Industrial Crops and Products 31 (2010) 92–98

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

A rapid *in vitro* multiplication system for commercial propagation of pharmaceutically important *Cyclea peltata* (Lam) Hook & Thoms. based on enhanced axillary branching

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ARTICLE INFO

Article history:
Received 29 June 2009
Received in revised form 22 August 2009
Accepted 11 September 2009

Keywords:
Hardening
Medicinal plant
Menispermaceae
Nodal cutting
Repeated subculture

ABSTRACT

An efficient and quick *in vitro* propagation of *Cyclea peltata* by repeated subculture of nodal cuttings has been standardized. The nodal cuttings were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations (0.5–7 mg/l) of 6-benzylaminopurine (BA) or kinetin (Kn) alone or in combination with indole-3-acetic acid (IAA; 0.5 mg/l) for culture initiation. Optimum response in terms of percent cultures responding, days to bud break and average shoot length was observed on MS medium fortified with 3 mg/l BA and 0.5 mg/l IAA. On this medium 90% cultures responded with 6.1 cm long unbranched vigorous solitary shoots in 45 days. The proliferated primary shoot emerged from the culture initiation step was incised into small nodal explants measuring a size of about 1.5 cm in length containing a single node and subcultured on multiplication medium supplemented with BA (0.5–7.0 mg/l) and IAA (0.5 mg/l). This process was repeated for another three subcultures (each of 45 days) to study the effect of subculturing on shoot multiplication. At the end of third passage 100% of nodal explants produced an average number of 14.2 healthy green shoots on MS medium supplemented with 3 mg/l BA and 0.5 mg/l IAA. The multiplied shoots were harvested and used for rooting on half-strength MS medium containing indole-3-butyric acid (IBA; 1–7 mg/l) or naphthalene acetic acid (NAA; 1–7 mg/l) for 45 days. The best rooting response was achieved on half-strength MS medium supplemented with 5 mg/l IBA. Here 96% cultures responded with an average number of 4.1 roots per shoot. Of the 40 plants transplanted to soil 28 survived (70%). This cost effective protocol will help the mass multiplication of *C. peltata* for commercial propagation.

1. Introduction

Plants play a key role in traditional medicine around the world and it provides crucial contribution to the pharmaceutical industry (Fowler, 1983). About 40% or more of the pharmaceuticals currently used in Western countries are fully or partially derived from natural sources (Rout et al., 2000). However, indiscriminate, extensive and nonregulated collections and harvest of plant materials on a mass scale beyond sustainable limits from the natural habitats always lead to the depletion of plant resources, destruction of plant habitat and extinction of natural populations.

Cyclea peltata is a member of the Menispermaceae family, commonly known as 'Padathaali' or 'Padakkilangu'. The plant grows throughout India and Sri Lanka, up to 800–900 m elevation. It is slender twining shrub, frequently climbing up on tall trees with tuberous roots, peltate leaves, greenish yellow flowers and drupaceous fruits. *C. peltata* is pungent and bitter in taste, and it has antipyretic and astringent properties (Purohit et al., 2003). The plant is used in traditional Ayurvedic medicine and the root of the plant is employed as an important ingredient of 'Hinguvachadi Choornam' which is used to treat gastric ulcer and allied stomach ailments (Lalithamma, 1996). The root of *C. peltata* is also used to treat jaundice and digestive disorders (Valiathan, 2003). The plant parts are also used against malarial disease (Willcox et al., 2004). Due to the high medicinal value of this plant, National Medicinal Plant Board of India identified this plant as "medicinal plant species in high trade sourced from tropical forests" (Ved and Goraya, 2007). Kurichiya tribal people in India use the tuberous roots of this plant along with a little salt to treat stomach pain (Ramachandran and Nair, 1981). There are several reports of the use of various parts of *C. peltata* by Indian indigenous communities like the Kani and Siddis tribes for various medicinal purposes to cure several diseases (Bhandary et al., 1995; Kingston et al., 2007; Vijayan et al., 2007a,b).

Pharmacological study of *C. peltata* was carried out by Kupchan et al. (1961) and isolated *d*-tetrandrine, *dl*-tetrandrine, *d*-isochondrodendrine, and fangchinoline from the roots and found

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doi:10.1016/j.indcrop.2009.09.011

that these compounds have activity similar to that seen with *d*-tubocurarine. In another study, Kupchan et al. (1973) isolated five bisbenzylisoquinoline alkaloids from the roots of this plant. This includes cycleapeltine, cycleadrine, cycleacurine, cycleanorine, and cycleahornine chloride. They determined the structures of these compounds also.

A study conducted to evaluate the protective effect of 70% methanolic leaf extract of *C. peltata* on cisplatin-induced renal toxicity showed that the extract significantly changed the increased malonyldyaldehyde (MDA) level and decreased glutathione (GSH) levels found in rats treated with cisplatin alone. Further the results indicate that the post-treatment of *C. peltata* extract might effectively ameliorate the oxidative stress parameters observed in cisplatin-induced renal toxicity and could be used as a natural antioxidant against cisplatin-induced oxidative stress (Vijayan et al., 2007a,b). In another study the modulatory effect of the root of *C. peltata* on stone formation induced by ethylene glycol treatment in rats is described (Christina et al., 2002). Recently the gastric antisecretory effect of the ethanol extract of *C. peltata* and its antiulcer activities on ethanol-induced, ethanol and indomethacin-induced gastric ulceration and pylorus ligation was investigated in rats (Shine et al., 2009).

There is an increase in interest during the last several years in *in vitro* culture techniques which offer a feasible tool for rapid clonal propagation and germplasm conservation of rare, endangered and threatened medicinal and aromatic plants. Additionally, genetic manipulation is another method to enhance the drug-yielding capacity of the plant (Tejavathi and Shailaja, 1999). Conventionally *C. peltata* is propagated by seeds and poor seed set and seed germination is the major constraint for the conventional propagation of this plant. Thus it is essential to develop an efficient micropropagation technique for *C. peltata* to quickly propagate elite clones once they are identified. However, there is no published information on the successful *in vitro* micropropagation of *C. peltata*. The present investigation describes a reproducible and quick method for the *in vitro* propagation and multiplication of *C. peltata* through high-frequency axillary bud proliferation from nodal cutting explants and the successful establishment of regenerated plants in soil.

2. Materials and methods

2.1. Plant material and culture initiation

Young, actively growing *C. peltata* vines with 4–5 nodes were harvested from about one-year-old plants being maintained in the botanical garden of St. Thomas college, Pala. The nodal cuttings from vines measuring a length of 2.0–2.5 cm long, were rinsed in running tap water for three times and washed in a 2% (v/v) Tween 20 detergent solution for 10 min. The explants were then dipped in 1% savlon germicide for 15 min and washed thoroughly in sterile distilled water for 3 min. Finally the explants were surface-sterilized in a solution of 0.1% aqueous mercuric chloride solution for 8 min and rinsed 3 times with sterilized distilled water. Such surface-sterilized nodal cuttings were slightly trimmed at the cut ends and used as explants. Unless mentioned otherwise, cultures for all experiments grew one explant per test tube, which contained 12 ml of the culture medium. MS medium was selected as basal medium for all experiments.

2.2. Culture conditions, subculture and statistical analysis

Culture tubes were capped and autoclaved at 121 °C for 15 min. Explants were inoculated vertically in culture tubes containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The medium was supplemented with 6-benzylaminopurine (BA; 0.5–7.0 mg/l) or kinetin (Kn; 0.5–7.0 mg/l) alone or in combination with indole-3-acetic acid (IAA; 0.5 mg/l). The shoots developed from the culture initiation step were incised after 45 days into small nodal explants containing a single node measuring a size of about 1.5 cm in length and subcultured on multiplication medium which consists of BA (0.5–7.0 mg/l) and IAA (0.5 mg/l). This process was repeated for another three subcultures (each of 45 days) to study the effect of subculturing on shoot multiplication. After 45 days each culture with explants were observed for shoot multiplication.

The pH of the medium was adjusted to 5.8 before adding 0.7% (w/v) agar and autoclaved at 120 °C and 104 kPa for 15 min. After the explant transfer, the test tubes were placed upright and maintained at 25 ± 2 °C under 16-h photoperiod with 35 μmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent tubes. Each treatment consisted of at least 24 cultures and all experiments were repeated three times. Analysis of variance and Duncan's multiple range test were used for comparison among treatment means.

2.3. Rooting and hardening

In the first experiment, elongated shoots measuring a size of 2.5–3.0 cm taken from the third subculture stage, which exhibited maximum shoot multiplication, were excised and transferred to half-strength MS medium supplemented with 1–7 mg/l indole butyric acid (IBA), or 1–7 mg/l naphthalene acetic acid (NAA) individually. In a separate set of experiments, shoots harvested from the culture initiation stage to the fourth subculture of shoot multiplication were used for rooting on medium containing 5 mg/l IBA for 45 days. In each experiment minimum 24 cultures were raised for each treatment.

Plantlets having 3–4 well-developed roots were taken out from the culture vials and after a thorough washing under running tap water, plantlets were transferred to plastic cups (6 cm diameter) containing autoclaved sand and garden soil (1:1). The pots were initially irrigated with half-strength MS basal salt solution for 4 wk. To maintain 90% humidity, porous polythene sheets were employed to cover the potted plantlets for 1 month. A total number of 40 plants were transferred to the pots. The plants were transferred to shade house after 2 months and eventually to field.

3. Results and discussions

3.1. Culture initiation and shoot formation

Even though the problem of contamination was low during the culture initiation; some cultures showed leaching of phenolic compounds from cut ends of nodal explants into medium. About 12% of cultures showed medium browning after 5 days of inoculation. This was overcome by transferring the explants to new culture tubes in every week until the browning disappeared.

There was no shoot development on MS basal medium. Whereas bud break and growth of single axillary bud development occurred on all cultures in presence of plant growth regulators during the culture initiation step. The percentage of nodal explants sprouting was increased with increasing concentration of cytokinins for both BA (up to 3.0 mg/l) and Kn (up to 5.0 mg/l). However, the percentage cultures responding were low when BA (0.5–7.0 mg/l) or Kn (0.5–7.0 mg/l) alone was employed for shoot proliferation. BA was comparatively better than Kn in terms of percent response. The superiority of BA over Kn for bud break as well as shoot growth and multiplication has been reported in several systems including *Syzygium alternifolium* (Sha Valli Khan et al., 1997) and *Pterocarpus marsupium* (Chand and Singh, 2004a,b). The number of shoots per explants remained to be invariability one in all the treatments. The shoot length of axillary shoots varied from 1.4 to 2.1 cm when