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Chapter 2

Biotechnological advances in *Opuntia*

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1 Introduction

Biotechnology today is part of numerous agricultural and industrial processes, contributing through its ability to increase speed, scale production, while also acting at localized levels such as cells, organelles, or genetic information, and providing clean products that contribute to environmental preservation.

The demonstrated usefulness of *Opuntia* spp. for obtaining products of various kinds, as well as the ability of plants from this genus to grow in arid lands, making the most of scarce nutrients and limited water, with high biomass production per unit of area, have led to research aimed at maximizing these properties through biotechnological techniques.

Some of the research findings on the application of biotechnology in *Opuntia* spp. are already being implemented, such as those related to the mass multiplication of plants through in vitro culture techniques (El Finti et al., 2013; Rodríguez & Ramírez-Pantoja, 2020; Mabrouk et al., 2021; Portillo and Soltero, 2021). Genetic studies have been developed to an acceptable level, but their application has not gone beyond the field of phylogenetics. Other approaches, such as transgenesis, are still being refined (Felker et

al., 2018; Angulo-Bejarano et al., 2019). The most important advances achieved in recent years are reviewed below.

1.1 *In Vitro* Micropropagation

The multiplication of *Opuntia* species can be carried out relatively easily from cladodes or seeds, in the latter case when aiming to obtain genetic variability. However, *in vitro* culture methods (micropropagation) have also been tested in this genus for several reasons:

- Propagation through botanical seeds does not guarantee genetic stability. Additionally, the seeds of most *Opuntia* species are demanding in terms of light and humidity conditions for germination. Finally, some species, such as *Opuntia ficus-indica*, the most extensively cultivated species worldwide, exhibit a germination rate barely exceeding 5% (Stambouli-Essassi et al., 2017). This problem may have a solution through *in vitro* culture techniques, which in this species allow for the production of over a thousand plants in one year from an only one explant (Rodríguez & Ramírez-Pantoja, 2020).
- Cladode propagation, although effective, can be slow when aiming to obtain plants on a large scale. In contrast, *in vitro* culture allows the obtainment of thousands of plants in a small space and in less time, reducing costs and preserving the genetic identity of the multiplied materials (Mabrouk et al., 2021).
- Many *Opuntia* species can be affected by diseases transmitted through cuttings. Among these is the so-called "macho disease," which consists of excessive thickening of the cladodes, apparently caused by an umbravirus (Felker et al., 2019). Other viruses and viroids have been reported recently (Ortega-Acosta et al., 2024). Fungal diseases, such as scabby canker caused by *Neofusicoccum batangarum*, also affect them (Schena et al., 2018; Aloi et al., 2020). Finally, the major pest insect of *Opuntia* spp. is *Dactylopius opuntiae*, a cochineal that wreaks havoc on the plants (El-Aalaoui & Sbaghi, 2022). The selection and micropropagation of healthy material can help in obtaining plants free from sanitary issues.

- The multiple uses of *Opuntia* spp. (human and animal food, pharmaceuticals, cosmetics, industrial uses) have led to their overexploitation. This, combined with the occupation of areas where these species naturally grow for other purposes (urbanization, industrial development), threatens the existence of some of them. According to a report by a group of experts for the International Union for Conservation of Nature (IUCN) Red List Categories and Criteria, 31% of cacti are at risk (Goettsch et al., 2015). *In vitro* culture can be beneficial for the conservation and multiplication of endangered species (Bouzroud et al., 2022), making it an alternative in this situation.

In vitro propagation of cacti, according to Lema-Rumińska & Kulus (2014), began about 60 years ago, but here only the most recent results will be discussed. Most research aimed at establishing *in vitro* multiplication technologies for *Opuntia* spp. has been developed with *Opuntia ficus-indica* L., possibly because it is the most recognized and commercially exploited species.

In Morocco, numerous efforts have been made to optimize propagation protocols for this species. El Finti et al. (2012) used cladode fragments containing the basal structures where the glochidia emerge (areoles) as explants, and tested various concentrations of kinetin (KIN) and benzyladenine (BA), combined or not with naphthalene acetic acid (NAA) at 0.5 mg L^{-1} to induce shoot formation. BA at 5 mg L^{-1}, alone or together with NAA, produced 15.25 and 13.5 shoots/explant, respectively. For rooting, they tested indole-3-acetic acid (IAA), NAA, and indole-3-butyric acid (IBA) at concentrations of 0.5 mg L^{-1}, obtaining the best results with IBA. These results were later successfully used in the *in vitro* propagation of three cultivars of this species (El Finti et al., 2013).

Bougdaoua and El Mtili (2020), on the other hand, used seeds from two Moroccan genotypes (Tétouan and Al-Hoceima) of *Opuntia ficus-indica* L., disinfected with mercury chloride and germinated on Murashige and Skoog (1962) medium. In the shoot proliferation stage, they used only BA and obtained the best results with 0.5 mg L^{-1} of this cytokinin, reaching 12.28 shoots per bud in Al-Hoceima and 7.73 for Tétouan; for rooting, they used IBA at three concentrations (0.1, 0.2, and 0.5 mg L^{-1}), without finding any significant differences in its effect.

Mabrouk et al. (2021) tested different types of explants and hormonal combinations to optimize propagation conditions for *Opuntia ficus-indica* L. The best variant turned out to be using areoles with intact glochidia as explants, disinfected for 10 minutes with 4.23% calcium hypochlorite and 0.4% Tween 20. For shoot formation, the combination of 5 mg L-1 of 6-benzylaminopurine (BAP) + 0.5 mg L^{-1} of NAA produced the best results.

The use of BAP to promote shoot formation also yielded positive results for Bouchiha & Mazri (2022); concentrations between 2 and 3 mg L^{-1} of this cytokinin generated between 16 and 21 new shoots per explant. The subsequent removal of cytokinins from the culture medium favored shoot elongation, and the vigor reached by the plants ensured a survival rate of over 80% during the acclimatization phase. Similarly, Marhri et al. (2023) obtained more than 19 shoots per explant with 5 mg L^{-1} of BAP. Interestingly, although auxins are traditionally used to stimulate rooting (Shehu et al., 2016), these authors report that adding 1.5 mg L^{-1} of KIN reduced rooting time and increased both the quantity and length of the roots.

In Mexico, Rodríguez & Ramírez-Pantoja (2020) created a protocol for the large-scale propagation of *O. ficus-indica* L. from areoles. To achieve this, they tested different concentrations of two cytokinins (KIN and BA) and two auxins (IAA and NAA) in MS (Murashige & Skoog, 1962) medium. The mixture of 2 mg L^{-1} BA + 0.2 mg L^{-1} NAA produced the highest number of shoots and the largest diameter of these; for rooting, the greatest results were achieved with 6 mg L^{-1} KIN + 2 mg L^{-1} IAA. The researchers estimated that with this methodology, more than 1200 new plants can be obtained after 14 months from each areole established in the laboratory.

Regarding the possibility of conserving genotypes of interest *in vitro*, Zoghlami et al. (2012) analyzed the genetic stability of *O. ficus-indica* L. plants maintained for 5 years *in vitro*, using randomly amplified polymorphic DNA markers (RAPD). The genetic variation found after such a long conservation period was very low (2.79%), indicating that this biotechnology could be useful for preserving endangered individuals.

Other *Opuntia* species have also been the theme of *in vitro* propagation due to their importance and application in various fields of production. In *Opuntia lanigera* Salm–Dyck, the highest proliferation was achieved with 6-γ,γ-dimethylalylaminopurine (DAP)

at concentrations between 5 and 7 mg L^{-1} (8 shoots per explant). The use of gibberellic acid (300 ppm) during the acclimatization phase was favorable for increasing seedling height (Estrada-Luna et al., 2008).

Gutiérrez & Portillo (2017) studied the *in vitro* response of *O. ficus-indica*, *O. joconostle*, *O. schickendantzii*, and *O. cochenillifera* to various concentrations of KIN, BA, and 2-isopentenyl adenine (2ip). As expected, there was an influence of genotypes on the results; however, in general, BA was the cytokinin that most stimulated shoot formation. Portillo & Soltero (2021) summarized the advances made at the University of Guadalajara in Mexico and Michael Technology in California, USA, in the propagation of various *Opuntia* species. They suggest using BA to stimulate shoot formation, followed by a multiplication phase without growth regulators using activated charcoal (2 g L^{-1}), and a similar phase for rooting. This contrasts with the approach proposed by Verma et al. (2020), who highlight the different responses of genotypes to growth regulators.

Ferreira et al. (2022) successfully replaced the KNO_3 in Mursahige & Skoog (1962) medium, where 1.9 g L^{-1} of this substance is used, with a smaller amount of potassium fertilizer (1.0 g L^{-1}), resulting in better growth of *Opuntia stricta*. This also represents a considerable cost-saving and resource-saving measure, as in several countries such as Brazil, high-quality reactive KNO_3 is regulated under strict government permits due to its explosive nature. Another interesting approach is the possibility of using pectin and mucilage from *Opuntia* spp. residues as substitutes for agar in culture media (Sánchez-Gutiérrez et al., 2023).

In summary, the accumulated experience in the micropropagation of *Opuntia* spp. is now extensive. There is no consensus among authors regarding the need to use growth regulators, the most suitable substances, their optimal concentrations, or the genotype responses to these conditions. However, the results demonstrate the feasibility of propagating these species in the laboratory in abundant quantities, and the usefulness of this biotechnology in the accelerated multiplication and conservation of species within the genus.

1.2 Somatic Embryogenesis

As in other species, in *Opuntia* spp., somatic embryogenesis has been approached as a method for mass reproduction and as a route for obtaining plants resulting from genetic transformation protocols. In this regard, efforts have been made to obtain embryos from calli as well as to rescue embryos from apomixis (the production of seeds without fertilization), which is a relatively common phenomenon in this genus (Mondragón-Jacobo, 2002).

Pinheiro da Costa et al. (2001) attempted to induce somatic embryo formation from immature seed tissues of *O. ficus-indica*. Using the B5 medium (Gamborg et al., 1968) supplemented with KIN and 2,4-dichlorophenoxyacetic acid (2,4-D) to the medium, they achieved the formation of globular structures, but upon transferring them to fresh medium, they did not progress to higher developmental stages.

From stem apices obtained from areoles of *O. ficus-indica* grown *in vitro*, Gomes et al. (2006) succeeded in obtaining somatic embryos on MS (Murashige & Skoog, 1962) medium with 4 mg L^{-1} of 4-amino-3,5,6-trichloropicolinic acid (Picloram) as an auxin-like growth regulator. The embryos matured and developed into plants, although with a very low conversion frequency (12.5%). It was also observed that the age of the shoots and the concentration of sucrose in the culture medium influenced embryo formation, which occurred more frequently in 10-15-day-old shoots and sucrose concentrations of 10-40 g L^{-1}.

Bouamama et al. (2011) worked with immature anthers, cultured in the dark on a medium containing 2.0 mg L^{-1} of 2,4-D and 2.5 mg L^{-1} of Thidiazuron (TDZ). The efficiency of embryo formation was around 60% for the two cultivars tested. The plants obtained were successfully acclimatized.

Kaaniche-Elloumi et al. (2015) used ovules extracted from *O. ficus-indica* flowers 10 days before anthesis as explants. These authors reported that prior application of GA_3 (500 ppm) to the flowers favored somatic embryo formation. In a new study by the same research group published simultaneously, Jedidi et al. (2015) obtained somatic embryos from this same type of explant on MS (Murashige & Skoog, 1962) medium, to which they added 87 mM fructose and 3 μM GA_3. Plants from somatic embryos were formed in a similar medium with 0.3 μM GA_3, although the authors did not report the conversion

frequency. Later, Jedidi et al. (2021) clarified that somatic embryogenesis occurs from the funicular cord tissues, which connect the embryo to the maternal tissue.

Apomixis is an obstacle to conventional breeding programs, which is why Carra et al. (2023) analyzed the frequency of apomictic and zygotic embryos rescued from ovules of four different cultivars of *O. ficus-indica* from 20 to 40 days post-anthesis. The immature ovules were cultured on MS (Murashige & Skoog, 1962) medium, where the embryos germinated and were subsequently allowed to continue developing into plants. The genetic constitution of the plants derived from the embryos was compared with that of the mother plants using molecular markers based on inter-sequence single repeat (ISSR) primers. The observed frequency of zygotic embryos varied with genotype but was higher in plants from embryos obtained at 35 days post-anthesis. The method appears to be a promising approach to support genetic improvement programs through ovule culture for rescuing zygotic embryos from crosses.

Immature ovules seem to be the most reliable source of tissue for somatic and zygotic embryogenesis in *Opuntia ficus-indica*. However, much remains to be done in terms of standardizing the cultivation methods and extending them to other species of the genus.

1.3 Transgenesis and Genetic Studies

Genetic transformation in *Opuntia* has been attempted using two methods: biolistics and *Agrobacterium tumefaciens*-mediated transformation.

The work by Llamoca-Zárate et al. (1998, 1999) proved the possibility of transforming the genome of *O. ficus indica* through biolistics, using calli, cell suspensions, and *in vitro* cultured plant fragments. The transformation efficiency for plant fragments was approximately 35% for the three genes transferred. In contrast, Cruz et al. (2009) achieved only 4.1% transformation efficiency through biolistics, using a mutant gene for resistance to the herbicide Imazapyr as the transfer material.

Angulo-Bejarano et al. (2019) conducted their studies on micropropagated plant fragments through biolistics. Two transgenes, *nptII* and *uidA* (coding for neomycin phosphotransferase II and for β-glucuronidase enzymes, respectively), were expressed in the transformed cells, and their presence in the genome was confirmed by Real Time-

Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR) assays, showing a transformation efficiency of 23%.

Silos-Espino et al. (2006) were the first to achieve transformation of *O. ficus indica* with *Agrobacterium tumefaciens*; they worked with meristematic tissue fragments, injecting the bacteria with a syringe, and obtained plants resistant to kanamycin, but with a transformation efficiency of only 3.2%. Felker et al. (2018) were unable to replicate these results and instead immersed 5 mm calli in a solution of *A. tumefaciens* for 30 minutes. Transformation was achieved, but plant regeneration from the calli was not attained.

Transgenesis protocols in *Opuntia ficus indica* have made modest progress, with transformation efficiency being medium to low compared to other species. There remain uncertainties about which transformation method is more efficient overall, in terms of achieving both adequate expression of foreign genes and good regeneration of the transformants.

In terms of genetic studies in *Opuntia* spp., Franco et al. (2022) point out that these have primarily focused on phylogenetic and population genetics. Compared to those conducted in other genera within the Cactaceae family, they are not very abundant. Table 1.1 shows the main results obtained in this area of knowledge.

Table 1.1 Genetic studies in *Opuntia* spp.

Topic	Main results	References
Population genetics	RAPD confirm that *O. prolifera* originated from the hybridization between O. *alcahes* and O. *cholla.*	Mayer et al. (2000).
	Microsatellite markers reveal high polymorphism in *O. echios*, *O. gigantea* and *O. zacana* from Galapagos archipelago.	Helsen et al. (2007).
	Microsatellite markers reveal high variability in *O. echios* from Galapagos archipelago, supporting the hybridization hypothesis rather than the clonal propagation hypothesis.	Helsen et al. (2011).

	In *Opuntia* Mexican germplasm the genetic diversity was reveal through SSR markers.	Samah et al. (2015).
	Morphological and molecular studies confirm the presence of *Opuntia bonaerensis* - previously considered endemic to Argentina- in Brazil and Uruguay.	Köhler et al. (2020a).
	Plastome studies define three well-differentiated tribes within the subfamily *Opuntioideae: Opuntieae, Cylindropuntieae,* and *Tephrocacteae.*	Köhler et al. (2020b).
	Morphological and PCR studies confirm *O. joconostle* and *O. matudae* as two distinct species.	Martínez-González et al. (2020).
	Molecular markers (RAPD) reveal the genetic differences and similarities among eight *Opuntia* species growing in Egypt.	Rabeh et al. (2020).
Phylogeny	Based on its morphological and molecular characteristics, *O. schickendantzii* should be reclassified as belonging to the genus *Salmonopuntia.*	Köhler et al. (2021a).
	O. cristalensis is the result of hybridization between *O. rioplatensis*, a native species of Argentina, and *O. ficus-indica*, introduced from North America.	Köhler et al. (2021b).
	The analysis of 18 sequences from the ITS, matK, and trnL-F regions demonstrates that *Opuntia tehuacana* and *Opuntia olmeca* are the same species.	Martínez-González et al. (2021).
	Morphological and molecular analyses reveal that *Opuntia lasiacantha* and *Opuntia rzedowskii* are different species.	Martínez-González & Morales-Sandoval (2021).

The search for genomic information that supports genetic improvement through conventional or biotechnological methods, including genetic engineering, is still quite scarce. Two exceptions are the research of Köhler et al. (2020b) and Liu et al. (2024).

The first one, although its primary focus is the phylogenetic differentiation of three tribes within the subfamily Opuntioidae, provides a set of markers that could be useful in genetic improvement. The second is a sequencing of the mitochondrial genome of *Opuntia cochenillifera*, which contributes to the mitochondrial genetics of *Opuntia* spp. and to improvement through genetic engineering.

1.4 Obtaining Metabolites and Other Processes

The extraordinary value of the metabolites produced by *Opuntia* spp., and the possibilities of utilizing them in the manufacture of various useful products, have driven the progress of biotechnological methods as a means to mass-produce these substances. It is known that species of *Opuntia* have high potential as animal feed due to their water content, energy molecules, minerals, and vitamins (Abidi et al., 2009, 2013). However, their protein content is low, so feed must be enriched to achieve a proper nutritional balance for animals. Araújo et al. (2005) developed a fermentation in solid-state system with *Saccharomyces cerevisiae* and cladodes of *O. ficus indica* for this purpose, obtaining 26% protein in the biomass, similar to or higher than that provided by other animal feeds. Flores et al. (2019) obtained over 36% protein from cladodes of *O. megacantha* with *Saccharomyces cerevisiae*, using a semi-solid fermentation system and 1% urea and 0.1% ammonium sulfate as nitrogen sources.

The residues from prickly pear production, due to their high content of organic acids and sugars, can be used to prepare media for growing growth-promoting bacteria in liquid fermentation. This was demonstrated by Magarelli et al. (2022), who successfully cultured a consortium of *Bacillus amyloliquefaciens*, *Rahnella aquatilis Azotobacter chroococcum*, *Pseudomonas fluorescens*, and *Burkholderia ambifaria*, in a medium consisting of 15% *O. ficus indica* cladode juice + 0.2% sucrose, achieving microbial growth at a similar level to that reached in a synthetic medium. The fermentation in solid-state of *O. ficus indica* peel residues with *Aspergillus niger* produces tannins with antioxidant activity and the ability to inhibit the growing of harmful bacteria and fungi (Coronado-Contreras et al., 2023).

To obtain biofertilizers, the residues of *Opuntia heliabravoana* are very suitable because they provide several types of bacteria (phosphorus-solubilizing, sulfur processors, starch

and cellulose degraders), and cellulolytic fungi. Additionally, the fermentation process for biofertilizers production also generates biogas. *O. heliabravoana* shows great promise in this area due to its high biomass yield and elevated water content, which saves this important resource in bioreactors (Quintanar-Orozco et al., 2018). The co-digestion of slaughterhouse wastewater and *O. ficus indica* cladodes can also be used for biogas production (Panizio et al., 2020).

Another promising application lies in the area of plant growth-promoting microorganisms (PGP). Several authors have demonstrated the existence of microorganisms in the rhizosphere and aerial parts of *Opuntia* spp., that stimulate plant growth and development processes, and these potentials can be harnessed through microbial biotechnology. *Opuntia* spp. biomass can also be used as a substrate for the development of beneficial microorganisms.

Twelve isolates of endophytic diazotrophic bacteria, belonging to the genera *Azospirillum*, *Bacillus*, and *Methylobacterium*, were identified by Da Silva et al. (2015), which may be useful for biofertilizer production and bioremediation. In the endosphere of seeds from *O. robusta*, *O. lasiacantha*, and *O. albicarpa*. Zelaya-Molina et al. (2021) isolated 18 morphotypes of bacteria; one of them, obtained from *O. albicarpa*, exhibited phosphate-solubilizing activity and produced indoleacetic acid and siderophores, making it potentially useful as a biofertilizer.

Govindasamy et al. (2022) obtained 179 isolates of endophytic bacteria from the roots of *O. ficus indica*. Of these, 10 belonged to the genus *Streptomyces spp.*, according to sequencing and phylogenetic analysis, and they demonstrated growth-stimulating activity on the roots and/or stems of wheat seedlings.

Córdova-Rojas et al. (2022) identified 100 bacteria of the *Bacillus sp.* genus and 53 of the *Pseudomonas sp.* genus from the rhizosphere of *Opuntia quitensis*. Approximately 40% of these increased the germination of corn seeds and showed ACC deaminase activity (which controls ethylene production), phosphate solubilization, and indole production, making them potential growth promoters. Recent studies analyzed a total of 246 bacterial isolates from these three genera, obtained from the rhizosphere of *O. ficus-indica* growing in a semi-arid region. Of these, 16% demonstrated the ability to survive

under drought conditions and acted as stimulators of vegetative growth in *Capsicum annuum* L. (Shreshtha et al., 2025).

Conclusions

Biotechnology in *Opuntia spp.* offers broad possibilities, some of which have already been utilized while others are still in the research and adjustment phases.

In vitro vegetative propagation has proven to exponentially increase the production of biological material for expanding these species to new areas, recovering land affected by various causes, conserving germplasm, and rescuing endangered species. Somatic embryogenesis, although with less consolidated results, is seen as a way to reduce propagation costs and provide starting material for other processes like transgenesis.

Advances have been made in transgenesis protocols, although there are still questions about the most suitable method, one that can combine adequate transformation efficiency with the regeneration of modified plants and the expression of introduced genes. Genetic studies supported by molecular biology have contributed to clarifying the origin of species in the genus; more research is needed to provide genome information, clarify its role in the production of useful metabolites, and support transgenesis.

The potential of *Opuntia spp.* to obtain various useful metabolites can be increased if they are integrated into biotechnological processes that promote their mass production while saving physical space and other resources. Some key applications of these processes include the preparation of low-cost culture media, the production of substances with inhibitory activity against harmful microbes, biogas generation, and the manufacture of bio-stimulants from plant growth-promoting microorganisms.

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