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Micropropagation of an endangered medicinal plant *Anaphyllum wightii* schott. through nodal culture

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Abstract

Micropropagation pave the way for multiplication of the plant, which are rare, endangered, threatened and are hard to multiply in natural condition. It plays a vital role in the conservation of plants which are under risk (rare, endangered). *Anaphyllum wightii* Schott. is an endemic and endangered plant species in Western Ghats of Kanyakumari district belonging to the family Araceae. It is unexplored and known for its therapeutic properties against poisonous bite and inflammation. Tribal communities use these plants as food and also prepare an oil with the addition of some more plants to treat snake bite.

Keywords: micropropagation, *Anaphyllum wightii* schott, rhizome, endemic, endangered, conservation

Introduction

Plant tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants (Thorpe, 2007) [1]. Tissue culture protocols have been developed for a wide range of medicinal plants, which includes endangered, rare and threatened plant species to ensure its conservation. Due to deforestation, urbanization, pollution, forest fire, overexploitation and other human activities, plant species are drastically decreasing every day. Australian forest fire in year 2019-2020 has destroyed large numbers of flora and fauna. At least half a billion animals and countless trees and plant have been killed, since the fire began in September 2019. More than 6.3 million hectares (63,000 sq km or 15.6 million acres) of forest land have been burned and many endemic plants may have been extinct. The Red list of threatened species, prepared by the International Union for Conservation of Nature (IUCN), has listed 132 species of plants and animals as Critically Endangered, from India. Plants seemed to be the most threatened life form with 60 species being listed as Critically Endangered and 141 as Endangered. Tissue culture plays an important role in conservation of these endangered species. *Anaphyllum wightii* is an endemic, endangered medicinal plant species belonging to the family Araceae distributed in the Southern Western Ghats, used to cure scabies and to prepare medicine for snake bite. Kanis of Kanyakumari district use the rhizome of *A. wightii* to treat snake bite and scabies but its medicinal property remains unexplored except the tribal community. The present study was aimed to multiply *A. wightii* using nodal explants to ensure its conservation status through micropropagation techniques.

Materials and methods

Selection and Sterilization of Plant Material Nodal portion was selected to be used as a source of explants. Nodes were trimmed from the rhizome and washed several times using running tap water then surface sterilized with detergent Tween 20 and washed with distilled water for about 20 minutes. Constant shaking was done during this period to get thorough sterilization. Later these explants were washed with double-distilled water for 10 min. Then explants were sterilized with 0.1% mercuric chloride for 3-5 minutes and rinsed with sterile distilled water to remove all traces. After that the explants were treated with 0.1% bavistin (Fungicide) and washed with distilled water for another 3 minutes. After the final wash, explants were spread on the sterilized petri dishes lined with sterile Whatman No.1 filter paper inside a laminar airflow chamber. Edges were trimmed using sterile surgical blade.

Culture Media

Murashige and Skoog medium commonly called as MS medium was selected as the optimal culture medium (Murashige and Skoog, 1962) [2]. Stock solutions of plant growth regulator was prepared using standard procedure. About 4.406 g powdered medium was dissolved in 900 ml of double distilled water in a 2-litre beaker. To this heat stable supplements such Sucrose 30 gm (3% per litre) and myoinositol 100mg was added. The solution was made-up to one litre by adding double distilled water. The made-up solution was divided into required part according to the hormone planned. The pH was adjusted with a single electrode electronic pH meter at 5.7, adjustment was done with 0.1N HCL and 0.1N NaOH. After adjusting pH, 0.8% agar was added to the medium and was heated (60°C) to dissolve the agar. Medium was dispensed into the culture vessels in sterile condition and kept in laminar flow hood before it gets solidify. The culture vessels plugged with cotton plug and bundled as 10-15 culture tubes per bundle. The bundles were wrapped with newspaper to avoid the unplugging of cotton during autoclave due to the pressure. The media was autoclaved for 20 minutes at 121oC. After autoclaving, culture tubes were placed in stands and kept under room temperature. These were then left to cool and

solidify. After solidification, it was stored inside the laminar flow hood.

Inoculation
The materials used for the inoculation were kept in the laminar flow hood under UV light for 15 minutes to ensure the proper sterilization. The explants were carefully transferred into the culture tubes using sterile forceps.

Incubation
All the cultures were maintained in an air- conditioned culture room at a temperatureof 25 ± 2°C. The source of illumination consisted of 2.5 feet wide fluorescent tubes (40 watt) and incandescent bulb (25 watt). The intensity of illumination was 3500 lux at the level of cultures and a 16-hour light followed by 08-hour darkness regime. A minimum of 10 culture tubes were raised for each combination and all experiments were performed 3 times.

Hardening and acclimatization
Developed plantlets were carefully removed from culture tubes and washed with double distilled water to remove all traces of medium. Then the plants were immersed in 1% solution of bavistin to avoid any fungal infection. The plantlets were transferred to poly cups containing a mixture of sand, sterilized soil and vermicompost in the ratio of 1:1:1. After proper acclimatization, the plants were gradually exposed to sunlight for attaining normal growth.

Results and discussion
Explants can be of various types used in the regeneration such as nodal segments, apical meristems, roots, cotyledons, embryo, leaf disc, leaf blade, pedicle, petiole, anther, ovary etc. (Aruna *et al*., 2012) [3] used various explants from *Caralluma lasiantha* for multiplication, among them only those of node and shoot tip showed positive morphogenic response. Various efficient micropropagation protocols were developed by excised nodes from various plants such as *Pistacia vera, Bacopa monnieri, Toddalia asiatica*, *Sida cordifolia* and *Boerhaavia diffusa* (Benmahioul *et al*., 2016; Dixit and Thakur, 2017; Anand *et al*., 2015; Sivanesan and Jeong, 2007; Biswas *et al*., 2009) [4, 5, 6, 7, 8]. In the present study, endemic and endangered medicinal plant *Anaphyllum wightii* was multiplied using nodal explant (Figure. 1). The shoot regeneration efficiency of nodal explants through direct organogenesis using three different cytokinins *viz* BAP, TDZ and Kin at different concentrations (0.2-0.5 mg/l) was studied. Totally 10 explants are studied in each and every concentration. The emergence of adventitious shoot bud was observed 8-10 days after inoculation. The results obtained are shown in Table. 1. Based on the observation it was found that BAP enhances more shoot initiation rather than TDZ and Kin. The nodal segments are placed on to the MS medium supplemented with 0.3 mg/l BAP shows maximum percentage of response 90 % when compared to all other different concentrations and hormones. Least percentage of response value 40 % was observed in 0.3 mg/l kin concentration and MS medium supplemented 0.4 and 0.5 mg/l kin shows no response. Initiation of shoot buds was noticed within 7 days. These results are in agreement with the results of (Rambabu *et al*., 2016; Sarma and Tanti, 2017; Mahipal *et al*., 2015) [9, 10, 11] they also reported that BAP shows efficient percentage of shoot induction than the other hormones. Sarma and Tanti (2017) [10] reported the highest mean number of shoots per culture were 6.2 ± 0.44 in combination of BAP and NAA, but the highest shoot length was found 4.02 ± 0.1 in 2.0 mg/l BAP. In the present study, among all other hormones average shoot length was found more in explants treated with BAP 0.4 mg/l 3.9±0.6. In TDZ, hormone highest shoot length value 3.0±1.3 was found in 0.4 mg/l and minimum 1.9±1.1 was found in 0.2 mg/l concentration. The kin hormone supplemented explants show moreover similar value in 0.2 and 0.3 mg/l concentration that is 2.3±1.2, 2.2±1.6 and no response was in 0.4 and 0.5 mg/l concentration. (Yucesan *et al*., 2007) [12] documented BAP was nearly twice more effective than Kin in terms of both the mean number of shoots per explant and the mean percentages of explants producing shoots. Similar findings were noticed in the present study, the data collected from this study is in agreement with the above report. In the present study also, BAP shows great potential of shoot induction than other hormones and least values were observed in Kin concentration. The explants treated with BAP shows maximum number of shoot induction in (4.0±0.8) 0.4 mg/l concentration. There is no response in explants which are treated with kin 0.4, 0.5 mg/l concentration, and minimum 1.0±0.3 was observed in 0.3 mg/l concentration of kin. Compared to BAP and TDZ, Kin shows decreased number of shoots. The results of this study were coinciding with (Prasad *et al*., 2004; Izabela Weremczuk-Jeżyna, 2018) [13, 14] they have been observed that BAP is more effective for shoot proliferation than other cytokinin. The root regeneration efficiency was examined using different cytokinin's *viz* IBA (0.2, 0.3, 0.4, 0.5, 0.8 mg/l), NAA and IAA (0.2, 0.5 1.0, 1.2, 1.5 mg/l) at different concentrations. The shoots supplemented with 0.4 mg/l of IBA hormones shows maximum 80% response and minimum 20% was observed in 0.2 mg/l of IBA. Increased concentration of IBA shows (Above 0.8) poor response. The results obtained are shown in Table. 2. Hormone applications in different doses shows great impact on the root induction. Similarly, (Anand *et al*., 2015; Sarma and Tanti, 2017; Otroshy *et al*., 2011; Haddadi and Aziz, 2010) [6, 10, 15, 16] documented among different concentration of various hormones IBA shows maximum potential in root induction values than the others. (Yucesan *et al*., 2007) [12] noticed increasing concentrations of both IAA and IBA, however, resulted in steady decreases for all of the parameters recorded. Callus development was also observed in this experiment, IBA induces more callus than IAA. The data obtained from this study also coincide with above report of Yucesan. Among all the hormones IBA shows maximum number of root induction followed by IAA and NAA. The highest number of root induction 3.8 ± 1.8 was acquired in IBA 0.4 mg/l concentration. However, the increased concentration of IBA (Above 0.8 mg/l) drastically affect the root induction. Increased concentration of IBA leads to the callus formation instead of root induction. The results revealed that lower concentration of IBA shows more effectiveness in the root induction. Lowest number of root induction 1.1 ± 0.7 was observed in 0.2 mg/l concentration of NAA. (Yucesan *et al*., 2007) [12] studied different concentration of IAA and IBA, on the rooting percentages of *Cichorium intybus*, among them IAA showed to be much more effective than IBA. In the present study also average root length was found increased in explants

supplemented with IBA hormone. The highest root length 3.5±0.2 was found in plants which are treated with 0.4 mg/l of IBA and minimum 1.1±1.0 was in 0.8 mg/l concentration. Shoots treated with NAA shows maximum value of root length 1.9± 1.5 at 1.2 mg/l concentration and minimum 0.8± 0.3 was in 0.2 mg/l concentration. The maximum value of root length found in IAA hormone is 2.1± 1.5 in 1.2 mg/l and minimum value 1.0± 0.3 was in 0.2 mg/l concentration.

The well rooted plantlets are carefully taken out from the culture vessel, washed with the tap water, then transferred to soil and subjected to a hardening process at green house condition for a period of one week at 28ºC with 90% humidity.

Regenerated plants were successfully acclimatized to soil and about 65% of the regenerates survived under natural conditions.

Table: 1 Effect of PGRs on shoot multiplication from the nodal explants of *Anaphyllum wightii*

S.No	Plant Growth Regulators (mg/l)			Number of explants studied	% of response	Average Number of shoots per explant	Average shoots length (cm)
	BAP	TDZ	Kin				
1	0.0	0.0	0.0	10	-	-	-
2	0.2	0.0	0.0	10	65%	2.8±1.2	3.1±0.1
3	0.3	0.0	0.0	10	90%	3.0±1.1	3.5±1.2
4	0.4	0.0	0.0	10	80%	4.0±0.8	3.9±0.6
5	0.5	0.0	0.0	10	70%	3.3±1.4	2.4±1.6
6	0.0	0.0	0.0	10	-	-	-
7	0.0	0.2	0.0	10	60%	1.8±1.2	1.9±1.1
8	0.0	0.3	0.0	10	76%	1.0±1.5	2.4±1.5
9	0.0	0.4	0.0	10	80%	2.5±1.0	3.0±1.3
10	0.0	0.5	0.0	10	70%	1.5±1.5	2.5±1.5
11	0.0	0.0	0.0	10	-	-	-
12	0.0	0.0	0.2	10	55%	1.0±0.8	2.3±1.2
13	0.0	0.0	0.3	10	40%	1.0±0.3	2.2±1.6
14	0.0	0.0	0.4	10	-	-	-
15	0.0	0.0	0.5	10	-	-	-

Table 2: Effect of PGRs on roots induction from *in vitro* derived shoots of *Anaphyllum wightii*

S.No	Plant Growth Regulators (mg/l)			Percentage of response	Average Number of roots induction per shoot	Average roots length (cm)
	IBA	NAA	IAA			
1	0.2	0.0	0.0	20	2.7 ±0.7	1.1± 1.3
2	0.3	0.0	0.0	40	3.0± 0.3	2.7±1.0
3	0.4	0.0	0.0	80	3.8± 1.8	3.5±0.2
4	0.5	0.0	0.0	70	2.3±1.4	2.4±1.6
5	0.8	0.0	0.0	60	1.8±0.1	1.1±1.0
6	0.0	0.2	0.0	40	1.1 ±0.7	0.8± 0.3
7	0.0	0.5	0.0	50	1.3± 0.3	1.0± 1.1
8	0.0	1.0	0.0	70	2.8± 0.6	1.5± 0.3
9	0.0	1.2	0.0	60	2.2±0.2	1.9± 1.5
10	0.0	1.5	0.0	50	1.2±0.3	1.0± 1.6
11	0.0	0.0	0.2	40	1.4±1.05	1.0± 0.3
12	0.0	0.0	0.5	50	2.2±1.03	1.0± 1.1
13	0.0	0.0	1.0	50	2.6±1.14	1.8± 0.3
14	0.0	0.0	1.2	60	2.9±1.23	2.1± 1.5
15	0.0	0.0	1.5	40	1.9±1.16	2.0± 1.6

Fig 1: *In vitro* micropropagation of *Anaphyllum wightii* Schott. through nodal culture.

a-d & g) *in vitro* shoot induction and elongation; e & f) Root induction & elongation; h & i) Hardening

Conclusion

Tissue culture plays a vital role in the conservation of rare, endangered and critically endangered species, which is hard to multiply by natural methods. This study will be the seed for breeding and conservation of *Anaphyllum wightii*.

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