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Micropropagation and *in-vitro* conservation of elite genotype of cactus pear (*Opuntia ficus indica* Mill.)

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Abstract

Micropropagation techniques of cactus pear for a thorn-less, vegetable type elite genotype, which was collected under germplasm collection programmes, was developed successfully through single bud explant. Physiologically mature buds on cladodes were collected and single bud segment was used for culture initiation. Maximum multiple shoots (8 shoots/ explant) formation was achieved on MS medium supplemented with 30 g sucrose, 8 g agar and 2 mg 6- Benzylaminopurine (BA) + 0.1 mg alpha-Naphthalene acetic acid (NAA) per liter. Multiple shoot clump were further subjected to culture for shoot elongation medium devoid of plant growth regulators. The elongated shoots were rooted cent per cent under *in vitro* conditions. The rooted plantlets were successfully acclimatized under three step hardening procedure. Under *in vitro* conservation study, *in vitro* rooted plantlets were used for further conservation by reducing growth of the plantlets and by minimizing sub-culturing process. The rooted plantlets were transferred on agar solidified MS medium supplemented with activated charcoal 3 g/l and different combinations of BA (0, 0.5, 1.0 mg/l) and NAA (0, 0.5, 1.0 mg/l). The higher concentration of BA (1.0 mg/l) was found to increase shoot proliferation and was detrimental to root growth of the *in vitro* plantlets whereas higher concentration of NAA (1.0 mg/l) was found to influence both shoot and root growth positively. This technique of *in vitro* conservation was found effective in maintaining cultures continue for more than six years. Further, proliferated growth of *in vitro* maintained plantlets were harvested and transferred to new containers for increasing number of stock materials.

Key words: Micropropagation , *in-vitro* conservation , Cactus pear

Introduction

Cactus pear (*Opuntia ficus indica* Mill.) belongs to family Cactaceae has specialized photosynthesis mechanism known as Crassulacean Acid Metabolism (CAM) and high water use efficiency (WUE) even in areas with annual rainfall as low as 120-150 mm. It is well adapted plant of Indian dessert and largely utilized as bio-fencing to protect vegetable farm or garden from wild animals and other herbivores by the farmers. Moreover, this crop is gradually gaining popularity as fruit and vegetable crops in hot arid regions of the world. Both spine and spineless cultivars are available in Mexico, but most of the Indian genotypes are thorny. The developed Nations such as Mexico, Chile, and America have recognized this crop as fruit, vegetable, animal feed and industrial raw material (Nobel, 1995). This crop is also commercially used in cosmetics and dye industry. Visualizing the horticultural, industrial and commercial potential of cactus pear, Food and Agriculture Organization has successfully implemented a cactus pear network with the aim to promote cactus pear as an important fruit crop world wide (Pimienta *et al.*, 1993). In this regard efforts were made in India in introduction, collection and conservation of both indigenous and exotic genotypes (Nath *et al.*, 1999). As a results of intensive efforts made at CIAH, Bikaner, about 35 genotypes having elite characteristics have been conserved in field repository. Some of the promising elite genotypes identified, now require large number of propagules for further research and extension purposes. Conventionally, cactus pear is propagated either through seed or cladodes; however, both methods are either not desirable or inefficient (Malda *et al.*, 1999). Vegetative method of multiplication through cladodes also suffers seriously with fungal infection of foot rot disease which leads to poor survival of plantlets (Mauseth, 1979). Therefore, there is an urgent need for propagation of cactus pear through tissue culture technique to build up large population of plant materials of superior and desired genotypes. Although, micropropagation has been successfully attempted for several members of Cactaceae family (Johnson and Emino 1979; Mauseth, 1979; Escobar *et al.* 1986; Clayton *et al.* 1990 and Khalafalla *et al.* 2007). However, no attempts were made for large scale multiplication

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of thornless cactus pear. Further, conservation of elite type genotypes is a prerequisite for improvement of crops. Some cactus genotypes which are spineless and of vegetable types are severely damaged by various insects pests, herbivores and diseases. Therefore, safe conservation technology viz., *in vitro* conservation is also highly desirable for sustainable and efficient resource management of germplasm. Keeping in view the above reasons, the present investigation was planned with the objective of faster multiplication and *in-vitro* conservation of superior genotypes of vegetable type cactus pear for its utilization in arid agro ecosystem of India.

Materials and methods

(A) Micro propagation study

Preparation of mother plant and collection of explants

Selected genotype of Cactus pear (*Opuntia ficus indica* Mill.) was maintained in green house irrigated and fertilized as per general recommendation with proper plant protection measures. Actively growing buds from cladodes were collected and single bud segment with adjacent tissues were used for culture initiation .

Explant preparation and sterilization

The segments of cladodes containing single bud (areole) of 2 x 2 cm size were collected from mother plants and subjected to washing in running tap water and rinsed in mild concentration of detergents followed by gentle rinsing in tap water. Thereafter, explants were treated with 0.1% $HgCl_2$ for 5-7 minutes. The surface sterilized explant were thoroughly washed several times with sterile water under laminar flow hood and sized to 1 x 1 cm before inoculation.

Culture media and conditions for cladodes induction

Surface sterilized explants were inoculated vertically on 30 ml Murashige and Skoog (1962) MS medium supplemented with 30 g sucrose, 8.0 g agar and various concentration of BA (0, 1, 2 and 4 mg/l) alone or in combination of NAA 0.1 mg/l. The pH of the medium was adjusted to 5.7 and sterilized by autoclaving at 121°C and 15 lbs for 15 minutes. The cultures were incubated at 26 ± 2° C temperature and 16 hrs photoperiod provided by cool fluorescent tube with 2000 lux light intensity.

Culture media for rooting

After third subculture period the multiple shoot clumps were subjected for elongation to hormone free medium for three weeks. Thereafter, microshoots were transferred to rooting medium containing MS medium supplemented with 30 g of sucrose, 8.0 g agar, 3 g activated charcoal and varied levels of NAA and IAA at concentration of (0,0.5,1.0 or 2.0 mg/l). The cultures were incubated at 26 ± 2° C temperature and 16 hrs photoperiod provided by cool fluorescent tube with 2000 lux light intensity.

Hardening of *in-vitro* produced plantlets

Under arid agro-ecosystem where both low and high temperatures are extreme, atmospheric humidity is very low and wind speed is very high, thus hardening and acclimatization of plantlets are very challenging component of micropropagation. The hardening of *in vitro* produced plantlets were taken under three step hardening procedure i.e. i) Transfer of plantlets to acclimatization hood inside the culture room. ii) Transfer of plantlets in environment control green house. iii) For further growth and development in glass house equipped with intermittent fogging device, different combination of potting mixture such as vermiculite, coco peat or vermi compost, were tried for survival and acclimatization of plantlets to pots.

(B) In vitro conservation study

Rooted plantlets developed under micropropagation study were used for further investigation of *in vitro* conservation, maintenance, growth inhibition and subsequent survival components. Micropropagated cladodes were transferred to MS medium with 8 g agar 3 g activated charcoal supplemented with 0.0, 0.5, 1.0, mg BA and 0, 0.5, 1.0 mg NAA/l for maintenance of the culture and growth inhibition. The observations on different parameters such as shoot and root growth, percentage of vitrified cultures and promotive/inhibitory effect on root growth were recorded after six month of culture incubated for *in vitro* conservation. The cultures were incubated at 26 ± 2° C temperature and 16 hrs photoperiod provided by cool fluorescent tube with 1000 lux light intensity.

Results and discussion

Effect of BA and NAA on the multiple shoot induction :

Observation recorded with respect to morphogenetic responses of cladode explant to medium supplemented by BA alone or in combination with NAA are summarized in Table 1. Explant inoculated on medium devoid of BA or NAA alone did not show any response. However, different level of BA and NAA produced a remarkable effect on the percentage of explant with shoots. The mean maximum percentage of explants with shoot (80%) were obtained with higher concentration of BA 4 mg/l either alone or in combination of NAA 0.1 mg/l. All concentrations of BA with higher concentration of NAA 1 mg/l found a decreasing influence on the shoot induction.

A perusal the data revealed that the different levels of BA and NAA resulted in significant variation on number of days taken for axillary bud breaking in inoculated cladode explant under *in vitro* condition. Higher concentration combined treatment of BA and NAA resulted in earliest bud break. Initially after first subculture performed after incubation of culture for four weeks period. There was no difference in induction of number of shoots among

Table 1. Effect of BA and NAA on the multiple shoot induction in cactus pear

Treatments			Days taken for bud break	Percentage of explant with shoot	No. of shoot/explant after I subculture	No. of shoot/explant after III subculture	Shoot length (cm)
	BA	NAA					
T_1	0.0	0.0	00	00	0.0	0.0	0.0
T_2	1.0	0.0	35.1	70	1.0	6.2	3.4
T_3	2.0	0.0	32.4	75	1.0	7.1	3.1
T_4	4.0	0.0	30.2	80	1.0	7.6	2.0
T_5	0.0	0.1	00	00	0.0	0.0	0.0
T_6	1.0	0.1	30.4	70	1.0	7.1	3.1
T_7	2.0	0.1	28.1	80	1.0	8.6	2.9
T_8	4.0	0.1	26.5	80	1.0	8.1	2.5
T_9	0.0	1.0	00	00	0.0	0.0	0.0
T_{10}	1.0	1.0	24.5	20	1.0	3.2	1.9
T_{11}	2.0	1.0	22.1	30	1.0	3.8	1.7
T_{12}	4.0	1.0	19.4	38	1.0	4.2	1.1
SEm.			0.29			0.223	0.14
C.D.			0.81			0.623	0.382

Table 2. Effect of NAA and IAA on the rooting of microshoot of cactus pear

	Treatment	Concentration (mg/L)	MS Media Rooting	No. of roots (%)
T_1	Control	0.0	33.33	1.7
T_2	NAA	0.5	80.00	2.2
T_3	NAA	1.0	100.00	4.4
T_4	NAA	2.0	66.67	4.6
T_5	IAA	0.5	60.00	2.2
T_6	IAA	1.0	73.33	2.4
T_7	IAA	2.0	100.00	3.1
SEm.				0.22
C.D.				0.613

all treatment of both BA and NAA. However, there was remarkable difference in number of shoot per explant after third subculture. The treatment without BA or with only NAA failed to induced shoot in the explants. Whereas, in all the treatments of BA alone or in combination with NAA produced shoots which were varied to different treatments. The differentiation of multiple shoots was maximum (8.6 shoots/explant) after third subculture with T_7 treatment (2 mg/l BA + 0.1 mg/l NAA). The influence of T_7 treatment was at par with T_8 treatment. Except the treatment T_{10}, T_{11} and T_{12}, all treatments were found to produce rootable size of microshoot. This may be due to the effect of higher concentration of NAA in these treatment .All the concentrations of BA alone or in combination of NAA produced promotive influence axillary bud breaking which gave rise to differentiation of multiple shoots in the cladode explants. These results are in agreement with the findings of Escobar *et al.* (1986), Mohamed-Yaseen *et al.* (1995), Giusti *et al.* (2002) and Garcia-Saucedo *et al.* (2005). The less percentage of explants with shoots was observed with treatment higher level of NAA 1 mg/l in combination of BA. These results are in close conformity of the results obtained by Khalafalla *et al.* (2007) who observed no improvement in shoot multiplication when NAA 0.5 mg/l used in combination of BA on kinetin in cactus. Thus, the synergetic influence of NAA at higher concentration did not improve the number of shoots per explant. This may be due to higher concentration of NAA with respect to BA must have suppressed stimulative effect of BA.

Effect of NAA and IAA on rooting in microshoots

The data given in Table 2 show effect of NAA and IAA on rooting percentage and number of roots in microshoot when transferred to MS medium containing different concentration of NAA and IAA. The mean maximum rooting percentage (100%) was obtained in T_3 and T_7 treatments. However, significantly higher number of roots (4.4) was recorded with NAA 1 mg/l.

In the present experiment, it was observed that rooting percentage in microshoots was better with NAA 1 mg/l. Auxin mediated induction of roots in cacti is well documented. *In vitro* studies by Johnson and Emino (1979), Mohmed-Yasseen *et al.* (1995) and Garcia-Saucedo *et al.* (2005) also support the present findings. These results indicate that the higher concentration of auxins have better stimulative influence on root initiation whereas lower concentration exhibited delayed rooting which may be due to in-sufficient quantity for root induction. Similar observations were reported by Mata-Rosas *et al.* (2001) with Mexican cactus *Turbinicarpus laui* and Jaurez and Passera (2002) with *Opuntia ellisiana* Griff. Zeng *et al.* (2003) obtained root formation with MS medium + 0.1 mg IBA l^{-1} after 10 days of culture with *Opuntia dillenii* cv. *Milpa Alta.*

Acclimatization of the plantlets

In vitro propagated plantlets were successfully acclimatized by transferring them into small pots of plastic, containing potting mixture of different medium. The rate of survival of the plantlets (Table 4) in the pots was 100% with vermiculite + cocopeat mixture of 3:1 (v/v) which was followed by another mixture i.e. vermiculite + vermicompost (60%). The lowest survival of plantlets (50%) observed with two step procedure of hardening. The higher percentage of survival of plantlets with vermiculite along with either cocopeat may be due to the complementary good properties of vermiculite and cocopeat in terms of porosity, high cation exchange capacity (CEC), highly decomposed, stable and good water holding capacity. Preece and Sutter (1991) also emphasized that certain environmental conditions are necessary for acclimatization of plantlets using controlled environmental facilities.

Similar achievements (100% survival of plantlets) were reported by Johnson and Emino (1979), Vyaskot and Jara (1984), Mohamed-Yasseen *et al.* (1995), Giusti *et al.* (2002) and Garcia-Saucedo *et al.* (2005).

In vitro conservation of plantlets

Under *in vitro* conservation study, *in vitro* rooted plantlets were used for further conservation by reducing growth of the plantlets and by minimizing subculturing process. The effect of BA, NAA and their combinations on the *in vitro* growth , injury and survivability of the cultures was evaluated. The rooted plantlets were transferred on agar solidified MS medium supplemented with activated charcoal 3g /litre and different combinations of BA (0, 0.5, 1.0 mg/l) and NAA (0, 0.5, 1.0 mg/l). The higher concentration of BA (1.0 mg/l) was found to increase shoot proliferation and was detrimental to root growth of the *in vitro* plantlets whereas higher concentration of NAA (1.0 mg/l) was found to influence both shoot and root growth positively (Table 3). This technique of *in vitro* conservation was found effective in maintaining cultures continue for more than six years. Further, proliferated growth of *in vitro* maintained plantlets was harvested and transferred to new containers for increasing numbers of stock materials. Addition of auxin in the medium had optimal positive influence on shoot and root growth. This is due to physiological action of NAA in cell division and stimulation of apical dominance in the micro-shoots and adventitious root formation including cell elongation.The study conducted on auxin induced morphogenetic responces in long-term *in vitro* subcultured *Mammillaria san-angelensis* Sanchez-Mejorada (Cactaceae) by Rubluo *et al* (2002) and concluded that auxin may be involved in *in vitro* morphogenetic response in Cactaceae. Further ,these results may be supported by the findings of Sajid et al (2006) who studied the effect of diverse hormonal regimes on *In vitro* growth of grape germplasm.

Table 3. Effect of BA, NAA and their combination on the in vitro growth, injury and survivability of the cultures

Treatments	BA	NAA	Increase in Shoot length cm.	Increase in Root length cm.	Percentage of vitrified culture	Root growth promotive/inhibitory effect
T_1	0.0	0.0	1.0	1.0	33.33	Promotive
T_2	0.0	0.5	2.6	1.6	13.33	Promotive
T_3	0.0	1.0	3.1	2.6	13.33	Promotive
T_4	0.5	0.0	1.1	0.0	40.00	Inhibitory
T_5	0.5	0.5	0.5	0.0	53.33	Inhibitory
T_6	0.5	1.0	0.4	0.0	53.33	Inhibitory
T_7	1.0	0.0	0.6	0.0	60.00	Inhibitory
T_8	1.0	0.5	0.5	0.0	73.33	Inhibitory
T_9	1.0	1.0	0.6	0.0	80	Inhibitory
SEm.			0.102	0.11		
C.D.			0.29	0.32		

Table 4. Influence of hardening procedure on survival percentage of plantlets

Hardening step	Survival % of plantlets	
	Vermiculite : Cocopeat (3:1)	Vermiculite : Vermicompost (3:1)
Two step procedure: Transfer to Environment control greenhouse for 8-10 days and subsequent transfer in shade house equipped with intermittent fogging.	80	50
Three step procedure: Primary hardening in acclimatization hood kept in culture room for 10-12 days and subsequent transfer to Environment control greenhouse for 8-10 days, thereafter to shade house equipped with intermittent fogging.	100	60

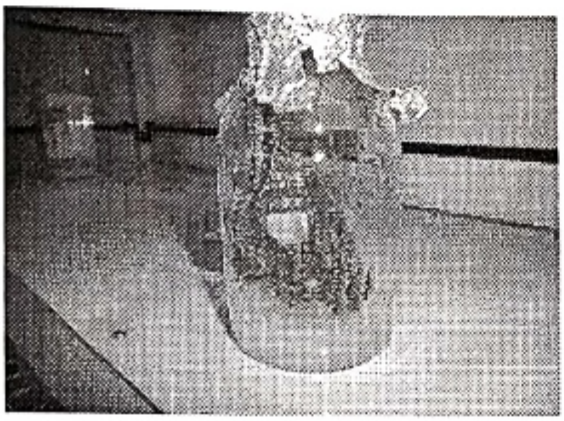

Fig. 1. Micropropagated cladode of Cactus pear

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