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Research Article

Opuntia ficus-indica (L.) Mill.: Cladode explants with several areoles, forchlorfenuron and indole-3-butyric acid for optimized *in vitro* culture and NPK controlled release fertilizer for direct *ex vivo* rooting

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Abstract

The genus *Opuntia* includes more than 300 species, with the most economically important being *Opuntia ficus-indica*. In this study, the *in vitro* propagation potential of the prickly pear cactus (*O. ficus-indica* (L.) Mill.) was evaluated. For shoot initiation (6 weeks, MS medium, 25 ± 1 °C, 24 h dark), three cladode explant types based on areole number (none, one, several), five BA (2, 3, 4, 5 and 6 mg L^{-1}) and four IAA concentrations (0, 1, 1.5 and 2 mg L^{-1}) were tested. The results showed 100% shoot induction by cladodes with several areoles under 6 mg L^{-1} BA + IAA (1-2 mg L^{-1}), compared to cladodes with one (63.33-66.67%) or none areole (26.66-30%). For multiplication of *in vitro* shoots derived from areole stimulation, four cytokinins [6-benzyladenine (BA), kinetin (Kin), thidiazuron (TDZ), forchlorfenuron (CPPU)] alone and combined with two auxins [indole-3-acetic acid (IAA), 2,4-dichlorophenoxy acetic acid (2,4-D)] were tested (8 weeks, Murashige-Skoog medium, 25 °C, 16h photoperiod). These results showed significantly higher shoot number (5.10) and length (6.19 cm) under 5 mg L^{-1} CPPU and 5 mg L^{-1} CPPU + 0.5 mg L^{-1} 2,4-D, respectively. For *in vitro* rooting, three auxins [indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA)] were applied (5 weeks, MS medium, 25 °C, 16-h photoperiod) and the results showed higher root number (19.06) with 0.5 mg L^{-1} IBA. For acclimatization, rooted microshoots (*ex vitro*) placed in a peat: vermiculite substrate and non-rooted microshoots (*ex vivo*) to the same substrate but supplemented with different concentrations (0, 0.8, 1.2, 1.6, 2 g $alveoli^{-1}$) of a controlled release fertilizer (CRF, 15%N-9%P_2O_5-12%K_2O). After 10 weeks in the greenhouse (24 ± 5 °C/ 18 ± 5 °C), 100% rooting (*ex vivo*) and survival (*ex vitro*, *ex vivo*) were recorded, nonetheless 1.2 g $alveoli^{-1}$ CRF gave higher *ex vivo* root number (23.05).

Keywords: acclimatization methods; areole activation; auxins, cladode explants; cytokinins; micropropagation; prickly pear

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Introduction

Cacti are distributed and cultivated worldwide mainly in semi-arid, arid and Mediterranean-climate regions, constituting one of the most important and diversified groups of vascular plants (Bouzroud *et al.*, 2022). Plants of the genus *Opuntia* are evergreen, perennial, succulent and belong to the Cactaceae family of the order Caryophyllales. They are considered one of the most overlooked plant genera, their classification being difficult, as they show easy hybridization in nature, creating continuous morphological variations (Ahmed *et al.*, 2021). Apart from *O. ficus-indica*, which is used for human consumption and as animal feed, other species are *O. megacantha, O. amyclaea, O. streptacantha* and *O. robusta*, which are cultivated for fruit consumption. The most important commercial varieties are found in Mexico and belong to the species *O. ficus-indica* and *O. megacantha* (Mazri, 2021). Prickly pear fruits are produced from several *Opuntia* species (Ramadan *et al.*, 2021) and show great genetic diversity (Mazri, 2021). In addition to being consumed by humans and animals, *Opuntia* can still be used as a living hedge and as a fuel (Gebretsadik *et al.*, 2013). *Opuntia* plants can also reduce soil erosion and degradation by enhancing vegetation restoration (Casas and Barbera, 2002). Still, they can be used as bioaccumulators by absorbing hazardous environmental pollutants, as happened in bioremediation projects in lead-contaminated waters (Shedbalkar *et al.*, 2010; Kumar *et al.*, 2018). They originate from Central America and especially the region of Mexico. Over the years their cultivation spread mainly for their fruits, which are consumed fresh or dried (Casas and Barbera, 2002). The fruit is a fleshy spherical-ovoid berry, consisting of peel, pulp and seeds, which may show different colors during ripening (Mazri, 2021). The fruits are also called tunas, while cladodes, being tender at an early stage, can also be eaten as vegetables (Ahmed *et al.*, 2021). The areoles found in cladodes can develop new axillary buds, spherical floral buds or oblong vegetative buds (Kumar *et al.*, 2018).

Opuntia species are rich in phenolic compounds, fibre, vitamins C and E, amino acids (such as taurine) and minerals (such as Fe, K, Mg, Ca, Na and P) (Ramadan *et al.*, 2021). Recently, alternative green extraction techniques have been employed to enhance the recovery of antioxidant compounds from these species (Parí *et al.* 2024). They also contain carotenes, ascorbic acid, quercetin and betalains (Shedbalkar *et al.*, 2010). The different *Opuntia* parts such as flowers, seeds, cladodes and fruits are rich in phenolic acids, flavonoids and organic acids (Durazzo *et al.*, 2021). Cladodes are rich in vitamins, polyphenols, polyunsaturated fatty acids, amino acids, pectin, mucilage, antioxidants and flavonoids, which promote liver, cardiovascular and intestinal health (Kumar *et al.*, 2018). They are considered to help in the treatment against diabetes, colon cancer, gastric ulcer, coronary artery disease and obesity (Ahmed *et al.*, 2021). Prickly pear in traditional medicine has been used for its hypoglycemic action, topically for wound healing, and as a soothing agent for inflammation and insect bites (Shedbalkar *et al.*, 2010). The nutritional and medicinal properties of *Opuntia* species are largely related to their content of phenolic compounds that can mitigate oxidative damage caused by diabetes, cancer and cardiovascular diseases (Nassrallah *et al.*, 2021).

Various cactus pear species exhibit anatomical, morphological, and physiological traits that enable them to adapt to diverse soil types and environmental conditions (Mazri, 2021) and are highly resistant to high temperatures (Alam-Eldein *et al.*, 2021). They are an important source of vegetables, fruits and fodder in semi-arid zones where other crops may be difficult to establish. They are also used as an alternative crop in arid climates (Escobar-Araya *et al.*, 1986) for more than 35 years. They have even been proposed by FAO as a crop in areas affected by water scarcity (Ramadan *et al.*, 2021). Cactus pears are among the plants with Crassulacean acid metabolism (CAM). During the day the leaf stomata remain closed and open during the night to collect CO_2. This photosynthetic mechanism reduces evapotranspiration and allows plants to adapt to dry conditions (Kumar *et al.*, 2018). Compared to C3 and C4 plants, they exhibit higher water use efficiency (Ramadan *et al.*, 2021). Under drought conditions, water loss is prevented through the thick and waxy outer surface cuticle. In addition, wind drying effects are mitigated due to the presence of thorns which shade the plant. Their root system is shallow, allowing direct absorption of rainwater (Glimn-Lacy and Kaufman, 2006), stored in the parenchyma through high production of mucilage, even under adverse climatic conditions (Kumar *et al.*, 2018).

Due to their stress tolerance, cacti could be a gene pool for other crops (Shedbalkar *et al.*, 2010) and could potentially become the food of the future (Kumar *et al.*, 2018). The spread of scientific knowledge, along with technological advancements, will enable broader utilization of these versatile species, encouraging their application in food systems, biotechnology, pharmaceuticals, and medicine (Coqueiro *et al.*, 2024).

Opuntia species are conventionally propagated by seed or vegetative propagation via rooted offshoots or grafting; however, these methods are not considered as adequate for mass propagation as *in vitro* techniques (Bouzroud *et al.*, 2022). Due to cross-pollination and natural hybridization, there is continuous genetic segregation in seed propagation, resulting in the production of seedlings that are genetically and phenotypically heterogeneous (Gebretsadik *et al.*, 2013). The longer juvenile phase and slower growth are also among the disadvantages of seed propagation (Escobar-Araya *et al.*, 1986). In many *Opuntia* species, such as *O. ficus-indica*, the hard lignified sheaths surrounding the embryo inhibit root protrusion resulting in low germination (Shedbalkar *et al.*, 2010). However, the seeds are used in breeding programs (Alam-Eldein *et al.*, 2021). Asexual propagation is done using 5-6 months old cladodes, the selection of which depends on whether it is intended for consumption as a vegetable, fruit or fodder (Kumar *et al.*, 2018). For propagation via cladodes, rooting can be achieved from stored water, without the limiting factor of soil moisture (Alam-Eldein *et al.*, 2021). Propagation techniques through cuttings on the one hand have increased the productivity of modern crops. However, large scale cultivation cannot be achieved in a short period of time as is done by applying biotechnology (Gebretsadik *et al.*, 2013).

Histocultivation has the potential to produce healthy plants, free of pathogens (Kumar *et al.*, 2018). *In vitro* techniques for the production of large-scale cacti for food could provide important incentives in areas where other vegetables cannot be grown (García-Saucedo *et al.*, 2005). In addition, *in vitro* techniques have the potential to increase the production of bioactive compounds that give *Opuntia* their nutritional and medicinal properties (Bouzroud *et al.*, 2022). Due to low availability of planting material, micropropagation techniques have been used for species such as *Opuntia ellisiana* in crops intended for animal feed (Alam-Eldein *et al.*, 2021). As referred to propagation, the metabolic and chemical profile of *Opuntia* depends on the species, growth stage, growing conditions and harvest season. Auxins can affect plant growth, embryo formation, cell growth and callus induction (Astello-García *et al.*, 2013). In addition, cytokinins and especially kinetin and BA have optimal results in proliferation at low concentrations (García-Saucedo *et al.*, 2005; Gebretsadik *et al.*, 2013).

In the framework of the aforementioned, the main goal of this study was to develop an efficient protocol for *in vitro* propagation of *Opuntia ficus-indica* (L.) Mill. by *in vitro* culture of cladodes through areole stimulation. The objectives of this research were to: a) scrutinize the most appropriate explant type for initiation of *in vitro* culture using cladodes without areoles, cladodes with one areole and cladodes with several areoles; b) determine the growth regulator (BA as cytokinin, IAA as auxin) concentration, able to induce *in vitro* shooting; c) determine the ideal concentration of different cytokinins applied alone and combined with different auxins for *in vitro* shoot multiplication (number and length); (d) evaluate the effects of different auxins on *in vitro* rooting and subsequent acclimatization to *ex vitro* greenhouse conditions; and (e) evaluate the effect of different concentrations of a CRF added to the substrate on direct *ex vivo* rooting of non-rooted *in vitro* shoots and survival rates under greenhouse conditions.

Materials and Methods

Plant material and initiation of culture

Prickly pear cladodes were collected from Diwaniya's experimental field (Figure 1A). The site is in the center of Iraq and has been the focus of disinfection and *in vitro* cultivation studies. Young cladodes removed from a parent plant (Figure 1B). The identification process was carried out through detailed morphological analysis by a trained botanist specializing in Cactaceae. Key diagnostic features—including the characteristic

flat, pad-like cladodes, specific spine size and shape, and floral morphology—correspond closely with the established traits of *Opuntia ficus-indica* (L.) Mill. The juvenile cladodes were 8 to 20 cm long after three to four weeks of growing on the mother cladode. To sterilize the juvenile cladodes, they were rinsed in running tap water for 20 min and sprayed with 70% ethanol. After that they were dried and immersed in a 30% solution of sodium hypochlorite (NaOCl) for 30 min (Figure 1C). Finally, they were rinsed three times in sterile distilled water. Sections of 2 cm^2 were cut from sterile cladodes (Figures 1D and 1E).

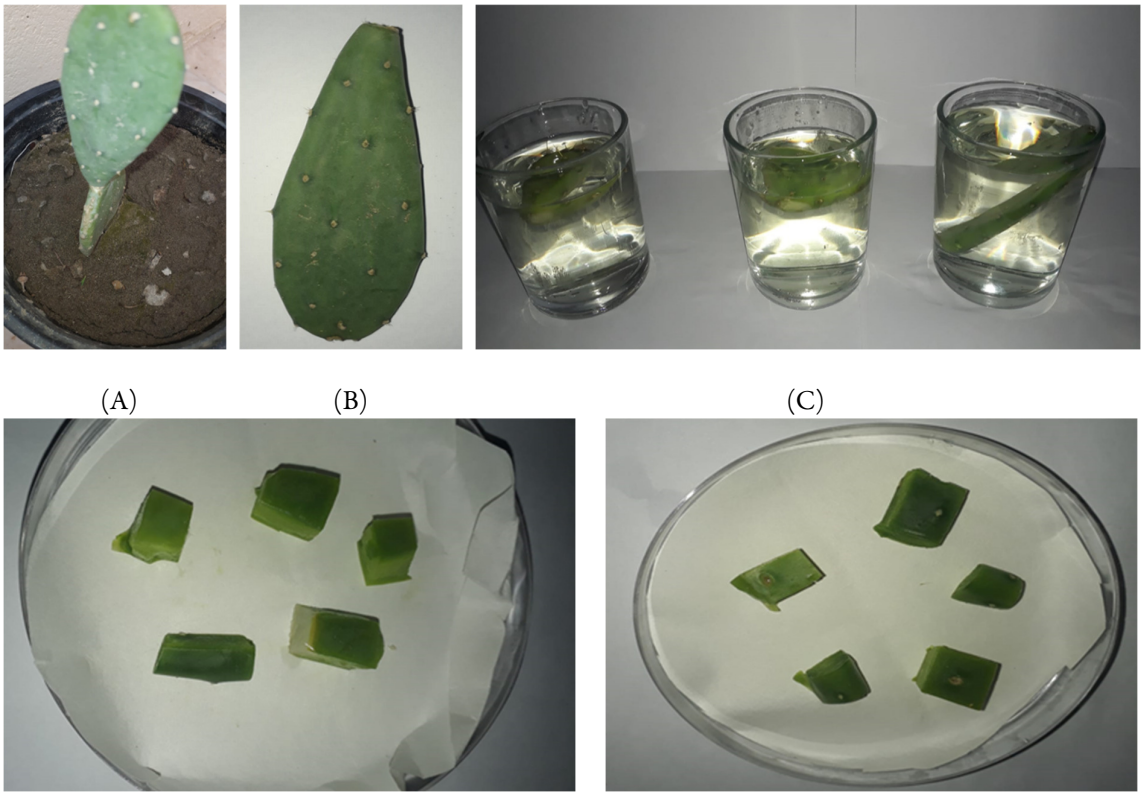
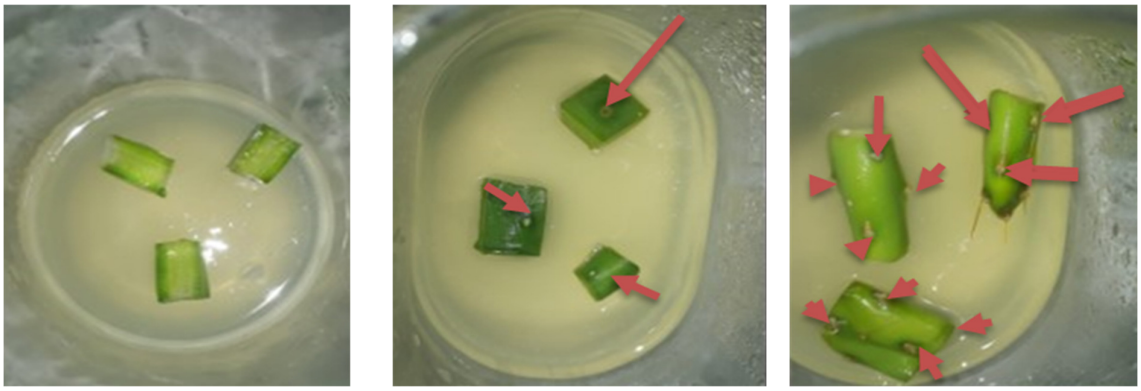


Figure 1. Mother *Opuntia ficus-indica* (L.) Mill. plant in the field, preparation of explants, disinfection of cladodes and establishment of *in vitro* culture; (A) Mother plant within pot containing soil; (B) A young cladode removed from its parent plant; (C) Sterilization of young cladodes with 30% solution of sodium hypochlorite (NaOCl) for 30 min; (D-E) Sections of 2 cm^2 were cut from sterile cladodes and used as source explants

According to the type of cladode, there were three different explant types: cladodes without areoles (Figure 2A), cladodes with one areole (Figure 2B), and cladodes with several areoles (Figure 2C). To induce areole stimulation and shoot formation, sterilized explants were grown in a MS (Murashige and Skoog, 1962) medium supplemented with indole-3-acetic acid (IAA) (0, 1, 1.5 and 3 mg L^{-1}) and 6-benzyladenine (BA) (2, 3, 4, 5 and 6 mg L^{-1}) in different concentrations and combinations. The control treatment was devoid of any plant growth regulator (PGRs-free). Three explants were placed vertically in a petri dish containing 10 ml of medium. Each treatment was consisted of 30 in total explants divided into 10 petri dishes x 3 explants/petri dish. The cultures were incubated at 25 ± 1 °C under dark conditions for 6 weeks of culturing.

Shoot multiplication

Shoots (2 cm long) initiated during the areole stimulation stage (*i.e.* exclusively derived from cladodes that have one or more areoles, not from cladodes lacking areoles) were separated and cultured in the proliferation medium, which contained MS media supplemented with 5 mg L^{-1} of cytokinins (BA, Kin, CPPU, and TDZ) alone or combatant with 0.5 mg L^{-1} of auxins (IAA and 2,4-D). The control treatment was PGRs-free. Each treatment was consisted of 30 explants in total divided into 10 culture vessels x three explants per vessel. Shoot number and shoot length per explant were measured after 8 weeks of culture at 25 ± 1 °C under a 16 h photoperiod (16 h light / 8 h dark).



(A) (B) (C)

Figure 2. Initiation of *in vitro* culture of *Opuntia ficus-indica* (L.) Mill. using three explant types: (A) Cladodes without areoles; (B) Cladodes with one areole; (C) Cladodes with several areoles. Red-colored arrows in sub-figures B and C show the presence of areoles (one, several) in each cladode placed horizontally into the culture medium, respectively

In vitro rooted shoots and *ex vitro* acclimatization (Experiment 1)

To encourage root formation, elongated shoots (4-7 cm long) were divided and cultured on MS media supplemented with 0.5 mg L^{-1} auxin (IAA, IBA, or NAA) (25 ± 1 °C, 16 h light/ 8 h dark). All of the utilized culture media were adjusted to pH 5.5 using NaOH 1N, before being sterilized at 121 °C for shoot initiation, shoot multiplication, and rooting experiments. The goal of this experiment was to find the best auxin type and concentration for the rooting and the *ex vitro* acclimatization. Ten shoots per treatment were used for the rooting experiment; five weeks later, the number of formed roots per explant was tallied in each treatment. The rooted plantlets (*vitro* plants) were placed in autoclaved soil and acclimated for six weeks under plastic coverings. Following this, the plantlets were moved to the greenhouse. After ten weeks of transplantation, the survival rates were calculated.

Ex vivo direct rooting of non-rooted *in vitro* shoots and acclimatization (Experiment 2)

Ex vivo rooting was carried out by placing the shoots directly on a substrate of peat and vermiculite (1:1), to which a controlled-release fertilizer (CRF) was added. The CRF used in this experiment was Osmocote Plus, produced by Scotts Company. It features an active nutrient formulation of 15-9-12 (N-P_2O_5-K_2O) [*i.e.* 15% total nitrogen (N): 8.4% Ammoniacal nitrogen (N-NH_4) and 6.6% Nitrate nitrogen (N-NO_3), 9% Phosphorus pentoxide (P_2O_5), 12% Potassium oxide (K_2O)]. Other nutrients included in the composition of this CRF are the following: 1.3% magnesium (Mg) of which 0.8% is soluble in water, 5.9% sulfur (S), 0.02% boron (B), 0.05% water soluble copper (Cu), 0.046% iron (Fe) of which 0.09% water soluble and 0.006% Fe-EDTA, 0.06% manganese (Mn) of which 0.02% water soluble, 0.02% molybdenum (Mo), and 0.05% zinc (Zn) of which 0.011% water soluble. This CRF is designed to release nutrients gradually over approximately six months under typical conditions. Its' release mechanism relies on polymer-coated resin technology, which controls the diffusion of nutrients into the soil. According to the manufacturer's datasheet, the fertilizer initially releases

nutrients over the first 2-3 weeks, followed by a consistent release rate of about 3-5% of the total nutrients each week. This ensures a steady supply of nutrients throughout the plant's growth period.

In this study, different dosages—0.8, 1.2, 1.6, or 2.0 grams per alveolus—were applied, each providing specific amounts of nutrients based on the fertilizer's formulation. Five various treatments were used: control (the plants were not nutritionally supplemented) and four amounts of CRF (0.8, 1.2, 1.6, and 2.0 g alveoli^{-1}). A total of 100 plants (20 plants/ treatment, five treatments) were used. Shoots were conducted with a natural photoperiod and irradiance of 24 ± 5 °C/18 ± 5 °C. The approach for acclimatization was the same as in Experiment 1. The following data were collected after ten weeks of growing *ex vivo* in the greenhouse: the *ex vivo* survival rate (%) (number of survival plants/number of acclimatized plants × 100) and the number of *ex vivo* roots.

Statistical analysis

All the experiments carried out followed the completely randomized layout. Analysis of variance (ANOVA) was performed using the IBM® SPSS® Statistics Version 21.0 package and the Duncan's multiple range test at a 5% significance level ($p \leq 0.05$).

The experimental layout of the shoot induction experiment was a 3 × 4 × 5 factorial one with three cladode explant types (without areoles, with one areole, with several areoles), four IAA concentrations (0, 1, 1.5 and 2 mg L^{-1}) and five BA concentrations (2, 3, 4, 5 and 6 mg L^{-1}), plus the three control (PGRs-free) treatments (*i.e.* one control for each explant type), thus included 63 treatments in total with 30 replications/ treatment (10 culture vessels x three explants/vessel). Mean separation and differences among the 63 treatments were estimated using three-way ANOVA, and General Linear Model was adopted to determine the effect of the main factors [explant type (A), BA concentration (B), IAA concentration (C)] and theirs interactions [A×B, A×C, B×C, A×B×C] on shoot induction %. In addition, two-way ANOVA and General Linear Model were performed for each cladode explant type to evaluate the effect of the main factors [BA concentration (A), IAA concentration (B)] and their interaction (A×B) (*i.e.* 21 treatments: five BA concentrations x four IAA concentrations, plus the control).

The shoot multiplication experiment was 4 × 3 factorial involving four cytokinin types (BA, Kin, CPPU, TDZ) and three auxin types (none, IAA, 2,4-D), plus the control (PGRs-free) treatment, thus included 13 in total treatments with 30 replications/treatment (10 culture vessels x three explants/vessel). The effect of the two main factors [cytokinin type (A), auxin type (B)] and their interaction effect [cytokinin type × auxin type (A×B)] on shoot number and length were determined by General Linear Model/2-way ANOVA.

The *in vitro* rooting experiment included four treatments (auxins-free, IBA, IAA, NAA) with 10 replicates (*i.e.,* explants) per treatment. One way-ANOVA applied for mean separation and evaluation of the significance of auxin type on *in vitro* root number, *in vitro* rooting %, and *ex vitro* survival % of acclimatized microshoots to greenhouse conditions.

The *ex vivo* direct rooting experiment, related to different concentrations of the fertilizer, included five treatments (0, 0.8, 1.2, 1.6, 2 g alveoli^{-1}) with 20 replicates (*i.e.,* explants) per treatment. One way ANOVA applied for mean separation and evaluation of the effect of CRF concentration on *ex vivo* root number, rooting % and survival % of non-rooted microshoots.

Results

Shoot induction under different areole-number cladode explants, BA and IAA concentrations

Shoot induction (23.33-100%) was observed in all three explant types (cladodes without areoles, cladodes with one areole, cladodes with several areoles) under the combinational treatments of the highest BA concentration of 6 mg L^{-1} with IAA (1, 1.5 and 2 mg L^{-1}), while no shoot formation occurred in the other treatments including the control. In the case of cladodes without areoles, shoot induction at a 23.33-30% rate

occurred under 6 mg L^{-1} BA combined with IAA (1, 1.5 and 2 mg L^{-1}), without differing significantly. The percentage of shoot induction for cladode explants with one areole ranged at similar non-significant levels (63.33-66.70%) in medium supplemented with 6 mg L^{-1} BA, regardless IAA concentration. Shoot induction was optimum (100%) when cladodes with several areoles were used as explants and cultured in medium composed of 6 mg L^{-1} BA and 1-2 mg L^{-1} IAA (Figure 3; Table 1).

In treatments where shoot induction was observed (6 mg L^{-1} BA + 1, 1.5 and 2 mg L^{-1} IAA), the comparison among the three explant types showed significantly higher shoot induction percentages (100%) for cladode explants with several areoles and significantly lower ones (23.33-30%) for cladodes without areoles. Cladodes with one areole exhibited intermediate shoot induction percentages (63.33-66.70%) differing significantly from the other two explant types (Figure 3).

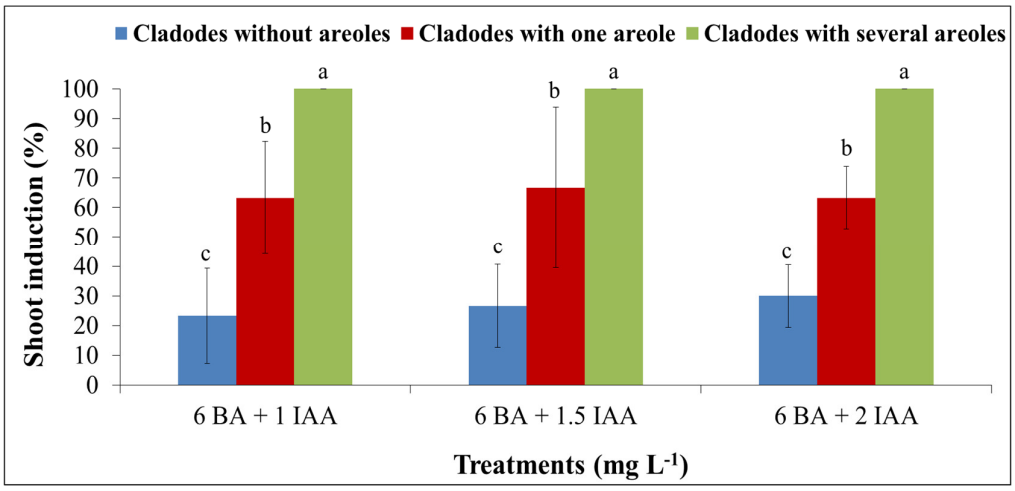


Figure 3. Shoot induction of *Opuntia ficus-indica* (L.) Mill. using three explant types (cladodes without areoles, cladodes with one areole, cladodes with several areoles) under the highest 6-benzyladenine (BA) concentration of 6 mg L^{-1} combined with three indole-3-acetic acid (IAA) concentrations (1, 1.5 and 2 mg L^{-1}) after a 6-week *in vitro* culture (MS medium, 25 °C, dark). Error bars are standard deviations (S.D.). Column bars accompanied by different letters denote significant differences among the nine treatments derived from the combined effect of three explant types × three IAA concentrations + 6 mg L^{-1} BA (Duncan test, $p \leq 0.05$)

For each explant type, General Linear Model and two-way ANOVA revealed that the effect of the main factors (*i.e.* BA concentration, IAA concentration) and their interaction on shoot induction % was significant at a 0.1% level ($p = 0.000 \leq 0.001$). Thus, cladodes with several areoles appeared as the most appropriate explant type for shoot induction. General Linear Model and three-way ANOVA revealed that the effect of the main factors (*i.e.* explant type, BA concentration, IAA concentration) and theirs amongst interactions on shoot induction % was significant ($p = 0.000 \leq 0.001$) (Table 1).

Regarding the "shoot number" parameter, the cytokinin type CPPU applied at 5 mg L^{-1} gave significantly higher value, specifically 5.10 shoots/explant. Considering the individual effect of cytokinins (BA, kinetin, CPPU, TDZ) in the absence of auxins (IAA, 2,4-D), CPPU proved to be the most effective cytokinin type related to shoot number, followed by TDZ and then by kinetin or BA, while the control (cytokinins- and auxins-free) was the least effective. In the combinational plant growth regulator (PGR) treatments, 5 mg L^{-1} CPPU + 0.5 mg L^{-1} IAA or 2,4-D exerted significantly higher shoot numbers as compared to the other treatments – 5 mg L^{-1} BA/Kin/TDZ + 0.5 mg L^{-1} IAA or 2,4-D. Considering two-way ANOVA and General Linear Model, shoot number was significantly affected by the cytokinin type (A) as a main factor and the interaction of cytokinin type x auxin type (A×B) at a 0.01% level ($p = 0.000 \leq 0.001$), as well as by auxin type (B) at an 1% level ($p = 0.001 \leq 0.01$) (Table 2; Figure 4).

Table 1. Combinational effect of explant type (cladodes without areoles, cladodes with one areole, cladodes with several areoles), 6-benzyladenine (BA) concentration (2, 3, 4, 5 and 6 mg L^{-1}) and indole-3-acetic (IAA) concentration (0, 1, 1.5 and 2 mg L^{-1}) on shoot induction (%) of *Opuntia ficus-indica* (L.) Mill. after a 6-week *in vitro* culture (MS medium, 25 °C, dark)

Treatment (mg L^{-1})	Shoot induction (%)		
	Cladodes without areoles	Cladodes with one areole	Cladodes with several areoles
Control	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
2 BA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
2 BA + 1 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
2 BA +1.5 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
2 BA + 2 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
3 BA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
3 BA + 1 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
3 BA + 1.5 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
3 BA + 2 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
4 BA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
4 BA + 1 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
4 BA + 1.5 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
4 BA + 2 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5 BA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5 BA + 1 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5 BA + 1.5 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5 BA + 2 IAA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
6 BA	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
6 BA + 1 IAA	23.33 ± 16.10 a	63.33 ± 18.92 a	100.00 ± 0.00 a
6 BA + 1.5 IAA	26.66 ± 14.05 a	66.70 ± 27.13 a	100.00 ± 0.00 a
6 BA + 2 IAA	30.00 ± 10.54 a	63.33 ± 10.54 a	100.00 ± 0.00 a
p-values (2-way ANOVA/General Linear Model) for each cladode explant type (without areoles, with one areole, with several areoles)			
BA concentration (A): 0.000***			
IAA concentration (B): 0.000***			
(A) × (B): 0.000***			
3-way ANOVA/ General Linear Model			
Explant type (A): 0.000***			
BA concentration (B): 0.000***			
IAA concentration (C): 0.000***			
(A) × (B): 0.000***			
(A) × (C): 0.000***			
(B) × (C): 0.000***			
(A) × (B) × (C): 0.000***			

Means (n= 30, 10 culture vessels x 3 explants/vessel) ± standard deviation (S.D.) accompanied by different letters in each column denote significant differences (Duncan test, $p \leq 0.05$). Effect of main factors [BA concentration (A), IAA concentration (B)], and their interaction *i.e.* BA concentration x IAA concentration (A × B) on shoot induction [21 treatments/explant type (General Linear Model). Effect of main factors [Explant type (A), BA concentration (B), IAA concentration (C)], and theirs interactions (A × B, A × C, B × C, A × B × C) [63 treatments: three explant types × 21 growth regulators, General Linear Model]. ***$p \leq 0.001$: significant effect at a 0.1% level

Table 2. Combinational effect of cytokinin type [6-benzyladenine (BA), kinetin (Kin), thidiazuron (TDZ), forchlorfenuron (CPPU)] each applied at 5 mg L^{-1}, and auxin type [indole-3-acetic acid (IAA), 2,4-dichlorophenoxy acetic acid (2,4-D)] each applied at 0.5 mg L^{-1} on shoot number/explant and shoot length (cm) of *Opuntia ficus-indica* (L.) Mill. after an 8-week *in vitro* culture (MS medium, 25 °C, 16 h light/ 8 h dark)

Treatments (mg L^{-1})	Shoot number/explant	Shoot length (cm)
Control	1.00 ± 0.00 f	1.71 ± 0.46 i
5 BA	1.77 ± 0.63 e	2.94 ± 0.13 h
5 Kin	1.83 ± 0.70 de	2.91 ± 0.14 h
5 CPPU	5.10 ± 0.84 a	5.25 ± 0.45 de
5 TDZ	2.27 ± 0.74 c	4.30 ± 0.39 g
5 BA + 0.5 IAA	2.00 ± 0.53 cde	4.87 ± 0.41 f
5 BA + 0.5 2,4-D	2.33 ± 0.80 c	5.11 ± 0.23 e
5 Kin + 0.5 IAA	2.17 ± 0.59 cd	5.67 ± 0.35 c
5 Kin + 0.5 2,4-D	2.17 ± 0.91 cd	5.38 ± 0.56 d
5 CPPU + 0.5 IAA	3.43 ± 0.50 b	5.30 ± 0.81 de
5 CPPU + 0.5 2,4-D	3.37 ± 0.56 b	6.19 ± 0.56 a
5 TDZ + 0.5 IAA	2.07 ± 0.69 cde	5.65 ± 0.56 c
5 TDZ + 0.5 2,4-D	2.13 ± 0.68 cde	5.95 ± 0.47 b
p-values (2-way ANOVA/ General Linear Model)		
Cytokinin type (A)	0.000***	0.000***
Auxin type (B)	0.001**	0.000***
(A)*(B)	0.000***	0.000***

Means (n=30, 10 culture vessels x 3 explants/vessel) ± standard deviation (S.D.) accompanied by different letters in each column among the 13 treatments for each parameter (shoot number, shoot length) denote statistically significant differences at a 0.5% level (Duncan test, $p \leq 0.05$). **$p \leq 0.01$: significant effect at an 1% level and ***$p \leq 0.001$: significant effect at a 0.1% level regarding main and interaction effect of factors [cytokinin type (A), auxin type (B), cytokine type x auxin type (A×B)] on shoot number and length (General Linear Model)

Accordingly, shoot length was significantly higher (6.19 cm) in medium supplemented with 5 mg L^{-1} CPPU + 0.5 mg L^{-1} 2,4-D, differing significantly from the other 12 treatments. Compared to control (PGRs-free) (1.71 cm), the other 12 treatments led to significant increases in shoot length (2.91-6.19 cm). Considering the individual effect of cytokinins (BA, kinetin, CPPU, TDZ) in the absence of auxins (IAA, 2,4-D), BA and kinetin proved to be the least effective cytokinin types related to shoot length. In cytokinin + auxin treatments, the auxin 2,4-D resulted in significantly higher shoot lengths than the auxin IAA when added to medium with a specific cytokinin type (BA, Kin, CPPU or TDZ). General Linear Model and two-way ANOVA revealed that the effect of the main factors (*i.e.* cytokinin concentration, auxin concentration) and their interaction on shoot length was significant at a 0.1% level (p= 0.000) (Table 2; Figure 4).

Microshoots were rooted at a 100% rate (100% *in vitro* rooting percentage) in all treatments including the control, regardless auxin type (IAA, IBA, NAA). *In vitro* root number was found significantly higher (11.9-19.6) in all three auxin type treatments as compared to the control (3.9 roots), being maximized (19.6 roots) under 0.5 mg L^{-1} IBA. Among the three auxin types, IBA proved the most effective in terms of *in vitro* root number, NAA the least effective, while IAA gave intermediate results between the other two types (Table 3; Figure 5A). During the acclimatization stage to the greenhouse, the *ex vitro* survival rate of the rooted microshoots was 100%, regardless the *in vitro* culture medium derived from (control, IAA, IBA, NAA) (Table 3; Figure 5B). Therefore, considering altogether *in vitro* rooting and *ex vitro* acclimatization stages of prickly pear rooted microshoots, IBA proved the most effective auxin type (Table 3; Figures 5A and 5B). One-way ANOVA revealed that the effect of auxin type on *in vitro* root number is significant at a 0.1% level ($p= 0.000 \leq 0.001$) (Table 3).

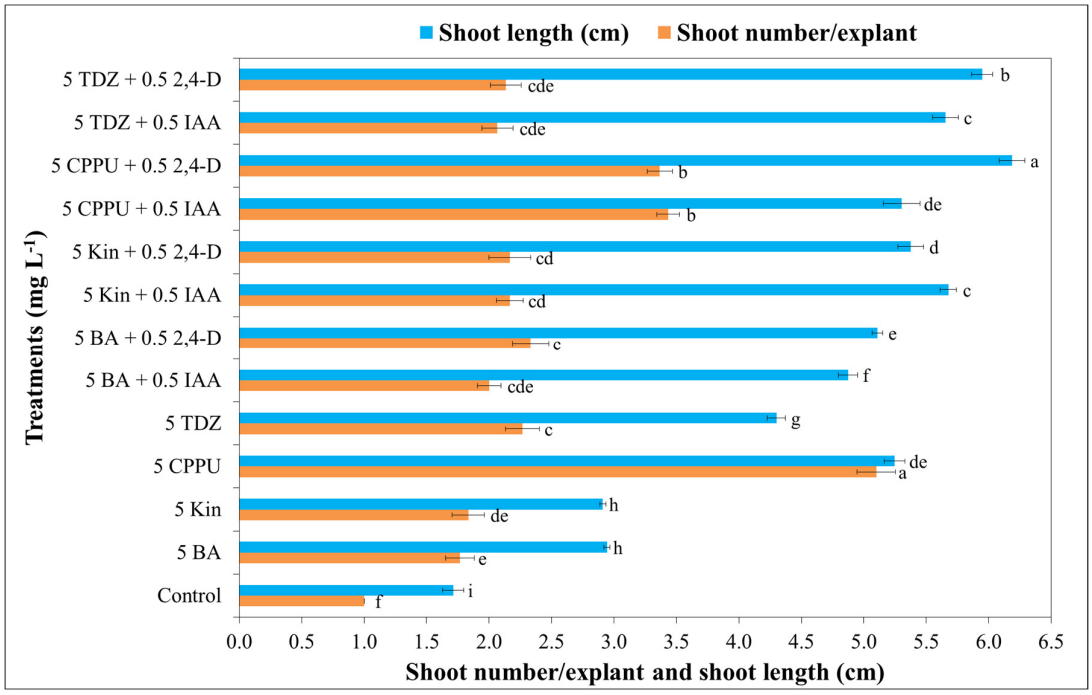
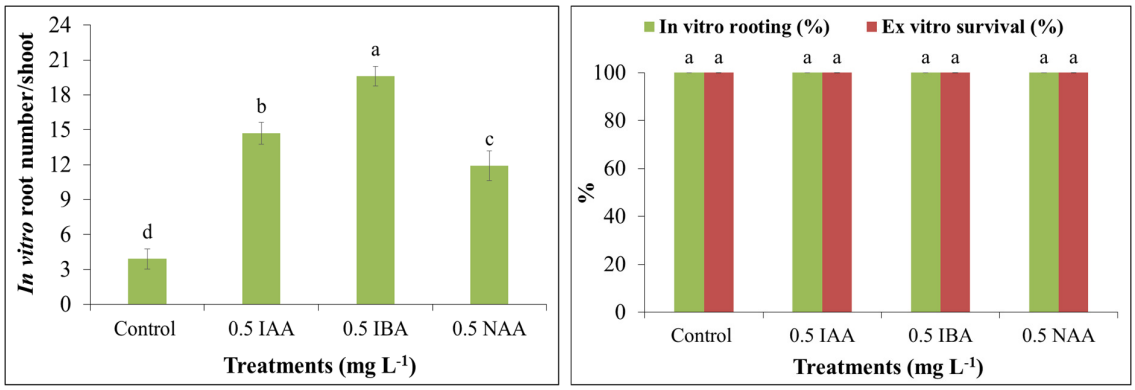


Figure 4. Shoot number/explant and shoot length (cm) of *Opuntia ficus-indica* (L.) Mill. during shoot multiplication stage under the combinational effect of four cytokinin types [6-benzyladenine (BA), kinetin (Kin), thidiazuron (TDZ), forchlorfenuron (CPPU)] each applied at 5 mg L^{-1} either alone or combined with two auxin types [indole-3-acetic acid (IAA), 2,4-dichlorophenoxy acetic acid (2,4-D)] each applied at 0.5 mg L^{-1}, after 8 weeks of *in vitro* culture (MS medium, 25 °C, 16h light/ 8h dark) in comparison to the control treatment devoid of any plant growth regulator (PGR's-free). Error bars are standard deviations (S.D.). For each parameter (shoot number or shoot length) separately, column bars accompanied by different letters denote significant differences among the 13 treatments (Duncan test, $p \leq 0.05$)

Table 3. Effect of auxin type [indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA)] each applied at 0.5 mg L^{-1} compared to control (auxin-free) on *in vitro* rooting (root number/microshoot and rooting %) and *ex vitro* survival of acclimatized rooted microshoots of *Opuntia ficus-indica* (L.) Mill. under greenhouse conditions

Treatment	*In vitro* root number/ microshoot	*In vitro* rooting (%)	*Ex vitro* survival of acclimatized rooted microshoots (%)
Control	3.90 ± 0.88 d	100.00 ± 0.00 a	100.00 ± 0.00 a
0.5 mg/L IAA	14.7 ± 0.95 b	100.00 ± 0.00 a	100.00 ± 0.00 a
0.5 mg/L IBA	19.6 ± 0.84 a	100.00 ± 0.00 a	100.00 ± 0.00 a
0.5 mg/L NAA	11.9 ± 1.29 c	100.00 ± 0.00 a	100.00 ± 0.00 a
p-values (one-way ANOVA)	0.000***	1.000 ns	1.000 ns

Means (n=10) ± standard deviation (S.D.) accompanied by different letters in each column among the four treatments for each parameter (*in vitro* root number, *in vitro* rooting %, *ex vitro* survival %) denote statistically significant differences at a 5% level (Duncan test, $p \leq 0.05$). ns: non-significant difference ($p > 0.05$); ***: significant difference at $p \leq 0.001$



(A) (B)

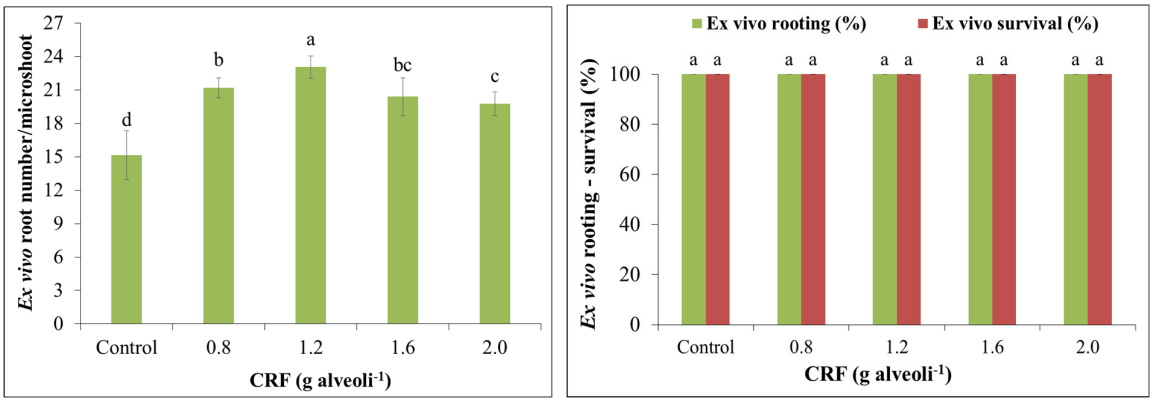
Figure 5. Effect of auxin type (IAA, IBA, NAA) each applied at 0.5 mg L^{-1} compared to control (auxin-free) on *in vitro* rooting and *ex vitro* survival of acclimatized rooted microshoots of *Opuntia ficus-indica* (L.) Mill. under greenhouse conditions; (A) *In vitro* root number/shoot; (B) *In vitro* rooting (%) and *ex vitro* survival (%). Error bars are standard deviations (S.D.). In diagram of figure 5A, column bars accompanied by different letters denote significant differences among the four treatments (Duncan test, $p \leq 0.05$). In figure 5B, column bars per parameter accompanied by the same letter denote non-significant differences (Duncan test, $p > 0.05$)

The CRF fertilizer (0.8-2 g $alveoli^{-1}$) resulted in significant increases in the *ex vivo* root number (19.75-23.05 roots/ shoot) as compared to the control (15.15 roots/ shoot). However, 1.2 g $alveoli^{-1}$ was the optimum concentration of the fertilizer leading to the significantly highest *ex vivo* root number (23.05) (Table 4; Figure 6A; Figure 7B). Non-rooted *in vitro* shoots rooted *ex vivo* in a peat: vermiculite (1:1) substrate supplemented with CRF, regardless of its concentration (0, 0.8, 1.2, 2 g $alveoli^{-1}$), exhibiting a 100% *ex vivo* rooting rate. After a 10-week period in the greenhouse (24 ± 5 °C/18 ± 5 °C), the *ex vivo* survival rates of these plants in the different CRF-concentration substrates were 100% (Table 4; Figure 6B; Figure 7C). Thus, 1.2 g $alveoli^{-1}$ of CRF best promoted *ex vivo* rooting of microshoots (Table 4; Figures 6A and 6B).

Table 4. Effect of five different amounts (0, 0.8, 1.2, 1.6 and 2 g $alveoli^{-1}$) of the controlled release fertilizer (CRF) added to the peat: vermiculite (1:1) substrate in comparison to the control (non-nutritionally supplemented), on *ex vivo* root number/microshoot, rooting (%) and survival (%) of non-rooted *in vitro* shoots of *Opuntia ficus-indica* (L.) Mill. under greenhouse conditions

CRF (g $alveoli^{-1}$)	*Ex vivo* root number /microshoot	*Ex vivo* rooting (%)	*Ex vivo* survival (%)
0.0 - Control	15.15 ± 2.21 d	100.00 ± 0.00 a	100.00 ± 0.00 a
0.8	21.20 ± 0.89 b	100.00 ± 0.00 a	100.00 ± 0.00 a
1.2	23.05 ± 1.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
1.6	20.40 ± 1.70 bc	100.00 ± 0.00 a	100.00 ± 0.00 a
2.0	19.75 ± 1.07 c	100.00 ± 0.00 a	100.00 ± 0.00 a
p-values (one-way ANOVA)	0.000***	1.000 ns	1.000 ns

Means (n=20) ± S.D. accompanied by different letters in each column among the five CRF concentration treatments for each parameter (root number, rooting %, survival %) denote significant differences at $p \leq 0.05$ (Duncan test). ns: non-significant difference ($p > 0.05$); ***$p \leq 0.001$: significant difference at a 0.1% level



(A) (B)

Figure 6. Effect of five different amounts (0 – Control, 0.8, 1.2, 1.6 and 2 g alveoli^{-1}) of a controlled release fertilizer (CRF) added to the peat: vermiculite (1:1) substrate in comparison to the control (non-nutritionally supplemented), on *ex vivo* root number/microshoot, rooting (%) and survival rate (%) of non-rooted *in vitro* shoots of *Opuntia ficus-indica* (L.) Mill., under greenhouse conditions (24 ± 5 °C/ 18 ± 5 °C) for 10 weeks. Error bars are standard deviations (S.D.). In diagram of figure 6A, column bars accompanied by different letters denote significant differences among the five CRF concentrations (Duncan test, $p \leq 0.05$). In figure 6B, column bars per parameter accompanied by the same letter denote non-significant differences among the five CRF concentrations (Duncan test, $p > 0.05$)

(A) (B) (C)

Figure 7. *In vitro* propagation and *ex vitro* acclimatization of *Opuntia ficus-indica* (L.) Mill.; (A) *In vitro* shoot multiplication after 8 weeks of culture in MS medium supplemented with 5 mg L^{-1} forchlorfenuron (CPPU) (best treatment); (B) Rooting *ex vivo* of non-rooted *in vitro* shoots after six months in a peat: vermiculite substrate supplemented with 1.2 g alveoli^{-1} of the controlled release fertilizer (CRF) (best treatment); (C) Acclimatization of plants derived from all treatments in the greenhouse (*ex vitro*) after a 6-month period

The different developmental steps for prickly pear (*O. ficus-indica*) followed in this study and their progress with the elapse of culture period is elaborately described below using a timeline in weeks for every stage (Table 5).

Table 5. Detailed description of each stage and creation of a timeline that summarizes the development steps of this study

Week	Step Description
0	Collection of mother plant and young cladodes
0-1	Disinfection and preparation of explants (cut into 2 cm^2)
1-7	*In vitro* initiation on MS medium with PGRs (dark, 6 weeks)
8	Transfer to shoot proliferation medium (light, 8 weeks)
16	Separation of shoots (~4–7 cm), root induction on MS + auxins (5 weeks)
21	*Ex vivo* rooting on peat/vermiculite with CRF (10 weeks)
31	Acclimatization in greenhouse (initial 6 weeks, then gradual exposure)
37	Final assessment of survival rate and plant growth

Discussion

Shoot induction from different cladode explants (without areoles, with one areole, with several areoles)

In cactus species, areoles are highly specialized axillary buds comprised of meristematic tissues. It has been reported that new and lateral multiple shoots are induced through areoles, when cultured *in vitro* in medium enriched with cytokinins, alone or in tandem with low concentrations of auxins. This is a well-known and prompt proliferation method for their mass propagation (Lema-Rumińska and Kulus, 2014; Pérez-Molphe-Balch *et al.*, 2015). The effectiveness of this areole activation method in a single culture cycle based on the number of shoots produced per explant (from 2-3 up to 30 shoots) and is commonly hinge on the plant species, explant type (*i.e.* presence or not of areoles, number of areoles per cladode) and the applied treatment (Pérez-Molphe-Balch *et al.*, 2015). According to Pérez-Molphe-Balch and Dávila-Figueroa (2002), culturing areoles in cytokinins-supplemented medium resulted in shoot induction and subsequently in secondary proliferation of these shoots to new sprouts, enabling the production of even 120 shoots per explant. This is based on the stimulating impact of cytokinins individually and combined with auxins on apical dormancy removal and boost of axillary buds' sprouting and shoot multiplication from areoles (Ngezahayo and Liu, 2014). The propagation method via areole activation is considered more advantageous, since it implicates the induction and growth of new shoots from preexisting meristems rather than cell dedifferentiation of already differentiated cells (Grafi *et al.*, 2011). The induction of direct organogenesis (*i.e.* shoots or roots from cultured explants barren of the median callus formation stage) in cactus species is triggered by plant growth regulators (PGRs), cytokinins (predominantly BA, but kinetin, 2-isopentenyl-adenine (2-iP), zeatin and TDZ in a lesser degree) and auxins (IBA, NAA, IAA, 2,4-D), depending on the PGRs type-concentration combination applied (Lema-Rumińska and Kulus, 2014). According to a study on establishment of callus from *Opuntia robusta*, the addition of 3 mg L^{-1} IAA achieved callus formation in 100% of explants, however with a slow growth rate, while elicitation with jasmonic acid (JA), increased phenolic acids by 1.3 times and flavonoids by 3 times compared to the control (Astello-García *et al.*, 2013).

In this study with *O. ficus-indica*, 30-100% shoot induction was obtained by cladode explants, regardless of areole presence and number but only under the highest BA concentration of 6 mg/L and when combined with IAA (1, 1.5 and 2 mg L^{-1}), pointing out the critical balance that must exist between endogenous and exogenous cytokinins for activation or inhibition of *in vitro* axillary buds (Pérez-Molphe Balch *et al.*, 2015), considering that no shoot initiation occurred in the control (PGRs-free) and lower BA concentrations (1-5 mg L^{-1}) with IAA (0, 1, 1.5 and 2 mg L^{-1}) treatments. Despite the competence of cytokinins for areole activation, various studies underscore the stimulating effect of high BA concentrations with low auxin concentration (0.0625-0.5 mg L^{-1} NAA or 0.1-1 mg L^{-1} IAA) on axillary shoot induction in some prickly pear species (Bouzroud *et al.*, 2022). Cladodes with several areoles proved the most appropriate explant type of the studied prickly pear cactus for shoot induction (100%) and cladodes without areoles the least appropriate (23.33-30%),

while cladodes with one areole were of intermediate competence for shoot induction (63.33-66.70%), emphasizing the importance of areoles presence and areoles number in the cladode. In accordance with the finding presented in this study, BA has been demonstrated as the most beneficial cytokinin type for 100% shoot induction percentages from areoles in *O. ficus-indica* in a wide concentration range, including 0.1 mg L^{-1} (Garcia-Saucedo *et al.*, 2005), 0.5 mg L^{-1} (Zoghlami *et al.*, 2012) and 2.5-4.5 mg L^{-1} (Bouchiha and Mazri, 2022).

In *in vitro* plant tissue culture, there are some cases in which the cytokinin BA (irrespective of applied concentration) alone, especially without auxins might not be enough to initiate shoot formation, (Hnatuszko-Konka *et al.*, 2021), because the absence of auxins can impede the growth of a proper shoot meristem, which is essential for shoot induction (Soleimani *et al.*, 2025) a response that is consistent with the outcomes of this study with *O. ficus-indica* cladode explants regardless areole number (none, one, several) cultured in 2-6 mg L^{-1} BA-supplemented media. Despite the enhancement of shoot induction with cytokinins such as BA, their competence is frequent boosted or even hinge on the presence of auxins (*i.e.* IAA, herein) due to antagonism between cytokinins and auxins, as a balanced concentration ratio of auxins to cytokinins (high cytokinin/low auxin) in the culture medium is crucial for optimal shoot induction (Siddique *et al.*, 2015), as evidenced in this study with the three *O. ficus-indica* explant types in media enriched with 6 mg L^{-1} BA + 1-2 mg $^{-1}$ IAA. In this study, the inhibitory impact of BA (2-6 mg L^{-1}) alone highlights the significance of optimizing the cytokinin (6 mg L^{-1} BA) - auxin (1-2 mg L^{-1} IAA) hormone balance for specific plant species and explant types (Soleimani *et al.*, 2025). In plant tissue culture, BA is a vital component playing a significant role in cell division, shoot induction because of break apical dominance, micropropagation, and overall plant development, therefore is an essential tool for plant scientists and researchers working with *in vitro* plant propagation and genetic improvement for plant regeneration from genetically modified cells or tissues and development of new traits and improved varieties (Mangena, 2020). Notwithstanding, the possible drawbacks of the cytokinin BA particularly when used in excess or over long periods in plant tissue culture systems are inhibition of root development due to a decrease in absorption rate of water and nutrients from the medium, reduced shoot quality and hyperhydricity, and somaclonal variation (Mangena, 2020). The negative aspects of BA application can be mitigated by optimizing BA concentration and duration of use, using alternative cytokinins (*i.e.* kinetin, 2-iP), supplementing the medium with other growth regulator (*i.e.* auxins), controlling the concentration of gelling agent and the overall culture environment (*i.e.* temperature), as well as alternating between hormone-free media and media containing cytokinins during long-term cultivation (Polivanova and Bedarev, 2022). As a key component in plant physiology and a natural (*i.e.* endogenous) auxin hormone, IAA can be utilized in various agricultural and horticultural practices including *in vitro* culture as medium supplement to enhance rooting success, callus formation, organ development, and overall plant health by influencing cellular processes (cell elongation and differentiation) and enhancing plant tolerance to various stresses (Bhat *et al.*, 2020).

Shoot multiplication under different cytokinin and auxin type-concentration combinations

In plant tissue culture, cytokinins like BA, kinetin, 2-iP, TDZ, and CPPU play crucial roles in shoot regeneration, multiplication, and overall plant development, in particular BA and kinetin are commonly used adenine-type cytokinins, TDZ and CPPU are synthetic phenylurea derivatives with potentially higher activity, while 2-iP is another adenine-type cytokinin used in some applications (Subotić *et al.*, 2009). Miscellaneous cytokinins have been employed for shoot proliferation of prickly pear (Mohamed-Yasseen *et al.*, 1995; Llamoca-Zárate *et al.*, 1999; Juárez and Passera, 2002; Khalafalla *et al.*, 2007). Since shoot regeneration and proliferation can be influenced by the type and concentration of the PGRs used, notably the cytokinins, because of their active participation in cell division and organogenesis processes, liable to produce higher shoot numbers (Aremu *et al.*, 2017). Except plant species and experimental conditions, the different responses (number, length) of the *O. ficus-indica* microshoots to different cytokinin types herein could be ascribed to their different activity, linked to variations in uptake rate from nutrient medium, translocation rate toward

meristematic tissues, and stability in metabolic processes, as they may degrade or merge with amino acids or sugar to produce biologically inactive compounds (Gan and Amasino, 1996).

In the studied prickly pear species, among the different cytokinins (BA, kinetin, CPPU, TDZ) applied at 5 mg L^{-1} alone or combined with two different auxins (IAA, 2,4-D) at 0.5 mg L^{-1}, the CPPU at 5 mg L^{-1} appeared as the most effective cytokinin type in terms of shoot number (5.10 shoots/explant) in the absence of auxins. The assertive impact of CPPU on shoot number of prickly pear could be attributed to its high cytokinin activity as a phenylurea, because of its relative tolerance to the endogenous cytokinin oxidases as the key enzymes of cytokinin degradation and its potentiality to increase the levels of endogenous cytokinins (Arinaitwe *et al.*, 2000). In plant tissue culture, *N*-2-(chloro-4-pyridyl)-*N*-phenyl urea (CPPU; forchlorfenuron) is a cytokinin-like molecule and synthetic cytokinin with functions similar to that of zeatin (an endogenous cytokinin) in higher plants, being valuable for promoting shoot proliferation, enhancing shoot regeneration by overcoming the inhibitory effects of auxins on bud outgrowth, improving overall plant growth and development (photosynthetic pigments, soluble sugar and free proline accumulation) thus contributing to healthier and more vigorous plantlets due to increased tolerance to abiotic stress, and in micropropagation protocols especially for recalcitrant species or when increased shoot multiplication is desired (Gashaw *et al.*, 2014). However, when using non-purine, synthetic phenylurea compounds with strong cytokinin-like activity such as thidiazuron (TDZ, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea) and forchlorfenuron (CPPU, N-(2-chloro-4-pyridyl)-N-phenylurea), it is essential to ascertain their optimum levels and methods of application as over exposure can lead to negative effects, viz. hyperhydricity, poor shoot quality and loss of rootability (Gashaw *et al.*, 2014). Consistent with the outcomes of this study, CPPU was associated with augmented shoot numbers in the endangered cactus, *Obregonia denegrii* Frič. (Cardarelli *et al.*, 2010). Nevertheless, in our experiment, BA mainly followed by kinetin proved the least effective cytokinins for shoot multiplication. Several other studies carried out on the same prickly pear species demonstrate the greater effectiveness of BA over other cytokinins (kinetin, 2-iP) on *in vitro* shoot multiplication of cultured areoles (De Medeiros *et al.*, 2006; Khalafalla *et al.*, 2007; Estrada-Luna *et al.*, 2008; Marhri *et al.*, 2023), a response which comes in contradiction to the results herein, as well as to other *Opuntia* spp. (*O. amyclaea, O. streptacantha, O. robusta, O. leucotricha*, and *O. cochinera* (Escobar-Araya *et al.*, 1986; Estrada-Luna, 1988; Juárez and Passera, 2002). Previous studies on the same species revealed that shoot number per explant was found significantly increased under 0.5 mg L^{-1} BA (Garcia-Saucedo *et al.*, 2005; Khalafalla *et al.*, 2007; Zoghlami *et al.*, 2012), 5 mg L^{-1} BA combined with 0.25-1 mg L^{-1} IAA (Ghaffari *et al.*, 2013), 2 mg L^{-1} BA + 0.1 mg L^{-1} NAA (Mohamed-Yasseen *et al.*, 1995), and 6 mg L^{-1} kinetin + 0.2 mg L^{-1} NAA (Rodriguez and Ramirez-Pantoja, 2020). On the contrary, kinetin (1-2 mg L^{-1}) was displayed as the most favorable cytokinin type, exhibiting higher shoot numbers in *O. ficus-indica in vitro* cultures (Bouchiha and Mazri, 2022), which is not in coherence with our study outcomes. The superior shoot multiplication performance (number, length) of *O. ficus-indica* microshoots with the CPPU cytokinin in comparison to the other cytokinin types (BA, Kinetin, TDZ) was proved and is reported for the first time in this study, since no previous study exists indicating the suitability of the other cytokinins (BA, TDZ, Kinetin, 2-iP, zeatin) in favor of CPPU. Besides being more expensive to purchase, zeatin and TDZ are more heat-sensitive cytokinins and should be added to the medium after autoclaving to avoid degradation in comparison to other cytokinins such as BA, kinetin, and 2-ip (Hart *et al.* 2016). In plant tissue culture, CPPU is a synthetic cytokinin known for its heat stability under autoclaving conditions, can be less prone to genetic variation during direct organogenesis methods (shoot multiplication, in this study) compared to callus-mediated indirect regeneration, and even more expensive than some other cytokinins like BA, its effectiveness in certain horticultural applications, like promoting fruit size, self-life, and delaying senescence, may justify the cost (Grzegorczyk-Karolak *et al.*, 2021).

Shoot elongation can be more facile triggered by the exogenous application of low to moderate concentrations of cytokinin, alone or altogether with low auxin concentration to the culture medium. However, their simultaneous use is demanded for *Opuntia* genus for obtaining higher shoot lengths (Bouzroud *et al.*, 2022). Despite the highest shoot number achieved herein under 5 mg L^{-1} CPPU, shoot length was best

enhanced (6.19 cm) similarly under 5 mg L^{-1} CPPU, but only when combined with 0.5 mg L^{-1} 2,4-D. Forchlorfenuron (CPPU) is a synthetic cytokinin, which means molecule that has cytokinin-like activity, thus promoting shoot multiplication at a greater degree in comparison to other cytokinins (Arinaitwe *et al.*, 2000). A number of studies conducted on *O. ficus-indica* evidence optimum shoot elongation performance from *in vitro* cultured areoles under different cytokinin types than CPPU, including BA applied alone (Mohamed-Yasseen *et al.*, 1995; El Finti *et al.*, 2010; El Finti *et al.*, 2012; Zoghlami *et al.*, 2012) or combined with an auxin type (NAA, IAA) (El Finti *et al.*, 2012; Rodriguez and Ramirez-Pantoja, 2020), and kinetin used alone (Gebretsadik *et al.*, 2013; Bouchiha and Mazri, 2022) or combined with IAA (Rodriguez and Ramirez-Pantoja, 2020). Similarly, to shoot number herein, BA and kinetin proved the least efficient cytokinins for elongation of prickly pear microshoots. The significantly lower lengths of *O. ficus-indica* microshoots in BA-supplemented medium compared to the other PGRs treatments excluding control, may be related to the toxic impact of BA on plants, owing to the sluggish cumulation and release of metabolites to other plant parts, provoking growth and rooting inhibition (Aremu *et al.*, 2017).

In this study, a micropropagation protocol was established for *O. ficus-indica*, including the generation of shoots exclusively from cladode explants that contained areoles, therefore this method can maintain the genetic fidelity of the regenerated progeny. Using pre-existing buds in plant tissue culture (cladode explants with areoles, herein with prickly pear cactus) helps maintain genetic fidelity (*i.e.* more stable genome) of regenerated plants and minimizes the risk of somaclonal variation which can occur during regeneration from undifferentiated cells. By utilizing meristematic tissues like axillary or apical buds, which have a higher degree of genetic stability, the chances of introducing genetic changes during propagation are significantly reduced. Maintaining genetic fidelity is crucial for ensuring that the regenerated plants are true to the original plant, especially in applications like clonal propagation for horticulture, conservation of plant genetic resources, and genetic improvement programs (Ioannidis *et al.*, 2022). In some species, CPPU, as a synthetic cytokinin mimics the effects of natural plant hormones that promote fruit development, thus it is a valuable tool for inducing parthenocarpy (development of seedless fruits without fertilization), especially in crops where pollination is unreliable or absent, since CPPU strengthens cell division and enlargement, resulting in larger fruit size (Li *et al.*, 2025). Among the benefits of CPPU for parthenocarpic fruit production are the seedless fruits, the reduced reliance on pollinators, and the increased yields, whereas potential drawbacks can be the adverse effect on fruit quality such as depleted vitamin C and sugar levels, less aroma, and augmented bitterness (Li *et al.*, 2025). In *in vitro* shoot multiplication stage, 2,4-D, a synthetic auxin, can have both positive (*i.e.* callus initiation for the production of secondary metabolites, shoot induction, plant regeneration) and negative effects (*i.e.* shoot proliferation inhibition, medium browning, decline of plant growth and development, somaclonal variation, cytotoxicity, herbicide resistance) at high concentrations or with prolonged exposure (Carsono *et al.*, 2021). The optimal concentration and application of 2,4-D in shoot proliferation protocols depend on the specific plant species, the type of explant, and the desired outcome, therefore it is essential to carefully optimize these parameters to maximize the positive effects of 2,4-D while minimizing its potential negative impacts (Carsono *et al.*, 2021).

In vitro rooting under different auxin types

The auxins IBA, IAA and NAA have been reported to promote root formation in *Opuntia* species (Escobar-Araya *et al.*, 1986; Mohamed-Yasseen *et al.*, 1995; El Finti *et al.*, 2010; Zoghlami *et al.*, 2012; Mabrouk *et al.*, 2021), for instance in *O. ficus-indica,* 100% rooting has been obtained with either IBA or IAA applied at 0.5 mg L^{-1} (El Finti *et al.*, 2010; Gebretsadik *et al.* 2013), 1-2 mg L^{-1} IBA (Mohamed-Yasseen *et al.*, 1995), 0.5 mg L^{-1} IAA (Khalafalla *et al.*, 2007), and 0.5 mg L^{-1} NAA (Bouchiha and Mazri, 2022). Similarly to a previous study on the same species (El Finti *et al.*, 2012) the microshoots derived from our experiment were rooted *in vitro* at a 100% rate in all treatments, irrespective auxin type (IBA, IAA, NAA) and concentration. A possible explanation for this response could be that the exogenous application of auxins in the *in vitro* culture medium can lead to elevated levels of endogenous auxins, thus rooting can occur spontaneously in medium

PGRs-free as has been illustrated in many *Opuntia* species (Escobar-Araya *et al.*, 1986; Clayton *et al.*, 1990; Khalafalla *et al.*, 2007; Bouchiha and Mazri, 2022). However, in some cases, if the endogenous auxins far exceed the optimum level, root formation inhibition can occur (*i.e.* lower rooting % and root number, shorter roots) (Khalafalla *et al.*, 2007; Ghaffari *et al.*, 2013). In accordance with the findings presented in our study, in several *Opuntia* spp. (*O. ficus-indica*, *O. robusta*, *O. amyclaea*), 100% rooting has also been recorded in media fortified with low-to-moderate concentrations of IBA, IAA or NAA (0.5-2 mg L^{-1}). Nevertheless, in *O. ellisiana*, root induction can mere be amplified by high auxin concentration (5 mg L^{-1} IBA) (Juárez and Passera, 2002).

In the current study used *O. ficus-indica*, a 3- to 5-fold increase was achieved in the root number (11.9-19.6) of the *in vitro* cultured shoots, under the effect of 0.5 mg L^{-1} of IBA, IAA and NAA regardless auxin type, compared to the control (3.9 roots), being maximized (19.6 roots) in IBA-supplemented medium. The superior performance of IBA at 0.5 mg/L on the *in vitro* rooting of the studied *O. ficus-indica*, mainly in root number, than that of IAA and NAA has been noticed in previous works with *Opuntia* species (Escobar-Araya *et al.*, 1986; Clayton *et al.*, 1990; Hubstenberger *et al.*, 1992; Mohamed-Yasseen *et al.*, 1995; García-Saucedo *et al.*, 2005; Estrada-Luna *et al.*, 2008; El Finti *et al.*, 2012), including three *O. ficus-indica* genotypes (Blanco sin Espina, Milpa Alta and Villa Nueva) under 1 mg L^{-1} IBA (Garcia-Saucedo *et al.*, 2005). Different outcomes in terms of auxin type efficiency have been depicted in other studies undertaken on the same species as herein, where root numbers/microshoot were remarkably elevated either with IAA at 0.5 mg L^{-1} (Khalafalla *et al.*, 2007) or in auxin-free medium (Bouchiha and Mazri, 2022). Possible reasons for the higher efficiency of IBA in rooting of prickly pear microshoots than IAA and NAA could be: (1) the higher stability of IBA than IAA, since the latter is 5-fold more facile to photo-oxidation, more rapid to decomposition, and of lower tolerance to enzymatic degradation (Ludwig-Muller *et al.*, 2005), (2) the finely tuned metabolic conversion of IBA to IAA (Skůpa *et al.*, 2014), (3) the poor translocation and longer persistence of IBA close to the application site and its relatively slow degradation by the enzyme IAA oxidase (Hartmann *et al.*, 2002), (4) the slower metabolic conversion rate and greater difficulty of NAA to be converted by some IAA auxin-conjugating enzymes as compared to IBA (Hosek *et al.*, 2012; Peat *et al.*, 2012), (5) the naphthalene ring of a NAA molecule occupies a larger space than the indole system of an IAA, making it less well absorbed (Flasiński and Hąc-Wydro, 2014), and (6) IAA is often less effective than NAA due to its rapid breakdown within the plant tissue (Eliwa *et al.*, 2025). It is assumed that the differences in rooting attributes may be the discrepancies in the rate of metabolism, uptake and transport of the three auxin types (IBA, NAA, IAA) (Barpete *et al.*, 2014).

In plant tissue culture, the auxin IBA promotes root induction (%) and rooting efficiency (number, length) for rapidly multiplying shoots, stimulates the formation of adventitious roots that emerge from non-root tissue, reduces transplant shock as it assist plants recover more quickly when transplanted from tissue culture to soil by promoting root growth (*i.e.* higher survival rates), improves plant tolerance to environmental stresses by influencing nutrient uptake and antioxidant activity, and is more stable than other auxins like IAA in tissue culture media and during autoclaving, making it a reliable choice for rooting protocols (Bai *et al.*, 2020). However, excessive IBA concentrations can lead to detrimental effects, like inhibiting root growth, causing tissue damage, stimulating ethylene production, and overgrowth resulting in excessive callus formation or other abnormal growth patterns (Bai *et al.*, 2020). When used on crops, IBA contributes to better flowering, fruiting, and overall yield, boosting agricultural profits because of versatility in its usage (powders, solutions, sprays). At low concentrations, IBA is non-toxic and poses no significant risk to human health, as it biodegrades quickly and does not persist in the environment, thus is of high environmental safety (Justamante *et al.*, 2022). The auxin NAA at low concentrations can promote root formation in cuttings, prevent premature fruit drop, influence plant shape, and increase plant tolerance to stress, while excessive NAA concentrations can inhibit root growth, reduce fruit size, delay fruit softening by inhibiting the enzymes responsible for cell wall breakdown, induce negative phototropism (bending away from light), and even cause plant toxicity (Wang *et al.*, 2022). Besides its involvement in apical dominance, root formation, and plant's defence mechanisms against pathogens, IAA is the most prevalent and significant auxin in plants, which influences cell division, elongation, tissue differentiation, and responses to environmental cues like light (*i.e.* phototropism) and gravity (*i.e.*

gravitropism) (Fu *et al.*, 2015). Roots are particularly sensitive to fluctuations in IAA levels, with different concentrations affecting root growth differently. Some plant pathogens can manipulate the plant's auxin metabolism to their advantage, potentially promoting disease development by increasing the levels of conjugated IAA forms like IAA-Asp (González-Lamothe *et al.*, 2012). While IAA is a natural plant hormone, IBA and NAA are synthetic auxins that are frequently used to enhance root development in plants and to regulate their growth. The specific effects of each auxin can vary depending on the plant species, concentration, and application method (Tien *et al.*, 2020). While the U.S. Environmental Protection Agency notes that IBA, due to its low application rates and similarity to natural compounds, poses minimal environmental risk, other synthetic auxins could have more significant ecological consequences if not handled carefully (EPA, 1992).

Acclimatization of in vitro rooted shoots and ex vitro survival

After a 6-week period of acclimatization of the rooted *O. ficus-indica* microshoots in autoclaved soil and under plastic coverings followed by a 10-week period after transplantation in the greenhouse, 100% *ex vitro* survival was recorded, regardless of the *in vitro* rooting medium (auxins-free, IBA, IAA, NAA) originated from, exhibiting healthy and active growth. Prickly pear cacti are known to have a high success rate (100%) in acclimatizing (Clayton *et al.*, 1990; Mohamed-Yasseen *et al.*, 1995; Hartmann *et al.*, 1997; Pérez-Molphe-Balch *et al.*, 1998; Juárez and Passera, 2002; Estrada-Luna *et al.*, 2008) including the *O. ficus-indica* (Khalafalla *et al.*, 2007; Angulo-Bejarano and Paredes-López, 2011; El Finti *et al.*, 2012). The high survival and low loss rates of micropropagated cacti plants during *ex vitro* acclimatization could be due to the existence of functional roots, the well-developed root system of Opuntiaceae linked to high rehydration capacity, the adequate cuticle, and the ability to regulate stomata function, thus diminishing transplant shock caused by immoderate water loss and enabling a constant and enduring plant growth (Hartmann *et al.*, 1997). For *ex vitro* acclimatization, auxins like IBA, NAA, and IAA can be used to promote root development in plantlets that have been propagated *in vitro*, allowing the plantlets to better absorb water and nutrients from the soil, especially synthetic auxins (mainly IBA followed by NAA) as the natural occurring IAA auxin is more prone to photo-oxidation and degradation by enzymes and therefore of lower stability, nevertheless the choice of auxin and its concentration can depend on the plant species and the specific protocol being used (Lakho *et al.*, 2023). It worth mentioning that despite the 100% *ex vitro* survival of *O. ficus-indica* rooted microshoots in both control (auxins-free) and auxins (IBA, NAA, IAA)-supplemented substrate, auxins like IBA and NAA are valuable tools for promoting further root development and improving the success of *ex vitro* acclimatization of micropropagated plantlets.

Ex vivo rooting and survival of non-rooted in vitro shoots under different CRF concentrations

The success of the acclimatization process equivalent to high survival rates is depended on various factors including the functionality of the adventitious roots influencing water and nutrients absorption, substrate composition and mineral nutrition (Gonçalves *et al.*, 2014). The controlled release fertilizers (CRFs) can ensure synchronized nutrient release that coordinate with plant growth requirements at different developmental stages, given that they control the rate, pattern, and duration of the nutrient release because granules are coated or encapsulated by organic or inorganic materials (Liu *et al.*, 2014; Santos *et al.*, 2020). The success degree of plant establishment in the field can be contingent on the development and architecture of the root system, seeing that higher root biomass is closely related to higher competence of water absorption, support, productivity and adaptation to unfavorable environmental conditions, thus plants of higher photosynthetic capacity and better performance to the field (Almeida *et al.*, 2005; Behling *et al.*, 2014; Salgado Pirata *et al.*, 2022). Among the advantages derived from the use of CRFs are the shortened production cycle of plantlets with desirable traits to fulfil the market requirements, the diminishment of labor, the enhancement in plant quality and optimization of production costs (Smiderle *et al.*, 2020). The CRF (15% total N, 9% P_2O_5, 12% K_2O) used in this study resulted in 100% *ex vivo* rooting and survival of the non-rooted *in vitro O. ficus-indica* shoots, similarly to the control substrate (not supplemented with the fertilizer), pointing out the low

nutritional requirements of prickly pear cactus species (Ferreira *et al.*, 2022) and corroborating the nutritional information of this species as evaluated by Lopes *et al.* (2010), and this way of acting is likewise noticed in *ex vitro* growth. The beneficial end result of CRF, especially in the concentration of 1.2 g/alveoli, in the number of *ex vivo* roots and further vegetative growth could be ascribed to the fact that N is a key element in the performance of plant metabolic activities and absorption of other nutrients from the environment (Rezende et al., 2008). According to Santos *et al.* (1990), the leading upward order of various nutrients at the end of the production cycle was mentioned to be nitrogen (N) < phosphorous (P) < potassium (K) < calcium (Ca), emphasizing the higher necessity of supplementing the substrate with nitrogen for better plant growth. It has been postulated that CRFs when applied at low doses neither alter the absorption of macronutrients including N, K, calcium (Ca), and Mg nor meddle in the metabolic processes (Taiz and Zeiger, 2013). Similarly, in the prickly pear cactus *Opuntia stricta* (Haw.) Haw. (Cactaceae), it was found that the *in vitro* growth and development of the species can be substituted by the use of a commercial potassium nitrate (KNO_3) fertilizer, reducing the amounts of nitrogen provided by the *in vitro* nutrient MS culture medium in the form of ammonium nitrate and potassium nitrate salts (Ferreira *et al.*, 2022). It is reported that CRFs containing N, P, and K can significantly promote root growth, including the development of more and denser lateral roots, and increased root hair formation, thus this enhanced root system improves nutrient and water uptake, ultimately benefiting plant growth and yield (García-Ilizaliturri *et al.*, 2025). For instance, N plays a vital role in overall plant growth, including root development, promoting the formation of new roots and enhancing their length and density, P is crucial for root growth, particularly the development of lateral roots and root hairs, which significantly increase the plant's ability to explore the soil for nutrients and water, while K is essential for overall plant health and vigor, influencing root growth, nutrient transport, and stress tolerance (Bharath-Kumar *et al.*, 2025).

Controlled release fertilizers (CRFs) are a promising tool for *ex vivo* plant propagation, offering a steady supply of nutrients to developing roots and reducing the risk of nutrient imbalances or toxicity associated with traditional, quick-release fertilizers. The coating in CRFs is designed to release nutrients based on factors like temperature or moisture, further enhancing the synchronization with plant needs. The benefits of CRFs in *ex vivo* rooting and subsequent survival of plants have been shown to be several including: (1) the provision of a steady supply of essential nutrients like N, P, and K, which are crucial for root development, (2) CRFs minimize the risk of nutrient burn or deficiencies, which can be detrimental to young, developing roots, (3) the simplified application of CRFs, typically incorporated into the rooting medium during preparation, eliminating the need for frequent fertilizer applications, and reducing labor costs, (4) the enhanced root development is associated to increased root length, biomass, and overall root system health, and (5) the potential for reduced transplant shock by providing a consistent nutrient supply during the acclimatization phase, CRFs may help plants better adapt to the transition from *in vitro* to *ex vitro* conditions (Salgado Pirata *et al.*, 2022). Except of the positive aspects of CRFs, these fertilizers face several limitations, including high costs, variability in nutrient release, potential for environmental impact, and challenges with scalability and long-term field performance. Genotype-specific responses to CRFs and the need for further research on long-term effects also present hurdles (Kelly *et al.*, 2023). CRFs are generally more expensive than conventional fertilizers, which can limit their adoption in large-scale agricultural operations, specifically the cost of the polymer coatings used in CRFs is a significant factor (Vejan *et al.*, 2021). While CRFs are used, their overall market share is still relatively small compared to traditional fertilizers (Davidson and Gu, 2012). Scaling up the production of CRFs to meet the demands of large-scale agriculture can be challenging, especially for novel formulations or bio-based CRFs (Fadiji *et al.*, 2024). The economic benefits of using CRFs need to be clearly demonstrated, especially in terms of increased yields and profitability, to encourage wider adoption (Rubel and Wei, 2025). Different plant genotypes may respond differently to CRFs, potentially due to variations in nutrient uptake mechanisms or root architecture, therefore research is needed to understand how CRFs can be optimized for specific crop genotypes and how to breed for improved nutrient use efficiency with CRFs, considering that soil health factors and management practices can also influence how CRFs perform, requiring further research to understand

these complex interactions (Kelly *et al.*, 2023). In long-term field conditions, the long-term fate of CRF coatings in the environment is not fully understood (Fadiji *et al.*, 2024), CRF residues, particularly polymer coatings, may accumulate in the soil over time, potentially impacting soil health and nutrient cycling (Kelly *et al.*, 2023), not to mention that CRFs can affect soil microbial communities, thus long-term studies are needed to understand these effects (Fadiji *et al.*, 2024). The durability of CRF coatings under field conditions, especially in extreme weather, needs further investigation (Singh *et al.*, 2022; Fadiji *et al.*, 2024), while ensuring consistent nutrient release over time, especially under fluctuating environmental conditions, is crucial (Fadiji *et al.*, 2024; Kumar *et al.*, 2024). Considerations that should be addressed when using CRFs for *ex vivo* rooting, survival and subsequent growth of plants are: (1) the nutrient ratio deeming the necessity to select a CRF with a balanced nutrient ratio proper for the specific plant species and developmental stage (Savvas and Gruda, 2018), (2) the release rate, which should be appropriate for the duration of the rooting process (Ren *et al.*, 2025), (3) the medium incorporation as CRFs are typically mixed into the rooting medium before planting, ensuring even distribution (Salgado Pirata *et al.*, 2022), and (4) the dosage following manufacturer recommendations to avoid over-fertilization (Araújo *et al.*, 2020).

Conclusions

A successful micropropagation protocol was established for prickly pear cactus (*O. ficus-indica* (L.) Mill.), considering different cladode explant types based on number of areoles, cytokinin types, auxin types and concentrations, plus acclimatization processes. *In vitro* shoot initiation was recorded for all cladode explant types (without areoles, with one areole, with several areoles) under the highest BA concentration (6 mg L^{-1}) with IAA (1-2 mg L^{-1}). The cladodes with several areoles proved to be the most suitable source of explants for shoot induction (100%). In the subsequent shoot multiplication stage, 5 mg L^{-1} CPPU was the most ideal cytokinin type for obtaining higher number of shoots, while 5 mg L^{-1} CPPU + 0.5 mg L^{-1} 2,4-D exhibited more elongated microshoots. Following that, IBA (0.5 mg L^{-1}) was the ideal auxin type promoting best *in vitro* rooting, resulting in higher root numbers. During acclimatization, 100% *ex vitro* survival rate was achieved for rooted *in vitro* shoots. Adding CRF (15-9-12, N: P_2O_5: K_2O) to the peat: vermiculite substrate led to 100% *ex vivo* rooting of the non-rooted microshoots, exhibiting a 100% survival rate, irrespective of concentration, nonetheless 1.2 g alveoli^{-1} was the optimum fertilizer concentration that further stimulated the number of *ex vivo* roots. Despite several previous studies in different *Opuntia* spp. using different types and concentrations of PGRs, the novelty of this study lays in the use of different cladode explants investigating the effectiveness of areole presence or absence and number of areoles per cladode on shoot initiation of *in vitro* culture. The greater the number of areoles in the cladode, the higher shoot induction percentage obtained. Besides other cytokinins such as BA, kinetin, 2-iP, and TDZ, commonly tested in previous works, the application of CPPU for shoot multiplication in a plant species of *Opuntia* genus was tested for the first time in this study and proved to be the most effective. More research is needed to fully understand the long-term impact of CPPU on fruit quality and development of seedless-parthenocarpic fruits for the *ex vitro* acclimatized prickly pear cactus (*O. ficus-indica*) *in vitro* plantlets in this study, to optimize its application for specific crops, as has already been demonstrated for greenhouse-grown melons, and other crops like cucumber, tomato, kiwifruit, and other cucurbits (Li *et al.*, 2025). Another new aspect is the development of different rooting and acclimatization procedures both *in vitro* in auxin-supplemented media (common) and directly *ex vivo* to the greenhouse in a peat:vermiculite CRF-supplemented substrate. In this study, the replacement of the *in vitro* rooting stage and culturing of explants in auxins-supplemented media with the feasibility of using CRFs at small amounts for direct rooting *ex vivo* is clearly supported, by shortening propagation cycle, reducing production costs and encountering the requirements of the *in vitro* culture of the tested prickly pear cactus species under the specific microclimatic and environmental conditions of Iraq. This is achieved without any alteration in plant physiology features during acclimatization and after acclimatization stages (Ferreira *et al.*, 2022), since *O. ficus-*

indica can easily root *in vitro* (even in medium auxins-free) and *ex vivo* (in substrate with no CRF). The necessity of establishing an effective micropropagation protocol to fulfill the constantly raising demand of *Opuntia* spp. for human consumption as fruit, animal feed, ecological restoration in semi-arid and arid zones, conservation and preservation of endangered or threatened cacti is well-defined (Bouzroud *et al.*, 2022). Plant regeneration via haploids and secondary metabolites production using organs, tissues or cell cultures from cacti constitute future biotechnological applications (Pérez-Molphe-Balch *et al.*, 2015). In on-going studies, molecular techniques like RAPD, ISSR, and SSR markers can be used to assess the genetic stability of the *in vitro*-regenerated *O. ficus-indica* plants through areole activation of cladode - axillary bud explants, and confirm the same genetic makeup of the regenerated plants to the mother plant (Ioannidis *et al.*, 2022).

Authors' Contributions

Conceptualization: HEM; Data curation: HEM; Formal analysis: TTZ and VS; Funding acquisition: HEM; Investigation: HEM, TTZ, VS and TT; Methodology: HEM; Project administration: HEM, TTZ and VS; Resources: HEM, TTZ, VS and TT; Software: VS; Supervision: HEM and VS; Validation: HEM and VS; Visualization: VS; Writing - original draft: HEM, TTZ, VS and TT; Writing - review and editing: TTZ, VS and TT. All authors read and approved the final manuscript.

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Conflict of Interests

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