Journal of Genetic Engineering and Biotechnology (2015) 13, 215–219

Academy of Scientific Research & Technology and
National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ARTICLE

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

B.S. Bhau *, **A.K. Wakhlu**

Plant Tissue Culture Laboratory, Department of Botany, University of Jammu, Jammu I80 006, Jammu & Kashmir, India

Received 24 January 2015; revised 30 April 2015; accepted 4 July 2015
Available online 8 August 2015

KEYWORDS

Coryphantha elephantidens;
Elephant tusk cactus;
In vitro micropropagation;
Explant type;
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 μM 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.

© 2015 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.

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

* Corresponding author at: Plant Genomics Laboratory, MAEP Division, CSIR-North-East Institute of Science & Technology (NEIST) – Jorhat (Formerly RRL), Jorhat 785 006, Assam, India. Tel.: +91 376 2370117/2370121; fax: +91 376 2370011.
E-mail address: bsbhau@gmail.com (B.S. Bhau).
Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2015.07.003
1687-157X © 2015 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.

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 $NaOC1_2$ (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 mgl^{-1}) of cytokinin (BAP and KN) alone or in combination with different concentrations (0.1–1.0 mgl^{-1}) of auxins (IAA – Indole-3-acetic acid, IBA – Indole-3-butyric acid, NAA α-Napthaleneacetic 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 mgl^{-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 $mmol^{-2}$ s^{-1} m 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

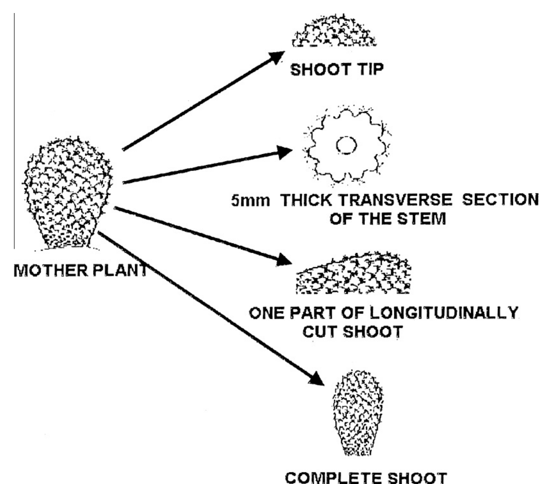

Figure 1 Diagrammatic representation of the different types of explant preparations from the young shoot.

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

Cytokinin	Concentration (mgl^{-1})	Explant type			
		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	0.0	2.0^{a}	2.0^{a}	1.0^{a}	1.0^{a}
BAP	0.1	2.2^{a}	2.4^{a}	2.0^{a}	1.0^{a}
	0.5	3.6^{b}	5.6^{bc}	2.2^{a}	1.2^{a}
	1.0	4.3^{bc}	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	0.1	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^{a}	6.6^{d}	3.6^{b}	1.5^{a}
	1.5	4.1^{bc}	7.2^{d}	3.8^{b}	1.5^{a}
	2.0	3.5^{b}	4.2^{b}	3.2^{b}	1.2^{a}

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.

containing 1.5 mgl^{-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 mgl^{-1} and KN < 1.5 mgl^{-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 mgl^{-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 superoptimal cytokinin level in the tissue as reported in *O. amyclaea* [21] and *Opuntia ficus-indica* [22], which resulted in the decline of shoot proliferation rate. Addition of auxins to BAP (1.5 mgl^{-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 mgl^{-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 mgl^{-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 mgl^{-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. Martínez-Vázquez and Rubluo [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. elephantidens*, 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.

In vitro formed shoots from all the explants of *C. elephantidens* after 4 weeks were transferred to a fresh MS basal medium for rooting. Within 10 days, all cultured shoots showed the initiation of roots (Fig. 2.6). The percentage of rooting on MS basal medium was 100%. Roots were thick and robust, and plantlets were removed after 5 weeks of their transfer to the media. Rooting of the shoots on MS basal medium is in

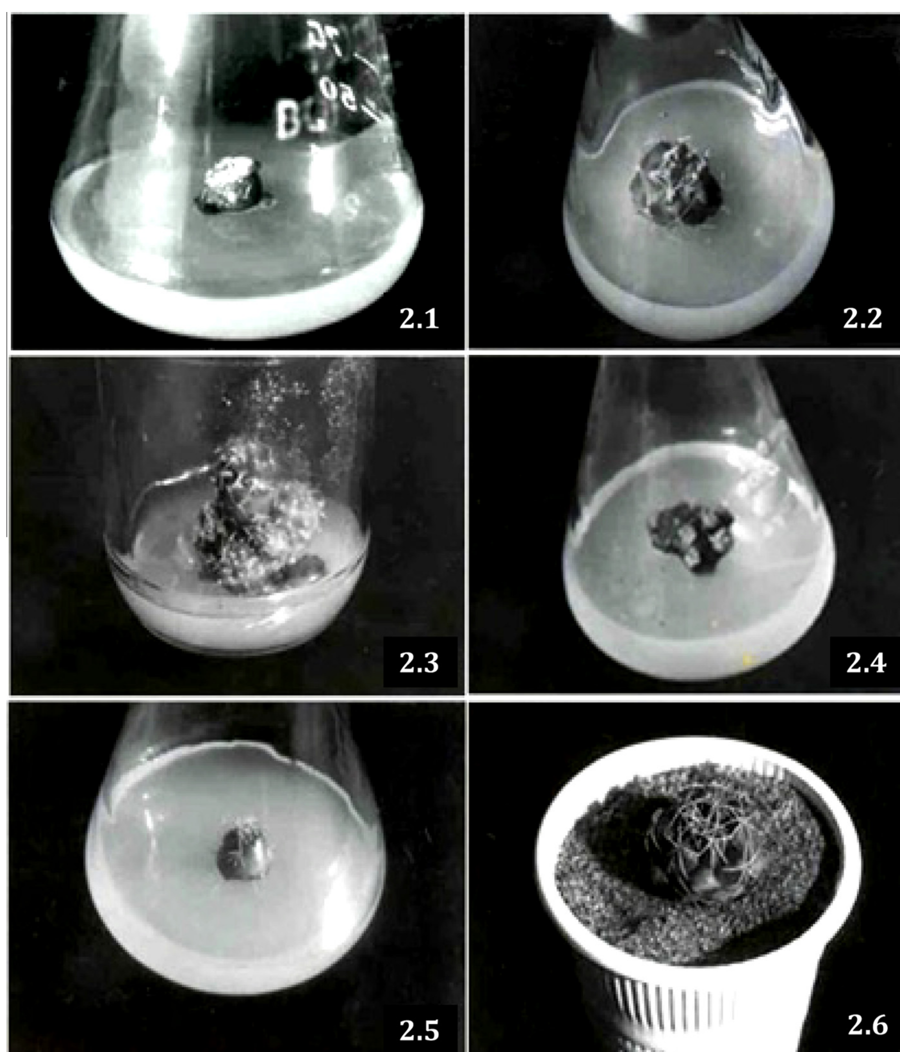

Figure 2 Multiple shoot formation and rooting from *Coryphantha elephantidens* explants. (2.1) Multiple shoot formation from a longitudinally cut shoot explant on MS medium containing 1. 5 mgl^{-1} BAP. (2.2) Multiple shoot formation from a transversely cut shoot explant on MS medium containing 1.5 mgl^{-1} BAP. (2.3) Multiple shoot formation from the shoot explant on MS medium containing 1.5 mgl^{-1} BAP. (2.4) Multiple shoot formation from the whole shoot tip explant on MS medium containing 1. 5 mgl^{-1} BAP. (2.5) Rooted shoot on MS basal medium after 2 weeks. (2.6) Acclimatized plant in the pot containing garden soil, sand and brick powder.

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	6.2^{a}	6.6^{b}	5.5^{a}
3	24.5^{b}	17.4^{d}	20.3^{b}
6	45.3^{c}	9.2^{c}	28.4^{b}
9	57.4^{d}	5.2^{b}	39.1^{c}
12	46.3^{c}	3.5^{a}	42.9^{c}

* 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.

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

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.

References

[1] D.A. Hegwood, HortScience 25 (1990) 1515–1516.
[2] M.A. Hershkovitz, E.A. Zimmer, Taxon 46 (1997) 217–232.
[3] C. Metz, A. Nerd, Y. Mizrahi, Hort. Sci. 35 (2000) 22–24.
[4] Nobel P. Preface, in: P.S. Nobel (Ed.), Cacti: Biology and Uses, University of California Press, Berkeley and Los Angeles, California, 2002, p. 9.
[5] J. Lema-Rumińska, D. Kulus, Haseltonia 19 (2014) 46–63.
[6] J.F. Hubstenberger, P.W. Clayton, G.C. Phillips, in: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, Vol. 20 High Tech and Micropropagation IV, Springier Verlag, Berlin Heidelberg, 1992, pp. 49–68.
[7] B.S. Bhau, J. Ind. Soc. Cacti Succul. 1 (1999) 12–15.
[8] B.S. Bhau, Sci. Hort. 81 (1999) 337–344.
[9] M. Jenkins, Traffic Eur. (1993) 3–17.
[10] M.R. King, J. Cact Succul. 29 (1957) 102–104.
[11] J. Lema-Ruminska, D. Kulus, Haseltonia 19 (2014) 46–63.
[12] M.F. Fay, J. Gratton, Bradleya 10 (1992) 33–48.
[13] G. Palomino, J. Dolezel, R. Cid, I. Brunner, I. Mendez, A. Rubluo, Plant Science 141 (1999) 191–200.
[14] R.M. Llamoca-Zárate, C. Studart-Guimarães, J. Landsmann, F.A.P. Campos, Plant Cell Tissue Organ Cult. 58 (1999) 155–157.
[15] O. Martinez Vazquez, A. Rubluo, J. Hort. Sci. 64 (1989) 99–105.
[16] R. Slaba, in: The Illustrated Guide to Cacti, Chancellor Press, London, 1992, pp. 40–41.
[17] T. Murashige, F. Skoog, Physiologia Plantarum 15 (1962) 473–497.
[18] W.G. Cochran, G.M. Cox, Experimental Designs, John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore, 1957.
[19] G.W. Snedecor, W.G. Cochran, Statistical Methods, Oxford and IBH Publishing Co., Pvt. Ltd, New Delhi, Bombay, Calcutta, 1968.
[20] D.B. Duncan, Biometrics 11 (1955) 1–42.
[21] H.A. Escobar, M. Vitor, A. Villalobos, A. Villegas, Plant Cell Tissue Organ Cult. 7 (1986) 269–277.
[22] Y. Mohamed-Yasseen, S.A. Barringer, W.E. Splittstoesser, R.J. Schnell, Plant Cell Tissue Organ Cult. 42 (1995) 117–119.
[23] J.E. Lazarte, M.S. Gaiser, O.R. Brown, Hort. Sci. 17 (1982) 84.
[24] J.M. Ribaut, P.E. Pilet, Physiologia Plantarum 81 (1991) 156–162.
[25] R. Galzy, D. Compan, Plant Cell Tissue Organ Cult. 31 (1992) 239–244.
[26] H. Lipavska, D. Vreugdenhil, Plant Cell Tissue Organ Cult. 45 (1996) 103–107.
[27] A. Tholakalabavi, J.J. Zwiazek, T.A. Thorpe, In Vitro Cell Dev. Biol. Plant 30P (1994) 164–170.
[28] R.H. Smith, P.J. Burdick, J. Anthony, A.A. Reilley, Hort. Sci. 26 (1991) 315.
[29] R. Infante, Plant Cell Tissue Organ Cult. 31 (1992) 155–159.