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STANDARDIZATION OF PROTOCOL FOR MICROPROPAGATION OF NYCTANTHES ARBORTRISTIS THROUGH TISSUE CULTURE TECHNIQUE

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ABSTRACT

An efficient protocol for shoot induction and multiplication of *Nyctanthes arbortristis* was achieved from apical and axillary meristems on Murashige and Skoog (MS) basal medium supplemented with 1.0–3.0 mg/l 6-benzylamino (BAP), and 3% (m/v) sucrose. Addition of Naphthalene acetic acid (NAA) along with BAP and Adenine sulphate shows maximum rate of shoot multiplication. Maximum mean number of microshoots per explant (6.65) was achieved on the MS medium supplemented with 2.0 mg/l BAP, 100 mg/l Ads and 0.5 mg/l NAA after 4 weeks of culture. The elongated shoots rooted within 13 to 14 days on strength MS medium supplemented either with naphtylacetic acid (NAA) with 3% sucrose. Maximum percentage of rooting was obtained on medium having 0.25 mg/l IBA, 0.1 mg/l IAA and activated charcoal. About 70% of rooted plantlets survived in the greenhouse. The *in vitro* raised plants were grown normally in the soil condition. This result will facilitate the conservation and propagation of the important medicinal plant.
Keywords: *in vitro*; shoot multiplication; growth regulators; medicinal plants

INTRODUCTION

The India Ayurveda is the oldest system of medicinal system has reported alone used 2,000 plants species followed by Siddha and Unani medicinal system. Approximately 25 % of plants drugs and other synthetic drugs prepared from prototype compounds derived from plant species in modern pharmacopoeia. In India the medicinal plants of high valued have been more exploited and was declined from the plant population over the years. Use of natural resources, as medicinal purpose also causes the medicinal plant in threatened. The growing of social and economical importance of biological resources on one hand and decreasing the survival on the other hand, makes imperative for the government to manage the important floras as a national treasure, must be protected and conserved.

The plant *Nyctanthes arbortristis* belongs to family Oleaceae distributed throughout India, Bangladesh, Indo-pak subcontinent and south east, grows naturally in Indo Malayan region, Burma, Thailand and Celon is a small woody tree, commonly grows up to 10 meter in height mainly grown in home gardens as an ornamental plant for its fragrant white flowers. The plant is also known as Parijata, Shefali, Harshringar, Kannika, Coral Jasmine and Night. The plant is native to India, widely distributed from sub Himalayan regions to Gogavari. (***Wikipedia* 2012, *Sandhar H.K., Kaur M.* 2011**).

The plant *Nyctanthes arbor-tristis* contains high amount of alkaloids specially in leaves and roots. The important active principles of this plant are iridoid arbortristoside has well known for the treatment of cancer as shows pronounced anti cancer activity (***Iyer et al***; 1999). It is investigated that the plant contains phenyl ethanoid derivatives and iridoid glycosides (***Jensen et al***; 2002). The whole plant and leaves also contains many important useful phyto chemicals like β-sitosterol, nyctanthic acid, olenoic acid, ascorbic acid, saponins, flavaonoids, anntannins and also many glycosides like D-mannitol, Astragaline, Nicotiflorine, flavonoglycosides, which shows anticancer and antidiabetic activities. **(*Iyer et al*; 1999, *Nanu et al;* 2008).**

Nyctanthes can propagate by seeds but due to poor seed germination on account of the presence of Phenolic compounds and alkaloids in the pericarp and seed coat (***Bhattacharya et al***; 1999), and due to the death of young seedlings under natural conditions (Anon 1998). Also due to destruction of habitat the natural population of this medicinal plant has been considerably reduced. The different approaches used for propagation *in vitro* culture provides new means of rapid propagation and conserving of valuable, rare and endangered medicinal plants (***Karuppusamy and Pullaiah 2007***). Thus the present study has been designed to develop an efficient and reproducible protocol for *in vitro* propagation which could be used for mass multiplication, conservation and to meet the increasing requirement.

MATERIALS AND METHODS

Collection of explants:

Collection of plant material is the first step for the micropropagation starts with the selection of plant tissues (explant) from a healthy, vigorous mother plant. The shoots of ***Nyctanthes arbortritis*** were collected in the month of January - February from the plant grown in the garden of medicinal park.

Nodal segments, for axillary and apical meristems of 5-10 centimeter length were excised and washed under running tap water and then washed thoroughly in sterile double distilled water (DDW). These explants were then kept in 1.0% Bavistin (BVN) a fungicide for 30 minutes followed by 5.0% (v/v) Teepol (Qualigens Fine Chemicals, India), a liquid detergent for 30 minutes by continuous shaking method. The treated explants were washed in sterile double distilled water (DDW) 4 to 5 times to remove the chemical inhibitors. Surface sterilization treatment was conducted in a laminar air flow chamber with 0.1% mercuric chloride solution for 5-10 minutes. After this procedure rinse with sterile distilled water for two to three times and were cut into 0.5 cm long segment and inoculated on different mediums.

For shoot initiation experiment from mature explants the surface sterilized nodal explants were inoculated on Murashige and Skoog's (MS) media supplemented with different concentrations (1.0-3.0 mg/l) of BAP (6-benzylaminopurine) and (1.0- 5.0 mg/l) KN (kinetin) either alone or with different combinations of (0.5 mg/l) NAA (α-naphthaleneacetic acid), IBA (Indole Butric acid)**.** The cultures were incubated in a culture room at 25±2^{0}C under 16 hours photoperiod provided by cool white fluorescent tubes **(Phillips India).**

Experiments were carried out to check the effect of different concentration of cytokinins BAP (6-benzylaminopurine) with 25-100 mg/l AS (Adenine sulphate) either alone or with different combination of auxins like (0.5 mg/l) IBA (Indole-3-butyric acid), (0.5 mg/l NAA) (indole-3-aceticacid) and (0.5 mg/l). The growth response of explants was studied at weekly interval. The parameters were taken as the average number of shoots initiated and multiplied, and the length of regenerated and multiplied shoots were recorded.

For root induction after multiplication phase the regenerated multiplied shoots with apex were separated, isolated and transferred on MS half strength medium containing different concentration of auxins like IAA (indole-3-aceticacid) (0.5-1.5 mg/l), 0.5-2.0 mg/l NAA (α-naphthalene acetic acid) and 0.5-1.5 mg/l IBA (Indole-3-butyric acid) with the addition of activated charcoal (100 mg/l) The growth parameters were observed as per percentage of root initiation, length in centimeters (cms) and number of roots formed. For hardening of *in vitro* raised plants of ***Nyctanthes arbortritis*** the well developed rooted shoots /plantlets were taken out from the culture medium flasks and washes thoroughly with running tap water to remove all traces of medium attached to the roots. Finally the plants were planted in pots containing mixture of soil, sand and farmyard in the ratio of (1:2:1) for acclimatization and were maintained in green house.

RESULTS AND DISCUSSION

Shoot Induction Experiments

Experiments were conducted to test the effect of cytokinins like BAP (6-benzylaminopurine) KN (Kinetin) alone and also along with auxins NAA (α-naphthalene acetic acid) supplemented in Murashige and Skoog (MS) medium on shoot bud induction, response from axillary and apical meristem explants.

Apical and first three node explants from mature plants are more active showed 80% sprouting within 10-15 days. The axillary buds of the newly sprouted branches, which were nearer to the apical bud, were more responsive. After the growth of about three or four axillary buds, the stems become woody and such buds showed less response in shoot initiation culture. The standardized time duration for surface sterilization of explants with 0.1% $HgCl_2$ solution was optimized at 4 - 5 minutes, shows 70-80% sterilized cultures.

Shoot induction was stimulated by the media with BAP (6-benzylaminopurine) concentrations ranging from 1.0-3.0 mg/l. Among all concentrations of BAP (6-benzylaminopurine) tested, maximum shoot induction (65.0%) was achieved on medium fortified with 1.0 mg/l BAP (6-benzylaminopurine) alone. The percentage of bud break, number of shoots and mean shoot length decreased by using concentration below or above 1.0 mg/l BAP (6-benzylaminopurine) results decrease in the rate of shoot initiation ability with the formation of callus on lower sides of explants**.**

Shoot induction was stimulated on the MS media containing KN (kinetin) concentrations ranging from (1.0-3.0 mg/l). The addition of KN shows not very effective in shoot initiation, maximum shoot induction (50.0%) was achieved within 20 days when fortified with KN (kinetin) 1.0 mg/l about 2.5±0.7 number of shoots were initiated and shoots were 3.5±0.9 centimeters in length**.** By using concentration above (1.0 mg/l) results decreases in the rate of shoot initiation ability with the formation of callus on lower sides of explants**.**

While BAP (6-benzylaminopurine) and KN (Kinetin) along with auxin NAA (α-naphthalene acetic acid) showed a significant variation in terms of bud break, number of shoot induced per explant.

The axillary buds of the newly sprouted branches, which were nearer to the apical bud, were more responsive. Maximum (70.0%) shoot induction was observed on medium supplemented with 1.0 mg/l BAP (6-benzylaminopurine) when combined with 0.5 mg/l NAA (α-naphthalene acetic acid) of 3.0±0.8 number of shoot buds were observed having length of 6.8±0.2 centimeters within 20 days culture. **(Table. 1, Figure: 18, 19).**

The initiated shoots were further transferred to fresh medium for multiplication experiment.

Table 1. Effect of MS Medium and Growth Regulators on Shoot Induction from axillary and apical meristem of N. *arbortristis.*

S. No	MS Medium+PGR			% of shoot induction	No. of shoots per explants	Number of shoots per explants (Mean± SE)	Shoot length in cm	Shoot Length in cm (Mean± SE)
	BAP	KN	NAA					
NI_1	0.5			**60**	**2**	3.0±2.45	2	2±0.35
NI_2	1.0			**65**	**3**	4.0±0.21	2	5.0±0.2
NI_3	2.0			**60**	**3**	4.0±2.47	2	2±0.35
NI_4	2.0		0.5	**50**	**4**	3.0±0.8	2	2±0.26
NI_5	3.0		0.5	**50**	**2**	2.7±0.8	2	2±0.46
NI_6	3.0			**40**	**2**	1.7±0.8	1	2.4±0.1
NI_7		1.0	0.5	**60**	**2**	4.0±2.0	2	2±0.32
NI_8		2.0	0.5	**50**	**2**	3.0±1.0	1	1±0.38
NI_9		3.0		**50**	**2**	2.0±1.2	1	1±0.36
NI_{10}		3.0	0.5	**60**	**3**	3.0±1.24	1	1±0.30
NI_{11}	1.0		0.5	**70**	**2**	4.0±1.05	3	1±0.25
NI_{12}		2.0	0.5	**50**	**2**	3.5±0.8	1	1±0.20
NI_{13}	2.0		0.5	**65**	**1**	3.7±0.2	3	1±0.23
Values Represent Mean± SE- Standard Error								

Shoot Multiplication Experiment:

The effect of BAP (6-benzylaminopurine) alone or in combinations with adenine sulphate was studied on multiplication and growth response of shoot clusters. Cultures showed best shoot multiplication directly from the meristem without any callus formation.

The maximum multiplications of axillary shoots were observed on medium supplement with BAP and AS compared to BAP alone. About 70% of cultures showed multiple shoot formation in the medium having 2.0 mg/l BAP with 50mg/l of AS.

Whereas **5**0.0 % of shoot multiplication was achieved on medium fortified with 1.0 mg/l of BAP alone in which only 5.64±0.11 number of shoots of 6.2±0.06 centimeters in length were observed. (**Table. 2, figure. 20-23)**

Cultures showed highest percentage (80.0%) of shoot formation with an average of 15.3±0.9 adventitious shoots, directly from the nodal region, without any callus formation on medium supplemented with 2.0 mg/l BAP (6-benzylaminopurine), 100 mg/l AdS (adenine sulphate) and 0.5 mg/l NAA (Napthalene acidic acid). Shoots formed in the average number of **18.0±0.1** were remarkable healthy and achieved the length of 11.2±0.8 centimeters in 25 days. (**Table. 2, Figure.**).

Rooting Experiments

To obtain full plants the shoots must be transferred to a rooting medium, which is different from the shoot multiplication medium, especially in its hormonal composition.

In the present study, the role of different auxins like IAA (indole-3-aceticacid), NAA (α-naphthalene acetic acid) and IBA (Indole-3-butyric acid) were tried in the root induction.

MS full medium without growth regulators did not promote any root induction. The induction of roots has been observed in every medium tried. Maximum (90.0%) root induction has achieved directly from the base of the shoots on medium supplemented with 50 mg/l **(AC)** Activated Charcoal having average length 9.0±0.5 centimeters within 15 days. **(Table. 8, Figure. 43- 48)**. Minimum (35%) percentage of root induction was observed on higher (1.0mg/l) concentration of IAA (Indole-3-butyric acid). Among the various concentrations and combinations of Murashige and Skoog (MS) basal medium supplemented with 50 mg/l Activated Charcoal and 1.0-2.0 mg/l IBA (Indole-3-butyric acid) shows best root formation protocol for healthy roots within minimum time period.

Table. 2. Effect Of MS Media And Growth Regulators on Shoot Multiplication of *N. arbortristic*

Media code	MS+Auxin/cytokinin (mg/l).				%age of shoot multiplication.	No. of days.	Mean No. of shoots.	Shoot length in cm.±SE.
	BAP	AS	NAA	IBA				
Nm_1	2.0	0.0			**50.0**	**20**	5.64±0.11	6.2±0.06
Nm_2	1.0	50			**55.0**	**25**	5.0±0.08	6.0±0.9
Nm_3	1.0	100			**60.0**	**15**	9.6±0.92	6.4±0.08
Nm_4	1.0	0.0			**50.5**	**20**	5.3±0.1	6.3±0.9
Nm_5	1.0	100			**55.5**	**25**	6.0±0.7	5.0±0.5
Nm_6	1.0	100	0.5		**70.0**	**30**	4.5.±0.5	3. 2 ± 0.8
Nm_7	1.0	100		0.5	**60.0**	**25**	15.0±0.9	10.7±0.6
Nm_8	2.0	100		0.5	**70.0**	**30**	3.8±0.5	4.5±0.0
Nm_9	2.0	50		0.5	**30.0**	**30**	6.5±0.9	7.5±0.4
Nm_{10}	2.0	100	0.5		**80.5**	**35**	18.0±0.1	11.2±0.8
Nm_{11}	2.0	50	0.5		**70.0**	**30**	5.56±0.10	10.5±0.3

Table. 8. Effect of MS Media and Growth Regulators on Root Induction of *Nyctanthes arbortristis:*

Media code	MS+ Growth Regulaors. (mg/ l).				% age of Root formation.	No. of Days.	Mean Root length in cm.±SE.
	IAA	IBA	NAA	AC			
Nr_1	0 .25	0.0			**60.0**	**20**	**4.0±0.3**
Nr_2	0.50	0.0			**70.0**	**15**	**7.0±0.6**
Nr_3	1.0	0.0			**60.2**	**20**	**5.3±0.8**
Nr_4		0.25			**50.0**	**25**	**3.5±0.0**
Nr_5		0.50			**70.0**	**20**	**6.5±0.6**
Nr_6		1.0			**55.0**	**25**	**3.8±0.2**
Nr_7			0.25		**40.5**	**30**	**2.5 ±0.7**
Nr_8			0.50		**65.0**	**30**	**5.0±0.1**
Nr_9			1.0		**53.0**	**25**	**4.0±0.2**
Nr_{10}				25	**50.0**	**30**	**2.0±0.2**
Nr_{11}				50	**90**	**15**	**9.0±0.5**
Nr_{12}				100	**60**	**15**	**2.5 ±0.7**

4.6. Hardening and Planting of Tissue Cultured Raised Plants

Healthy plants with well developed roots (5-7 cm) from the shoot multiples were taken out of the culture room and kept for pre- acclimatization at room temperature under diffused sunlight for a period of 2 days.

The transfer of plants from the culture flasks to the soil requires a careful, stepwise procedure. After 25-30 days of culture on rooting media, the rooted plantlets were transplanted to pots or trays for hardening prior to their final transfer to soil. From the 35 plants transferred to mixture of sand, soil and farmyard (2:1:1), more than 25 plants survived and hardened in 3 weeks. A minimal survival rate of 50% was recorded during the months of July and August. However, the plants taken out after October showed a substantial increase in survival percentage.

The axillary meristems proved the bet explants were also used by ***Rout et al;*** (2007) ***Jahan et al;(*****2011)** and ***Bansal S. et al****;* (2012*)* to develop a rapid shoot multiplication protocol. Murashige and Skoog (MS) basal medium with 1.0 - 1.5 mg/l 6-benzyladenine (BA), 50 mg/l Adenine sulphate (Ads) and 3% (m/v) sucrose. Here also inclusion of auxin indole-3-acetic acid (IAA) promoted a higher rate of shoot multiplication. Maximum frequency of root initiation on MS half strength with 2% sucrose and NAA (10.74 µM) has reported by ***Bhansal S. et al****;* (2012*)* while ***Jahan et al;(*****2011)** in 2.0 mg/l of IBA with MS medium.

The different approaches used for propagation *in vitro* culture provides new means of rapid propagation and conserving of valuable, rare and endangered medicinal plants (***Karuppusamy and Pullaiah 2007***). Thus the present study has been designed to develop an efficient and reproducible protocol for *in vitro* propagation which could be used for mass multiplication, conservation and to meet the increasing requirement.

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Figure: 1. Plant in field used for explants collection. 2 & 3. Shoot bud break and initiation on MS + 1.0 mg/l BAP and 0.5mg/l NAA. 4,5,6. Shoot multiplication on MS+ 2.0mg/l BAP,100mg/l AS and 0.5mg/l NAA. 7. Rooted shoots on MS+ 50 mg/l **(AC)** Activated Charcoal . 8. Hardened plant of *N. arbortristis.*

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