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Micropropagation, Isolation and Characterization of Berberine from Leaves of *Naravelia zeylanica* (L.) DC.

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Abstract: An *in vitro* regeneration protocol was standardized using leaf explants of *Naravelia zeylanica* (Ranunculaceae) a rare medicinal plant of the Western Ghats, India. The adventitious shoot buds were organized directly from mid veins and margin of the excised leaf explants cultured on MS-medium fortified with a range of 2.0 to 3.0 mg L^{-1} BAP and 0.3 to 0.7 mg L^{-1} IBA. The frequency of shoot bud organogenesis was the highest (14.9±0.27 shoots per explants) at the concentration of 2.5 mg L^{-1} BAP and 0.5 mg L^{-1} IBA. The excised micro shoots were pretreated in 0.5 mg L^{-1} IBA and transferred to MS half strength semisolid medium induced root initials from the cut ends. A mean of 13.5±0.30 root intact plantlets was recovered per explants showed morphological similarity with the *in vivo* plants. Two hundred and fifty gram of the powdered leaves from *in vivo* plants of *N. zeylanica* was subjected to Soxhlet extraction using methanol. Ten gram of each of the extracts were used for total alkaloid isolation and the yield was 270 mg. An alkaloid berberine was isolated by preparative TLC method using the solvent system methanol, water and ammonium hydroxide in the ratio 8: 1: 1 v/v. The characterization of the constituent was confirmed by IR, ^{1}HNMR and Mass spectral studies.

Key words: *Naravelia zeylanica*, medicinal climber, leaf culture, caulogenesis, berberine

INTRODUCTION

Naravelia zeylanica (L.) DC. (Ranunculaceae) is a woody climber distributed in the Western Ghats of India (Saldanha, 1984). It is reported to be threatened in Southern India (Ravikumar and Ved, 2000). In the Indian system of medicine, Ayurveda the plant has been used in the treatment of pitta, helminthiasis, dermatopathy, leprosy, rheumatalgia, odontalgia, colic inflammation, wounds and ulcers (Praveendhar and Ashalatha, 2003). The root and stem have a strong penetrating smell (Warrier *et al*., 1995) and is used to relive malarial fever and headache. While, root and stem paste is applied externally for psoriosis, itches and skin allergy (Harsha *et al*., 2003). In Kerala, India *N. zeylanica* is used as a source of drug for intestinal worms, skin disease, leprosy and toothache (Sivarajan and Balachndran, 1958). The traditional medicine practitioners residing in the vicinity of Bhadra Wild Life Sanctury, Karnataka, India are using the leaf and stem juices for treating psoriasis and dermatitis. Many pharmaceutical industries in India (Hindustan Liver Ltd., Mumbai; Himalayan Drug House, Bangalore) are engaged in the production of skin ointments from this plant. Biosystematically this species relive much importance, the genus *Naravelia* consists of only two species in Karnataka (Saldanha and Nicolson, 1976). Destruction of the natural habitat and commercial exploitation of this species from natural resources has resulted in the dwindling of populations in the Central Western Ghats of India.

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In vitro propagation is a promising tool for the rapid multiplication of threatened and endangered medicinal plants either through direct organogenesis (Rout *et al*., 2000; Vespasiano *et al*., 2003; Biondo *et al*., 2004) or indirectly through the calli (Faisal, 2003; Li *et al*., 2004; Emma *et al*., 2005). A literature survey indicated that an *in vitro* protocol has not yet been standardized for this rare climber. Recently bioactive compounds isolated from plants relive much importance for the discovery of safe drugs. The earlier investigators (Gilani and Janbaz, 1992; Anis *et al*., 2001; Biswas and Mukherjee, 2003; Pi and Tsai, 2004) isolated berberine from *Berberis aristata* of Berberidaceae. The bioactivity of berberine was pharmacologically screened for antimutagenic, anticancer, antimicrobial activity, diarrhea, intestinal parasite infections, ocular trachoma infections (Lau *et al*., 2001; Marta *et al*., 2002).

In view of its medicinal importance, threatening status, lack of tissue culture reports, the present investigation was undertaken to standardize the protocol for micropropagation and isolation of alkaloid, Berberine from the leaves of *N. zeylanica*.

MATERIALS AND METHODS

Plant Material and Explant Source

Tender leaves of *N. zeylanica* were collected from a healthy plant growing in the University Medicinal plant garden located in Bhadra Wild Life Sanctuary, Karnataka, India. The leaves were thoroughly washed under running tap water for 25-30 min and then rinsed in a solution containing the surfactant Tween-20 (two drops in 100 mL solution). Subsequently, they were surface-sterilized with 0.1% (w/v) $HgCl_2$ solution for 2-3 min, followed by three to five rinses with sterile distilled water in a clean air cabinet. The surface-sterilized explants were aseptically trimmed into transverse segments of 10 to 15 mm than blotted with a sterilized blotting paper and were carefully inoculated on the culture medium.

Culture Media and Culture Conditions

The nutrient medium consisted of MS salts and vitamins of Murashige and Skoog (1962), containing sucrose (3% w/v) as the carbon source and agar (0.8%) as the gelling agent (Hi-Media, India). The pH of the medium was adjusted to 6.0-6.2. About 50 mL of molten medium was dispensed into sterilized 10.5×6.5 cm culture bottles (Varsha Storage Racks, Bangalore, India) and autoclaved at 121°C at 15 Psi (1.06 kg cm^{-2}) pressure for 20 min. All cultures were incubated for 16 h photoperiod provided at 25±2°C under a light intensity of 30 μmol m^{-2} sec^{-2} provided by cool-white fluorescent lamps with 55% relative humidity.

Acclimatization

After twelve weeks of culture root intact plantlets recovered were washed with sterile distilled water and transferred to plastic pots with sterile vermiculite: Perlite: Peat moss (1:2:3 v/v/v) (Dugar Industries, India) for hardening. The plantlets were placed in a growth chamber at 70% relative humidity, 28±2°C under a 12 h photoperiod for acclimatization. The acclimatized plantlets were transferred to the soil and their survival rate was recorded.

Statistical Analysis

For each treatment consists of 10 replicates and a complete randomized design was used in all experiments. The number of shoots per explant and the number of root intact plantlets recovered were statistically evaluated by Analysis of variance carried out using Duncan's Multiple Range Test (Duncan, 1955). Statistical significance was determined at the 5% level, using statistical software SPSS Inc. (Chicago, USA).

Extraction and Isolation of Berberine

Healthy leaves were collected from the *in vivo* plants, dried at shade and powdered mechanically. 100 g of the powdered leaves was extracted with methanol in a Soxhlet apparatus for 48 h. The extracts were concentrated under reduced pressure using rotary flash evaporator (Büchi, Flawil, Switzerland); 10 g of extracts were separately acidified with 2% HCl. The filtered acidic solution was extracted with Et_2O to remove neutral materials and made basic (pH 8-9) with Na_2CO_3 and extracted with CH_2Cl_2 The CH_2Cl_2 solution was concentrated and dried in the desiccator. The crude extract was tested qualitatively for the presence of alkaloids and chromatographed by TLC method using the solvents methanol: Water: Ammonium hydroxide in the ratio of 8:1:1. The low polar fraction was separated by preparative method and their weight was measured. The characterization of berberine was carried out by subjecting the eluted fractions for IR, ^{1}H NMR and MASS spectral studies. The IR spectrum was recorded with KBr pelletes on a Perkin-Elmer 1710 FT-IR spectrophotometer. The ^{1}H NMR spectra was obtained on a Bruker AMX (400 MHZ) Spectrophotometer and the FAB mass spectrum was recorded on a JEOL SX102 Mass Spectrophotometer at Central Drug Research Institute, Lucknow, India.

RESULTS AND DISCUSSION

The leaf segments inoculated onto MS medium augmented with 2.0-3.0 mg L^{-1} BAP and 0.3-0.7 mg L^{-1} IBA showed caulogenic response without interveining callus phase. The type and concentration of cytokinin would have an immense effect on shoot bud organogenesis. In many species cytokinin alone or in combination with lower concentration of auxin provoked direct organogenesis from the leaf explants (Bansal and Pandey, 1993; Sriskandarajah *et al*., 2001). In the present study also synergetic effect of cytokinin and auxin induced adventitious shoot organogenesis from the leaf explant.

The explants retained their photosynthetic activity and became enlarged to thrice of their optimal size. After 15 days of incubation pale greenish photosynthetic protuberances were organized from the margin which were later grew up into shoots without intervening the callus phase (Fig. 1A). Similar mode of organogenesis of the shoot buds directly along the edge of the leaf explant was reported on *Coffea bengalensis* and *Embelia ribes* (Mishra and Sreenath, 2003; Shankarmurthy *et al*., 2004).

The effect of interaction of higher levels of BAP (2.0-3.0 mg L^{-1}) with lower levels of IBA (0.3-0.7 mg L^{-1}) on adventitious shoot organogenesis is depicted in Table 1, in terms of mean number of shoots and mean number of root intact plantlets. However, at increased concentration of IBA (above 0.6 mg L^{-1}) the shoot organogenic potentiality of the leaf explant was hindered. The presence of other hormones, 2,4-D (0.5-2.5 mg L^{-1}), NAA (0.5-1.0 mg L^{-1}) and Kn (0.5-3.0 mg L^{-1}) induced only callus initiation from the explant. One of the possible roles of higher concentration of auxin in the organogenic stage is to nullify the effects of cytokinin on shoot bud organogenesis and elongation. The interaction of BAP with IBA at the concentration of 2.5 and 0.5 mg L^{-1}, respectively proved to be an optimal condition for adventitious shoot bud organogenesis from the leaf explants. In four weeks old culture, shoot buds sprouted from the margin of the explant were grow up well with large photosynthetic leaves (Fig. 1B). After six weeks of incubation small photosynthetic protuberances were arose all over the surface of the lamina and were developed into multiple shoots (Fig. 1C). A mean of 14.9±0.27 shoots were organised per explant. The initial leaves are simple pubescent with dentate lamina, later leaves showed trifoliate characteristic with modification of terminal leaflet into tendril.

The micro shoots when transferred on to auxins supplemented media showed dedifferentiation shoot base into callus mass and from this callus soft root initials with tuft of brownish hairs were organized. But due to lack of root intact vascular connection between the shoot and root system. They were failed to establish into complete plantlets on the contrary, pretreatment of micro shoots on 0.5 mg L^{-1} IBA for 24-72 h and then transferred into MS half-strength medium, showed rhizogenesis

Fig. 1: (A) Adventitious shoot bud organogenesis from the margin leaf explant on MS + 2.5 mg L^{-1} BAP and 0.5 mg L^{-1} IBA, (B) Four weeks old culture showing elongated adventitious shoots with large leaves, (C) Organogenesis of multiple shoots all over the surface of excised lamina, (D) 0.5 mg L^{-1} IBA pretreated micro shoots showing profuse root system and establishment of root intact plantlets and (E) A Hardened and soil acclimatized regenerants

without intervening callus phase. The effectiveness of IBA on root induction has been reported in many of the medicinal plant species (Scaramuzzi and Emerico, 2003; Rani and Grower, 1999; Manickam *et al*., 2000). According to Jutta (2000) the transport velocity of IBA was markedly slower when compared to other auxins like IAA and NAA. This slow movement and slow degradation of IBA facilitates its localization near the site of application and thus, it functions better in inducing roots (Nickell and Kirk, 1982).

After five weeks of incubation the pretreated micro shoots established into a root intact plants with large trifoliate leaves with a terminal leaflet modified into tendril and much branched root system (Fig. 1D). The root intact plantlets recovered were washed with sterile distilled water and transferred to plastic pots with sterile vermiculite: perlite: peat moss (1:2:3 v/v/v) (Dugar Industries, India) for

Table 1: Effect of BAP and IBA on adventitious shoot bud induction and regeneration of plantlets through leaf explant culture of *Naravelia zeylanica*

Growth regulators (mg L^{-1})		No. of shoot buds per explant	No. of rooted plantlets per explant
BAP	IBA	Mean±SE	Mean±SE
2.0	0.3	1.7±0.21^{i}	1.2±0.13^{i}
2.0	0.4	3.1±0.23^{h}	2.2±0.20^{h}
2.0	0.5	5.4±0.33^{f}	3.6±0.30^{g}
2.0	0.6	8.2±0.32^{e}	7.1±0.31^{e}
2.0	0.7	10.1±0.34^{d}	9.3±0.21^{d}
2.5	0.3	12.4±0.30^{c}	11.4±0.22^{b}
2.5	0.4	13.5±0.42^{b}	12.2±0.24^{b}
2.5	0.5	14.9±0.27^{a}	13.5±0.30^{a}
2.5	0.6	13.4±0.40^{b}	12.1±0.37^{b}
2.5	0.7	11.7±0.21^{c}	10.4±0.30^{c}
3.0	0.3	8.9±0.17^{e}	6.8±0.71^{e}
3.0	0.4	8.1±0.23^{e}	5.5±0.22^{f}
3.0	0.5	5.8±0.46^{f}	4.1±0.58^{g}
3.0	0.6	4.1±0.34^{g}	2.3±0.21^{h}
3.0	0.7	1.9±0.17^{i}	1.4±0.16^{hi}
F value:		199.88	165.20

In each column the mean value with different alpha^{b}etical letter(s) are significantly different ($p<0.05$). Uses harmonic mean sample = 10

hardening, subsequently they were transferred to the field condition with the survival rate of 90%. Morphologically they showed all the similarity with the *in vivo* plants (Fig. 1E).

In vitro regeneration is an efficient means of *ex situ* conservation of plant diversity and it assists sustainable maintenance of the present day rapidly dwindling germplasm on long-term basis, especially for the medicinal plants. With this employed technology, many threatened medicinal plants can be quickly propagated and preserved from a minimum of plant material and with little impact on wild populations. Moreover direct organogenesis has the unique advantage of maintaining the genetic stability of a desired taxon. The above protocol is applicable for the *ex situ* conservation of this threatened medicinally important plant species and mass propagated with in a abort time for the production of microbe free plants for the commercial purpose.

The Soxhlet extraction of 100 g of dried leaves yielded 12 g of crude methanolic extract. Ten gram of the crude extract yielded 270.0 mg of total alkaloids from the leaves extract. The presence of the alkaloid confirmed qualitatively by Dragendorff's, Mayer's and Wagner's tests. (Kokate *et al*., 1990) The separation of berberine was done by preparative TLC method and the yield was 70 mg from *in vivo* leaf extract. The constituent was characterized by subjecting it to IR, ^{1}H NMR and MASS spectral studies.

Spectral Characterization of Code RA

IR (Kbr): 2820 cm^{-2} (C-H str)
1597 cm^{-2} (C = C, C = N str)
1354-1383 cm^{-2} (C-H deformation)
1060 cm^{-2} (C-O str) (Fig. 2)

^{1}H NMR ($CDCL_3$): δ 1.75 (m, 2H,-CH_2-)
δ 2.4 (m, 2H, N^{+}-CH_3)
δ 3.9 (s, 6H, 2 X OCH_3)
δ 6.0 (s, 2H, O-CH_2-O)
δ 6.6-9.4 (m, 6H, Ar-H) (Fig. 3)

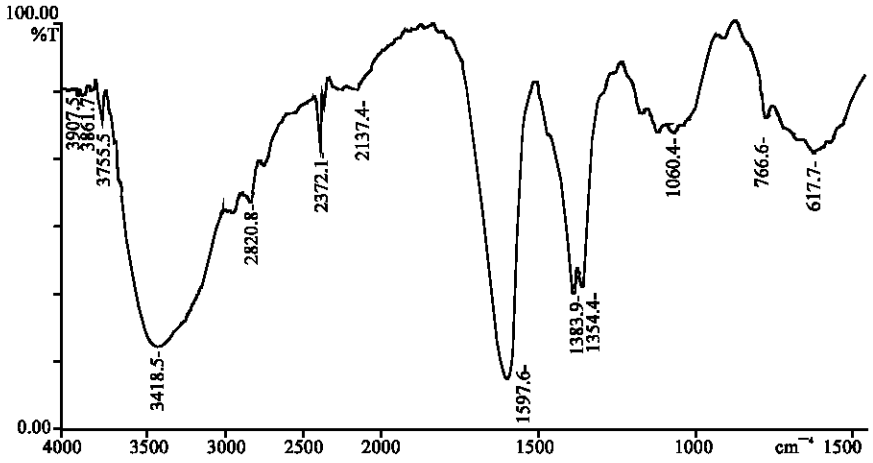

Fig. 2: IR spectrum of berberine

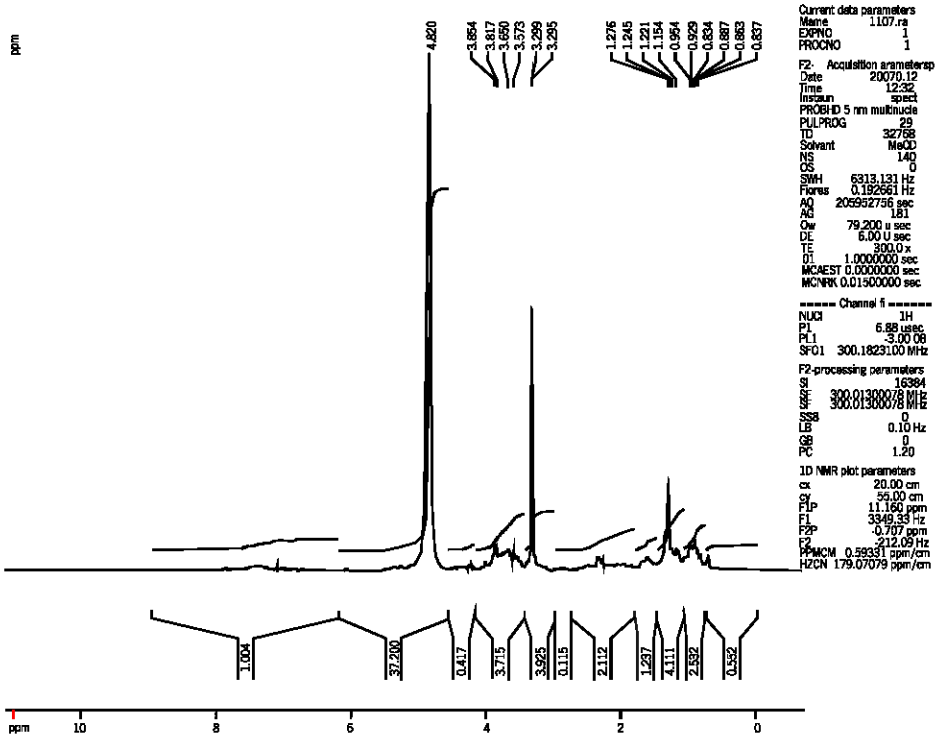

Fig. 3: 1H NMR spectrum of berberine

Mass spectra (EI-MS):

Molecular formula : C20H19NO4

Molecular weight : 336.37

EIMS: (m/z) : 336 (m^+, 20%), 322 (35%), 281 (50%), 267 (38%), 207 (76%), 147 (100%) (Fig. 4)

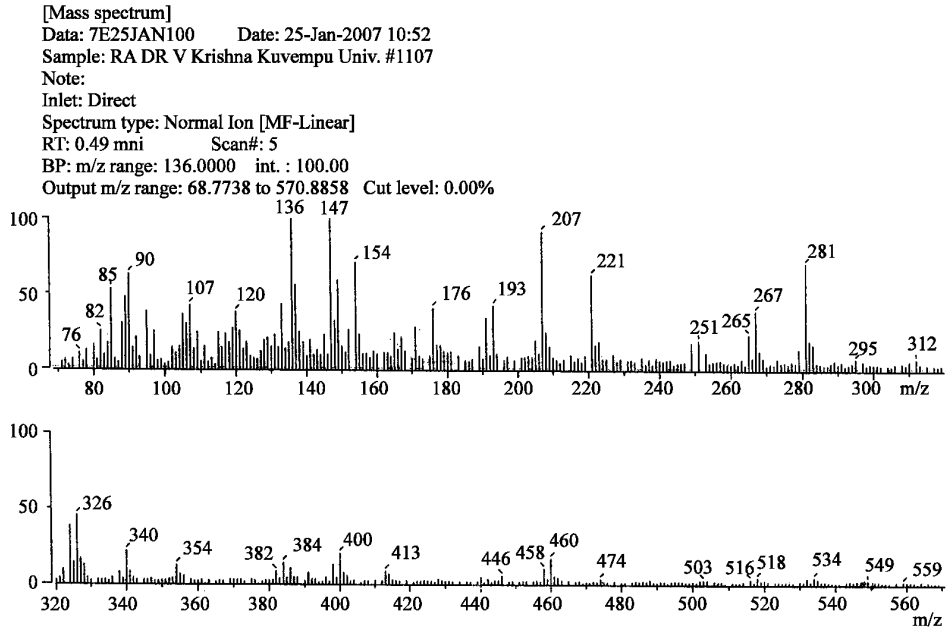

Fig. 4: Structure of berberine

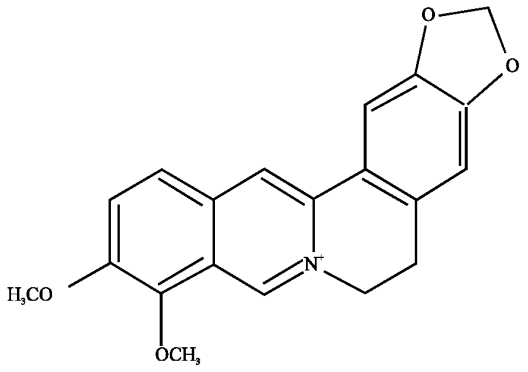

Fig. 5: Mass spectrum of berberine

Based on the above data the compound coded RA was identified as Berberine and it has the following chemical structure (Fig. 5).

Plants are the complex chemical storehouses, which contain array undiscovered biodynamic compounds with unrealized potential for use in modern medicine. It is believed that herbal drugs are relatively safe and exhibit a remarkable efficacy in the treatment of chronic ailments. The therapeutic properties of the medicinal plants are due to the presence of active principles, which has to be extracted and screened for medicinal properties. Due to over exploitation and destruction of natural habitat many of the endemic and threatened medicinal plants species are in the verge of extinction. To circumvent further deterioration of the species in contrast *in vitro* culture offers a sustainable and viable tool for rapid propagation and storage of germplasm. The protocol reported here could be used for the *ex situ* conservation of this endemic and threatened medicinal plant and extraction of anticancerous alkaloid, Berberine from the leaves of *N. zeylanica*. The earlier investigators isolated Berberine from

many plant species and its bioactivity was pharmacologically screened for antimutagenic, anticancer, antimicrobial activity, diarrhea, intestinal parasite infections, ocular trachoma infections and lowers Low Density Lipoproteins (LDL). Further research is under progress for the evaluation of pharmacological activities of Berberine.

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