Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.com

Document heading doi:10.12980/APJTB.4.2014C529

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

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PEER REVIEW

Peer reviewer

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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.
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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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Foundation Project: Supported by the Urmia University, Urmia, Iran (research Grant No. 477-15).

Article history:
Received 2 Feb 2014
Received in revised form 12 Feb, 2nd reviesed form 22 Feb, 3rd revised form 29 Feb 2014
Accepted 19 Mar 2014
Available online 5 Apr 2014

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.

Figure 1. Effect of plant growth regulators on shoot regeneration and root induction of *C. copticum* L.

A: Apical bud explant of *C. copticum* L. isolated from *in vitro* grown seedlings; B: Shoot regeneration from apical bud explants after 3 weeks of culture; C&D: Regenerated normal shoots on MS medium supplemented with 4.4 μmol/L BAP and 0.5 μmol/L IAA; E: Root formation of *in vitro* regenerated shoots on rooting media; F: Acclimatized plantlet growing in a pot containing sterilized perlite.

Table 1

In vitro shoot regeneration from apical bud explants of *C. copticum* L. on MS medium fortified with different plant growth regulators.

Treatments (μmol/L)		Shoot regeneration (%)	Average number of shoots
BAP	+ IAA		
0.0	0.0	0.00^{o}	0.00^{h}
2.2	0.0	8.82^{h}	1.80^{h}
4.4	0.0	47.05^{de}	9.60^{de}
8.8	0.0	30.29^{f}	6.20^{f}
0.0	0.5	0.00^{o}	0.00^{h}
2.2	0.5	17.64^{g}	3.60^{g}
4.4	0.5	97.50^{a}	19.90^{a}
8.8	0.5	36.76^{f}	7.50^{f}
0.0	1.1	0.00^{o}	0.00^{h}
2.2	1.1	29.55^{f}	6.03^{f}
4.4	1.1	47.24^{de}	9.80^{d}
8.8	1.1	46.47^{de}	9.50^{de}
0.0	2.2	0.00^{o}	0.00^{h}
2.2	2.2	38.23^{ef}	7.80^{ef}
4.4	2.2	62.64^{c}	12.80^{c}
8.8	2.2	71.47^{b}	14.60^{b}

Means in each column followed by the same letters are not significantly different at $P \leqslant 0.05$ by DMRT.

The statistical analysis showed, however, that the applied concentrations of BAP alone or in combination with IAA, made a significant difference in response of apical buds in terms of number of regenerated shoots per explant on the media containing 0.0-8.8 μmol/L BAP and different concentrations of IAA (Table 1). Maximum average number of shoots (19.90 shoots per explant) was observed on MS media containing BAP 4.4 μmol/L and IAA 0.5 μmol/L. On the other hand, reduction of BAP concentration decreased the shoot proliferation rate significantly (Table 1). The minimum shoot regeneration frequency (0.00%) was observed on hormone free media or media without BAP levels. In general, comparison of different treatments pointed out that explants on MS media containing BAP and IAA responded better, in terms of shoot regeneration frequency and number of regenerated shoots per explant, than those supplemented with BAP alone.

Our results confirmed the positive effect of hormones on adventitious shoot regeneration. In this study, the highest regeneration rate (97.5%) was achieved on medium containing 4.4 μmol/L of BAP in combination with 0.5 μmol/L IAA whereas the lowest regeneration rate (0.0%) was recorded for control samples, cultured on media containing only MS media and 0.5, 1.1 and 2.2 μmol/L IAA.

3.2. Rooting of shoots and acclimatization of plantlets

The analysis of variance indicated that auxins used in the experiment had a possitive effect on *in vitro* rooting of regenerated shoots of *C. capticum* L. Root formation was observed in all the media which shoots were regenerated. Root primordial emerged from the shoot bases after two weeks of culture on hormone free medium or medium supplemented with various concentrations of IBA and IAA (Figure 1E). ANOVA results revealed that auxins (IAA and IBA) promoted rhizogenesis of *C. capticum* L. (Table 2), however, rhizogenesis of ajowan was best promoted by IBA and IAA in high concentrations (6 μmol/L) where root regeneration was induced in 100.00% and 98.00% of shoots, while the rooting rate was reported 4.30% for control samples (Table 2). The maximum average numbers of roots (41.80 and 41.20 per shoot) were developed in both above-mentioned treatments respectively (Table 2). Similar results (35.30 and 32.00 per shoot) were obtained on 1/2 MS media fortified with IBA (3 and 6 μmol/L) concentration. The least root formation rate (4.30%) and average number of roots per shoot (1.8 roots per shoot) were exhibited by control samples. These results revealed that using MS medium results better than 1/2 MS medium.

Table 2

Effect of different combinations and concentrations of auxins on root induction of *C. copticum* L.

Rooting medium	Root formation (%)	Average number of roots	Length of root (cm)
MS	4.30^{g}	1.80^{e}	2.84^{a}
MS+3 μmol/L l IAA	33.32^{f}	13.93^{d}	2.45^{b}
MS+6 μmol/L IAA	98.00^{a}	41.20^{a}	1.26^{cd}
MS+3 μmol/L IBA	50.30^{e}	21.06^{c}	1.74^{c}
MS+6 μmol/L IBA	100.00^{a}	41.80^{a}	0.60^{d}
1/2 MS+3 μmol/L IAA	51.43^{e}	21.50^{c}	2.50^{b}
1/2 MS+6 μmol/L IAA	59.33^{d}	24.80^{c}	2.07^{c}
1/2 MS+3 μmol/L IBA	76.55^{c}	32.00^{b}	2.30^{b}
1/2 MS+6 μmol/L IBA	84.44^{b}	35.30^{b}	2.07^{c}

Means in each column followed by the same letters are not significantly different at $P \leqslant 0.05$ by DMRT.

For root induction and its development in regenerated shoots of *C. copticum* L., MS and 1/2 MS media supplemented with various concentrations of IAA and IBA, were compared. The results showed the superiority of 6 μmol/L IBA and IAA in high concentrations and their significant effect on root formation rate, average number of roots per shoot and length of roots (Table 2).

All the regenerants acclimatized well in the greenhouse and then under outdoor conditions (Figure 1F). Survival rate of 90% was achieved when the rooted shoots were transferred to pots containing sterile perlite and irrigated regularly with 1/2 MS salt-solution and tap water. The acclimatized plants did not show any visible variations from the mother plants.

4. Discussions

4.1. Effect of hormonal combination on shoot regeneration and proliferation

Various factors including genotype, explant types and different combinations of growth regulators can influence the successful *in vitro* regeneration of a plant[13-15]. In general, the most effective explant type for direct shoot bud formation is organized explants, *e.g.*, shoot tips, axillary buds, and zygotic embryos. Explants were used for micropropagation in *Manihot esculanta* and *Phyllanthus maderaspatensis* L.[16,17]. Results of other experiments showed that explants obtained from shoot tips were more responsive than those obtained from meristem segments of *Telfairia occidentalis*[18]. It was observed that both applied explant types induced shoots but those from shoot tips developed earlier and responded better than those from meristem. This is one of the advantages of explants over those from the meristem, and it is largely due to the fact that explants are larger in size than meristems[18]. Preliminary experiments using different explants of ajowan documented that apical buds, excised from 2-week old *in vitro* germinated shoots, were more suitable for regeneration than explants obtained from other parts of seedlings (such as hypocotyls, cotyledons or nodal segments).

In vitro morphogenic responses of cultured plant tissues were affected by different components of culture media, especially concentration and combination of plant growth regulators; therefore it is important to evaluate their effects on plant regeneration. It has been reported that a combination of cytokinin and auxin is well suitable for the shoot regeneration and morphogenesis of the annual *Medic* species[19]. Combinations of cytokinin such as BAP and Kin with low level of auxin (*e.g.* IAA or 1-Naphthaleneacetic acid) have also been used to induce shoot formation in numerous plant species[20]. The result of this experiment showed that the intermediate levels of BAP (4.4 μmol/L) along with lower concentrations of IAA (0.5 μmol/L) had a favorable effect on direct shoot regeneration of ajowan plantlets using apical buds as explants. According to Hassani *et al.*[21] the use of auxin in combination with cytokinin, leads to rapid cell division, forming a large number of relatively small and undifferentiated cells.

Our results confirmed the positive effect of combination of BAP and IAA on adventitious shoot regeneration. These results are in agreement with other findings on various medicinal plants[16,22,23]. These findings showed disagreement from the investigation by Namli *et al.* for *Hypericum retusum*[24], where the highest number of shoots was obtained on a medium supplemented with BAP alone. Combination of BAP and IAA was used for shoot culture in *Adhatoda vasica*[25], shoot multiplication in *Hypericum maculatum*[26].

4.2. Rooting of shoots and acclimatization of plantlets

Application of auxins for micropropagated shoots may increase the number of regenerated roots by mounting the endogenous contents of enzymes[27]. Liu *et al.*[28] reported that auxin induced the complicated process of lateral root formation through repetitive cell division. George *et al.*[29] suggested that auxins were essential for the maintenance of polarity of the plants.

In this experiment, high concentrations of auxins are effective for root induction, root formation rate, average number of roots per shoot and length of roots. The same result was obtained in *Hibiscus sabdariffa* L. which highest level of rooting was obtained on MS medium supplemented with 1 mg/L IBA[30]. Many researchers have obtained similar results in some herbaceous plants[9,26,31].

In this study, a simple and reliable regeneration and acclimatization protocol for *C. copticum* has been presented. Low concentration of IAA (0.5 μmol/L) combined with intermediate level of BAP (4.4 μmol/L) showed better results as compared to other concentrations used in regeneration media. The shoot regeneration rate and maximum shoot number were obtained on media containing BAP (4.4 μmol/L) and IAA (0.5 μmol/L). Rooting of ajowan shoots were promoted on MS media supplemented with 6 μmol/L IBA or IAA. 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.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are thankful to Dr. hassani at the Horticulture Department, Urmia University, Urmia, Iran for providing the seeds. The authors gratefully acknowledge the encouragement and support of Bioscience and Biotechnology Research Center of Urmia University, in carrying out the research work. This research is supported by the Urmia University research grant No. 477-15.

Comments

Background

Ajowan is an important medicinal plant containing pharmaceutical bioactive compounds that can be used for possible scale-up production of its chemical compounds. Thus, development of a reliable and simple protocol for *in vitro* mass production of the plant is required.

Research frontiers

The present research work describes, for the first time, a simple and reliable protocol for *in vitro* regeneration of ajowan, an important medicinal plant. This protocol can be used for *in vitro* studies especially for genetic transformation of this species in the future.

Related reports

Recently, several researchers have also used apical bud explant for efficient regeneration of various medicinal plants. In these works apical explant has been showed as highly responsive part of the plants for *in vitro* propagation. Also, suitable recombinations of auxins and cytokinins have been recommended for efficient regeneration of medicinal plants by various workers.

Innovations and breakthroughs

In this paper, for the first time, authors have reported an efficient *in vitro* regeneration in ajowan, an important medicinal plant.

Applications

The protocol described in the present study can be used for agrobacterium-mediated transformation of ajowan for improvement of its phytochemical contents.

Peer review

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.

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