instruments are implemented sporadically.

The total support worldwide is estimated to be $20 billion in 2009. Most of the support goes to ethanol (more than $13 billion in 2009), and the largest share is carried by the United States ($8.1 billion), followed by the European Union ($7.9 billion), and Brazil ($2.6 billion) [25].

Biofuel Mandates

Biofuel mandates have become a preferred policy mechanism to induce biofuel production and have been introduced in at least 30 countries [33]. The main principle of the mandate is to establish a minimum content of a biofuel relative to a fossil fuel (gasoline, diesel) used. A biofuel mandate is used in two forms: a

consumption (or quantity) mandate (e.g., in the United States, Japan) or a blend mandate (e.g., in the European Union,[4] Brazil, Argentina, Australia, Canada).

The consumption mandate establishes a fixed amount of biofuel (which can vary from year to year) to be blended with a fossil fuel. Because the demand for biofuel is perfectly inelastic in this case, the price of biofuel is, in principle, determined in the feedstock (e.g., corn) market and is thus susceptible only to shocks in that market (unless an oil price change significantly affects the production costs of the biofuel feedstock). Although straightforward in theory, the practical implementation of the quantity mandate is more difficult. For example, it is challenging to justify the assignment of mandatory levels of a biofuel to be blended with a fossil fuel for individual fuel blenders. In practice, the quantity mandates are therefore typically implemented as blend mandates, whereby each fuel blender is required to mix the biofuel and fossil fuel in a certain proportion. This is, for example, how the biofuel mandate is implemented in the United States.

The countries using a biofuel blend mandate implement it in three forms.[5] For example, in the United States, the Environmental Protection Agency (EPA) is responsible for developing and implementing regulations called Renewable Fuel Standard (RFS) to ensure that transportation fuel sold in the United States contains at least a predefined volume of a renewable fuel. The RFS was introduced by the Energy Policy Act of 2005, but the quantitative mandates were later expanded considerably by the Energy Independence and Security Act (EISA) of 2007. For ease of implementation, the EPA annually converts the consumption mandate into its blend equivalent based on a prediction of the annual U.S. fossil fuel consumption. Hence, the U.S. blend mandate is effectively the ratio of the volume of a biofuel and fossil fuel (gasoline or diesel). For instance, the blend equivalent of the U.S. biofuel consumption mandate was set to 7.95 % in 2011 [34] and 9.23 % in 2012 [35].[6] The EPA requires 15.2 billion gallons of renewable fuel to be blended into the domestic fuel supply in 2012. From this amount, 1.0 billion gallons of biomass-based diesel, 2.0 billion gallons of advanced ethanol, and 8.65 million gallons of cellulosic biofuels were blended. The EISA originally envisioned 500 million gallons of cellulosic biofuels for 2012, but the EPA, due to the lack of sufficient commercial capacity for cellulosic biofuel production, has reduced this requirement to 8.65 million gallons [8]. The current RFS also envisions the total amount of biofuel to increase to 36 billion gallons by 2022. Since the mandate is set

[4] The EU mandate is termed a "target."

[5] The differing approaches to implementation of blend mandates mean that, for example, a 10 % volumetric mandate in the United States will have different effects on the market outcome compared to a 10 % energy blend requirement in the European Union. This happens, other things equal, because a lower energy content of biofuels relative to fossil fuels makes an energy blend mandate translate into a higher volumetric blend mandate.

[6] Because under the blend mandate the quantity of biofuel is proportional to consumption of fuel which in turn depends on the oil price, the price of biofuel is determined by the interaction between the fuel and feedstock markets. Contrast this with how the biofuel price is determined under a consumption mandate.

for the use of biofuels, some biofuels can be imported rather than produced domestically.

In Brazil, the blend mandate determines the share of the volume of ethanol in the total volume of fuel (i.e., anhydrous ethanol and gasoline).[7] Since 1975, Brazil has mandated that anhydrous ethanol be blended with all gasoline sold. The Brazilian ethanol mandate can vary between 18 % and 25 %. In October 2011, it was reduced from 25 % down to 20 % because of bad weather adversely affecting sugarcane production (the primal ethanol feedstock in Brazil) and because of rising global prices for sugar due to high sugar demand [36]. Apart from mandating ethanol use, the Brazilian government also requires that 2 % of biodiesel be blended with diesel, and this share is to increase to 5 % in 2013 (and even reach 10 % in 2020).

The way the biofuel mandate is implemented in the European Union is similar to Brazil, with the exception that the quantities of biofuel and total transportation fuel are expressed in energy equivalents. The EU Directive 2009/28/EC set the reference target to 5.75 (energy) percent for the share of biofuels in transport fuel consumption by 2010, and the target was to be increased to reach at least the mandatory 10 (energy) percent by 2020. At least 20 (40) percent of the 2015 (2020) targets were supposed to come from "nonfood and feed-competing" second-generation biofuels or from cars running on green electricity and hydrogen. The EU targets for biofuels are subsumed in the 2009 Energy and Climate Change Package, whose goals are summarized by the "20/20/20 objective": a 20 % greenhouse gas (GHG) emission reduction, a 20 % increase in energy efficiency, and a 20 % share of renewable energy (e.g., solar or wind, in addition to biofuels) in the EU total energy consumption, all by 2020 [37].

To be counted toward the EU targets, biofuels must reduce at least 35 % carbon emissions relative to the fossil fuel they are assumed to replace – a saving to be increased to 50 % and 60 % for existing and new installations, respectively, in 2017. In comparison, the U.S. EPA requires at least 20% carbon emission reductions for corn-based ethanol. The ethanol that did not meet this sustainability standard was not eligible for the blender's tax credit (an ethanol consumption subsidy), but could be counted toward the U.S. mandate.

Worldwide, the magnitude of the blend mandate ranges between 1 % and 25 % of the fuel consumption, depending on the country and time horizon. The 25 % mandate is the maximal share allowed in Brazil. Other countries' mandates are significantly lower, however, especially because of insufficient infrastructure for the E85 gas stations (a fuel blend containing up to 85 % of ethanol) and vehicles. The level of currently enforced mandates in countries other than Brazil thus ranges between 1 % and 7 %.

In general, the mandates are gradually being increased until a predefined level is achieved (but notice the recent proposal for a reduction in the first-generation

[7] There are two types of ethanol used in Brazil: hydrous (contains water) and anhydrous (water-free). Gasoline can only be blended with anhydrous ethanol. The use of hydrous ethanol is not mandated.

biofuel targets in the European Union).[8] Indeed, many countries have set their future biofuel strategy up to 2015 (e.g., Canada), 2020 (e.g., the European Union, Brazil, and the United States), or even 2030 (e.g., Japan). Thus, for instance, Canada increased the biodiesel blend mandate from 5 % to 7 % and is considering increasing it further to at least 10 % by 2015 [38].

The envisaged maintenance and expansion of mandates over one or two decades is an important signal toward the biofuel industry. It reduces industry's uncertainty about future policy changes. It allows biofuel producers to better plan investments in refiners and research and development in new technologies. It is interesting to note that developed countries (e.g., Australia, Canada, the European Union) implement predominantly mandatory blend requirements, whereas some less developed and developing countries (e.g., Fiji, Kenya, Malawi, Nigeria) tend to rely on voluntary blends.

Consumption Subsidies

Biofuels are almost perfect substitutes to fossil fuels. Because the latter are generally highly taxed, especially in oil-importing countries, to support biofuel consumption (and hence production), some countries provide reductions in the fuel tax for biofuels. Depending on its administration, the consumption subsidies take two forms: a blender's tax credit and a tax exemption.[9] The two policies have identical market effects in a closed economy: they raise the market price of biodiesel by the amount of the subsidy [12, 39]. However, with international trade, their effects differ, depending on which country and policy determines the biofuel price [see 40, 41 for details].

The United States was the first country to apply a tax credit by providing a tax reduction to fuel blenders. The initial stimulus came from the 40 cents per gallon federal subsidy established by the Energy Tax Act of 1978. It increased early on to reach 60 cents per gallon with the Tax Reform Act of 1984 but was being gradually adjusted downward since 1990. The federal blender's tax credit was last decreased to 45 cents per gallon as of January 2009 to be finally phased out at the end of December 2011 [8]. Until its expiration on December 31, 2011, biodiesel blenders enjoyed a tax credit of $1 per gallon of biodiesel blended with regular diesel (it was temporarily suspended in 2010 but reenacted retroactively in December 2010). In

[8] The European Union is currently considering introduction of a 5 to 7 percent cap on the amount of first-generation biofuels in the EU's 2020 transportation mix. This would be a reduction from the 10 (energy) percent target discussed earlier.

[9] Technically, with the tax credit blenders pay the full fuel tax on the fuel sold, regardless of the biofuel's share. At the end of the fiscal year, they are subsequently reimbursed for the discount on the biofuel (proportional to the biofuel's share in total fuel volume) provided to the fuel consumers throughout the year. With the tax exemption, the blender directly collects a lower tax on the volume of biofuel in the fuel mixture.

addition to federal tax credits, some U.S. states apply additional tax credits/exemptions. For example, the state level tax credit for corn ethanol averaged $0.048/gal in 2009 [42].

In Brazil and the European Union, member states are allowed to grant tax exemptions for renewable fuels. A tax exemption represents a reduction in the fuel excise tax collected at the pump. The level of the tax exemption varies across EU countries and between biofuels, but it is declining as governments wish to recoup fiscal revenues from fuel taxes that were foregone because of the exemptions. For example, a tax exemption for biodiesel in Germany declined from €0.47 per liter to €0.29 per liter between 2005 and 2009. For Brazil, Kliauga, de Gorter, and Just [41] report the (consumption weighted) average tax exemption of R$ 0.67 per liter which was approximately 2.7 times the U.S. tax credit.

Subsidies to Feedstock Production

Subsidies to feedstock production directly stimulate supply of crops used for production of biofuels. They target biofuel crops, such as corn, soybean/rapeseed, or sugarcane, either specifically contracted for use in biofuel production or regardless of the crop use. Both types of subsidies reduce the cost of production of biofuels, thus expanding their supply and lowering the market prices [16]. For example, the European Union used to provide €45 per hectare to farmers who were producing feedstock used for production of biofuels (energy crops) or to generate heat or power. The set-aside land was eligible for production of feedstock for biofuels or for generation of heat or power. However, the Common Agricultural Policy's (CAP) revision, called "Health Check," abolished the energy crop premium and the set-aside scheme in January 2009.

In Brazil, sugarcane growers were eligible for the Regional Producer Subsidy in the amount of R$ 5.00 per metric tonne of sugarcane up to 10,000 tonnes regardless of the use of sugarcane (ethanol or sugar). Similarly, the subsidies for the U.S. corn production are estimated to reach $4.6 billion in 2011 [43].

The subsidies to biomass production usually represent an integral part of the general support system to agriculture in most countries. Agricultural subsidies are most often not directed specifically toward biofuel crops but are rather applied to all agricultural production and include various policy instruments such as direct income payments to farmers, input subsidies, price support, trade measures and production quotas, or environmental payments.

Agricultural subsidization is in particular prevalent in developed countries. For example, the agricultural support, known as Producer Support Estimate (PSE),[10] calculated by the OECD represented 20 % of the total value of OECD countries'

[10] The Producer Support Estimate is an indicator of the annual monetary value of gross transfers to farmers.

farm production in 2010. It varied from 61 % in Norway to 1 % in New Zealand. In non-OECD countries, the support level tends to be lower. For example, in 2010 the PSE represented 21 %, 17 %, and 4 % of the value of farm production in Russia, China, and Brazil, respectively [44].

The agricultural policies may affect the prices of biofuel crops in both directions, depending on a policy instrument [7]. For example, input subsidies, such as subsidized fertilizers, water, or fuel, encourage production of biomass (feedstock), reduce their production costs, and thus improve profitability of the biofuel industry relative to fuel production from fossils.[11] Trade protectionary measures (e.g., import tariffs) have an opposite impact on biofuels, as they increase domestic biomass prices and reduce profitability of the biofuel production relative to the fossil fuel production.

Import Tariffs

Biofuel trade shows relatively high dynamics, representing 1,630 million gallons at global scale in 2011. Most biofuel trade occurs between Brazil, the United States, the European Union, and Argentina. Brazil is the main net exporter of ethanol, whereas the European Union is the main net importer of ethanol and biodiesel. The United States is a significant exporter of biodiesel, and for ethanol it turned from a net exporter to a net importer in 2010. Argentina has become the main biodiesel exporter in recent years.

Governments often restrict trade by using tariffs and quotas aimed at protecting domestic producers against foreign competition. In the United States, a specific import duty of $0.54 per gallon combined with an out-of-quota ad valorem import tariff of 2.5 % for ethanol used to be in effect until January 1, 2012 (when it expired along with the tax credit) [46]; it was meant to protect domestic ethanol producers by preventing ethanol imports from more efficient ethanol producers, like Brazil, who were also eligible for the tax.

The European Union uses an import tariff on denaturated and undenaturated ethanol imports of €10.20 and €19.20 per hectoliter, respectively, which is an equivalent of 39 % and 63 %, respectively, in ad valorem terms [47]. Although most countries permit blending gasoline with undenaturated ethanol only (such that their domestic market is protected by the higher tariff rate), some do allow blending gasoline with denaturated ethanol, too, implying a lower trade protection level (e.g., the United Kingdom, the Netherlands, Finland, Denmark, the Czech Republic, or Slovakia).

[11] This does not hold in general, however. Under a binding tax credit, the biofuel market price is directly linked to the oil price; hence, it does not respond to any changes in the feedstock market [16, 45]. The incidence of the feedstock subsidies is then to increase the quantity of biofuel produced.

The EU import tariff on biodiesel is 6.5 %, while it is only 3.2 % on vegetable oil for biodiesel production (soybean, sunflower, rapeseed). In response to the U.S. "splash & dash" program, in March 2009 the European Union imposed antidumping and countervailing duties on imports of U.S. biodiesel [40]. The antidumping duty rates range between €68.60 and €198 per tonne (equivalent to approximately €0.09–0.25 per liter). The countervailing duty rates range between €211.20 and €237 per tonne (€0.24–0.27 per liter) [33]. Nevertheless, some European trading partners already benefit from duty-free access for biofuels under the Everything But Arms Initiative, the Cotonou Agreement, the Euro-Med Agreements, and the Generalised System of Preferences Plus [24].

Brazil applies a 20 % import tariff (temporarily suspended in 2010 and 2011) on denaturated and undenaturated ethanol and a 14 % import tariff on biodiesel.

Other Biofuel Support Instruments

Other support instruments targeted at the biofuel sector include grants and loans, price support, research and development subsidies, support for distribution and use, and subsidies targeted on biofuel production. These instruments are less widespread and may have direct and indirect and long-term impacts on the biofuel sector.

Grants and loans are directed toward supporting biofuel industry by providing investment grants to build ethanol refineries and biodiesel production plants. These grants reduce investment costs. By the same token, subsidized loans also reduce the cost of investment and therefore support the growth of the biofuel industry. Some countries created a scheme which allows the investor to reduce its taxable income when biofuel plants are constructed. For example, the Government of Canada has invested C$200 million in the "ecoAGRICULTURE Biofuels Capital Initiative," a program providing loans (repayable contributions) with the aim to encourage producer equity/ownership in biofuel facilities [38].

Price support can be also used to enhance production of biofuels. The government sets a guaranteed price above the market price. Biofuel distributors therefore pay a higher price to the producer of biofuel than they would pay without the price support. For example, the incentive for farmers in Germany to invest in biogas digesters is a guaranteed feed-in tariff for the generated electricity which is considerably higher than that of electricity generated from fossil fuels, natural gas coal, or nuclear sources [48].

Research and development subsidies aim at supporting technological innovation of biofuel production. Often, this assistance is provided for research and development related to biomass conversion into biofuels. However, the focus of these programs is increasingly shifting toward second-generation biofuels as they may have more favorable economic and environmental effects (e.g., related to land use, food price effects, environmental implications) than conventional biofuels. For example, the IEA estimates direct government spending on new biofuel-related

research and development to be more than $1 billion in the Unites States, $430 million in Canada, and $12 million in Australia [25].

Support for distribution and use targets at assisting the distribution or consumption of biofuels. The distribution of biofuels may imply additional costs related to building of new installations to retailers. Higher costs may also be borne by consumers as they may need to adjust vehicles and other equipment because fuel with high share of biofuels may require engine and technology adaptation, which implies additional (fixed) costs to consumers. This has often led policymakers to provide assistance to cover the additional costs related to distribution or consumption of biofuels [49].

Subsidies targeted at biofuel production directly lower the cost of producing biofuels. For example, the U.S. ethanol production subsidies are estimated to be $1.35 billion in 2008 alone [42]. By driving a wedge between the biofuel prices received by producers and blenders, the biofuel production subsidies simultaneously increase the market price of the biofuel feedstock and reduce the fuel price paid by consumers [16].

Market Impacts of Biofuel Policies

In this section, we provide brief discussion of the implications of biofuel policies for biofuel price formation, food prices, and land use. As discussed in the previous section, biofuel policies are a key driver of biofuel markets and thus impact biofuel price formation. Biofuel prices together with the prices of feedstocks determine profitability of biofuel production and demand for biofuel feedstock. The interlinkage between biofuels and their feedstocks is reflected in food price responses and land use adjustments. These two issues are of primary concern to society. The recent increases in food prices [50] have sparked a lively debate and controversy about the contribution of biofuels and biofuel policies to these developments. High and more volatile food prices affect food security not only in developed but especially in low-income developing countries. The impact of biofuels on land use is a second societal concern; biofuels may have unintended consequences on the environment by releasing more carbon emissions due to the expansion of land cultivation around the world.[12]

[12] The indirect land use change is just one form market leakage of biofuel policies. According to Drabik and de Gorter [18], the leakage in the fuel market itself is much bigger.

Biofuel Price Interlinkages and the Role of Biofuel Policies

The main focus in the literature studying the nexus between the biofuel price formation, the role of biofuel policies, and where the biofuel prices are determined has been on the United States, Brazil, and the European Union, as these countries are the largest biofuel producers, accounting for 90 % of global biofuel production. de Gorter and Just [11, 51] developed a theoretical model explaining how the biofuel price is determined under alternative policies: a blender's tax credit, a biofuel mandate, or their combination. They show that the price of the biofuel is determined either by a tax credit (tax exemption) or a binding (consumption or blend) mandate, but never by both at a time.

If the tax credit, t_c (or a tax exemption), is the binding biofuel policy, meaning it determines the biofuel market price, then the ethanol market price P_E is given by [51]:

$$P_E = \lambda P_G - (1-\lambda)t + t_c \tag{18.1}$$

where the coefficient λ represents miles traveled per gallon of ethanol relative to a gallon of gasoline,[13] P_G is the gasoline (oil) price, and t is the volumetric fuel consumption tax. An implication of Eq. (18.1) is that any change in the tax credit (fuel tax) is directly transmitted into the ethanol price with positive (negative) correlation between them. Although the market ethanol price is linked to gasoline (oil) price, the ethanol price can be lower, higher, or equal to the gasoline price, depending on the magnitudes of other parameters in Eq. (18.1).

Although the economics of a consumption mandate differs somewhat from that of a blend mandate [11],[14] a common outcome of the two forms of the mandate is that – unlike with a blender's tax credit – the biofuel price is not fully linked to the gasoline (oil) price: the link is completely severed under a consumption mandate (because the biofuel market price is determined by the intersection of the ethanol supply curve and a fixed mandate level), and it is partially severed under a blend mandate insofar as a change in the gasoline (oil) price affects the fuel demand. The intuition behind this result is that the biofuel price is determined more by the biofuel supply than by the gasoline price for a given mandate quantity. In the case of a blend mandate, only with an inelastic biofuel supply is the biofuel price tightly linked to the gasoline price.

Identifying which country determines world biofuel prices is more complex. This price determination is sensitive to market conditions in a given country and at a given time. Kliauga et al. [41] and de Gorter et al. [40] hypothesize that only one

[13] For ethanol, $\lambda \approx 0.70$, while for biodiesel $\lambda \approx 0.90$.

[14] It is important to note that the impact of biofuel policies (tax exemptions, tax credits, or price premia due to biofuel mandates) on biofuel prices is not additive: the market price of a biofuel is not determined by the sum of each country's tax exemption, tax credit, or a mandate price premium.

country's policy and market situation determines the world biofuel price. The world biofuel market price is linked to the gasoline/diesel (oil) price through a tax credit (or tax exemption) and is determined in the country with a combination of the highest consumer price paid for gasoline (diesel) and the highest net subsidy (the combination of the lowest fuel tax and highest biofuel tax credit/tax exemption). Alternatively, the world biofuel market price can be determined by a binding mandate in the price leading country. This happens if the induced biofuel price under the mandate is higher than under the tax credit (tax exemption). In addition, this price has to be the highest internationally.

In other words, the price leading country sets the world biofuel price if its biofuel policy generates a price that is higher than in other countries. This is because the net subsidy on a biofuel in the price leading country provides the most favorable biofuel price at the world level; this price is expected to be followed by other countries. The arbitrage in biofuels will lead to equalization of prices across countries, up to transportation costs and tariffs (which lower the biofuel price in non-price leading countries). These costs do not, however, affect the direction of causation of the price relationships between countries; they may only weaken these relationships [52]. For example, if the United States is the price leader for ethanol, then ethanol prices in other countries are likely to be lower by the sum of transportation costs and tariffs or may be independent of the U.S. price if the transportation costs or tariffs are prohibitive. Naturally, the price leader can be only a large country, such as the United States, Brazil, or the European Union, able to absorb large amounts of biofuels.

The empirical evidence on the biofuel price formation and on the price leadership (partially) confirms these theoretical predictions. However, it also suggests that the price leadership may switch between countries, depending on which policy is binding. Rajcaniova et al. [52] empirically find that the U.S. and Brazilian ethanol polices (mostly the U.S. blender's tax credit and Brazilian tax exemption) have historically shared the price leadership, but the Brazilian impact appears to be stronger. Similarly, for biodiesel, the authors support the prediction of de Gorter et al. [40] about the European Union's price leadership.

Biofuels' Impact on Food Prices

Bioenergy has a direct effect on agricultural sector and food prices because biomass is used as feedstock for biofuel production. The demand for agricultural commodities for biofuels competes with the food demand; given the limited availability of agricultural land, this competition leads to price escalation especially in years when supply shocks occur in agricultural production (e.g., the drought of 2012).[15] Key

[15] It is expected that the second-generation biofuels may have lower impact on food prices than first-generation biofuels because of having less intensive demand for agricultural land due to their

factors affecting the transmission from biofuel price to food prices – due to exogenous shocks, such as bad weather or an increase in food demand due to an income growth – are food demand elasticities, availability of land, and crop yield improvements.[16] The food price response to biofuel prices decreases with higher food demand elasticities because a higher demand elasticity implies less agricultural price responsiveness to supply change. In a special situation when food demand is perfectly elastic, food prices would not be affected by the biofuel price. Similarly, price transmission decreases with land availability and crop yield improvement – higher land supply availability and higher yields allow for feedstock production expansion, thus reducing pressure on food price increase.

It is important to note that because of limited availability of land, biofuels increase prices of all agricultural commodities, including those not directly used as inputs to biofuel production. The expansion of biofuels induces higher production of biofuel feedstocks (e.g., corn or soybeans), which in turn increases agricultural factor prices such as land. Higher factor prices push up agricultural production costs, leading to lower production of non-biofuel agricultural commodities (e.g., rice or coffee), hence the increase in prices of these commodities. However, the price transmission onto non-biofuel agricultural commodities may be delayed because of various institutional and market rigidities present in rural factor markets (e.g., land rental contracts or constrained access to capital). Biofuel feedstocks are likely to respond to biofuel policies first, followed by other commodities when the adjustments in the factor markets occur [1, 10, 39, 46, 56–58].

The boom in biofuel production coincides with the significant increase in food grain/oilseed prices worldwide. This is shown in Fig. 18.4 where the FAO food price index increased by 70 % in 2008 relative to 2005, and this increase was even more marked in 2011 (94 %). Rausser and de Gorter [61] and de Gorter et al. [36, 45] give a detailed account of the relationship between food, ethanol, and oil prices in the period 2006–2012.

higher biomass yields and/or due to using residues from agriculture. As a result, second-generation biofuels may lead to lesser competition between biofuels and food demand for agricultural commodities, hence posing lower pressure on food price increase [53]. However, this result may not hold in general. In particular, it depends on the origin of feedstock used for the second-generation biofuels. Havlik et al. [54] find that if second-generation biofuels are produced on agricultural land, they result in higher food price increase than the first-generation biofuels, whereas if second-generation biofuels are sourced from traditional forests or marginal lands, then they result in lower food price increases. In this section, we focus on the first-generation biofuels' impact on food prices as there is more research done in this direction and the second-generation biofuels' effects are more difficult to be empirically evaluated given that they are not commercially exploited at a larger scale yet.

[16] de Gorter and Just [51] derive a theoretical link between ethanol and corn prices, where a $1/gal increase in the ethanol price results in a $4/bushel increase in the corn price. Using cash prices, Drabik [16] shows this theoretical link holds also empirically. Mallory et al. [55] lent support to this relationship using futures prices.

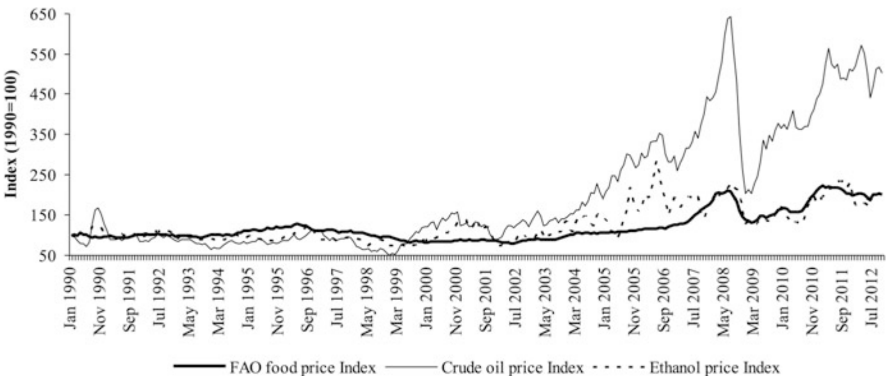

Fig. 18.4 Food, crude oil, and ethanol price developments, 1990–2012 (Based on data from Refs. [1, 59, 60])

Various approaches have been used to estimate biofuel policies' contribution on food prices.[17] Although the findings tend to confirm the inflationary effect of biofuel policies on food prices, the magnitude of this effect is subject to significant uncertainty. For example, although the European Commission acknowledges the energy input channel (i.e., higher oil prices) as the contributor to the recent increase in food prices, it argues that the impact of biofuels is rather small [6, 65]. Similarly, Yu et al. [62] and Zhang and Reed [66], employing a vector autoregressive model, find an insignificant impact of higher crude oil prices on food.[18]

The studies that find a significant biofuel price effect are more prevalent. For example, in their recent paper, Roberts and Schlenker [70] find that the 2009 Renewable Fuel Standard caused corn, rice, soybean, and wheat prices to increase by 20 %. Analyzing the U.S. corn market only, Drabik [16] also finds a substantial increase in corn prices due to corn ethanol policies – an estimated increase of 33–46.5 % in the period 2008–2011. Baier et al. [15] explored the role of the food price increase in the period 2006–2008. Their estimates suggest that the increase in biofuel production had a sizeable impact on corn, sugar, barley, and soybean prices but a much smaller impact on global food prices. According to their estimates, the increase in worldwide biofuel production over the period 2006–2008 accounted for just over 12 % of the rise in the IMF's food price index. The increase in U.S. biofuel production accounted for roughly 60 % of this effect, while Brazil accounts for

[17] Two types of approaches have been followed in the empirical literature. First, time series econometric analyses were performed to estimate the long-run relationship between fuel and food prices [7, 50, 62, 63]. Second, partial and general equilibrium models have been developed to simulate the interdependencies between agricultural, biofuel, and energy markets [e.g., 64]. Further, studies either investigate directly the impact of biofuels (production or prices) on food prices or indirectly through crude oil prices. The studies using the second approach may overstate the overall effect because crude oil price impacts agricultural sector not only through the biofuel channel but also through the indirect input channel.

[18] Other studies that find a lower impact of biofuels on food prices include [67–69].

14 %, and the European Union accounts for 15 %. Rosegrant [71] shows that the biofuel growth accounted for 30 % of the food price increase from 2000 to 2007. The biggest impact was on corn prices, 39 %, and rice prices, 21 %.

The estimates of Ciaian and Kancs [50] indicate that both biofuel and non-biofuel agricultural commodity prices are affected by biofuels but that there is a delayed price transmission for non-biofuel agricultural commodities.[19] According to their estimates, the magnitude of the price transmission elasticities between crude oil and agricultural commodity prices ranges between 0.04 and 0.27 but is higher for biofuel agricultural commodities (sugar, soybeans, corn, and wheat), between 0.13 and 0.27, than for non-biofuel commodities (banana, cotton, sorghum, rice, tea), between 0.04 and 0.06. Similar range of estimated long-run price transmission elasticities for rice and soybeans, 0.16 and 0.32, respectively, is provided by Rahim et al. [17].

Land Use

An important aspect of biofuels relates to land use change and associated environmental implications. In fact, one of the main reasons behind the policy support of biofuel is to decrease the dependence on fossil fuels, thus decreasing the greenhouse gas emissions. Although a direct effect of biofuels may be to reduce greenhouse gas emissions, if indirect effects are taken into account, this may not hold true anymore. One channel through which negative environmental impacts may emerge is leakage effects from biofuels on land use. Due to price interdependencies between biofuel and agricultural markets, biofuel support policies may have far-reaching environmental effects by leading to the expansion of agricultural production into forest, idle, or high-value land. At the same time, land use relocation from food crops to biofuel crops may have implications for food security.

The total world agricultural area allocated to biofuel production represented 14 million hectares in 2006 and 30 million hectares in 2011, and it is expected to reach 100 million hectares in 2050, representing 1%, 2%, and 6 %, respectively, of the total world arable area [9]. However, when looking at specific crop sectors for main biofuel-producing countries, the land devoted to biofuel production is much more significant. For example, in 2010 the area devoted to biofuel feedstock production covered between 60 % and 75 % of the rapeseed area in the European Union, 48 % of the sugarcane area in Brazil, and 31 % of the corn area in the United States [9].

Theoretical models provide two explanations for biofuel policies' impact on land use: a direct land use change impact and an indirect land use change impact. The direct impact on land use change captures the agricultural land switched to

[19] Their findings indicate a small and statistically insignificant transmission between crude oil price and agricultural commodity prices through the indirect input channel.

producing biofuel crops, that is, biofuel policies cause substitution in land use between food and biofuel crops. The indirect impact on land use change captures the total land cultivation expansion, implying that new land, which previously was not used for agricultural production (such as idle land or forest land), is converted into arable land [1, 10, 11, 39].

The empirical literature on the land use change impacts of biofuels mostly relies on partial and general equilibrium simulation models [14, 20, 64, 71–74]. In general, these studies find a positive impact of biofuels on land use, but the effects vary strongly across models, depending on model assumptions and simulated scenario. For example, Searchinger et al. [20] used a global model to analyze the land use change impact of an ethanol increase in the United States. They estimate that ethanol increase of 56 billion liters (14.8 billion gallons) brings 10.8 million hectares of additional land into cultivation worldwide: 2.8 million hectares in Brazil, 2.3 million hectares in China and India, and 2.2 million hectares in the United States. Their results also show that new crops do not necessarily replace all corn diverted to ethanol (12.8 million hectares), because the ethanol coproduct replaces roughly one third of animal feed, which otherwise would be diverted away in the absence of feed coproduct.

Tyner et al. [74] apply a general equilibrium model to estimate land use change impact of the U.S. corn ethanol production. According to this study, the estimated land use changes heavily depend on model assumptions, such as yields, population growth, and base year. Their results imply that producing 50 billion liters of ethanol requires between 1.72 and 2.96 million hectares of additional land. In contrast, Darlington [39] finds that the expansion of corn ethanol production to 56.8 billion liters per year by 2015 is unlikely to result in the conversion of nonagricultural land, arguing that yield improvements will offset the global demand for cropland to meet the corn ethanol production growth.

Piroli, Ciaian, and Kancs [19] apply time series analytical mechanisms and show that biofuels may have both direct and indirect land use change impacts in the United States. Their results show that wheat, barley, and corn areas expand due to biofuels (between 4.3 and 14.8 thousand hectares per $1/barrel increase in the fuel price), whereas rice and soybean contract (between 27.0 and 1.2 ha per $1/barrel increase in the fuel price). The indirect land use changes are also found to be significant, that is, total land use expands up to 56.3 thousand hectares per $1/barrel increase in the fuel price. Similarly, Diermeier and Schmidt [75] estimate a vector autoregressive model to analyze the global impact of crude oil on land use. They find significant global impacts of the oil price on the areas used for production of corn, soybean oil, sugar, and wheat. However, for other commodities (rice, sunflower, cereals), the effects were not significant.

Conclusions

This chapter provides an overview of international biofuel polices and their economic impacts on agricultural commodity prices and land use. There has been a tremendous increase in biofuel production in the recent period (more than 600 % just over the past two decades). This rapid expansion of biofuel production would likely not have occurred without government assistance. Biofuel policies are widely implemented in most developed as well as many developing countries.

A review of the biofuel policies shows that government support is implemented at all stages of the biofuel production and use chain, from growing agricultural biomass to consumption of the end product. The policies used internationally show a number of commonly used measures. In particular, most countries implement mandates for the blending of biofuels with fossil fuel. This instrument targets consumption side of the biofuel market by making obligatory consumption of a certain quantity of biofuels. The second most commonly applied instrument represents biofuel consumption subsidies (tax credits or tax exemptions). However, due to the ongoing financial crisis, consumption subsidies are being phased out or at least reduced in many countries. In contrast, mandates are being gradually expanded, thus making this instrument the key driver for the future development of the biofuel sector.

Other important set of instruments represents trade protective measures and subsidies to feedstock production. These two instruments tend to target the production side as the former protects domestic producers against foreign competition, whereas the latter attempts to reduce the cost of feedstock to biofuel producers. However, subsidies to feedstock production often form an integral part of the general agricultural support system targeted at the whole agricultural sector and not only at biofuel crops. The last group of instruments less frequently used includes grants and loans, price support, research and development subsidies, and support for distribution and use. They are targeted on different stages of the biofuel chain.

The direct impacts of biofuel policies are reflected in the functioning of the biofuel market itself, as they affect incentives of biofuel producers and consumers. The main focus in the literature studying the nexus between the biofuel price formation, the role of biofuel policies, and where the biofuel prices are determined has been on the United States, Brazil, and the European Union, as these countries are the largest biofuel producers, accounting for 90 % of global biofuel production. Biofuel mandates and consumption subsidies were found to determine the biofuel prices. Empirical findings from the literature suggest that the U.S. and Brazilian ethanol polices (mostly the U.S. blender's tax credit and Brazilian tax exemption) have historically shared the price leadership, whereas the European Union appears to have been price leader in biodiesel market [40, 52].

The transition of biofuel policies is further reflected in agricultural market adjustments. Biofuel prices determine profitability of biofuel production and the use of agricultural commodities for biofuel feedstock. This interlinkage between

biofuel and agricultural markets exerts pressure on food markets. Given the limited availability of agricultural land, this competition leads to price escalation especially in years when supply shocks occur in agricultural production. Although empirical findings tend to confirm the inflationary effect of biofuel policies on food prices, the magnitude of this effect is subject to significant uncertainty. The earlier studies were less supportive of the existence of the causal link between biofuel and food prices; the recent literature tends to support it.

One of the main reasons behind the policy support of biofuel is to decrease dependence on fossil fuels, thus decreasing the greenhouse gas emissions. Although direct effect of biofuels may lead to a reduction in greenhouse gas emissions, if indirect land use effects are taken into account, this may not hold any more. Due to price interdependencies between biofuel and agricultural markets, biofuel policies may have far-reaching environmental effects by leading to the expansion of agricultural production into forest, idle, or high-value land. Empirical evidence tends to confirm that this is indeed the case and that biofuels lead to both direct land use changes (land substitution away from non-biofuel crops to biofuel crops) and indirect land use changes (expansion of total land use).

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Chapter 19
Partnerships, Future, and Emerging Technologies

Thomas D. Foust

Abstract This chapter will cover the current state and possible futures of the biofuels industry. Technology options for the production of cellulosic ethanol will be explained with comparative economics given for representative biochemical and thermochemical cellulosic ethanol conversion processes. This will be followed by a description of the current state of the biofuels industry with possible future directions outlined and what needs to happen both from a technical and business perspective to provide the best chances of success. Finally, advanced biofuel (hydrocarbon fuels) conversion options will be discussed with preliminary economics provided.

Keywords Biofuels • Bioproducts • Biofuels industry • Cellulosic ethanol • Advanced biofuels • Comparative economics

Overview

The future of industrial crops for biofuels and bioproducts is highly dependent on the current and future direction of the biofuels and bioproducts industry. The biofuels industry is very much in a state of transition with many possible futures. After significant growth in the 2000s decade, first-generation biofuels have entered a period of minimal to stagnant growth due to a variety of factors such as market conditions, concerns about land use, sustainability concerns, and decreasing government policy support. Conventional wisdom was that the stagnation of first-generation biofuels industry would lead to the natural transition to the second-generation biofuels industry, utilization of lignocellulosic crops.

However, the transition to the second-generation biofuels industry is occurring at a much slower pace than anticipated again to a variety of factors such as market and economic conditions as well as renewed debate about the proper role of biofuels in a sustainable world future on food and fuel supplies. Hence, the biofuels and bioproducts industry is currently in a state of flux with many possible future scenarios that are both dependent on technologies and markets.

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This chapter will explore several possible scenarios for the future of the second-generation biofuels industry and the implications that this will have on approaches for industrial crop breeding for bioenergy and bioproducts. For first-generation biofuels, the relationships between desired crop characteristics and biofuel or bioproduct type are fairly straightforward, i.e., high sugar or starch content correlates to higher yields and better economics. However, this relationship for second-generation biofuels and bioproducts is much more complex and is highly conversion-type specific. For example, for the same product, cellulosic ethanol, high carbohydrate low lignin crops would be preferred if a fermentation-based conversion technology is utilized. However, if a thermal conversion route such as a gasification/catalytic fuel synthesis conversion route is utilized, relative carbohydrate/lignin composition percentages are not that important, but inorganic trace constituents are very important.

Cellulosic Ethanol

Cellulosic ethanol has been the historical focus point of second-generation biofuels. Cellulosic ethanol development efforts date back to the early 1980s [1, 2], with some initial work beginning in the late 1970s [3]. Cellulosic ethanol is the logical biofuel to initially focus on for two main reasons: it builds upon the corn and sugar ethanol industry and it addresses the gasoline market, which is the biggest fuel market worldwide.

Cellulosic ethanol shows a good deal of promise for overcoming many of the limitations of first-generation ethanol technologies that utilize sugar or starch crops as the feedstock. Since cellulosic crops are utilized as feedstocks, the issue of direct competition with food production is negated [4], although land completion issues still exist [5].

Environmental and Sustainability Benefits

Cellulosic ethanol has long been touted for its environmental and sustainability benefits over first-generation ethanol technologies. The two biggest producers of first-generation ethanol are Brazil from sugarcane and the USA from corn. True environmental benefits and effects on food supply and food prices are concerns that are commonly brought up about first-generation ethanol. For example, at the 2011 US production rate of 52.8 billion liters/year, almost 40 % of the US corn crop is utilized for ethanol production [6]. There is considerable debate about the effect this amount of corn crop consumption for ethanol production has on world food prices and supplies, but in any case further significant growth is unlikely.

Cellulosic ethanol also has significant potential greenhouse gas (ghg) emission reduction benefits over first-generation ethanol technologies [7]. Figure 19.1 shows

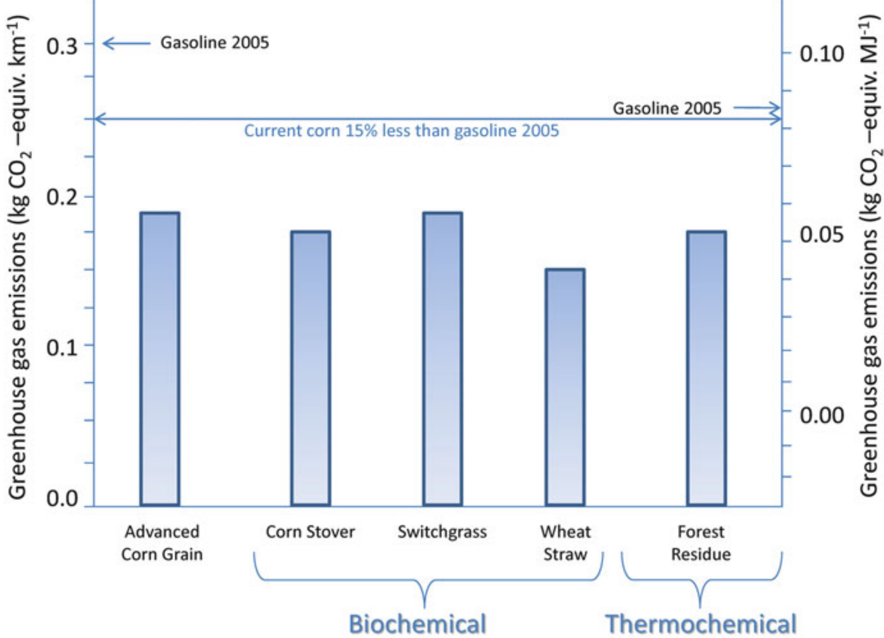

Fig. 19.1 GHG benefits of cellulosic ethanol compared to first-generation ethanol

potential ghg benefits of various cellulosic ethanol technologies, current first-generation corn ethanol, and an advanced future state of the corn ethanol industry as they compare to gasoline based on values given by Hsu et al. [8]. These ghg values incorporate using the lignin component to supply the heat and power needs of the conversion process in the cellulosic ethanol cases and an increased biomass component in the advanced corn ethanol state [9]. Another benefit of cellulosic ethanol over first-generation ethanol is that cellulosic ethanol has a much-improved net energy balance [10], and similar to the ghg emission benefits, much of this benefit comes from utilizing the lignin component to supply the heat and power needs for the conversion process.

Conversion Technologies

Although there are a multitude of variations, there are essentially two main technology approaches for producing cellulosic ethanol from lignocellulosic biomass. The first route, commonly referred to as the biochemical route, utilizes a sugar intermediate, which is fermented into ethanol, and the second route utilizes a syngas intermediate, which can either be fermented or catalytically converted to ethanol. Both routes have received considerable amount of public and private

support to develop the technologies to a commercially viable state, and both routes have the potential to make a significant contribution to the world's supply of transportation fuels.

Biochemical

A schematic of a representative biochemical conversion process for cellulosic ethanol is shown in Fig. 19.2.

The biochemical conversion process can essentially be categorized into two main subcomponents: the liberation of the sugars from the biomass and the fermentation of these sugars to ethanol. The efficient liberation of sugars from the carbohydrate portion of the biomass "saccharification" is a significant challenge given the recalcitrant nature of biomass [11]. Saccharification research has received considerable attention over the past couple of decades with significant improvements made in both the efficiency and the cost of the process [12].

Saccharification can essentially be either a chemical process where a concentration acid process or multiple stages of dilute acid are utilized to liberate both the hemicellulose and cellulose sugars [13] or a two-step approach involving a pretreatment step and a enzymatic hydrolysis used to liberate some to most of the hemicellulose sugars and condition the biomass to a state that is amenable for enzymatic hydrolysis [14].

The US Department of Energy (DOE) evaluated the long-term potential [15] of these two approaches and determined that the pretreatment/enzymatic hydrolysis approach had the best potential for efficient conversion at low cost for wide-scale applicability [16]. Although this is true at the macroscale, chemical saccharification technologies such as concentrated acid or multistep dilute acid approaches are certainly viable for special niche applications.

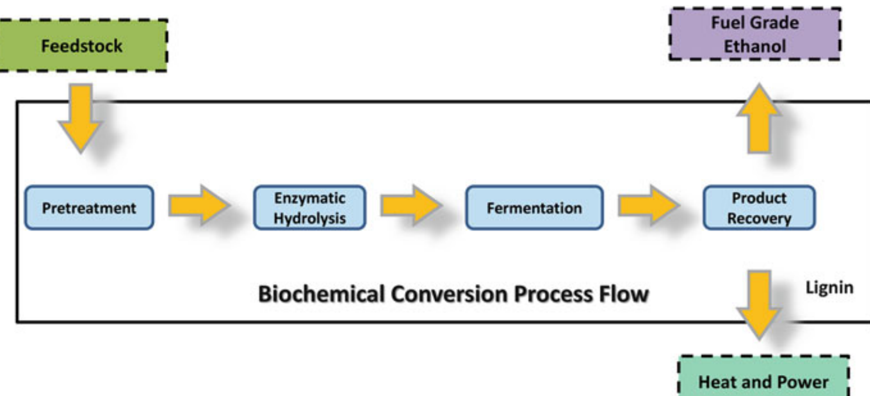

Fig. 19.2 Biochemical conversion process schematic

When effectively coupled, pretreatment and enzymatic hydrolysis can be a very efficient, cost-effective way to liberate monomeric sugars from the carbohydrate portion of biomass with some organizations reporting over 90 % conversion of structural polysaccharides to monomeric sugars [17]. There are a multitude of pretreatment approaches being pursued that cover the gamut of pH ranges from acidic to alkaline approaches as well as temperature ranges (~140–210 °C). All of these approaches have their plusses and minuses when compared on an efficiency and cost basis [18]. However, comparison work has shown that there is really not a "one size fits all" when it comes to a pretreatment approach across the spectrum of suitable lignocellulosic feedstocks. In general, the alkaline or higher severity approaches such as wet oxidation [19] that are more aggressive at depolymerizing the lignin component tend to perform better on higher lignin feedstocks such as softwoods, while the lower severity approaches such as dilute acid or hot water [20] tend to perform better on the low lignin herbaceous feedstocks such as corn stover or switchgrass.

Enzyme development with the goal of low cost-efficient hydrolysis to monomeric biomass sugars has been an area of extensive focus with considerable progress being made [21]. This progress has been critically important in moving the biochemical conversion process towards its goal of economic competitiveness; however, further progress in both specific activity and costs to produce the enzymes is still possible and desirable.

Similarly, with enzyme development, organism development for the cost-effective efficient fermentation of biomass sugars to ethanol has been an area of significant focus, again with impressive, high-impact progress being made [22]. Humbird [15] reported for pilot scale results very effective fermentation results of greater than 90 % of total biomass sugars to ethanol, which would correspond to a total ethanol yield of 330 l/tonne of biomass. At these yields and efficiencies, the overall economics compare favorably to first-generation ethanol technologies. However, this needs to be caveated with the fact that these are pilot plant numbers and the technology still needs to be proven out at commercial scale.

The final step in the biochemical lignocellulosic ethanol conversion process is product recovery, which is envisioned to be standard fractional distillation to the ethanol-water azeotrope, followed by molecular sieve concentration to anhydrous ethanol. These techniques will be very similar to what is currently used in first-generation ethanol processes and hence is a well-proven technology.

Since cellulosic ethanol technology utilizes only the carbohydrate portion of the biomass, the lignin component of the biomass is available for other uses. Initial process designs put forward by a number of organizations [23] dry the lignin and then use it as a fuel for heat and power needs of the conversion process. The Renewable Fuel Standard (RFS) requires that advanced biofuels show a 60 % ghg reduction when compared to conventional gasoline to quality for the RFS credit. Utilization of the lignin component as opposed to using a fossil fuel such as natural gas or coal contributes significantly to ghg reductions for cellulosic ethanol [24].

Feedstock Considerations

Since the biochemical lignocellulosic ethanol conversion process hinges on high sugar conversion to ethanol, desirable feedstocks will have a high carbohydrate content that is amenable to conversion to monomeric sugars that in turn are readily fermented to ethanol. Although this statement is fairly straightforward and may be a bit obvious, it is in reality overly simplistic and may not be a good metric for judging the relative value of feedstocks. Lignocellulosic feedstocks have a great variety as to both physical and chemical properties, and these variations can have significant impacts on the yields and efficiency of the conversion process.

In the early days of biochemical conversion process development, the process was commonly referred to as the sugar platform and organizations and researchers focused on cost of sugars [25]. Research predominantly focused on maximizing sugar yields per mass unit of feedstock. However, since the ultimate goal in any conversion process is to produce the final product at the highest efficiency and yield at the lowest possible cost, history and experience has shown that this singular focus on sugar yields could be misleading. Fermentation inhibitors such as furfural, hydroxymethylfurfural (HMF), or other inhibitory compounds can significantly affect the fermentability of the sugar solution [26]. The presence and concentration of these inhibitory compounds is very much a function of the feedstock type, the pretreatment technology, and finally the severity of the pretreatment. Since some inhibitory compounds such as furfural and HMF arise from thermal degradation of sugars, the higher severity pretreatment approaches that lead to higher sugar yields also lead to high inhibitory compound concentrations. Hence final ethanol yields could actually be lower for higher sugar concentration hydrolysates with these compounds present, then they would be for lower sugar concentration hydrolysates without these compounds being present. Although these inhibitory compounds can be reduced or eliminated by hydrolysate conditioning [27], these processes add cost and complexity to the process as well as lead to sugar losses; hence, it is best to avoid these processes if possible.

For these reasons, there is really not a preferred feedstock since the best choice will be the feedstock that is available at the desired quantities at the lowest cost. The conversion process will need to be well suited for the feedstock. Adding to the complexity will be that feedstock availabilities and cost are geographically dependent as well as weather and time of year dependent. Therefore, it might be cost effective to build robustness into the process conversion plant so that a range of feedstocks can be accommodated based on weather or seasonal variations. Simply building a plant capable of processing the predominant feedstock in the geographical area (i.e., corn stover in the US Midwest or Sugar Cane Bagasse in Brazil) may make the plant difficult to operate economically year in and year out over the 30+ year life of the plant if the feedstock availability drops significantly due to a prolonged drought or other condition that affects the availability of that particular feedstock.

Economics

Since ethanol like any transportation fuel is a commodity with no price differentiation in the market place, price parity with first-generation ethanol as well as ultimately price parity with conventional petroleum-based gasoline on an energy-adjusted basis needs to be achieved before demand-driven market penetration can occur. Achieving price parity has proven to be a very difficult challenge, and fortunately after decades of focused effort, significant encouraging progress is being made. Several companies are going forward with commercial cellulosic ethanol facilities, but private companies are typically resistant or even prohibited, depending on the country regulations where they are located from publicly disclosing feedstock or production costs. Hence, the only method to get production cost numbers is from the open literature. This tends to be somewhat of a mixed bag depending on the source of the numbers and the rigor that was used to develop the production cost numbers as well as the underlying motivation for publishing the numbers. Press releases [28] and other popular press articles [29] exist that show economically competitive production costs, which if taken at face value would indicate that price parity has been achieved or even exceeded. However, peer-reviewed literature on economics of cellulosic ethanol production tends to be more conservative on the costs [30]. Hence, why it is difficult to state a number or even a range of numbers is that range is likely to be so broad that it ceases to be meaningful.

With all these said, probably the best source of public numbers on the production cost of cellulosic ethanol from a biochemical production route is available from the US DOE via the National Renewable Energy Laboratory (NREL). Humbird et al. [22] published a case where a fully loaded production cost of $2.15/gallon (2007 dollars) could be achieved based on technology demonstrated at the pilot plant for a 2,000 tonnes/day commercial plant for an nth plant case.

At these production costs, cellulosic ethanol produced via a biochemical conversion route compared very favorably with first-generation ethanol production costs from sugar or corn. A noticeable difference is the percentage of overall production costs represented by the feedstock component. In the case of first-generation ethanol, feedstock costs represent about 70 % of overall production costs, whereas in the case of cellulosic ethanol, feedstock costs only represent 30 % of overall production costs [29]. Analyses that have been performed on a range of technically mature conversion processes have shown that typically feedstock costs represent 30–50 % of production costs for commodity products [31] such as ethanol. Hence, this would indicate that the long-term potential for further cost reductions is higher for cellulosic ethanol than it is for first-generation ethanol.

Thermochemical

A schematic of a representative thermochemical conversion process for cellulosic ethanol is shown in Fig. 19.3. This conversion route is based upon utilization of catalytic fuel synthesis for ethanol production. An alternative to catalytic fuel synthesis would be fermentation of the syngas to ethanol [32]. The catalytic fuel synthesis option will be covered here primarily because better public data exists for this option.

Similarly to the biochemical conversion process, the thermochemical ethanol conversion process can essentially be categorized into main components: the gasification of the biomass to syngas (H_2 and CO) and then the catalytic conversion of this syngas to ethanol. Biomass gasification is an early-stage commercial technology that has been deployed at a few locations [33]. Biomass gasification technologies cover a fairly broad gamut of simple to sophisticated approaches with, as would be intuitively expected, the sophisticated approaches having higher costs, both from an operating and capital perspective. The simplest biomass gasification technologies, updraft or downdraft air-blown approaches, produce a syngas highly diluted with N_2 that is generally not well suited for catalytic conversion to fuels. Oxygen blown or direct gasification and indirect gasification do not introduce air into the gasification process and hence do not have the N_2 dilution issue; thus, they are the two best gasification technology choices for producing a syngas suitable for catalytic fuel synthesis. Phillips, Dutta, and coauthors did a series of studies [34–36] where they looked at dry ash and slagging direct biomass gasification approaches compared to indirect biomass gasification for mixed alcohol fuel synthesis and concluded that for the scales of biomass (2,000 tonnes/day), indirect gasification was the preferred route on both a cost and an efficiency basis.

A drawback of indirect biomass gasification is the amount of tars and light hydrocarbons produced during the gasification process [37]. Light hydrocarbons and tars are problematic since they represent a carbon and hence an efficiency loss,

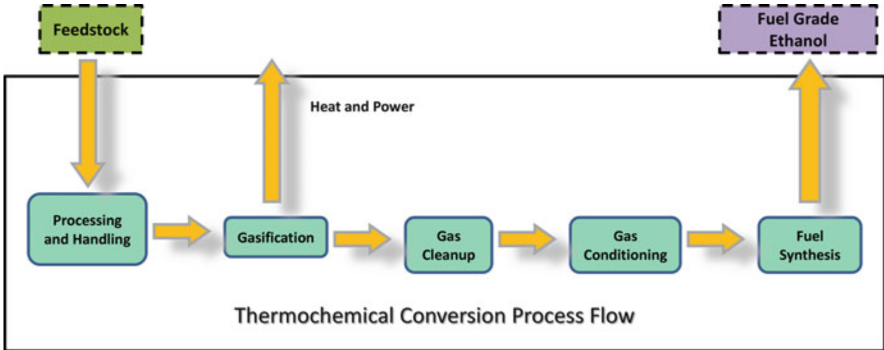

Fig. 19.3 Thermochemical conversion process schematic

but tars are especially problematic since they have the additional detrimental effect of fouling downstream fuel synthesis catalysts and system.

Proven technology approaches for removing the light hydrocarbons and tars from the syngas is a two-step process: water quenching to remove the tars and other particulates from the syngas stream followed by steam methane reforming to change the methane and other light hydrocarbons to additional syngas. Although water quenching is effective for removing tars and heteroatoms from the syngas, it represents a carbon and efficiency loss since the carbon and energy contained in the tars would be lost to the system. However, even more problematic is the large toxic wastewater stream that water quenching would create. Steam methane reforming does not represent a carbon or energy loss or create a problematic waste stream, but it does add additional cost and complexity to the process.

A preferred approach to this two-step process would be to perform integrated tar and light hydrocarbon reforming in a catalytic single-step process. This would have the benefits of increasing the carbon and energy efficiency of the process, reducing process steps and hence the cost and complexity of the conversion process, and finally eliminating a large volume toxic wastewater stream. Although several researchers have published results showing that a number of catalysts look promising for tar and light hydrocarbon reforming [38], the challenge is to maintain the activity in the presence of sulfur. This area has been the focus of considerable effort over that past several years, and a number of researchers and organization are reporting some encouraging results [39].

The next major step in the process after the syngas has been cleaned and conditioned is to perform the catalytic fuel synthesis. Several organizations have developed mixed alcohol synthesis catalysts over the past decade or so [40, 41]. In order to be commercially viable, mixed alcohol synthesis must have good selectivity to the desired product, in this case ethanol, as well as good CO conversion. Several researchers have reported significant improvements in mixed alcohol synthesis catalyst performance [42]. Improvements in catalyst performance that increase single-pass conversion and ethanol productivity are particularly beneficial since these improvements have the added benefit of simplifying the process by requiring fewer recycle loops, hence improving both costs and efficiencies.

Feedstock Considerations

Biomass composition affects thermochemical processing differently than biochemical processing. Unlike biochemical conversion, which only converts the carbohydrate portion, gasification converts the entire organic component of biomass, both carbohydrate and lignin fractions, into syngas, light hydrocarbons and some tars. Trace inorganics predominantly sulfur, salts, and alkaline earth metals can be problematic in a variety of ways. Potassium can be problematic in the gasifier for fluidized bed gasifiers. The potassium interacts with the silica in the system to form K_2SiO_4 which has a low melting point of ~ 500 °C, and its formation will lead to the

bed media becoming sticky which in turn leads to agglomeration and defluidization of the bed media.

Sulfur that gets converted into H_2S and alkaline earth metals such as Ca or Na can be significant catalyst poisons for both the tar reforming catalyst and the fuel synthesis catalyst. Depending on their concentration in the feedstock and the sensitivity of the catalysts being utilized, they may need to be reduced or eliminated from the syngas stream by water scrubbing or catalyst guard beds. Although both of these techniques are well-proven technologies, they do add cost and complexity to the process.

Economics

The thermochemical conversion process has not received the same degree of focus as has the biochemical conversion process, and therefore there are less references in the literature on the economics of the process. Similar to the biochemical conversion process, probably the best public source of production cost numbers is NREL. Dutta et al. published a case where a fully loaded production cost of $2.05/gallon (2007 dollars) could be achieved based on technology demonstrated at the pilot plant for a 2,000 tonnes/day commercial plant for an nth plant case [43].

Given these two independent technology approaches for producing cellulosic ethanol, the logical question is how they compare. Several studies have looked at this particular question, and one study [44] specifically did a rigorous comparison of these two technologies based on 2007 reported numbers. Table 19.1 provides some direct comparison numbers for the two processes based on the latest reported results from NREL referenced above.

As can be seen from the values in Table 19.1, the economics of the two processes are very similar. The thermochemical process has a slightly lower MESP (5 % lower) but a higher required capital investment (22.1 % higher). The thermochemical process does have slightly higher yields since the lignin portion is utilized for fuel production as well. Average return on investment is almost identical.

An important point of distinction is that the biochemical results are presented for corn stover, whereas the thermochemical results are for pine. The authors of the referenced studies choose the feedstocks that tended to give the best performance and economics for their conversion technology. Although conversion economics do not exist for pine feedstocks for the biochemical conversion process or corn stover for the thermochemical conversion process, poorer conversion economics would be expected for these cases due to lower carbohydrate content for the pine feedstock for biochemical conversion and higher ash content for the corn stover feedstock for thermochemical conversion. This illustrates the earlier point that there does not appear to be a clear superior conversion technology in terms of yields and/or economics. Hence, the best approach is to best match the conversion technology to the predominant feedstock. Since feedstocks tend to be local, the best conversion technology choice will most likely be feedstock dependent or regionally specific.

Table 19.1 Comparative economics for cellulosic ethanol production

	Biochemical	Thermochemical
MESP[a]	$2.15/gallon	$2.05/gallon
Ethanol yield	79.0 gal/dry ton	83.8 gal/dry ton
Delivered feedstock/cost	$58.50/dry ton (corn stover)	$61.57/dry ton (pine)
Total installed equipment cost	$232 MM	$296.5 MM
Total capital investment	$422.5 MM	$515.85MM
Average return on investment	56.6	57.5
Current yield (actual/theoretical)	76.0 %	40.0 %

[a]MESP defined as a fully loaded production cost with a 10 % IRR

State of Industry

After rapid growth in the 2000s decade, further expansion of first-generation ethanol technology has been fairly stagnant. Future growth in first-generation ethanol production will most likely continue to be slow mainly due to concerns about the percentages of the sugar and corn crop being directed to ethanol production, true environmental benefits, as well as market saturation.

Many companies are proceeding forward with plans to commercially deploy cellulosic ethanol in many parts of the world with several commercial production plants in construction. Since pioneer plants or first-of-a-kind plants have higher costs than mature nth plant cost, many countries such as the USA [45] have incentives in place for the initial commercial production of cellulosic ethanol to offset this initial higher cost.

An important point to make is that since the biofuels industry and especially the cellulosic ethanol industry is in an immature state and as any immature industry, it is highly dynamic and will change and evolve over time. For this reason, mentioning any companies' or organizations' plans is not that useful since it will quickly become outdated. A review of the recent history of the biofuels industry from 2006 to 2012 clearly illustrates this point. In the 2006–2008 timeframe, driven by record high prices for crude oil and aggressive government policies for biofuels development and deployment, there was tremendous growth in the biofuels and bioproducts sector with more than 135 companies [46] being active in the "second-generation" biofuels space. This growth period continued until 2011–2012 where there has been a consideration contraction of the industry. A specific illustrative example of this point is that in the US public company valuations for 13s generation biofuels companies are down more than 66 % in 2012 which translates into a $4B USD decrease in value. Many companies have delayed or even shelved plans to proceed forward with commercial plants leading to layoffs across the industry. Therefore, the industry is clearly in a critical state where it can move on to a successful future state or continue downward to an uncertain future. For the industry to be successful, it must focus on sustainable value creation and learn from and move on from past mistakes.

Initially, the biofuels sector was following the business model set by the biotech and software industries. These industries operate on the investment/capital-lite potentially very high revenues model. A highly successful example of this is Google, which went public in 2004 with only $25MM of total external funding and generating $3.2B in revenues in its first year. Although this is an extreme example for even the software industry, this type of revenues/investment ratio is completely outside the realm of possible for the biofuels industry. Investments in R&D to develop the technology plus the required capital to build and operate a pilot plant to demonstrate the technology will easily run into the $100 s of millions to get the technology to a commercially viable state. Estimates for a pioneer commercial plant are around $500MM or higher so the total investment to get to a commercial state could be around $1B to produce a commodity product that will compete with well-established first-generation ethanol and conventional gasoline. The time period required to go from R&D stage to commercial production can easily be 10 plus years, which also serves as an impediment. Granted government R&D funding or incentives can be leveraged to offset some of the costs as well as help on the revenue side, still the promise of biotech or software industry types of returns on investment simply is not realistic.

The cellulosic ethanol industry needs to abandon this unrealistic model, which has led to overhyping and overpromising, and endorse a more realistic business model based on sustainable value production over the long term. Given the large amounts of resources and time required to develop the technology to commercialization, the industry will need to pool assets either by strategic alliances or consolidation to form strong players with the required resources and strong business positions to be successful. The many small players with weak business plans, poor strategic and intellectual property (IP) positions, and incomplete assets and capabilities are plaguing the industry with too many failures and few to any major commercial successes. This is giving the industry a black eye with the investment community, government policy makers, as well as the fuel industries with a reputation of being long on promises and short on delivery.

To focus on sustainable value production, the biofuels industry needs to reduce costs and risk and focus on ways to increase revenue over both the near and long terms. Pooling expertise, IP, and capital through consolidation and/or strategic alliances can reduce risks. Since the industry grew out of R&D roots, there is too much focus and redundancies in R&D to develop unique processes for unit operations such as pretreatment in biochemical conversion that in reality has minimal impact on overall costs and yields and ultimately business position. Research and development resources would be better spent on ways to increase revenue by creating higher value products that can be sold into chemical, materials, or other higher value markets to maximize potential for commercial success. The industry also needs to look into innovative ways to reduce time to market since failure to deliver on past commitments is adversely impacting the industry.

Limitations of Cellulosic Ethanol

Although ethanol has many benefits as a fuel, i.e., it is readily blended into gasoline the predominant worldwide transportation fuel, it can be used in most cars at low blend levels (<15 % ethanol) without modification, and it has favorable production costs. Compared to conventional gasoline, it does have some drawbacks that have limited its growth as a transportation fuel. Ethanol has only 2/3 of the energy content of gasoline and hence will deliver less mileage on a volumetric basis than gasoline. Additionally ethanol has only limited compatibility with the existing infrastructure, thus requiring new investments in pipeline or refining infrastructure for large-scale deployment.

However, the biggest drawback of ethanol has proven to be limited markets. In the USA, the world's biggest producer and user of ethanol, almost all ethanol is utilized as E10 with E85 usage being almost negligible [47]. Currently, in the USA almost 97 % of motor gasoline is E10; hence, the E10 market is essentially saturated. This situation is commonly referred to as the "blend wall" and is the primary reason potential developers of cellulosic ethanol cite as limiting the deployment of cellulosic ethanol. This is interesting because the common perception is it lacks commercially viable cellulosic ethanol technology, which in reality is not the case. The US Environmental Protection Agency (EPA) did approve the use of E15 in model 2000 and newer cars [48] to address this issue, but the adoption of E15 has been slow due to a number of logistical factors. Brazil has not faced the blend wall issue due to a different fuel strategy that is unique to Brazil [49]. Two fuels are offered in Brazil E25 and E100, and since 2003 most new vehicle sales are rapidly trending towards "flex" vehicles, which can accommodate any amount of ethanol. Transportation experts are mixed in their opinion if this strategy to avoid the blend wall issue could be used outside of Brazil.

Although ethanol is a good blend component for gasoline, it is not a good blend component for diesel and completely not suitable for jet fuel. Since diesel and jet fuels are growth fuels and gasoline demand is leveling off or declining especially in the EU and USA, biofuels that do not face the market issues of ethanol and are suitable for the diesel and jet fuel markets are highly desirable.

Advanced Biofuels

The nomenclature of "advanced biofuels" is somewhat used inconsistently in the literature. As it is used here, it refers to hydrocarbon fuels or "drop-in" fuels that can be directly utilized in the existing gasoline, diesel, and jet fuel pools. Producing advanced biofuels from biomass has a number of advantages over ethanol. Since they are essentially substantially similar to current gasoline, diesel, and jet fuels, they would be completely compatible with the existing fuel distribution and vehicle infrastructure. This compatibility could be extended all the way to processing inside

existing petroleum refineries, which will be explained in more detail in the following sections.

Another significant advantage of advanced biofuels is that they do not face any market or new fuel acceptance issues. The challenge to get a new fuel certified can be very significant in a large part due to emission and air quality concerns. For instance, in the USA, the US EPA requires a very rigorous testing program to verify that a new fuel or even a blend limit change, i.e., E10 to E15, does not adversely affect emissions. Additionally, vehicle manufacturers and engine manufacturers are very leery of new fuels or blend limit changes because of possible impacts on performance or reliability. For advanced biofuels, none of these issues or concerns would come into play since the fuels would be simply gasoline, diesel, or jet, which are currently certified and accepted by vehicle and engine manufacturers.

However, in the context of nothing is as simple as it first appears, fuel liability issues would exist and need to be addressed. Most country's fuel liability provisions are structured in a manner that the fuel supplier accepts all liability issues associated with the use of the fuel. Therefore, thorough testing would most likely be required for advanced biofuels before fuel suppliers would be willing to accept them as part of the fuel supply and accept the liability associated with their sale.

The categorization of conversion routes into biochemical routes and thermochemical routes roughly holds for advanced biofuels as it does for cellulosic ethanol with the distinction that the biochemical route is more of a sugar intermediate route because the upgrading to fuels can be by either a fermentation route or a catalytic route.

Sugar Intermediate Routes

Figure 19.4 illustrates the overall process for advanced biofuel conversion via a sugar intermediate. The first part of the process, converting the carbohydrate portion of the biomass to a sugar intermediate, is essentially the same pretreatment/enzymatic hydrolysis process that was explained in detail in the biochemical cellulosic ethanol conversion section. From the sugar intermediate, the upgrading to an advanced biofuel can take two dramatically different routes, either a fermentative route to an isoprenoid [50] that can be upgraded to a diesel fuel in a fairly straightforward manner or a catalytic route that involves a number of catalytic and upgrading steps to produce an advanced biofuel [51].

Feedstock Considerations

The feedstock considerations are similar to biochemical cellulosic ethanol conversion. In general feedstocks with high carbohydrate content and correspondingly lower lignin concentration tend to have better performance. This tends to favor the

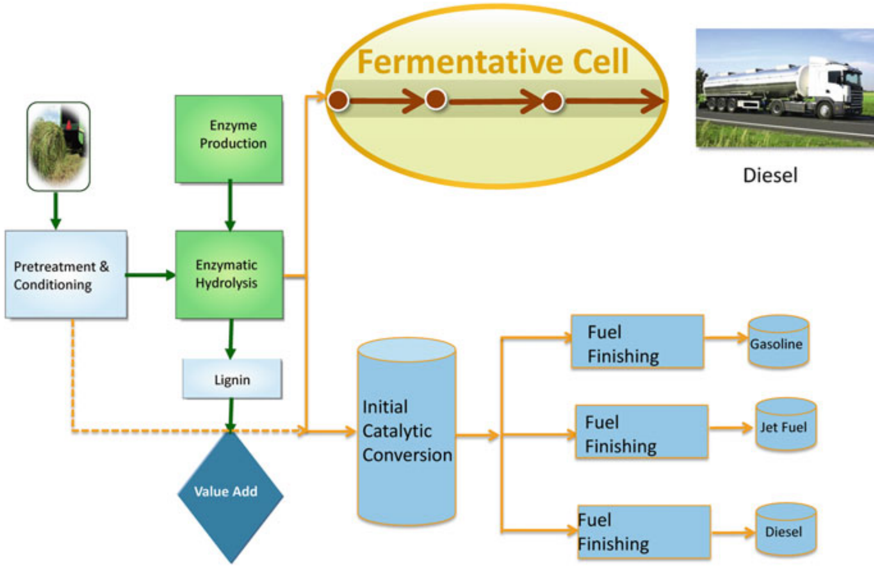

Fig. 19.4 Advanced biofuel routes via sugar intermediate

herbaceous feedstocks such as corn stover or switchgrass. For catalytic conversion, sulfur and alkaline earth metal concentrations can be a significant issue since these compounds can be significant catalyst poisons.

Economics

The economics for conversion to advanced biofuels are much less developed than they are for cellulosic ethanol primarily because the technology is in an earlier state of development. Since the biomass deconstruction to intermediate sugars component is essentially the same as it is for biochemical cellulosic ethanol, these costs are well quantified. However, much less cost specificity exists for the advanced biofuels production step. For the fermentative route, it is fair to say that this will be a higher cost fermentation than ethanol fermentation since isoprenoid fermentations are aerobic which are higher cost both from a capital and operating perspective than anaerobic ethanol fermentations. The final product, hydrocarbon diesel fuel, has higher value than ethanol, so this cost/benefit ratio will determine how the economics compare to cellulosic ethanol. For the catalytic upgrading route, the challenge most likely will be the number of process steps required. Multiple processing steps increase capital cost as well as operationally complexity. Since fuel production like any commodity process requires high plant online time, simpler processes will have the advantage.

Thermal Routes

Thermal routes can be categorized onto three areas based on the intermediate they produce, liquefaction, pyrolysis, or gasification. Figure 19.5 shows the intermediate as a function of severity of deconstruction.

The highest severity thermal deconstruction technique is gasification at temperatures of 600 °C and above, depending on the type of gasification technology used [52]. Once a syngas has been produced and appropriately cleaned and conditioned, there is a multitude of fuel synthesis routes for producing an advanced biofuels [53]. Some of these routes such as Fischer-Tropsch synthesis are well-proven technologies that are being practiced commercially in certain countries such as South Africa. Others are more in the development stage such as single-step olefinic gasoline production that shows better potential for being economically viable at the scale of biomass.

The mid-severity thermal deconstruction technique is pyrolysis which is performed at temperatures in the range of 300–600 °C [54] at atmospheric pressure. Pyrolysis processes produce oil commonly referred to as "bio-oil" or "pyoil" that somewhat resembles crude oil in appearance but has dramatically different physical and chemical properties. The initial biomass pyrolysis process developed was fast pyrolysis. This process is well proven and relatively efficient, but the oil produced has some very undesirable properties that present some significant challenges for upgrading to advanced biofuels [55]. Many of these desirable properties are directly related to the high oxygen content of the bio-oil.

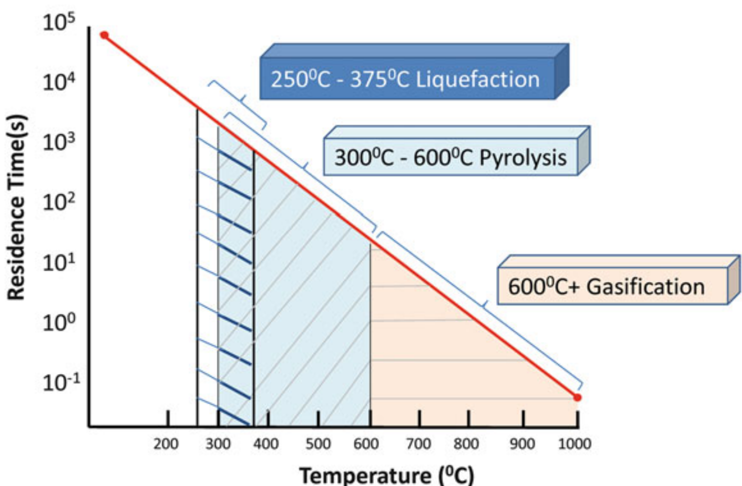

Fig. 19.5 Thermal intermediate as a function of temperature and residence time

To address this issue, several researchers and organizations have developed processes that deoxygenate the bio-oil during the production process such as catalytic fast pyrolysis [56] or hydropyrolysis [57]. Since these processes produce oil considerably lower in oxygen content, the oil has much better properties that make it more amenable for upgrading to an advanced biofuel. These processes are complex from both a physical and chemical perspective, so some technical challenges will need to be overcome before they can be deployed commercially.

The lowest severity deconstruction technique is liquefaction or hydrothermal processing at temperatures in the range of 250–375 °C [58]. Hydrothermal processing requires high pressures in the range or 600–3,200 psi to maintain the water in a liquid state at these temperatures and as shown in Fig. 19.5 requires considerable longer residence times than pyrolysis or gasification. Hydrothermal processing produces oil considerably lower in oxygen content that is more amenable for upgrading to an advanced biofuel.

Economics

Since gasification technologies had been under development for some time, a number of studies exist in the literature on the economics of gasification processes to advanced biofuels. There has been considerable interest over the years to pair biomass gasification with Fischer-Tropsch synthesis since they are both relatively developed technologies, and this pairing could represent a fairly streamlined path to commercialization. Unfortunately, most studies have shown that the economics of this paring are not that attractive primarily due to the fact that Fischer-Tropsch synthesis is a capital-intensive technology and the economies of scale do not match well with the scales of biomass [59]. Studies that have looked at paring biomass gasification with less capital-intensive advanced fuel synthesis routes have shown potentially attractive economics [60].

The economics of pyrolysis processes or liquefaction processes to advanced biofuels are not as well developed since the technology is in an earlier stage of development. A very enticing possibility of these routes is to perform a significant portion of the upgrading to an advanced biofuel inside existing petroleum refineries. Figure 19.6 shows one possible scenario for this. The economic advantages of this are potentially significant. If a large portion of the upgrading could be accomplished utilizing existing petroleum refineries, the capital investment required would be dramatically reduced. A study estimated that a cumulative investment of $95B [61] would be required in new processing facilities to meet the RFSII goals of 21 billion gallons of cellulosic ethanol and/or advanced biofuels by 2022. Although a definitive estimate does not exist as to how much this required investment could be reduced by biofuels processing inside existing petroleum refineries, primarily because this technology is in too early of a stage of development to make this determination, it is fair to say the potential is very significant and warrants further investigation.

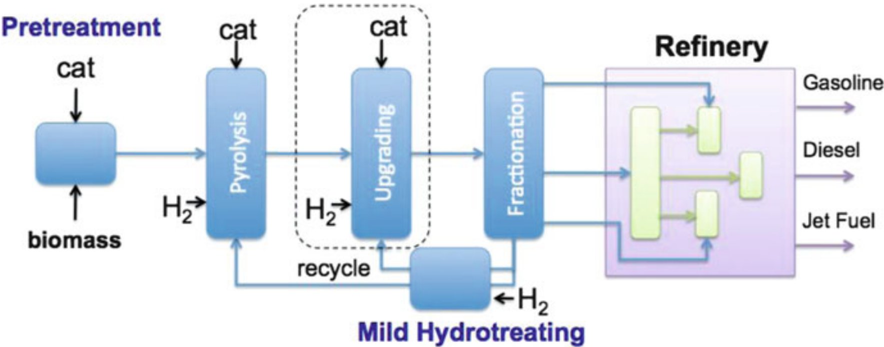

Fig. 19.6 Advanced biofuels integration with petroleum refining

State of Industry

For the most part, the comments made about the cellulosic ethanol industry hold for the advanced biofuels industry as well. One important distinction is that if the approach for processing inside petroleum refineries develops to a state where it is commercially viable, several large petroleum refinery industrial players have stated they would be interested in adopting the technology. This would be a dramatic shift in the biofuels industry because it would constitute a change from many small companies to a few well-established large players. These larger players would address the issue of not having adequate resources to take the technology to commercial deployment.

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