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DEVELOPMENT OF MICROPROPAGATION PROTOCOL FOR MEDICINALLY IMPORTANT *PERIPLOCA HYDASPIDIS*

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ABSTRACT

The present study represents protocol optimization for in vitro micropropagation of Periploca hydaspidis a weed which is medicinally important. The plant is locally used as purgative. The induction of the shoots was carried out from nodal shoot explants using Murashige and Skoog (MS) media added with different concentrations and combiantions of benzyalminopurine (BAP) and Nephthalene acetic acid (NAA). The effect of different concentrations of BAP and NAA on shoot number, shoot length and per cent shoot induction of explant was noted. The sprouting of shoots started within 7-8 days of inoculation. The combination of BAP 4 mg/L and NAA 1 mg/L induced maximum shoot length (6.25 cm) while minimum induction was noted in combination of BAP 2 mg/L and NAA 0.1 mg/L (1.75 cm). The maximum rooting (4.25 cm) was shown by the combination of IBA 3mg/L and NAA 1.5 mg/L while minimum (1.90 cm) was exhibited by the combination of IBA 2mg/L and NAA 1 mg/L. In vitro micropropagated small plants having healthy roots were transferred to soil through successive acclimatization, which yielded 80 per cent survival rate.

Key words: *Periploca hydaspidis,* BAP, NAA, IBA.

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INTRODUCTION

P. hydaspidis belongs to the family *Asclepiadaceae*. It is a twining shrub, usually leafless; branches are smooth green and are 1.5 mm in diameter. Leaves (when present) are linear or lanceolate; nerves in leaves are obscure, petiole 1-2 mm long. Flowers are in lax axillary trichotomous cymes. Bracts are ovate, deciduous or persistent. Calyx is 5-lobed and the lobes are 1-1.5 mm long, ovate, blunt, ciliate. Corolla lobes are 2.5 mm long, yellow, oblong-lanceolate, glabrous outside, villous inside, corona yellow, of five filiform hairy processes

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Follicles are 7.5 cm long. Seeds are present in amount of 10-50 in a coating. Flowering is perennial and usually occurs in September-October (Appendix 1). Locally the plant is called as *Taroona* and is used as purgative (personal communication with local people). This plant is usually found in Swat, Pakistan at altitude of 850m to 1600m. Other species of the family like *P. aphylla, P. calophylla* are found in Kashmir and India (Ali, 1983).

Many medicinal plant species are endangered now and some are at the verge of extinction. The data on the preservation condition of these plants have variation (Nasir, 1991, Choudry and Qureshi, 1991). The rate of extinction of these plants species is increasing more due to anthropogenic activities then from natural phenomenon (Akeroyd, 2002; Bramwell, 2002; Alam *et al.,* 2009). In Pakistan many medicinal plant species are endangered. Red list of IUCN (2008) reported 19 endangered flowering plants. Ali and Qaiser (2012) declared 21 plants threatened in Pakistan. According to IUCN Red List Categories and Criteria (Anonymous, 2008), no significant studies have been carried out on endangered plants of Pakistan as very little information is available on this subject.

Micro-propagation is a fast and rapid tissue culture technique and for the commercialization of important medicinal and eatable plants like apple banana, cardamom, pears, strawberry, and many ornamentals plants like Orchids (Sheeba *et al.,* 2010). The techniques of micro-propagation are preferred over the traditional asexual propagation techniques because of the accompanying reasons: (a) in the micro-propagation technique, very small amount and size of tissue is necessary for production of millions of clonal plants in a year, (b) it is also helpful in introducing desirable changes in many species, (c) *in-vitro* stock can be rapidly multiplied because it is time independent, and (d) storage for long period of vital germplasm is conceivable.

Tissue Culture techniques mainly micropropagation is efficiently used for the designing of protocols to ensure the safety of endangered species of plants (Ahmed *et al.,* 2010). This technique is also significant in swift initial release of novel varieties former to multiplication by conventional methods (Drew, 1980). This technique is also used for germplasm storage to maintain the disease free stock in controlled environmental condition (Kartha *et al.,* 1980).

Because of the disproportionate growth in the population of human, industrialization, and over utilization of nonrenewable resources of nature, the natural population of the plants in certain regions is promptly vanishing (Alam and Ali, 2009). *P. hydaspidis* faced the same situation and It is now going to be rare and endangered in the specific areas of Pakistan because of its limited availability and over use (Ali, 1983). Hence, it is crucial to protect the indigenous flora from

vanishing by taking proper actions (Ashraf and Akram, 2009; Gilani *et al.,* 2009). The tissue culture of plants is one of the most imperative techniques used in biotechnology for the protection of threatened and medicinally important plant species. To protect the biodiversity, a micro propagation protocol was successfully optimized in this study for large scale production of the plant.

MATERIALS AND METHODS

The plants of *P. hydaspidis* (collected from District Swat KPK, Pakistan) were retained in containers under controlled conditions in greenhouse at Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan in winter 2014. These plants functioned as the source of explants for the present study. Shoot nodal explants were used as experimental material. The plants were cut at nodal sites to produce explants ranging from 2-3 cm and these were inserted in the medium up to nodes. The surface of the nodes was scratched a bit for better absorption of the nutrients. The process was done with extreme precautions to prevent damage to explants. For the sterilization of the surface, the explants were subjected to washing for 30 min in running tap water to remove dust particles and were soaked in detergent for 30 min (0.1% Tween-20). Running tap water was again used for washing of the samples to remove any remaining of detergent. The cleaned explants were further sterilized with 70% ethanol (v/v) for 1 min and the explants were again surface sterilized with 0.1% mercuric chloride (w/v) for 4 min. Following the surface sterilization, explants were washed five times with autoclaved, double distilled water in laminar flow unit. Commercially available MS medium (Murashige and Skoog, 1962) was used to culture the explants. The medium was supplemented with sucrose (3%), agar (0.8%) and Growth hormones. For preparation of 1L MS medium, 4.43g of the commercially available medium was dissolved in 1L of distilled water.For shooting and rooting different concentrations of the growth hormones were prepared. BAP, IBA and NAA were prepared in 2, 3 and 4 mgl^{-1}, 1, 2 and 3 mgl^{-1} and 0.1, 0.5 and 1 mgl^{-1} respectively. The pH of prepared medium was adjusted to 5.8 by using 1N KOH or 1N HCl and then agar was added to the medium at the rate of 0.8% w/v for solidification of the medium. The media was poured into test tubes and were tightly capped. The media was autoclaved at 121°C for 20 min at 15psi pressure. Under aseptic conditions, explants of appropriate size were inoculated in autoclaved culture tubes having basal MS medium with diverse concentrations and combinations of growth hormones. Following inoculation, the entrance of culture tubes was flamed quickly, capped firmly and appropriately sealed with kiln film to keep it

away from external air. The culture tubes were transferred to growth room after proper labeling.

For shoot propagation, different concentrations of BAP and NAA were used in three different combinations (2+0.1 mgl^{-1}, 3+0.5 mgl^{-1} and 4+1 mgl^{-1} BAP and NAA respectively) with full strength MS medium. For control full strength MS medium without any growth hormone was used. Under sterile conditions, the explants with freshly formed shoots were taken out after 28 days of culture period. Data was recorded after 28 days of culture by measuring the length of the shoots in centimeters. Every possible care was taken to prevent any further contamination. The explants with freshly formed shoots were moved to media for rooting having different concentrations and combinations of IBA and NAA (1+0.5 mgl^{-1}, 2+1 mgl^{-1} and 3+1.5 mgl^{-1}), respectively. Data was noted after 15 days of culture for rooting.

All types of Culture were performed under definite conditions of air circulation, light quality, temperature and humidity for definite time. Culture room was set at a temperature of 25±3°C with12 hrs photoperiod with intensity of light at 2000-2500 flux.

Plantlets with small freshly formed roots were taken out from the culture tubes using sterilized forceps with extreme care to avoid injury to the plantlet and freshly formed roots. The plantlets were dipped in warm water to wash off any remaining of agar. The plantlets were washed with 1% (w/v) solution of Bavistine for preventing the infection of fungi to newly propagated plantlets. After this treatment the new small plants were cautiously planted in polyethylene house having farmyard manure and mixture of soil in 1:1. The plantlets were watered thoroughly and were kept under poly ethene house for the period of ten days at 31°C with 80% humidity. During this duration the plantlets were watered thoroughly with the help of sprayer to retain the essential level of moisture. Before the final transfer to the field after rooting, the plantlets were transferred to plastic pots for their hardening and acclimatization. The plantlets were moved to green house with lower humidity level having indirect sunlight and were watered twice daily to prevent wilting.

Statistical Analysis

Data was analyzed using statistix 8.1. The one- way analysis was done for statistical comparison, and p values< 0.05 were considered significant.

RESULTS AND DISCUSSION

The data recorded during the experiment indicated that explants soaked in 0.1% mercuric chloride for 7 min showed 100% mortality rate. When the time was reduced to 4 min, the survival rate increased to 75% and the mortality rate was decreased to 25% having

the contamination rate of 12% while the mortality rate was further reduced to 0% when the time was reduced to 2 min. The contamination rate at the present treatment was 10 % (Table-1). After one week of the inoculation of the explants in MS media, shoot induction was noted in all of the treatments. However, maximum shoot proliferation was noted in the combination of BAP 4 mg/L and NAA 1 mg/L (6.25 cm) while minimum was noted in combination of BAP 2 mg/L and NAA 0.1 mg/L (1.75 cm). The maximum percent shoot induction was 89% (Table 2). Further increase in the concentration of the shooting growth hormones negatively affected the shooting of the plant. Similar results were also reported by Sheeba et al. (2010) and Subathraa and Poonguzhali, (2012) who reported that increasing concentration of the BAP beyond certain levels negatively affect the shoot formation. The data further suggested that high percent of shoot induction was observed in combination of BAP 4 mg/L + NAA 1 mg/L which was 33% while minimum percent shoot induction was observed in BAP 2 mg/L + NAA 0.1 mg/L. The culture tubes containing no growth hormones showed no shoot propagation (Soliman *et al.*, 2011). After 28 days of the inoculation, the healthy cultured shoots shifted to fresh culture tubes having media for rooting. The data on rooting indicated that maximum rooting (4.25 cm) was shown by the combination of IBA 3mg/L and NAA 1.5 mg/L while minimum (1.90 cm) was exhibited by the combination of IBA 2mg/L and NAA 1 mg/L (Table-3). These results are in agreement with Intzaar *et al.* (2013). During our study it was noted that increase in the concentration growth hormones of the rooting media beyond these levels have negative effects on rooting as reported by (Sheeba *et al.*, 2010).

The most critical step in the process of tissue culture is acclimatization of newly generated plantlets as these are extremely sensitive and face difficulties for their establishment when directly exposed to standard environmental circumstances (Bhojwani and Razdan, 1992). The mortality rate of the freshly developed plantlets depends on its hardening stages which are mainly characterized as two stages. The first stage of hardening is most vital where the plantlets are transferred to polyethylene houses and nurtured for ten days while the survival rate at the second hardening stage is enhanced as the plantlets are pretty hardened in the first stage. After 40 days of the culture on roots proliferation media, the small plantlets with good conditions were transferred to plastic pots having farmyard manure and soil with 1:1 for the purpose of hardening (Sheeba *et al.*, 2010). The plantlets were retained in polyethene houses for ten days, and the suitable moistness (80%-85%) was maintained by continuous provision of water. The data indicated that plantlets under these conditions exhibited 50% survival rate in polyethylene house. The

plantlets were then transferred to greenhouse having controlled conditions of humidity, temperature and sunlight. The plantlets were watered twice a day in the green house. The plantlets showed progress in growth, the shoots gained weight and overall survival rate of the plant in natural environment after the process of micropagation was noted to be 75%.

CONCLUSION

Periploca hydaspidis is a medicinal herb of substantial importance. It is found in district Swat in Pakistan. It is also present in Kashmir and India. Because of the disproportionate proliferation in population of humans, industrialization and urbanization, and over consumption of the plants, *P. hysaspidis* has entered to the list of endangered species. In the present study we successfully optimized the greenest and effective mircropropagation protocol for production of the plant in large quantity, and to maintain the biodiversity of this medicinally important, endangered plant.

Table-1. Sterilization of the nodal explants

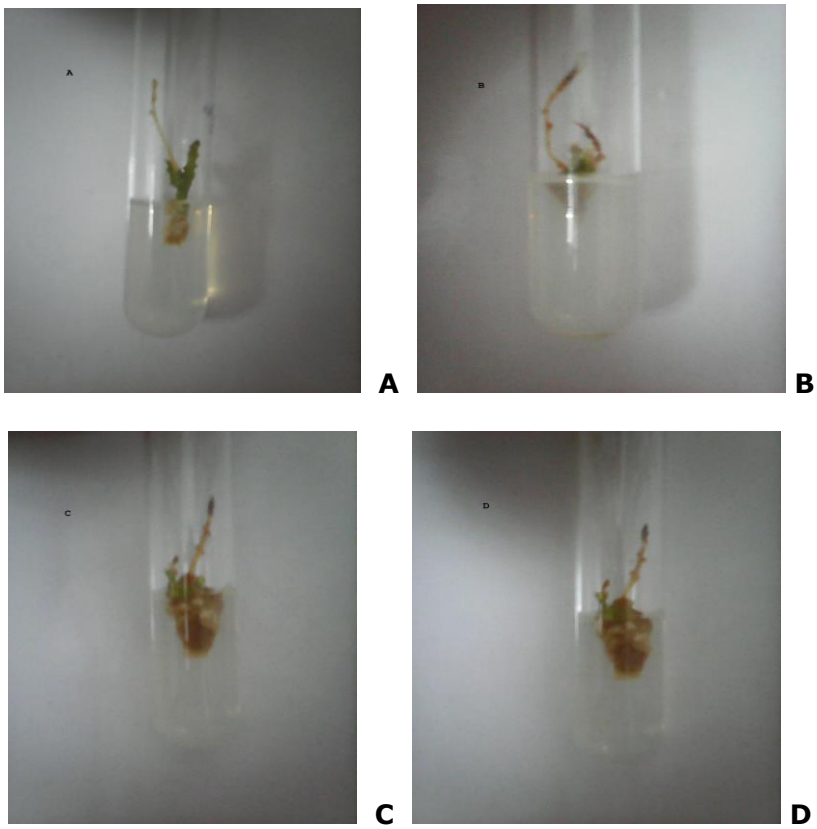
Explant	Percent concentration of mercuric chloride	Time exposure	Percent Response		
			Contamination	Mortality	Survived
Nodal shoot parts	0.1	7.00	---	100.00	0.00
	0.1	4.00	12.00	25.00	75.00
	0.1	2.00	10.00	0.00	100.00

Table-2. Shoot propagation of nodal explant of *P. hydaspidis*

Growth Hormones		number of shoots			Length of Shoots (cm)			% shoot induction
BAP+NAA (mg l^{-1})	No. of treatments	Min	Max	Mean ±SD	Min	Max	Mean ±SD	
2.00+0.10	30.0	1.0	1.0	1.00±0.00	1.2	2.3	1.75±0.77	78.00
3.00+0.50	30.0	1.0	2.0	1.50±0.70	2.3	3.5	2.90±0.84	82.00
4.00+ 1.00	30.0	2.0	3.0	2.50±0.70	6.0	6.5	6.25±0.35	89.00
5.00+ 1.00	30.0	1.2	2.5	1.85±0.91	5.5	6.0	5.75±0.35	85.00
Control	30.0	0.0	0.0	0.00±0.00	0.0	0.0	0.00±0.00	0.00

Table-3. Roots propagation of nodal explant of *P. hydaspidis*

Growth Regulator		Roots number			Roots length (cm)			% roots induction
IBA+NAA (mg l^{-1})	No. of treatments	Min	Max	Mean ±SD	Min	Max	Mean ±SD	
1.00+0.50	30.00	0.0	0.0	0.00±0.00	0.0	0.0	0.00 ±0.00	0.00
2.00+1.00	30.00	1.0	1.0	1.00±0.00	1.3	2.5	1.90±0.84	58.00
3.00+1.50	30.00	1.0	2.0	1.50±0.70	3.0	5.5	4.25±0.76	67.00
4.00+2.00	30.00	1.0	1.0	1.00±0.00	2.0	3.5	2.75±1.06	63.25
Control	30.00	0.0	0.0	0.00±0.00	0.0	0.0	0.00±0.00	0.00



Picture 1: (A) and (B) represents shooting while (C) and (D) represents rooting. Roots being Chiral and small are not properly visualized.

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