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In vitro shoot multiplication, rhizogenesis, and ex vitro acclimatization protocol for spineless cactus (*Opuntia ficus indica*)

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Abstract
Spineless cactus pear is a robust, water-efficient xerophyte essential for forage security and soil conservation in drought-prone agro-ecosystems. However, traditional vegetative propagation via cladodes is limited by low multiplication rates and risks of disease dissemination. This investigation aimed to establish an efficient downstream micropropagation system encompassing in vitro shoot proliferation, adventitious rooting, and ex vitro acclimatization from established areole cultures. Shoots initiated from areoles were cultured on Murashige and Skoog (MS) media containing diverse plant growth regulator combinations. Maximum shoot multiplication (6.25 ± 0.05 shoots per explant) was obtained on MS medium fortified with 1.5 mg/L 6-benzylaminopurine (BAP), 0.25 mg/L α-naphthaleneacetic acid (NAA), and 0.5 mg/L kinetin (KIN). For root induction, full-strength MS medium supplemented with 0.5 mg/L indole-3-acetic acid (IAA) and 30 gm/L sucrose produced the highest rooting percentage (85%) and the fastest root initiation (13.37 ± 0.52 days). Micropropagated plantlets were successfully acclimatized using a sequential two-stage hardening process. Primary hardening in pure cocopeat resulted in 60% survival, while secondary hardening in a 1:1 soil and sand mixture achieved 100% plantlet survival. This standardized protocol provides a dependable, scalable method for mass propagation and clonal production of high-quality planting material.

Keywords: *Opuntia ficus indica*, Micropropagation, Shoot multiplication, Adventitious rooting, Acclimatization, Auxins, Cytokinins

Introduction

Opuntia ficus indica (L.) Mill., a member of the Cactaceae family, is an arborescent succulent widely distributed throughout arid and semi-arid regions of the world. Operating via Crassulacean Acid Metabolism (CAM), *O. ficus indica* exhibits exceptional water-use efficiency, thriving under minimal annual rainfall (120-150 mm) and extreme high-temperature regimes. In dryland regions, particularly across India, livestock rearing forms a critical economic pillar; however, chronic green forage shortages during post-monsoon periods pose severe challenges. Spineless cactus pear has emerged as a valuable drought-proof emergency forage crop, carbon sink, and biological windbreak to combat land degradation and desertification.

Despite its vast agronomic potential, conventional propagation using cladode cuttings is constrained by slow multiplication rates, high land requirement, and potential disease transmission across mother stands. Seed propagation is equally problematic due to low seed germination rates, physical dormancy, and substantial genetic segregation that undermines clonal uniformity. Plant tissue culture serves as an effective biotechnological tool for mass propagation, offering rapid production of uniform, disease-free true-to-type plants.

Although initial explant establishment and areole bud break have been explored, standardizing optimal downstream steps specifically mass shoot proliferation, adventitious rooting, and ex vitro acclimatization remains vital for practical applications. Plant growth regulator (PGR) balances severely regulate organogenic responses in cacti. Furthermore, ex vitro hardening represents a major bottleneck where high transplant mortality often occurs.

Therefore, the present investigation was conducted to formulate an optimized protocol for in vitro shoot multiplication, adventitious root development, and ex vitro acclimatization of *O. ficus indica*.

Materials and methods

Study location and micropropagation media

The research was carried out at the Tissue Culture Unit, Biotechnology Laboratory, Botany Division, Govt. College of Agriculture, Pune, Maharashtra, India. In vitro shoot cultures previously established from areole explants (sourced from AICRP on Forage Crops, MPKV, Rahuri) served as the primary material. Full-

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strength and half-strength Murashige and Skoog (MS) media were used, enriched with 100 mg/L myo-inositol, 30 g/L sucrose, and 0.8 (w/v) plant tissue culture grade agar (HiMedia, Mumbai, India). The medium pH was adjusted to 5.8 ± 0.02 prior to autoclaving at 121 °C and 15 psi (103.4 kPa) for 20 minutes. Cultures were kept at 25 ± 2 °C under a 16-hour light photoperiod (2500 lux intensity, cool white fluorescent tubes).

Adventitious rooting protocol

Elongated shoots (> 2 cm) were transferred to distinct rooting media:

- T1: Half-strength MS + 0.5 mg/L IBA + 30 g/L sucrose
- T2: Half-strength MS + 10 g/L sucrose
- T3: Full-strength MS + 1.5 mg/L KIN + 30 g/L sucrose
- T4: Full-strength MS + 0.5 mg/L IAA + 30 g/L sucrose

Rooting success (%) and the number of days required for root emergence were monitored over 4 weeks.

Hardening and acclimatization

Rooted plantlets were carefully removed from vessels, rinsed with double-distilled water to clear agar traces, and dipped in 0.1 % (w/v)} Bavistin fungicide for 5 minutes.

- Primary Hardening: Plantlets were planted into cell trays containing pre-sterilized cocopeat substrate under high relative humidity (70-80%) inside a shaded greenhouse structure for 14 days.
- Secondary Hardening: Plantlets were subsequently transplanted into polybags filled with a soil and sand mixture (1:1 v/v) for 30 days under natural daylight conditions. Survival percentages were recorded for both steps.

Statistical design

Experiments followed a Completely Randomized Design (CRD) with 4 replications per treatment (n = 20 explants per replication). Data were analyzed for standard error and Critical Difference (CD) at ≤ 0.05 according to standard procedure.

Results

In vitro shoot proliferation

Varying concentrations and combinations of cytokinins and auxins significantly influenced shoot multiplication parameters (Table 1). Treatment T3 (MS + 1.5 mg/L BAP + 0.25 mg/L NAA) + 0.5 mg/L KIN) produced the highest shoot regeneration, averaging 6.25 ± 0.05 shoots per explant. Treatment T1 (MS + 0.5 mg/L BAP) yielded 5.40 ± 0.05 shoots, whereas treatment T4 (MS + 2.0 mg/L BAP + 0.2 mg/L NAA) generated 4.40 ± 0.05 shoots per explant. The lowest shoot proliferation rate (3.15 ± 0.05 shoots per explant) was observed in treatment T2 (MS + 1.0 mg/L BAP).

MS Media + 1.0 mg/l BAP



Plate 1b. Multiplication

Plate 1c. Multiplication
MS Media + 1.5 mg/l BAP + 0.25 mg/l NAA + 0.5 mg/l Kinetin

Plate 1d. Multiplication
MS Media + 2.0 mg/l BAP + 0.2 mg/l NAA

Plate 1a. Multiplication
MS Media + 0.5 mg/l BAP

Table 1. Effect of plant growth regulators on in vitro shoot multiplication of *Opuntia ficus indica*

Treatment No.	Media Combination	Number of Shoots Regenerated
T1	MS Media + 0.5 mg/l BAP	5.40c
T2	MS Media + 1.0 mg/l BAP	3.15a
T3	MS Media + 1.5 mg/l BAP + 0.25 mg/l NAA + 0.5 mg/l Kinetin	6.25d
T4	MS Media + 2.0 mg/l BAP + 0.2 mg/l NAA	4.40b
S.E. (m) ±		0.052

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Treatment No.	Media Combination	Number of Shoots Regenerated
C.D. at 5%		0.161

Note: Treatment means having common superscripts are statistically non-significant (

$$p > 0.05$$

). Treatment means having different superscripts are statistically significant (

$$p \leq 0.05$$

).

Adventitious root induction

Auxin supplementation enhanced root induction metrics in microshoots (Table 2). Treatment T4 (MS + 0.5 mg/L IAA + 30 g/L sucrose) induced the highest rooting percentage (85%) and required the lowest mean days to root initiation (13.37 ± 0.52 days). Treatment T1 (Half-strength MS + 0.5 mg/L IBA + 30\ g/L sucrose) achieved an 80% rooting success within 15.11 ± 0.52 days. Treatment T2 (Half-strength MS + 10 g/L sucrose) yielded a 65% rooting response (17.81 ± 0.52 days), whereas treatment T3 (MS + 1.5 mg/L KIN + 30 g/L sucrose) exhibited the lowest rooting percentage (55%) and delayed root initiation (20.60 ± 0.52 days). The overall variation in rooting percentage across treatments was statistically non-significant ($p > 0.05$), while days to root induction differed significantly ($p \leq 0.05$).



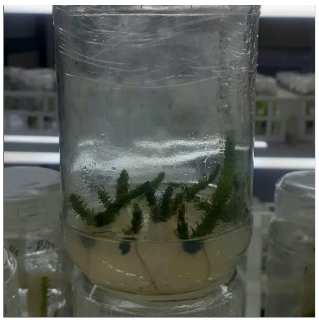






Plate 2a. Rooting

½ MS media + 0.5 mg/l IBA + 30 gm/l Sucrose
Plate 2b. Rooting

MS media + 0.5 mg/l IAA + 30 gm/l Sucrose
Plate 2c. Rooting

½ MS media + 10 gm/l Sucrose
Plate 2d. Rooting

MS media + 1.5 mg/l Kinetin + 30 gm/l Sucrose

Table 2. Effect of different growth regulators on rooting of microshoots

Treatment No.	Media Combination	Percent Rooting (%)	Days to Root Induction
T1	½ MS media + 0.5 mg/l IBA + 30 gm/l Sucrose	80a	15.11b
T2	½ MS media + 10 gm/l Sucrose	65a	17.81c
T3	MS media + 1.5 mg/l Kinetin + 30 gm/l Sucrose	55a	20.60d
T4	MS media + 0.5 mg/l IAA + 30 gm/l Sucrose	85a	13.37a
S.E. (m) ±		7.22	0.515
C.D. at 5%		NS	1.586

Note: Treatment means having common superscripts are statistically non-significant (

$$p > 0.05$$

). Treatment means having different superscripts are statistically significant (

$$p \leq 0.05$$

).

Hardening and plantlet survival

A systematic two-stage hardening process was evaluated for acclimatizing rooted plantlets (Table 3). Primary hardening in cocopeat substrate supported initial plantlet survival at 60%. Upon transferring

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surviving plantlets to secondary hardening in a 1:1 soil and sand potting mixture, a 100% plantlet survival rate was achieved.

Table 3. Survival percentage of *Opuntia ficus-indica* plantlets during ex vitro hardening

Type of Hardening	Potting Mixture	Survivability (%)
Primary Hardening	Cocopeat	60
Secondary Hardening	Sand + Soil (1:1)	100

Discussion

The morphogenic responses observed in *Opuntia ficus indica* microshoots highlight the key role of hormonal cross-talk between cytokinins and auxins. Solitary BAP application at low concentration (0.5 mg/L, T1) proved superior (5.40 shoots/explant) to a higher BAP concentration (1.0 mg/L, T2), indicating potential supra-optimal cytokinin suppression. Combining BAP (1.5 mg/L) with kinetin (0.5 mg/L) and trace NAA (0.25 mg/L) (T3) achieved maximum shoot proliferation (6.25 shoots/explant). This synergistic enhancement aligns with findings reported by Bahulikar *et al.* , who recorded maximum shoot multiplication in *O. ficus indica* (6.10 shoots/explant) on MS medium containing identical growth regulator ratios. The combined action of BAP and kinetin likely enhances cell division by stimulating multiple cytokinin signaling cascades, while low auxin concentrations reduce apical dominance.

During the rhizogenesis phase, auxin type played a primary role in regulating the speed of root primordia development. Indole-3-acetic acid (0.5 mg/L IAA, T4) induced root formation significantly faster (13.37 days) than IBA or cytokinin-dominated media. This rapid response with IAA aligns with previous reports noting efficient adventitious rhizogenesis in prickly pear microshoots treated with low-strength auxins. Cytokinin-rich media (T3) delayed root initiation (20.60 days), consistent with the established inhibitory effect of cytokinins on root apical meristem organogenesis.

The ex vitro hardening phase represents a vital transitional stage where tissue-cultured plantlets adjust from heterotrophic in vitro conditions to autotrophic greenhouse environments. Cocopeat served as an effective primary substrate due to its high moisture retention, protecting young plantlets from initial desiccation. Transitioning to a porous 1:1 soil and sand mixture provided physical support and root zone aeration, eliminating excess moisture retention and achieving 100% survival during secondary hardening. Similar substrate combinations have been shown to facilitate root development and ex vitro establishment in succulent species.

Conclusion

This study establishes a complete protocol for the downstream micropropagation stages of spineless cactus (*Opuntia ficus indica*):

1. Shoot Proliferation: MS basal medium fortified with 1.5 m/ L BAP + 0.25 mg/L NAA + 0.5 mg/L KIN promoted optimal shoot multiplication (6.25 shoots/explant).
2. Adventitious Rooting: Full-strength MS medium supplemented with 0.5 mg/L IAA and 30 g/L sucrose induced optimal rooting (85% success in 13.37 days).
3. Hardening Protocol: A two-stage acclimatization technique using cocopeat for primary hardening followed by a 1:1 sand and soil mixture achieved 100% plantlet survival.

This micropropagation workflow offers a reliable method for mass clonal propagation, germplasm conservation, and nursery supply of spineless cactus fodder crops.

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