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Regeneration of plants from callus cultures of *Origanum vulgare* L.

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ABSTRACT Investigations were undertaken to achieve rapid multiplication and improvement of *Origanum vulgare* (a herbaceous, ornamental plant well known for its aromatic and medicinal value) through plant regeneration from callus. The explants (cotyledons, hypocotyl and root segments) excised from 15 d old aseptic seedlings were cultured on Gamborg's B_5 medium supplemented with 2,4-D, NAA and BAP individually and in various combinations (at concentrations of 0, 10^{-7},10^{-6} and 10^{-5} M). Best callus induction was noted on medium with 10^{-7} M 2,4-D alone. The cotyledonary explants proved to be the best source for compact and nodulated callus. The subcultured cotyledonary calli showed shoot induction when transferred onto media supplemented with BAP alone or in combination with 10^{-7} M or 10^{-6} M NAA. However, 10^{-5} M NAA completely suppressed the shoot inducing ability of BAP. In general, NAA promoted root induction from all explants used including cotyledonary callus. Best shoot induction was obtained on medium supplemented with 10^{-6} M BAP + 10^{-6} M NAA. Both IBA and NAA at 10^{-6} M proved to be equally effective in induction of roots from the cut ends of 15-20 mm long shoots (excised from callus) in half-strength B_5 liquid medium. Rooted shoots were successfully re-established in soil under controlled conditions.

ABBREVIATIONS 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; NAA, α -naphthaleneacetic acid; BAP, 6-benzylaminopurine.

INTRODUCTION

In vitro culture of plant cells and organs on chemically defined media offers the opportunity to regenerate and select plants with desirable characters (by taking advantage of somaclonal variations) which are otherwise difficult to obtain by traditional plant breeding (Larkin and Scowcroft 1981). It is not an easy task to propose a generalized medium for achieving totipotency in all plants or even in all cells of the same plant, as the kind and balance of growth regulators needed for various morphogenetic phenomena vary from tissue to tissue and cell to cell depending on their metabolic status (Tisserat 1985, Hussey 1986). So far no success has been reported on regeneration in *Origanum vulgare* L., although callus formation has been reported (Becker 1970). This species is an underexploited aromatic cum medicinal plant (Douglas 1969), a source of oil of *Origanum*. The oil of *Origanum*, obtained from all the aerial parts of the plant (flowering heads being a rich source), is used in high grade flavour preparation, perfumery, cosmetics and liquor industries. As the oil possesses carminative, stomachic, diuretic, diaphoretic and emmenagogue properties, it is used in the treatment of chronic rheumatism, toothache, earache, whooping cough and bronchitis (Shah and Gupta 1976, Vaidya 1977, Uniyal et al. 1982, Singh et al. 1983, Balandrin and Klocke 1988). The present study was undertaken to standardize media for rapid multiplication of this plant through regeneration of plants from callus.

MATERIALS AND METHODS

Seeds of *Origanum vulgare* were procured from the Department of Botany, Andhra University (Visakhapatnam, India). Seeds were treated with 0.2% Cetavenol (ICI India Ltd., Madras, India) and washed thoroughly with distilled water. These were then surface sterilized with 0.05% $HgCl_2$ (for 5 min), rinsed with sterile distilled water, and inoculated onto 0.8% (W/V) agar to obtain seedlings. All cultures were incubated for 28 d at 28±2°C with a light/dark cycle of 12 h/12 h. White fluorescent light of an intensity of 3 W m^{-2} (Philips, India) was used.

Various explants, viz. cotyledons, hypocotyl and root segments, were excised from 15 d old seedlings and cultured onto B_5 semi-solid medium (Gamborg et al. 1968) supplemented with and without an auxin (NAA, 2,4-D) and/or a cytokinin (BAP) at concentrations

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0, 10^{-7}, 10^{-6}, 10^{-5} M. Sequestrene 330Fe (28 mg/l) of B_5 was replaced by $FeSO_4.7H_2O$ and $Na_2.EDTA.2H_2O$ (27.8 mg/l and 37.3 mg/l respectively). After addition of all ingredients including plant growth regulator(s), the pH of the medium was adjusted to 6.0 with 0.1 M NaOH, and autoclaved for 15 min at 1.05 Kg cm^{-2} in culture tubes (150 x 15 mm Borosil tubes), each holding ~20 ml medium.

The cotyledonary calli from the best callus induction medium (B_5 supplemented with 2,4-D 10^{-7} M) were multiplied for 3-4 passages (each for a period of 28 d) on fresh medium of the same composition. The calli were cut into ~2.5-3.0 mm^3 pieces and subcultured onto media supplemented with NAA and/or BAP at concentrations 0, 10^{-7}, 10^{-6}, 10^{-5} M.

Rooting of the shoots was attempted on Whatman No.1 filter paper bridges using half-strength B_5 liquid media with 1% sucrose, and NAA or IBA at concentrations 0, 10^{-7}, 10^{-6}, 10^{-5}M.

The hardening of plantlets was done using half-strength B_5 medium for 18 d followed by their transfer into half-strength B_5 medium deprived of sucrose and other organics for 28 d. These plantlets were transferred to pots containing peat: vermiculite: soil (1:1:2) covered with perforated polythene bags, and kept in a culture room at 28±2°C under continuous fluorescent light. These seedlings were irrigated with half-strength liquid B_5 medium minus sucrose and other organics. After 18 d, the polythene bags were removed.

At least 24 tubes/replicates were used for every treatment, and all experiments were repeated at least three times. The data presented here are averages of three independent experiments.

RESULTS AND DISCUSSION

Callus Formation and Shoot induction The percentages of cultures of each explant source showing callusing and rooting in the B_5 media supplemented with and without NAA or 2,4-D are summarized in Fig. 1. NAA or 2,4-D promoted both callus induction and root initiation. The percentages of cultures showing callus induction and root initiation increased with an increase in NAA concentration in the medium. However, all cultures of each of the three explants showed callusing in medium supplemented with 2,4-D at the lowest concentration (10^{-7} M) used. The percentage of cultures showing callus induction decreased with an increase in concentration of 2,4-D to 10^{-5} M (Fig. 1). The extent of callus proliferation from cotyledonary explants was relatively meager on the B_5 medium. Among the different concentrations of NAA used, the best callus production was seen at 10^{-5} M where callus proliferation was moderate with profuse root induction all over the callus surface. However, the best callus initiation and proliferation from all explants was obtained on medium with 2,4-D at 10^{-7} M. Although 2,4-D promoted root initiation in some cultures, the number of roots was low.

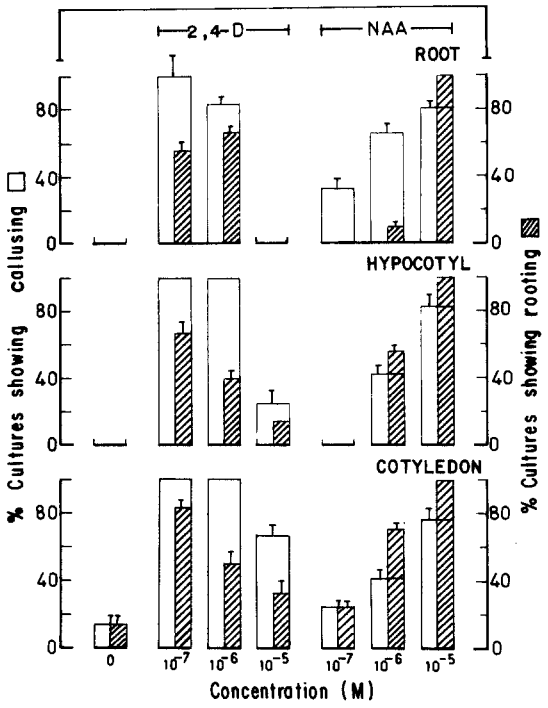

Fig. 1 Percentage of root, hypocotyl and cotyledon explant cultures of *Origanum vulgare* showing callusing and/or rooting on B_5 media supplemented with 2,4-D or NAA. The vertical lines above the bars represent standard deviation of the mean values of three independent experiments (each with 24 replicates).

Calli obtained in media supplemented with 2,4-D varied from compact, nodular, friable, to gelatinous depending on the concentration of 2,4-D. At higher concentrations of 2,4-D (10^{-5} M) calli were friable and gelatinous. On medium supplemented with 10^{-7} M 2,4-D, hypocotyl and root explants became swollen either from the cut ends or over the entire surface by day 7, but cotyledonary explants enlarged twice their original size during the same period. Callus initiation was noted from the cut end and margins of cotyledons, and from one or both ends of root and hypocotyl segments within 10-12 d. This was followed by the proliferation of the callus from over the entire surface of the explants, and the rate of proliferation of callus increased with time. Earlier Becker (1970) achieved callus induction from stem segments of field grown *Origanum vulgare* plants, on MS (Murashige and Skoog 1962) media supplemented with different auxins (IAA, NAA, 2,4-D). He also noted 2,4-D to be better at stimulation of callus formation and proliferation.

In the present study it was noted that the rate of multiplication of calli obtained on medium with 10^{-7} M 2,4-D increased considerably when subcultured onto medium supplemented with 2,4-D at the same level, resulting in a profuse callusing within 15 d. The rate of multiplication of the callus during the course of second, third and fourth subcultures remained similar to that noted after the first subculture.

As cotyledons proved to be a better source of compact and nodulated callus (Fig. 2A), further experiments were conducted with cotyledonary callus only. When calli multiplied through the three (i.e. 84 d) to four (i.e. 112 d) subcultures were transferred onto media supplemented with 2,4-D alone or in combination with BAP (0, 10^{-7}, 10^{-6}, or 10^{-5} M), only callus proliferation with sporadic rooting was obtained. However, when the calli from the third or fourth subcultures were transferred onto media with BAP (0, 10^{-7}, 10^{-6}, 10^{-5} M) alone or in combination with NAA, formation of localized green patches occurred on the calli which developed into shoots within 15-20 d. No marked difference in the response of calli from the third or fourth subcultures to the different treatments was noted. The calli subcultured onto media supplemented with NAA alone proliferated as callus, and also exihibited root induction, which was most profuse at the highest concentration of NAA used (Figs. 2B, 3). Although BAP alone resulted in shoot induction, as reported earlier in some members of Labiatae, viz. *Lucosceptrum cannum* (Pal et al. 1985), a significantly better induction of shoots was noted on media supplemented with BAP and NAA (Fig. 3), suggesting that the cytokinin to auxin ratio plays a critical role in shoot induction in *Origanum vulgare*. This is consistent with earlier reports in *Lavendula latifolia, L. angustifolia* (Calvo and Segura 1989) and four species of *Ocimum* (Ahuja et al. 1982). With an increase in the concentration of BAP the percentage of cultures showing shoot induction as well as the number of shoots per culture increased (Fig. 3). Among different combinations of BAP and NAA used, the best shoot induction was seen on medium with 10^{-6} M NAA + 10^{-6} M BAP (Figs. 2C, 3). The shoot inducing ability of the calli remained similar even after 20 subcultures (~18 months).

It was not possible to achieve shoot induction from all the subcultured calli in any of the media used (Fig. 3). A reduction in the percentage and extent of cultures showing root induction were observed with an increase in the BAP concentration (Fig. 3), as documented in other systems (Hussey 1986). Infact, during the present investigations, BAP at 10^{-5} M totally suppressed the root inducing capacity of NAA used at 10^{-7} or 10^{-6} M (Fig. 3). On the other hand, an increase in the level of NAA in the media to 10^{-5} M resulted in total

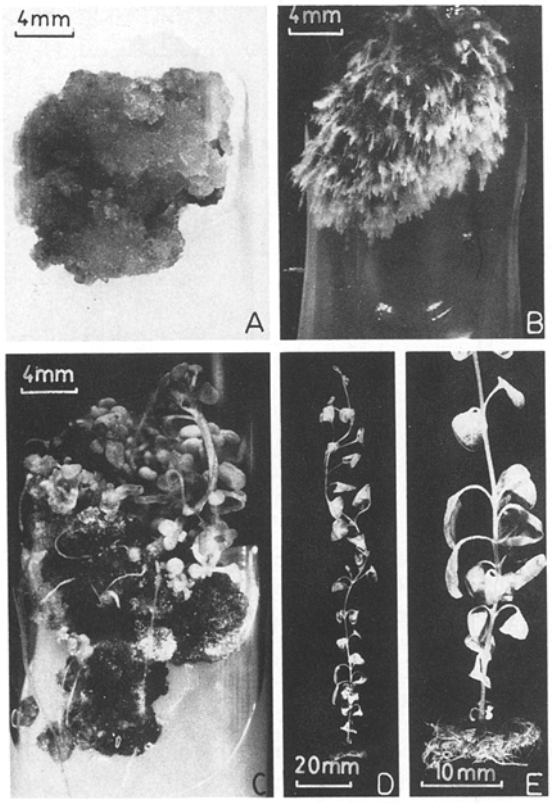

Fig.2 A. Cotyledonary callus of *Origanum vulgare* grown on B_5 supplemented with 2,4-D at 10^{-7}M.
B. Rhizogenesis from cotyledonary callus on B_5 supplemented with NAA at 10^{-5} M.
C. Caulogenesis from cotyledonary callus on B_5 supplemented with 10^{-6} M NAA + 10^{-6}M BAP.
D. Elongated shoot regenerated from callus and rooted on half-strength B_5 supplemented with IBA at 10^{-6} M.
E. Lower portion of regenerated shoot showing rooting on B_5 supplemented with IBA at 10^{-6} M.
(All photographs were taken 30 d after culture).

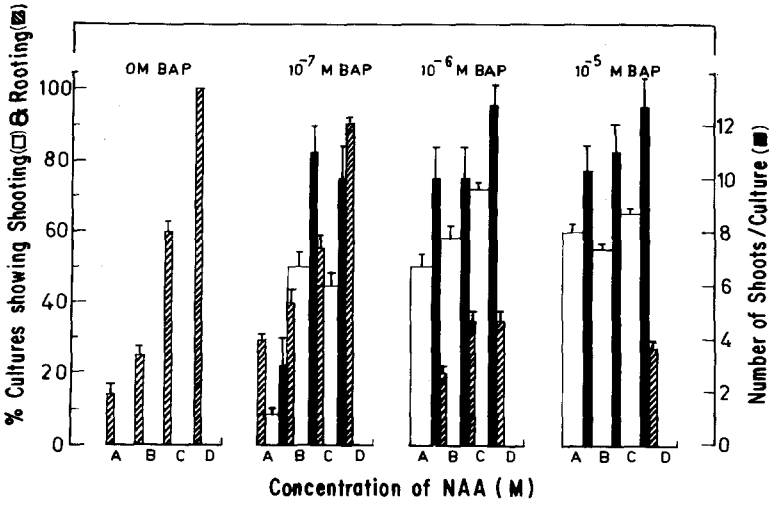

Fig. 3 Effectiveness of BAP and/or NAA for inducing shoots and/or roots from cotyledonary callus of *Origanum vulgare*. The vertical lines above the bars represent standard deviation of the mean values of three independent experiments (each with 24 replicates). A, B, C, and D represent 0, 10^{-7} M, 10^{-6}M, and 10^{-5} M of NAA, respectively.

inhibition of shoot induction, even in the presence of the highest concentration of BAP used.

Rooting of shoots Auxins like NAA and IBA are widely employed to induce rooting (Hussey 1986, Wakhlu et al. 1989). In the present investigation, individual shoots excised from regenerating callus cultures were transferred onto half-strength B_5 liquid medium supplemented with either IBA or NAA at concentrations ranging from 10^{-7} M to 10^{-5} M. Both IBA and NAA promoted initiation of roots, at all concentrations tested, within 5-7 d. Best root induction and formation of healthy roots was obtained with either auxin at 10^{-6} M (Figs. 2D, E). A simultaneous elongation of shoots was also achieved in these media, both due to an increase in the length of internodes and the number of nodes (Fig. 2D). However, both the auxins induced callusing from cut ends, as well as rooting, when used at 10^{-5} M. About 60% of the plantlets which were transferred into soil in pots are presently being maintained under controlled conditions in a culture room.

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