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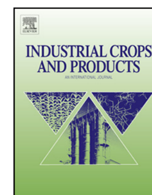

Thidiazuron induced *in vitro* multiplication of *Mentha arvensis* and evaluation of genetic stability by flow cytometry and molecular markers

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ABSTRACT

An efficient and reproducible system for the development of genetically uniform clones of a highly valuable essential oil producing crop *Mentha arvensis* was developed by pre-conditioning of nodal explants with thidiazuron (TDZ) supplemented liquid Murashige and Skoog (MS) media. Multiple shoot formation was achieved by pretreating of nodal explants with 5, 15, 25 or 50 μM TDZ for 24 h, followed by their transfer on TDZ free semi-solid MS media. The maximum response in terms of percent regeneration (92.6 ± 3.65), average number of shoots per explants (23.7 ± 1.45), and greatest shoot length (5.2 ± 0.72) were obtained from nodal explants treated with 15 μM TDZ for 24 h followed by culture of explants on TDZ free MS medium for 8 weeks. Elongated shoots when transferred to half-strength MS medium supplemented with 0.5 μM indole-3 butyric acid (IBA) resulted in 91 ± 2.91% rooting. *In vitro* regenerated plantlets with well-developed shoot and roots were successfully established in pots containing garden soil with a 90% survival rate. Genetic stability of micropropagated plantlets was assessed and compared with mother plant using flow cytometry and molecular markers. No changes in plant genome size/ploidy level, and in directed amplification of minisatellite-region (DAMD) and inter simple sequence repeat (ISSR) profiles were found among the micropropagated plants, which were similar to that of the donor plant, illustrating their genetic uniformity and clonal fidelity. This confirms that clonal propagation of this plant using axillary shoot buds can be used for commercial exploitation of the selected genotype where a high degree of fidelity is an essential prerequisite.

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1. Introduction

Mentha arvensis Linn. (Field mint, wild mint or menthol mint) belonging to family Lamiaceae is a perennial, glabrous, strongly scented herb native to the temperate regions of Europe and western and central Asia, east to the Himalaya and eastern Siberia, and North America (Anonymous, 2003). Traditionally, wild mint used as a domestic herbal remedy because of its antiseptic properties and beneficial effect in digestion. The whole plant is anesthetic, antispasmodic, antiseptic, aromatic, and has agents that counteract inflammation, that relieve and remove gas from the digestive system, induce sweating, promote or assist the flow of menstrual fluid, promote secretion of milk, relieve fever and thirst, give strength and tone to the stomach, and is a stimulant (Chopra et al., 1986). Now it is an important industrial crop for the production of menthol oil that is widely used in cosmetic, pharmaceutical, food and flavoring industries. The menthol oil is a colourless, pale yellow or greenish yellow liquid with a strong odour. Mint extracts and menthol-related chemicals are used in food, drinks, cough medicines, creams and cigarettes. The most common use of wild mint today is as flavoring agent in a variety of oral products including toothpaste, chewing gum and after-dinner mints (Boon and Smith, 2004). Natural resurgence of this plant is mainly by shoot cuttings because of poor seed setting and seed viability. Besides this, in dry places and during summer season, the propagation of this plant is difficult (Maity et al., 2011). Thus an effective strategy is required for rapid and large-scale multiplication of this potential medicinal plant.

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Tissue culture is a potential tool for rapid mass multiplication of plants and holds great promise for controlled production of useful secondary metabolites, but the occurrence of cryptic genetic deficiencies arising by somaclonal variation in regenerated plants can seriously limit the advantage of this system (Larkin and Scowcroft, 1981; Gould, 1986; Smykal et al., 2007; Bhatia et al., 2009; Rathore et al., 2011). It is, therefore, essential to establish genetic uniformity of tissue culture plantlets to suggest the quality of plantlets for its commercial value. Molecular techniques are at present powerful and valuable tool for establishing genetic uniformity of the micropropagated plantlets. Of the various markers polymerase chain reaction (PCR)-based techniques such as directed amplification of minisatellite DNA (DAMD), developed by Heath et al. (1993) and intersimple sequence repeat polymorphic DNA (ISSR) developed by Zietkiewicz et al. (1994) are immensely useful in establishing the genetic uniformity of *in vitro*-regenerated plantlets in many crop species (Ashmore, 1997; Aronen et al., 1999; Silva et al., 2001; Joshi and Dhawan, 2007; Faisal et al., 2012). There are few reports on *in vitro* multiplication of *M. arvensis* via axillary bud sprouting (Chisti et al., 2007; Maity et al., 2011), but there is still no report on the effect of TDZ for its micropropagation and genetic stability. Although, the existing protocols gave very low number of shoots. Recently, thidiazuron (TDZ; (N-phenyl-1,2,3 thidiazol-5-ylurea)), a substituted phenylurea with cytokinin like activity, has been extensively used for plant regeneration (Huetteman and Preece, 1993). TDZ is found to be as or more efficient than 6-benzyl adenine (BA) for shoot induction (Van Nieuwkirk et al., 1987). In the present study, a new approach of preconditioning the nodal explants with thidiazuron supplemented liquid media for enhanced shoot regeneration and plant establishment has been attempted. Furthermore, the genetic stability of the *in vitro* regenerated plants was also assessed first time by flow cytometry and molecular markers (DAMD and ISSR).

2. Materials and methods

2.1. Plant material and surface sterilization

Young, healthy shoots of *M. arvensis* collected from an identified plant were washed under running tap water for 30 min and then soaked in a 5% (v/v) detergent, labolene, for 5 min. After thorough washing, their surfaces were sterilized with 0.1% (w/v) $HgCl_2$ for 3 min and finally rinsed four times with sterile distilled water. Shoot segments measuring 4–6 mm were cut into single node explants and cultured in sterile nutrient media.

2.2. Culture media and culture conditions

The basic nutrient medium consisted of Murashige and Skoog (MS; 1962) salts and vitamins with 3% (m/v) sucrose. Liquid MS medium was supplemented with various concentration of TDZ (5, 15, 25 and 50 μM). The pH of the medium was adjusted by 1 N NaOH or 1 N HCl. The media were solidified with 0.7% (m/v) agar. The culture vials containing the media were autoclaved at 121 °C at 1.06 kg cm^{-2} for 20 min. All the cultures were maintained at 24 ± 2 °C under a 16-h photoperiod with a photosynthetic photon flux density of 50 μmol m^{-2} s^{-2} provided by cool white fluorescent lamps (Philips, Poland).

2.3. Shoot induction and multiplication

MS liquid medium containing different concentration of TDZ (5, 15, 25 and 50 μM) were used for shoot induction. MS medium lacking growth regulators served as control. After an induction period 24 h on TDZ enriched medium, the explants were transferred to the semi-solid medium without TDZ. All cultures were transferred to fresh medium every 2–3 weeks. Different basal media were also examined and compared to detect best medium suitable for maximum shoot regeneration. Woody plant medium (Lloyd and McCown, 1981; WPM), B5 (Gamborg et al., 1968), and Schenk and Hildebrandt (SH; 1972) medium were used for *in vitro* regeneration from nodal explants pretreated with 15 μM of TDZ. The percentage of explants forming shoots; number of differentiated shoots per explant and shoot length was recorded after 8 weeks of transfer to TDZ free media.

2.4. Rooting and acclimatization

For rooting, shoots about 4–5 cm long were excised and transferred to 1/2MS medium supplemented with IAA or IBA (0.1, 0.5, 1 and 2 μM). The percentage of root formation and the mean number of roots per shoot were recorded after 4 weeks. After rooting, regenerated plantlets were washed carefully and transferred to pots containing sterile soilrite under diffuse light (16:8 h photoperiod). Potted plantlets were covered with a transparent polybags to ensure high humidity and watered every 3 days with half-strength MS salt solution for 2 weeks. Polybags were opened after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, these plants were removed from the planting substrates and transferred to pots containing normal garden soil and maintained in a glasshouse under normal day length conditions.

2.5. Flow cytometry analysis

Ploidy level was determined using the Beckman Coulter flow cytometer (Coulter Epics XL/Xl-MCL, USA and System II Software, Version 3.0). Nuclei from approximately 100 mg fresh weight tissue were mechanically isolated by chopping with a sharp scalpel blade no. 21 in 1 ml of Galbraith buffer (Galbraith et al., 1983) (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0). The resulting nuclear suspensions were filtered using double layered 28 μm nylon meshes and supplemented with 50 μg/ml of propridium iodide (PI, Sigma, USA) for DNA staining. RNase (50 μg/ml) (Sigma, USA) was added to prevent staining of double stranded RNA. Samples were incubated for 10–15 min before analysis. All flow cytometer assessments were repeated at least three times for each sample and included a minimum of 5000 nuclei per run.

2.6. DNA extraction and PCR amplification

Genomic DNA was isolated from fresh leaf tissues of micropropagated plantlets and the donor plant using modified cetyltrimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1987). Purified total DNA was quantified and its quality verified by spectrophotometry (6705 UV/vis. spectrophotometer, Jenway, UK), and each sample was diluted to 25 ng/μl in sterile Milli-Q water and stored at 4 °C. Regenerated plantlets were tested for genetic stability using 5 DAMD and 9 ISSR primers ((Gene Link, New York, USA) for their unambiguous and reproducible band patterns.

PCR amplifications were carried out as described by Williams et al. (1990). PCR was performed in a volume of 20 μl containing 25 ng total DNA, 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U Taq DNA polymerase (Fermentas, GmbH, Germany) and 5 pmol primer (DMAD or ISSR). The PCR amplification was performed by using Thermal cycler (T-100™ Thermal Cycler, Bio-Rad, USA). PCR reactions were performed with initial DNA denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C (30 s) for DNA denaturation, 46–57 °C (45 s) for primer annealing, 72 °C (90 s) for primer extension and final extension at 72 °C for 7 min was performed. All the amplified PCR products obtained from DAMD and ISSR markers

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