# Mutation Breeding in Date Palm (*Phoenix dactylifera* L.)

# 25

Djamila Yatta El Djouzi, Hafida Khelafi, Shri Mohan Jain, and Jameel M. Al-Khayri

## Abstract

Date palm (*Phoenix dactylifera* L.) is one of the oldest fruit crops grown in the arid regions of the Arabian Peninsula, North Africa, and the Middle East. The date palm fruit is pivotal of the economy and the social life of the date palm producing regions. Currently, there are various challenges opposed date palm development, processing, and marketing such as the presence of low-quality cultivars, poor farm management, insect and diseases pest infestations control, deficiencies in harvesting, processing, shortage of qualified and national trained staff and laborers, insufficient research and development activities. To overcome these challenges, there is a need for alternative approach to conventional plant breeding. Genetic enhancement of the date palm by radiation induced mutagenesis represents a major opportunity since it increases the genetic variability to sustain food security. Gamma-irradiated date palm callus regenerated plants resistant to Bayoud toxin isolated from the causal fungus *Fusarium oxysporum* f.sp. *albedinis*. Several selected putative mutants resistant to Bayoud disease have maintained resistance underfield conditions. This chapter presents an overview of induced mutation and their application date palm improvement.

D. Yatta El Djouzi · H. Khelafi
I.N.R.A. Biotechnology and Plant Breeding Division CRP Mehdi Boualem, Alger, Algeria
e-mail: djamila.yatta@inraa.dz; hafida.Khelafi@inraa.dz

S. M. Jain
Department of Agricultural Sciences, PL-27, University of Helsinki, Helsinki, Finland
e-mail: mohan.jain@helsinki.fi

J. M. Al-Khayri (✉)
Department of Agricultural Biotechnology, College of Agriculture and Food Sciences, King Faisal University, Al-Ahsa, Saudi Arabia
e-mail: jkhayri@kfu.edu.sa

## 25.1 Introduction

Date palm (*Phoenix dactylifera* L.), is a monocotyledonous fruit tree belonging to the family Palmaceae (Arecaceae), which inhabit tropical and subtropical habitats where the majority of palms are found. It is an economically important fruit crop widely cultivated in arid and semi-arid regions including the Middle East and North Africa (Al-Khateeb 2006). More than 5000 date palm cultivars are spread worldwide differing in nutritional value, morphological and genetic attributes, although commercial cultivars are limited in number (Abul-Soad et al. 2017). According to FAO statistics (FAOSTAT 2018), the world's largest producer was Egypt ranking first with a total production 1,501,799 metric tons (mt), followed by Iran (1,083,720 mt), Saudi Arabia (1,065,032 mt), Algeria (848,199 mt) and Iraq (6828 mt).

Date palm production faces serious problems such as low yields as well as marketing constraints. These include diseases like Bayoud (*Fusarium oxysporum* f.sp. *albedinis*) and insect pest infestations like red palm weevil (*Rhyncophorus ferrugineus*) and inadequate integrated pest management (Al-Khayri et al. 2018). Furthermore, there are problems as well as deficiencies in harvesting, processing, and marketing practices, shortage of qualified and national trained staff and laborers in addition to insufficient research and development activities (Erskine et al. 2003). The major causes of low date productivity are the large number of the old trees, the existence of many low-quality and undesirable cultivars, lack of sufficient number of offshoots to establish new orchards or even renew the old ones, and the increase in cost of offshoots of good-quality cultivars (Abul-Soad et al. 2017).

Since the spontaneous mutation rate is very slow, induced mutation is crucial to improve the rate of genetic diversity so that breeders can develop the diverse varieties in plant breeding programs (Jatt et al. 2019; Aly et al. 2019).

Mutation breeding is based on the fine art of selecting the "one" genotype, which can bring solution to a given situation, such as tolerance and/or resistance to drought, high salinity content, diseases, and pests. Mutagenesis is method by which sudden heritable changes in the genetic material of an organism induced by mutagens such as chemical, physical or biological agents (Bradshaw 2016; Roychowdhury and Tah 2013). Mutations can be induced at a higher frequency by exposing cells to mutagens (Foster 1991; Miler et al. 2021).

This chapter provides an overview of conventional breeding limitation in date palm and the role of mutation technology. In addition, it presents a case study of mutation induction, date palm in vitro improvement for resistance to Bayouddisease and mutant assessment.

### 25.1.1 Limitations of Conventional Breeding

The date palm is a diploid, cross-breeding species, where male and female inflorescences develop on separate trees. It is conventionally propagated both asexually (vegetatively) and sexually (seeds). The main advantage is the simplicity of its application and in obtaining a great morphological and genetic variation, thus producing a huge number hydrids and creating a massive reservoir of genetic diversity (Jain 2012). Therefore, this technique is very practical in genetic enhancement programs for the selection of genotypes resistant to abiotic and biotic stresses as well as appreciable characteristics (Jain 2012; Hazzouri et al. 2020; Yatta El Djouzi et al. 2020).

Seed propagation provides progeny comprising about 50% male and 50% female (Yatta et al. 2013; Yatta El Djouzi et al. 2014; Abed et al. 2014). Nevertheless, this method cannot be used to propagate elite cultivars with desirable traits or selected genotypes due to high heterozygous characters of the date palm (Tisserat 1982). The inconvenience of this method is long vegetative phase, 4–8 years for the first inflorescences depending on cultivar and growth conditions. Only after the onset of flowering the gender of the date palm tree can be identified and fruit quality can be assessed following fruit maturation (Al-Khayri 2007; Naik and Al-Khayri 2016). Farmers select among palm trees produced by seeds and produced the best dates. The chosen clones are propagated vegetatively.

The process of vegetative propagation is initiated by the development of axillary buds, arising base of the stem, which gives rise to offshoots (Al-Khayri 2007; Yatta El Djouzi et al. 2014; Behnaz et al. 2018). However, the date palm produces only a limited number of offshoots during its life on an average 10–40 offshoots depending on the cultivar (Naik and Al-Khayri 2018, 2020).

This traditional technique remains slow and inefficient to meet the great demand for the rapid expansion of palm groves. This method is also very slow and takes at least 30 years to obtain a few thousand palms from offshoots plantation, and in addition to that, offshoot constitutes a means of dissemination of diseases such as Bayoud (Jain 2012).

The spontaneous mutations occur at low rate (varying from $10^{-5}$ to $10^{-8}$) which is insufficient to be utilized for enhancing variability in crop improvement (Suprasanna and Jain 2017). Induced mutagenesis using physical and chemical mutagen treatment enhance genetic variability in crop improvement programs (Jain and Suprasanna 2011).

### 25.1.2 Role of Mutation Breeding

Food security can be ensured by improving crop tolerance to different environmental stresses (Suprasanna and Jain 2017). Traditional plant breeding techniques, mutagenesis, and genetic engineering, have an essential role in harnessing available germplasm resources to increase the genetic variability and to develop improved Crops (Hallerman and Grabau 2016). Induced mutagenesis is very efficient tools for

the enhancement of crops (Ahloowalia and Maluszynski 2001; Maluszynski et al. 2004; Miler et al. 2021). It helps to expand genetic variation and crop diversity. It contributes to increasing the potential of phylogenetic resources and allows breeders to create more efficient varieties contributing to food security. For this, a large number of mutagenized crop species have been released to farmers and commercialized, thus indicating the economic value of this approach (Suprasanna and Jain 2017). Genomics tools and bioinformatics could be helpful to select suitable mutagenesis techniques heavy ion beams comparing with physical mutagen can be used to create new cultivars with selected target traits without troubling exist characters of parent cultivar (Suprasanna et al. 2015). Precise confirmation individuals and screening of mutants with desirable traits are most importants points in variation breeding (Suprasanna and Jain 2017). Mutant screen is a way for detecting desired selection criteria in putative mutants. Mutant reevaluation and their confirmation need a large mutated population under a restricted environment.

Mutagenesis technique has advantages to obtain mutants in genomic variation with the trait of interest that may possibly be excellent to cultivate under the global change in climatic conditions (Jain 2010).

The Food and Agriculture Organization of the United Nations/International Atomic Energy Agency—Mutant Variety Database reports on the developed and officially released mutants, a total of 3275 accessions from 225 species (FAO/IAEA-MVD 2021).

## 25.2    Mutation Sources

### 25.2.1  Somaclonal Variation

"Somaclonal variation" has been used to refer to genetic variation found in "somaclones" of plants regenerated from any type of tissue cultured in vitro. This concept has been extended to all genotypic and phenotypic alterations occurring during tissue propagation processes. However molecular markers were used to detect variability and screenpositifs variants. They are therefore neutral indicators of genetic variabilty which make it possible to identify polymorphism between species, varieties, populations and even between individuals. Its helps to increase genetic variation and crop diversity. Its contributes to allow breeders to produce more efficient varieties contributing to food security.

In date palm, somaclonal variation and in vitro mutation induction make it possible for select genotypes with new agronomic traits, stress tolerance or quality characteristics (El-Hadrami et al. 2011; Ahloowalia and Maluszynski 2001; Jain 2001; Jain and Maluszynski 2004; Bradshaw 2016), allows the expansion of the date palm genetic base, using micropropagation and tissue culture.

The high rate of desired somaclonal variant can be obtained by introducing selection gents to the tissue propagated in vitro (Jain 2014; Bradshaw 2016). During in vitro tissue propagation, several factors produce genetic variability in these plant tissues. These changes depend on genotype, origin explant, age the culture and

number of subculture cycles used; growth regulators concentrations, in vitro multi-plication technique type, ploidy level (Jain 2011; Krishna et al. 2016). In date palm, the growth regulators depend on genotypes, and sensitivity to certain auxins often trigger variations during in vitro tissue regeneration (Jain 2012). Cytogenetic Studies have reported different number of chromosome pairs and karyotype also reveals changes structure of chromosomes. Various research in date palm have shown that the chromosome number ranging from $2n = 26, 32, 34, 36$ (Al-Salih et al. 1987; Jatt et al. 2019).

However, the information available on these variants remains unknown, sparse, and inconclusive. It is therefore complex to estimate the percentage of this genetic variability and to verify its impacts on this species (El Hadrami et al. 2005). No comparison between these two in vitro techniques has been made due to the long method of tissue cultures (Bradshaw 2016). Note that some date palm cultivars can produce a high rate of genotype conformity, whereas others are more prone to variation. It has also been observed that certain cultivars propagated by tissue culture present a certain level of recalcitrance; particularly for those with desired production and resistance qualities (Bradshaw 2016).

## 25.2.2 Induced Mutation

Induced Mutation is used to enhance the probability of creating a favorable genetic variation in plant improvement and the Mutants are generally obtained by mutagenic agents (Mba et al. 2010; Bradshaw 2016; Miler et al. 2021). The concept of the artificial induction of mutations by ionizing radiation began in the twentieth century, and Mutations can be induced at a higher frequency.

It is used successfully for crop improvement and complements conventional methods (Jain 2010; Amin et al. 2015). For this, several types of mutagens are widely used for genetic variability creation for improving crops (Jain 2010, 2012).

Induced mutations involve exposing seeds, stem cuttings, buds, and tubers to mutagens. The success of mutagenesis is dependent on the genetic purity of parental lines. The intensity of the dose, the type, and the concentration of mutagen varies according to plant material used (Suprasanna et al. 2015; Miler et al. 2021).

There are currently numerous chemical agents (e.g., alkylating agents, azide, hydroxylamine, antibiotics, nitrous acid, acridines, and base analogs) capable of inducing mutations in plant species; however, only a limited number have been used in experimental plant mutagenesis and the selection of plant mutants (Wani et al. 2014; Feldman et al. 2017). The consequence of chemical mutagens on plants is often observed as minimally invasive. Alkylating agents, azide, and hydroxylamine react with bases by adding methyl or ethyl groups. The degradation of the alkylated base can go as far as the production of basic sites to cause mutations through DNA replication.

Nitrous acid causes oxidative deamination in which amino groups are converted to ketone groups; cytosine residues are thus converted into uracil which can pair with adenine rather than guanine. Similarly the deamination of adenines to Cytosine

rather than Thymine. Antibiotics are caused chromosomal aberrations and cytoplasmic male sterility. Acridines intercalate between the bases of the DNA, creating deformations which can cause deletions or insertions leading to shifts in the reading frame. Base analogs can be incorporated into DNA during replication. Thus bromouracil, an analog of cytosine hybridizes preferentially to Adenine, and 2 aminopurine an analog of adenine pairs with cytosine.

The ratio of mutational changes to unwanted changes is usually higher for chemical mutagens than for physical mutagens (Viana et al. 2019).

Since the discovery that physical radiation (such as gamma rays, X-rays, and UV radiation, as well as particulate radiation, fast and thermal neutrons, α and β particles) have mutagenic properties, interest in the use it has grown increasingly to induce genetic variation in crop species.

The mutagenic dose applied is important in any mutagenesis program. The lethal dose-50 (LD50) gives an inspiration of the optimal mutagenic dose which produces the maximum number of mutations with the minimum risk. An overdose of mutagens causes plants death, while a low dose will show lower mutation. The mutagenic dose depends on the concentration, the time and the temperature during the treatment.

The gamma radiation of radioactive cobalt ($^{60}$Co) is widely used; the majority of mutant varieties have been developed (Jain 2011; Suprasanna et al. 2015). Radiation technology and colored polyethylene packaging are recommended for shelf-life, sanitary and phytosanitary purposes of palm date (Mohammadzai et al. 2010) and to for improving fruit quality of date palm (El-Beltagi et al. 2019).

In date, X-rays is used to enhance plant growth and also to improve understanding of the physiological responses imposed by irradiation stress (Jain 2011; Al-Enezi et al. 2012). In recent decades, ion beams have been widely used as replacements of gamma and X-rays and neutrons (Jain 2011; Feldman et al. 2017). Radiation causes chromosomal aberrations, lethality the damage caused by ion beams to double-stranded DNA is less repairable due to the removal of DNA fragments of different sizes (Feldman et al. 2017).

In vitro screening of mutants, usually the selection pressure varies and it is better to determine LD50 dose (Jain 2010). Induced mutations in plant breeding changes in the nuclear DNA, cells, organelles, resulting mutations which are interest to breeders to choose useful mutant; In date palm induced mutations is possible at this time due to a dependable plant regeneration system-induced mutations through somatic embryogenesis and organogenesis.

In date palm, there is any publication done on mutation induction, excepting FAO/IAEA Coordinated Research Project on development of date palm mutant varieties resistant to Bayoud disease in North Africa (Jain 2005, 2006). In date palm, mutant plants were selected in the greenhouse using isolated toxin from *Fusarium oxysporum* f.sp. *albedinis* fungus causal agent (Patade and Suprasanna 2008; Jain 2006) and were transferred to infected field for evaluation, resistance confirmation and selection of other agronomic traits, such as yield and date quality. Actually only 1 mutant of cv. Deglet Nour of 14 putative mutants transferred to a disease-infected field was found to be true resistant after 12 years in the field.

For the putative mutants of cv. Teggaza, 11 vitroplants showing resistance to artificial inoculation of the pathogen were transferred in to infested field planting at INRAA Station, Adrar, Algeria. So far, they are growing very well.

## 25.3   In Vitro Selection

In vitro selection is among the biotechnology techniques frequently used in date palm breeding for tolerance to different stresses, i.e., drought, salinity, cold and diseases and pests (Jain 2011; Nikam et al. 2015; Schaart et al. 2016).

It can shorten the selection time for desirable characters in vitro and can complement field screening (Jain 2012).

Different in vitro culture tools such as suspension cultures and protoplast cultures can be extensively chosen to have a genetic uniformity by adding the selective agents reducing the growth to select tolerant plants.

Mutants have been induced and improved in several plant species (Viana et al. 2019). During in vitro selection, two selections strategies can be used (single-step selection and multi-step selection) (Suprasanna et al. 2012a, b). In the single-step selection, the minimum inhibitory concentration (MIC) is added into the culture medium and cultures are maintained for several subcultures and then resultant surviving tissues are isolated and plants regenerated. In a multi-step selection method, the in vitro cultures exposed to sub-lethal concentration in order to develop and in the following subcultures, a gradual increase in inhibitor level is maintained.

It has been argued, with this process, that selected mutant traits are frequently stable, while variant cells are in stable exposure to improving levels of inhibitor.

### 25.3.1  Abiotic Stress Agents

Cold stress is the most important abiotic stresses that directly affect crop growth and development. Heat stress has a direct impact on the crop resulting in a loss of yield and quality at harvest. Indeed, Heat stress-induced affect the floral organs and the formation of the fruit. Just as meiosis and the filling phase of the seed are sensitive to the rise in temperature. High temperature stress influences the protein synthesis as well as photosynthetic system dysfunction (Brar and Jain 1998; Akter and Islam 2017; Muhammad et al. 2020). Salt stress affects the reduction in the number of leaves, stomatal conductance, protein synthesis, energy metabolism and photosynthesis. The presence of NaCl in the culture medium limits the supply of the plant with major cations, such as potassium ($K^+$) and calcium ($Ca^{2+}$). Bressan et al. (1985) obtained salt-adopted tobacco cells, which were grown for at least 25 generations in 25 g $L^{-1}$ sodium chloride.

Using in vitro tissue cultures to study abiotic stress responses is based on the information that in vitro cultivated cells behave correspondingly to cells of intact plants subjected to abiotic stress situations such as water deficit and salinity stress (Attree et al. 1991).

In vitro culture selection induced by selection pressure alone or associated with mutagenesis is a process to be exploited to enhance genetic variability allowing the creation of new cultivars of cultures (Predieri 2001). This is based on the in vitro culture of plant cells, tissues, or organs on a medium supplemented with selective agents, making it possible to select genotypes one resistant to different abiotic stresses (Pérez-Clemente and Gómez-Cadenas 2012). Drought stress has been caused leaf wilting, a reduction in leaf area, and also raising the rate of photosynthesis. Mainly cases, selection is applied to tissue cultures such as callus, cell suspension, protoplast cultures adding growth inhibitory levels of selection agent in culture medium (Widholm 1972).

In vitro researches in the date palm were conducted to recuperate plants improved tolerance to salinity and drought stress in arid areas. Al Mansoori et al. (2007) examined the effect of sodium chloride (NaCl) on tissue callus issued from immature embryos of local date palm cultivars and they concluded that salinity much affects date palm immature embryos. Al-Mulla et al. (2013) utilized the cvs. Kasab, Barhee and Khalas on in vitro plants to examine their tolerance to salinity under greenhouse conditions. They concluded that both soil salinity and water stress affects date quality and yield. Al-Rokibah et al. (1998) reported a difference in response to salt stress among date palm cultivars grown in Saudi Arabia. Al-Khayri and Al-Bahrany (2004) showed that in response to in vitro drought stress induced by PEG-8000, endogenous free proline content of date palm callus increased gradually in response to increase PEG-concentration. Further research is necessary to optimize in vitro selection and plant regeneration processes to ensure the viability and conserve selected cells totipotency issue from selected cell lines for tolerance to abiotic stress (Al-Khayri and Ibraheem 2014).

Al Kharusi et al. (2017) suggested that there is variation in tolerance to high salinity between different cultivars of date palm. Shoot $Na^+$ exclusion, photosynthesis, and membrane stability are apparently the main determinants of tolerance and can be used in salinity tolerance selection of cultivars. Heat stress affects the growth of plants and productivity in agricultural crops plants. Climate change is predictable to lead to decrease date palm adaptation. This can be restored by escalating the photosynthesis on a leaf area basis provided that the intercept of solar radiation does not modify significantly. The phytohormone abscisic acid (ABA) plays a main role through plant adaptation to different stresses (high salinity, drought, and extreme temperature) (Jain et al. 1998; Zhu 2002).

## 25.3.2 Biotic Agents

Biotic stresses in plants are caused by various pathogens such as fungi, bacteria, viruses, etc. diverse agents lead to plant death such as infections caused by fungi and insects. Others affect photosynthesis per leaf area such as virus infections. Selection systems to separate tolerant lines have been planned using screening with culture filtrates, chemicals and toxins.

Culture filtrates (CF) have been successfully used and corresponds a simple process of selection by adding into the culture media at suitable concentrations. Mutants resistant have been screened by using culture filtrate in vitro and disease resistant plants have been regenerated in many crops (Molot et al. 1984; Ahloowalia and Maluszynski 2001). First reported by Carlson (1973), in vitro selection was conducted in tobacco against *Pseudomonas syringae* for disease resistance. Chawla and Wenzel (1987) used callus cultures of barley and of wheat for selection against *Helminthosporium sativum* and the screening with pathotoxins treatments showed in 6–17% of surviving calli. In vitro selection has huge possible for fast production of helpful mutants resistant to biotic stress (Chandra et al. 2010). Callus and regenerated plants were confirmed for resistance by contact to selection agent of *A. alternate*.

Jain (2010) have been reported results on gamma ray irradiation of strawberries grown in vitro using *Phytophthora cactorum* crude extract; where 5% of the plants survived and they were able to resist drought for 5–6 days.

Jain (2006) have been noted that mutant plants of date palm were screened in the greenhouse after infection with bayoud toxin isolated from fungus causal *Fusarium oxysporum* f.sp. *albedinis*.

Bayoud disease is rampant in North Africa, whereas red palm weevil (*Rhynchophorus ferrugineus*) devastates in the Middle East Asia and Mediterranean regions (Jain 2012). In addition, 34 various fungal and Oomycetes have been found to be associated with root diseases of date palm (Saaidi et al. 1981).

## 25.4  Mutation Induction in Developing Bayoud Resistance

The Bayoud disease, caused by the ascomycete imperfecta fungus *Fusarium oxysporum* special form *albedinis*, is the most serious enemy of Date palm (*Phoenix dactylifera* L.). It has disturbing impact lopsided the ecology of a number of areas and posed serious problems of human, social, and economic problems. It has already destroyed more than ten million palm trees in Morocco and nearly three million in Algeria. The problem is aggravated by pathogen attack on productive cultivars and high commercial values.

Various control strategies are implemented to deal with the disease. However, genetic control remains the most recommended way to reduce or stop the progression of this *Fusarium*. This source of resistance can be found in varieties currently cultivated or in natural populations or induced by gamma irradiation treatment. Confirmation of this resistance must be evaluated by rapid and reliable tests such as the use of toxins or culture filtrates or by artificial inoculations with *F.o.a.* fungus in vitro cultured explants; and mutant evaluation in the glasshouse. Several selected putative mutants were transferred in the field and have maintained tolerance to vascular fusariosis under field conditions. Actually, the resistance is evaluated in contaminated field to obtainBayoudresistant palms, with high-quality dates. Newly developed date palm mutants with Bayoud resistance and high date fruit quality are grown to repopulate the palm groves, devastated by the disease, in the South West

**Table 25.1** Date palm mutant development phases and procedural steps of each phase

| Phase | Procedural steps numbered in a consecutive order | | |
|---|---|---|---|
| Introduction of offshoots for callus induction and irradiation | 1. Obtaining actively growing callus | 2. Irradiation 60 Cobalt of Embryogenic Cultures (200 embryogenic calluses/Dose) | 3. Radio-sensitivity test (Performed on 200 embryogenic callus) (0, 10, 15, 20, 25, 30 Gy) for determining optimal mutagenic dose |
| Selection in vitro using a culture filtrate or toxin | 4. Callus Irradiation (Minimum 1000 callus) with the optimal dose $\leq LD_{50}$ | 5. Callus proliferation (1: M1V1, 2: M1V2, 3: M1V3, 4: M1V4) | 6. In vitro screening of calluses against *F.o.a.* with culture filtrate or fusaric acid. |
| | 7. In vitro selection of somatic embryos against *F.o.a.* with culture filtrate | 8. In vitro selection by detached leaves | 9. Regeneration of putative mutants |
| Evaluation of mutants (Confirmation of Resistance in vivo and in field) | 10. Transfer of germinated plantlets to the acclimatization phase | 11. Greenhouse Evaluation (Treatment of mutant plants with Fusarium toxin and the *F.o.a.* culture filtrate at 35%) | 12. Screening and evaluation using flow cytometric and molecular markers |
| | 13. Field evaluation of the mutant plants (Confirmation of resistance in field and selection of other agronomic traits (date quality and others)) | | |
| Micropropagation of mutant plants | 14. Micropropagation of desired resistant mutant plants exhibiting high date quality | | 15. Multi-site trials |

and center. The use of the mutants obtained in the repopulation of the affected and threatened palm groves by Bayoud would contribute to the protection of the oasis ecosystem, and increase in genetic diversity also they will contribute to the strengthening of the employment policy and to the guarantee food security. The date palm mutant development processes are shown in Table 25.1.

## 25.4.1 Establishment of Embryogenic Cultures

Somatic embryogenesis is the most efficient way huge scale clonal propagation of date palm. It has several supplementary advantages, such as the production of virus-free plants, the capacity to produce large numbers of plants, facilities for change of genetic material at different levels (national, regional, and international), automation potential, opportunities for synthetic seed production, cryopreservation, and genetic manipulations (Jain 2012; Yatta El Djouzi et al. 2014).

**Fig. 25.1** Date palm explants preparation. (**a**) Date palm offshoot isolated from mother plant, (**b**) Removal of leaves and fiber sheets of the out layers, (**c**) Isolated shoot tip, (**d**) Disinfected shoot tip, (**e**) Isolated explants from the shoot tip region, (**f**) Explants cultured on tissue culture medium. (Photos by Al-Khayri JM)

Three date palm cultivars were tested including two cultivars obtained from Adrar oasis, Southwest Algeria (cvs. Taquerbucht and Teggaza), the former is resistant to Bayoud but have low fruit quality, whereas the latter is susceptible but has a good fruit quality. The third cultivar was obtained from Touggourt, Southeast Algeria (cv. Deglet Nour), which is known for high fruit quality but highly susceptible to this disease. Eight healthy offshoots (Fig. 25.1a) weighing 3–5 kg were harvested per cultivar from well-irrigated groves. Outer dry and fibrous leaf sheaths were removed until the inner shoot tip is reached (Fig. 25.1b). Shoot tip region consists of apical meristem bordered by white young leaves surrounding the meristem. The shoot tip region was isolated using a curved knife and trimmed to approximately 10 cm long

**Table 25.2** Composition of culture medium used for callus initiation (M1 supplemented 100 mg L$^{-1}$ of 2,4D) and (M2 contained 12.5 mg L$^{-1}$ of Picloram) and for establishment of cell suspension (M3 contained 5 mg L$^{-1}$ of Picloram)

| Component | Media designation | | |
|---|---|---|---|
| | M1 | M2 | M3 |
| Murashige and Skoog macroelements salts (Murashige and Skoog 1962) | | | |
| Ammonium nitrate NH$_4$NO$_3$ | 1650 mg L$^{-1}$ | 1650 mg L$^{-1}$ | 1650 mg L$^{-1}$ |
| Potassium nitrate KNO$_3$ | 1900 mg L$^{-1}$ | 1900 mg L$^{-1}$ | 1900 mg L$^{-1}$ |
| Potassium phosphate monobasic KH$_2$PO$_4$ | 170 mg L$^{-1}$ | 170 mg L$^{-1}$ | 170 mg L$^{-1}$ |
| Calcium chloride CaCl$_2$, 2H$_2$O | 440 mg L$^{-1}$ | 440 mg L$^{-1}$ | 440 mg L$^{-1}$ |
| Magnesium sulphate heptahydrate MgSO$_4$·7H$_2$O | 370 mg L$^{-1}$ | 370 mg L$^{-1}$ | 370 mg L$^{-1}$ |
| Murashige and Skoog microelements salts (Murashige and Skoog 1962) | | | |
| Boric acid H$_3$BO$_3$ | 6.2 mg L$^{-1}$ | 6.2 mg L$^{-1}$ | 6.2 mg L$^{-1}$ |
| Cobalt chloride CoCl$_2$·6H$_2$O | 0.025 mg L$^{-1}$ | 0.025 mg L$^{-1}$ | 0.025 mg L$^{-1}$ |
| Cupricsulphate CuSO$_4$·5H$_2$O | 0.025 mg L$^{-1}$ | 0.025 mg L$^{-1}$ | 0.025 mg L$^{-1}$ |
| Manganese sulphate MnSO$_4$·7H$_2$O | 16.9 mg L$^{-1}$ | 16.9 mg L$^{-1}$ | 16.9 mg L$^{-1}$ |
| Molybdic acid sodium salt Na$_2$MoO$_4$·2H$_2$O | 0.25 mg L$^{-1}$ | 0.25 mg L$^{-1}$ | 0.25 mg L$^{-1}$ |
| Potassium iodide KI | 0.83 mg L$^{-1}$ | 0.83 mg L$^{-1}$ | 0.83 mg L$^{-1}$ |
| Zinc sulphate ZnSO$_4$·7H$_2$O | 8.6 mg L$^{-1}$ | 8.6 mg L$^{-1}$ | 8.6 mg L$^{-1}$ |
| Iron (Fe-EDTA) source | | | |
| Ferrous sulphate FeSO$_4$·7H$_2$O | 27.8 mg L$^{-1}$ | 27.8 mg L$^{-1}$ | 27.8 mg L$^{-1}$ |
| Na$_2$-EDTA | 37.3 mg L$^{-1}$ | 37.3 mg L$^{-1}$ | 37.3 mg L$^{-1}$ |
| Vitamins MS (1962) | | | |
| Thiamine | 1 mg L$^{-1}$ | 1 mg L$^{-1}$ | 1 mg L$^{-1}$ |
| myo-Inositol | 100 mg L$^{-1}$ | 100 mg L$^{-1}$ | 100 mg L$^{-1}$ |
| Plant Growth Regulators (Hormones) | | | |
| Dimethylallylamino purine (2iP) | 3 mg L$^{-1}$ | 1 mg L$^{-1}$ | 1 mg L$^{-1}$ |
| 2,4-Dichlorophenoxyacetic acid (2,4-D) | 100 mg L$^{-1}$ | – | – |
| 4-Amino-3,4,6 tricloropicolinic acid (Picloram) | – | 12.5 mg L$^{-1}$ | 5 mg L$^{-1}$ |
| Others additives | | | |
| Monosodium phosphate NaH$_2$PO$_4$ | 170 mg L$^{-1}$ | 170 mg L$^{-1}$ | 170 mg L$^{-1}$ |
| Potassium phosphate monobasic KH$_2$PO$_4$ | 100 mg L$^{-1}$ | 100 mg L$^{-1}$ | 100 mg L$^{-1}$ |
| Ammonium citrate | 200 g L$^{-1}$ | 200 g L$^{-1}$ | – |
| Glutamine | 100 mg L$^{-1}$ | 100 mg L$^{-1}$ | 200 mg L$^{-1}$ |
| Adenine | 40 mg L$^{-1}$ | 40 mg L$^{-1}$ | 40 mg L$^{-1}$ |
| Activated charcoal | 3 g L$^{-1}$ | 0.2 g L$^{-1}$ | – |
| Polyvinyl pyrrolidones (PVP) | – | – | 2 g L$^{-1}$ |
| Carbohydrates source | | | |
| Sucrose | 45 g L$^{-1}$ | 45 g L$^{-1}$ | 45 g L$^{-1}$ |
| Solidifying agent | | | |
| Agar | 7 g L$^{-1}$ | 7 g L$^{-1}$ | – |
| pH | 5.84 | 5.84 | 5.84 |
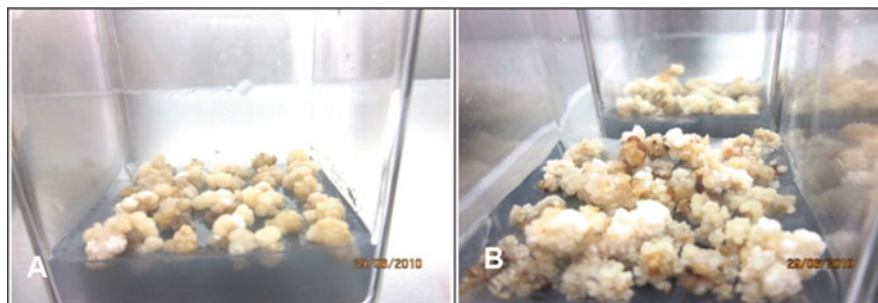
**Fig. 25.2** Compact embryogenic calli (**a**) and friable embryogenic calli exhibiting prolific growth after 6 months the shoot tip explants cultured (**b**). (Photos taken in 2010 by Yatta El Djouzi D, Khelafi H and Abed F)

and 5 cm diameter (Fig. 25.1c). This tissue was disinfected in 3 g L$^{-1}$ Benomyl solution for 45 min, and then sterilized for 20 min in 12% sodium hypochlorite (750 mL) solution containing 100 mg L$^{-1}$ potassium permanganate and 2 mL L$^{-1}$ Tween 20. The shoot tip was then rinsed with sterilized distilled water three times, 5 min each (Fig. 25.1d). Under aseptic conditions, a layer of the out-leaf tissue near the shoot tip was detached and meristem region fragmented into 8–12 explants approximately 1 cm$^3$ (Fig. 25.1e). The shoot tip explants were cultivated on two modified MS media (Murashige and Skoog 1962) designated M1 and M2 (Fig. 25.1f). Their compositions are listed in Table 25.2.

The pH adjusted to 5.7 before autoclaving at 120 °C for 20 min. The cultures were incubated in darkness under a thermoperiod of (28 ± 2 °C for 16 h and 22 ± 2 °C for 8 h) for six to eight subcultures at 5 weeks intervals until callus was produced. Explants which have increased in volume were fragmented into several fractions. The enlargement of the explants was observed between 5 and 9 weeks after their cultivation. As soon as the explants presented small white lumps of a nodular appearance, they were isolated from explant and transferred to new culture medium. The duration of appearance of the first embryogenic strains for the cv. Deglet Nourwas 9 months. The embryogenic calli obtained were of two types; compact and friable (Fig. 25.2a, b).

## 25.4.2 Irradiation of Embryogenic Cultures

The first step in vitro mutagenesis protocols is to initiate the multiplying calli cultures with high regeneration capacity. Gamma rays are ionizing electromagnetic radiation used on calli culture to induce the mutations. The objective of this work was to test the different doses of cobalt 60 on embryogenic callus of the Deglet Nour and Teggaza cvs. of the date palm to define the threshold of radiosensitivity and determine the optimal dose of irradiation. Embryogenic Calli (Minimum 1000 callus), 1–2 mm in size, were irradiated to diverse levels of gamma rays from a
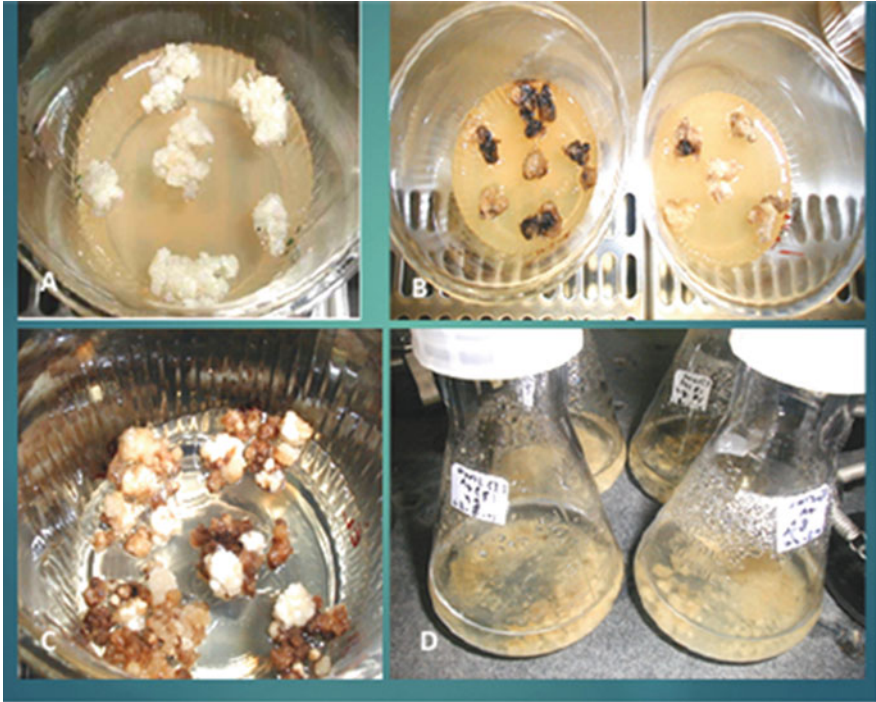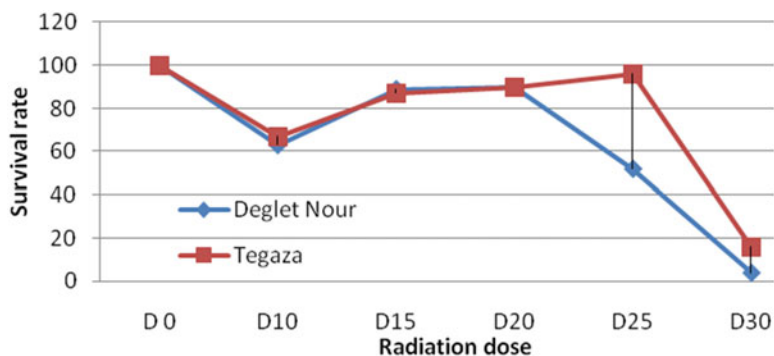
**Fig. 25.3** Embryogenic callus irradiated (**a**) embryogenic callus necrosis with living calli in white and dead in brown (**b**), proliferation of survival calli (**c**) and cell suspension cultures (**d**). (Photos by Jain SM)

Cobalt 60 source at dose of 0, 10, 15, 20, 25, and 30 Gy, which was conducted at National Nuclear Center Research (CNRA), Algiers, Algeria. Each treatment consisted of 200 embryogenic calli and 20 embryogenic calli non-irradiated were used as controls. Instantly after irradiation, the embryogenic calli were placed onto fresh solid culture medium (Fig. 25.3a).

After 1 month, the $LD_{50}$ was determined with counting the number and the percentage of survival (white) and death (brown) calli (Fig. 25.3b, c). Cultivars Deglet Nour and Teggaza varied in their percentage survival at various irradiation doses (Fig. 25.4). The percentage survival was constantly elevated in cv. Teggaza than cv. Deglet Nour. The survival rate of both cultivars decreased with dose increases.

These strains were completely necrotic (95.84%) and were found to be incapable of reacting with the exception of one single strain. At the 25 Gy dose, nearly half of the strains do not survive (47.83%). For the 10, 15, and 20 Gy doses, browning of the calluses is reversible and the majority of the strains survive (Table 25.3). We noted, however, an irreversible necrosis of 7 strains at the 10 Gy dose, 2 strains at the 15 Gy dose, and 1 strain at a dose of 20 Gy, which respectively represents a mortality rate of 36.85%, 11.12%, 5.56%, 11.12% and 5.5%.

**Fig. 25.4** Percentage of survival irradiated embryogenic calli of Deglet Nour and Teggaza after 1 month of culture

### 25.4.2.1 Gamma Room Cobalt 60 Characteristic

The self-protected irradiator is suitable for processing samples for research and for the calibration of dosimeters. This is type B01 equipment (Transelektro Ltd, Budapest) integrating 24 Cobalt 60 source pencils with an overall activity of around 9604.65 Ci (at the date of irradiation), of a conveying system of the irradiation container. An automatic device allows the irradiation duration to be programmed. The flow dose and the transit reference point dose were determined by the reference dosimeter of Fricke positioned at National Nuclear Center Research (CNRA), Algiers, Algeria. The glass samples were irradiated at doses of, a distance of 20 cm and a dose rate of 11.21 Gy min$^{-1}$ at room temperature. Dosimetry is performed using ionization chambers of varying volumes connected to Fricke Dosimeter. The instrument and ionization chambers are calibrated by the Instrument Calibration Section of the Radiation Biology and Health Physics Branch, against National Research Council of Algeria standards.

### 25.4.2.2 Post-irradiation

The multiplication of the irradiated calli was carried out on modified MS media of the same composition as those of the induction step and placed in the dark. Subcultures take place every 2 months, the time necessary to observe callus proliferation. Four subcultures were carried out to arrive at the stage which was qualified as M1V4, i.e., 8 months after irradiation (Table 25.4).

The Irradiation of multicellular structures such as seed, meristem tissue or offshoots can lead to chimeras in regenerated plants which would require dissociation by plant multiplication up to the M1V4 generation (Jain 2012, 2010).

In the case of the palm tree, data about the next generation of irradiated sources is not available at this time. In our experiment, we used callus proliferation, i.e., cell divisions or cell proliferation from the irradiated mother cell as a generation. The secondary callus could have been considered a generation, but it was only encountered in the case of the 30 Gy dose, where the necrotic strain.

**Table 25.3** Effects of gamma irradiation doses on the embryogenic calli of *Phoenix dactylifera* after one 1 month of culture

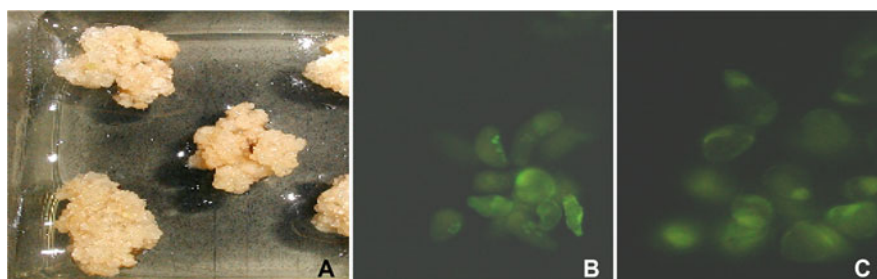| Deglet Nour | | | | Teggaza | | | |
|---|---|---|---|---|---|---|---|
| Irradiation dose | Number of irradiated calli | Survival number of embryogenic calli | % of embryogenic calli necrosis | Irradiation dose | Number of irradiated calli | Survival number of embryogenic calli | % of embryogenic calli necrosis |
| 0 | 16 | 16 | 0 | 0 | 30 | 30 | 0 |
| 10 | 19 | 12 | 36.85 | 10 | 30 | 20 | 33.33 |
| 15 | 18 | 16 | 11.12 | 15 | 30 | 26 | 13.33 |
| 20 | 20 | 18 | 5.56 | 20 | 30 | 27 | 10 |
| 25 | 23 | 12 | 47.83 | 25 | 30 | 29 | 3.33 |
| 30 | 24 | 1 | 95.84 | 30 | 30 | 5 | 83.33 |

Source: Yatta and khelafi (2012); Yatta et al. (2004)

**Table 25.4**  Proliferation of embryogenic calli from cv. Deglet Nour after fourth subculture

| Irradiation dose (Gy) | Number of initial irradiated calli | Number of strains remaining at the first subculture | Number of strains obtained after 4 subcultures | Particularity of the calluses obtained |
|---|---|---|---|---|
| 0 | 16 | 16 | 30 | Friable callus + or − vitrified calluses |
| 10 | 19 | 12 | 16 | Heterogeneous calluses: vitrified + or − friable + nodular calluses |
| 15 | 18 | 16 | 22 | Heterogeneous calluses: vitrified + or − friable + nodular calluses |
| 20 | 18 | 17 | 67 | Friable and grainy calluses |
| 25 | 23 | 12 | 25 | Friable and grainy calluses |
| 30 | 24 | 01 | 01 | Loose-textured secondary calluses |

Source: Abed et al. (1999)



**Fig. 25.5**  Obtaining irradiated calli from Deglet Nour at stage M1V4 (**a**) Viable Calli of Deglet Nour (**b**) and of Teggaza (**c**). (Photos by Yatta El Djouzi D)

### 25.4.2.3  Optimal Dose of Mutagen

In our mutagenesis process, first main step is to establish the suitable mutagen doses to achieve best results. Dose 30 Gy can be considered as the lethal dose ($LD_{100}$). At the 25 Gy dose, half of the strains do not survive and is considered as the $LD_{50}$. The optimal dose for the irradiation of the strains is estimated at 20 Gy of gamma for Deglet Nour and 25 Gy of gamma irradiation for cultivars Teggaza. Four subcultures were carried out to reach the M1V4 (from stage, i.e., 8 months later irradiation). After treatment with mutagenic, The viability of irradiated calli was assessed by Fluorescein di acetate (FDA) under an inverted microscope (Fig. 25.5a–c). The observation of these cellular masses reveals large cellular aggregates of variable size. The irradiated calluses of more or less compact texture give rise to aggregates of amyliferous cells capable of dividing and mixed with elongated non-amyliferous

cells. The increase in the number of cells and aggregates, and the maintenance of a certain level of viability (80–99%) indicate the favorable action of the culture medium used for cell multiplication.

### 25.4.2.4 Proliferation of Irradiated Callus

The number of subcultures depends mostly on the genotype, $LD_{50}$ dose and on other factors, such as propagation calli rate. After their transfer to germination medium, the well-differentiated embryos germinated and gave seedlings morphologically identical to the control seedlings. Irradiation of embryogenic calli of date palm made it possible to determine the threshold of sensitivity. After 1 month of culture, the embryogenic calli irradiated at a dose 30 Gy were completely necrotic and proved incapable of reacting except for one, which reveals that the toxic dose or close to the lethal ($LD_{100}$) is from 30 Gy. After the 25 Gy dose, half of the calli do not survive. The $LD_{50}$ would be this dose. For doses 10 and 15 Gy browning of the callus is reversible and the majority of the calli survive, which shows that the calli are resistant to these doses. It was observed that different sensitivities of the embryogenic calli to the irradiation doses. On the doses 10 and 15 Gy the percentage of mortality varied respectively from 36.85% to 11.2%, whereas for a higher dose 20 Gy 5.56% was recorded (Table 25.4). The proliferating irradiated callus appears to be heterogeneous. These results obtained during the proliferation of irradiated callus have shown that with dose 10 and 15 Gy, the proliferation rate is 1.3, whereas with doses 20 and 25 Gy, the proliferation rate is respectively 36.85% and 11.12%. This leads us to suppose that the irradiated callus exhibited heterogeneity in the stages of cell differentiation. The sensitivity of the callus to decreasing dose can be explained by different cell diversity, or by fact that some calli are in the multiplication phase or in the differentiation phase. Indeed, Devreux et al. (1986) showed that protoplasts with high cell density are less radiosensitive than those at low density. Furthermore their results also demonstrated unambiguously that differentiating callus are more sensitive than those that are only actively multiplying.

### 25.4.2.5 Histological Analysis of Calli

Irradiated callus fragments were fixed in solution containing in 100 mL, 4 mL 25% glutaraldehyde solution, 50 mL phosphate buffer at pH 7.2, 20 mL of 10% paraformaldehyde solution, 1 g caffeine, and 26 mL distilled water (Schwendiman et al. 1988). The fixation is carried out for 48 h with a passage of 2–3 h under vacuum to facilitate the penetration of the fixer and eliminate the air contained in the tissues. The samples are then rinsed and preserved in alcohol. Dehydration is an operation that is done by successive passages of the calluses in alcohol baths at increasing concentrations. Impregnation of the organs is done by placing the organs in an impregnation mixture composed of 100% alcohol and toluene for 45 min. The samples are then placed in three successive baths of toluene, 1 bath of toluene and paraffin and 1 bath of paraffin. Impregnation of the organs is done by placing the organs in an impregnation mixture composed of 100% alcohol and toluene for 45 min. The samples are then placed in three successive baths of toluene, 1 bath of toluene and paraffin and 1 bath of paraffin. The samples are placed in molds
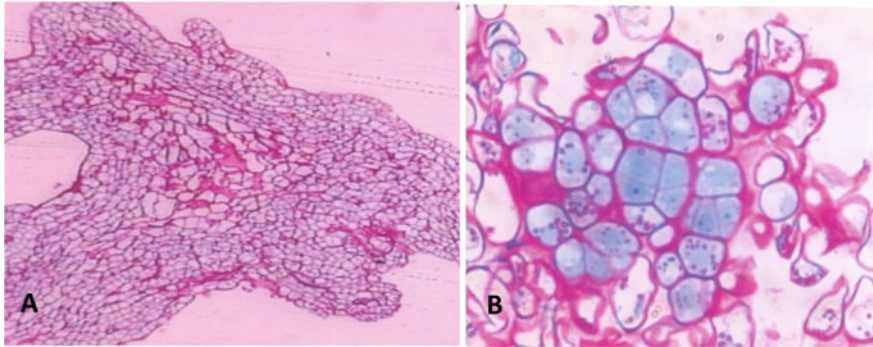
**Fig. 25.6**  Histology of calli on the floral explants (**a**). Early stage of cell suspension (**b**). (Photos by Yatta El Djouzi D and Abed F)

containing a quantity of approximately 1.5 mL of paraffin. Polymerization takes place at room temperature overnight. The sections are made using a microtome (Leica RM 2125RT) at a thickness of 10 μm and spread in the form of ribbons on glass slides using a brush. The sections are dried on a plate set at 40 °C.

The sections are stained by a double stain with Periodic Acid Schiff (PAS) which stains the polysaccharides of the walls in red, the mucus and the starchy reserves (cellulose, pectic compounds, hemicelluloses, etc.) in purplish pink. Naphthol Blue Black stains the cytoplasm (soluble proteins) light blue, the nucleus and nucleolus dark blue and the protein bodies (protein reserves) dark blue. After 6 months of culture in the Murashige and Skoog (MS) medium containing 100 mg L$^{-1}$ of 2,4-D, the histology shows that after culturing show the presence of compact granular calli which consist of numerous independent spherical globules with a diameter varying between 300 and 500 μm. They are meristematic and well individualized near the vascular tissues. These meristematic cells of the nodules present a nucleus and a dense cytoplasm very rich in protein reserves intensely stained in black by Naphthol Blue Black (Fig. 25.6a). They are surrounded by dense cell layers that are visible on the periphery of the nodules. These cellular areas are the place of numerous divisions and function as a cambium (pseudo cambium) which is at the origin of the growth of nodules. Nodules have three concentric cell layers—a central zone (ZC) formed of meristematic cells, an intermediate zone made up of pseudo-cambial cells in active division, a peripheral zone (ZP) formed of several less active cellular layers. Compact calluses consist of the juxtaposition of more or less voluminous nodules, linked together by inactive cells forming light mucilage which allows the cohesion of the whole. They actively divide between the 6th and 12th month of culture, giving rise to embryogenic stem calluses which are made up of embryogenic cells containing large nuclei very rich in soluble proteins (Fig. 25.6a). These observations are analogous to those already reported for palmaceae such as *Elaeis guineensis* (Chwendiman et al. 1988). Callus proliferation near vascular tissues has also been observed with leaf explants of Cocos nucifera (Buffard-Morel et al. 1992), and in other species such as *Gossypium hirsutum* (Gawel et al. 1986).

The histological sections made during the evolution of the embryogenic cell masses. After 1 month of culture in liquid medium, made it possible to distinguish single cells or groups of embryogenic cells surrounded by a thick outer wall intensely colored in pink, and which allows them to be separated from the surrounding degenerative tissues. These observations were also highlighted by Verdeil et al. (2001). Also, the suspensions are accompanied by a significant accumulation of reserve substances of a starchy and/or lipoprotein nature which are good indicators of tissue development towards embryogenesis (Chwendiman et al. 1988; Verdeil et al. 2001). Unlike the date palm, obtaining a friable callus is not essential for the establishment of cell suspensions in some species. This is the case for cassava and alfalfa. Cell suspensions can be directly initiated by transfer of leaf fragments in liquid medium (Raemakers et al. 1993).

### 25.4.3  In Vitro Resistance Screening

#### 25.4.3.1  Extraction and Fraction of Fusarium Toxin

The aggressive strain 133 of *Fusarium oxysporum* f.sp. *albedinis* was grown on PDA medium (Potato: 250 g; 20 g: glucose and 15 g agar) for 5 days in the fungal culture was flooded with sterile water to prepare conidial suspension and the concentration was attuned to $10^6$ spore mL$^{-1}$. One millilitre of the conidial suspension was transferred into Erlen Meyer flasks containing 200 mL Czapeck medium (2 g NaNO$_3$, 1 g K$_2$HPO$_4$, 0.5 g KCl, 0.5 g MgSO$_4$·7H$_2$O, 0.01 g FeSO$_4$ 7H$_2$O, 30 g sucrose, 1000 mL distilled water) and shaken at 200 rpm on a rotary shaker at 25 °C for 10 days. The cultures were filtered with Whatman N°1 filter paper, the filtrate was centrifuged at 4200 g for 20 min, and the supernatant was used for further toxin extraction. The extraction was performed on the *F.o.a.* culture filtrate according to the method of Pringle and Scheffer (1963), modified by El Fakhouri et al. (1996).

Five liters of culture filtrate were evaporated under vacuum below 45 °C pending its initial volume was reduced to 200 mL. An equal volume of methanol was added and the mixture was placed at 4 °C for 48 h. After this time, the precipitated material was removed by filtration, washed two times with a cold methanol/water (1:1, V/V) mixture, and discarded. The resulting filtrate was vacuum-evaporated at 45 °C and the entire volume was reduced to 150 mL. This liquid was poured on a glass column (3 × 40 cm) of Norite mixed with Celite mixed and equilibrated with water. The column was then washed with distilled water until the eluate was completely clear. The absorbed fractions were then eluted by three successive steps of mixture of 10%, 30%, and 50% pyridine in water that generated three fractions: FI, FII, and FIII, respectively (Sedra et al. 1993; El Fakhouri et al. 1996; Sedra and Lazrek 2011). The fraction FII was used for the selection because it showed the most phytotoxic activity during biological assays (Sedra et al. 2008).

The process of infecting a plant with a pathogen begins with the establishment of contact between the two protagonists of the parasitic relationship. The pathogenic agent may use several modes of action to penetrate into plant tissue to overcome mechanical, chemical and/or physiological barriers of the host plant. Pathogenic

fungi often damage their host plants by producing toxic metabolites, which causes various symptoms toxins these metabolites could be involved in the pathogenicity (Berestetskiyis 2008). Phytotoxics are classified into two broad groups, those which are nonspecific and those which are host-specific toxins. Some of the toxins have general phytotoxic properties and are active on a wide range of plant species. These are non-host-specific toxins. They contribute to the virulence or development of symptoms of the disease in which they occur, but are not the primary determinants of the host range. Other toxins are host specific and affect only certain plant varieties (Osbourn 2001). Specific toxins play a role in determining the host range of specificity of plant pathogens and can act as a virulence agent for these pathogens. The majority of the special forms of *Fusarium oxysporum* and *Fusarium* species, in general, produce toxins which are derived from primary or secondary metabolism, the nature of which is very diverse: protein (Mussel 1972), terpenic (Casinovi 1972). The first toxic substance, of peptide nature, which was isolated and purified from cultures of *Fusarium oxysporum* f.sp. *lycopesici* is lycomarasmin (Messiaen and Cassini 1968).

The first work carried out on the toxins of *Fusarium oxysporum* f.sp. Surico and Graniti (1977), who isolated fusaric acid and anydro-aspergillomasarin A and B. *F.o. a.*, secretes into a liquid culture media numerous substances like toxins (Mokhlisse 1987; Sedra et al. 1993, 1997); and enzymes (Amraoui et al. 2004). Some of them have been chemically and biologically well characterized, such as fusaric acid and its derivatives which were isolated from culture filtrates (Mokhlisse 1987; Sedra et al. 1993, 1997). Fusaric acid may play an important role in the early stage of date palm infection by *F.o.a.* (Bouizgane et al. 2004; Sedra et al. 2008).

The extraction of secondary metabolites was performed on *F.o.a.* culture filtrates according to the method of Pringle and Scheffer (1963). During extraction, the adsorbed fractions were diluted by three successive steps of mixtures of 10%, 30%, and 50% pyridine in water (v/v) that, respectively, gave three collected fractions labeled FI, FII, and FIII. These fractions were evaporated until dry and a yellowish powder was obtained for FI, while whitish ones were obtained for the FII and FIII fractions. However, the FII (*F.o.a.*) fraction contains the fusaric acid and other toxic sub-fractions (H3, H4, H5 and H6).

### 25.4.3.2 In Vitro Selection of Irradiated Materials

In vitro screening of irradiated materials is based on the use of purified or crude culture filtrates of the pathogen as a selective agent (culture filtrate from *F.o.a.*). After the radio-sensitivity test, the irradiated calli were irradiated with a 20-Gy dose for Deglet Nourand a 25-Gy dose for Teggaza, then were transferred in the same fresh medium until they reach the M1V4 stage. In our experiment, we used callus proliferation, i.e., cell division or cell proliferation from the irradiated calli as a generation. The secondary callus could have been considered a second generation, but it was only encountered in the case of the 30 Gy dose, where the necrotic embryogenic callus screening of 500 calli using 10% *F.o.a.* filtrate culture. Surviving calli have been selected using a culture medium containing 10% culture filtrates were transferred on liquid medium (Fig. 25.7).
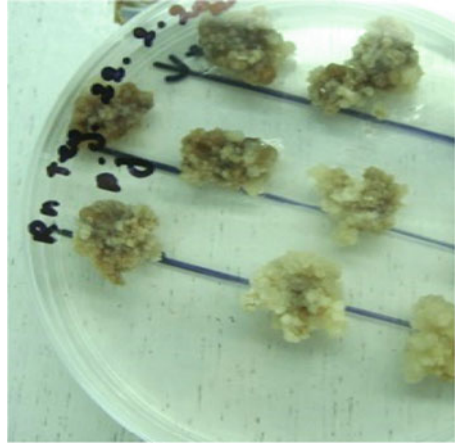
### 25.4.3.3 Embryogenic Suspension Cultures

Cell suspension cultures are useful for date palm genetic enhancement based on in vitro selection studies (Jain 2012). A number of researchers successfully obtained in vitro plant regeneration using date palm embryogenic cell suspension cultures (Al-Khayri 2012; Naik and Al-Khayri 2016). These studies proved that cell suspension culture is a prolific source of somatic embryos, suitable for mass propagation of several date palm cultivars.

Embryogenic cultures of "Deglet Nour" and "Teggaza" were transferred in liquid initiation medium in 125 mL Erlenmeyer flasks (40 mL medium per flask) on a rotary shaker (110 rpm), and maintained as suspension cultures and sub cultured into fresh medium at 2-week intervals.

A growth curve was established based on fresh weight of cell suspensions over time. Weighing was carried out regularly before and after each subculture, and estimate the increase of the fresh weight of cell suspensions. The Fig. 25.8 expresses the growth cell suspensions development curve in two cultivars. From the beginning time of suspension culture, the lag phase was indiscriminate and translated into a latency phase, this is not observable. It started during first week of cell culture, between the first and 6 days and embryogenic masses started to isolate from mother callus tissue. The diameter of the particles varied from 2 to 3 mm. From the second week in a liquid medium, the callus weight doubled. The exponential phase, where the highest growth rate occurred, was started 1 week after culture initiation.

After 5 weeks, growth deceleration had occurred and lasted until week 10 and a stationary phase that starts from sixth subculture. At this level, the growth appears nil, this is due to a loss of activity where the multiplication rate is equivalent to that of mortality.

After 3 weeks of the culture, the clusters formed were removed by sieving through a 380 μm sterile sieve to keep only small embryogenic cell suspension. The cultures were subculturing weekly by fresh liquid medium until obtain small pro-embryonic cell aggregates. Embryogenic cultures of Deglet Nour and Teggaza
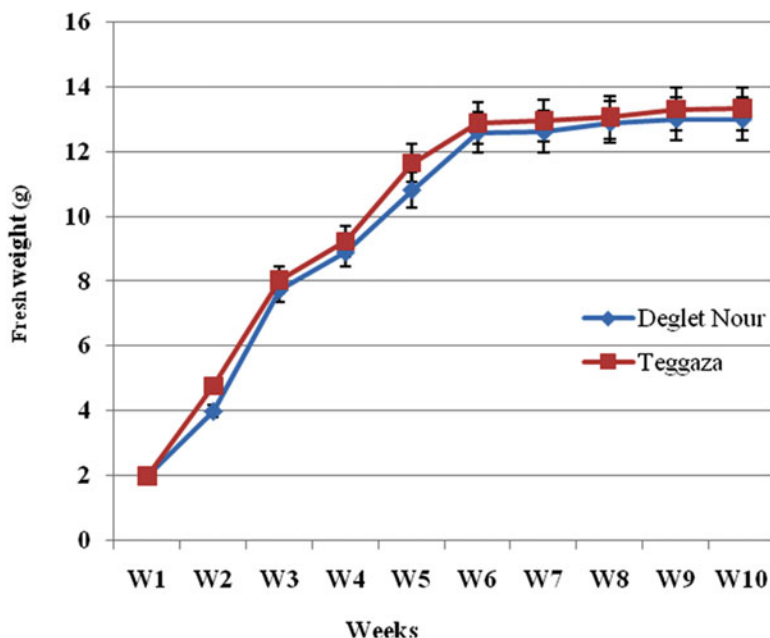
**Fig. 25.8** Growth cell suspension evolution in two cultivars Teggaza and Deglet Nour. (Constructed byYatta El Djouzi D)



**Fig. 25.9** Somatic embryos (**a**) and pro-embryonic cell aggregates (**b**) obtained in liquid media M3. (Photos by Yatta El Djouzi D and Abed F)

in suspension had similar morphologies, and consisted of small pro-embryonic cell aggregates (heap retained on the filter >380 μm) (Fig. 25.9a). Suspension cultures of Teggaza on the other hand, consisted entirely of pro-embryonic cells and small aggregates of pro-embryonic cells (Fig. 25.9a, b).

In date palm, several researchers successfully obtained in vitro plant regeneration using cell suspension cultures (Bhaskaran and Smith 1992). These studies proved

that cell suspension culture is a productive source of somatic embryos, suitable for mass propagation of several date palm cultivars.

### 25.4.3.4 In Vitro Somatic Embryos Selection

Compact spherical nodules correspond to proembryos. They are maintained under the same culture conditions. These nodules or proembryos evolve into small elongated shapes, varying in size between 7 and 8 mm and are characteristic of somatic date palm embryos. These latter can be isolated. 5200 embryogenic somatic embryos obtained were transferred on a medium culture containing culture filtrates concentration at 35% and 1800 somatic embryos have been selected (Fig. 25.10a–d). The embryogenic somatic embryos are located on germination medium devoid of growth substance at a photoperiod of 16 h. After 6 months of culture, the well-differentiated embryos developed and gave rise to plants. At doses 10 and 15 Gy, the well-differentiated embryos germinated to give green seedlings. Callus is observed which evolves either in roots (46% and 45%) or completely necrosis without any evolution (Table 25.5).

The synchronization of somatic embryo germination is observed for the control cultures as well as for the treated samples. The green plants obtained are not morphologically different from those obtained with the control. However, certain abnormalities were observed during germination in embryos irradiated at different doses. Forty-five percent of germinating somatic embryos gave roots only on doses 10 and 15 Gy, although this phenomenon was not observed at dose 30 Gy. Rooted
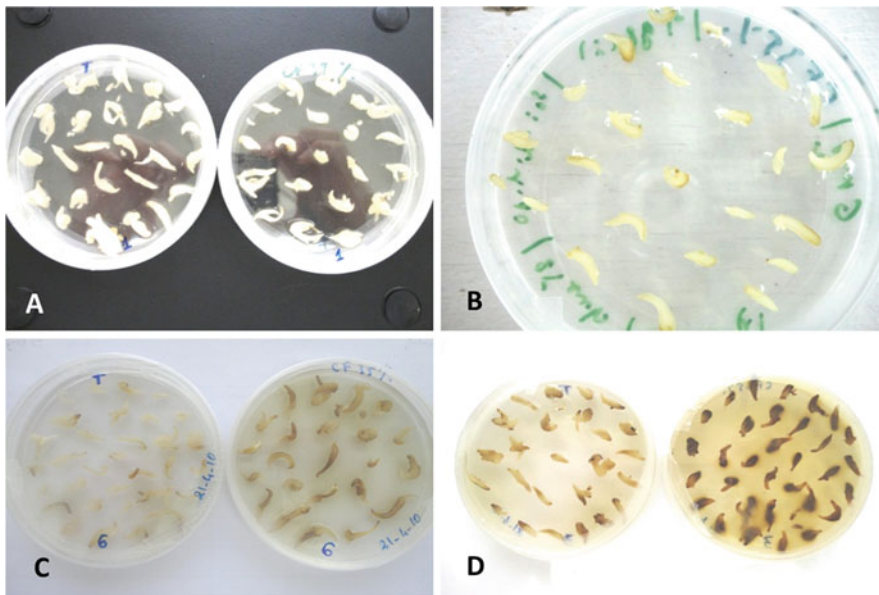


**Fig. 25.10** In vitro selection of somatic embryos using the Fusarium culture filtrate at 35% (**a–d**). (Photos by Khelafi H, Abed F and Yatta D)

**Table 25.5** Effect different irradiation doses on the germination of somatic embryos

| Dose (Gy) | Number of somatic embryos germinating | Number of germinated somatic embryos | | Green shoots and roots | | Cotyledons and roots | | Roots only | |
|---|---|---|---|---|---|---|---|---|---|
| | | N** | %*** | N | % | N | % | N | % |
| 0 | 15 | 10 | 66.6 | 9 | 60 | 1 | 66 | 0 | |
| 10 | 15 | 08 | 53.3 | 9 | 60 | 7 | 46.6 | 7 | 46.6 |
| 15 | 22 | 10 | 45.5 | 7 | 31.8 | 10 | 45.4 | 10 | 45.5 |
| 20 | 50 | 20 | 40 | 20 | 40 | 13 | 26 | 0 | |
| 25 | 15 | 10 | 66.6 | 10 | 66.6 | 4 | 26.6 | 0 | |
| 30 | 01 | 0 | | 0 | | 0 | | 0 | |

Table constructed by Khelafi H

N.S.E.G: Number of somatic embryos germinating, N.E.S.G: Number of germinated somatic embryos, P.V.R: Green plants + Root, C.R: Cotyledons + Roots, N**: Number of germinated somatic embryos, %***: percentage of Number of germinated somatic embryos

fused cotyledons were 45% at dosages 10 and 15 Gy, 26% at dosages 20 and 25 Gy, and 66.6% in the control. The 20 Gy dose was determined to be the optimal irradiation dose for the irradiation of date palm callus.

Similar studies in vitro cultures of date palm in Morocco and Tunisia, on different cultivars, different steps of differentiation, showed that the irradiation dose was around 20 Gy (Jain 2011). Donini (1991) locate the irradiation dose at the Gamma ray close to the $LD_{50}$ dose, where 50% of the irradiated material survived, which confirms the results obtained. The aim of this work is to arrive at determining the radiosensitive dose, and to follow the effect of the irradiation doses on the stages of somatic embryogenesis of Deglet Nour. The lethal dose in which irreversible necrosis of all the embryogenic calli is observed is 30 Gy and represents the $LD_{100}$.

The $LD_{50}$ dose where half of the irradiated calli survive is 25 Gy. Remaining strains proliferate and the germinating somatic embryos have given rise to whole plants. Following these results, additional studies are to be carried out to improve the process for obtaining homogeneous embryogenic strains and to monitor work aimed at obtaining variants combining date quality and resistance to Bayoud.

### 25.4.3.5 Regeneration of Putative Mutants

The irradiated and selected calli gradually were transferred into the germination medium to permit the induction of the somatic embryos and their germination. The culture medium was used is the MS base medium (PGRs-free MS4) medium supplemented with 60 g $L^{-1}$ sucrose, 1 mg $L^{-1}$ Thiamine HCl, and 40 mg $L^{-1}$ adenine. The embryogenic strains are maintained in darkness under a thermoperiod of 28 ± 2 °C for 16-h and 22 ± 2 °C for 8 h. The transfer to a new medium is done 2-months intervals. Somatic embryogenesis goes through three stages, namely: somatic embryos induction, somatic embryos into seedlings and development of the root system. The proembryos which are compact spherical nodules are maintained under the same culture conditions. They then evolve into small,
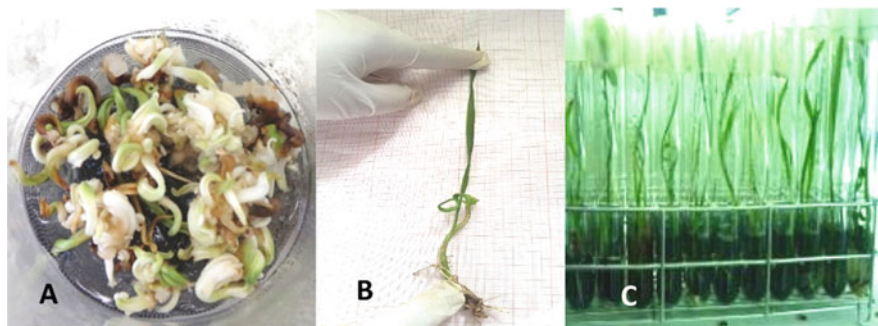
**Fig. 25.11** Somatic embryos Maturation and Embryos germination of Deglet Nour (**a**), somatic seedlings (**b**) and seedlings shoot and roots (**c**). (Photos by Yatta El Djouzi D)

elongated shapes and are typical of somatic date palm embryos. The proembryos may appear separate or remain in clumps. The first somatic embryos appeared after 6 months of their transfer.

Embryos evolve and germinate under the same culture conditions. The cotyledon and radical appear. There is a lengthening of the cotyledon which has at its base the cotyledonary slit from which a first leaf emerges (Fig. 25.11a). Embryos evolve and germinate under the same culture conditions (Fig. 25.11b, c).

### 25.4.3.6 In Vitro Selection by Detached Leaves

Leaves, 20–25 cm in size, were collected from 20 in vitro-plants, issued from irradiated callus, and evaluated for their confrontation to *Fusarium* wilt. Two concentrations, 25 and 50 µg mL$^{-1}$ toxin fractions FII were used. Sterilized water was used as a control. The standard concentration used to screen resistant from sensitive materiel was 50 µg mL$^{-1}$ (Sedra et al. 1993). This concentration can be increased to 100 µg mL$^{-1}$ if large size detached leaves are used. There is a relation between the weight of leaves and the quantity of toxin used (Sedra et al. 1998). The leaves were dried first using cotton, soaked with ethyl alcohol and then with sterile water. They were immersed in test tubes containing 25 mL toxin fraction FII. For each treatment, three leaves per concentration and per vitro-plants were used. For each trial, the treatment is repeated three times. Tubes carrying leaves were maintained under daylight, at an ambient temperature between 22 and 27 °C. The symptoms were observed by measuring the progression of necrosis the petiole every 3 days for 22 days.

### 25.4.3.7 Test of Artificial Inoculation of Plants

Twenty vitro-plants, previously tested against toxin, were inoculated with 100 mL *F. o.a.* conidia suspension, at the concentration of $10^6$ sp mL$^{-1}$. All tests were carried out in the greenhouse. One Deglet Nourvitro-plant was used as a control, grown in 100 mL water. Weekly assessment was carried out on the mortality of inoculated

plantlets. From sensitive plants, re-isolation of the *F.o.a.* from roots was performed on the P.D. A medium (Potato dextrose agar).

### 25.4.3.8 Effect of Fraction FII of *F.o.a.* Toxin on Detached Leaves of Vitro-Plants

Three days after immersion of the leaves in different concentrations of toxin, fraction FII of *F.o.a.*, browning was observed at the base of the petiole of all treated leaves (Fig. 25.12a–c). This was a wound response to sectioning of the petiole. After 5–6 days, the necrosis progressed in leaves soaked in fraction FII of toxin.

At toxin concentration of 25 µg mL$^{-1}$, the necrosis varied between 1 and 11 mm in length, whereas it varied between 3 and 40 mm in response to 50 µg mL$^{-1}$ toxin fraction. In the control, necrosis did not exceed 2 mm in length (Table 25.6). The response to the use of 25 and 50 µg mL$^{-1}$ FII fraction of *F.o.a.* toxin on detached leaves of 20 plantlets regenerated from irradiated callus of Deglet Nourcultivar showed that the size of the necrosis increased with increasing dose of the toxin.

The differences in necrosis size varied between 1 and 37 mm in the presence of toxin concentrations 25 and 50 µg mL$^{-1}$. The necrosis size of vitroplants (M7, M12, M13, M17, and M19) did not exceed 1 mm. Other five vitroplants (M9, M10, M14, M15, and M16) had necrosis size between 2 and 5 mm. The size of detached leaves of eight vitroplants (M1, M2, M4, M6, M8, M11, M18, and M20) varied between 7 and 15 mm. However, necrosis size was 19 mm for M5 and 37 mm for M3 vitroplants.

A correlation between vitroplants noted that showed resistance to artificial inoculation andthe size of the necrosis caused by the treatment of FII fraction of *F.o.a.* toxin on detached leaves (Fig. 25.13). The size of necrosis on detached leaves treated with 25 µg mL$^{-1}$ toxin varied between 1 and 7 mm in vitroplants that had resistance to the fungus, whereas it varied between 3 and 17 mm in response to 50 µg mL$^{-1}$ toxin.

The sensitive date palm vitroplants showing symptoms of *Fusarium* wilt after artificial inoculation had a positive response to the toxin on their detached leaves for both doses. The effect of toxin doses was clearer for the mutants tested. The size of the necrosis varied between 2- and 11-min in the presence of 25 µg mL$^{-1}$and
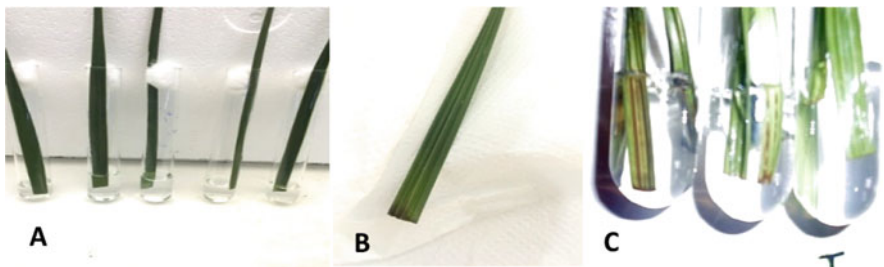


**Fig. 25.12** Immersion of leaves (**a**), browning at the base of the petiole (**b**), Progression of necrosis of leaves in the in the solution of FII toxin of *F.o.a.* presence of the fraction FII of *F.o.a.* toxin (**c**). (Photos taken in 2015 by Khelafi H and Yatta El Djouzi D)

**Table 25.6** Different responses of date palm mutants to *F.o.a.* based on resistance evaluation test indicated by the size of necrosis developed at the base of the petioles (mm: millimeter) in response to inoculums

|  | Size of necrosis at the base of the petioles (mm: millimeter) | | |
|---|---|---|---|
| Lot | 0 µg mL$^{-1}$ | 25 µg mL$^{-1}$ | 50 µg mL$^{-1}$ |
| VM1 | 1 | 11 | 20 |
| VM2 | 1 | 10 | 25 |
| M3 | 1 | 3 | 40 |
| VM4 | 1 | 1 | 12 |
| VM5 | 2 | 11 | 30 |
| VM6 | 1 | 1 | 10 |
| VM7 | 2 | 12 | 30 |
| VM8 | 1 | 6 | 17 |
| VM9 | 1 | 7 | 10 |
| VM10 | 1 | 3 | 8 |
| VM11 | 1 | 4 | 11 |
| VM12 | 2 | 4 | 5 |
| VM13 | 1 | 5 | 6 |
| VM14 | 1 | 3 | 6 |
| VM15 | 2 | 1 | 3 |
| VM16 | 2 | 6 | 8 |
| VM17 | 1 | 2 | 3 |
| VM18 | 1 | 2 | 15 |
| VM19 | 1 | 5 | 6 |
| VM20 | 1 | 2 | 15 |

Constructed by Khelafi H and Yatta El Djouzi D
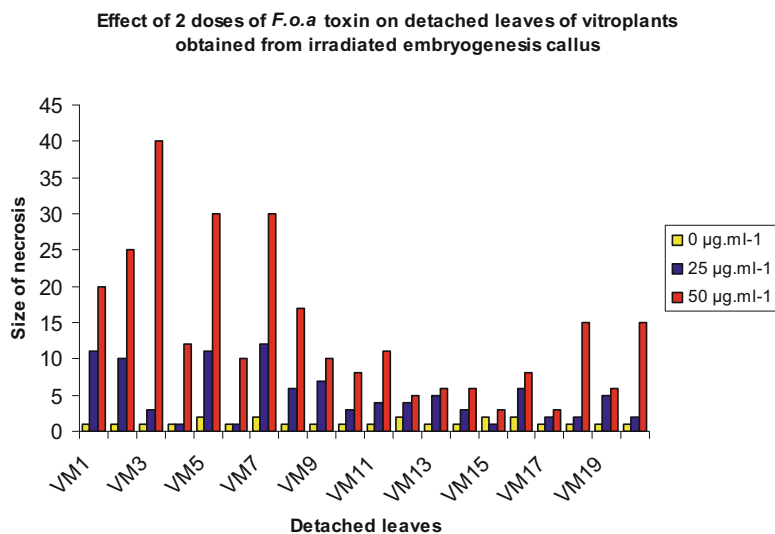This evaluation is made on the scale between 0 and 4 cm
1: −1 cm ; 2: <2 cm; 3: +2 cm; 4: 3 cm or larger

between 6 and 40 mm in the presence of 50 µg mL$^{-1}$ toxin. This result is consistent with the work obtained by Asnaghi et al. (2007) and Browne et al. (2005) using detached leaves screening for rusts disease resistance (Xie and Mew 1998). In addition, Twizeyimana et al. (2007) evaluated the detached leaves for selection *Musa* species for resistance to black leaf streak.

For all inoculated vitroplants, necrosis on detached leaves exceeded 11 mm except two vitroplants where the necrosis did not exceed 6 mm when applying 50 µg mL$^{-1}$ toxin. One month after artificial inoculation, leaves started drying in two vitro plants. After 6 months, 9 vitroplants of Deglet Nour among toxin-treated twenty plants sowed symptoms of Bayouddisease and decayed. The control inoculated with water showed no symptoms of *Fusarium* wilt.

The roots of inoculated vitroplants showed the development of fine, curly salmon pink mycelium after their transfer onto a culture medium. This confirmed wild form *F.o.a.*, which is responsible for *fusarium* wilt of date palm.

The most excellent approach to overcome *Fusarium*-wilt difficulty is to obtain resistant palms trees. The mass production of plants using in vitro technique and

**Effect of 2 doses of *F.o.a* toxin on detached leaves of vitroplants obtained from irradiated embryogenesis callus**

**Fig. 25.13** Effect of two doses of filtration of *F.o.a.* toxin on detached leaves of vitroplants obtained from irradiated embryogenic callus. (Constructed by Khelafi H and Yatta El Djouzi D)

screening resistant cultivars was recommended for a long time (Louvet and Toutain 1973; Saaidi et al. 1981). It was noted that fusaric acid isolated from culture filtrate of *Fusarium oxysporum* f.sp. *albedinis* (Amraoui et al. 2005), played an essential role in early phases of date palm contamination by *F.o.a.* (Bouizgane et al. 2004). The selection of mutants is done by detached leaf technique by using FII toxin of *F. o.a.* The pathogen secrets in the medium several toxic substances of various kinds including fusaric acid, anhydrous Aspergillomarasmine-B, and peptidic compounds. These compounds from which, three toxin fractions FI, FII and FIII were determined and the study of their toxicity. Fraction FII showed the most toxic for the date palm (Sedra and Lazrek 2011; Sedra et al. 2008). Amraoui et al. (2005) also tested fusaric acid on date palm leaves to examine its toxic effect with that of the FII (*F.oxysporum* f.sp. *albedinis*) and HPLC—purified fractions. However, they established that fusaric acid and some portions purified from FII (*F. oxysporum* f.sp. *albedinis*), i.e., H (3), H (4) and H (5), were toxic on detached leaves. They showed that FII (*F.o. a.*) fraction contains new toxins, different from enniatins or fusaric acid by their specificity and their elution time in HPLC, respectively. These molecules could act independently or synergistically with fusaric acid to induce the characteristic symptoms of the Bayoud disease.

For these biological tests, several researchers tested whole plants or only a fragment of plant to study the effects of fungal toxins. Molot et al. (1984) used the asparagus plantlets to evaluate the effect of toxins secreted by *Rhizoctonia violacea*. Tomato plantlets were also used to evaluate the effect of toxins secreted by *Pyrenochaeta lycopersici* while Pinon (1984) tested toxins *Hypoxylum mammatum* on poplar leaves.

## 25.5 Evaluations of Mutants

### 25.5.1 In Vivo Evaluation of Putative Mutants

Vitro plant acclimatization is an important step after in vitro cultivation. The acclimatization conditions must be well identified to avoid the loss of valuable plant material given the long time the date palm takes to react in vitro culture. The seedlings from the first irradiations and the control seedlings were acclimatized and raised in a greenhouse, in plastic bags containing a mixture of sand and compost in equal volume (1/2). The irradiated seedlings chosen for the acclimatization stage must have a robust structure and a well-developed root system, with at least four roots of a minimum length of 10 cm.

Started drying in two vitroplants. After 6 months, nine vitro-plants among toxin-treated 20 plant sowed symptoms of Bayoud disease and decayed. The control inoculated with water showed no symptoms of *Fusarium* wilt.

The roots of inoculated vitro-plants showed the development of fine, curly salmon pink mycelium (Fig. 25.14) after their transfer onto a culture medium. Fourteen plants of Deglet Nour showed tolerance levels to toxins to the resistant cultivars Taquerbucht. The plants were grown under sheltered shade until reaching the optimal stage for planting in the field.

The infection symptoms development was observed much earlier on detached leaves with the toxin was observed much earlier on detached leaves with the toxin of *F.o.a.* than vitro-plants inoculated with the actual pathogen. The artificial inoculation with liquid form of *F.o.a.* was most efficient in the selection of date palm where concentration was $10^6$ sp mL$^{-1}$.

A similar artificial screening method was used by Ando et al. (1984) to choose plants resistant to fusariosis using soil inoculation with a fungus suspension of $4 \times 10^5$ spores mL$^{-1}$.

Also, the result shows the effect toxic of culture filtrate of the *Fusarium subglutinans* to provide a process of in vitro selection cultivars of pineapple (*Ananas comosus*) for resistance to the disease, as has already been tried in some other host-pathogen system (Ludwig et al. 1992; Song et al. 1994).

**Fig. 25.14** Development of mycelium on PDA medium. (Photo by Khelafi H)

### 25.5.1.1 Acclimatization of Putative Mutants

In vitro selection of 70 seedlings putative mutant using the *F.o.a.* culture filtrates at 35% (Fig. 25.15a–c).

### 25.5.1.2 Greenhouse Evaluation

Inspection of the morphological quality of plants showed that the acclimatization step went well and Seedlings issues from the first irradiations and control seedlings grown vigorously. Indeed, the evaluation of the survival of the plants during the first week of acclimatization showed that all Seedlings survived (100% survival). The mutant plantlets selected and healthy adapted in vitro are evaluated in order to select a solid mutant before transfer to the infested field. The screening pressure could be applied 2–3 times to make sure that the preferred putative mutant are resistant (Fig. 25.16a, b). The mutant plantlets were treated with toxin and the *F.o.a.* culture filtrate at 35%. Ten mutant plants were selected from 70 seedlings putative mutant in
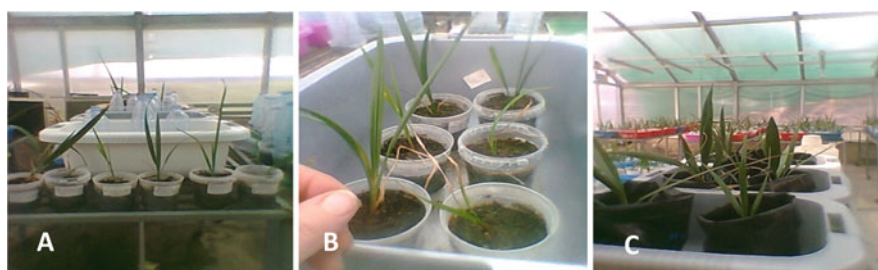


**Fig. 25.15** Inoculation with *F.o.a.* (**a**, **b**) Selection with respect to the *F.o.a.* and development of cultivars Teggaza (**c**). (Photos by Abed F)
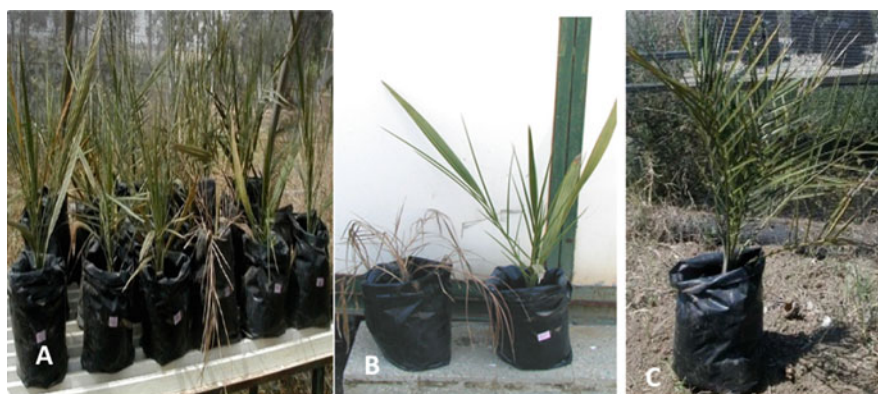


**Fig. 25.16** Artificial inoculation test on irradiated tissue culture plants obtained from embryogenic callus. The mutant plants treated with *Fusarium* toxin and the *F.o.a.* culture filtrate at 35% (**a**). The mutant plant infected and mutant plant tolerant after treated with *F.o.a.* culture filtrate (**b**). The mutant plantlets selected tolerant before transfer to the field evaluation (**c**). (Photos by Team date palm INRAA and Jain SM)

the greenhouse, which later on transferred in the contaminated field (Fig. 25.16c). They showed tolerance levels to toxins similar to the resistant cultivars cv. Taquerbucht. The plants were grown under sheltered shade until reaching the optimal stage for planting in the field.

### 25.5.1.3  Field Evaluation

Transfer plantlets (14 plants showed tolerance levels to toxins to the resistant cultivars Taquerbucht) from irradiated calli, plantlets from non-irradiated calli, (control), and resistant cultivar Taquerbucht (as control) to infested field in order to confirm resistance of mutants, research of interesting agronomic traits and Increase genetic variability of date palm.

However, the field screening of date palm is laborious, expensive, and site specific. Consequently, selection should be increased by the development of mass screening methods at the in vitro level.

The field evaluation of the mutant plants, based on survival rate and other agronomical such as yield and date quality, was carried out in 2008. We choose the Field planting (3 locations in Ghardaia). The controls are sensitive (Deglet Nour), and resistant (Taquerbucht). After 1 year, only 8 plants from 14 plants showed tolerance levels to toxins similar to the resistant cultivars Taquerbucht. We have detected mutant infected by *F.o.a.* after 8 years transplantation in contaminated field and M3 mutant of DN infected with Bayoud, confirmed the sensitivity after isolation of *F.o.a.* from the sampled. The putative mutants of cv. Deglet Nour have been transferred to a disease-infected field 12 years ago. Actually only 1 mutant of Deglet Nour were found to be really resistant after 12 years in Farmer field (Fig. 25.17a–d). This mutant show well development of the tree, emission of four offshoots, and higher bunch with good quality of date palm fruit.

For the putative mutants of cv. Teggaza, in 2017 we have chosen the infested Field planting in south at INRAA Station Adrar (Fig. 25.18a–f). Eleven vitro-plants showing resistance to artificial inoculation of the pathogen were transferred to a plot infested with Bayoud disease also we have transferred the sensitive Controls (Deglet Nour), and resistant Controls (Taquerbucht) in order to study of the behavior of their comportment against *Fusarium oxysporum* f.sp. *albedinis*. This confirmed wild form *F.o.a.*, which is responsible for *Fusarium* wilt of date palm.

Confirm resistance of mutants, research of interesting agronomic traits and Increase genetic variability of date palm.

### 25.5.2  Molecular Characterization

Molecular approaches were used to understand the mechanisms within the process of regeneration by, genetic stability of plantlets and also to discriminate the genetic variability between the mutant and the mother plants through characterizing the variations at DNA level. Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single-strand conformational polymorphism (SSCP), microarray, differential display, targeting induced local lesions in
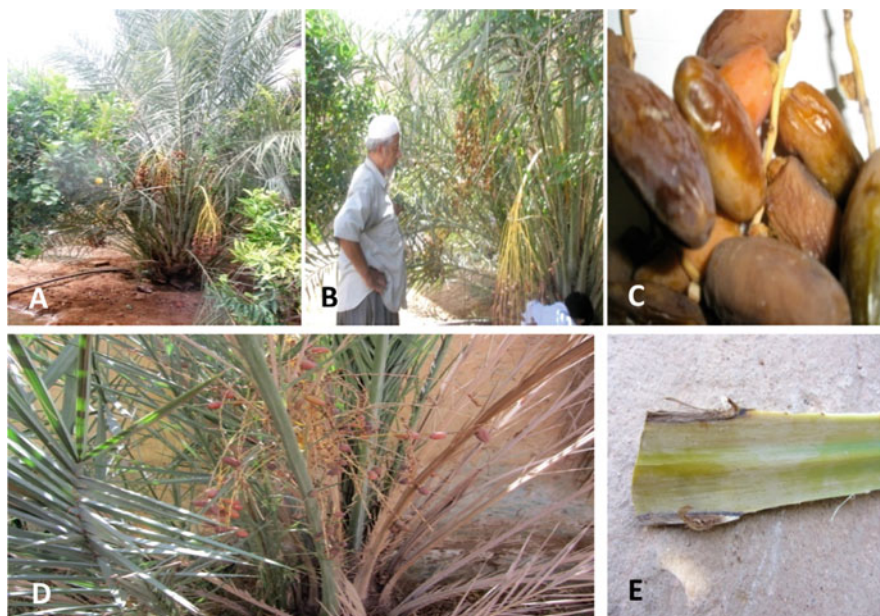
**Fig. 25.17** Only 1 mutant of Deglet Nour were found to be resistant after 10 years in Farmer (**a**, **b**) and mutant M2 fruit of Deglet Nour (**c**). Mutant infected by *F.o.a.* after 8 years transplantation in contaminated field (**d**, **e**) (M3 mutant of DN infected with Bayoud, Confirmation of the sensitivity after isolation of *F.o.a.*). (Photos taken byAbed F and Yatta El Djouzi D)



**Fig. 25.18** Transfer in contaminated field of 13 Teggaza mutants selected in vitro and in the greenhouse (**a**, **b**), Teggaza mutants after 1 year (**c**), inoculation technics in field (**d**, **e**) and Teggaza mutants were found to be resistant after 30 months in contaminated field (**f**). (Photos by Yatta El Djouzi D and Abed F)

genome (TILLING) and high-resolution melt (HRM) permi tquick and profoundly analysis of mutational variations (Penna et al. 2012).

Sianipar et al. (2015) have been used RAPD method to identify the genetic variability between the mutant plantlets enhanced from physical mutagens rodent tuber calli. They obtained 69 fragments from 11 mutant plantlets by using 10 RAPD.

ISSR technique was used to discriminate the drought-tolerant sugar beet mutant improved using irradiated shoot tip explants by gamma radiation (Sen and Alikamanoğlu 2012). These authors reported the presence 91 polymorphic bands of 106 PCR fragments with 19 inter simple sequence repeat (ISSR) primers.

In coffee plants, after mutagenic treatment through sodium azide, the genetic variability was determined using RAPD and AFLP markers (Vargas-Segura et al. 2019). They obtained 46 fragments with 12 RAPD primers of which 34 were polymorphic bands (74%). The amplification with AFLP, 6 selective primer combinations revealed detection of 36 polymorphic bands polymorphisms with a percentage of 17.8. The study showed that both $NaN_3$ and EMS induced genetic variability within the DNA regions amplified with AFLP and RAPD markers.

In date palm, Quenzar et al. (2001) identified two circular plasmid-like DNAs (S and R) in the mitochondria through a PCR-based approach; the presence of R plasmid and absence of S plasmid showing that could be considered as a reliable molecular marker of Bayoud disease resistance. Date palm has been spontaneous mutation induced by mutagen treatments and selected for resistance to Bayoud disease by magnetic fields (Jain 2011).

Molecular tools were used to understand the mechanisms within the process of regeneration, genetic stability of plantlets and also to compare the ploidy level and polymorphism mutant improved and control vitro-plants of the same variety (Figs. 25.19 and 25.20). DNA was isolated form leaf tissues of the plantlet
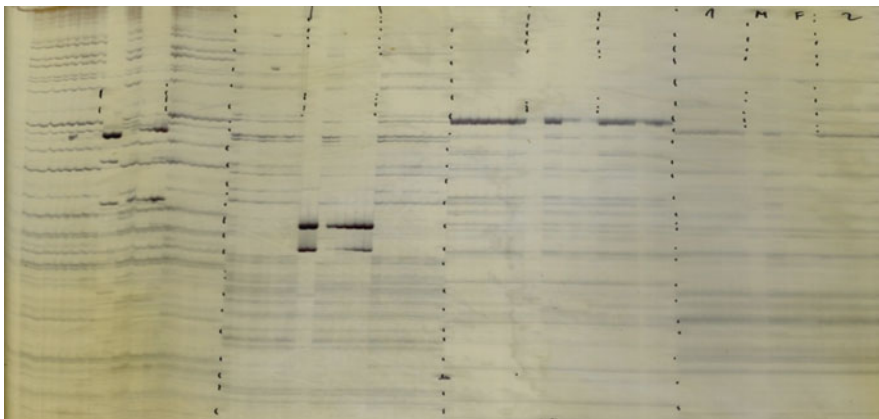


**Fig. 25.19** Portion of gel produced with the pairs of primers E-AAC—M-AAG, E-AAG—M-AAG, E-ACG—M-ACG, E-AAC—M-AGT showing the specificity of the AFLP profiles in putative mutant of Tgazza and their control vitro plants. M (putative mutant) and VPC (control vitro plants). (Photo by Yatta El Djouzi D)

**Fig. 25.20** Portion of gel produced with the pairs of primers E-AAC—M-AAG, E-AAG—M-AAG, E-ACG—M-ACG, E-AAC—M-AGT showing the specificity of the AFLP profiles in putative mutant of Deglet Nour and their control vitro plants. M1 to M10 (putative mutant) and VPC (control vitro plants). (Photo by Khelafi H)

regenerated by somatic embryogenesis and their mother plants using the mixed alkyltrimethylammonium bromide (MATAB) method (Gawel and Jarret 1991) contained (0.35 M sorbitol, 0.1 M Tris, 1.25 M NaCl, 0.005 M EDTA, 0.5% Na bisulfite, Matab to 4% pH 8). Amplified fragment length polymorphism (AFLP) analysis to be performed in order to verify level polymorphism between irradiated vitroplants of cvs. Deglet Nour, Teggaza and mother plants.

Six primer pairs tested through 17 offered better readability in this study; these are identified as follows: E-AAC—M-AAG, E-AAC—M-AGT, E-AAC—M-ACA, E-AAG—M-AAC, E-AAG—M-AAG, E-AAG—M-ACT. Results of cv. Deglet Nour showed the presence of systematic bands specific to vitroplants control with two pairs of primers E-AAC—M-ACA, E-AAG—M-AAC, and bands specific to irradiated vitroplants. The *mutagen* is a chemical or physical *agent* primers combination selected reveal polymorphic bands with a percentage of 35% for Deglet Nour and 40% for Teggaza.

The electrophoretic profiles showed a number of monomorphic bands equal to 120 between the irradiated and control vitroplants, which corresponds to 65% for Deglet Nour and 10% for Teggaza.

Mutants created by mutagen physic agent at 20, 30 and 40 Gy improved by 20 random primers showed a low polymorphism level compared with the control. Other studies have also shown the efficacy of measure genetic polymorphism using molecular markers for determining mutation procedures in date palm (Saker et al. 2006) and other species such as in peach (Hashmi et al. 2017), soybean (Gesteira et al. 2008; Atak et al. 2004), and rice (Rashid et al. 2009).

However, site-specific mutagenesis with the CRISPR/Cas9 system will allow scientists to analyze the gene expression in date palm genome.

In date palm, CRISPR/Cas9 offers a number of benefits well-suited to modification of dioecious tree crops (Kole et al. 2015), to discover genetic markers for sex determination, to develop flowering, juvenility control and manipulation of fruit ripening, fruit quality, nutritional value and production of biofuel from the date palm wastes (Sabir et al. 2014). As well, CRISPR/Cas9 system can be also explored to curtail phytoplasma diseases. The supplementary applications may help in regulating secondary metabolites production from date palm fruits.

### 25.5.3 Flow Cytometric Analysis

Flow cytometry protocol was developed for polyploidy detection, dissociating chimeras monitoring of cytochimera dissociation, and chromosomal stability. Moreover, application of DNA flow cytometry is quite straightforward. This technique is based on the use of a specific DNA fluorochrome (propidium iodide) and on the quantification of the relative intensity of the fluorescence of the labeled nuclei. DNA content is correlated with ploidy level. During this experiment, *Zea mays* L. (CE 777) (2C = 5.43 pg of DNA) was used as reference internal standards.

Samples for analysis of the quantity of nuclear DNA are taken from four types of tissues leaves of vitroplants raised from irradiated callus of cv. Teggaza and their (1 cm), vitroplants of cv. Taquerbucht (approx. 300 mg).

These samples were placed in a Petri dish held on an "ice block" containing 1 mL of extraction buffer (Doležel et al. 1997) of the following composition: 15 mM TRIS, 2 mM $Na_2EDTA$, 0.5 mM spermine 4HCl, 80 mM KCl, 45 mM $MgCl_2$, 30 mM sodium citrate and 40 mM $Na_2SO_3$ at pH 7.5. They were then chopped very finely using a razor blade. The suspension, which includes isolated nuclei, is then removed using a 3 mL Pasteur pipette and filtered using a 30 μm polyethylene sieve (Partec Celltries). Propidium iodide (IP) at 330 μg $mL^{-1}$ (IP4170; Sigma) is added to the filtrate then the mixture vortexed and left to stand for 30 min in the dark before analysis by flow cytometry (CyFlow Partec II NOT). Propidium iodide (PI), a nucleic acid intercalating agent, will bind to DNA molecules contained in the cell nucleus.

The DNA assay is carried out using a Partec II PAS (Particle Analyzing System) flow cytometer (Partec GmbH, Münster, Germany) whose laser radiation with a wavelength of between 454 and 515 nm passes through the nucleus and excites by fluorescence the IP bound by the DNA molecule. The quantity of fixed IP is correlated to the quantity of DNA contained in the nuclei and therefore to the ploidy level of the sample. For each sample, 800 nuclei were analyzed on average. The distribution of fluorescence intensities (relative DNA content) obtained by flow cytometry is generally expressed in arbitrary units (C). The 1C value represents the DNA content of a haploid (*n*) chromosome set. To determine the ploidy level, this scale should be calibrated against a reference. In this study, we used *Zea mays* (CE 777) (C = 5.43 pg DNA) as an internal reference standard.

Using flow cytometry, to estimate the nuclear DNA content of *P. dactylifera* L. cv. Teggaza, and analyzed the stability for this factor in regenerated putative mutants. In this case, non-irradiated vitroplant of cv. Teggaza was used as a control for the vitroplants issued from irradiated of cv. Teggaza. The resulting values of the relative Nuclear DNA content of *P. dactylifera* L. cv. Teggazawas 1.68 ± 0.05 pg. However, the significant differences (ca. 60%) in genome size were found in some vitroplants issued from irradiated calli of cv. Teggaza (Fig. 25.18a, c). This is showing that at least some plants could be considered as a mutants induced by irradiation. Molecular approaches were used to understand the mechanisms within the process of regeneration by, genetic stability of plantlets and also to compare the ploidy level and polymorphism in vitro cultures of irradiated vitroplants and no-irradiated control vitroplants of the same variety. The establishment of screening and evaluation using flow cytometric and molecular markers allowed us the selection of date palm lines with good quality and disease resistance.

## 25.6    Conclusions and Prospects

Date palm plays considerable role socio-cultural economy. Mainly the date palm trees are very old and becoming more vulnerable to various biotic and abiotic constraints. Biotechnological techniques including induced mutation and in vitro selection propose a huge potential for the development of mutants exhibiting resistance to biotic stress in plant breeding programs. However, studies in date palm mutation induction are scarce. Somatic embryogenesis proved to be an ideal approach for mutation-based breeding programs. Selection systems to isolate tolerant lines have been designed using selection for resistance to culture filtrates of the fungal pathogen and toxins in vitro screening of calluses, selection of somatic embryos and regeneration of putative mutants; in vivo evaluation and confirmation of resistance initially in the greenhouse and finally in infested field, and selection of other agronomic useful traits. Flow cytometric and molecular markers were helpful in the selection and confirmation of date palm lines with good quality and Bayoud resistance. They are applied to big numbers of plant cells in culture to establish mutations and the successive regeneration of whole plants offers enormous prospect for dipping the cost of breeding programs.

The next important step is micropropagation of resistant mutants and their multi-location trials for evaluating resistance in contaminated fields to obtain Bayoud resistant high-quality palms. The development of new cultivars with excellent characteristics is a major target of date palm-breeding programs. Other approaches are useful and could be promoted for breeding programs such as genome editing with the CRISPR-Cas9 system, genetic manipulation, and the applications of high-through genome sequencing may develop date palm biotechnology. It possible to repopulate the palm groves already devastated by the disease, in the South West with Teggaza variety and in the center with the Deglet Nour variety. The use of the mutants obtained in the repopulation of the affected and threatened palm groves by Bayoud would contribute to oasis preservation, and increase in genetic diversity also

they will contribute to the strengthening of the employment policy and to the guarantee food security.

# References

Abed F, Saka H, Amara A, Kermiche A (1999) Effet de differentes doses de cobalt 60 sur les cals embryogènes de Deglet nour (*Phoenix dactylifera* L.). Recherche Agronomique 5:73–80

Abed F, Yatta D, Amara B et al (2014) Somatic embryogenesis from offshoot of different cultivars of date palm (Phoenix dactylifera L.) of three regions of southern Algeria. In: Farooq SA, Abed R, Baqir S (eds) Biotechnology and conservation of species from arid regions, vol 1. NOVA publishers, New York, pp 275–286

Abul-Soad AA, Jain SM, Jatoi MA (2017) Biodiversity and conservation of date palm. In: Ahuja MR, Jain SM (eds) Biodiversity and conservation of woody plants. Sustainable development and biodiversity, vol 17. Springer, Cham, pp 313–353

Ahloowalia BS, Maluszynski M (2001) Induced mutations – a new paradigm in plant breeding. Euphytica 118:167–173

Akter N, Islam MR (2017) Heat stress effects and management in wheat. A review. Agron Sustain Dev 37:37

Al Kharusi L, Dekoum VM, Assaha et al (2017) Screening of date palm (*Phoenix dactylifera* L.) cultivars for salinity tolerance. Forests 8:136

Al Mansoori TA et al (2007) Evaluation techniques for salt tolerance in date palm. Acta Hortic 736:301–306

Al-Enezi NA, Al-Bahrany AM, Al-Khayri JM (2012) Effect of x-irradiation on date palm seed germination and seedling growth. Emir J Food Agric 24(5):415–424

Al-Khateeb AA (2006) Role of cytokinen and auxin on the multiplication stage of date palm (*Phoenix dactylifera* L.) cv. Sukry. Biotechnology 5:349–352

Al-Khayri JM (2007) Micropropagation of date palm *Phoenix dactylifera* L. In: Jain SM, Haggman H (eds) Protocols for micropropagation of woody trees and fruits. Springer, Berlin, pp 509–526

Al-Khayri JM (2012) Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. Emir J Food Agric 24(5):444–455

Al-Khayri JM, Al-Bahrany AM (2004) Genotype-dependent in vitro response of date palm (Phoenix dactylifera L.) cultivars to silver nitrate. Sci Hortic 99(2):153–162

Al-Khayri JM, Ibraheem Y (2014) *In vitro* selection of abiotic stress tolerant date palm (*Phoenix dactylifera* L.). Emir J Food Agric 26(11):921–933

Al-Khayri JM, Naik PM, Jain SM, Johnson DV (2018) Advances in date palm (*Phoenix dactylifera* L.) breeding. In: Al-Khayri JM, Jain SM, Johnson DV (eds) Advances in plant breeding strategies: fruits, vol 3. Springer, Cham, pp 727–771

Al-Mulla L, Bhat NR, Khalil M (2013) Salt-tolerance of tissue-cultured date palm cultivars under controlled environment. Int J Food Vet Agric Eng 7:468–471

Al-Rokibah AA, Abdalla MY, El-Fakharani YM (1998) Effect of water salinity on Thielaviopsis paradoxa and growth of date palm seedlings. J King Saud Univ Agric Sci 1:55–63

Al-Salih AA, Al-Najjar NR, Al-Mashhadani AN (1987) A study on the chromosome number of two specific female date palm cultivars. Date Palm J 5:134–143

Aly AA, Eliwa NE, Abd El-Megid MH (2019) Stimulating effect of gamma radiation on some active compounds in eggplant fruits. Egypt J Rad Sci Appl 32:61–73

Amin R, Laskar RA, Khan S (2015) Assessment of genetic response and character association for yield and yield components in lentil (*Lens culinaris* L.) population developed through chemical mutagenesis. Cogent Agric 31(1):1000715

Amraoui H, Sedra MH, Hamdaoui A (2004) Etude des sécrétions protéiques et enzymatiques du *Fusarium oxysporum* f. sp *albedinis:* agent causal de la fusariose vasculaire du palmier dattier. Al Awamia 109(110):47–62

Amraoui H, Lazrek HB, Sedra MH (2005) Chromatographic characterization and phytotoxic activity of *Fusarium oxysporum* f. sp. *albedinis* and saprophytic strain toxins. J Phytopathol 153:203–208

Ando A, Menten JOM, Tulmannn Neto A et al (1984) Obtençao de mutantes resistentes à fusariose en pimento-do-reino (*Peper nigrum* L.). In: Proceedings of the seminario regional sobre Tecnicas nucleares naproducao de plantasagricolaq, Piracicaba. CENA/USP, Sao Paulo, Brazil, pp 47–49

Asnaghi C, D'Hont A, Glaszmannand JC, Rott P (2007) Resistance of sugarcane cultivar R 570 to Puccinia melanocephala isolates from different geographic locations. Plant Dis 85(3):282–286

Atak C, Alikamanoğlu S, Açík L, Canbolat Y (2004) Induced of plastid mutations in soybean plant (*Glycine max* L. *Merrill*) with gamma radiation and determination with RAPD. Mutat Res 556(1–2):34–44

Attree SM, Moore D, Sawhney VK et al (1991) Enhanced maturation and desiccation tolerance of white spruce [*Picea glauca* (Moench) Voss] somatic embryos: effects of a non-plasmolysing water stress and abscisic acid. Ann Bot 68:519–552

Behnaz A, Zarghami R, Salari A (2018) The effects of agno3 and 2ip (n6-(2-isopentenyl) adenine) on different stages of somatic embryogenesis in date palm (*Phoenix dactylifera* L.) (Cv. medjool) Pakistan. J Bot 50:495–502

Berestetskiyis AO (2008) A review of fungal phytotoxins: from basic studies to practical use. Appl Biochem Microbiol 44(5):453–465. https://doi.org/10.1134/S0003683808050013

Bhaskaran S, Smith RH (1992) Somatic embryogenesis in date palm (Phoenix dactylifera L.), Forestry Sciences Chapter book series, vol 44–46. FOSC

Bouizgane B, Brault M, Pennarum AM (2004) Electrophysiological responses to fusaric acid of root hairs from seedlings of date palm susceptible and resistant to *Fusarium oxysrporum* f.sp *albedinis*. J Phytopathol 152:321–324

Bradshaw JE (2016) Mutation breeding plant breeding: past, present and future. Springer International Publishing, Switzerland. ISBN: 978-3-319-23284-3

Brar DS, Jain SM (1998) Somaclonal variation: mechanism and applications in crop improvement. In: Jain SM, Brar DS, Ahloowalia BS (eds) Somaclonal variationand induced mutations in crop improvement. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 15–38

Bressan RA, Singh NK, Handa AK et al (1985) In: Plant genetics-UCLA symposium on molecular and cellular biology. In: Freeling M (ed), vol 35. Liss, New York, pp 755–769

Browne RA, Murphy JP, Cooke BM et al (2005) Evaluation of components of *Fusarium* head blight resistance in soft red winter wheat germplasm using detached leaf essay. Plant Dis 89: 404–411

Buffard-Morel J, Verdeil JL, Pannetier C (1992) Embryogenèse somatique du cocotier (Cocos nucifera L.) à partir d'explants foliaires: étude histologique. Can J Bot 70:735–741

Carlson PS (1973) Methionine sulfoximine-resistant mutants of tobacco. Science 180:1366

Casinovi CG (1972) Chemistry of the terpenoid phytotoxins. In: Wood RKS, Ballio A, Graniti A (eds) Phytotoxins in plant diseases. Academic, New York, pp 105–125

Chandra R, Kamle M, Bajpai A, Muthukumar M, Kalim S (2010) In vitro selection: a candidate approach for disease resistance breeding in fruit crops. Asian J Plant Sci 9(8):437–446

Chawla HS, Wenzel G (1987) Regeneration potential of callus from wheat and barley. Arch. Zuchtungforsch., Berlin 17(6):337–343

Chwendiman J, Pannetier C, Michaux-Ferriere N (1988) Histology of somatic embryogenesis from leaf explants of the oil palm *Elaeis guineensis*. Ann Bot 62:43–52

Devreux M, Magnien E, Dalschaert X (1986) Cellules végétales *in vitro* et radiations ionisantes. In: Nuclear techniques and *in vitro* culture for plant improvement. Proceedings of a symposium, Vienna 1985. International Atonic Energy Agency (AIEA/FAO), Vienna, pp 93–100

Doležel J, Greilhuber J, Suda J (1997) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2(9):2233–2244

Donini B, Mannino P, Ancora A, Sonnino A et al (1991) Mutation breeding programmes for the genetic improvement of vegetatively propagated plants Italy. In: Plant mutation breeding for crop improvement, Proceedings of a Symposium, vol l. IAEA and FAO, Vienna, pp 237–255

El Fakhouri R, Lazrek HB, El Bahraoui E, Rochat H (1996) Preliminary investigation on a peptidic toxin produced by *Fusarium oxysporum* f.sp *albedini*s. Phytopathol Mediterr 35:11–15

El Hadrami H, El Idrissi-Tourane A, El Hasni M et al (2005) Toxin-based *in vitro* selection and its potential application to date palm for resistance to the Bayoud *Fusarium* wilt. Plant Biol Pathol C R Biol 328:732–744

El-Beltagi HS, Aly A, El-Desouky W (2019) Effect of gamma irradiation on some biochemical properties, antioxidant and antimicrobial activities of Sakouti and Bondoky dry dates fruits genotypes. J Rad Res Appl Sci 12:437–446

El-Hadrami A, Daayf F, Elshibli S et al (2011) Somaclonal variation in date palm. In: Mohan S, Al-Khayri JM, Johnson DJ (eds) Date palm biotechnology. Springer, New York, pp 183–203

Erskine W, Moustafa AT, Osman AE et al (2003) Date palm in the GCC countries of the Arabian Peninsula. Euphytica 118:167–173

FAO/IAEA-MVD (2021) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency – mutant variety database. https://mvd.iaea.org/#!Search. Cited 30 Jan 2021

FAOSTAT (2018) Website of the Food and Agriculture Organization of the United Nations. Available online http://www.fao.org/faostat/en/data/QC. Accessed on 1 August 2018

Feldman AB, Leung H, Baraoidan M (2017) Increasing leaf vein density *via* mutagenesis in rice results in an enhanced rate of photosynthesis, smaller cell sizes and can reduce interveinal mesophyll cell number. Front Plant Sci 8:1–10

Foster PL (1991) In vivo mutagenesis. Methods Enzymol 204:114–125

Gawel NL, Jarret RL (1991) A modified CTAB DNA extraction procedure of Musa and Ipomoea. Plant Mol Biol Rep 9:262–266

Gawel NJ, Rao AP, Robacker CD (1986) Somatic embryogenesis from leaf and petiole callus cultures of Gossypium hirsutum L. Plant Cell Rep 5(6):457–459

Gesteira AS, Otoni WC, Barros EG, Moreira MA (2008) RAPD-based detection of genomic instability in soybean plants derived from somatic embryogenesis. Plant Breed 121(3):269–271

Hallerman E, Grabau E (2016) Crop biotechnology: a pivotal moment for global acceptance. Food Energy Secur 5(1):3–17

Hashmi G, Hashmi S, Sen S et al (2017) Polymorphism in heat shock protein gene (hsp70) in entomopathogenic nematodes (rhabditida). J Therm Biol 22(2):143–149

Hazzouri KM, Flowers JM, Nelson D et al (2020) Prospects for the study and improvement of abiotic stress tolerance in date palms in the post-genomics era. Front Plant Sci 11:293. https://doi.org/10.3389/fpls.2020.00293

Jain SM (2001) Tissue culture-derived variation in crop improvement. Euphytica 118:153–161

Jain SM (2005) Major mutation-assisted plant breeding programs supported by FAO/IAEA. Plant Cell Tissue Org Cult 82:113–123

Jain SM (2006) Radiation-induced mutations for developing Bayoud disease resistant date palm in North Africa. In: Zaid A (ed) Proceedings of the international workshop on true-to-typeness of date palm tissue cultured-derived plants. Al Ain, UAE, Plant Tissue Culture Laboratory, UAE University, pp 31–41

Jain SM (2010) Mutagenesis in crop improvement under the climate change. Rom Biotechnol Lett 15(2):88–106

Jain SM (2011) Radiation induced mutations for date palm improvement. In: Mohan S, Al-Khayri JM, Johnson DJ (eds) Date palm biotechnology. Springer, New York, pp 271–286

Jain SM (2012) *In vitro* mutagenesis for improving date palm (*Phoenix dactylifera* L.). Emir J Food Agric 24(5):386–399

Jain SM (2014) *In vitro* culture and mutagenesis in genetic improvement of date palm (*Phoenix dactylifera* L.). In: Proceedings of the fourth symposium on date palm in Saudi Arabia, King Faisal University, Alahsa, 5–8 May 2007, pp 693–705

Jain SM, Maluszynski M (2004) Induced mutations and biotechnology on improving crops. In: Mujib A, Cho M, Predieri S, Banerjee S (eds) *In vitro* applications in crop improvement: recent progress. IBH-Oxford, India, pp 169–202

Jain SM, Suprasanna P (2011) Induced mutations for enhancing nutrition and food production. Geneconserve 40:201–215

Jain SM, Ahloowalia BS, Veilleux RE (1998) Somaclonal variation in crop plants. In: Jain SM, Brar DS, Ahloowalia BS (eds) Somaclonal variation and induced mutations in crop improvement. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 203–218

Jatt T, Markhand GS, Rayburn L et al (2019) Karyotype variations among date palm (*Phoenix dactylifera* L.) cultivars of Sindh, Pakistan. Sarhad J Agric 35(3):708–718

Kole C, Muthamilarasan M, Henry R et al (2015) Application of genomics-assisted breeding for generation of climate resilient crops: progress and prospects. Front Plant Sci 6:563

Krishna H, Alizadeh M, Singh D et al (2016) Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech 6:54

Louvet JJ, Toutain G (1973) Recherches sur les fusarioses VIII. Nouvelles observations sur la fusariose du dattier et précisions concernant la lutte. Ann Phytopathol 5:35–52

Ludwig AC, Hubstenberger J, Phillips F (1992) Screening of Allium tester lines in vitro with Pyrenochaeta terrestris filtrates. Hort Sci 27:166–168. https://doi.org/10.21273/HORTSCI.27.2.166

Maluszynski M, Szarejko I, Maluszynska J (2004) Mutation techniques. Encyclop Appl Plant Sci 1(3):186–201

Mba C, Rownak A, Souleymane B, Jain SM (2010) Induced mutagenesis in plants using physical and chemical agents. In: Davey MR, Anthony P (eds) Plant cell culture, essential methods. Wiley-Blackwell, Chichester, Sussex). ISBN: 978-0-470-68648-5, pp 111–130

Messiaen CM, Cassini R (1968) Recherches sur les fusarioses. IV. La systématique des *Fusarium*. Ann Epiphytes 19(3):387–454

Miler N, Jedrzejczyk I, Jakubowski S, Winiecki J (2021) Ovaries of Chrysanthemum irradiated with high-energy photons and high-energy electrons can regenerate plants with novel traits. Agronomy 11(6):1111

Mohammadzai IU, Ziarat S, Ihsan I et al (2010) Effect of gamma irradiation, packaging and storage on the nutrients and shelf life of palm dates. J Food Process Preserv 34:622–638

Mokhlisse N (1987) Contribution à l'identification et à l'étude de la toxicité des différents constituants de la toxine sécrétée par le *Fusarium oxysporum* f. sp. *albedinis*. Thèse de 3ême cycle, Faculty des Sciences-Semlalia Marrakech Morocco

Molot PM, Conus M, Cotta J, Ferrière H (1984) Mise en évidence d'une toxine dans les filtrats de culture de *Rhizoctonia violaceae*. Relation entre la sensibilité des plantules d'asperge à cette toxine et aux contaminations par mycélium. Rev Cytol Biol Végét Bot 7:205–217

Muhammad I, Raheem S, Al-Harrasi A et al (2020) Silicon and gibberellins: synergistic function in harnessing ABA signaling and heat stress tolerance in date palm (Phoenix dactylifera L.). Plants (Basel) 9(5):620

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Mussel HW (1972) Toxic proteins secreted by cotton isolates of *Verticillium albo-atrum*. In: Wood RKS, Ballio A, Graniti A (eds) Phytotoxins in plant diseases. Academic, New York, pp 443–445

Naik PM, Al-Khayri JM (2016) Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) through cell suspension culture. In: Jain SM (ed) Protocols for in vitro cultures and secondary

metabolite analysis of aromatic and medicinal plants, Methods in molecular biology, 2nd edn. Humana, New York, pp 357–366

Naik PM, Al-Khayri JM (2018) Cell suspension culture as a means to produce polyphenols from date palm (*Phoenix dactifera* L.). Ciênc e Agrotecnologia 42(5):464–473

Naik PM, Al-Khayri JM (2020) Influence of culture parameters on phenolics, flavonoids and antioxidant activity in cell culture extracts of date palm (*Phoenix dactylifera* L.). Erwerbs-Obstbau 62:181–188

Nikam AA, Devarumath RM, Ahuja A et al (2015) Radiation induced *in vitro* mutagenesis system for salt tolerance and other agronomic characters in sugarcane (*Saccharum officinarum* L.). Crop J 3:46–56

Osbourn AE (2001) Tox-boxes, fungal secondary metabolites, and plant disease. Proc Natl Acad Sci U S A 98(25):14187–14188

Patade YV, Suprasanna P (2008) Radiation induced *in vitro* mutagenesis for sugarcane improvement. Sugar Tech 10:14–19

Penna S, Vitthal SB, Yadav PV (2012) *In vitro* mutagenesis and selection in plant tissue cultures and their prospects for crop improvement. Bioremed Biodivers Bioavailab 6:6–14

Pérez-Clemente RM, Gómez-Cadenas A (2012) In vitro tissue culture, a tool for the study and breeding of plants subjected to abiòtic stress conditions. In: Recent advances in plant in vitro culture, pp 91–108

Pinon J (1984) Propriétés biologiques de la toxine d'hypoxylonmammatum parasite des peupliers de la section Leuce. Rev Cytol Biol Végét Bo 7:271–277

Predieri S (2001) Mutation induction, tissue culture in improving fruits. Plant Cell Tissue Organ Cult 64:185–210

Pringle RB, Scheffer RP (1963) Purification of the selective toxin of *Periconia circinata*. Phytopathology 53:785–787

Quenzar B, Trifi M, Bouachrine B, Hartmann C et al (2001) A mitochondrial molecular marker of resistance to Bayoud disease in date palm. Theor Appl Genet 103(2–3):366–370

Raemakers CS, Schavemaker CM, Jacobsen E, Visser RG (1993) Improvements of cyclic somatic embryogenesis of cassava (Manihot esculenta Grantz). Plant Cell Rep 12:226–229

Rashid U, Anwar F, Ansari TM et al (2009) Optimization of alkaline transesterification of rice bran oil for biodiesel production using response surface methodology. J Chem Technol Biotechnol 84:9

Roychowdhury R, Tah J (2013) Mutagenesis a potential approach for crop improvement. In: Hakeem KR, Ahmad P, Ozturk M (eds) Crop improvement: new approaches and modern techniques. Springer, New York, pp 149–187

Saaidi M, Toutain G, Bannerot H, Louvet J (1981) La sélection du palmier dattier (*Phoenix dactylifera* L) pour la résistance au Bayoud. Fruit 4(36):241–249

Sabir JSM, Arasappan D, Bahieldin A et al (2014) Whole mitochondrial and plastid genome SNP analysis of nine date palm cultivars reveals plastid heteroplasmy and close phylogenetic relationships among cultivars. PLoS One 9:e94158. https://doi.org/10.1371/journal.pone.0094158

Saker MM, Adawy SS, Mohamed AA, EL-Itriby HA (2006) Monitoring of cultivar identity in tissue culture-derived date palms using RAPD and AFLP analysis. Biol Plant 50(2):198–204

Schaart JG, van de Wiel CCM, Lotz LAP, Smulders MJM (2016) Opportunities for products of new plant breeding techniques. Trends Plant Sci 21(5):438–449

Schwendiman J, Pannetier C, Michaux-Ferriere N (1988) Histology of somatic embryogenesis from leaf explants of the oil palm Elaeis guineensis. Ann Bot 62:43–52

Sedra MH, Lazrek HB (2011) *Fusarium oxysporum* f. sp. *albedinis* toxin characterization and use for selection of resistant date palm to Bayoud disease. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date palm biotechnology. Springer, Cham, pp 253–270

Sedra MH, El Fakhouri R, Lazrek HB (1993) Recherche d'une méthode able pour l'évaluation de l'effet des toxines secrétées par *Fusarium oxysporum* f. sp. *albedinis* sur le palmier dattier. Al Awamia 82:89–104

Sedra MH, El Fakhouri R, Lotfi F, Lazrek HB (1997) Activités des toxines secrétées par Fusarium oxysporum f. sp. albedinis, agent causal du bayoud du palmier dattier et d'autres formes spéciales du Fusarium oxysporum. Al Awamia 98:57–65

Sedra MH, Lazrek H, Lot F, Rochat H (1998) Fusarium oxysporum f. sp. albedinis toxin isolation and use for screening of date palm plants for resistance to the bayoud disesase. In: Proceedings of XXV International Horticultural Congress (IHC), 2–7 Aug 1998, Brussel, Belgium

Sedra MH, Lazrek HB, Amraoui H, Nour S (2008) Pathogen toxins of Bayoud disease on date palm: *in vitro* selection and biological and specificity activities. J Plant Pathol 90 (Suppl):53–57

Sen A, Alikamanoğlu S (2012) Analysis of drought-tolerant sugar beet (*Beta vulgaris* L.) mutants induced with gamma radiation using SDS-PAGE and ISSR markers. Mutat Res 738–739:38–44

Sianipar N, Ariandana F, Maarisit W (2015) Detection of gamma-irradiated mutant of rodent tuber (Typhonium Flagelliforme Lodd.) in vitro culture by RAPD molecular marker. Procedia Chem 14:285–294

Song HS, Lim SM, Widholm JM (1994) Selection and regeneration of soybeans resistant to the pathotoxic culture filtrate of *Septoria glycine*. Phytopathology 84:948–951

Suprasanna P, Jain SM (2017) Mutant resources and mutagenomics in crop plants. Emir J Food Agric 29(9):651–657

Suprasanna P, Jain SM, Ochatt SJ et al (2012a) Applications of *in vitro* techniques in mutation breeding of vegetatively propagated crops. In: Shu QY, Forster BP, Nakagawa H (eds) Plant mutation breeding and biotechnology. CABI International, Wallingford, pp 371–385

Suprasanna P, Vitthal SB, Yadav PV (2012b) *In vitro* mutagenesis and selection in plant tissue cultures and their prospects for crop improvement. Bioremed Biodivers Bioavail 6:6–14

Suprasanna P, Mirajkar SJ, Bhagwat SG (2015) Induced mutations and crop improvement. In: Bahadur B, Rajam VM, Sahijram L, Krishnamurthy KV (eds) Plant biology and biotechnology, Plant diversity, organization, function and improvement, vol I. Springer, India, pp 593–617

Surico G, Graniti A (1977) Produzione di tossine da Fusarium oxysporum Schl. f. sp. albedinis. Phytopathol Mediterr 16:30–33

Tisserat B (1982) Factors involved in the production of plantlets from date palm callus cultures. Euphytica 31:201–214. https://doi.org/10.1007/BF00028323

Twizeyimana M, Ojiambo PS, Tenkouano A et al (2007) Rapid screening of *Musa* species for resistance to black leaf streak using *in vitro* plantlets in tubes and detached leaves. Plant Dis 9:308–314

Vargas-Segura C, López-Gamboa E, Araya-Valverde E et al (2019) Sensitivity of seeds to chemical mutagens, detection of dna polymorphisms and agro-metrical traits in M1 generation of coffee (*Coffea arabica* L.). J Crop Sci Biotechnol 22:451–464

Verdeil JL, Hocher V, Huet C et al (2001) Ultrastructural changes in coconut calli associated with the acquisition of embryogenic competence. Ann Bot 88:9–18

Viana VE, Pegoraro C, Busanello C, Costa de Oliveira A (2019) Mutagenesis in rice: the basis for breeding a new super plant. Front Plant Sci 10:1326

Wani MR, Kozgar MI, Tomlekova N (2014) Mutation breeding: a novel technique for genetic improvement of pulse crops particularly Chickpea (*Cicer arietinum* L.). In: Parvaiz A, Wani MR, Azooz MM, Lam-son PT (eds) Improvement of crops in the era of climatic changes. Springer, New York, pp 217–248

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Techol 47:189–194

Xie GL, Mew TW (1998) A leaf inoculation method for detection of Xanthomonas oryzae Pv. Oryzicola from rice seed. Plant Dis 82:1007–1010

Yatta D, khelafi H (2012) Mise en oeuvre des biotechnologies: cals embryogenes irrradiés, suspensions cellulaires en vue d'amélioration de palmier dattier à la résistance au bayoud. Célébration de la manifestation du 50eme anniversaire de l'indépendance Nationale organisée par la DGRSDT (la Recherche Scientifique et le Développement Technologique). 07 à 20 juillet, 2012 au Palais des expositions des Pins maritimes à Alger

Yatta D, khelafi H, Abed (2004) Effect of gamma rays doses on the embryogenic calli of date palm (Phoenix dactylifera L.). Regional workshop organised by A.I.E.A / F.A.O sur «Cost effective up scale in vitro plant production and long – term storage of mutant plant material» Sfax (Tunisia) 29 November to 3 December 2004

Yatta El Djouzi D, Abed F, Amara B et al (2014) The genomic DNA structure in somatic embryogenesis of date palm (*Phoenix dactylifera* L.) by flow cytometric, RAPD and AFLP analysis, vol 1. NOVA Publishers, New York, pp 241–252

Yatta El Djouzi D, Al-hayri JM, Bouguedoura N (2020) Plant regeneration from cell suspension-derived protoplasts of three date palm cultivars. Propag Ornam Plants 20(1):28–38

Yatta D, Abed F, Amara B et al (2013) Protoplast isolation from cellular suspension of two Algerian cultivars of date palm (Takerbucht and Teggaza). Acta Hortic 994:323–329

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273