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Research Article

IN VITRO MULTIPLICATION OF *MAJORANA HORTENSIS* MOENCH – AN AROMATIC MEDICINAL HERB

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ABSTRACT
An efficient protocol for mass multiplication of *Majorana hortensis* is developed through nodal cultures. Surface sterilized explants were inoculated on Murashige and skoog's and Phillips and Collins media fortified with BAP and KIN to raise the cultures. Both direct and indirect organogeneses were obtained depending on the hormonal concentrations and combinations. Presence of AS in the medium promoted direct proliferation of the shoots from the nodal region while other cytokinins favored indirect regeneration of shoots from the callus. Repeated subcultures to the same media enhanced the number of multiple shoots from the cultures. Thus obtained shoots were transferred to the rooting medium containing IBA. The healthy regenerated plants were hardened in hardening mixture- Vermiculite and then transferred to pots containing sand: soil: manure in 1: 1: 1 proportion. The acclimatized plants were shifted to the field with 50% survival frequency.

Key Words: *Majorana Hortensis, Oil of Sweet Marjoram, Nodal Cultures, Mass Multiplication*

INTRODUCTION
Majorana hortensis Moench belonging to the family Lamiaceae is one of the important aromatic medicinal plants, widely used in indigenous systems of medicine. Steam distillation of leaves and flower heads yields a volatile oil known commercially as the "oil of sweet Marjoram" which is used to cure various human ailments. Further, the plant is reported to possess antibacterial properties (Ben *et al.,* 2001; Farooqi and Sriramu, 2004). An increasing demand for the essential oil in the Pharma and cosmetic industry has necessitated large scale production of this valuable taxon. Large quantities of the source material for the preparation, processing and testing of the product have led to the aggravation of the issue. Though the plant is usually propagated by stem cuttings, survival efficiency is not very significant because of poor rooting. Perusal of literature on the *in vitro* studies has revealed that this taxon is underexploited (Yashoda *et al.,* 2011). In the present study, an attempt is made to develop an effective and rapid propagation method for this valuable medicinal plant through direct and indirect regeneration using nodal explants.

MATERIALS AND METHODS
Procurement of Plants
Healthy plants were procured from The University of Agricultural Sciences, GKVK, and Bangalore and maintained in the polyhouse of the Department of Botany, Bangalore University. Nodal segments of 1 to 1.5 cms length were excised from 3 to 4 month old healthy plants and used as explants.
Surface Sterilization
The nodal explants were washed in tap water and then in liquid detergent Tween-20 for 5 to 10 minutes. After thorough washing in distilled water, the explants were treated with a fungicide – Bavistin (1%) for 5 to 10 minutes. After several washes in distilled water, the explants were surface sterilized with 70% alcohol for 10 seconds followed by washes in sterile water. They were then sterilized with $HgCl_2$ (0.1%) inside the laminar airflow chamber for about 2 minutes followed by repeated washes in sterile water to remove traces of $HgCl_2$.

Culture Medium

The surface sterilized explants were inoculated on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) and Phillips and Collins (L2) medium (Phillips and Collins, 1979) supplemented with different concentrations of various cytokinins like BAP, Kinetin (KIN), Adenine Sulphate (AS), Thidiazuron (TDZ) and 2- iso pentynyl adenine (2-ip) to induce multiple shoot formation. Media fortified with various auxins like IAA, IBA, NAA, 2, 4-D and gibberellins like GA_3 at different concentrations were also tried to study their effects. L- Glutamine (100 mg/l) was also used as a supplement along with plant growth regulators. Sucrose (3%) was the carbon source and Agar (0.8%) of PTC grade was used as a gelling agent. pH of the medium was adjusted to 5.8 and then autoclaved for 15 minutes at 121^0C.

Culture Condition

The cultures were maintained at 25 ± 2^0C under cool fluorescent lights with a photoperiodic regime of 16 h light and 8 h darkness with a light intensity of about 25 $\mu molm^{-2}s^{-1}$.

Multiple Shoot Induction

Multiple shoots were produced from the nodal region of the explants directly or from the callus produced at the base of the explants inoculated on MS and L2 media fortified with different concentrations of cytokinins. After 5 to 6 weeks of incubation, healthy shoots were sub cultured on fresh media with the same concentrations of plant growth regulators for further multiplication and elongation of the shoots. Experiments were repeated thrice and observations recorded at regular intervals.

Rooting

Healthy, elongated shoots of about 4-5 cms were transferred to full and half strength MS and L2 media supplemented with various concentrations of NAA and IBA for root induction.

Hardening and Acclimatization

The *in vitro* regenerated plants with well developed roots were subjected to hardening under controlled environmental conditions. The regenerated plants were washed in sterile water to remove any adhering medium and transferred to plastic pots containing various autoclaved hardening mixtures like perlite, vermiculite and soilrite, singly and in combination of 2 or more mixtures. After about 30 days they were transferred to earthen pots containing 1:1:1 soil: sand: manure and maintained in the polyhouse for acclimatization.

Histological Studies

The organogenic callus was fixed in FAA (Formalin: Acetic acid: Alcohol 90: 5: 5) for 24 hrs. Customary paraffin technique was followed. Microtome sections of about 20-25 μm thickness were taken and stained with Haidenhain's haematoxylin and counter stained with orange G / Eosin / Erythrosine. Microphotographs of stained sections under Nikon binocular microscope were taken with a canon camera.

RESULTS AND DISCUSSIONS

The *in vitro* production of plants through nodal cultures is the most reliable method to get uniform plants of selected genotypes (George and Sherrington, 1984). The surface sterilized nodal explants of *Majorana hortensis* were cultured on MS and L2 media supplemented with different concentrations of various growth regulators to study their effects on the induction of multiple shoots. L2 medium fortified with various hormones showed better response than MS medium with the same hormonal combination. This is in conformity with the previous reports on *Macrotyloma uniflorum* (Tejavathi *et al.*, 2010) and *Sauropus androgynous* (Tejavathi and Padma, 2011).

Callus started initiating from the basal part of the explants after two weeks of culture on L2 medium supplemented with auxins (George and Sherrington, 1984; Reinert and Bajaj, 1997; Tejavathi *et al*., 2010).The type of callus varied with the type of auxin used in the medium. IAA induced greenish callus where as rhizogenic, greenish callus was obtained on NAA/IBA supplemented medium. However, grayish-green, nodular callus was initiated from the basal part of the explants on 2, 4-D supplemented medium. A few axillary shoots were formed from the nodal region on L2 medium supplemented with IAA (22.84 μM) / IBA (14.70 μM) and NAA (21.48 μM) (Table1). When the callus derived from the

basal part of the explants was sub cultured on various combinations of media, no morphogenesis was observed. However, presence of cytokinins in the media resulted in both direct and indirect organogenesis from the cultures. Multiple shoots were formed either directly from the node or from the basal callus depending on the type, concentration and combination of the cytokinins. Presence of AS (16.29 μM) induced direct proliferation of multiple shoots from the nodal region without any callus induction. Rapid increase in the number of multiple shoots was observed when sub cultured to fresh medium of same combination. Very high concentration of AS (30mg/l) promoted the proliferation of multiple shoots from the nodal cultures of Pomegranate (Patil *et al.*, 2011). Presence of Kin, BAP, TDZ and 2-ip in the medium promoted the initiation of profuse creamish callus from the basal part of the explants after 2 weeks of culture. Greenish shoot buds started emerging from the callus after 4-5 weeks of culture. Such callus mediated shoot morphogenesis has been accomplished in several medicinal plants (Agarwal and Sardar, 2006). Exogenous supply of cytokinins is required to bring out the morphogenetic potential from the shoot cultures since the quantity of the cytokinins produced by the shoots is very less compared to roots which are the principle sites of cytokinin synthesis (Kodo and Okozava, 1980). Among the cytokinins supplemented, kinetin proved to be the most efficient in initiating a large number of shoots from the callus. Kinetin at the concentration of 9.30 μM promoted the formation of the highest number of multiple shoots 38.8 ± 0.78 per culture.

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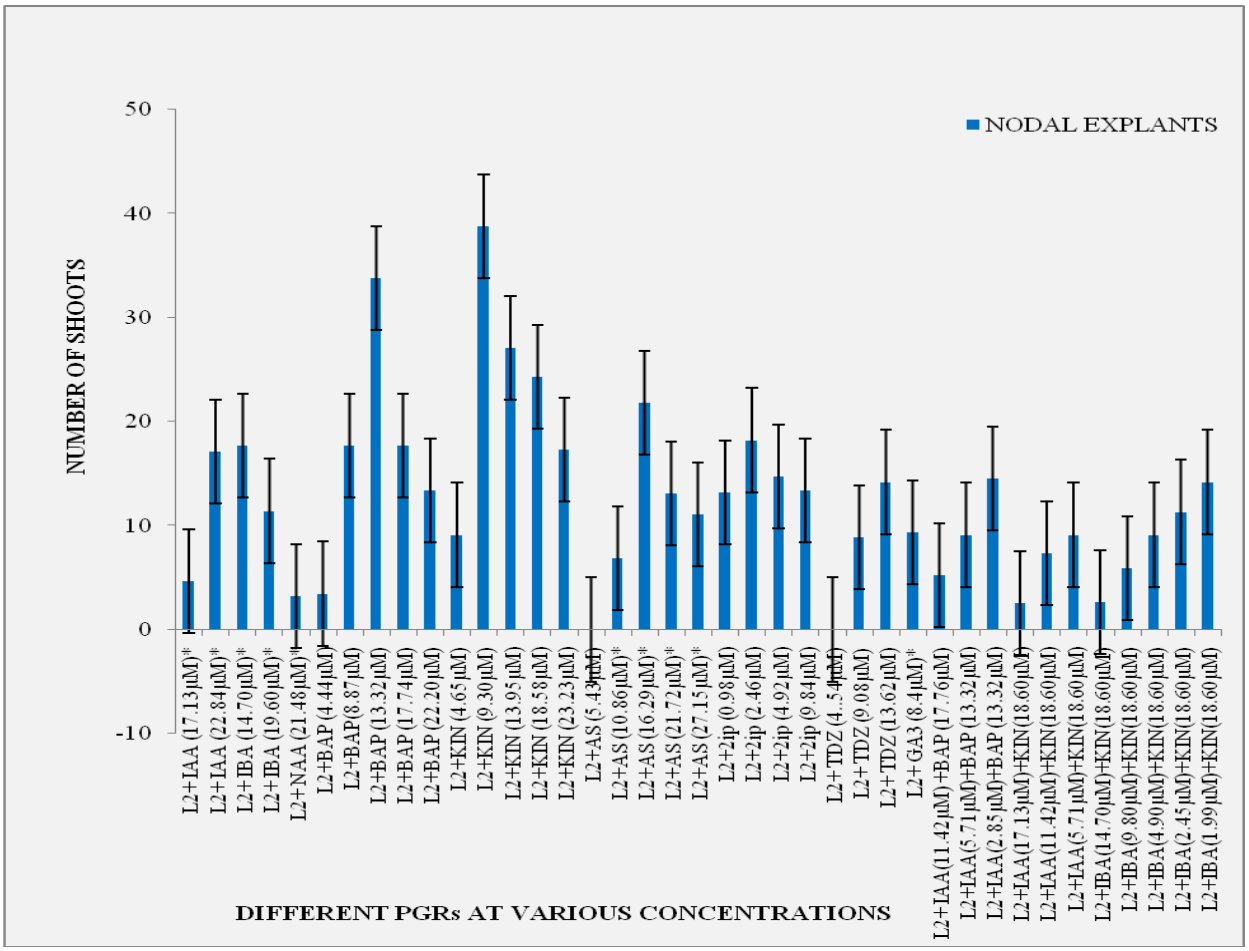

Figure 1: Effect of various plant growth regulators on multiple shoot formation from nodal explants of *Majorana Hortensis*

Table 1: Effect of various plant growth regulators on multiple shoot formation from nodal explants of *Majorana Hortensis*

S. No.	Treatment	Number of Shoots/Explant (Mean±Std)
1	L2+ IAA (17.13µM)*	4.66±0.5
2	L2+ IAA (22.84µM)*	17.1±1.05
3	L2+ IBA (14.70µM)*	17.7±0.97
4	L2+ IBA (19.60µM)*	11.4±0.52
5	L2+ NAA (21.48µM)*	3.22±0.83
6	L2+BAP (4.44µM)	3.44±0.52
7	L2+BAP(8.87µM)	17.7±0.83
8	L2+BAP (13.32µM)	33.8±0.92
9	L2+BAP (17.74µM)	17.7±0.83
10	L2+BAP (22.20µM)	13.4±0.88
11	L2+KIN (4.65µM)	9.11±0.92
12	L2+KIN (9.30µM)	38.8±0.78
13	L2+KIN (13.95µM)	27.1±0.92
14	L2+KIN (18.58µM)	24.3±0.70
15	L2+KIN (23.23µM)	17.3±0.70
16	L2+AS (5.43µM)	0.00±0.00
17	L2+AS (10.86µM)*	6.88±0.78
18	L2+AS (16.29µM)*	21.8±0.92
19	L2+AS (21.72µM)*	13.1±0.92
20	L2+AS (27.15µM)*	11.1±0.78
21	L2+2ip (0.98µM)	13.2±0.83
22	L2+2ip (2.46µM)	18.2±0.97
23	L2+2ip (4.92µM)	14.7±0.83
24	L2+2ip (9.84µM)	13.4±0.88
25	L2+ TDZ (4..54µM)	0.00±0.00
26	L2+ TDZ (9.08µM)	8.88±0.92
27	L2+ TDZ (13.62µM)	14.2±0.83
28	L2+ GA3 (8.4µM)*	9.33±0.70
29	L2+ IAA(11.42µM) +BAP (17.76µM)	5.22±0.83
30	L2+IAA(5.71µM)+BAP (13.32µM)	9.11±0.78
31	L2+IAA(2.85µM)+BAP (13.32µM)	14.5±0.52
32	L2+IAA(17.13µM)+KIN(18.60µM)	2.55±0.52
33	L2+IAA(11.42µM)+KIN(18.60µM)	7.33±0.70
34	L2+IAA(5.71µM)+KIN(18.60µM)	9.11±0.78
35	L2+IBA(14.70µM)+KIN(18.60µM)	2.66±0.5
36	L2+IBA(9.80µM)+KIN(18.60µM)	5.88±0.33
37	L2+IBA(4.90µM)+KIN(18.60µM)	9.11±0.78
38	L2+IBA(2.45µM)+KIN(18.60µM)	11.3±0.70
39	L2+IBA(1.99µM)+KIN(18.60µM)	14.2±0.66

**Direct Organogenesis*

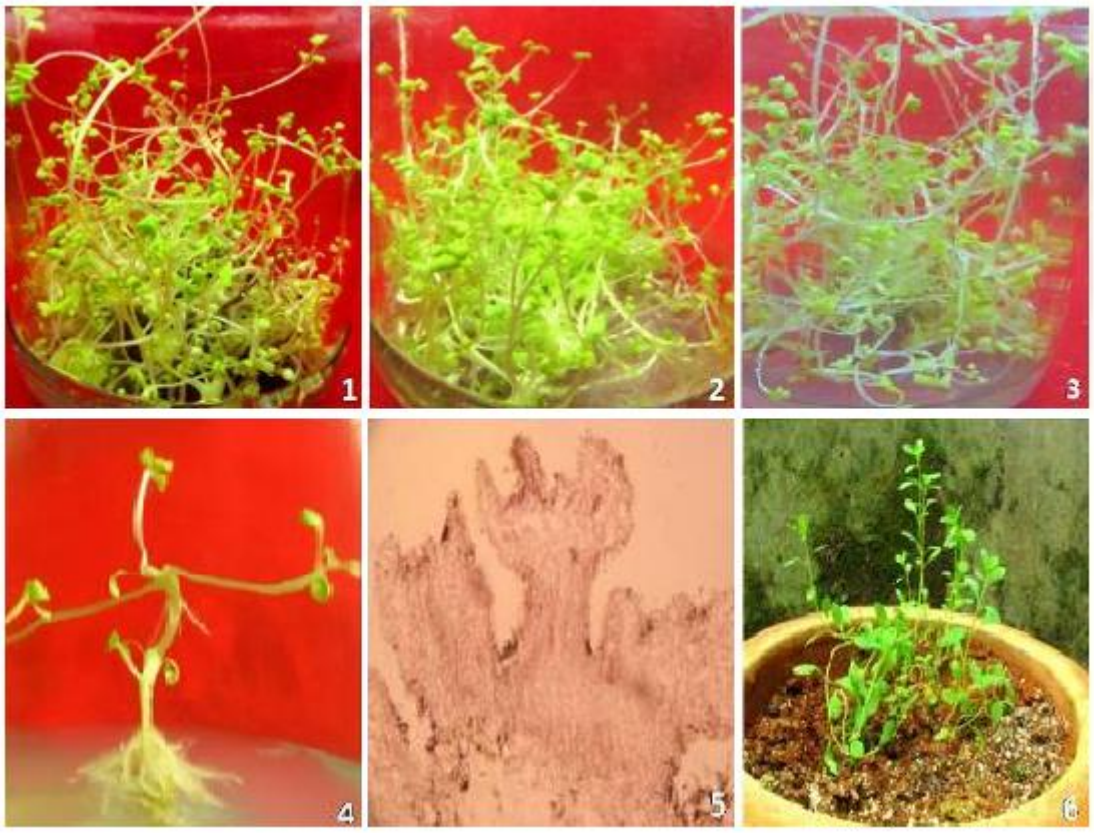

Figure 1 to 6: 1- Multiple shoots through indirect organogenesis on L2+BAP (13.20µM); **2-** Multiple shoots through indirect organogenesis on L2+KIN (9.29µM); **3-** Multiple shoots through direct organogenesis on L2+AS (16.29µM); **4-** Rooting on L2+IBA (4.92µM); **5-** Histological sections showing the multiple shoots through indirect organogenesis; **6-** Hardened regenerated plant.

BAP is considered a potential hormone to induce multiple shoots in several taxa (Leelavathi 2009, Karthikeyan *et al.*, 2009; Sharma *et al*., 2010 and Tejavathi and Indira, 2011). Maximum number of 33.8 ± 0.92 shoots per culture was recorded on BAP (13.32μM) supplemented medium. Yashoda Bai *et al.*, (2011) also opined that Kin was more efficient in this taxon than BAP. Similar observations were made in axillary bud cultures of *Lavendula dentate* (Anamaria *et al.*, 1998) and *Solanum surattense* (Ramaswamy *et al.*, 2004). In the present study when the concentration of KIN and BAP were increased beyond 9.30 μM and 13.32 μM respectively, the rate of shoot multiplication gradually reduced. Similar effects of higher concentrations of cytokinins were observed in *Solanum surattense* (Ramaswamy *et al.*, 2004), *Ruta graveolens* (Bohidhar *et al.*, 2008; Tejavathi and Manjula, 2010). Thidiazuron is considered a more effective cytokinin than purine type cytokinins (Lu, 1993). The dual effect of TDZ in bringing out both direct and indirect organogenesis is established in *Nothapodytes foetida* (Tejavathi *et al.*, 2012) depending on the type of media and combinations. However, in the present taxon, TDZ can induce a few multiple shoots from the callus formed at the base of the explant. Indirect organogenesis was also observed when the explants were cultured on media containing a combination of auxins and cytokinins revealing the synergistic effect of plant growth regulators. Further, keeping the concentration of cytokinin constant and reducing the concentration of auxin increased the number of shoots. Maximum number of 14.5±0.52 shoots per culture was obtained on L2+IAA (2.85µM) +BAP (13.32µM). A low concentration of auxin in combination with a high concentration of cytokinins is the most suitable for induction of multiple shoots (Tejavathi and Indira, 2011).

Better morphogenetic response was noticed in the cultures upon the addition of L-Glutamine to the culture medium. L-Glutamine is a potent amino acid frequently used in tissue culture studies to enhance morphogenetic potential of the explants (George and Sherrington, 1984). Mohamed (1996) is of the opinion that cultured cells are normally capable of synthesizing all the required amino acids, yet the addition of amino acid/s may stimulate cell growth and facilitate plant regeneration. L-Glutamine can also serve as a source of nitrogen which can be absorbed by tissues more rapidly than inorganic nitrogen (Thom *et al.,* 1981). In the present study, the effect of L-Glutamine was evident in better quality of shoots in respect of growth of shoots and morphology of leaves. The shoots were more elongated and the leaves were much greener and broader. Tejavathi *et al.,* (2010) have reported similar effects of L-Glutamine in *Nothapodytes foetida*. L-Glutamine is known to be an effective chelating agent and when added together with micronutrients, is said to facilitate absorption and transportation of micronutrients inside the plant.

Histological studies of the organogenic callus revealed the presence of shoot buds emerging from the peripheral region of the callus characterized by actively dividing cells. Similar type of indirect organogenesis was reported in *Piper nigrum* (Sujatha *et al.,* 2003), *Agave Vera* Cruz (Tejavathi and Gayathramma, 2005), *Andrographis paniculata* (Tejavathi *et al.,* 2008) and *Sauropus androgynous* (Tejavathi and Padma, 2011).

The shoots obtained from direct and indirect organogenesis were excised and transferred to a medium containing auxins at various concentrations. Root initiation is an important and crucial step in the regeneration of plants which is usually achieved by treatment with auxins (Bohidhar *et al.,* 2008). This is in conformity with the observations made by Torrey (1965) that auxins have a role in root development. Role of IBA as an effective root inducing hormone is well established in several taxa (Jha and Jha, 1989; Whakhlu and Barna, 1989; Lal and Ahuja, 1989; Bensal and Pandey, 1993; Sivaprakash *et al.,* 1994; Thiruvengadam and Jayabalan, 2000; Raman and Jaiwal, 2000; Santos *et al*., 2003 ; Cheepala *et al*., 2004; Bohidhar *et al*., 2008; This is in contrast to the results of Madhavan and Balu (1995) in *Widelia chinensis* where IBA promoted multiple shoot formation. In the present study, transfer of multiple shoots to media fortified with auxins like IBA and NAA induced rooting. Similar results were obtained by Tavares *et al.,* (1996) in *Melissa officinalis* and by Andrade *et al*., (1996) in *Lavendula Vera* using MS medium fortified with IBA or NAA. In *Majorana hortensis* best rooting was induced on full strength L2 medium fortified with IBA (4.92 μM). This is in accordance with the observations of Arockiasamy *et al*., (2003) in *Solanum trilobatum*, Jeyakumar and Jayabalan, (2002) in *Psoralea corylifolia,* Arya *et al*., (2008) in *Pluchea lanceolata*, Bohidhar *et al*., (2008) in *Ruta graveolens*, Sharma *et al*.,(2010) in *Bacopa monnieri* and Tejavathi and Indira(2011) in *Drymaria cordata* that IBA is a more efficacious hormone for root induction than other auxins. The slow movement and degradation of IBA facilitates its localization near the site of application and thus it functions better in inducing roots (Nickll and Kirk-Othmer, 1982). However, it differs from the results obtained by Tejavathi *et al*., (2010) in *Sauropus androgynous* where better rooting is seen on half strength medium fortified with NAA. Yashoda Bai *et al*., (2011) reported that in *Oreganum majorana* MSBM fortified with KN, IBA and NAA is the most suitable rooting medium. Presence of L-glutamine is also said to promote root induction in microshoots (Witrzens *et al*., 1988).

The regenerated plants with healthy, delicate roots were hardened under controlled environmental conditions in the polyhouse and vermiculite proved to be the best hardening mixture indicating the requirement of less water and good aeration for better establishment. The hardened plants acclimatized better in potting mixture containing soil: sand: manure in the ratio 1:1:1. They were transferred to the field with 50% survival frequency.

CONCLUSIONS

Effects of various growth regulators at different concentrations have been analyzed and suitable protocols for rapid multiplication through direct and indirect regeneration of *Majorana hortensis* have been

developed. The protocol reported is reproducible and has the potential for being utilized for the conservation of the germplasm.

Large scale production of this valuable medicinal and aromatic plant can be achieved through micropropagation and the regenerates can be utilized for the extraction of the essential oil which is much in demand in the pharmaceutical, flavor and fragrance industry. Further, the study can be utilized to introduce variations in the plant for better yield and the elite variants can be selected for further propagation in the field.

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