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A highly efficient *in vitro* propagation protocol for elephant tusk cactus: *Coryphantha elephantidens* (Lem.) Lem.

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**KEYWORDS**

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Carbon source

**Abstract**  
Background: Elephant tusk cactus *Coryphantha elephantidens* (Lem.) Lem. is an important attractive ornamental cactus. The plant produces offshoots from tubercles very rarely, and the seedlings exhibit slow growth and susceptibility to damping off. Slow growth and high demand in the cactus industry lead to finding an alternate fast propagation method.

Results: An innovative *in vitro* technique based on axillary bud proliferation has been developed for an ornamental cactus *C. elephantidens* (Lem.) Lem. Four different explant types formed multiple shoots on Murashige and Skoog (MS) medium. Of the two cytokinins, 6-Benzylaminopurine (BAP) and Kinetin (KN), BAP proved to be more effective for multiple shoot induction and shoot growth from different explant types. Longitudinally cut stem explants, when cultured on MS medium supplemented with 6.6 \( \mu \text{M} \) BAP give maximum axillary shoot proliferation (12.4 shoots). Type of explant significantly influenced the micropropagation rate. Type of carbon source used in the medium imparted a profound effect on shoot growth and dry weight. The maximum dry weight gain of the shoot was observed with 9\% sucrose.

Conclusion: Development of an efficient micropropagation protocol which can be used to produce more than 10,000 rooted plantlets in 150 days from a single longitudinally divided shoot explant.

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1. Introduction

*Coryphantha elephantidens* (Lem.) Lem. also known as elephant tusk cactus, is an important ornamental cactus. The plant produces offshoots from tubercles very rarely, and the seedlings exhibit slow growth and susceptibility to damping off [16]. In contrast, the development of *C. elephantidens* shoots *in vitro* can be extremely rapid in comparison with traditional propagation methods. There is however, no report
on in vitro clonal propagation of *C. elephantidens* via axillary shoot proliferation method. With approximately 1500 species in c. 100 genera, Cactaceae represents one of the most conspicuous and diverse angiosperm families originated in warm, arid America and played an important role in their life. Cacti belonging to different species have been extensively used in the production of food and fodder, medicines, cosmetics and hedges [1–5]. Its diversity is most obvious in vegetative habitat, which encompasses leafy shrubs and trees, columnar, barrels, prickly pears, Christmas cacti, and elaboration of photosynthetic stems into forms resembling palm fronds, making them an important group of plants in worldwide horticultural trade. It comprises of many important ornamental species. The conventional methods of propagation are often inadequate to meet the commercial demands of cacti in the world market [6–8]. Commercial production of cacti by different conventional methods is laborious and a costly affair. Moreover, plants raised from seeds are very slow growing and prone to many soil born pathogens [9]. These reduce the production and increase the cost of the plants. The development of tissue culture protocols for different cacti is imperative for successful application of this technology in commercial production.

First attempt to mass propagate cacti in vitro was made about 50 years ago [10]. Since then, there have been numerous reports on in vitro culture of various cacti [6,11]. However, the techniques for in vitro culture of cacti are still not well developed, and in vitro morphogenetic behavior is not well understood [12–14]. Explant type and position on the plant impart a strong influence on the in vitro morphogenetic response [8,15]. Although additional species will require the identification of individual growth regulator treatments, continued optimization of micropropagation techniques promises to clarify the conditions required for the commercial production of cacti to fulfill the international market demand. Moreover, micropropagated plants retain the genetic constitution of the mother plant with clonal fidelity and are disease free [16].

This paper provides evidence based on the consideration that, although multiple shooting can be induced in *C. elephantidens* using different types of explants, some are more competent over other types. The effects of different carbon sources were also studied to optimize the required carbon source for micropropagation. This report describes for the first time a reproducible method for the commercial propagation of *C. elephantidens* through tissue culture. The present work also provides a basis for a rational approach to conservation of members of the Cactaceae family, which are threatened due to over collection from the natural habitat.

2. Materials and methods

Young shoots about 2 cm long of *C. elephantidens* were obtained from plants in the Cactus garden of the University of Jammu, Jammu, India. They were washed thoroughly with tap water and 1% detergent. Shoots were surface sterilized by immersion in 70% ethanol for 1 min, followed by immersion in NaOCl (5% available chlorine) for 15 min. They were again washed with sterilised-distilled water 5–6 times. Disinfected shoots were rinsed 4–5 times with sterile double distilled water and dried on a sterile filter paper. Sterilised shoots were cut longitudinally, transverse strips (5 mm), without shoot tip, shoot tip (5 mm) and whole shoot as 4 different types of explant (Fig. 1).

Different types of explants were cultured on MS (Murashige and Skoog) medium [17] supplemented with 3% sucrose (Qualigens Fine Chemicals, Mumbai, India). The media were solidified by 0.8% Difco Bacto agar (Hi Media, India). The pH of the medium was adjusted to 5.7 before autoclaving at 121 °C, 104 KPa for 15 min. Different concentrations (0.1–2.0 mg l⁻¹) of cytokinin (BAP and KN) alone or in combination with different concentrations (0.1–1.0 mg l⁻¹) of auxins (IAA – Indole-3-acetic acid, IBA – Indole-3-butyric acid, NAA α-Napthalenacetic acid) were tested for shoot proliferation. To study the effect of carbon source (sucrose, fructose, and mannitol) on shoot development and dry weight (DW) gain, the shoots growing on 3% sucrose supplemented media (MS + 1.5 mg l⁻¹ BAP) after 10 days were again transferred to media with different types of carbon sources (0–12%). Average values of 10 samples (5 shoot each) were evaluated per treatment. Shoots were oven-dried at 60 °C for 48 h before recording their DW. Cultures were maintained in a culture room at 25 ± 2 °C with 16/8 h light/dark regime (45 mmol · m⁻² · s⁻¹ light intensity).

All treatments for each explant type and growth regulator type consisted of 20 explants. Each experiment was repeated at least 3 times. The results were recorded at a regular interval of 4 weeks of culture and analyzed by analysis of variance using randomised block design method [18]. Data taken in percentage were subjected to arcsine transformation for proportions before analysis and converted back to percentages for presentation in tables [19]. Means were compared using Duncan’s new multiple range test [20].

3. Results and discussion

Multiple shoot induction was readily achieved for different types of studied explants after culturing on MS medium supplemented with BAP or KN. Shoots start emerging from the explants within 7 days of culture. Explants cultured on a medium lacking cytokinin formed few shoots. BAP was superior to KN for multiple shoot induction and proliferation. Medium
containing 1.5 mg l\(^{-1}\) BAP induced maximum number of shoots (12.4) for longitudinal cut shoots. In *Mammillaria san-angelensis* [15] and *Opuntia amyclaea* [21] optimal medium for *in vitro* initiation and multiplication of shoots was similar as observed in this study. Higher cytokinin levels (BAP > 1.5 mg l\(^{-1}\) and KN < 1.5 mg l\(^{-1}\)) in the medium resulted in the decline in the shoot number for all different explants. Shoots cultured on MS medium supplemented with 1.5 mg l\(^{-1}\) BAP showed more vigorous growth than the shoots growing on high concentrations of BAP except shoots produced from shoot tip explants because, it is not significant. It is possible that a higher concentration of exogenous BAP, in addition to the endogenous cytokinin, reached a super-optimal cytokinin level in the tissue as reported in *O. amyclaea* [21] and *Opuntia fuscus-indica* [22], which resulted in the decline of shoot proliferation rate. Addition of auxins to BAP (1.5 mg l\(^{-1}\)) supplemented medium promoted the formation of profuse callus and inhibited the shoot proliferation response (Data not shown). This is in agreement with the earlier report for *Epiphyllum chrysocardium* [23] in which addition of 1.0 mg l\(^{-1}\) IAA to the BAP supplemented medium inhibited the rate of multiple shoot formation.

The effect of explant type was significant on shoot proliferation, although BAP at 1.5 mg l\(^{-1}\) was the best cytokinin concentration in all the three-explant types tested (Table 1). Longitudinally cut shoot explants showed the maximum number of shoots (12.4) formed per explant (Fig. 2.2). The shoot tip explants formed a maximum of 5.2 shoots (Fig. 2.4), whereas the transversely cut shoot explants produced a maximum of 6.9 shoots per explant (Fig. 2.3). Whole shoots when cultured on the medium containing 1.5 mg l\(^{-1}\) BAP formed 1.0 shoots (Fig. 2.5). In all explant types, high concentration of BAP leads to a decline in the shoot proliferation rate and shoot length (Data not presented). It was also observed that lateral buds developed into shoots after the removal of the apical meristem in the presence of BAP. All the explant types produced multiple shoots, with varying degrees of efficiency. Martinez-Vázquez and Rublou [15] obtained similar results in different explant types of *M. san-angelensis*.

This study also reveals that growth of *in vitro* cultured explants is strongly influenced by the exogenous carbon source in the medium. At high sucrose concentrations (higher than 9%), a decline in DW of the shoots was observed. With increasing concentration of sucrose, the DW of the shoots increased, reaching a maximum (57.4 mg) at 9% level (Table 2). Shoots cultured on a medium supplemented with fructose showed an increase in DW, with the maximum (42.9 mg) at 12% level. Medium supplemented with mannitol as a carbon source leads to a decrease in the DW of shoots, with a maximum (17.4 mg) at the 3% level. With the increase in sucrose concentration up to 9%, the shoots showed vigorous growth. At a high sucrose level (12%), growth of shoots was inhibited. There is a positive correlation between the carbon source in the medium and total DW gain. This relationship is however valid for the response at low sugar levels. At high levels a decrease in DW is observed in Maize, vitis and potato plants *in vitro* [24–26]. The decrease in DW of shoots at high sucrose levels may be due to a decrease in water potential of the medium. The DW of shoots decreased in response to different mannitol levels tested in the medium. Mannitol is a sugar alcohol that is produced by some plants as a primary photosynthetic product and some plants can metabolize it. In *C. elephan-tidens*, we observed a decline in DW of the shoots on mannitol-supplemented medium, which is in agreement with the earlier report [27]. They found that mannitol was not taken up by the cells of poplar even after 12 days of culture.

### Table 1: Effect of explant source and cytokinin type on the number of axillary shoots produced per explant of *Coryphantha elephan-tidens* after 4 weeks of cultivation on MS supplemented with 3% sucrose.

| Cytokinin Concentration (mg l\(^{-1}\)) | Transversely cut shoot No. of shoots formed/explant (mean) | Longitudinal cut shoot No. of shoots formed/explant (mean) | Shoot tip No. of shoots formed/explant (mean) | Shoot No. of shoots formed/explant (mean) |
|----------------------------------------|----------------------------------------------------------|----------------------------------------------------------|-----------------------------------------------|------------------------------------------|
| None                                   | 2.0\(^a\)                                                | 2.0\(^a\)                                                | 1.0\(^a\)                                    | 1.0\(^a\)                                 |
| BAP                                    | 2.2\(^b\)                                                | 2.4\(^c\)                                                | 2.0\(^a\)                                    | 1.0\(^a\)                                 |
| 0.5                                    | 3.6\(^b\)                                                | 5.6\(^c\)                                                | 2.2\(^a\)                                    | 1.2\(^a\)                                 |
| 1.0                                    | 4.3\(^c\)                                                | 7.5\(^d\)                                                | 3.5\(^b\)                                    | 1.4\(^a\)                                 |
| 1.5                                    | 6.9\(^d\)                                                | 12.4\(^e\)                                               | 5.2\(^c\)                                    | 1.0\(^a\)                                 |
| 2.0                                    | 2.5\(^a\)                                                | 4.6\(^b\)                                                | 5.2\(^c\)                                    | 1.0\(^a\)                                 |
| KN                                      | 2.0\(^a\)                                                | 2.5\(^a\)                                                | 2.0\(^a\)                                    | 1.0\(^a\)                                 |
| 0.5                                    | 2.2\(^a\)                                                | 4.8\(^b\)                                                | 2.4\(^a\)                                    | 1.3\(^a\)                                 |
| 1.0                                    | 2.7\(^b\)                                                | 6.6\(^b\)                                                | 3.6\(^b\)                                    | 1.5\(^a\)                                 |
| 1.5                                    | 4.1\(^c\)                                                | 7.2\(^d\)                                                | 3.8\(^b\)                                    | 1.5\(^b\)                                 |
| 2.0                                    | 3.5\(^b\)                                                | 4.2\(^b\)                                                | 3.2\(^b\)                                    | 1.2\(^b\)                                 |

Means followed by the same letter in a column are not significantly different from each other at 5% level by Duncan’s [20] new multiple range test.
concurrence with the earlier report in *C. macromesis* [28] and *Mediocactus coccineus* [29]. Rooted plants were transferred in pots containing sand and vermiculite (1:1). They were kept in the greenhouse with high humidity for 14 days. Ninety-five percent of (238 plants survived from the total of 250) plants were hardened (Fig. 2.6). After 1 month of hardening, these plants were transferred to pots containing a mixture of garden soil, sand and brick dust.

Whatever the process, micropropagation is characterized by the efficiency of the multiplication rate. The protocol that is reported here fulfills this attribute. Conventional propagation of *C. elephantidens* leads to the production of 10–15 tubercles per plant each year [16]. In contrast, the micropropagation protocol reported can lead to a production of a large number of plants within a short period of time. This is possible because one longitudinal shoot explant can produce 12 shoots and each shoot provides two explants i.e. 24 shoots after 30 days. Each of these explants will develop an average of 12 shoots in

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**Table 2** Effect of different carbon sources on an increase in dry weight of shoots of *Coryphantha elephantidens* after 4 weeks of culture on MS media supplemented with 6.6 µM BAP.

| Concentration (mgl⁻¹) | Carbon source | Sucrose Increase in DW (mg) | Mannitol Increase in DW (mg) | Fructose Increase in DW (mg) |
|-----------------------|---------------|-----------------------------|-----------------------------|-----------------------------|
| 0                     | 6.2a          | 6.6b                        | 5.5e                        |
| 3                     | 24.5b         | 17.4d                       | 20.3b                       |
| 6                     | 45.3c         | 9.2e                        | 28.4b                       |
| 9                     | 57.4d         | 5.2b                        | 39.1e                       |
| 12                    | 46.3c         | 3.5a                        | 42.9e                       |

* Means followed by the same letter in a column are not significantly different from each other at 5% level by Duncans [20] new multiple range test.
30 days of culture. By repeating this process we can produce more than 10,000 plants after 150 days of culture. A complete micropropagation protocol for *C. elephantidens* was achieved by selecting the best explant (longitudinally cut shoot) type from shoots of greenhouse grown plants. It is thus our contention that better knowledge of requirement for specific explant types and carbon sources can notably enhance *in vitro* propagation.

**Author contributions**

B.S.B. proposed the theoretical frame, conceived, designed the experiments and wrote the paper: A.K.W. Contributed reagents/materials/analysis tools and helped in finalising the MS.

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