In vitro clonal propagation of *Nyctanthes arbortristis* Linn. – a medicinal tree

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ABSTRACT: Rapid shoot multiplication of *Nyctanthes arbortristis* was achieved from axillary meristems on Murashige and Skoog (MS) basal medium supplemented with 1.0–1.5 mg/l 6-benzyladenine (BA), 50 mg/l adenine sulfate (Ads) and 3% (m/v) sucrose. Inclusion of indole-3-acetic acid (IAA) in the culture medium along with BA + Ads promoted a higher rate of shoot multiplication. Maximum mean number of microshoots per explant (6.65) was achieved on the MS medium supplemented with 1.5 mg/l BA, 50 mg/l Ads and 0.1 mg/l IAA after 4 weeks of culture. The elongated shoots rooted within 13 to 14 days on ½ strength MS medium supplemented either with indole-3-butyric acid (IBA), IAA or naphtylacetic acid (NAA) with 2% sucrose. Maximum percentage of rooting was obtained on medium having 0.25 mg/l IBA, 0.1 mg/l IAA and 2% sucrose. 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

Nyctanthes arbortristis Linn. is a valuable medicinal plant which belongs to the family Oleaceae, and grows in the Indo-Malayan region. It is a small medicinal tree with scented white flowers. It is a native of India occurring wild in the Sub-Himalayan region. It is cultivated in gardens for its fragrant flowers (Anonymous 1988). It tolerates moderate shade and is often found as undergrowth in dry deciduous forests. This plant is otherwise called as night jasmine plant. Leaves, flowers and seeds are useful for their medicinal properties and are used for various diseases such as chronic fever, bronchitis, asthma, constipation, grayness of hair, baldness and skin diseases (Kirtikar, Basu 1981). The extracted juice of leaves acts as a cholagogue, laxative and mild bitter tonic. It is given with little sugar to children as a remedy for intestinal ailments. In several cases, it has been found to act efficaciously for malaria fever. The bright orange corolla tubes of the flowers contain a coloring substance nyctanthin, which is identical with ά-Crocetin ($C_{20}H_{24}O_4$) from Saffron. The corolla tubes were formerly used for dyeing silk, sometimes together with Safflower or turmeric. Seeds contain an essential oil similar to that of Jasmine. Nyctantic acid, friedelin, beta-sitosterol and oleanolic acid isolated from leaves were used for antiviral activity (Sharma 2003). However, pharmaceutical companies largely depend upon material collected from natural stands. Due to unrestricted large-scale exploitation of the natural resources, coupled with limited cultivation and insufficient attempts for its replenishment, the natural stock of this species has been markedly depleted. Propagation from seed is unreliable due to poor germination and death of many young seedlings under natural conditions (Anonymous 1988). *In vitro* culture is an alternative method for conservation and propagation of this species, but no report on this plant has been published so far. Hence, this investigation aimed to develop an efficient protocol for micropropagation of *Nyctanthes arbortristis* Linn., an important medicinal plant.

MATERIALS AND METHODS

Plant material: Elongated shoots (4–5 cm long) were collected from field-grown plants of *Nyctanthes arbortristis* and brought to the laboratory with cut ends dipped in distilled water. Stem without leaves were washed in a 2% (m/v) (Teepol; Qualigen, India) detergent solution. Subsequently, surface sterilization was performed in a 0.1% (m/v) aqueous mercuric chloride solution for 15 min. After rinsing 4 to 5 times with sterile distilled water, stems were cut into smaller segments (about 0.5 cm long), each with one node was used as explant source.

Culture medium and culture conditions: Nodal explants were placed on semisolid MS (Murashige,

Skoog 1962) mineral salts plus 555μM myo-inositol, 4.06μM nicotinic acid, 2.43μM pyridoxine-HCl, 0.296μM thiamine-HCl supplemented with various concentrations of cytokinins, i.e. 6-benzylaminopurine (BA: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), kinetin (Kn: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), adenine sulfate (Ads: 25, 50 and 100 mg/l) and auxins like indole-3-acetic acid (IAA: 0.0, 0.10, 0.25 and 0.5 mg/l), 1-naphthaleneacetic acid (NAA: 0.0, 0.10, 0.25 and 0.5 mg/l) for shoot multiplication. The pH of the medium was adjusted to 5.8 prior to autoclaving. Each treatment was represented by 20 cultures and the experiment was repeated three times. The cultures were incubated under a 16-h photoperiod having a light intensity of 55 $\mu E/m^2/s$ from cool, white fluorescent lamps at 25 ± 2°C.

Induction of rooting and acclimatization. Elongated shoots (2–3 cm long) were excised from the culture and transferred to ½ strength semisolid MS medium supplemented with different concentrations of IBA, IAA and/or NAA (0, 0.1, 0.25, 0.5 and 1.0 mg/l) and 2% (m/v) sucrose for root induction. One excised shoot was cultured in each tube (25 × 150 mm) with 15 ml of the culture medium. All the cultures were incubated at 25 ± 2°C under 16 h photoperiod with cool white fluorescent lamps. The percentage of shoots forming roots and the number of roots per shoot were examined periodically up to 4 weeks of culture.

Rooted micropropagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm earthen pots containing a mixture of soil, sand and dry cow-dung manure (1:1:1, m/v) and kept in the greenhouse for acclimatization. The plants were watered at 2-day intervals and were supplied with ¼ strength MS inorganic solution twice a week before being transferred to the open field. The survival rate was recorded one month after the transfer into pots.

Scoring of data and statistical analysis: All the cultures were examined periodically, and the morphological changes were recorded on the basis of visual observations. There were 20 cultures per treatment for shoot multiplication, and each experiment was repeated three times; subculture was carried out in a 4-week interval. The mean percentage of cultures producing multiple shoots and mean number of shoots per culture were recorded after 4 weeks. The percentage of rooting and the average number of roots per shoot were recorded after 4 weeks. The data were analyzed statistically by the Duncan's multiple range test (Harter 1960). Means followed by the same letter within columns were not significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

Shoot multiplication

Meristem growth and multiplication were initiated on the MS medium supplemented with different concentrations of BA, Kn and Ads alone or in combinations. The growth of axillary meristems and their subsequent multiplication could not be achieved on the medium without growth regulators. The media containing kinetin alone or in combination with Ads showed a low rate of shoot multiplication and inhibited shoot elongation as compared to BA alone or BA + Ads (Table 1). The combination of kinetin plus IAA or NAA did not influence the shoot multiplication. The medium having BA in combination with Ads enhanced the rate of shoot multiplication within 2 weeks of culture (Fig. 1A). The medium containing BA + Ads + IAA or NAA produced a significant number of multiple shoots as compared to BA + Ads (Table 1). The medium having IAA or NAA alone had no effect on shoot multiplication or growth. About 78% of cultures showed multiple shoots in the medium having 1.5 mg/l BA, 50 mg/l Ads and 0.10 mg/l IAA (Fig. 1B). The increase of IAA concentration higher than 0.25 mg/l suppressed the rate of shoot multiplication and stunted growth. The maximum number of multiple shoots (6.65) was obtained in the medium containing 1.5 mg/l BA, 50 mg/l Ads and 0.1 mg/l IAA after 4 weeks of culture initiation (Table 2). Similar observations indicating an effect of cytokinin and auxin on shoot multiplication were previously reported in *Clerodendrum colebrookianum* (Mao et al. 1995), *Plumbago* (Rout et al. 1999), *Lawsonia inermis* (Rout et al. 2001), *Ocimum gratissimum* (Gopi et al. 2006). The present findings suggest a high frequency of shoot production from axillary meristems by manipulating the growth regulators. There were differences between treatments both in the percentage of cultures with multiple shoots and in the mean number of shoots per culture. Many authors reported that cytokinin is required in optimal quantity for shoot proliferation in many genotypes but an inclusion of a low concentration of auxin along with cytokinin increases the rate of shoot multiplication (Sharma et al. 1993; Sharma, Singh 1997; Shasany et al. 1998; Rout et al. 2000; Rout 2005). A higher concentration of BA (> 2.0 mg/l) in the culture medium inhibited the growth of the shoots and stimulated small callusing at the basal end. The number of multiple shoots per explant varied from 3.74 to 6.65 between different treatments. The rate of multiplication increased as the number of subcultures increased. The number of multiple shoots

Table 1. Effects of different concentrations of cytokinins (BA, Kn, Ads) added to the MS medium on shoot proliferation from axillary meristems of *Nyctanthes arbortristis* after 4 weeks of culture

MS + growth regulator (mg/l) + 3% (w/v) sucrose			Percentage of cultures showing multiplication[a] ± SE (average number of multiple shoots per culture)
BA	Kn	Ads	
0.0	0.0	0	0
0.5	0.0	0	0
1.0	0.0	0	56.6 ± 1.2 f (2.32 ± 0.6)
1.5	0.0	0	68.2 ± 1.0 h (3.81± 0.7)
2.0	0.0	0	74.4 ± 0.8 i (3.88 ± 0.8)
0.0	1.0	0	24.4 ± 1.1 a (1.82 ± 0.5)
0.0	1.5	0	36.2 ± 1.0 c (2.12 ± 0.7)
0.0	2.0	0	42.6 ± 1.1 d (2.76 ± 0.8)
0.0	0.0	25	28.2 ± 0.8 b (2.11 ± 0.7)
0.0	0.0	50	45.6 ± 1.0 e (3.13 ± 0.8)
0.0	0.0	100	62.2 ± 0.8 g (3.43 ± 0.7)
1.5	0.0	25	76.8 ± 1.4 j (4.52 ± 0.8)
1.5	0.0	50	80.2 ± 1.1 k (4.22 ± 0.5)
2.0	0.0	50	78.4 ± 0.8 k (3.88 ± 0.6)
0.0	1.5	50	46.4 ± 0.8 e (1.85 ± 0.7)
0.0	2.0	50	41.8 ± 0.7 d (1.68 ± 0.6)

[a]Values are mean ± SE; 20 cultures per treatment, repeated three times; means having a letter within a column are not significantly different at 5% level by the Duncan's multiple range test

remained constant after 16th subcultures (data not shown). This was probably due to adaptation of the explants to *in vitro* conditions. Similar observations were reported for *Gentiana kurroo* (Sharma et al. 1993) and *Plumbago* species (Rout et al. 1999).

Induction of rooting and acclimatization

Elongated shoots were excised and placed in ½ strength MS medium supplemented with various concentrations of IBA, IAA or NAA for root induction. Full strength MS medium without growth regulators did not promote root induction; roots were observed in media containing ½ strength MS medium supplemented with NAA, IAA or IBA with 2% sucrose. However, optimal rooting and growth of microshoots were observed in medium containing 0.25 mg/l IBA, 0.10 mg/l IAA with 2% sucrose after 13–14 days of culture without intervening callus (Table 3). The percentage of shoots forming roots

Table 2. Effects of different concentrations of cytokinins and auxins on shoot multiplication from axillary meristems of *Nyctanthes arbortristis* after 4 weeks of culture

MS + growth regulator concentration (mg/l)				% of explants with multiple shoots (mean ± SE)[a]	Mean number of shoots/ explant (mean ± SE)[a]
BA	Ads	IAA	NAA		
0.0	0	0.00	0.00	0	0
1.0	25	0.10	0.00	65.8 ± 1.2 c	5.12 ± 0.5 c
1.5	50	0.10	0.00	78.4 ± 1.1 g	6.65 ± 0.6 d
1.5	50	0.00	0.10	72.6 ± 1.0 f	4.78 ± 0.7 b
1.5	50	0.00	0.25	62.2 ± 1.1 b	4.86 ± 1.0 b
1.5	50	0.25	0.00	66.4 ± 0.8 c	5.34 ± 0.8 c+
2.0	50	0.25	0.00	70.2 ± 1.0 e+	5.28 ± 0.6 c+
2.0	50	0.00	0.25	68.3 ± 0.9 d+	5.12 ± 0.7 c
2.0	100	0.10	0.00	58.8 ± 1.2 a+	3.74 ± 0.6 a+
2.0	100	0.00	0.10	62.1 ± 0.8 b+	3.80 ± 0.8 a+

[a]Values are mean ± SE; 20 cultures per treatment, repeated three times; + callusing at the basal end; means having a letter within a column are not significantly different at 5% level by the Duncan's multiple range test

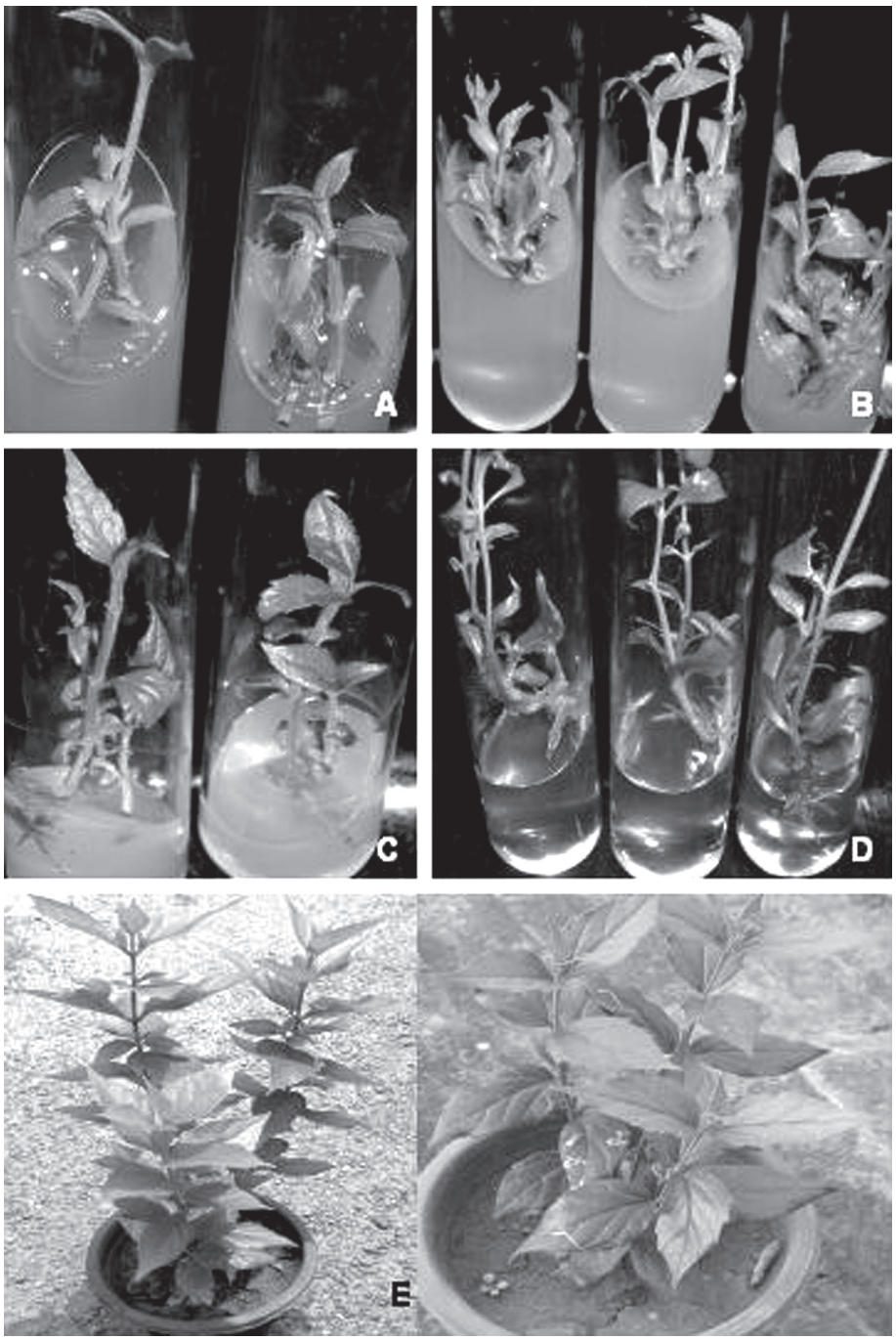

Fig. 1. *In vitro* propagation of *Nyctanthes arbortristis*: (A) Development of axillary shoots from nodal explants of *N. arbortristis* on MS medium supplemented with 1.5 mg/l BA, 50 mg/l Ads after 2 weeks of culture. (B) Development of multiple shoots from nodal explants of *N. arbortristis* on MS medium supplemented with 1.5 mg/l BA, 50 mg/l Ads, 0.1 mg/l IAA and 3% sucrose after 4 weeks of subculture. (C, D) Induction of roots from microshoots of nodal explants of *N. arbortristis* on half strength MS medium supplemented with 0.25 mg/l IBA, 0.1 mg/l IAA and 2% sucrose after one week (C) and two weeks (D) of culture. (E) *In vitro* raised plantlets grown normally in the soil

and number of roots per shoot significantly varied depending on concentrations of IBA. The maximum percentage of rooting (72.6%) was obtained in the medium containing 0.25 mg/l IBA and 0.1 mg/l IAA (Figs. 1C and 1D). Root development was, however, slow at higher concentrations of IBA or IAA. The rooted plantlets were transferred into pots for acclimatization. About 70% of the rooted plantlets survived in the pot one month after the transfer. The plants were grown normally (Fig. 1E).

In conclusion, a successful production of multiple shoots and *in vitro* root formation were dependent on the nutrient medium and the culture conditions. This study might provide new opportunities for

Table 3. Effects of different concentrations of auxins on rooting response of *Nyctanthes arbortristis* after 4 weeks of culture

½ MS + 2% sucrose + growth regulator (mg/l)			Percentage of cultures showing root (mean ± SE)*	Average number of roots/ shoot (mean ± SE)*	Days to rooting
IAA	IBA	NAA			
0.00	0.00	0.00	0	0	0
0.10	0.00	0.00	28.2 ± 0.8	2.32 ± 0.3	18
0.25	0.00	0.00	52.8 ± 1.0	3.24 ± 0.4	16
0.50	0.00	0.00	54.6 ± 0.8+	3.35 ± 0.5	15+
0.00	0.10	0.00	36.4 ± 0.7	2.12 ± 0.6	15
0.00	0.25	0.00	68.4 ± 0.8	4.22 ± 0.7	14
0.00	0.50	0.00	56.3 ± 1.0+	3.54 ± 0.6	14+
0.00	1.00	0.00	+	+	+
0.00	0.00	0.10	0	0	0
0.00	0.00	0.25	26.2 ± 0.8	2.14 ± 0.4	17
0.00	0.00	0.50	34.4 ± 1.0+	2.84 ± 0.5	20+
0.00	0.00	1.00	+	+	+
0.10	0.10	0.00	34.6 ± 0.5	3.25 ± 0.6	15
0.10	0.25	0.00	72.6 ± 0.4	4.32 ± 0.7	13
0.00	0.25	0.1	68.4 ± 0.8	4.38 ± 0.3	14
0.00	0.10	0.25	42.8 ± 0.6	3.18 ± 0.6	17

*Mean of three replications of 15 cultures each; + basal callusing at the cut end

clonal propagation and germplasm conservation of *Nyctanthes arbortristis*.

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In vitro klonové množení léčivé dřeviny *Nyctanthes arbortristis* Linn.

ABSTRAKT: Rychlá multiplikace (zmnožení) výhonů u *Nyctanthes arbortristis* byla dosažena z axilárních meristémů na základním Murashige a Skoog (MS) médiu doplněném o 1,0–1,5 mg/l benzyladeninu (BA), 50 mg/l adenin sulfátu (Ads) a 3% sacharózu. Přítomnost IAA (kyselina indolyloctová) v kultivačním médiu s BA a Ads podpořila vysokou míru multiplikace výhonů. Nejvyšší průměrný počet mikrovýhonů na explantát (6,65) byl obdržen na MS médiu doplněném 1,5 mg/l BA, 50 mg/l Ads a 0,1 mg/l IAA po čtyřech týdnech kultivace. Prodloužené výhony zakořeňovaly v rozmezí 13–14 dnů na ½ MS médiu, doplněném buď o IBA (kyselina indolylmáselná), IAA nebo NAA (kyselina naftyloctová) a 2% sacharózu. Nejvyšší procento zakořenění bylo získáno na médiu obsahujícím 0,25 mg/l BA, 0,1 mg/l IAA a 2% sacharózu. Ve skleníku přežilo kolem 70 % zakořeněných rostlin. *In vitro* vypěstované rostliny rostly v půdních podmínkách standardně. Dosažené výsledky usnadní zachování a rozmnožování významných léčivých rostlin.

Klíčová slova: *in vitro*; multiplikace výhonů; růstové regulátory; léčivé rostliny

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INFORMATION

1st International Scientific Conference on Medicinal, Aromatic and Spices Plants

Date and venue of this conference: 5th–6th Dec. 2007 (Wednesday, Thursday), Slovak University of Agriculture in Nitra

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Description: The conference is organised for the professional community in the sphere of production, processing and use of medicinal, aromatic and spice plants in order to present the latest scientific knowledge. **English** will be the official language.

XIII Specialised Seminar with international participation: **Actual Aspects of Growing, Processing and Use of Medicinal, Aromatic and Spice Plants** will be a part of this scientific conference

Date and venue of this seminar: 7th Dec. 2007 (Friday), Slovak University of Agriculture in Nitra

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Description: The seminar is organised for growers and processors of medicinal, aromatic and spice plants in order to present the latest professional and practical experience. **Slovak** and **Czech** will be the official languages.