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Original Article

Sexual Propagation of the Tunisian Spinescent *Opuntia ficus-indica* (L.) Mill., Morphogenetic Deployment and Polymorphism

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Abstract

The most common means of *Opuntia ficus-indica* (L.) Mill. propagation is through the use of asexually methods without genetic recombination which has been extensively studied for commercial purpose or to reduce erosion and desertification. However, seeds are important units to be considered for maintaining the species genetic variability. With the aim to undrestand the morphogenetic polymorphism deployment of the Tunisian spinescent *Opuntia ficus-indica* from the juvenile to the advanced stages, we search, firstly, the optimal conditions permetting to ensure the highest germination rate during the shortest period with sulphuric acid scarification in ten levels time of dipping. The best germinated rate (68.0%) is reached within 30 min of treatment exposure. Some germinated seeds showed polyembryony phenomenon. The highest rate (29.3%) was recorded after 25 and 30 min of pretreatment. Occurrence of morphogenetic abnormalities: tricotyledony, dissymmetried, coiled and adhered cotyledons in percentages varying from 12.8 to 20.0% was also reported. Secondly, a morphogenetic tracking of *Opuntia ficus-indica* young seedlings to adult plants during seven years (2009-2015) from the germination of seeds to the production of seeds was performed. This essay allowed us to report major seedling characteristics, their growth and phenotypic polymorphism: vertical succession of varying numbers of basal cladodes, future plant trunk formation, development and arrangement of secondary cladodes in the space, spinescence polymorphism and the first flowering and fruiting.

Keywords: abnormalities, cactus, germination, morphogenetic tracking, polyembryony

Introduction

Species from the genus *Opuntia* are cultivated worldwide as fruit and vegetable crops (Inglese *et al.,* 2002) and have gained economic importance in many countries of Europe, America, Asia and Africa (Basile, 2001). *Opuntia ficus-indica* (L.) Mill. (Cactaceae, Opuntioideae), called prickly pear or Nopal, is the widely distributed cactus in the world (Nobel, 2002). It is endemic to Mexico, but becoming an interesting alternative as a fruit and forage crop for semi-arid areas of the world (Cruz-Hernández and Paredes-López, 2010). The species is a multipurpose plant which is very important for the people who live in the production areas (Vigueras and Portillo, 2001; Cruz-Hernández and Paredes-López, 2010). Nearly, 1 million has been planted in the last 50 years in Algeria, Morocco and Tunisia where the Nopal is cultivated for human consumption as vegetable and for the production of prickly pear fruits called barbary figs (Selmi *et al.,* 2002). In Tunisia, *Opuntia ficus-indica* cultivars cover an area of about 600 000 ha distributed mainly in western central Tunisia (Kasserine, Thala, Sidi Bouzid and Gafsa) (Monjauze and Le Houérou, 1965; Nefzaoui and Ben Salem, 2002). In the Zelfene region of Kasserine province, there are an estimated 16 000 ha of commercial plantations for fruit production (Selmi *et al.*, 2002).

Since the early 1900s, various strategies have been adopted to reduce erosion and desertification in northern Africa (Nefzaoui and Ben Salem, 2001) where *Opuntia ficus-indica* varieties have been planted to reduce water and wide erosion and rangeland degradation. Among them the variety *inermis,* which is drought and erosion tolerant, is being advantageously and widely established in Tunisia and Algeria (Nefzaoui and Ben Salem, 2001). More recently, there is interest in boosting the productivity of cactus-growing areas to provide higher quality cladode and fruit and additional commercial areas could be planted (FAO, 2013). Despite its widespread, Barbary figs culture is considered as a marginal one since it has poor commercial values comparatively to other fruit crops furthermore, the plantations are seriously menaced by drastic genetic erosion due to biotic and abiotic stresses particularly the intensive urbanization (Bendhifi *et al.*, 2013).

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The ecological success of *Opuntia* can be explained in part based on the diversity of its methods of propagation (Reyes-Agüero *et al.*, 2006). According to Rojas-Aréchiga and Vázquez-Yanes (2000), cactus propagation can be done in three ways: through *in vitro* tissue culture, by means of vegetative propagation, or by seed germination. Vegetative propagation of Cactaceae has been extensively studied. It can be achieved *via* shoots and cuttings, by division of caespitose specimens, and by grafting. All of these are asexual methods without genetic recombination and have been used extensively for many species. The most common means of propagation in *Opuntia* is through the use of cladodes (Vélez-Gutiérrez and Rodríguez-Garay, 1996). Nopal is propagated asexually for commercial purposes, but seed propagation is essential for breeding (Cruz-Hernández and Paredes-López, 2010) and it is an important method because it allows genetic diversity of populations. Seeds are important units to be considered for genetic improvement and germplasm conservation because they are easily stored for long-term use (Vélez-Gutiérrez and Rodríguez-Garay, 1996).

Studies on seed germination and seedling establishment are important for understanding reproductive strategies. There is an urgent need for more studies on propagation for the conservation and the production of genetically unique individuals, which contribute to maintaining the species genetic variability. Propagation by seed of prickly pear species is used for scientific research to study the genetic variability and the factors affecting the germination process (Rojas-Aréchiga and Vázquez-Yanes, 2000). In fact, many species of the Cactaceae have prolonged seed dormancy with low or null germination (Rojas-Aréchiga and Vázquez-Yañes, 2000). Abnormalities and polyembryony were previously reported by authors (Gates, 1910; Went, 1944; Haskell, 1954; Harrison, 1964; Dessureaux, 1967; Kerr, 1985; Rajora and Zsuffa, 1986; Pimienta-Barrios, 1990; Asker and Jerling, 1992; Perez, 1993; Mondragon and Pimienta-Barrios, 1995; Garcia and Pimienta-Barrios, 1996; Nieddu and Chessa, 1996; Vélez-Gutiérrez and Rodríguez-Garay, 1996; Conway and Poethig, 1997; Negron-Ortiz, 1998; Vernon *et al.*, 2001; Mondragon, 2001; Graz, 2001; Azumi *et al.*, 2002; Nagesh and Kardam*, 2004*; Hu *et al.*; Khan 2006; Madishetty *et al.,* 2006; Korekar *et al.*, 2012; Narantsetseg, 2014; Shveta and Veenu, 2015) and tracking the evolution of the obtained seedlings allows the report of those phenomenons in our study. On the other hand, among the main modifications in the course of the development of *O. ficus-indica* were the modified stems (cladodes) which have undergone changes towards succulency, anatomy and physiology (Sudzuki Hills, 1995). Woody stems extension provide the necessary support that gives rise to the typical shape of an *O. ficus-indica* tree. Nevertheless stems extension of the arborescent cactus leading to a vertical growth over several years have never been reported or followed.

The purpose of this study was, firstly, to search the optimal conditions permetting to ensure the highest germination rate of seeds for *Opuntia ficus-indica* var. *spinescent* during the shortest period. The polyembryony occurrence and the morphologic abnormalities were reported. Secondly, we investigated during seven years, from 2009 to 2015, the morphogenetic polymorphism deployment of *O. ficus-indica* from the juvenile to the advanced stages, particularly their ramification and occupation of space, before the installation of the arborescent adult cactus marked by the first flowering and fruiting.

Materials and Methods

Plant material

Mature fruits were collected in august of 2009 on vigorous *O. ficus-indica* var. *spinescent* (spines at the areolas) mother plants planted in a private field in Thala, the coldest region in Tunisia (35° 35′ N; 8° 40′ E), located in Kasserine governorate, west central Tunisia, (Fig. 1). The locality is under a semi-arid climate with a cold winter and environmental conditions were an average minimum temperature of 10 °C and an average maximum temperature of 20 °C per year. The pluvial precipitation varied from 150-450 mm per year (PREDD, 2015).

Seed extraction

Fruits are purplish red coloured and contain an average of 80 seeds per fruit. At the laboratory, seeds were manually removed from fleshy pulp and washed with tap water followed by distilled water. The broken, deformed and insect damaged seeds were discarded. The average seed weight is 0.017 g. Seeds were air-dried for two weeks, and stored in glass bottles at room temperature (23-25 °C) and at a relative humidity of 50%.

Sterilization and chemical treatment of seeds

Uniformly shaped and sized seeds (≥2 mm) were sterilized first by soaking in a 5% solution of hypochlorite sodium for 15 min, then, with mercury chloride ($HgCl_2$) for 10 min and washed three times with sterile distilled water. In order to study the effects of sulphuric acid on germination and early seedling growth in *O. ficus-indica* var. *spinescent*, seeds (1,500) were divided into ten lots and an experiment was conducted using a completely randomized design in six replications of 25 seeds for each treatment. The scarification effect was evaluated in ten levels time of dipping in sulphuric acid (98%, 36N) H_2SO_4 (0, 5, 10, 15, 20, 25, 30, 40, 45 and 60 min). Subsequently, the seeds were washed thoroughly under running tap water, followed by three rinses with distilled water to remove the acid from the seeds completely.

Fig. 1. Location map of Kasserine governorate (Thala region, Tunisia)

Seed germination, polyembryony and morphogenetic abnormalities

The seeds under different pre-sowing treatment conditions were subjected for a germination test. Twenty five seeds were put in each Petri dish (9 cm diameter) on 5 filter papers moistened with sterile distilled water in six replications for each respective time treatment (a total of 150 seeds by treatment). The filter paper was always kept wet with distilled water. The Petri dishes were covered to prevent the loss of moisture by evaporation, and subsequently put in a climatic growth chamber temperature-controlled for 30 days at 26 °C (8 h obscurity/16 h light) and 65% relative humidity. Seeds were kept under observation till complete germination of the seeds. Every 24 hours after soaking, the number of germinated seeds was reported. Emergence counts were made daily for one month. Seeds were considered germinated when the emergent radicle reached 2 mm length.

After 30 days of incubation, germination percentages were recorded. The germination percentage was calculated by using the following formula: Germination % = (Number of germinated seeds/Total number of seeds sown) × 100. Polyembryony was evaluated daily by direct observations during the germination tests and by counting the seeds which develop two or more plantlets during the seedling emergence. For morphogenetic abnormalities, 400 seeds were treated during 30 min with the sulphuric acid and are kept to germinate in the same conditions. The number of plantlets which show morphogenetic abnormalities was reported and percentages of polyembryony and morphogenetic abnormalities were calculated.

Seedling transplantation and acclimatization of plants

After emergency of the embryonic root, the hypocotyl developed and the cotyledons emerged. In order to follow the development of seedlings and since the Petri dishes were narrow we proceed the transplantation of seedlings aged of one month, to larger containers consisting of 6 cm diameter and 750 cm^3 volume Jiffy-pots, previously prepared and continually watered, containing peat commercial soil substrate, one plantlet per pot. The Jiffy- pots were placed in a climatic growth chamber temperature-controlled (26 °C, 8 h obscurity/16 h light). Plantlets continue growth and form a more substantial root system with a hypocotyl increasing in height, cotyledons open and gemmule lengthens and forms the cylindrical epicotyl (2-3 cm length). Plantlets aged of 4 months, considered as young plants since they are an independent autotrophic organisms capable of photosynthesis, were transferred in 16 cm diameter and 2000 cm^3 volume pots, filled with a mixture of vegetal soil (2/3) and fine sand (1/3) and placed under a greenhouse at 23 °C day/18 °C night temperature. To keep the substrate moist, the pots were irrigated twice a week with 100 mm of tap water during 8 months. After eight months, plants aged of 1 year were transferred in larger pots (30 cm diameter and 3800 cm^3 volume), filled with the same mixture soil placed outside the greenhouse in the shade and irrigated once a week with tap water in autumn/winter and twice a week in spring/summer.

Tracking of morphogenetic polymorphism deployment of seedlings to adult plants

A morphogenetic tracking of *Opuntia ficus-indica* young seedlings to adult plants during seven years (2009-2015) from the germination of seeds to the production of seeds, by reporting major phenotypic polymorphism, was performed. Seedling characteristics, their growth and phenotypic variation were studied.

Statistical analyses

The number of germinated seeds (radicle ≥2 mm long) was recorded daily. Germination percentage was based on the percentage of viable seeds for each species. Data collected from ten independent experiments were subjected to analysis of variance (ANOVA) using SPSS 13.0 for Windows (SPSS Inc Chicago, Il, USA). The percentages of seed germination, polyembryony and morphogenetic abnormalities were transformed using arcsin square-root (arcsin $\sqrt{x}$) before ANOVA. Means were separated at the 5% significance level by a least significant difference test (*Student's test*).

Results and Discussion

Scarification influence on seed germination, polyembryony and abnormalities in seedlings

Seed germination started after two weeks, the radical grows and bursts through the testa and hypocotyl begins to elongate. The rapid growth of the hypocotyl pulls the cotyledons out of the testa. The gemmule was still between the cotyledons. The hypocotyl straightens and the cotyledons separate, exposing the gemmule. The seeds of *O. ficus-indica* have an epigeal germination. For each replicate, cumulative germination percentages were recorded after 30 days. Soaking of *Opuntia ficus-indica* seeds in concentrated sulphuric acid at different duration had significant effect ($p \leq 0.5$) on the final germination percentage (Table 1). *Opuntia* seeds germination was very low and takes between two weeks to one month.

The seeds without any treatment show a week percentage of germination (5.3%). This percentage increased with the time of sulphuric acid soaking and germination peaked at 68.0% within 30 min of treatment exposure. When pre-treatment time exceeds 30 min, the germination percentage decreased (40.0% and 10.6% after 40 and 45 min of scarification, respectively). Seeds exposed 60 min in H_2SO_4 did not germinate. Thus, a long period of treatment damaged seeds and causes the embryo death. Acid scarification is known to be highly effective in improving germination of species with hard seed coats (Youssef, 2008).

Table 1. Effect of the duration of scarification with H_2SO_4 on the germination of *O. ficus-indica* seeds and polyembryony detected

Duration of scarification with H_2SO_4 (min)	Germination percentage at day 30th	Polyembryony percentage
0 (control)	5.3±0.8ab	0.0±0.0a
5	24.0±2.3c	17.9±6.3c
10	30.6±3.0cd	7.4±3.7b
15	48.0±3.8e	6.1±3.6b
20	50.6±3.5f	17.6±3.9c
25	60.0±6.3g	29.3±8.1e
30	68.00±5.2h	29.3±3.6e
40	40.0±2.3d	23.3±8.0d
45	10.6±2.8b	22.2±10.2d
60	0.0±0.0a	0.0±0.0a

Note: Means ± SD with the same letter (s) in the same column are not significantly different ($p > 0.05$) (*Student's* multiple range test) (n = 150 seeds per treatment)

The seeds of *O. ficus-indica* derived from an anatropous ovule, as has been reported for the first time by Bâillon (1887). In the Opuntioideae subfamily, the hardness of the *Opuntia* seed covers was related to a strongly lignified funicular tissue which surrounds and completely encloses the seed (Archibald, 1939; Bregman and Bouman, 1983; Stuppy, 2002).

Many authors have studied the effect of scarification on the germination of cactus seeds. *Opuntia ficus-indica* seeds, as for many other *Opuntia* species, show low germination capacity due mainly to their hard lignified integuments. The most inward of these was the funiculus that envelops the embryo, obstructing radicle protrusion (Altare *et al.*, 2006). Then, immersion in concentrated sulphuric acid increased germination in some species of *Opuntia* (Potter *et al.*, 1984; Khan, 2006). Beltran and Rogelio (1981) obtained an increase in germination speed and in rate through scarification, which can be a mechanical or a chemical scarification with HCl at 20% for 24 hours. Altare *et al.* (2006) reported that scarification of *Opuntia ficus-indica* f. *inermis* (Web.) Le Houér. Seeds with H_2SO_4 for 5 min followed by their incubation in H_2O_2 solution (from 1 to 5%), under photoperiodic regime 14/10 h (light/darkness), gave the maximum germination percentage in the shortest time (80.0-85.3%). For Olvera-Carrillo *et al.* (2003), the scarification period varied; *O. tomentosa* seeds collected in 2000, needed a short scarification (5 min) to germinate in higher percentages, while seeds of the same age, collected in 1998, required 90 min in sulphuric acid to reach similar germination percentages.

Up to the present, seed dormancy has been found in 28 *Opuntia* species from which the *O. ficus-indica* (Wang *et al.*, 1996; Altare *et al.*, 2006; D'Aubeterre *et al.*, 2006). It's a very common adaptive plant strategy in arid and semi-arid landscapes (Jurado and Flores, 2005). The *Opuntia* species hard seed covers (Aguilar *et al.*, 2003; Orozco-Segovia *et al.*, 2007) act as mechanical protection of the embryo, instead of being an impermeable element (Werker, 1997). Seed dormancy was considered a very common adaptive plant strategy in unpredictable or harsh environments, such as arid and semi-arid landscapes (Jurado and Moles, 2003; Jurado and Flores, 2005). Reyes-Agüero *et al.* (2006) noted that this characteristic was quite common in *Opuntia* seeds and was associated with the impermeability of the tegument, funicular hardening, and with their capacity to survive long periods of dormancy (up to 15 years or more). It has been suggested that *Opuntia* spp. seeds have both physiological dormancy (a period after-ripening to break seed dormancy) and mechanical dormancy (their embryos have a low growth potential) (Orozco-Segovia *et al.*, 2007; Delgado-Sánchez *et al.*, 2010). Although several authors have also suggested that *Opuntia* seeds have physical dormancy (Olvera-Carrillo *et al.*, 2003; Reyes-Agüero *et al.*, 2006).

Polyembryony phenomenon

Opuntia ficus-indica seedlings were phenotypically non-uniform, slow growing with long juvenile period (Pimienta-Barrios, 1990). The majority of germinated seeds developed a single plant (monoembryony). However, some ones showed polyembryony phenomenon, with two seedlings emanating from the same seed (Fig. 2 a-f). The obtained results were summarized in Table 1. Polyembryony, as large as 4 seedlings per seed of *Opuntia*, as reported in literature (APAT, 2003), has not been reported in our studies.

Polyembryony percentages of germinated seeds varied according to the treatment duration. The highest rate (29.3%) was recorded after 25 and 30 min of treatment. Also, high rates were recorded after 40 and 45 min of treatment (23.3% and 22.2%, respectively). The polyembryony phenomenon was reported for many *Opuntia* species (Pimienta-Barrios, 1990; Asker and Jerling, 1992; Mondragon and Pimienta-Barrios, 1995; Garcia and Pimienta-Barrios, 1996; Negron-Ortiz, 1998; Mondragon, 2001) and for *Opuntia ficus-indica* (Mondragon and Pimienta-Barrios, 1995; Nieddu and Chessa 1996; Velez and Rodriguez, 1996). This phenomenon can occurs during the multiplication by seed inducing two or more embryos formation. One of them is a zygotic embryo of sexual origin derived from fertilization of the embryo sac. The other, was called apomictic, derived directly from nucellar tissue (sporophytic agamospermy) (Pimienta-Barrios, 1990) or from unfertilized egg (diplospory-parthenogenesis) (Garcia and Pimienta-Barrios, 1996; Velez and Rodriguez, 1996; Mondragon, 2001). In Central Mexico, polyembryony was found in four of the seven *Opuntia* species studied, and it appears to be more frequent in seeds from dry sites of the gradient. *Opuntia robusta* seeds show the highest percentage (20.7%) (Romo-Campos *et al.*, 2010). The polyembryony causes are numerous. Environmental variations can affect plant characteristics such as morphology seed (Giménez-Benavides *et al.*, 2005; Luzuriaga *et al.*, 2005; Lemos *et al.*, 2008). According to Batygina and Vinogradova (2007), ecological conditions were essential in the polyembryony induction: high or low temperatures and humidity. A common feature of all apomicts was the autonomous development of embryos and the generation of progenies that were exact genetic replicas of the mother plant (Carneiro *et al.*, 2006). Polyembryonic seeds were common in most of the wild and cultivated *Opuntia* ssp. growing in semi-arid central Mexico (Perez, 1993). Two and on rare occasions three embryos were detected for many wild *Opuntias* seeds (Trujillo and Gonzalez-Espinosa, 1991). Tisserat *et al.* (1979) reported apomixis in several *Opuntias* among them *O. ficus-indica.* According to Perez (1993), the presence of polyembryonic seeds in germination tests varied from 10.9 to 18.5% for *O. streptacantha* and its hybrids, 3.6 to 24.7% for *O. robusta*, 0 to 14.3% for *O. cochinera*, 0 to 6.7% for *O. leucotricha* and 0 to 50% for *O. rastrera*.

To the best of our knowledge, there were no studies recording this phenomenon for the *Opuntia* cultivated in various countries from North Africa and specially in Tunisia for *O. ficus-indica.*

Morphogenetic abnormalities

To study morphogenetic abnormalities, 400 seeds soaked during 30 min, in sulphuric acid were kept to germinate in the same conditions. A total of 270 seeds germinated (67.5%). Seedlings of one month showed four different morphogenetic abnormalities; tricotyledony, dissymmetried, coiled or adhered cotyledons in percentages varying from 12.8 to 20.0% (Table 2; Fig. 2). The majority of seedlings have two cotyledons but few of them (44 in 270 seedlings i.e., 15.9%) exhibited tricotyledony (Fig. 2g).

An important cotyledon polymorphism was also noted,

Table 2. *Opuntia ficus-indica* morphogenetic abnormalities frequency

Type of abnormality	Tricotyledony	Adhered cotyledons	Coiled cotyledons	Dissymmetried cotyledons
*Number of seedlings with abnormality	44	53	34	39
Abnormality (%)	15.9±2.6b	20.0±2.4c	12.8±2.7a	16.0±3.2b

*400 seeds were kept to germinate, 270 seedlings were obtained

Fig. 2. Seedlings at different stage of development showing polyembryony and morphogenetic abnormalities. Polyembryony was reported for seedlings aged of three (a) or four (b, c) weeks, and for plantlets aged of three (d, e) or four months (f). Tricotyledony (g) dissymmetried (h, i), coiled (j) or adhered (k, l) cotyledons were observed for seedlings aged of two weeks (h), one (g, j, k, l) or two (i) months

mainly the adhered cotyledons (Figs. 2k and 2l) and reached the highest percentage of 20.0%. The other abnormalities: coiled cotyledons (Fig. 2j) and dissymmetry of the two cotyledons (Figs. 2h and 2i) were reported at percentages of 12.8 and 16.0%, respectively.

According to Conner and Agrawal (2005), intraspecific variation in cotyledon number occurs in some dicotyledonous plants. This phenomenon producing three cotyledons has been referred to as tricotyledony or tricotyly and has been reported from hundreds of species in over 15 families of plants (Gates, 1910; Went, 1944; Haskell, 1954; Harrison, 1964; Dessureaux, 1967; Rajora and Zsuffa, 1986; Graz, 2001). Tricotyledony referred to the presence of three cotyledons in dicotyledonous plants was well known as a rare phenomenon sporadically present in dicotyledonous plants, it has apparently been treated in the past as a rare and somewhat trivial abnormality (Holtorp, 1944; Shveta and Veenu, 2015). Holtorp (1944) have been unable to trace any evidence of its being regarded as heritable.

Researchers have documented this phenomenon of tricotyledonous mutant in many plant species (Holtorp, 1944; Reynard, 1952; Kerr, 1985; Nagesh and Kardam, 2004; Madishetty *et al.,* 2006). More generally, the production of an abnormal number of cotyledons has been referred to as pleiocotyly (Korekar *et al.*, 2012). Molecular studies have shown that a few mutated genes could produce the tricotyledonous in *Arabidopsis* (Azumi *et al.*, 2002; Vernon *et al.*, 2001; Conway and Poethig, 1997). For *Opuntia*, only Khan (2006) observed tricotyledonous seedlings in *O. ficus-indica* from northern areas of Pakistan. The author shows that majority of seedlings had two cotyledons but 7.86% of them, exhibited tricotyledony. Tricotyledonous seedlings did not differ significantly from dicotyledonous ones in radicle and hypocotyl growth, but had somewhat larger cotyledonary area per seedling. More recently Narantsetseg (2014) noted that appearance of more than two cotyledons might be related to genetic drift. This phenotype was controlled by a few recessive genes with about 50% penetrance (Hu *et al.*, 2005).

Low frequency of tricotyledony has also been reported in *Brassica oleracea* var. *capitata* (0.6%) (Gupta and Jain, 1980), *Crotolaria juncia* (1.05%) (Purkayastha, 1940) and *Raphanus raphanistrum* (0.53%) (Conner and Agrawal, 2005). Plants with three cotyledons are potentially useful for faster establishment of seedlings after planting and may serve as morphological characteristics for distinguishing cultivars (Hu *et al.*, 2006). Molecular studies have shown that a few mutated genes could produce tricotyledonous (Gupta and Jain, 1980). According to Olvera-Carrillo *et al.* (2009), polymorphism in seed germination observed in *O. tomentosa* was a consequence of the morphological features (hardness of the funicular envelope) and physiological features (physiological dormancy) of seeds. The extra cotyledon could help the seedlings to provide nutrients such as lipids, proteins and carbohydrates that the growing plant needs. Another interesting aspect was that the mutant has larger leaf area for photosynthesis (Hu *et al.*, 2005) and showed increased levels of polar auxin transport (Al-Hammadi *et al.,* 2003).

Tracking morphogenetic development of seedling during the first year

The seemingly simple process of seed germination initiates seedlings that undergo a long series of morphogenetic changes during one year and we charted the progress of seedlings as they germinated and grow (Fig. 3). After two weeks of sowing of seeds removed from fruits (Figs. 3a and 3b), the radicle emerged (Fig. 3c), followed by the hypocotyl elongation and the appearance of the two cotyledonary leaves (Fig. 3 d-f). After one month, the gemmule appeared (Fig. 3g). Four month later, seedlings were transplanted to Jiffy-pots. The gemmule reached an average length of 3 cm (Fig. 3h). After, it starts to flatten and lead to a globulous stem with areolas and small conical leaves, arranged according to a multihelical phyllotaxy of dextral and senestre winding, accompanied by glochids and trichomes (Fig. 3i). After six culture months, cotyledons turn yellow and fall (Fig. 3j) and the eight-month photosynthetically active seedlings characterized by the borough of cladode summital zone (Fig. 3k) were transferred in 2000 cm^3 volume pots. The number of areolas was relatively more important at this zone (Fig. 3l). Seedlings continue their growth until the age of one year.

Morphogenetic evolution and polymorphism of cactus plants from juvenile to adult (1-7 years) stage

During seven years, we followed and studied the development of *O. ficus-indica* plants. An important phenotypic polymorphism in advanced stages was reported (Fig. 4). Morphological characters analysis of the cladodes arrangement revealed considerable variability. In fact, we noted, during the first three years of culture, a vertical succession of varying numbers of basal cladodes (3 to 5) (Fig. 4a), that contribute, after lignification and suberisation, to the formation of the future plant trunk. During the fourth year of culture, the plants, transferred into larger pots (3800 cm^3 volume), showed a high variability in their ramifications which occurs most frequently from the areoles of the latest cladode apical zone with the formation of two or three secondary cladodes staggered in time, either in the same plan of the preceding cladode or arranged in orthogonal plan (Figs. 4b and 4c). Cladodes ramifications continue with the tertiary cladode development, generally arranged in the same plan during the fifth year (Figs. 4d and 4e). At the end of the fifth year, plants showed an important polymorphism concerning spinescence. Few plants remains spinescent, while for others, a partial or total falling of spines was observed (Fig. 4f). In the nature, intermediate forms were encountered from *Opuntia ficus-indica* var. *spinescent* to var. *inermis* (Walali Loudyi and Skiredj, 2003). In the sixth year of culture, all plants derived from seedlings were transferred to an experiment field of 200 m^2, in the area of the Faculty of Sciences of Tunis. Plants continue their ramification and become shrubs with an average size of

Fig. 3. Tracking morphogenetic development of spinescent *Opuntia ficus-indica* seedlings during the first year. Fruits on mother plants (a); seeds from the *spinescent* variety (b); seed germination and radicle elongation (c), cotyledonary leaves appearance and hypocotyl elongation (d-f); gemmule appearance (g); seedlings aged of one month transplanted and developed in Jiffy-pots (h); appearance of juvenile leaves and epicotyle flattening (i); cotyledons falling (j); plants aged of 4 months, transplanted in pots and borough of cladode summital zone (k) bearing an important number of areoles (l)

Fig. 4. Morphogenetic development of the *Opuntia ficus-indica* var. *spinescent* plants. Vertical succession of 3-5 basal cladodes and lignification of the basal cladodes during the first three years (a); development of two to three secondary cladodes during the fourth year culture (b, c); development of tertiary cladodes (d, e); plants aged of 5 years with partial or total falling of spines (f); after the 6th year *Opuntia* shrub (g) and final trunk formation (h); formation of the first flowers (i) and fruits after the 7th year (j, k)

about 2-3 m (Fig. 4g). Brownish basal rackets, suberized, very lignified and branched in their apical zone form a final trunk (Fig. 4h). At the seventh year, we attend the first flowering of adult plants during April/June period (Fig. 4i). Seedlings of *Opuntia ficus-indica* obtained from seeds show long juvenile phase. They take many years to develop and grow in a typical adult arborescent cactus. Flowering maturity was observed in an average of seven years. The first crop of ripe fruits occurs from august to October (Figs. 4j and 4k) with limited number of flowers and fruits which intensified in the following years.

Conclusions

The pretreatment of seeds by sulfuric acid during 30 min was essential and facilitate nursery production of *O. ficus-indica* seedlings. Abnormalities and particularly polyembryony were reported for the first time for *O. ficus-indica* cultivated in Tunisia and could be exploited in genetic programs amelioration. Although that seeds were harvested on *O. ficus indica spinescent* mother plants, after 5 years we obtained spinescent or *inermis* cactus plants and after seventh year, we attend the first flowering of adult plants and the first crop of ripe fruits. Basal rackets forming the future trunks and the development of ramification either in the same plan or in the orthogonal plane allow the maximum optimization of the space. The polymorphism of *O. ficus-indica* can be considered in selection programs for new and interesting agronomic characters.

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