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IN VITRO CONSERVATION OF *CADABA HETEROTRICHA* STOCKS, AN ENDANGERED SPECIES IN PAKISTAN

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Abstract

In vitro conservation protocol was developed for *Cadaba heterotricha*, an endangered species reported from Southern Pakistan. This species has been subjected to various threats including habitat loss and over-exploitation, need an urgent conservation measures. Seeds collected from the wild, were used as initiating material. While, nodal segments of *in vitro* germinated seedlings were employed to induce multiple shoot regeneration. Full strength MS medium supplemented with 2.5 mg/l BAP and 0.5 mg/l Kin along with 0.5 mg/l NAA helps in attaining the highest number (16.0) of shoots with 100% shoot regeneration frequency. The cultures maintained in good conditions with subculture after 6-7 months. IAA at the level of 3.0 mg/l gave maximum number (2.70) of rooting along with the highest rooting frequency (80%).

Introduction

Cadaba heterotricha Stocks (Capparaceae), is reported from Ethiopia, Kenya, Somalia, Oman and Yemen. While, in Pakistan it is restricted to Cape-monze and adjoining areas on rocky slopes, cliffs and gravel plains and is considered as rare (Jafri, 1958, 1973) and endangered (Nasir, 1991; Abbas *et al.*, 2010) species. Anthropogenic activities in the restricted habitat are causing multiple threats i.e. low population, habitat loss, fuel wood cutting, grazing and poultry business activities, for the taxon (Abbas *et al.*, 2010).

In situ environment is becoming unfavorable for its survival and there is a dire need for an urgent conservation effort for avoiding its extirpation. Although species conservation is achieved most effectively through the management of wild populations and natural habitats (*in situ* conservation), *ex situ* methods can be used to complement *in situ* methods and in some instances, may be the only option for some species (Maunder *et al*., 1998; Ramsay *et al*., 2000).

In vitro techniques and applications have been found to be useful in the conservation of a large number of rare (Bramwell, 1990; Holobiuc *et al*., 2009) and threatened (AmoMarco & Lledo, 1996; Dhar *et al*., 2000; Pence, 2005; Rajasekharan *et al*., 2009) species with poor and uncertain responses to conventional methods of propagation (Sarasan *et al*., 2006). According to Engelmann (1997 & 1998) standard culture environment can be effectively utilized for short to medium term *in vitro* conservation of plant germplasm, through increasing intervals between subcultures in slow growing species.

The lack of published methods for *in vitro* conservation of wild taxa and the limited amount of experimental plant material make the choice and development of initial culture medium for rare and threatened plants somewhat arbitrary (Krogstrup *et al.*, 2005). There are no reports on the *in vitro* conservation of *Cadaba heterotricha*. The prime focus of the present investigation was to establish a short term *in vitro* conservation protocol for this species.

Materials and Methods

a. Plant material: Fresh seeds of *Cadaba heterotricha* Stocks, were collected from the wild population and used as an initiating material for further study.

b. Sterilization of seeds: Seeds were thoroughly washed for 20 minutes under running tap water, followed by quick dip in 95% ethanol for 20 seconds. Thereafter, surface sterilization was performed using 10% commercial bleach (Sodium Hypochlorite, NaOCl) solution containing 3-6 drops of Tween 20 in 200 ml solution for 15 minutes. Sterilized seeds were rinsed 3 times with autoclaved distilled water before inoculating on nutrient media.

c. Media and culture procedure: Different concentrations of growth regulators {i.e. 6-benzylaminopurine (BAP), Kinetin (KIN), Naphthalene Acetic Acid (NAA), Indole-3 Acetic Acid (IAA) and Indole Buteric Acid (IBA)} were incorporated in the media along with 3% sucrose. 0.6% Phytagel (P8169-Phytagel, Sigma-Aldrich, St. Louis. Mo. USA) was used as a gelling agent. For shoot induction BAP was used at the level of 0.0, 0.5, 1.0, 1.5, 2.0 & 2.5 mg/l. While, in another experiment the various levels of BAP were used in combination with constant level of KIN (0.5 mg/l) and NAA (0.5 mg/l). In case of root regeneration IAA and IBA each was used at the level of 0.0, 1.0, 2.0 & 3.0 mg/l. The pH of the medium was adjusted to 5.8 prior to autoclave (121^{o}C for 15 minutes). The autoclaved media were properly stored in storage room till its use. Seeds were inoculated on specific media within laminar flow hood (Technico Scientific, Lahore, Pakistan) to avoid any contamination. Glass bottles with plastic caps were used for culture. After inoculating seeds on hormone free MS media (Mureshige & Skoog, 1962), seeds were incubated at 25^{o}C under 16 hours of photoperiod. Light was provided using 40 watts, normal cool white florescent tubes (Philips-TL40W 54). Intensity of light ranged from 2000 to 3000 lux of energy. Sterile seedlings were used for the collection of explants (nodal segments) for establishing *in vitro* cultures. The nodal segments were cultured on different levels of growth hormones for the shoot multiplication. Experiment was laid out according to completely randomised design (CRD) with 5 replicates per treatment. Established shoot cultures were sub-cultured after 4-6 months depending upon the growth and condition of media in the glass jars. Full strength MS media containing IAA or IBA, was used for root induction. The data was recorded on frequency (%) and number of shoots and roots in various combinations of media. The data was statistically analysed using Duncan's Multiple Range Test (DMR).

Results

i. Effect of various concentrations of BAP in MS medium on shoot regeneration: The results showed that various concentrations of BAP in MS medium had significant effect on shoot regeneration frequency (65%) and number of shoots per explant in *C. heterotricha* (Table 1). Means for frequency (%) of regeneration and number of shoots per explant ranged 1.0 to 65 and 0.20 to 6.50, respectively. Maximum number of shoots (6.50) per explant was recorded on MS medium containing 2.5 mg/l BAP. Negligible and significantly reduced numbers of shoots (0.20) per explant was recorded in the absence of plant growth regulators.

Table 1. Effect of various concentrations of BAP in MS medium on number of shoots per explant from nodal cuttings of seedlings of *C. heterotricha*.

BAP concentrations (mg/l)	Frequency (%) of shoot regeneration	Mean number of shoots per explant
0	1d	0.20d
0.5	23c	2.30c
1.0	27c	2.70c
1.5	33c	3.30c
2.0	46b	4.60b
2.5	65a	6.50a

Values within column followed by small letters are significantly different in accordance with Duncan's Multiple Range Test

Table 2. Effect of various concentrations of BAP - KIN - NAA in MS medium on frequency (%) and number of shoots per explant of *C. heterotricha*.

BAP (mg/l)	Kin (mg/l)	NAA mg/l	Frequency (%) of shoot regeneration	Mean number of shoots per explant
0.0	0.0	0.0	5.0d	0.50d
0.5	0.5	0.5	31.0c	3.10c
1.0	0.5	0.5	38.0c	3.80c
1.5	0.5	0.5	39.0c	3.90c
2.0	0.5	0.5	61.0b	5.50b

Values within column followed by small letters are significantly different in accordance with Duncan's Multiple Range Test

ii. Effect of various concentrations of BAP - KIN - NAA in MS medium on shoot regeneration: Results showed that various concentrations of BAP - KIN - NAA in MS medium had significant effect on shoot regeneration frequency (100%) and number of shoots per explant in *C. heterotricha* (Table 2). The results indicated that the means for frequency (%) of shoot regeneration and number of shoots per explant ranged 5 to 100% and 0.50 to 16.60, respectively. Maximum number of shoots (16.60) per explant was recorded on MS medium containing BAP (2.5 mg/l) - KIN (0.5 mg/l) - NAA (0.5 mg/l) (Fig. 1). A sharp decline in the mean number of shoots per explant was recorded in the absence of plant growth regulators (Table 2).

iii. Effect of various concentrations of IAA or IBA in MS medium on number of roots: Various concentrations of IAA or IBA in MS medium had significant effect on rooting frequency and number of roots per explant in *C. heterotricha* (Table 3). The results showed that the maximum rooting frequency (80%) was recorded on MS medium containing 3.0 mg/l IAA. Contrarily, IBA was not so promising and resulted in sharp reduced frequency of rooting on any concentration of IBA. Similarly, maximum number of roots per explant (2.70) was recorded on MS medium containing 3.0 mg/l IAA. In general, increase in the concentration of IAA resulted in corresponding increase in the number of roots per explant, with maximum roots at 3.0 mg/l of IAA (Fig. 2). IBA resulted in the decrease of roots per explant; even no roots were recorded on MS medium containing 1.0 mg/l IBA. Similarly, no roots were recorded on auxin free MS medium (control).

Fig. 1. Profuse shoot regeneration from nodal explant of *Cadaba heterotricha* after 25 weeks of culture on MS medium containing 2.5 mg/l BAP - 0.5 mg/l Kin - 0.5 mg/l NAA.

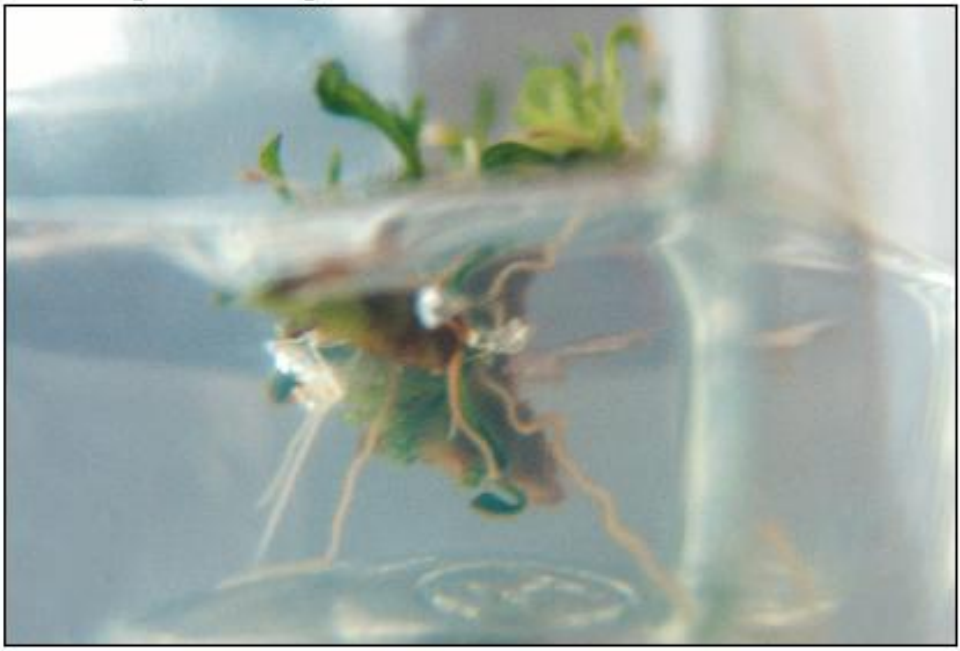

Fig. 2. Profuse rooting of *Cadaba heterotricha* after 16 weeks of culture on MS medium containing 2.0 mg/l IAA.

Table 3. Effect of various concentrations of IAA and IBA in MS medium on *In vitro* rooting of *C. heterotricha*.

Conc. mg/L	Frequency (%) of root regeneration		Number of roots per explants	
	IAA	IBA	IAA	IBA
0	0.0d	0.0c	0.00c	0.00b
1.0	2.0c	0.0c	0.20c	0.00b
2.0	15.0b	3.0b	1.14b	0.40ab
3.0	80.0a	25.0a	2.70a	0.80a

Values within column followed by small letters are significantly different in accordance with Duncan's Multiple Range Test

Discussion and Conclusion

A review of previous literature suggests that no published report exists on *in vitro* conservation of *C. heterotricha.* Shoot multiplication is considered one of the most promising ways for clonal multiplication of selected species (Wala & Jasrai, 2003), while nodal segments proved an excellent explant for multiple shoot regeneration (Kaur *et al.,* 1998).

Literature on *in vitro* culture of various plant species indicates that even different cultivars of the same species had a diverse response and growth patterns under *in vitro* or *ex vitro* conditions (Norton & Norton, 1985; Simpson & Bell, 1989). Fay (1992) recommended the use of seeds instead of vegetative material as the source of propagation material to maintain a wider genetic base.

Superiority and stimulating effect of BAP for bud break over other cytokinins has been reported *in vitro* conservation of medicinal, aromatic and shrubby plants (Kukreja *et al.,* 1990; Sen & Sharma, 1991; Bonga &Von-Aderkas, 1992; Purohit *et al.*, 1994; Bhatt *et al.,* 1995; Pattnaik & Chand, 1996; Sexana *et al*., 1997; Khalafalla & Hattori, 1999; Faisal *et al*., 2006).

The findings of our investigation conforms the findings of the results of the above studies, showing variable effects of different levels of BAP on multiple shoot regeneration in *C. heterotricha.* Sharon & Gupta (2009) recorded 86% shoot regeneration on explants taken from 7 days old seedlings, when lateral buds of *Laasiosipon eriocephalus* were cultured on MS medium containing 1.0 mg/l BAP. However, Brassard *et al.* (1996) and Figueriedo *et al.* (2001) observed that BAP had a stimulating effect on shoot proliferation but inhibits shoot elongation. They noted that 2.5 mg/l BAP induced the highest number of shoots (6.50) per explant. Where as a synergetic effect on shoot regeneration in a media containing combination of BAP and KIN along with 0.5 mg/l NAA was observed. Similar results were obtained by Kaur *et al*. (1998).

Regular subculture of the explant has been recommended as an effective method in the micropropagation of woody plants (Sharma & Chaturvedi, 1988; Aitken - Christie & Jones, 1987; Shekhawat *et al.,* 1993). Sturdy growth, shoot elongation along with the highest number of shoots (16.60) was recorded on the MS medium containing BAP (2.5 mg/l) - KIN (0.5 mg/l) - NAA (0.5 mg/l) in this study.

Rooting was induced on MS media containing IAA or IBA. The shoots failed to induce roots or root initials in the absence of auxins (control). IAA was found more efficient in inducing root initials.

The results of this study are in agreement with Sharon & Gupta (2009), who noted maximum rooting (50%) in *Lasiosipon eriocephalus* on MS medium containing IAA as compared to other auxins in the rooting medium. Hiregoudar *et al*. (2003), also recorded the similar results who induced 80% roots on half strength MS medium containing IAA.

Thus, it is concluded that *C. heterotricha* can successfully be conserved *in vitro* by using MS medium augmented with a combination of BAP (2.5 mg/l) - KIN (0.5 mg/l) - NAA (0.5 mg/l) for acquiring the highest level of shoot multiplication, while 3.0 mg/l IAA in the same medium found precise for root regeneration.

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