Indian J. Plant Physiol., Vol. 15, No. 2, (N.S.) pp. 168-172 (April-June, 2010)

SHORT COMMUNICATION

AN EFFICIENT METHOD FOR MICROPROPAGATION OF *OCIMUM BASILICUM* L.

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Received on 17 Sept., 2009, Revised on 3 May, 2010

An efficient plant regeneration protocol has been developed from nodal explants of *Ocimum basilicum* L., medicinally important herbaceous plant species belonging to the family Lamiaceae. Axillary shoot bud proliferation was initiated from nodal explants cultured on Murashige and Skoog (MS) medium supplemented with various concentration of benzyl adenine (BA) (0.25-2.0 mg 1^{-1}) and kinetin (KN) (0.25-2.0 mg 1^{-1}). Maximum numbers of shoots (6.2±0.1) with average length (3.7±0.0) were induced on medium containing 0.5 mg 1^{-1} BA. Shoots culture was established by repeated subculturing the original nodal explant on the same medium. Rooting of shoots was achieved on half strength MS medium supplemented with 1.0 mg 1^{-1} NAA. Well-developed complete plantlets were transferred to plastic pots containing a mixture of (1:1) soil and vermiculite showed 90 % survival rate.

Key words*: *In vitro* propagation, nodal segments, *Ocimum basilicum

Medicinal plants are valuable sources of traditional medicine and most of the people living in developing countries are almost completely dependent on traditional medical practices for their primary health care needs. Although synthetic drugs and antibiotics are essential for current medical practice, plants provide a major contribution to the pharmaceutical industry (Flower 1983). Rapid industrialization and urbanization has led to overexploitation and loss of valuable natural resources, including medicinally important herbaceous plants. Therefore, many species have become rare, non regulated collection, threatened with extinction or endangered (Arora and Bhojwani 1989, Purohit *et al*. 1994, Sudha and Seeni 1994).

Ocimum basilicum L. (sweet basil) is one of the most economically important medicinal plants in the world. It belongs to the family Lamiaceae, subfamily Ocimoideae and includes over 150 different species and varieties (Pushpangadan and Bradu 1995, Paton *et al*. 1999) distributed in tropical regions of Asia, Africa, Central and South America (Darrah 1988). The plant is stomatic, antihelmintic, antipyretic, diaphoretic, expectorant, carminative, stimulant and pectoral (Phippen and Simon 2000). It is also considered to be a source of aroma compounds and essential oils containing biologically active constituents that possess insect repellent, nematocidal, antibacterial, antifungal agents and antioxidants activities (Lee *et al*. 2005, Juliani and Simon 2002). It is also used as an anti-emetic agent (Caceres *et al*. 1990).

The major difficulty in the use of Lamiaceae species for pharmaceutical purposes lies in the genetic and biochemical heterogenecity (Dode *et al*. 2003). The conventional method of propagation of this species is the through seeds but the seed viability is very poor and low seed germination frequency restricts its propagation on a large scale and the seedling progeny also show cross-pollinated nature of the plant (Heywood 1978).

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In vitro micropropagation is an effective means for rapid multiplication of species in which it is necessary to obtain a high progeny uniformity. Different species of the genus *Ocimum* has been subjected to *in vitro* studies using different explants, like nodal segments (Ahuja *et al*. 1982, Sahoo *et al*. 1997, Begum *et al*. 2002), leaf segments (Phippen and Simon 2000, Gopi *et al*. 2006) and *in vitro* flowering (Sudhakaran and Sivashakari 2002). In the present study, we attempted to establish a reliable direct shoot regeneration protocol by using nodal explants for large scale production of *O. basilicum* L.

Nodal explants (1-2 cm) were collected from 2-3 month old plants of *O. basilicum* maintained in the medicinal and aromatic plant garden of the Department of Botany, University of Kalyani, Kalyani, India and washed thoroughly under running tap water and then treated with 5% Teepol (Qualigens, Mumbai, India) for 15-20 min followed by rinsing in several times with sterile distilled water. The explants were excised and surface disinfected with 0.1% aqueous mercuric chloride ($HgCl_2$) solution for 5-6 min and finally rinsed with sterile distilled water (five to seven times). The nodal segments were then trimmed at both ends prior to inoculation on culture media.

Initially shoot tips were cultured on MS (Murashige & Skoog 1962) basal medium containing 3% sucrose for culture initiation and served as explant sources for subsequent experiments. The pH of the medium (Supplemented with respective growth regulators) was adjusted to 5.7 with 1N NaOH or 1N HCl before gelling with 0.8% agar (Hi-Media, Mumbai, India). All the cultures were kept under cool fluorescent light (16 h photoperiod 40 µmol m^{-2} s^{-1}, at 25±2 ^{0}C) and 60-70 % relative humidity.

For axillary shoot bud proliferation, the nodal explants were placed on MS medium supplemented with BA (0.25-2.0 mg l^{-1}) or KN (0.25-2.0 mg l^{-1}) as a sole growth regulator. Each treatment was repeated three times with 15 replicates. Data were recorded after 4 weeks of culture.

For initiation of roots, the 8-10 weeks old individual regenerated shoots (approximately 2-3 cm in height) were cultured on half-strength or full-strength of MS basal medium supplemented with different concentrations of NAA (0.25-2.0 mg l^{-1}) or IBA (0.25-2.0 mg l^{-1}). The number of shoots that produced roots were recorded after three weeks of incubation.

The complete rooted plantlets with 7-10 fully expanded leaves were removed from the culture medium and the roots were washed gently under running tap water to remove agar. The plantlets were transferred to plastic pots containing a mixture of sterile soil and vermiculite in the ratio 1:1 and covered with transparent plastic bags to ensure high humidity. The growth chamber was maintained at 26±1^{0}C, 80-85% relative humidity with light intensity of 50 µmol m^{-2} s^{-2} on a 16 h photoperiod and gradually opened during an acclimatization period of 3 weeks. After acclimatization, plantlets were transferred to a green house with simulated habit and the percentage of survival was recorded.

The experiments were set up in a completely randomized design. Data were analyzed by one way analysis of variance (ANOVA) and the difference between means were scored using Duncan's Multiple Range Test $P \leq 0.05$ (Duncan 1955) on the statistical package of SPSS (Version 10).

Plant regeneration under *in vitro* conditions depends on various factors such as age of the explant, basal medium, growth regulators, genotypes, culture conditions, etc. (Thorpe 1994). When nodal explant from field-grown mature plants of *O. basilicum* were cultured on MS medium supplemented with different concentrations of BA and KN (0.25 to 2.0 mg l^{-1}), the emergence of the adventitious shoot buds was observed on the 12-15 days of inoculation (Fig. 1A). After a period of 4 weeks a thick mat of multiple shoot buds was spread over the surface with a maximum percentage (95.55) of response in MS media supplemented with 0.5 mg l^{-1} BA (Table 1, Fig. 1B). The maximum number of multiple shoots was obtained (6.2) in the medium containing 0.5 mg l^{-1} of BA. The shoots developed in this medium also attained maximum height of 3.7 cm after 4 weeks. The increase of BA concentration higher than 0.5 mg l^{-1} suppressed the rate of shoot length as well as number of multiple shoots. The hormone-free medium (Control)

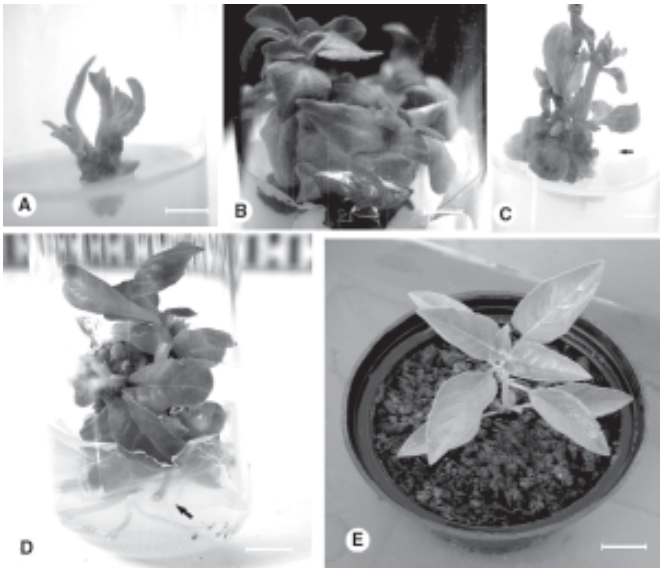

Fig. 1. (A) 12-15 d old culture showing shoot induction from nodal explant on MS medium supplemented with 0.5 mg l^{-1} BA (bar =0.12 cm); (B) Four weeks old culture showing multiple shoots on MS medium supplemented with 0.5 mg l^{-1} BA (bar =2.0 mm); (C) Regenerated multiple shoots – 3 weeks after transfer from fresh medium supplemented with 0.5 mg l^{-1} BA (bar =0.93 cm); (D) A rooted shoot on half strength MS medium supplemented with 1.0 mg l^{-1} NAA (bar = 1.6 cm) & (E) *In vitro* developed plantlets transferred to pot, (bar = 4.0 cm)

Table 1. Effect of different concentrations of benzyladenine (BA) and kinetin (KN) in MS medium for multiple shoot induction from nodal explants of *Ocimum basilicum* L. Each treatment (n=15) is repeated three times. Means± SE, means followed by the same letters are not significantly different at P≤0.05 (Duncan 1955).

Growth regulators (mg l^{-1})	Response [%]	No. of Shoots [explant^{-1}]	Average length of shoots [explant^{-1}] (cm)
BA			
0.25	57.77	2.9 ±0.6^{bc}	2.1±0.4^{bc}
0.50	95.55	6.2 ±0.1^{a}	3.7±0.0^{a}
1.00	75.55	4.0 ±0.6^{b}	3.0±0.4^{ab}
1.50	51.10	2.8 ±0.7^{bc}	2.1±0.5^{bc}
2.00	33.33	1.7 ±0.6^{c}	1.3±0.5^{c}
KN			
0.25	35.55	1.8±0.6^{c}	1.5±0.5^{bc}
0.50	51.10	2.5±0.6^{bc}	2.0±0.5^{bc}
1.00	55.55	3.1±0.6^{bc}	2.3±0.5^{bc}
1.50	71.10	3.4±0.5^{bc}	2.9±0.4^{ab}
2.00	55.55	2.9±0.6^{bc}	2.1±0.4^{bc}

*Basal medium= MS + Sucrose (3%) + Agar (0.8%)

did not show any response as the explants swelled and turned necrotic after 2 weeks of culture. Among the two cytokinins tested, BA was found to be the more effective for multiple shoot induction as compared to the KN. Similar response has been reported earlier in *Ocimum* species (Ahuja *et al*. 1982, Sahoo *et al*. 1997, Singh and Seghal 1999, Begum *et al*. 2002, Gopi *et al*. 2006). Similarly, many workers earlier reported stimulatory effect of BA on multiple shoot induction in other medicinally important plants species such as *Curcuma* spp. and *Zingiber officinale* (Balachandran *et al*. 1990) and *Piper* spp. (Bhat *et al*. 1995).

Multiplication of primary regenerated multiple shoots from nodal explants in the MS medium with 0.5 mg l^{-1} BA, developed new shoots after about 3 weeks of culturing. The shoot generation ability was maintained up to three subcultures on shoot induction medium by regular subculturing. Therefore, average number of 10-12 (Fig.1C) shoots could be obtained from single nodal explants after 7-8 weeks of culture (data not shown). A similar finding of subculturing the *in vitro* generated nodal explants to fresh shoot multiplication medium was reported in *Sesbania rostrata* (Jain *et al*. 2004), *Sophora flavescens* (Zhao *et al*. 2004). After the three sub-cultures the shoot multiplication rate declined. A similar result was recorded in *O. basilicum* (Sahoo *et al*. 1997) and *Crossandra* spp. (Girija *et al*. 1999). Seven to eight weeks old *in vitro* regenerated shoots were transferred in rooting medium and roots were obtained successfully after 1-2 weeks.

Elongated shoots were excised and placed on half - strength or full- strength MS medium supplemented with various concentrations of NAA or IBA for induction of roots. Full- strength MS medium containing auxins showed very poor response in rooting, but well developed roots were achieved on half-strength MS medium supplemented with NAA or IBA with reduced sucrose

Table 2. Effect of different concentrations of naphthyl acetic acid (NAA) and indole butyric acid (IBA) in MS medium for root induction from regenerated shoots of *Ocimum basilicum* L. Each treatment (n=15) is repeated three times. Means± SE, means followed by the same letters are not significantly different at P≤0.05 (Duncan 1955).

Growth regulators (mg l^{-1})	Response [%]	No. of Shoots [explant^{-1}]	Average length of shoots [explant^{-1}] (cm)
NAA			
0.25	48.88	2.1±0.6^{bc}	1.1±0.3^{b}
0.50	62.22	3.2±0.7^{abc}	1.4±0.3^{ab}
1.00	82.22	4.6±0.6^{a}	2.4±0.3^{a}
1.50	64.44	2.6±0.5^{bc}	1.3±0.2^{b}
2.00	48.88	2.2±0.5^{bc}	1.1±0.2^{b}
IBA			
0.25	15.55	1.2±0.6^{c}	0.8±0.3^{b}
0.50	46.66	2.2±0.6^{bc}	1.2±0.3^{b}
1.00	62.22	3.2±0.6^{ab}	1.5±0.3^{ab}
1.50	53.33	2.9±0.6^{abc}	1.6±0.3^{ab}
2.00	51.10	2.4±0.6^{bc}	1.1±0.3^{b}

* Basal medium= ½ MS + Sucrose (2%) + Agar (0.8%)

concentration (2%) within 15-20 days. However, optimum rooting (82.22%) was achieved on medium supplied with 1 mg 1^{-1} NAA (Table 2, Fig. 1D). In this medium a maximum number of 4.6 roots per explants attaining a length of 2.4 cm were obtained. Root development was however, slow at higher concentration of NAA or IBA. Begum *et al*. (2002) reported that for root initiation in *O. basilicum* NAA was effective, but these results were not in conformity with that of Sahoo *et al*. (1997). Many authors reported that NAA was best for root induction in other plant species such as *Rehum emodi* (Lal and Ahuja 1989), *Ruscus hypophyllum* (Jha and Sen 1985) and *Ocimum sanctum* (Begum *et al*. 2000).

For acclimatization, plantlets were removed from the rooting medium, thoroughly washed with water, and 115 plantlets were transferred to plastic pots containing a mixture of (1:1) soil and vermiculite (Fig. 1E). In the first week of transplantation, plantlets kept covered in a polythene tent for providing the condition of high humidity and sufficient light. The polythene cover was removed periodically and progressively whenever the leaves were appeared. Polythene covers were completely withdrawn after 2-3 weeks of hardening. After 3 weeks, plants were transferred to larger pots filled with soil and organic manure for further growth. The percentage of plant survival was calculated after two months. The *in vitro* rooted plantlets were successfully acclimatized with around 90% survival rate.

Hence, a reliable micropropagation protocol in *O. basilicum* L. from nodal explant, which can ensure large scale propagation, as well as *ex situ* conservation of this important aromatic and medicinal herb has been developed.

ACKNOWLEDGEMENTS

The authors are grateful to the University Grants Commission (UGC), New Delhi, India for financial assistance. Assistance rendered by Mr. Tulsi Dey in analysis of the data is thankfully acknowledged.

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