International Journal of Tropical Agriculture
Volume 40, Numbers (1-2) • 2022 : pp. 1-6
ISSN: 0254-8755

Shoot Regeneration on Hypocotyl Explants from *Carum copticum* L.

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Abstract: Shoot regeneration was established on hypocotyl explants from 7-10 day old in vitro seedlings of *Carum copticum*. Shoot regeneration frequency was 96.6% on Murashige & Skoog (MS) medium containing 6.66 µM N6-Benzyladenine (BA) and induced a maximum number of 11.8 shoots per hypocotyl explant with an average shoot length of 6.67 cm. Root induction frequency reached to 96.6 % on all the basal media comprising MS or Schenk & Hildebrandt (SH) or Nitsch & Nitsch (NN) supplemented with either 1.34 µM Naphthaleneacetic acid (NAA) or 1.44 µM Indole-3-acetic acid (IAA). Root induction was optimized (7.4 roots per shoot) on NN or SH medium supplemented either with 1.44 µM IAA or 1.34 µM NAA with a root length of 4.21cm. Regenerated shoots produced healthy roots in the soil with 100% acclimatization frequency. This regeneration method offers scope for enhancement of secondary metabolites for bioactive compounds of essential oil in *C.copticum*.

Keywords: Hypocotyls, Plant growth regulators, Root induction, Shoot regeneration

Introduction

Carum species of Apiaceae, are commercial important spices plants, hold therapeutic attributes for human health care systems. This plant is constantly sought for its unique use in traditional medicines being used in poor households for the treatment of many abdominal ailments including diarrhea [5]. The essential oil of seeds containing thymol as an essential component is famous for its pharmacological properties especially being used as analgesic and anti-asthmatic [6]. The increased inclinations towards the traditional medicines throughout the world attract scientific investigations to evaluate the therapeutic effects of medicinal spices [18]. Additionally, a viable alternative strategy to alleviate the problem of low multiplication and high infections on field plants includes the technique of direct shoot organogenesis from explants on synthetic medium under aseptic conditions. An added advantage of this method is the genetic fidelity of plantlets in all the successive generations in future.

Direct shoot organogenesis has been a powerful technique of multiplication of shoots irrespective of the seasonal and environmental influences [18]. This study describes efficient shoot regeneration from hypocotyl explants from in vitro seedlings of *Carum copticum*.

Seeds were collected from Central Institute of Agriculture Engineering, Bhopal, India. These seeds were washed with tap water, disinfected with 0.1% (w/v) $HgCl_2$ for 3 min and thoroughly rinsed thrice with autoclaved double distilled water. Surface disinfected seeds were cultured on MS [15], NN [16] and SH [22] media supplemented with various concentrations of Gibberellic acid (GA_3) (0.72, 1.44, 2.89, 3.33, 4.77 µM). Germination (%) of seeds was recorded after 20 days of culture.

Subsequently, different explants of seedlings were cultured on different media such as MS, NN

Article History: Received: 28 December 2021; Revised: 12 January 2022; Accepted: 27 January 2022

and SH containing sucrose (Merck, India) 30 g/l in MS, 20 g/l in NN and 25g/l in SH medium. Plant growth regulators were added to the medium such as different concentrations of cytokinins- BA (1.11, 2.22, 4.44, 6.66 µM), Kinetin (Kn) (1.16, 2.33, 4.66, 6.99 µM), 2-isopentenyladenine (2iP) (1.22, 2.45, 3.67, 5.17 µM) and auxins - IAA (1.44, 2.89, µM), Indole-3-butyric acid (IBA) (1.23, 2.46, 4.92 µM) and NAA (1.34, 2.69, 5.37 µM). The pH of all the media was adjusted to 5.8 with 1N NaOH/HCl prior to addition of 0.8% (w/v) agar (Merck, India).

All experiments were carried out in 25 × 150 mm glass culture tubes (Borosil, India) containing 15 ml of molten medium and autoclaved at 121°C for 20 minutes. All Culture were inoculated for 20 days under a photoperiod of 16h light and 8h dark at 35 µE $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (40 W. Philips) with 55% to 60% RH at 29 ± 2°C.

Hypocotyls and radical pieces of about 0.5 - 1.0 cm were inoculated on MS, NN and SH media supplemented with different concentrations of BA (1.11, 2.22, 4.44, 6.66 µM), Kn (1.16, 2.33, 4.66, 6.99 µM), 2iP (1.22, 2.45, 3.67, 5.17 µM) singularly or in combination of BA (1.11, 2.22, 4.44, 6.66 µM) with Kn (2.33 µM) or BA (1.11, 2.22, 4.44, 6.66 µM)) with 2iP (2.45 µM). Subculturing was performed every 20 day. Shoot regeneration (%), number of shoots per hypocotyl and mean shoot length were recorded after 45 days of culture.

In vitro shoots (2-3cm length) harvested after six weeks of culture were transferred into MS, NN and SH media supplemented with auxins - IAA (1.44, 2.89, µM) or IBA (1.23, 2.46, 4.92 µM) or NAA (1.34, 2.69, 5.37 µM) for root initiation. Root initiation was observed after second week of culture. After 45 days of culture, root induction (%), number of roots per shoot and mean root length were recorded. The rooted plantlets were washed under running tap water to remove the medium and then transferred to pots containing autoclaved vermicompost and soil (1:1). The pot was wrapped with polyethylene bags for two weeks to maintain high humidity. The hardened plants were transferred to bigger pots and maintained at medicinal garden of the institute.

Each experiment was repeated thrice with 10-12 replicates. Data were analyzed by using one way analysis of variance (ANOVA) and the means were scored using Tukey test on statistical package of SPSS (version 20) [13].Treatments were significantly different ($P < 0.01$).

Seed germination (100%) was observed either on NN medium supplemented with 2.89 µM GA_3 or on MS or SH medium supplemented with 1.44 µM GA_3 (Figure 1 and 4a - 4c). GA_3 at the level of 1.44 - 2.89 µM promoted seed germination of *C.copticum* on MS medium [12]. Similarly, GA_3 at low concentration (1.44 - 2.89 µM) stimulated seed germination in *Asparagus densiflorus* [22].

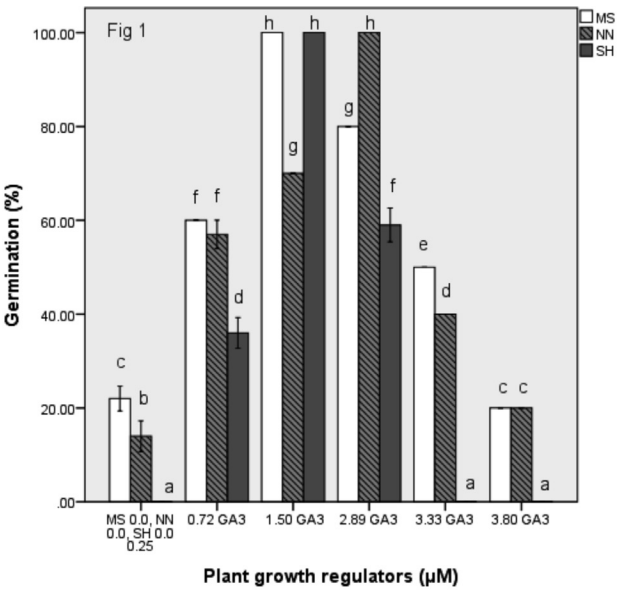

Figure 1: Effect of basal medium (MS, NN and SH) supplemented with different concentrations of GA_3 on seed germination (%) of *C. copticum* after 20 days of incubation. Different letters (s) indicate significant difference between treatments of $P < 0.05$ according to Tukey test

Hypocotyl explants were more responsive to adventitious shoot organogenesis than radical explants. Shoot regeneration was not observed on basal MS, NN and SH media without PGRs. Shoot regeneration frequency on MS, NN and SH media containing 6.66 µM BA was 96.6%, 92% and 82% respectively (Figure 2a and 4g - 4j). Maximum number of 11.8 shoots per hypocotyl explant with a shoot length of 6.67 cm was observed on MS medium supplemented with 6.66 µM BA whereas 10.8 shoots per hypocotyl explant with a shoot length of 5.25cm was observed on NN medium containing the same

concentration of BA. On the other hand, 11.05 shoots per hypocotyl explant with an average shoot length of 5.48 cm was obtained on SH medium containing the same concentration of BA (Figure 2b - 2c). The effectiveness of BA on the MS and NN medium for shoot initiation and multiplication corroborates with the studies in *Spermacoce hispida* [7] and *Carum copticum* [23]. The stimulating effects of BA on multiple shoot formation have been reported in earlier studies such as *Ocimum basilicum* L. [21], *Vitex trifolia*[9] and *Mentha piperita* [20], *Origanum sipyleum* [16], and *Salvia guaranitica* [7]. In the present study, shoot multiplication rate was declined after the fourth subculture which is in line with the previous reports of *Portulaca grandiflora* [10] and *Gardenia jaminoides* [8].

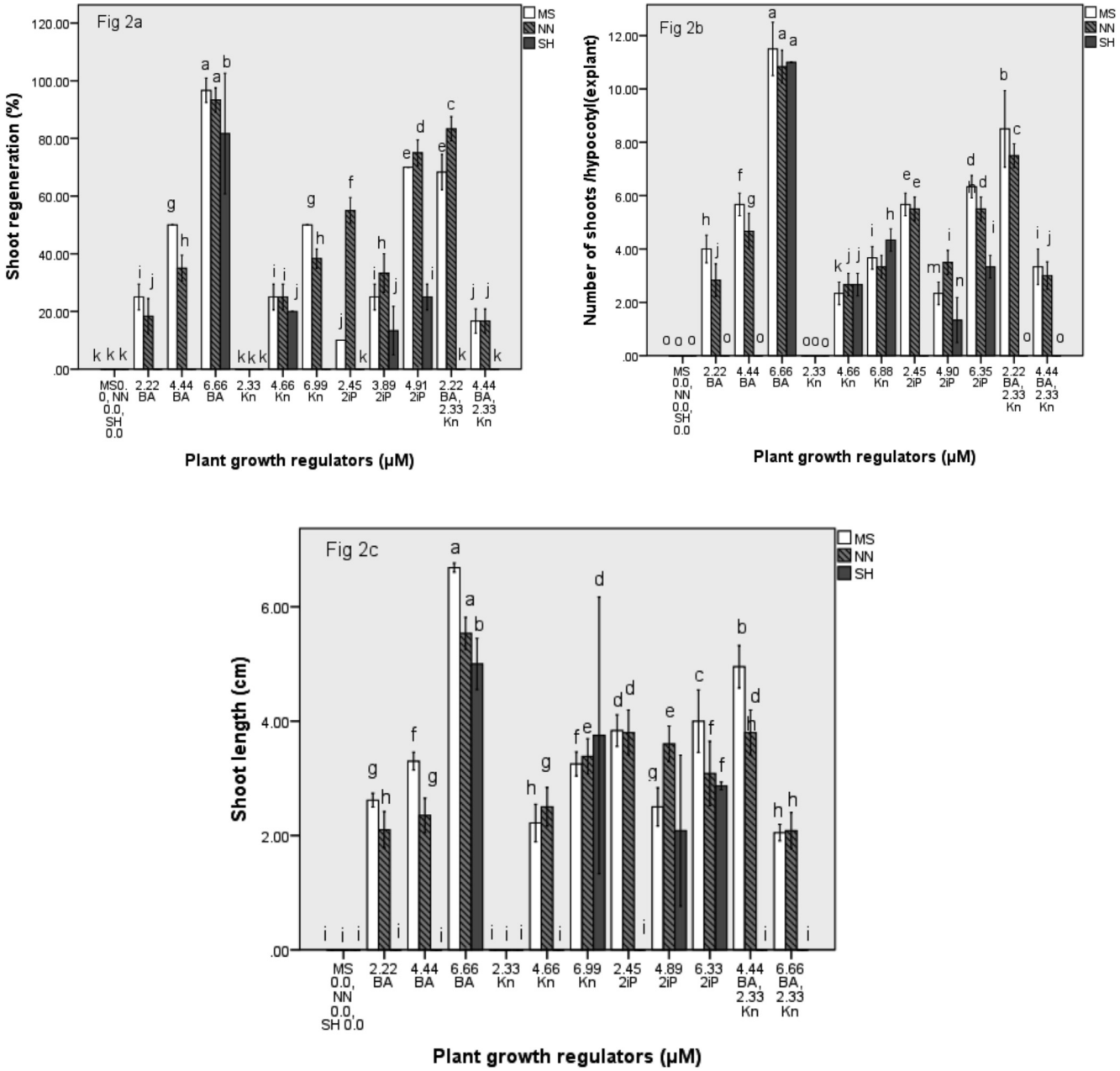

Figure 2 a-c Effect of basal media (MS, NN and SH) supplemented with different combinations and concentrations of BA, Kn and 2iP on shoot regeneration of *C.copticum* after 45 days of culture a, shoot regeneration (%). b, number of shoots per hypocotyl. c, shoot length (cm). Different letters (s) indicate significant difference between treatments of P < 0.05 according to Tukey test.

Regenerated shoots (2 - 3 cm) were excised and transferred to full strength MS, NN and SH media supplemented with various concentrations of auxins (IAA, IBA, and NAA) for root induction. Shoots were not rooted on basal MS, NN and SH media without plant growth regulators. Roots were developed from cut off surface of the roots with the induction frequency of 96.6% either on MS or NN or SH medium supplemented with 1.34 µM NAA or on NN medium containing 1.44 µM IAA (Figure 3a). There were 7.4 roots per shoot with an average root length of 4.21cm on NN medium supplemented with 1.44 µM IAA. However, a maximum of 6.2 roots per shoot with a root length of 4.1 cm was observed on MS medium containing 1.34 µM NAA, while 7.4 roots per shoot with a root length of 4.45 cm was obtained on SH medium containing the same concentration (1.34 µM) of NAA (Figure 3b - 3c and figure 4k-4m). Auxin effects on root induction varies upon types and concentrations used in different plant species. Similarly, in the previous studies auxins such as, NAA (1.34 – 2.69 µM) was the most suitable plant growth regulator for root induction in *Pulsatilla tongkangensis* [24], *Carum copticum* [13], *Aloe barbadensis* [4] and *Aloe vera* [1]. However,in this study NAA concentration at 1.34 µM not only influenced quality of roots but also decreased the number of roots which is commensurate with the earlier studies in *Thymus satureioides* [3] and *Prunella vulgaris* [19]. Regenerated plants were transplanted to pots containing soil and vermiculite (1:1) mixture and acclimatized under high humidity at room temperature with survivability of 100% (Figure 4n - 4p).

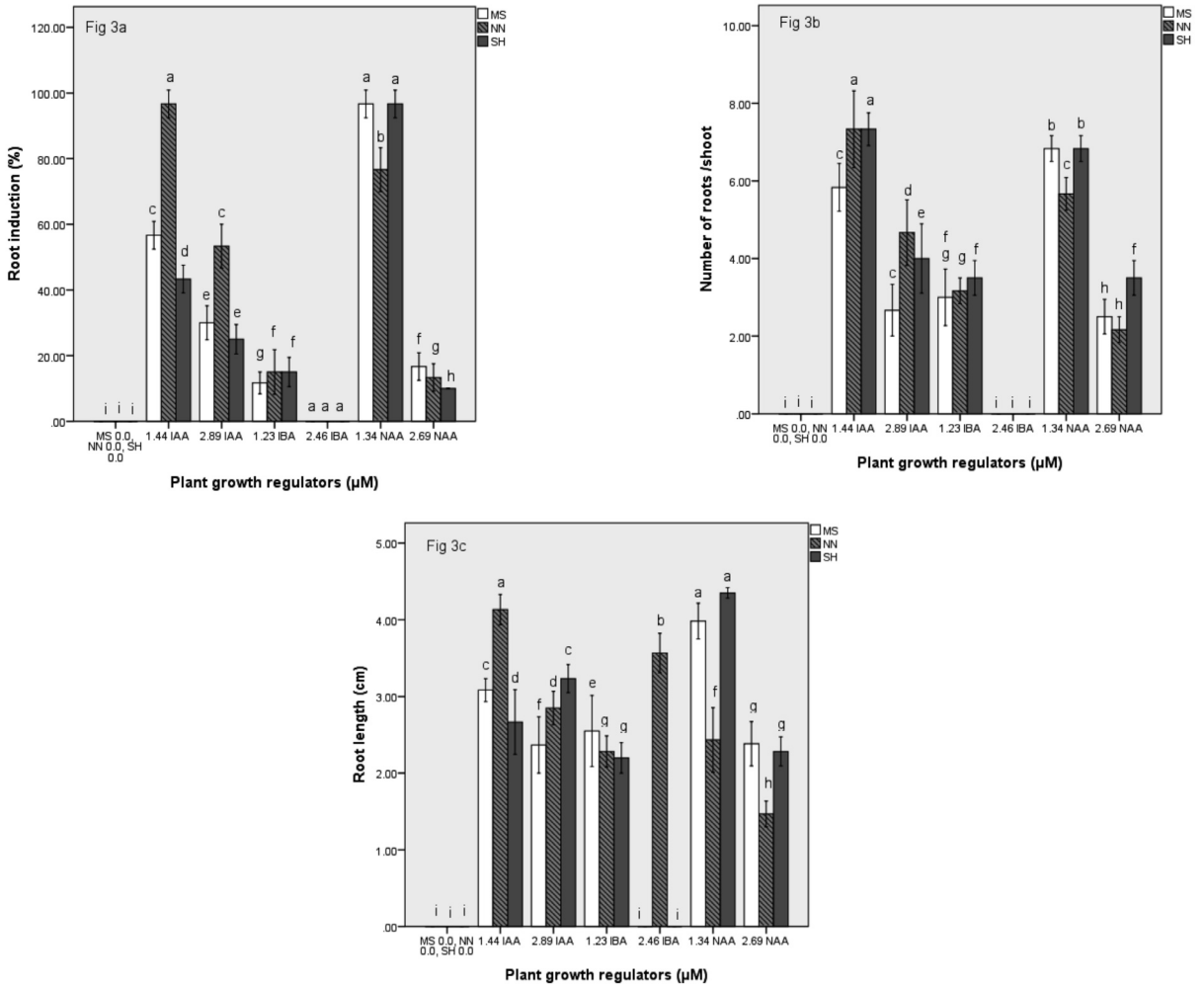

Figure 3 a-c: Effect of basal media (MS, NN and SH) supplemented with different concentrations of IAA, IBA and NAA on root induction of *C.copticum* after 45 days of culture: a, root induction (%) b, number of roots per shoot c, root length (cm). Different letters (s) indicate significant difference between treatments of P < 0.05 according to Tukey test.

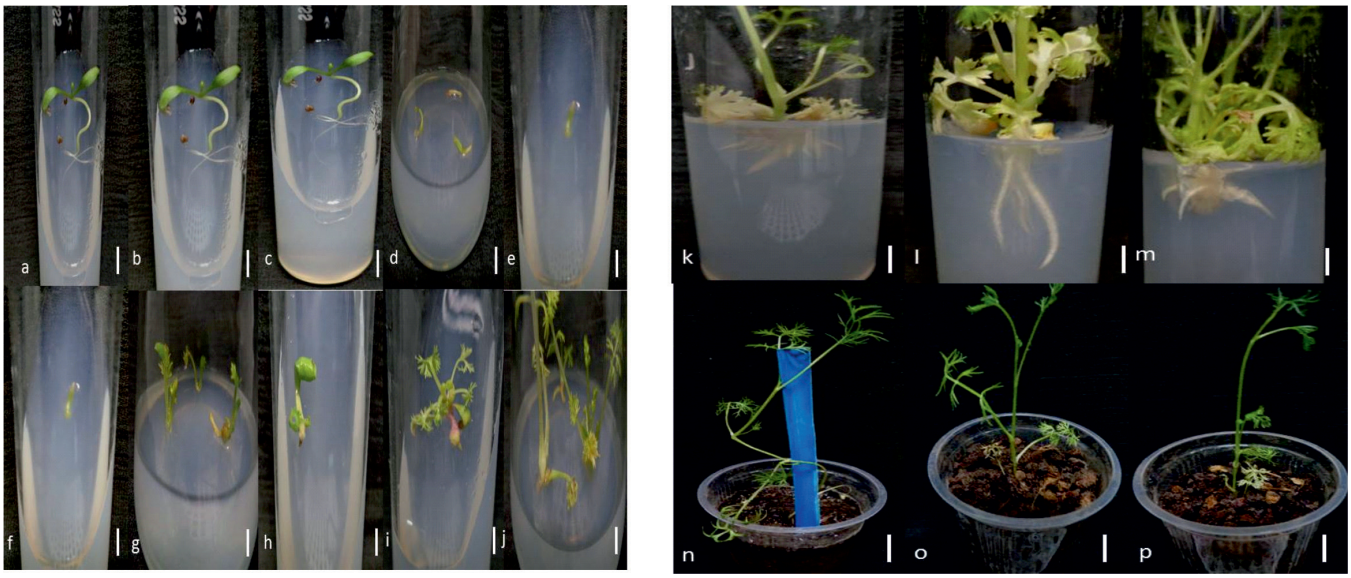

Figure 4 a-p Regeneration of shoots from hypocotyl explant of *C.copticum*. Seedling after 20 days on (a) MS medium with 1.44 µM GA_3, (b) NN medium with 2.89 µM GA_3, and (c) SH medium with 1.44 µM GA_3. Shoot regeneration from hypocotyl explants on (d) MS medium with 6.66 µM BA, (e) NN medium with 6.66 µM BA and (f) SH medium with 6.66 µM BA. Shoot regeneration and multiplication after 45 days of culture on (g, h) MS medium with 6.66 µM BA, (i) NN medium with 6.66 µM BA and (j) SH medium with 6.66 µM BA. Rooting of in vitro shoots after 45 days of culture on (k) MS medium supplemented with 1.34 µM NAA, (l) NN medium with 1.44 µM IAA and (m) SH medium with 1.34 µM NAA. (n - p) Acclimatized healthy plantlets of *Carum copticum* (two months) in soil. Bars in (a) - (j) represent 0.5 cm, Bars in (k - p) represent 1.0 cm

An efficient and reliable shoot regeneration protocol was established from hypocotyl explants derived from vitro seedlings of *Carum copticum*. This regeneration system ensures large scale shoot regeneration of genetically true to type plants for sustainable supply of plant materials to the pharmaceutical industries and conservation of germplasm as well.

Acknowledgements

The authors are grateful to Director, National Council of Educational Research and Training (NCERT), New Delhi and Principal, Regional Institute of Education, Bhopal for providing research support.

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