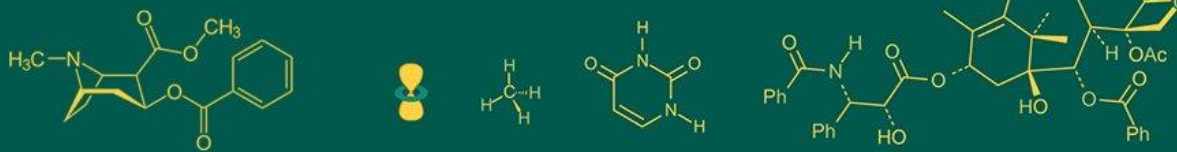


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De novo regeneration and micropropagation of *Opuntia ficus-indica* (L.) Mill.

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Abstract

This investigation presents a comprehensive *in vitro* regeneration protocol for *Opuntia ficus-indica* (L.) Mill., developed through a series of controlled experiments conducted at the Tissue Culture Laboratory, State Level Biotechnology Centre, MPKV, Rahuri (2023-2025). Three explant types were evaluated, and areoles from 2-3-month-old cladodes exhibited the highest regenerative capacity (76.66%), whereas older areoles responded poorly and cladode sections lacking areoles failed to regenerate. A stepwise sterilization protocol- 0.1% Tween-20 for 15 min (86.67% survival), Bavistin (0.1%) + Streptomycin (0.05%) for 20 min (93.33% survival), 0.1% HgCl₂ for 5 min (93.33% survival), 1% NaOCl for 3 min (66.67% survival) and 70% ethanol for 60 s (86.67% survival)- yielded maximum aseptic recovery. Hormonal optimization revealed that MS medium supplemented with 1.0 mg L⁻¹ BAP + 1.0 mg L⁻¹ 2,4-D produced the highest shoot induction (90% areole activation within 30 days). Shoot multiplication peaked on MS + 2.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ NAA, achieving 100% initiation and 10.3 shoots per explant, while combinations of 0.5 mg L⁻¹ NAA + 1.5 mg L⁻¹ KIN and 4.0 mg L⁻¹ KIN + 0.4 mg L⁻¹ IAA also achieved full initiation with 3.3 and 4.3 shoots per explant, respectively. Rooting was maximized on half-strength MS medium containing 0.5 mg L⁻¹ IAA (T₈), resulting in 100% root induction within 28 days. Primary hardening in soil: cocopeat: sand (1:1:1) ensured 100% survival, followed by 100% secondary survival in vermicompost: sand: soil (1:1:2). Issues such as phenolic browning and slow areole activation were resolved through pre-inoculation hydration and reduction of explant size. This fully detailed protocol provides a reliable, reproducible and scalable system for micropropagation, conservation and commercial deployment of *O. ficus-indica* in arid and semi-arid regions.

Keywords: *Opuntia ficus-indica*, micropropagation, areole, BAP, 2,4-D, IAA, sterilization, acclimatization, tissue culture

Introduction

Opuntia ficus-indica (L.) Mill., commonly known as prickly pear or nopal is a drought-tolerant cactus of the Cactaceae family native to Central America and now widespread in arid and semi-arid regions. Morphological traits such as fleshy cladodes, spines, cuticled epidermis and CAM photosynthesis enable survival under harsh climates (Slaba, 1992; Nobel, 1991) [21, 17]. Among more than 2000 cactus species it is the most economically important cultivated for fruits, pads, fodder, soil conservation and ecological restoration (Nobel, 2002; Casas and Barbera, 2002) [18, 3]. In India it is grown in Rajasthan, Gujarat, Madhya Pradesh and Punjab contributing to fodder supply and land reclamation (Khalafalla *et al.*, 2007) [9].

Even with high theoretical transmissibility, propagation under practical conditions remains limited. Seed germination is poor and genetically variable (Stambouli-Essassi *et al.*, 2017) [22], while cladode multiplication is slow and unsuitable for large-scale use (Mohamed-Yasseen *et al.*, 1995) [15]. Tissue culture offers a reliable alternative for producing uniform, disease-free plants (Lema-Rumińska and Kulus, 2014) [10], though regeneration efficiency depends on genotype, explant type and growth regulator balance (Escobar *et al.*, 1986; García-Saucedo *et al.*, 2005; Estrada-Luna *et al.*, 2008) [5, 8, 6]. Areoles are considered highly responsive, with cytokinin-auxin interactions playing a key role in morphogenesis (Skoog and Miller, 1957; Mabrouk *et al.*, 2021) [20, 12]. This study aimed to develop an efficient *de novo* regeneration protocol for spineless *O. ficus-indica* under Indian agro-climatic

conditions, focusing on explant source, sterilization, growth regulator regimes, rooting and acclimatization.

Materials and Methods

Plant material

The study (2023-2025) was conducted at the State Level Biotechnology Centre, MPKV, Rahuri, India. Spineless *Opuntia ficus-indica* accession 1280 (origin: Argentina, yellow-fruited) from the AICRP on Forage Crops collection was used. Young cladodes (10-15 cm) were excised and areoles (~1 cm²) served as explants.

Sterilization

Explants were disinfected using combinations of Tween-20 (0.1-0.2%), Bavistin + Streptocycline (0.1-0.2%), NaOCl (1%), ethanol (70%) and HgCl₂ (0.1-0.5%) for varying durations. The most effective treatment based on contamination percentage was used for subsequent cultures.

Culture medium and conditions

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962)^[16] with 3% sucrose, 0.8% agar and pH 5.8 was employed. Cultures were incubated at 25 ± 2 °C under a 16 h light/8 h dark photoperiod (1600 lux).

Shoot initiation

Areoles were cultured on MS medium with BAP (0.5-2.5 mg L⁻¹) either alone or combined with 2, 4-D (1.0-2.0 mg L⁻¹) and GA₃ (0.5 mg L⁻¹). Fifteen treatments were tested and days to shoot initiation and response percentage were recorded after 4 weeks.

Shoot multiplication

Regenerated shoots were transferred to MS medium containing BAP (1.0-3.0 mg L⁻¹) or kinetin (0.5-4.0 mg L⁻¹) combined with NAA (0.1-1.5 mg L⁻¹) or IAA (0.3-0.5 mg L⁻¹). Twenty-two treatments were evaluated for shoot number per explant and multiplication percentage after 8-9 weeks.

Rooting

Shoots (2-3 cm) were placed on half-strength MS medium with IBA (0.1-1.5 mg L⁻¹) or IAA (0.5-1.0 mg L⁻¹), with or without GA₃ (0.2-0.6 mg L⁻¹). Nine treatments were compared for rooting percentage, root length and days to root initiation after 3-4 weeks.

Acclimatization

Rooted plantlets were washed, treated with Bavistin (0.1%) and transferred to sterile soil: cocopeat (1:1), sterile soil: sand (1:1), or sterile soil: cocopeat: sand (1:1:1). After 5-6 weeks, plants were shifted to soil: vermicompost: sand (2:1:1) or sand: vermicompost: cocopeat (2:1:1) for secondary hardening in the greenhouse. Survival was recorded.

Results and Discussion

The present investigation, “*De novo* regeneration studies of *Opuntia ficus-indica*”, was conducted during 2023-2025 at the State Level Biotechnology Centre, MPKV Rahuri, to establish an efficient *in vitro* regeneration and propagation protocol. The results of explant response, sterilization, shoot initiation, multiplication, rooting and acclimatization are presented below.

Explant selection and initiation

For this experiment three explant types were tested *viz.* areoles from young (2-3 months) cladodes, areoles from mature (6 months) cladodes and cladode segments without areoles. Areoles from 2-3-month-old cladodes showed the highest regeneration potential with 76.7% induction (230/300 explants) whereas older cladode areoles responded at only 40% (Table 1). Cladode segments devoid of areoles failed to regenerate, confirming the areole as the key morphogenic site. These results align with previous findings that younger cactus tissues retain higher metabolic activity and meristematic competence (Finti *et al.*, 2013 and Rodríguez & Ramírez-Pantoja, 2020)^[7, 19].

Table 1: Explant response during initiation of *O. ficus-indica*

Explant type	No. cultured	No. responding	% Response
Areoles, 2-3 month cladodes	300	230	76.7
Areoles, 6 month cladodes	200	80	40.0
Cladode segments (no areole)	75	0	0

Sterilization protocol standardization

Surface sterilization was optimized using different sterilants. A combination protocol proved most effective: 0.1% Tween-20 for 15 min+ [Bavistin (0.1%) + Streptocycline (0.05%)] for 20 min+ 0.1% HgCl₂ for 5 min + 1% NaOCl for 3 min + 70% ethanol for 1 min, yielding > 90% survival and minimal contamination. Individually, HgCl₂ (0.1%, 5 min) gave 93.3% survival, while 1% NaOCl for 3 min gave 66.67% survival and ethanol (70%, 60 sec) supported 86.7%. Higher NaOCl or HgCl₂ concentrations reduced survival, consistent with reports of phytotoxicity at supra-optimal levels (Marhri *et al.*, 2023; Bouzroud *et al.*, 2022)^[13, 2]. Thus, a sequential sterilization regime ensured maximum recovery of viable, contamination-free explants.

Shoot initiation and areole activation

To induce areole activation, MS medium supplemented with BAP, 2,4-D and GA₃ was tested. The most effective response (90% induction within 30 days) occurred on MS + 1.0 mg/L BAP + 1.0 mg/L 2,4-D (Table 2). BAP alone supported only limited response (≤ 50%), while higher auxin concentrations (≥ 2.0 mg/L 2,4-D) suppressed shoot initiation, favoring callus formation instead. Addition of GA₃ enhanced elongation but did not substitute for auxin-cytokinin synergy. This confirms earlier studies showing that a balanced cytokinin-auxin ratio is crucial for cactus organogenesis (Linhares *et al.*, 2005; Mengesha *et al.*, 2016)^[11, 14].

Table 2: Effect of BAP, 2,4-D, GA₃ for shoot proliferation of *Opuntia (O. ficus indica)* explants on MS medium

Hormonal Treatment (mg/L)	% Response	Days Required	Remark
1.0 BAP	50% (10/20)	40	Moderate activation
1.5 BAP	45% (9/20)	42	Low to moderate
2.0 BAP	0%	-	No response
2.5 BAP	0%	-	No response
2.5 BAP + 0.5 GA ₃	45% (9/20)	40	GA ₃ improved induction
1.0 BAP + 1.0 2,4-D	90% (18/20)	30	Best induction treatment
1.5 BAP + 1.0 2,4-D	65% (13/20)	40	Good response
2.0 BAP + 1.0 2,4-D	45% (9/20)	45	Moderate
1.0 BAP + 1.5 2,4-D	50% (10/20)	42	Moderate
1.5 BAP + 1.5 2,4-D	40% (8/20)	40	Low-moderate
2.0 BAP + 1.5 2,4-D	30% (6/20)	42	Low response
1.0 BAP + 2.0 2,4-D	0%	-	No response
1.5 BAP + 2.0 2,4-D	0%	-	No response

Shoot multiplication and proliferation

After initiation, explants were subcultured on MS medium with varied cytokinin-auxin combinations (Table 3).

- **BAP + NAA was most effective:** 2.0 mg/L BAP + 0.5 mg/L NAA achieved 100% response with the highest multiplication rate (10.3 shoots/explant).

- KIN + auxin combinations produced moderate multiplication. The best response was 4.0 mg/L KIN + 0.4 mg/L IAA (100% response; 4.3 shoots/explant) and 0.5 mg/L NAA + 1.5 mg/L KIN (100%; 3.3 shoots/explant).

Table 3: Effect of hormonal combinations on shoot multiplication and proliferation of *O. ficus-indica*

Hormonal treatment (mg/L)	% Response	Avg. shoots/explant	Remark
2.0 BAP + 0.5 NAA	100%	10.3	Best multiplication
3.0 BAP + 0.1 NAA	100%	3.0	High initiation, low shoots
1.0 BAP + 0.1 NAA	66.7%	6.3	Moderate
0.5 NAA + 1.5 KIN	100%	3.3	Best KIN-NAA combination
4.0 KIN + 0.4 IAA	100%	4.3	Best KIN-IAA combination
3.0 KIN + 0.3 IAA	100%	2.0	Good initiation, fewer shoots
Other combinations	0-66.7%	1-2	Poor to moderate response

These findings highlight the complementary action of cytokinins and auxins in regulating cell division and differentiation. Similar results were reported by Bahulikar *et al.* (2022)^[1] and Rodríguez & Ramírez-Pantoja (2020)^[19].

Root induction

Rooting was achieved on half-strength MS medium with auxins. 0.5 mg/L IAA gave the best response, producing

100% rooting within 28 days with average root length of 0.95 cm. In contrast, IBA (0.5-1.0 mg/L) induced roots in only 57% of explants, though roots were longer (up to 2.1 cm). Higher auxin levels (> 1.0 mg/L) and GA₃-supplemented treatments inhibited rooting (Table 4). These results agree with Bouzroud *et al.* (2022)^[2] and El Finti *et al.* (2010)^[4], who found that IAA is superior for initiation, whereas IBA mainly enhances elongation.

Table 4: Root induction in regenerated shoots of *O. ficus-indica*

Treatment (½ MS + hormone)	Rooting%	Avg. root length (cm)	Days to rooting	Remark
0.5 mg/L IAA	100	0.95	28	Best treatment
0.5 mg/L IBA	57.1	1.3	39	Moderate response
1.0 mg/L IBA	57.1	2.1	32	Longer roots
Higher auxins / GA ₃	0	-	-	Inhibitory

Hardening and acclimatization

A two-stage hardening protocol was employed. In primary hardening, sterile soil: cocopeat: sand (1:1:1) supported 100% survival, compared to 60% in sterile soil: cocopeat and 40% in sterile soil:sand mixtures. For secondary hardening, vermicompost: sand: soil (1:1:2) resulted in 100% survival with vigorous growth, whereas vermicompost: sand: cocopeat (1:2:1) showed 60% survival. These findings emphasize the importance of nutrient-rich, well-aerated substrates for acclimatization, confirming earlier reports by Khalafalla *et al.* (2007)^[9].

Troubleshooting during micropropagation

Phenolic leaching and medium browning were minimized by soaking explants in sterile water for 1 h prior to

inoculation. Reducing cladode segment size to ~1 cm accelerated areole activation from 90 to 30 days. Contaminated cultures were salvaged using 70% ethanol and NaOH washes, enabling recovery of regenerating explants.

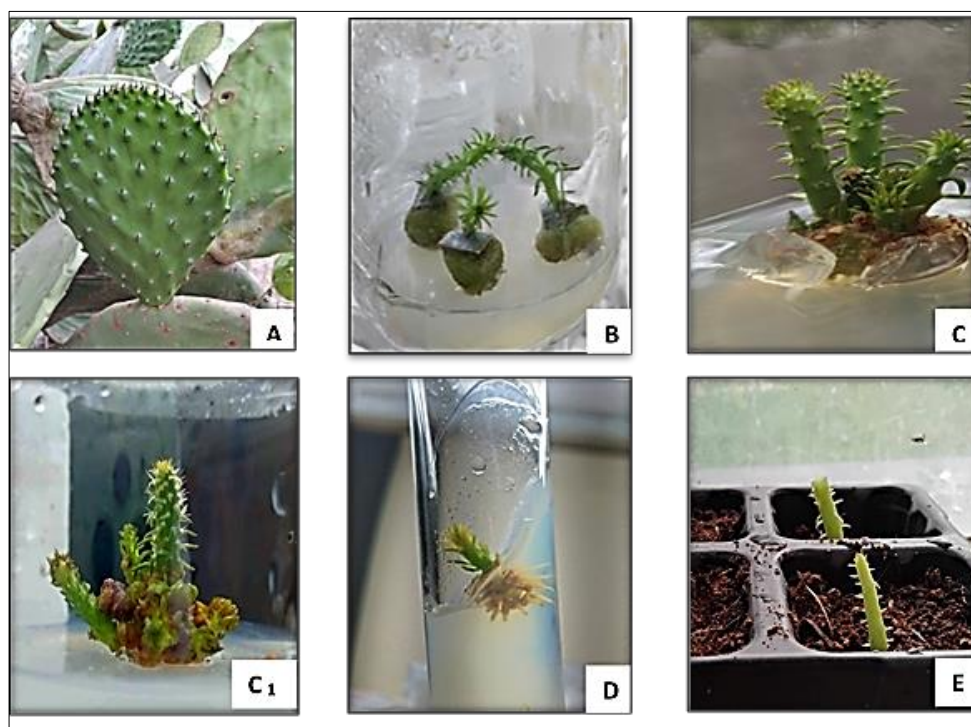
Discussion overview

This study demonstrated that regeneration of *O. ficus-indica* depends critically on explant age, sterilization efficiency, and precise hormonal balance. Young cladode areoles combined with BAP + 2,4-D for initiation, followed by BAP + NAA for multiplication and IAA for rooting, yielded a highly efficient and reproducible regeneration protocol.

The optimized pipeline from initiation (76% response), to multiplication (up to 10.3 shoots/explant), rooting (100%

success) and acclimatization (100% survival) - establishes a reliable platform for mass propagation and conservation. These results strengthen the potential of *O. ficus-indica* as a

multipurpose crop for arid and semi-arid regions, supporting food, fodder, and ecological restoration initiatives.



A) Areoles from 2-3 month-old cladodes **B)** Initiation (1.0 BAP + 1.0 2,4-D mg/l) **C)** Multiplication (BAP 1.0 + NAA 0.1 mg/l) **C₁)** Multiplication (BAP 2.0 + NAA 0.5 mg/l) **D)** Rooting ($\frac{1}{2}$ MS media + IAA 0.5 mg/l) **E)** Hardening [Primary hardening - soil: cocopeat: sand (1:1:1)]

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