Journal of Pharmacological Reports

Research Article Open Access

In Vitro Micropropagation of *Carum copticum L.*

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Abstract

An efficient clonal propagation protocol has been established from seedling explants of *Carum copticum*. Best seed germination frequency (100%) was observed on both Murashige and Skoog (MS) medium and Nitsch and Nitsch (NN) medium supplemented with 1.0 mg l^{-1} and 0.5 mg l^{-1} GA3 respectively. Shoot multiplication was initiated from hypocotyl and radical explants cultured on MS and NN media supplemented with various concentration and combination of N6-benzyladenine (BA), kinetin (Kn) and gibberellic acid (GA3). The highest shoot induction (96.66%) was observed from hypocotyl explants on NN medium supplemented with 1.5 mg l^{-1} BA whereas 93.33% shoot induction was observed on MS medium supplemented with 1.5 mg l^{-1} BA. The maximum of 11.5 shoots per hypocotyl explant with an average length of 6.68 cm was observed on MS medium supplemented with 1.5 mg l^{-1} BA whereas 10.83 shoots per hypocotyl explant with an average shoot length of 5.53 cm was observed on NN medium containing same level of BA concentration. Rooting of shoots was achieved on MS and NN media supplemented with various concentrations of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and napthaleneacetic acid (NAA). The highest root regeneration frequency (96.66%) was observed either on MS supplemented with 0.25 mg l^{-1} NAA or on NN medium with 0.25 mg l^{-1} IAA. The maximum number of 7.83 roots per shoot with an average root length of 3.98 cm was observed on MS medium supplemented with 0.25 mg l^{-1} NAA whereas 7.33 roots per shoot with an average root length of 4.13 cm was observed on NN medium supplemented with same level of NAA. Plantlets were acclimatized in garden soil and transferred to field. The method described here can be successfully employed for large scale production of sterile plants for pharmaceutical use and genetic transformation studies.

Keywords: *Carum copticum*; Micropropagation; Seed culture; Hypocotyls and radicals

Abbreviations: MS: Murashige and Skoog; NN: Nitsch and Nitsch; BA: N6-Benzyladenine; Kn: Kinetin; GA3: Gibberellic Acid; IAA: Indole-3-Acetic Acid; IBA: Indole-3-Butyric Acid; NAA: Napthalene Acetic Acid

Introduction

Carum copticum L. is a medicinal herb belonging to family Apiaceae. It is an annual and cross pollinated herb which grows in the east of India, Iran, Pakistan and Egypt. The plant (Ajwain) bears white flowers and small brownish fruits which on ripening being used as aromatic seed and thus holds promise for treatment of liver disorders [1]. Its seed is rich in thymol which is antispasmodic and antifungal [2]. Although this plant is easily propagated through seed, its propagation through *in vitro* micropropagation technology is advantageous to produce high quality disease free and true-to-type plant irrespective of seasonal influences.

All medicinal plants possess a multitude of secondary metabolites which impart an unprecedented variety of medicinal uses. Plant species finds use for treatment of a wide spectrum of health disorders in traditional and folk medicine [3]. There has been a tremendous urge for healthy diet and natural products leading to ever increasing demand for medicinal plants as raw material for pharmaceutical, food, cosmetics and other processing industries [4,5]. Extensive cultivation of spices crop plants resulted in concentration of their crops and consequently rising number of pathogens, which in turn cause considerable economic losses in yield and its quality. Although improvements in agronomic traits can be achieved through years of conventional breeding, novel traits can be inserted into the target through genetic transformation [6]. The success of genetic transformation in plant species for quality improvement necessitates the availability of reproducible and highly efficient in vitro regeneration system [7]. In Vitro shoot regeneration of *C. copticum* thus opens scope for efficient genetic transformation system which in turn would contribute to its improved performance and quality.

The present investigation was carried out to establish a protocol for in vitro micropropagation using intact seedling and hypocotyl and radical explants of *C. copticum* on MS and NN media supplemented with plant growth regulators at different concentrations.

Materials and Methods

Culture media and conditions

The culture media used for the micropropagation were MS (Murashige and Skoog) [8] and NN (Nitsch and Nitsch) [9]. The medium was supplemented with 0.25 or 0.5 or 1.0 or 1.5 or 2.0 mg l^{-1} N6-benzyladenine (BA) or kinetin (Kn) or gibberellic acid (GA3) either alone or in combination for seed germination and shoot induction. The medium was also supplemented with 0.25 or 0.5 or 1.0 mg l^{-1} IAA or IBA or NAA for root induction. The pH was adjusted to 5.8 with 1 N NaOH or 0.1 N HCl and gelled with 0.8% agar (w/v) (Qualigens, India) into 25 × 150 mm glass tubes and 100 ml conical flasks (Borosil India) and autoclaved at 121°C and 1.04 kg cm^2 for 20 minutes. All cultures were incubated in a culture room at 29°C ± 2°C under a 16 h photoperiod with a light intensity of 35 µE m−2s−1 provided by cool white fluorescent tubes (40 W Philips) and 8 h dark period with 55% to 60% RH. Seedlings were raised both in vitro and ex vitro in the nursery beds of medicinal garden.

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Received January 04, 2016; **Accepted** February 02, 2016; **Published** February 05, 2016

Citation: Mandal J, Sharma P (2016) *In Vitro* Micropropagation of *Carum copticum L.* J Pharma Reports 1: 108.

Disinfection of plant material grown *ex vitro*

Seeds were collected from Central Institute of Agriculture Engineering, Bhopal, India. The seeds were sown in nursery beds and seedlings were maintained in nursery beds for 10-20 days. The seedlings were carefully washed under running tap water for 30 min. Ex vitro seedlings were surface disinfected in a solution of 0.1% (w/v) $HgCl_2$ for 3 min and thoroughly rinsed three times in sterile double distilled water to remove any trace of $HgCl_2$. Hypocotyl and radical explants were excised for culture on MS and NN media supplemented with 0.25 or 0.5 or 1.0 or 1.5 or 2.0 mg l^{-1} GA3. Germination frequency was recorded after 10 days of culture.

Seed sterilization and establishment of seedling culture

The seeds were immersed in solution of 0.1% (w/v) $HgCl_2$ for 3 min and thoroughly rinsed three times with autoclaved double distilled water. Disinfected Seeds were cultured on MS and NN media supplemented 0.25 or 0.5 or 1.0 or 1.5 or 2.0 mg l^{-1} GA3. Germination frequency was recorded after 10 days of culture.

Shoot multiplication

Isolated 1 cm long hypocotyl and radical explants were inoculated on MS and NN media supplemented with 0.25 or 0.5 or 1.0 or 1.5 mg l^{-1} BA or 0.25 or 0.5 or 1.0 or 1.5 mg l^{-1} Kn or 0.25 or 0.5 or 1.0 or 1.5 mg l^{-1} GA3 either alone or in combination. Shoot percentage, number of shoots per hypocotyl and mean length of shoots were recorded after 30 days of culture.

Rooting of shoots and acclimatization

In Vitro shoots of 2-3 cm length derived after six weeks of culture were transferred to MS and NN media supplemented with 0.25 or 0.5 or 1.0 mg l^{-1} IAA or IBA or NAA for root induction. Root frequency, number of roots per shoot and root length per shoot were recorded after six weeks of culture. The rooted plants were washed with water to remove adhered medium and transferred to pots containing autoclaved vermicompost and soil (1:1) and kept jacketed with polyethylene bags for two weeks to maintain high humidity. Thereafter these hardened plants were transferred to bigger pots and maintained at medicinal garden of the Institute.

Statistical analysis

The Experimental set up was in completely randomized design; each experiment was repeated thrice with 10^{-1} 2 replicates. Data were analyzed by using one way analysis of variance (ANOVA) and the means were scored using Tukey test [10] on statistical package of SPSS (version 20) [11].

Results and Discussion

Seed germination and establishment of seedling culture

Seed sterilization is an essential step as to make available contamination free seedling as a source of explants for micropropagation [12]. Seed germination frequency was found low either on MS (17%) or NN (22%) medium when no plant growth regulator was added to the medium (Figure 1). The highest seed germination frequency (100%) was observed either on MS medium supplemented with 1.0 mg l^{-1} GA3 or on NN medium supplemented with 0.5 mg l^{-1} GA3 (Figure 2). Similarly, stimulating effects of GA3 at low concentration on seed germination by overcoming dormancy was found to be beneficial in many species such as *Asparagus densiflorus* [13] and *Withania somnifera* [14].

Shoot multiplication

The morphogenic response of hypocotyls and radicals from seedlings of *Carum copticum* after 2-3 weeks of culture initiation showed that shoot multiplication was not induced either on MS or NN medium without supplementation of plant growth regulator. Hypocotyls were observed to be better explants for multiple shoot induction than radicals Figure 2. Medium with different concentrations of BA, Kn and GA3 either alone or in combination facilitated shoot regeneration. The maximum shoot regeneration frequency of 96.66% ± 2.10% (Figure 3) was obtained on NN medium supplemented with 1.5 mg l^{-1} BA whereas 93.33% ± 2.10% shoot regeneration was observed on MS medium supplemented with 1.5 mg l^{-1} BA. The highest number of 11.5 ± 0.5 shoots per hypocotyl explant (Figure 3) with maximum shoot length of 6.68 cm ± 0.04 cm (Figure 3) was observed on MS medium containing 1.5 mg l^{-1} BA whereas on NN medium supplemented with same concentration of BA resulted 10.83 ± 0.30 shoots per hypocotyl explant with an average shoot length of 5.53 cm ± 0.14 cm (Figure 1). Among the plant growth regulators tested (BA or Kn or GA3), the cytokinin BA was found to be the most effective for shoot multiplication. Like the present study, BA concentration higher than 1.5 mg l^{-1} showed an inhibitory effect on shoot multiplication of *Ocimum basilicum* [15-17]. Shoot multiplication was not observed after third subculture in many species including *Ocimum basilicum* L. [15,18,19], *Mentha piperita* [20], *Vitex trifolia* [21] and *Cassia angustifolia* [7] which corroborates the observation with the present study.

Rooting of shoots

The regenerated shoots of 2 cm - 4 cm length were excised and transferred to MS and NN media supplemented with 0.25 or 0.5 or 1.0 mg l^{-1} of IAA or IBA or NAA for root induction. Rooting of shoots was neither observed on MS nor NN medium without plant growth regulator. Root induction of individual shoot cuttings was achieved when MS or NN medium was supplemented with various concentrations of IAA or IBA or NAA. Root induction frequency of 96.66% ± 2.10% was observed on MS medium supplemented with 0.25 mg l^{-1} NAA whereas NN medium supplemented with 0.25 mg l^{-1} IAA

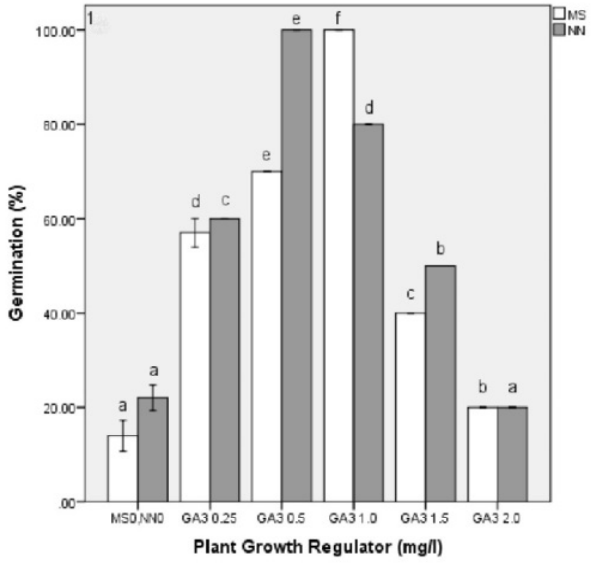

Figure 1: % Germination of *Carum copticum* on MS and NN media supplemented with different concentrations of GA3. Different letters(s) indicate a significant difference between treatments at P ≤ 0.05 according to Tukey test.

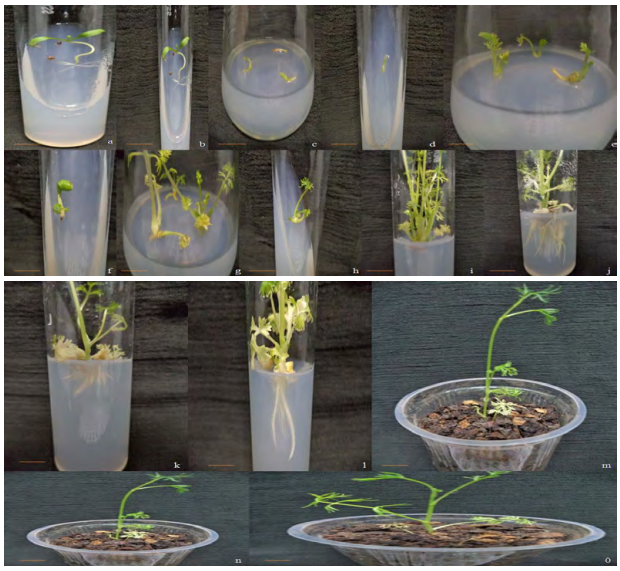

Figure 2: (a)-(o). *In vitro* plant regeneration of *Carum copticum.* (a- b) Germinated seedling (s) on MS and NN media supplemented with 1.0 mg l^{-1} GA3 and 0.5 mg l^{-1} GA3 respectively; (c-d) Response of hypocotyl explants on MS and NN media with same level of 1.5 mg l^{-1} BA for shoot multiplication, (e-f) stage of shoot initiation on MS and NN media respectively, (g) Multiple shoot induction on MS medium with 1.5 mg l^{-1} BA, (h-i) multiple shoot induction on NN medium with 1.5 mg l^{-1} BA; (j) Root initiation on MS medium containing 0.25 mg l^{-1} NAA, (k-l) Roots induction on NN medium supplemented with 0.25 mg l^{-1} IAA; (m-o) Stages of acclimatization of plantlets of 2 m old. Bars = 0.57 cm (a-e); 0.5 cm (f-g); 0.71 cm (k-m) and 0.35 cm (n-o).

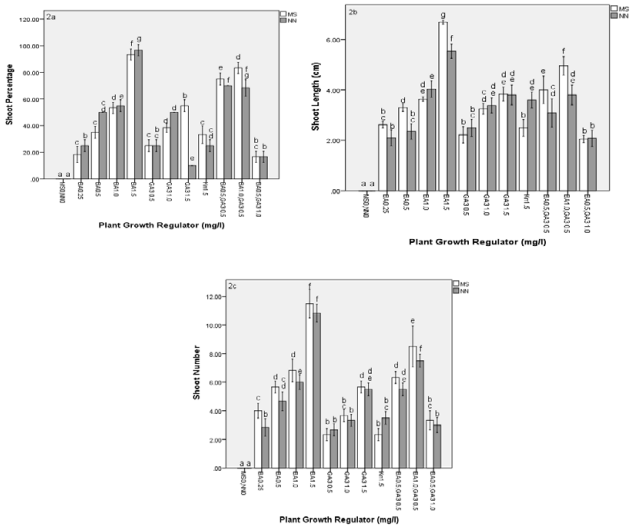

Figure 3: (a)-(c). Morphogenic responses of hypocotyls explants of *Carum copticum* on MS and NN media supplemented with plant growth regulators (Only responding plant growth regulator concentration shown) after 30 days. (a) % Shoot regeneration; (b) Mean shoot number;(c) Mean shoot length. Different letters(s) indicate a significant difference between treatments at P ≤ 0.05 according to Tukey test.

induced 96.66% ± 2.10 % root induction frequency (Figure 4). The maximum of 7.83 ± 0.16 roots per shoot (Figure 4) with an average root length of 3.98% ± 0.11 cm was observed on MS medium supplemented with 0.25 mg l^{-1} NAA whereas maximum root number of 7.33 ± 0.49 per shoot with an average root length of 4.13 cm ± 0.09 cm was

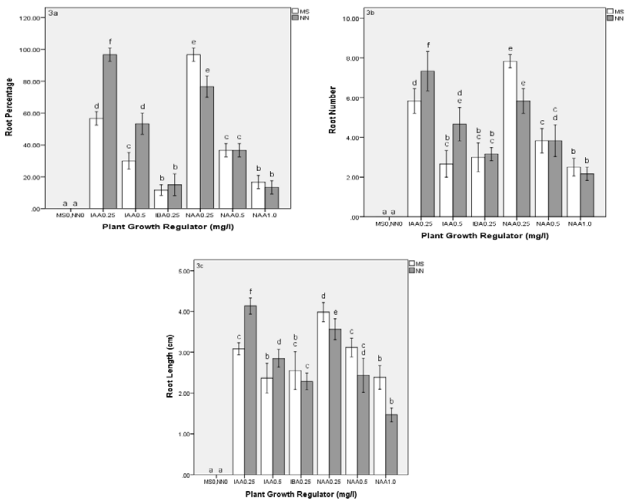

Figure 4: (a)-(c). Morphogenic responses of *in vitro* shoot explants of *Carum copticum* on MS and NN media supplemented with plant growth regulators (Only responding plant growth regulator concentration shown) after 30 d. (a) % Root regeneration; (b) Mean root number; (c) Mean root length. Different letters(s) indicate a significant difference between treatments at P ≤ 0.05 according to Tukey test.

observed on NN medium supplemented with 0.25 mg l^{-1} IAA (Figures 1 and 4). The presence of auxins at lower concentration was found to be best for root induction. Similarly, stimulatory effects of NAA or IAA at low concentration were reported in the studies of *Portulaca grandiflora* [22], *Picorrhiza kurroa* [21], *Heliotropium indicum* [23] and *Eclipta alba* [24,25].

Acclimatization

The rooted plantlets were washed with water to remove adhered medium and transferred to plastic pots containing a mixture of autoclaved vermicompost and soil (1:1) and kept jacketed with polyethylene bags for two weeks to maintain high humidity. These hardened plants were then transferred to bigger pots and maintained at medicinal garden of the institute with 90% survival of rooted plantlets.

Conclusion

The present study established an efficient and reliable micropropagation protocol for in vitro regeneration of *Carum copticum* from hypocotyl explants which can be used to propagate a large number of plants for pharmaceutical use and genetic transformation studies. Furthermore, this micropropagation system may also be used for selection of high yielding varieties of Carum sp. which usually produce a wide variety of pharmaceutical compounds of medicinal use.

Acknowledgement

Authors express gratitude to Director, NCERT, New Delhi and Principal, Regional Institute of Education, Bhopal for providing research facilities. Thanks are also due to Mr. Jose J.K. and Mr. Rohen Meetei for statistical analysis.

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