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INDIRECT ORGANOGENESIS OF *ANDROGRAPHIS ECHIOIDES* (L.) NEES

Dr. T. Francis Xavier and D. Sathish Kumar*

Department of Botany, St, Joseph's College, Tiruchirappalli-620 002, Tamilnadu, India.

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***Corresponding Author**
Dr. Sathish Kumar
Department of Botany,
St, Joseph's College,
Tiruchirappalli-620 002,
Tamilnadu, India.

ABSTRACT

In present study, suitable protocol for callus induction and regeneration for the valuable medicinal plant species, *Andrographis echioides* has been developed by micropropagation technique. Leaf and node explants inoculated on MS medium containing the growth regulator (2,4-D 3.0mg/L) + (NAA 2.0mg/L) respectively showed higher callus induction (Leaf explants 90%) and (node explants 95%). The amount of the callus responded for shoot formation grater was leaf callus explant 93% (28.14±4.05) and shoot callus explant 87% (22.30±3.30). When the sub cultured on to the MS medium containing BAP (3.0mg/L) the best shoot formation leaf callus explant 84% (22.07±4.55) and shoot callus explant 89% (29.85±6.93). When the sub cultured on to the MS medium containing KN (5.0mg/L) the elongation shoots were rooted well on MS medium supplemented with IBA (1.5mg/L) and IAA (2.0mg/L). Regenerated plantlet were successfully acclimatized and hardened off in green house condition with better survival rate of 70%.

KEYWORDS: *Andrographis echioides*, growth hormone, indirect organogenesis.

INTRODUCTION

Medicinal plants are the richest source of drugs for traditional medicines, nutraceuticals, food Supplements, folk medicines, pharmaceutical intermediates.[1] *In vitro* micro propagation technique provides many advantages over conventional propagation methods. Micro propagation through shoot tip culture, often utilized to maintain clonal fidelity would be a special advantage in this technique.[2] To over pass this difficulty and for species protecting the species from extinction *in vitro* propagation method was introduced and this technique plays an important role in supply of medicinal plant to pharmaceutical industries and contribute much to conservation of species.[3] Approximately 80% of the people in the

world's developing countries rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts.[4] *A. echioides* is an herb widely distributed in the dry districts of tropical India and Srilanka.[5] The whole plant extract is applied topically over fungal infections, to control hair fall, snake bite, cuts and wounds.[6] Leaf juice boiled with coconut oil used to control falling and graying of hair.[7] The most economically-successful tissue culture technique is the alternative means of plant vegetative propagation known as micropropagation. The most significant advantage offered by micropropagation over conventional methods is that in a relatively short time and space a large number of plants can be produced from a single individual independently of the seasons.[8] hence, the present research works on evaluating the seed germination under open and *in vitro* conditions and also to standardize the techniques for *in vitro* culture of *A. echioides* has been conducted.

MATERIALS AND METHODS

Plant collection and identification

Fresh seeds were collected from healthy mature plants and free symptoms of disease the plant ***Andrographis echioides* (L.) Nees** was collected from Thuraiyur region of Trichy District.

Seeds sterilization

Collected seeds were thoroughly washed with tap water for 15-20 minutes to remove any extraneous materials followed by immersion in detergent solution for five minutes. After washing with distilled water, explants were again washed in 70% alcohol for few seconds and rinsed three times with distilled water. The explants were brought to the inoculation chamber and surface sterilized with 0.1% $HgCl_2$ (Mercuric Chloride) for 3-5 min and again washed with sterile distilled water for 5- 7 times.

Culture Medium

Basal MS (Murashige and Skoog)[9] medium along with the various hormone composition were used. The pH of the medium was adjusted to 5.6 - 5.8 before adding agar (8.0gm/L).

Sterilization of Culture Media

The sterilized of MS medium with various concentrations of growth hormones were dispensed in culture tubes (20ml). The culture tubes were then plugged with cotton and autoclaved at 121ºC for 15 min. After autoclaving, the culture tubes were left undisturbed

until the medium was solidified. Then culture tubes were transferred to the inoculation chamber after inoculation.

Inoculation

Before starting inoculation, all the required equipment's /materials (sterilized forceps, petri plates, sterile blade, Scalpel, sterile distilled water and spirit lamp) were transferred to laminar air flow chamber. The surface and two sides of the chamber were swiped with alcohol and the door was tightly closed. Then the UV light was switched on for 15min. After that, the equipment's were sterilized by dipping in 95% alcohol followed by flaming and cooling. Before starting the inoculation, hands were cleaned with alcohol and the inoculation was carried out in vicinity of the flame. The sterilized explants were placed on the medium at the center of culture tubes.

Incubation

Incubated cultures containing vials were marked with necessary information regarding media, explants, date of inoculation etc. incubated on the culture room. Cultures were maintained at 25 ± 2ºC with a photoperiod of 16 hrs light and 8 hrs dark per day of fluorescent light (3000 lux) for all treatments. Subcultures were made once in 15-20 days.

Seed germination

Surface sterilized seeds were explanted on to MS culture medium supplemented with 3%sucrose, 100mg/L myo-inositol, 0.8%/L agar, culture were maintained on culture room at 25±2ºC and pH adjusted at 5.6-5.8 using 5-7 seeds per flask for germination under aseptic condition and the seedlings were allowed to grow for 35 days. Leaf and node were excised from these seedlings and used for micropropagation,

Callus induction

Leaf and nodal explants excised from 35-days seedlings germination grown *in vitro* were inoculated on MS media supplemented with different concentrations of 2,4-D and NAA in combination with each other (Table.1) for callus induction.

Multiple Shoot induction

Calli formed after 35-days of culture were sub cultured on MS media containing different concentrations/ combination of 6-BAP and Kinetin (Table.2) to induce multiple shoots.

Multiple root induction

Multiple shoots formed were transferred to MS media with different concentrations / combination of IBA and IAA (Table.3) to induce multiple roots formed.

Acclimatization

The *in vitro* regenerated plantlets with well-developed shoots and roots were washed thoroughly in running tap water and transferred to paper cup containing sand, red soil and vermicomposting (1:1:1).

RESULT AND DISCUSSION

Seed germination

The present study shows that seed germination started after 1 week and by 35 days 90% well germination was recorded (Fig.1). The reports suggest that successful seed germination in basal MS medium and shoots were elongated after 25 to 35 days. The enhance seed germination through *in vitro* culture was found useful in securing seedling when seeds are limited and the germination seeds are a good source of explant material for subsequent mass micropropagation.[10] The seedling (foliage leaf and node) used as explants for mass multiplication the foliage leaf samples were cut into small pieces and epicotyl node cut small pieces and transferred to MS medium supplemented with different plant growth hormone concentration.

Table 1: Different types of callus formation from leaf and nodal explants of *Andrographis echioides* (L.) Nees.

S. No.	Plant Growth Regulators (PGRs)		No. of explants Inoculated / Response		% of callus induction		Type callus color formation	
	NAA (mg/L)	2,4-D (mg/L)	Leaf	Nodal	Leaf	Nodal	Leaf	Nodal
1.	2.0	1.0	07/20	09/20	35	45	Friable yellow	Friable yellow
2.	2.0	2.0	11/20	12/20	55	60	green	White
3.	2.0	3.0	18/20	19/20	90	95	Friable yellow	Friable green
4.	2.0	4.0	16/20	15/20	80	75	Compact white	Compact green
5.	2.0	5.0	15/20	17/20	75	85	Friable green	Friable yellow

LEAF EXPLANT

Callus induction

Leaf explant when cultured on MS medium supplemented with different kind of auxins (2,4-D and NAA) in various concentration (1.0 – 5.0 mg/L) induced callus. The callus induction started from cut end of leaf explant after two weeks of inoculation. Although callus induction occurred two combination of auxins, maximum callus (80%) formation occurred in medium supplemented with 2,4-D (4.0mg/L) + NAA (2.0mg/L). 2,4-D and NAA in various concentration produced loosely arranged friable yellow callus. The response of leaf explant of *Andrographis echoides* different concentration of 2,4-D and NAA were observed. Callus initiation was achieved in shortest time (thirty five days) on the medium containing 3.0mg/L of 2,4-D + 2.0mg/L of NAA from explants and observed highest percentage of callus induction (90%) and the color of calli was friable green callus (Table.1). The presence of auxins and cytokinins is necessary for indirect organogenesis. The procedure for plant multiplication involves callus induction from explant and shoots stimulation and development.[11]

Shoot induction and elongation

Multiple shoots were initiation from callus using different concentration of BAP (1.0-5.0mg/L) and KIN (1.0-5.0mg/L). The best short induction (28.14±4.05) per explants for leaf was observed on BAP (3.0mg/L) and (22.07±4.55) explant observed on KIN (5.0mg/L).(Table.2). The elongations of shoots were achieved on the same medium. The highest number of shoot/callus from leaf explant similarly result were observed in *Triticum aestivum*.[12]

NODAL EXPLANT

Callus induction

Successful callusing in the most basic step in indirect organogenesis for callus induction MS medium supplemented with different concentration of 2,4 -D (1.0-5.0mg/L) combination of NAA (2.0mg/L) was used. The primary callus induced from nodal explants was sub-cultured. For further proliferation and developing embryonic callus the frequency of explants forming embryonic callus, degree of callus growth, color and texture were recorded after seven to ten weeks of culture. Repeated sub-culturing was done after every two weeks for maintained and proliferation of the calli. The quantitative and measurement of the callus growth was estimated in term of percentage of callus growth. MS medium supplemented with 2,4-D

(5.0mg/L) combination with NAA (2.0mg/L) produced maximum percentage of callus (80%), and increased the response (90%) suitable hormone concentration of 2,4-D (3.0mg/L) with NAA (2.0mg/L) (Table.2). several compositions of the culture medium have been formulated for different plant species. However, Murashige and Skoog medium (MS medium) supplemented with suitable plant growth regulators (PGR's) was found to be versatile in callus induction and growth.[13]

Shoot induction and Elongation

Shoot regeneration response was found to be dependent on the type of cytokinins as well as it concentration. The fast growing nodular calli produced on BAP (2.0mg/L) and KIN (3.0mg/L). started to develop green pigmentation and further nodulation within six days of transfer to the regeneration MS medium containing combination of first multiple shoots was recorded after 40 days.(Fig.1) in BAP (3.0mg/L) best shoots formation (87%) for nodal explants. Whereas maximum number of shots per culture (22.30±3.30) in KIN (5.0mg/L) best shoot formation (93%) for nodal explants, high number of shoots per culture (29.85±6.93) (Table.2). BAP was proved to be better to stimulate multiple shoot induction in number of plant species *Eclipta alba*.[14]

Table 2: Shoot Formation from Leaf and Nodal Explants of *Andrographis echioides* (L.) Nees.

S. No.	Plant Growth Regulators (PGRs)		No. of explants Inoculated / Response		% of shoot induction		No. of shoots per explants Mean ± SD	
	BAP (mg/L)	Kin (mg/L)	Leaf	Nodal	Leaf	Nodal	Leaf	Nodal
1.	1.0	00	6/15	5/15	40	33	29±4.85	24±2.73
2.	2.0	00	8/15	8/15	53	53	28.62±3.81	24.5±2.72
3.	**3.0**	**00**	**14/15**	**13/15**	**93**	**87**	**28.14±4.05**	**22.30±3.30**
4.	4.0	00	13/15	11/15	87	73	29.46±5.45	24.81±5.94
5.	5.0	00	09/15	07/15	60	47	26.22±4.43	24.14 ±4.94
6.	00	1.0	04/15	04/15	27	27	25±2.16	23.25±1.70
7.	00	2.0	07/15	06/15	47	40	24.14±2.41	23.81±3.65
8.	00	3.0	09/15	09/15	60	60	23.55±3.04	27.77±5.23
9.	00	4.0	11/15	11/15	73	73	23.36±2.01	26.54±5.55
10.	**00**	**5.0**	**13/15**	**14/15**	**87**	**93**	**22.07±4.55**	**29.85±6.93**

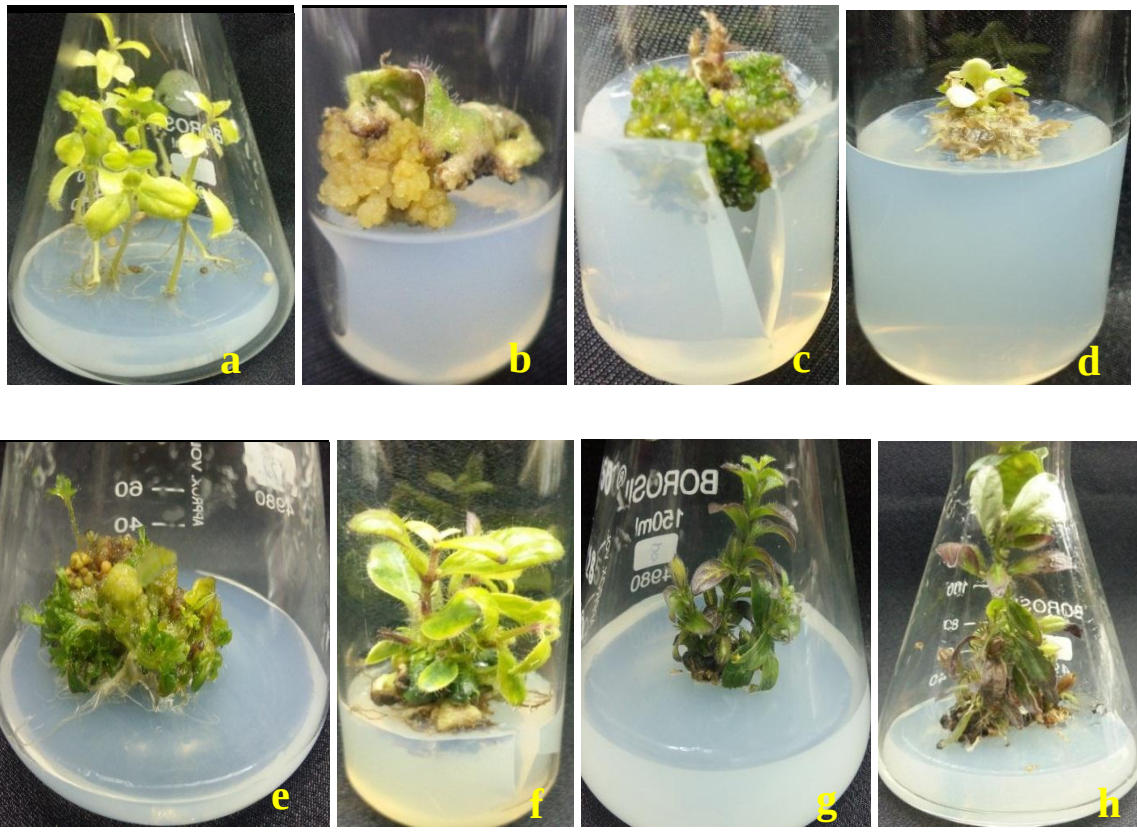
ROOTING

After elongation, the shoots were harvested and transferred to rooting medium. Well-developed shoots from various explants (leaf and nodal) were excised and transferred to

rooting medium MS containing IBA and IAA. Were used in the range of IBA 0.5 to 3.0 mg/L and IAA 1.0mg/L roots induction occurred in two weeks of culturing with best root induction on MS medium containing 1.5mg/L (IBA) combination with 1.0mg/L (IAA) (Table.3). Rooted *in vitro* shoots on the MS medium containing 1.0mg/L IBA and IAA.[15]

Table -3: Roots formation of *Andrographis echioides* (L.) Nees.

S. No.	Plant Growth Regulators (PGRs)		No. of explants Inoculated / Response		% of root induction		No. of roots/shoot Mean ± SD	
	IBA (mg/L)	IAA (mg/L)	Leaf	Nodal	Leaf	Nodal	Leaf	Nodal
1.	0.5	1.0	2/7	3/7	28	42	8.2±2.1	7.5±1.5
2.	1.0	1.0	3/7	4/7	42	57	9.3±1.4	8.1±2.0
3.	**1.5**	1.0	4/7	5/7	**57**	**71**	**10.4±3.4**	**9.2±1.0**
4.	2.0	1.0	5/7	6/7	71	85	10.2±1.1	6.3±2.1
5.	2.5	1.0	3/7	3/7	42	42	9.2±1.4	8.3±2.1



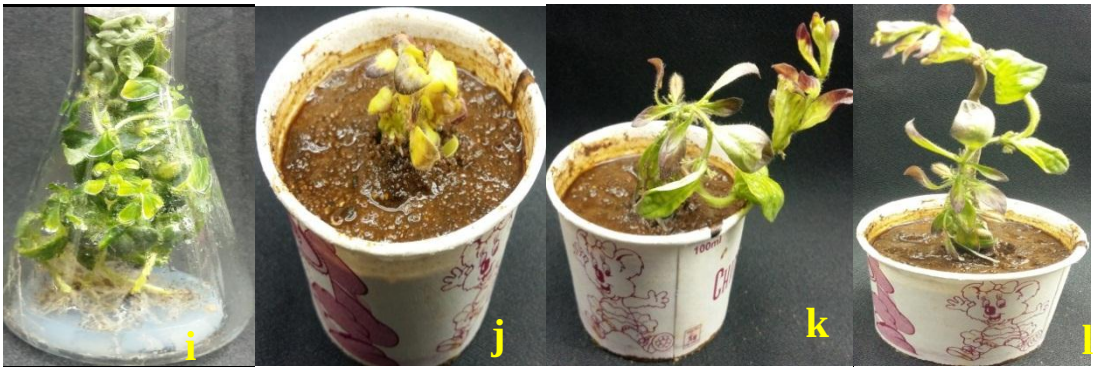


Fig -1: Indirect organogenesis of *Andrographis echioides* (L.) Nees a- Seed germination; b – Compact yellow leaf callus; c – Friable green nodal callus; d - Leaf callus explant shoot formation; e - node callus explant shoot formation; f-leaf explant shoot elongation; g and h- node explant shoot elongation; I - shoot elongation; j, k and l - acclimatization of the *in vitro* grown plant in natural soil.

ACCLIMATIZATION

The well-rooted plantlets were transferred to plastic cups containing sterile sand, red soil, and vermiculite (1:1:1) and covered with paper cup. The plantlets were maintained under controlled environmental conditions for fifteen days and were irrigated with water once in two days9 during this period to avoid wilting. Paper cup were gradually removed and the survived plantlets (70%) were subsequently transferred to clay pots containing composition mixture as mentioned above and grown in greenhouse (Fig.1). Plants are therefore, allowed to grow on rooting media for about 4 weeks after root initiation. During this phase the nutrients in the culture go on gradually depleting and plantlets become sturdy and easy to acclimatize in the greenhouse.[16]

CONCLUSION

An efficient *in vitro* propagation protocol has been developed for *A. echioides* in the present study. Callus formation and a very good number of shoots were multiplied and almost all the shoots were rooted using *ex vitro* method. It reduced time, energy and cost of production of micropropagated plantlets and increased the chances of survival in the field condition. This protocol can be used for mass scale production of superior and disease free plants which could be replanted in the forest area and supplied to the farmers at reduced cost as standard stock planting material.

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