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Rapid *in vitro* Clonal Propagation of *Lavendula officinalis* chaix A Multipurpose Plant of Industrial Importance

[1]Nahida Chishti, [2]Z.A. Kaloo, [1]A.S. Shawl and [1]Phalisteen Sultan
[1]Regional Research Laboratory, Sanat Nagar, Srinagar, J and K, India, 190005
[2]Department of Botany, University of Kashmir, Srinagar, J and K, India, 190006

Abstract: An efficient regeneration protocol has been developed for rapid and mass scale clonal propagation of *Lavendula officinalis* from shoot tip explants. For the induction of multiple shoot formation, the explants were cultured on Murashige and Skoog's (MS) basal medium supplemented with different concentrations of cytokinins viz., BAP (0.5-2.0 mg L^{-1}), Kn (0.5-2.0 mg L^{-1}) and on different cytokinin plus auxin combinations viz., BAP (1.0-2.0 mg L^{-1} + IAA (0.5-1.0 mg L^{-1}) and Kn (1.0-2.0 mg L^{-1}) + IAA(0.5-1.0 mg L^{-1}). These shoot tips produced multiple shoots, with the best result in BAP (2.0 mg L^{-1}) whereby (45±6.40) shoots were produced/explant in 80% cultures in 4 weeks time. The regenerated shoots were subcultured on the best induction medium (BAP 2.0 mg L^{-1}) after every 4 weeks so as to increase their number. These shoots rooted best in half strength MS basal medium fortified with IBA (1.0 mg L^{-1}) with 7-18 roots per shoot (Avg. 16±6.89) roots in 80% cultures in 4 weeks time. Rooted plantlets were shifted to the plastic pots containing mixture of sand:clay:vermiculite (1:1:1) and kept for two weeks under controlled conditions of temperature (25±2°C) and relative humidity 60%. Afterwards these plantlets were hardened in green house environment (Vista Biocell Limited) for one month and finally shifted to field conditions were 70% plants survived.

Key words: *Lavendula officinalis*, aromatic, clonal, shoot tips, organogenesis, *in vitro*

INTRODUCTION

Lavendula officinalis Chaix syn. *Lavendula angustifolia* Mill. (Genuine lavender, F Lamiaceae) is one of the most important aromatic plants in France, Spain, Bulgaria, Yugoslavia and Russia. The plant was introduced in Kashmir in 1983 and its cultivation and processing for essential oil and dried flowers was quite successful (Tajjudin *et al*., 1983). Lavender oil distilled from the flowering spikes is used in high grade perfumes and cosmetics. It is a component of many creations (Gimpsey and Porter, 1999). The oil is traditionally believed to have antibacterial, antifungal, antimicrobial, antidepressive, carminative activity and is effective for burns and insect bites (Grieve, 1931; Gattefosse, 1937). The spicy, vegetative nuances found in the chypre like perfumes is created by the addition of small amounts of natural lavender oil to the fragrance. The dry note associated with the lavender oil is famous for creating perfumes for men (Shawl and Kumar, 2000). The continuing popularity and commercial value of lavender was recently confirmed when it was named 'Herb of the year' 1999 by the Herb Growing and Marketing Network in USA (Anonymous, 1999).

The poor rooting ability of stem cuttings as well as the lack of selected clones restrain its industrial exploitation. Multiplication through seeds involves segregation which results drop in quality and quantity of the essential oil (Segura and Calvo, 1991). Limited tissue culture work has been done on *Lavendula* species.

Tissue culture studies on *Lavendula officinalis* have largely been restricted to monitor monoterpene synthesis in shoots regenerated from callus culture (Webb *et al*., 1984). Although micropropagation of *Lavendula vera* has been reported but that is through differentiation in leaf derived calli(Tsuro *et al*., 1999). During the present study an efficient procedure for mass scale clonal propagation of *Lavendula officinalis* from shoot tip explants has been developed.

MATERIALS AND METHODS

Plant material: The present study was carried out at Regional Research Laboratory, Sanat Nagar, Srinagar, Kashmir during the year 2004. Juvenile shoot tips (2 cm) of *Lavendula officinalis* were collected from the mature plants (6 years old) grown in the RRL farm at Pulwama Kashmir, India. These explants were washed thoroughly

Corresponding Author: Nahida Chishti, Regional Research Laboratory, Sanat Nagar, Srinagar, J and K, India, 190005
Tel: 2437953

with running tap water for at least 30 min followed by immersing in 5% (v/v) Tween-20 for at least 15 min. The explants were surface sterilized with 0.1% w/v $HgCl_2$ for 1 min. After 4-5 extensive rinses with sterile distilled water, these explants were then inoculated onto the culture medium.

MS basal medium Murashige and Skoog (1962) containing 3% (w/v) sucrose was used in all the experiments. The pH of the medium was adjusted to 5.8 prior to the addition of 1% w/v agar and autoclaving at 15 lbs pressure at 121°C for 15 min. The cultures were maintained at 25±2°C temperature with photoperiod of 16 h at 35 μE m^{-2} per flux density.

RESULTS AND DISCUSSION

Induction of multiple shoot formation: In order to induce multiple shoot formation from the shoot tip explants, the MS basal medium was fortified with a range of cytokinin (BAP and Kn) concentrations. These cytokinins were most effective at concentration of 0.5-2.0 mg L^{-1}(Table 1).

On MS basal medium augmented with BAP (0.5 and 1.0 mg L^{-1}) the shoot tips produced multiple shoots (15±5.48) and (25±6.08) in 30 and 60% cultures, respectively within four weeks of incubation. The best result was achieved on MS medium fortified with BAP (2.0 mg L^{-1}) which induced the production of as many as 44-47 shoots (Avg. 45±6.40) shoots/explant in 80% cultures within four weeks of incubation (Fig. 1a). Alternatively, Kn at concentration of (0.5-1.0 mg L^{-1}) induced multiple shoot differentiation only in 20 and 50% cultures respectively with the mean no. of shoots being (6±1.58) and (10±1.58) after four weeks of inoculation. Increasing the concentration of Kn from 1.0 mg L^{-1} to 2.0 mg L^{-1} resulted in a decrease in the rate of shoot regeneration ability (Table 1).

The shoots regenerated were subcultured on the best induction medium (MS + BAP 2.0 mg L^{-1}) after every four week so as to produce the shoots at a mass scale. The regenerated shoots were then transferred to the rooting medium.

Table 1: Effect of cytokinins on multiple shoot regeneration from shoot tip explants of *Lavendula officinalis Chaix* in MS basal medium after four weeks of culture

Treatments	Regeneration %	Mean No. of shoots/ explant±SD	Mean shoot length (cm)±SD
BAP (0.5 mg L^{-1})	30	15.0±5.48	3.5±1.07
BAP (1.0 mg L^{-1})	60	25.0±6.08	5.5±0.51
BAP (2 mg L^{-1})	80	45.0±6.40	6.5±1.08
Kn (0.5 mg L^{-1})	20	6.0±1.58	2.5±1.00
Kn (1.0 mg L^{-1})	50	10.0± 1.58	5.0±1.58
Kn (2.0 mg L^{-1})	30	6.0±1.58	4.0±1.58
Basal	-	-	-

Values represent mean±standard deviation of 10 replicates per treatment in three repeated experiments

Table 2: Effect of cytokinin plus auxin combinations on shoot tips *of Lavendula officinalis* Chaix in MS basal medium after four weeks of culture

Treatments	Regeneration %	Mean No. of shoots/ explant±SD	Mean shoot length (cm)±SD
BAP (1 mg L^{-1})+IAA (0.5 mg L^{-1})	60	20.0±4.18	5.5±1.25
BAP (2 mg L^{-1})+IAA (1.0 mg L^{-1})	70	35.0±5.61	4.0±1.58
Kn (1 mg L^{-1})+IAA (0.5 mg L^{-1})	30	8.0±1.58	2.5±1.11
Kn (2 mg L^{-1})+IAA (1.0 mg L^{-1})	20	4.0±1.58	3.5±1.15

Values represent mean±standard deviation of 10 replicates per treatment in three repeated experiments

Table 3: Effect of MS salt strength, IAA and IBA concentrations on root induction of the *in vitro* raised shoots of *Lavendula officinalis* Chaix after four weeks of culture

Treatments	Regeneration %	Mean No. of shoots/ explant±SD	Mean shoot length (cm)±SD
MS	30	4.0±1.58	1.0±0.70
1/2MS	50	10.0±2.24	1.5±0.90
1/2MS+IAA (0.5mg L^{-1})	40	3.0±1.58	1.0±0.07
1/2MS+IAA (1.0mg L^{-1})	60	5.0±2.92	1.5±0.47
1/2MS+IBA (0.5mg L^{-1})	60	8.0±1.58	1.8±0.86
1/2MS+IBA (1.0mg L^{-1})	80	16.0±6.89	2.1±1.24

Values represent mean±standard deviation of 10 replicates per treatment in three repeated experiments

Multiplication of the shoots: The synergistic effect of the cytokinins and the auxins also proved effective in inducing multiple shoot formation in the shoot tips (Table 2). MS basal medium augmented with BAP (2.0 mg L^{-1}) + IAA (1.0 mg L^{-1}) proved the best combination in which as many as 34-37 shoots (Avg. 35±5.61) shoots were produced per explant in 70% cultures within four weeks of inoculation (Fig. 1b). These regenerated shoots also produced roots when subcultured twice after every four weeks on the same media composition. The regenerated plantlets were then transferred to the jiffy pots for acclimatization.

Root induction: The *in vitro* raised shoot cuttings (3-4 cm) in length, were excised from the clump of shoots and inoculated on MS basal media of both full and half strength of inorganic nutrients as well as on MS basal medium augmented with auxins viz., IBA and IAA in different concentrations (Table 3). The maximum frequency of root differentiation (16±6.89) was achieved on half strength MS basal medium fortified with IBA (1.0 mg L^{-1}) with the average length of roots (2.1±1.24), respectively as recorded after four weeks of inoculation (Fig. 1c). Alternatively, IAA at a concentration of (0.5 and 1.0 mg L^{-1}) induced root induction in 40 and 60% cultures, respectively with the mean no. of roots (3.0±1.58); 5.0±2.92) produced per shoot after four weeks of inoculation (Table 3).

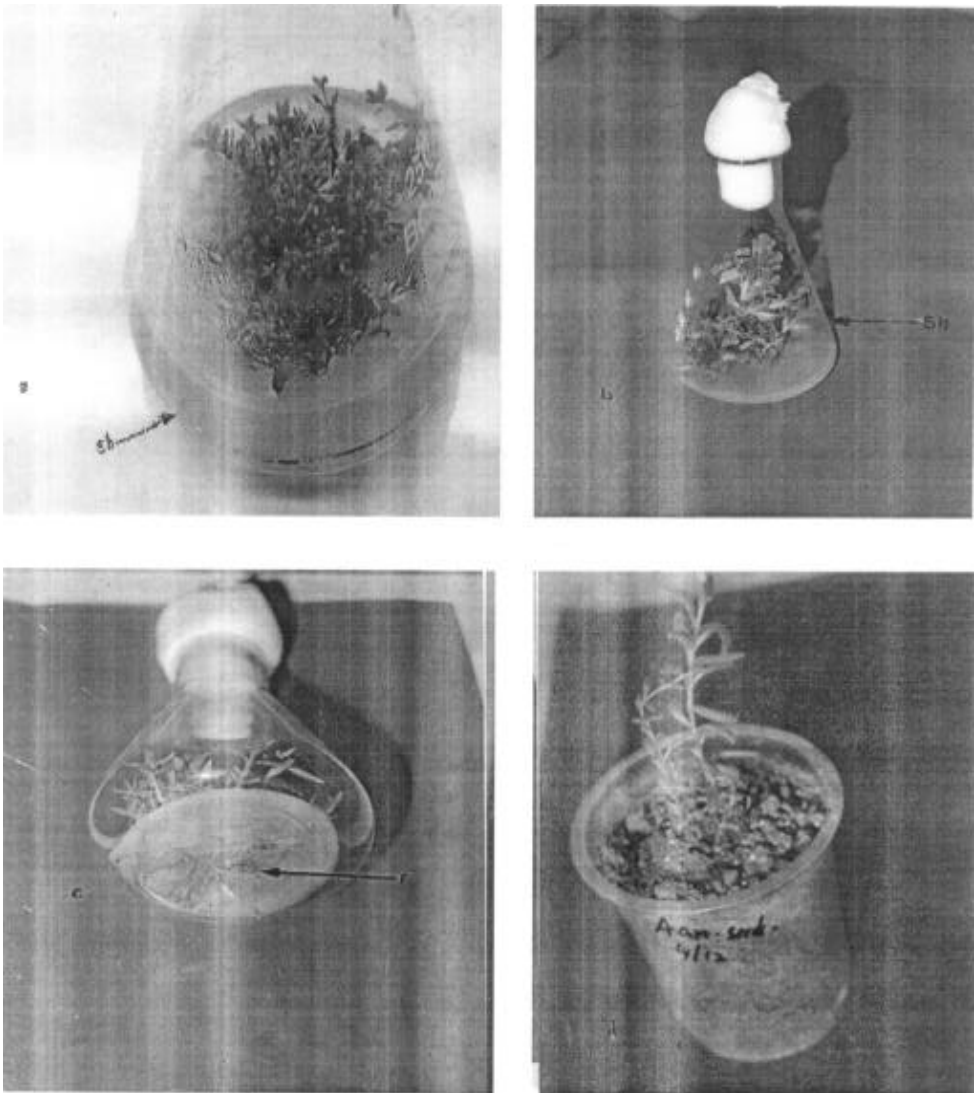

Fig. 1: a) Shoot tip explants producing multiple shoots on MS+BAP (2 mg L^{-1}), b): Shoot tip explants producing multiple shoots on MS+BAP (2 mg L^{-1})+IAA (1 mg L^{-1}), c) Regeneration of roots on 1/2MS+IBA (1 mg L^{-1}), d) Acclimatised potted plantlets, sh: shoots, r: rhizogenesis

Acclimatization: The rooted plants were thoroughly washed with running tap water to remove the adhering agar and planted in plastic pots containing sterile mixture of sand:clay:vermiculite in 1:1:1 for hardening at 16 h photoperiod conditions. The potted plantlets were covered with transparent polythene bags to ensure high humidity and watered alternatively with half strength MS salt solution (Fig. 1D). Once, the acclimatization was accomplished these plants were then transferred to greenhouse (Vista Biocell Limited) for one month and then shifted to field conditions where 70% of the plants survived exhibiting normal development with high uniformity.

For successful micropropagation shoot tip cultures were preferred as pre-existing meristem easily develop into shoots while maintaining clonal fecundity. The survival percentage and their subsequent development into shoots varied from 30-80% on BAP (0.5-2.0 mg L^{-1}) and 20-50% on Kn (0.5-2.0 mg L^{-1}). The frequency of sprouting buds was comparatively lower on Kn supplemented medium. Increasing the concentration of BAP from (0.5-2.0 mg L^{-1}) resulted in an increase in the rate of shoot regeneration ability. These results are in consonance with the earlier reports by Tsuro *et al.* (1999), Carlos *et al.* (1996) and Tsuro *et al.* (2001) in case of nodal explants of *Lavendula latifolia* and leaf derived

calli of *Lavendula vera* DC, respectively, where enhanced shoot multiplication was observed on MS supplemented with BA (5 or 20 μM) and BAP (4.4×10^{-7}- 4.4×10^{-5}, 4.4×10^{-6}M). The superiority of BAP over Kn in the present investigation is in conformity with the earlier reports on leaf explants of lavandin (*Lavendula× intermedia* Emeric ex Loiseleur), nodal explants of *Lavendula viridis* L'Her (Dronne *et al*., 1999; Dias *et al*., 2002) and on nodal segments of *Lavendula vera* DC (Andrade *et al*., 1999).

The shoots regenerated on BAP supplemented medium showed better growth. However, addition of IAA with optimal concentration of Kn or BAP significantly reduced the frequency of shoot formation (Table 2). This is in agreement with the earlier reports where instead of IAA; NAA was used as a source of auxin which decreased the shoots per culture in case of *Lavendula latifolia* (Carlos *et al*., 1996).

Among the various auxin cytokinin combinations, the best results in terms of shoot multiplication were recorded on BAP (2 mg L^{-1}) + IAA (1 mg L^{-1}) after 6-8 days of inoculation. In the next four weeks the microshoots in the same media composition showed enhanced shoot multiplication (35±5.24) along with (7±1.58) roots per explant. In these cultures an exudation of some blue pigments was also released into agar medium. Similar, results were reported by Nakahma *et al.* (1990) and Banthrope *et al.* (2001) in the immobilized cultured cells of *Lavendula vera* in the presence of L-cysteine and also in callus culture of *Lavendula angustifolia.*

The elongated shoots (2-3 cm) in length, were excised from shoot clump and transferred to different rooting media. These microshoots were rooted on auxin free MS basal medium which produced three to five roots per shoot tip. Root formation in auxin free medium may be due to the presence of higher quantity of endogenous auxin levels in the *in vitro* shootlets. Reduction of nutrients to half strength (1/2 MS) and addition of IBA in the rooting medium enhanced root differentiation frequency. Half strength MS basal medium fortified with IBA (0.5-1.0 mg L^{-1}) induced higher frequency of rooting (Table 3). Success of IBA for efficient root induction was also reported in *Lavendula vera* DC (Tsuro *et al*., 1999; Dronne *et al*., 1999) on lavandin. On the other hand in *Lavendula vera* DC Andrade *et al.* (1999) did not get any response with IBA as a rooting agent and reported NAA to be more effective rooting agent. While as increased sucrose concentration from 58.4 to 87.6 mM resulted in a significant increase in rooting frequency in *Lavendula viridis* L'Her (Dias *et al*., 2002).

Although micropropagation of *Lavendula vera* DC and lavandin has been achieved earlier by Carlos *et al*., (1996) and Tsuro *et al*. (2001) but the plants which were raised were from the leaf derived callus which are not a clone. In order to maintain the quality and to increase the quantity of the lavender oil, clonal propagation being highly desirable has been achieved in the present study were in a rapid protocol for large scale propagation (45±6.40) shoots/explant; 70% survival rate for *Lavendula officinalis* has been developed. Similar, results were also achieved in *Lavendula viridis* (Dronne *et al*., 1999) but the highest multiplication rate (11.69 shoot/node) obtained was comparatively low than that achieved in the present study.

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