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IN VITRO RESPONSE OF RUELLIA LINEARIBRACTEOLATA TO DIFFERENT GROWTH HORMONES - AN ATTEMPT TO CONSERVE AN ENDANGERED SPECIES

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Abstract

Ruellia linearibracteolata Lindau is an endangered taxon in the southern region of Pakistan. Being exposed to harsh environmental conditions and subjected to multiple threats including habitat loss, grazing activities and soil erosion, Hence an urgent conservation strategy is required to avoid its extirpation. In this connection an experiment has been conducted to study its response to different growth hormones. Seeds from the wild were germinated in vitro and then aseptically grown seedlings were used as a source of explant. The efficiency of MS medium was thoroughly examined by augmenting different growth hormones. The cultures maintained in appropriate conditions with subculture after 6-7 months. 1.5 mg/l BAP along with 0.5 mg/l Kin and 0.5 mg/l NAA produced the highest number (5.80) of shoots with 58.0% shoot regeneration frequency. While, IBA (1.0 mg/l) produced maximum number (2.2) of roots along with the highest rooting frequency (95%).

Introduction

Ruellia linearibracteolata Lindau (Acanthaceae), is a much branched perennial herb, up to 80 cm tall with bluish purple flowers, mostly found around the edges of calcareous rocky hills, cliffs, slopes and canyons (Malik & Ghafoor, 1988; Abbas, 2010, Abbas & Qaiser 2011). Previous workers, Ghafoor & Heine (1986) and Malik & Ghafoor (1988) reported it as *Ruellia sindica* in the Flora of Pakistan and classified it as an endemic to this region. However, in the current study it has been revealed that it is also reported from Somalia, Ethiopia and Kenya (Thulin, 2006) and Yemen (Kilian *et al*., 2004).

Habitat destruction and the continuous harvesting of native plants are the main factors affecting nearly all plant species (Malda *et al*., 1999; Beck & Dunlop, 2001) while, species rate of natural recruitment also has a direct influence on its extinction (Martin & Pardeep, 2003). According to Abbas (2010) and Abbas & Qaiser (2011) that anthropogenic activities i.e., habitat loss, grazing activities, soil erosion fuel wood cutting, poultry business and its fragmented, isolated and dispersed localities collectively occupied 1988.88 Km2 in the study area. As the *in situ* environment is becoming unfavorable for its survival along with low and fragmented population size, there is a dire need for an urgent conservation effort for avoiding its extirpation (Abbas, 2010; Abbas & Qaiser, 2011; Abbas *et al*., 2012). 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) but it is becoming impractical due to the disappearance of large wild areas (Engelmann, 1991; Seeni & Decruse, 2007). *Ex-situ* conservation has gained the international recognition through its inclusion in the ninth article of Convention on Biological Diversity (CBD).

In vitro techniques and applications have been found to be useful in the conservation of a large number of rare (De Langhe, 1984; Withers, 1989; 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. According to Martin & Pardeep (2003) it is the only feasible way to maintain a gene bank of plants for their future sustainable utilization. The lack of published methods for *in vitro* culture 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 culture of Ruellia linearibracteolata. The prime focus of the present investigation was to establish a fast in vitro conservation/micropropagation/multiplication protocol for this species.

In vitro conservation of endangered species can offer considerable benefits for the rapid cultivation of at risk species which have a limited or no reproductive capacity and exist in threatened habitats (Fay, 1992; Sarasan *et al*., 2006). Detailed field surveys revealed that the species has no natural recruitment, at the same time the population is declining due to anthropogenic activities. Hence, *in vitro* conservation seems to be an appropriate option for the protection of *Ruellia linearibracteolata* out of its natural habitat.

Materials and Methods

Plant material: Fresh seeds of *Ruellia linearibracteolata* were collected from the wild population and used as an initiating material for further study.

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.

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 & 3.0 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 autoclaved (121°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°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 randomized 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 analyzed using Duncan's Multiple Range Test (DMR).

Results

Effect of various concentrations of BAP-NAA in MS medium on shoot regeneration: The results showed that various concentrations of BAP-NAA in MS medium had significant effect on shoot regeneration frequency (46%) and number of shoots per explant in *Ruellia linearibracteolata* (Table 1). Means for frequency (%) of regeneration and number of shoots per explant ranged 2.0 to 46 and 0.20 to 4.50, respectively. Maximum number of shoots (4.50) per explant was recorded on MS medium containing 1.5 mg/l BAP along with 0.5 mg/l NAA. Negligible and significantly reduced numbers of shoots (0.20) per explant was recorded in the absence of plant growth regulators.

Table 1. Effect of BAP-NAA in MS medium on frequency and number of shoots per explant from nodal cuttings of seedlings of *Ruellia linearibracteolata*.

BAP concentrations (mg/l)	NAA concentrations (mg/l)	Frequency of shoot regeneration (%)	Mean number of shoots per explant
0	0	2.0d	0.20d
0.5	0.5	6.0cd	0.60cd
1.0	0.5	24.0b	2.28b
1.5	0.5	46.0a	4.50a
2.0	0.5	9.0c	0.90c
2.5	0.5	0.0d	0.00d
3.0	0.5	0.0d	0.00d

Values within column followed by similar letters are non-significantly different in accordance with Duncan's Multiple RangeTest

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 (58.0%) and number of shoots per explant in *Ruellia linearibracteolata* (Table 2). The results indicated that the means for frequency (%) of shoot regeneration and number of shoots per explant ranged 2 to 58.0 and 0.0 to 5.80, respectively. Maximum number of shoots (5.80) per explant was recorded on MS medium containing BAP (1.5 mg/l) - KIN (0.5 mg/l) - NAA (0.5 mg/l). A sharp decline in the mean number of shoots per explant was recorded in the absence of plant growth regulators (Table 2).

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

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	2.0c	0.20c
0.5	0.5	0.5	12.0c	1.00c
1.0	0.5	0.5	26.0b	2.40b
1.5	0.5	0.5	58.0a	5.80a
2.0	0.5	0.5	9.0c	0.90c
2.5	0.5	0.5	4.0c	0.40c
3.0	0.5	0.5	0.0c	0.00c

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

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 *Ruellia linearibracteolata* (Table 3). The results showed that the maximum rooting frequency (95%) was recorded on MS medium containing 1.0 mg/l IBA. Contrarily IAA was not so promising and resulted in sharp reduced frequency of rooting on any concentration of IAA. Similarly, maximum number of roots per explant (2.20) was recorded on MS medium containing 1.0 mg/l IBA. In general, increase in the concentration of IBA resulted in corresponding increase in the number of roots per explant, with maximum roots at 1.0 mg/l of IBA. IAA resulted in the decrease of roots per explant; even no roots were recorded on MS medium containing 3.0 mg/l IBA. Similarly, no roots were recorded on auxin free MS medium (control).

Table 3. Effect of various concentrations of IAA and IBA in MS medium on *in vitro* rooting of *Ruellia linearibracteolata*.

Conc. (mg/L)	Frequency (%) of root regeneration		Number of roots per explants	
	IAA	IBA	IAA	IBA
0	0.0b	0.0b	0.0b	0.0b
1.0	5.0a	95.0a	0.4a	2.2a
2.0	0.00b	5.0b	0.0b	0.2a
3.0	0.00b	0.00a	0.0b	0.0b

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

Discussion

Ruellia linearibracteolata, is, a rare and an endangered species for Pakistan (Abbas 2010; Abbas & Qaiser, 2011). *In vitro* culturing is a proven method for the efficient and reliable *ex situ* conservation of medicinal and aromatic species, because of their commercial exploitation due to hidden potential for valuable plant-based pharmaceuticals (Rout, 2002; Faisal *et al*., 2005; Abbas *et al*., 2012). Karuppusamy *et al*. (2009) suggest use of axillary buds for multiple shoot regeneration to maintain true to type clones, for efficient germplasm conservation. Combinations of BAP and NAA have been used to induce shoot formation in numerous species (Moore, 1986; Tripepi, 1997; Huang *et al*., 2000). According to Abdulaziz & Bahrany (2002) shoot multiplication was scarcely-rare in the absence of BAP, even in the treatments where supplemented with Kin, only a single shoot grew from either of the axillary buds of the node explants. They further added that level of NAA had no influence on shoot multiplication, in the absence of BAP. Above mentioned results are in agreement with the current investigation, where as greater number (5.80) shoot regeneration was obtained, when 1.5 mg/l of BAP was used along with 0.5 mg/l Kin and 0.5 mg/l NAA. The results of this study were also in agreement with the findings of Pathak *et al*., (2009), who concluded that addition of BAP along with Kin and NAA at the concentration of 0.5 mg/l was most suitable for shoot multiplication in sugarcane. Contrarily, Sajid *et al*. (2006), observed poor shoot regeneration at low concentration of BAP and auxins in grapes. However, they recorded higher number of shoots at high concentrations of BAP with auxin. During this investigation, higher levels of BAP along with Kin - NAA induced callusing with no shoot regeneration.

Karuppusamy *et al*. (2009) found 5.0 mg/l NAA in ½ strength MS medium a suitable level for the root induction in the presence of 50 g/l sucrose. While Swamy & Sahijram (1998) reported highest (47.6 %) rooting of *Bougainvillea* using 5 mg/l IBA in MS medium. In the study, greater number of roots was obtained at 1.0 mg/l IBA and same level gave highest rooting frequency (95%). In response to IAA, extremely week and negligible rooting was observed at 1.0 mg/l, while IAA at 2.0 and 3.0 mg/l caused callusing. IBA at 3.0 mg/l also callus induction. Results of Fidanci *et al*. (2008) are in agreement with the study, where they reported 95 to 100% rooting in cherry root stocks when MS medium was augmented with 1.0 mg/l IBA.

Comparison of the above results indicated that IBA could be preferred for rooting of *R. linearibracteolata* as compared to IAA.

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