Abstract
A procedure was developed for plant regeneration of *Hybanthus enneaspermus*, a rare ethnobotanical herb from the Deccan peninsula in India, through seed-derived callus. Seeds demonstrated a high induction frequency (69.4±2.8%) and a high yield (364.4 ±2.5 mg) of light-yellow friable callus on Murashige and Skoog’s (MS) medium containing 2.6 μM NAA and 2.2 μM BA within 4 weeks of incubation. After 1 year of subculture, yellow friable and light-green compact calli types were established from initial light-yellow friable callus. Shoot differentiation was achieved from light-green compact callus, but not from yellow friable callus. Shoot differentiation resulted when light-green compact callus was transferred to MS medium supplemented with 8.8 μM BA and 2.6 μM NAA; the highest percentage of calli forming shoots (66.6 ±4.8%) and the highest number of shoots (8.9 ±0.3) were achieved in this medium. Differentiated shoot buds elongated to 4–5 cm within 4 weeks. The addition of casein hydrolysate (500 mg/l) and more potassium phosphate (1.86 mM ) to the culture medium enhanced shoot differentiation. Rooting was achieved on the shoots using half-strength MS medium containing 4.8 μM IBA. About 70% of the plants were established in pots containing pure garden soil after 2 weeks of hardening. The regenerated plants were morphologically uniform and exhibited normal seed set.

Key words Plant regeneration · Seed-derived callus · *Hybanthus enneaspermus* · Ethnobotanical herb · Rare

Abbreviations MS Murashige and Skoog’s (1962) medium · NAA 1-naphthalene acetic acid · BA N6-benzyladenine · KN kinetin · 2-ip 2-isopentenyl adenine · CH casein hydrolysate · IBA indole-3-butyric acid

Introduction
*Hybanthus enneaspermus* L. Muell, a member of the Violaceae family, is a rare, perennial herb found in some of the warmer parts of the Deccan peninsula in India. Popularly called ‘Ratanpurus’ by the local Yanadi and Santal tribes, villagers and herbalists, this ethnobotanical herb is known to have unique medicinal properties. The preparations made from the leaves and tender stalks of the plant are used in herbal medicine for its aphrodisiac, demulcent and tonic properties. The root is diuretic and administrated as an infusion in gonorrhea and urinary infections (The Wealth of India 1959; Nagaraju and Rao 1996). The fruits and leaves are used as antidotes for scorpion stings and cobra bites by the Yanadi tribes (Raja Reddy et al. 1989; Sudarsanam and Sivaprasad 1995). However, the natural regeneration potential of this herb is very poor due to low seed viability. Because the seeds and developing capsules are often found on the ground, loss due to rodents and inundation is considerable. Increasing human and livestock populations have already affected the status of wild plants, particularly those used in herbal medicine. In view of its ethnomedical importance, there is a need to conserve the wild stock of *H. enneaspermus*. Plant tissue culture is a useful tool for the conservation and rapid propagation of rare and endangered medicinal plants (Saxena et al. 1997; Castillo and Jordan 1997; Sanyal et al. 1998). Plant propagation through callus or suspension cultures requires the induction of an organogenic or embryogenic callus type from explants. Organogenic or embryogenic callus is generally induced from meristematic tissues such as embryos, basal meristems or shoot tips (Bajaj 1992). However, there are a few reports on callus induction and plantlet regeneration using seeds as explants (Lin and Griffin 1992; Mii et al. 1994;...
Griffin and Dibble 1995). To our knowledge, there are only three reports on successful in vitro culture in the family Violaceae, especially on Viola patrinii DC (Han et al. 1990; Kwon et al. 1992; Sato et al. 1995). So far there have been no reports on in vitro culture and plantlet regeneration in the genus Hybanthus. This paper reports for the first time an in vitro procedure for plantlet regeneration from seed-derived callus of H. enneaspermus.

Materials and methods

Plant material and explant source

Mature capsules were collected during November and December from plants of H. enneaspermus growing at the Botanical Garden of the Department of Botany, Sri Venkateswara University, Tirupati, India. Seeds were removed from the capsules and washed with a 1% (v/v) Teepol detergent solution for 15 min. The seeds were then disinfected by immersion in 70% (v/v) ethanol for 1 min followed by immersion in 2% (v/v) sodium hypochlorite solution containing a few drops of Tween-20 for ten min. After five rinses in sterile distilled water, the seeds were transferred onto culture medium for callus formation.

Callus formation

Different concentrations of NAA either alone (1.3–5.3 μM) or in combination with BA (1.1 μM or 2.2 μM) were tested for their effect on callus formation from seeds. MS medium lacking growth regulators served as the control. Data on callus formation was recorded as the percentage of seeds forming callus. Seed-derived callus growth was determined as the final fresh weight after 4 weeks of incubation. Twelve seeds were used per treatment with three replications.

Shoot differentiation

Three sets of experiments were carried out on shoot differentiation. In the first set of experiments, two types of seed-derived calli (yellow friable callus and light-green compact callus) were transferred onto MS medium supplemented with BA either alone (4.4–16.6 μM) or in combination with NAA (2.6 μM) to determine shoot differentiation potential. MS medium lacking growth regulators served as the control. The second and third set of experiments were carried out with light-green compact callus. In these the effects of casein hydrolysate (0–1000 mg/l) and potassium phosphate (0.0–2.48 mM) were tested, respectively, on shoot differentiation during optimal growth regulator treatment. MS medium lacking casein hydrolysate and potassium phosphate served as controls for the Second and third set of experiments, respectively. For all experiments, 12 callus pieces (approximately 300 mg fresh weight) were used per treatment with three replications. Data on percentage of calli forming shoots and number and length of differentiated shoots were recorded after 4 weeks.

Root formation

In vitro differentiated shoots measuring 5–6 cm in length were excised from seed-derived light-green compact callus growing on optimal growth regulator treatment and cultured at four concentrations (1.2, 2.4, 4.8 and 9.8 μM) of IBA along with a control (no auxin) for rooting. Twelve shoots were used per treatment with three replications. Data were recorded on percentage of rooting, root number and root length after 4 weeks on rooting media.

Culture media and incubation conditions

Murashige and Skoog’s (1962) medium supplemented with 3% (w/v) sucrose was used for callus formation and shoot differentiation. Half-strength MS medium supplemented with 2% (w/v) sucrose was used for the rooting experiments. The media were solidified with 0.8% (w/v) agar (Bacteriological grade, Qualigen, India), and the pH was adjusted to 5.8 prior to autoclaving at 1.06 kg cm⁻² for 20 min. All cultures were incubated at 25°C ± 2°C and under a 16-h light/8-h dark photoperiod with a light intensity of 50 μE m⁻² s⁻¹ provided by cool-white fluorescent lamps in combination with incandescent bulbs. Healthy plantlets with 4- to 5-cm-long roots were individually removed from the culture tubes. After washing their roots carefully with tap water, plantlets were transplanted into 10-cm-diameter plastic pots containing a mixture (1:1 v/v) of autoclaved soil and vermiculite. The plants were watered with half-strength MS salts solution every week and covered with a polythene bag for 2 weeks. Afterwards, the hardened plants were gradually transferred to 20-cm pots containing pure garden soil and kept in the field for developing into mature plants.

Statistical analysis

All experiments were repeated thrice. The effects of different treatments were quantified and the data subjected to statistical analysis using ‘standard error of the mean’.

Results and discussion

Callus formation

The seeds of H. enneaspermus failed to produce callus on MS medium lacking growth regulators as well as on MS medium containing only BA (1.1 or 2.2 μM). In contrast, callus initiation was achieved from the seeds within 7 days of incubation on MS basal medium supplemented with NAA either alone (1.3–5.3 μM) or in combination with BA (1.1 μM or 2.2 μM). The callus appeared light-yellow in colour and friable in texture. MS medium containing only BA (1.1 μM or 2.2 μM) stimulated seed germination and produced only seedlings. The concentration of NAA, presence or absence of BA and their interactions influenced induction frequency and the growth of seed-derived callus (Table 1). Seeds showed a lower induction frequency and decreased callus formation on media containing NAA alone (1.3–5.3 μM) than on media supplemented with both NAA (1.3–5.3 μM) and BA (1.1 μM or 2.2 μM) (Table 1). This result supports the synergistic effect of NAA and BA as reported for callus formation from Psoralea corylifolia (Saxena et al. 1997). Media containing lower concentrations of NAA (1.3–2.6 μM) and BA (1.1 μM or 2.2 μM) yielded moderate to high amounts of callus (Table 1). On the other hand, media containing higher concentration (5.3 μM) of NAA in combination with BA (1.1 μM or 2.2 μM) induced decreased amounts of callus. Seeds demonstrated a high induction frequency (69.4±2.8%) and a high yield (364.4±2.5 mg) of light-yellow friable callus.