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High-frequency *in vitro* plantlet regeneration from apical bud as a novel explant of *Carum copticum* L.

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PEER REVIEW

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Comments

This is a good research work in which authors have investigated effects of phytohormonal treatments on regeneration of ajowan and determined the optimal levels of plant growth regulators for efficient shoot bud induction.
Details on Page S427

ABSTRACT

Objective: To develop an *in vitro* regeneration system to increase the recovery of *Carum copticum* L. plantlets as a part of developing a metabolic engineering program.
Methods: The efficacy of different concentrations and combinations of 6-benzyladenine, indole-3-acetic acid and indole butyric acid on direct shoot regeneration and rooting of ajowan from apical bud explants were assessed. All explants were cultured on Murashige and Skoog (MS) medium supplemented with different combinations of 6-benzyl amino purine (BAP) (0, 2.2, 4.4, 8.8 μmol/L) and indole-3-acetic acid (IAA) (0, 0.5, 1.1, 2.2 μmol/L).
Results: The maximum shoot regeneration frequency (97.5%) and the highest number of shoots produced from apical buds (34 shoots per explant) were obtained on MS medium fortified with BAP (4.4 μmol/L) and IAA (0.5 μmol/L). Low shoot regeneration frequency was observed in BAP free treatments. The effects of different strengths of MS medium and various concentrations of IAA and indole-3- butyric acid on rooting rate, length and average number of roots were also investigated. Application of indole-3- butyric acid (6 μmol/L) in full-strength MS medium, was more effective than IAA and resulted in highest shoot regeneration frequency with the rooting rate of 100% and highest mean number of roots per shoot (41.8). The rooted plantlets were acclimatized successfully in greenhouse conditions with a survival rate of 90%.
Conclusion: In this study, a simple and reliable regeneration and acclimatization protocol for *Carum copticum* has been presented. This protocol can be found very advantageous for a variety of purposes, including mass multiplication of *Carum* species, medicinal plant breeding studies and transgenic plant production.

KEYWORDS

Apical bud culture, *Carum copticum*, Direct shoots regeneration, Regeneration frequency, Root induction

1. Introduction

Ajowan [*Carum copticum* L. (*C. copticum*)] belongs to Umbelliferae family, growing around the Mediterranean Sea and in southwest Asia extending from Iraq to India[1]. Ajowan is one of the aromatic seed spices, generally used for medicinal purposes to treat liver disorders and as a digestive stimulant. This plant is also reported to have analgesic and antitussive effects[2,3] as well as antioxidant and antimutagenic activities[4]. Thymol, the major phenolic compound present in ajowan has been reported to be an antispasmodic and antifungal agent[5].

In recent years interests in tissue culture techniques which offer viable tools for mass propagation and germplasm conservation of threatened medicinal plants, were increased. The loss of biodiversity and plantations due to deforestation in combination to the demand from both domestic and export markets have led to the utilization of *in vitro* methods of propagation as tools to meet commercial needs. However,

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the successful genetic transformation of plants depends on an important pre-requisite, the establishment of efficient adventitious shoot regeneration systems in which somatic tissues can develop into whole plants[6].

Different types of explants have been used for *in vitro* direct regeneration of many medicinally important plants. Nodal, petiole, leaf and shoot tip explants were used for micropropagation in *Solanum sarrachoides*[7], *Dipteracanthus prostratus*[8], *Hypericum spectabile* and *Aloe vera*[9,10], respectively. The effects of different phytohormonal combinations and concentrations on shoot bud induction and frequency of shoot regeneration on different plant species were studied[7,9]. Gopi *et al*.[11] reported that using different concentrations and combinations of 6-benzyl amino purine (BAP), Kinetin (Kin), indole-3-acetic acid (IAA) and indole-3- butyric acid (IBA) for direct regeneration, maximum number of shoots (14.3±1.5) was observed on medium containing 0.5 mg/L BAP and 0.25 mg/L IAA after four weeks of culture. Akbas *et al*.[9] claimed that the highest shoot formation using leaf explants, was obtained on Murashige and Skoog (MS) medium containing 1 mg/L BAP and 1 mg/L Kin.

To our knowledge, this paper describes the first report on a successful protocol for regeneration in *C. copticum* L. using apical bud explants under *in vitro* conditions. We studied the effects of several plant growth regulators, including BAP, IAA and IBA, in order to obtain high shoot and root regeneration rate and survival percentage in this species.

2. Material and methods

2.1. Plant material and culture conditions

Seeds of *C. copticum* L., collected from medicinal plants garden of Urmia University, Iran, were surface-sterilized by submerging in ethanol (70%, v/v) for 60 seconds followed by continuous agitation in 5% commercial sodium hypochlorite for 10 min and rinsing three times with sterile distilled water. These seeds were then germinated on MS medium supplemented with sucrose (3%, w/v) and 7 g/L plant agar (Duchefa, The Netherlands) [12]. In all of the experiments, MS medium containing 3% (w/v) sucrose and 0.7% plant agar (Duchefa, the Netherlands) was used as basal medium. The pH of media was adjusted to 5.8 prior to the inclusion of agar and autoclaved for 20 min at 121 °C. IAA and IBA were added to the medium after autoclaving by filter sterilization (0.22 μm, Millipore). All the cultures were kept in growth chambers at (25±2) °C under a 16/8 h (light/dark) photoperiod at a photon flux rate of 60 μmol/m^2/s provided by cool daylight fluorescent lamps.

2.2. Induction of adventitious shoot buds from cultures

Apical bud explants from 2 weeks old *in vitro* germinated seedlings were isolated and inoculated on MS media supplemented with different concentrations of BAP (0.0, 2.2, 4.4 and8.8 μmol/L) and IAA (0.0, 0.5, 1.1 and 2.2 μmol/L). Each treatment contained three glass flasks, each containing ten explants. Explants were sub cultured at 3-week intervals. After 9 weeks of culture, regeneration rates, expressed as the percentage of responsive explants, and number of shoots per responsive explant, were evaluated for each treatment.

2.3. Root induction and acclimatization

For root induction and formation, elongated shoots (5 cm length) were excised and transferred to MS and 1/2 MS media containing different concentrations of IAA (3 and 6 μmol/L) and IBA (3 and 6 μmol/L). Rooting rate and length of roots were recorded for each treatment. Several shoots were maintained on MS and 1/2 MS auxin free medium as control. For *ex vitro* acclimatization, well developed plantlets were gently washed with tap water to remove the remnants of agar and then transferred to plastic boxes containing sterile perlite. The cultures were kept in a plant room with high relative humidity at (25±2) °C under a 16 h day/night photoperiod for 4 weeks. The acclimatized plantlets were finally transferred into greenhouse conditions.

2.4. Statistical analysis

All experiments were set up in a factorial completely randomized design. Three replicates per treatment with 10 explants for each replicate were used for shoot-bud induction. The percentage of regenerated shoots, the number of shoots per explants, the percentage of rooting and length of roots were recorded at the end of rooting experiment. Data were statistically analyzed using the SPSS statistical software. The means were compared using Duncan's multiple ranges tests (DMRT) at the 5% and 1% probability level. Graphs were plotted with the Excel program.

3. Results

3.1. Effect of hormonal combination on shoot regeneration and proliferation

Multiple shoot buds were induced on explants cultured on MS media supplemented with various plant growth regulators and shoots with developing trifoliate were visible after 3 weeks of culture (Figure 1B). Significant differences in regeneration frequency were observed among explants grown on different treatments ($P \leq 0.05$) (Table 1). Comparison of effects of different culture media on shoot induction revealed that the highest rate of shoot induction and regeneration (97.5%) was obtained on MS media containing BAP (4.4 μmol/L) and IAA (0.5 μmol/L) (Figure 1C and D). No shoot regeneration was observed in the absence of BAP or when IAA was used alone. These results showed that BAP free medium was not favorable for shoot formation in Ajowan but higher concentrations of BAP (8.8 μmol/L) also decreased shoot regeneration percentage. Intermediate concentration of BAP (4.4 μmol/L) was more effective for shoot regeneration in comparison to other BAP concentrations used alone or in combination with IAA.

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