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Somatic embryogenesis in *Cymbopogon pendulus* and evaluation of clonal fidelity of regenerants using ISSR marker

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ABSTRACT

An efficient plant propagation system through somatic embryogenesis was established in *Cymbopogon pendulus*, an aromatic grass followed by analysis of genetic status of regenerants using ISSR markers. Optimum embryogenic callus induction was observed on MS basal medium supplemented with 13.57 μM 2,4-dicholorophenoxyacetic acid (2,4-D) with 8.88 μM N^6-benzyladenine (BA). Subsequent culturing of embryogenic calli on MS medium containing 4.52 μM 2,4-D and 8.88–13.32 μM BA gave maximum number of somatic embryos. Addition of coconut water (CW) promoted induction, growth and differentiation of callus and somatic embryogenesis. Further development of embryos into plantlets was achieved on MS medium supplemented with lower concentration of biotin and calcium pantothenate (CaP) along with BA (4.44–13.32 μM) and kinetin (2.32–4.65 μM). The root meristems were established on half strength MS medium containing 2% sucrose and 2.46–9.84 μM Indole3-butyric acid (IBA) and successfully established in soil with 77.8% survival rate in field condition. Thirteen randomly selected regenerated clones were screened using six ISSR primers. Nine clones produced similar monomorphic amplification profiles while remaining clones showed minor variation with absence of certain parental bands and appearance of unique band. Majority of the regenerants maintained genetic fidelity with the generation of few variants as evidenced from similarity matrix estimates using Nei Li's coefficient of similarity data.

1. Introduction

Plant propagation via somatic embryogenesis not only helps to obtain a large number of plants irrespective of seasons, but also can act as a promising biotechnological tool for crop improvement. Moreover the induction of somatic embryogenesis for *in vitro* plant regeneration provides several advantages over traditional organogenesis (Wang and Bhalla, 2004). *Cymbopogon pendulus* also known as North Indian lemon grass is a rich source of citral. It is commercially propagated as modern cash crop for essential oil production. This seed grown species of *Cymbopogon* is a cross pollinator and permits the scope to harness the hybrid vigour through hybrids, synthetics or composites. However due to the cross pollinating behaviour of *C. pendulus*, field maintenance requires space, isolation barriers and labour. Plant tissue culture precludes these limitations and the specific inbred lines or populations can be cloned *in vitro* with relative ease, not only for an efficient plant propagation, but also to develop somaclonal variants and agronomically superior mutants. Although various documentation of tissue culture studies has been worked out for other species of *Cymbopogon* (Baruah and Bordoloi, 1989; Sreenath and Jagadishchandra, 1991; Nayak et al., 1996), no such reports appear for *C. pendulus*. Again assessment of micropropagated plants at an early stage of tissue differentiation is always a prerequisite criterion to identify important variants grown under tissue culture regime. Several DNA markers like Randomly amplified polymorphic DNA (RAPD) and Inter simple sequence repeat (ISSR), have been successfully employed to assess the genetic stability in regenerated plants including those with no obvious phenotypic alterations (Rani et al., 1995; Rahman and Rajora, 2001). ISSR markers, developed by Zietkiewicz et al. (1994) is based on amplification of a single primer containing a microsatellite "core" sequence anchored at the 3′ or 5′ end by a set of 2–4 purine or pyrimidine residues. This technique is more specific, offers a high degree of reproducibility and a rich level of polymorphism in a relatively simple and low cost procedure, overcoming common criticism against RAPD (Yang et al., 1996). Hence it has been widely used in assessment of genetic diversity, clonal identity (Bornet and Branchard, 2001) and cultivar identification (Prevost and Wilkinson, 1999).

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In the present communication the effects of explant type, growth regulation type, combination and concentration of plant growth regulators (PGR), growth adjuvants and vitamins on propagation via indirect somatic embryogenesis of *C. pendulus* were described. Assessment of clonal fidelity of 13 randomly selected embryo derived plants was also performed using 6 ISSR primers.

2. Materials and methods

2.1. Plant materials and culture condition

Rhizome and leaf segments (each approx. 3–5 mm long) of *C. pendulus* variety, procured from the Regional Research Laboratory (RRL), Bhubaneswar (Orissa), India were used as primary explants. Rhizomes were collected from 11-month-old potted green house plants, freed from roots and apical shoot portion and washed thoroughly with tap water to remove soil. Both mature and young leaf segments were used for culture initiation. The young leaves were light green in colour, smooth, glabrous. The mature leaves are dark greenish in colour, leaf blade 90 cm long, coarse textured due to silica deposition and have flat adaxial surface with frequently occurring papillae and prickle hairs all through the leaf area with abundant microhair. For surface sterilization, explants were treated with 0.1% (w/v) aqueous $HgCl_2$ solution for 3–5 min and rinsed thoroughly in sterile double distilled water and blot dried. The sterilized explants (rhizome and leaf segments) were cut obliquely to remove mature or damaged parts and placed horizontally in MS (Murashige and Skoog, 1962) nutrient agar medium (20 ml/tube and 50 ml/bottle) containing various concentrations and combinations of plant hormones along with other defined and undefined plant growth regulators (PGR) under aseptic condition in a laminar hood. The pH of the medium was adjusted to 5.8 before adding 0.8% agar (Bacteriological grade, Himedia) and prior to autoclaving at 1.1 kg cm^2 pressure (121 °C) for 15 min. All the cultures were incubated under cool white fluorescent light (16 h photoperiod 40 m m^{-2} s^{-1}, Philips, India) at 25 ± 2.0 °C and 60–70% relative humidity (RH).

2.2. Callus Induction and plant regeneration

Effects of various auxins namely 2,4-D and α-napthalene acetic acid (NAA), on callus induction were investigated. Callus induction was achieved on MS basal medium supplemented with various concentrations of 2,4-D (9.05–18.10 μM), NAA (1.34–16.11 μM), either alone or in combinations with BA (1.12–8.88 μM) and 10% CW. For development of somatic embryos approximately 6 weeks old, visually distinguishable, creamy white, nodular embryogenic calli were transferred onto tissue proliferation media [MS basal medium + 1.13–13.57 μM 2,4-D + 2.22–13.32 μM BA + 10% CW]. After another 4 weeks, embryogenic calli, having distinct globular somatic embryos, were subcultured onto the same medium for 2 subsequent weeks. The germination and regeneration of 12 weeks old somatic embryos were achieved on MS regeneration medium supplemented with 4.44–13.32 μM BA, 2.32–4.65 μM kinetin, 0.41–2.0 μM biotin and 0.21–0.63 μM calcium pantothenate (CaP). The regenerated plantlets were transferred to full, half and one-fourth strength MS medium containing 2.46–9.84 μM IBA with 2% sucrose for rhizogenesis. Each experiment was repeated thrice with 10 replications. Optimum regeneration of plantlets was achieved after 3 weeks of transfer of 12 weeks old somatic embryos into regeneration medium and plants were further subcultured for another 3 weeks. The regenerants were initially maintained in half strength liquid MS medium for 12 days under high humidity condition (95% RH). The well rooted shoots were gently removed from the culture vessels, washed under tap water to remove traces of medium, and transferred to plastic pots containing different sterilized potting mixtures, viz. soil:vermiculate:sand (2:1:1); soil:vermiculate:sand (2:2:1); garden soil:organic manure (1:1); sand:soil:compost (2:2:1); and sand:soil:compost (2:1:1), and kept under green house condition (27–30 °C, 85% RH) for 60 days. Thirty plantlets were initially transferred for each treatment combination and each treatment was repeated thrice. The survival percentage of the plants were registered after 10, 30 and 60 days of potting and final data (after 60 days) were statistically analysed using DMRT. Forty-five plants randomly selected from each treatment combination of potting mixtures were subsequently shifted to field condition. Each field establishment process was repeated thrice with 15 replicates each. Survival percentage of the plants were recorded after 45, 90 and 120 days of transferring to the field and final data (120 days) was subjected to statistical analysis by mean separator (DMRT).

2.3. Histological preparation of somatic embryos

Embryogenic masses for conventional light microscopy were prepared according to the method of Jeffree (Jeffree and Read, 1991). For histological confirmation on the initiation and development of somatic embryos, embryogenic callus and somatic embryos at different stages were fixed in aqueous 1% OsO_4 for 3 h. The fixed samples were washed for 30 min, thrice with double distilled water. After washing, the specimens were dehydrated through ethanol series treatment. The specimens were embedded in Spurr's resin (Spurr, 1969) and placed in an embedding oven (70 °C overnight) to cure and blocks were sectioned at 10 μm using a rotary microtome (*Leica*). The sections were mounted onto slides and allowed to dry for at least 10 min before staining. The specimens were stained with hematoxylin–eosin and counterstained with fast green for general histological examinations. Cover slips were mounted with Histoclad mounting medium and dried on a 40 °C hot plate. Permanent slides were examined on a *Zeiss* Universal microscope equipped with a camera.

2.4. DNA extraction and ISSR amplification

The Cetyl trimethyl ammonium bromide (CTAB) method, with certain modifications in the extraction procedure according to Khanuja et al. (1999) was used to extract genomic DNA from leaves and rhizomes of mother plant (used as the source of explants for embryogenesis) as well as from 13 regenerated plants. The regenerants were 18 weeks old and each derived from 13 separate embryogenic callus lines of *C. pendulus*. DNA was isolated at least three times from each regenerants and the quantity and quality of extracted DNA samples were estimated by comparing band intensities on 0.8% agarose gel. Genomic DNA was then PCR amplified using ISSR primers (3′ anchored). The annealing temperature was found to vary according to the base composition of the primers. Total 17 ISSR primers were screened initially out of which 6 ISSR markers were found to generate reproducible, unambiguous amplification profiles. Amplification reaction volumes were 25 μl, each containing 25 ng template genomic DNA, 1× PCR buffer, 130 μM dNTPs, 50 mM $MgCl_2$, 0.3 μl (3 U/μl) *Taq* DNA polymerase, 0.5 μl (50 μM) primer. Amplifications were performed in a thermocycler (*Perkin elmer 2400 gene Amp PCR system)* programmed for an initial denaturation at 94 °C for 4 min followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at a temperature 2 °C lower than melting point for each primer and 2 min extension at 72 °C with a final extension of 72 °C for 10 min (Wolfe et al., 1998). Control reactions containing water in place of genomic DNA were also performed along side in order to verify absence of contamination. The PCR products obtained were separated on 2% agarose gel (Sigma, USA), stained with ethidium