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Lavandula angustifolia L. (Lavender): An important aromatic medicinal shrub and its *in vitro* micro-propagation for conservation

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Abstract *In vitro* micro-propagation protocol for large scale multiplication of *Lavandula angustifolia* L. (Lavender) belonging to family Lamiaceae, was carried out aseptically in Laboratory for its conservation. Almost all the cultures got initiated after five days of culturing. The results which are quite noticeable were taken on 28th day of aseptic culturing. For results interpretation, number and growth of axillary branches and leaflets were measured. The maximum length of leaflets growth (118mm) was found at 15 mg/l of IBA with stem/shoot-tip explants. Whereas other concentrations like 0.2 mg/l, 1.0 mg/l, 5.0 mg/l of IBA showed total length of leaflets growth as 96mm, 102mm and 101mm respectively. So 15 mg/l of IBA was best concentration for *in vitro* culturing of *L. angustifolia.* On the other hand, the maximum length of leaflets growth (142mm) was found at 2.0 mg/l of BAP with stem/shoot-tip explants. Whereas other concentrations like 4.0 mg/l, 6.0 mg/l, 8.0 mg/l of BAP showed total length of leaflets as 119mm, 96mm and 84mm respectively. So 2.0 mg/l of BAP was best concentration for *in vitro* micro-propagation of *L. angustifolia.* But 2.0 mg/l of BAP concentration was best than 15 mg/l of IBA concentration comparatively as more leaf length growth was observed at this concentration and moreover, less quantity of BAP hormone was required than IBA hormone. Also, 2.0mg/l of BAP+15mg/l of IBA supplemented MS medium was a compassionate concentration for mass culture of callus for further multiplication.

Key words: Growth regulators, Micro-propagation, *Lavandula angustifolia,* Shoot initiation, 6-Benzylaminopurine

Introduction

Medicinal plants are very important for mankind. The extracts from these plants are used for the production of various remedies which can cure various

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life threatening diseases. Lavander is one of very important medicinal plant of Lamiaceae family which is used in various remedies. Lavender's essential oil is commonly used in aromatherapy and massage (Kokkalou, 1988; Hui *et al.,* 2010). Its major clinical benefits are on the central nervous system. Many studies conducted on both animals and humans support its use as a sedative and anxiolytic (mood modulator). Lavender oil has *in vitro* antimicrobial activity against bacteria, fungi and some insects also it exerts spasmolytic activity in smooth muscle *in vivo*, supporting its historical use as a digestive aid (Hui *et al.,* 2010; Cassella *et al.,* 2002). Particular chemical constituents of lavender have potent anticarcinogenic and analgesic properties. Lavender has some allergic reactions and it affects the central nervous system. People suffering from seizure disorders and using sedative medications should avoid the use of lavender. Although, lavender has traditionally been used to treat symptoms like restlessness and colic cramp in infants and children. Systematic studies have not been conducted to test the efficacy and safety of lavender use in infants and children or during pregnancy or lactation (Catherine and Kamper, 2001).

The name *lavender* is derived from the Latin "lavare", which means to wash. The fragrant flowers were used in ancient Rome and North Africa to scent public baths and were carried by the Roman army for use as a disinfectant. "Lavenders" in Medieval and Renaissance times were used for the storage of laundry. The Ancient Egyptians are said to have used the flower in the mummification process. Now-a-days, there is a rekindled interest for lavender aromatherapy (Kim *et al.,* 2007; Jin and Ha, 2005). In Traditional Chinese Medicine (TCM), lavender is used to treat several conditions including infertility, infection, anxiety, and fever. It has long been used in Arabic medicine to treat stomachaches and kidney problems. Lavender was commonly used as an aphrodisiac in Victorian times (Catherine and Kamper, 2001).

Various folk traditions have used the herb for a variety of other medicinal purposes ranging from giddiness to hair loss. Preparations from the plant have been used to increase bile flow, treat varicose ulcers and relieve carpal tunnel syndrome. It has been considered an antidepressant, antispasmodic, antiflatulent, antiemetic, diuretic and a general tonic. Lavender has also been recommended as a worm remedy and as a topical remedy for insect bites (Catherine and Kamper, 2001). Lavender is commonly used today in perfumes, soaps, bath and talc powders, candles andscented sachets. Small amounts are sometimes used to flavor teas and foods, such as in the French *herbs de Provence*. The flower's strong and pleasant fragrance has led to its popular use in aromatherapy, where it is considered one of the most versatile and useful essential oils (Jin and Ha, 2005). Aromatherapy with lavender oil has been recommended to treat a wide range of ailments including stress, anxiety,

depression, fatigue, motion sickness and hypertension. Often administered with massage in Europe, the oil is used to aid in relaxation, treat colic and stimulate the appetite. Massage with a combination of lavender and peppermint essential oils has been recommended to relieve tension headaches (Kim *et al.,* 2007; Hudson, 1996; Catherine and Kamper, 2001).

The lavender plants prefer full sun and nutrient rich soil for propagation. Most lavender species are indigenous to the mountain regions of the countries bordering the western Mediterranean, the islands of the Atlantic, Turkey, Pakistan and India. Native lavender species have also been found in northern and southern Africa, Micronesia, the Arabian Peninsula, Bulgaria, and Russia. Now the plants are extensively cultivated all over the world, particularly in France, Bulgaria, Russia, Italy, Spain, England, the United States, and Australia (Anonymous, 2009).

The chemical composition of lavender's essential oil depends largely on the species from which it was obtained. Steam distilled extracts have a characteristically higher ratio of alpha-terpineol and linalool to linalyl acetate compared to supercritical fluid extracts. Burning lavender oil does not affect its composition, implying that inhaling smoke from lavender aromatherapy candles may have the same impact as inhaling the vapour of the unheated essential oil. Allergic reactions to lavender have been reported. Potentially toxic compounds in lavender are D-limonene, geraniol, linalool and linalyl acetate (Jin and Ha, 2005).

Enough work has not been done on physiological studies and *in vitro* culturing of *Lavandula angustifolia*. Study were conducted already that say that *in vitro* clonal propagation of mature maditerrian *Lavandula viridis* was obtain from mature field grown plants. Single node explants were successfully established in MS media supplemented with 0.44µmM of 6 benzyl adenine. The higher rate is 11.69 shoot/node was obtain with 0.67µM 6 benzyl adenine in MS media with micronutrient with half concentration (Dias *et al.*, 2002). Chambdon (1992) had done analysis on micro-propagation of Lavenders and Lavandins and a tissue culture protocol was established for the mass propagation of disease free plantlets from commercial hybrid stock plants. When regenerants were planted in fields, quality characteristics, including essential oil concentrations, were similar to parent plants. Tissue cultured plantlets could therefore, be a good source of disease-free plantlets for regeneration purposes. In a study, carried out by Boyadzhieva *et al.* (1977) on the vegetative propagation of lavender by rooting of stem sections, 1 year old wood, various mixed wood and three-year-old wood stem sections were compared and the highest percentage of rooting was obtained from 8-10 cm long, 1-year-old cuttings in open beds in October-November. Some reports are

also available on callus formation of *Lavandula vera* (Tsuro *et al.*, 2001), somatic embryogenesis of *L. angustifolia* (Onisei *et al.*, 1999), bud regeneration of *Lavandula latifolia* (Calro *et al.*, 1989).

Revival of herbal medicine has led increased demand for identification of products. The business of trading in medicinal plants depends upon active ingredients. Thus, top priority should be given to conservation of existing plant species. Recently, Radha *et al.* (2011) and Sharma *et al.* (2011) had analyzed the *in vitro* propagation of medicinal plants like *Rubia cordifolia* L. and *Portulaca oleracia* L. respectively and established standard protocols on *in vitro* mass propagation. Thus, *in vitro* culturing is effective in generation of plants in large scale. Thus, there is necessity for the preservation of plants for use as genetically pure lines for effective culturing and efficient extraction of its raw material from pure and aseptic culture (Calvo and Segura, 1991; Jahan *et al.*, 2012). Thus, it is least known to people as a medicinal shrub, the main motive is to establish the *in vitro* culture protocol to propagate this plant so as to have large scale multiplication of this plant. As per literature review, there are some or very little reports on *in vitro* culturing of different species of *Lavandula* but there is no any report on *in vitro* propagation of *L. angustifolia* in the north-western Himalayan part of India. So in the present study, an optional regeneration protocol on *in vitro* micro-propagation of *L. angustifolia* has been described.

Material and methods

Sample collection: Plant material was collected from surrounding locations of UHF Nauni, Solan Himachal Pradesh, India during 2009-10. Explants from actively growing shoots having meristematic tissue were taken. Here explants from shoot tip/stem were taken from *Lavandula angustifolia.*

Working Stock: All the macro and micro nutrients are divided in to seven types of stocks A, B, C, D, E, F, and G. These included N P K source, Vitamins, Amino acids and Carbon source. Stock solutions were prepared according to Murashige and Skoog's (1962) composition (MS medium) in distilled water. Now 10 ml. of solution was taken from each stock and working solution was prepared and final volume was made 1000 ml. The working solution was kept and used for media preparation.

Sterilization of glassware: Sterilization of glassware and metallic instruments was carried out in dry heat for 3 hrs. at 160^0-180^0 C.

***MS medium* (*Murashige and Skoog's medium*) *or* (*Blank/control*)**: Growth media was prepared by adding 30g/l of sucrose and 8g/l of agar in working solution as mentioned above. In this solution, no IBA (Indole-3-butyric acid) and BAP (6-Benzylaminopurine) were added. This solution was

heated up to 15 minutes till it started boiling and there should be complete mixing of agar. This solution was then poured in to test tubes and autoclaved at 121^0C temperature with pressure of 15 psi for 15 minutes for further experimentation.

Preparation of MS medium supplemented with IBA (Indole-3-butyric acid) and BAP (6-Benzylaminopurine): Four different concentrations of MS medium i.e. 02mg/l, 1mg/l, 5mg/l and 15mg/l supplemented with IBA in working solutions were prepared. Similarly, another set of four concentrations of MS medium i.e. 2mg/l, 4mg/l, 6mg/l and 8mg/l supplemented with BAP in working solutions was prepared. The concentrations of BAP were slightly different from IBA concentrations as per the previous protocols for *in vitro* tissue culturing in literature. The clear MS media containing above mentioned growth regulators in respective concentrations was then poured in to test culture tubes. These culture tubes were then autoclaved at 121^0C temperature with pressure of 15 psi for 15 minutes and shifted to a sterile place/laminar air flow (Bottino, 1981; Doods and Roberts, 1995; Fowler, 2000; Streat, 1973; Sunderland and Cocking, 1978).

Preparation of MS medium supplemented with IBA+BAP: The MS medium supplemented with IBA and BAP was prepared with respective concentration which had shown best results in above experiments. In this case, both the hormones at 15mg/l of IBA and 2 mg /l of BAP together in the MS medium were used. So the concentrations of IBA and BAP which were showing best results in above media are mixed together and used for shoot multiplication from the callus.

Preparation of MS medium supplemented IAA (3-Indol acetic acid): IAA containing medium was used for root initiation in the tissue cultured shootlets/regenerants. The concentration of 1 mg/l of IAA was used in 250ml of working stock for root initiation. The medium containing IAA (1mg/l) for rooting was inoculated with the shootlets/regenerants which were already grown in IAA + BAP (15mg/l of IBA + 2 mg /l of BAP) medium.

Explants preparation and surface sterilization: Explants of stem and shoot were prepared by cutting with scalpel. These are firstly washed with tap water thoroughly and then in lab wash solution. Now explants were taken in to laminar air flow and rinse in 70% ethanol thrice and consequently wash with sterilized distilled water. Explants are surface sterilized with 0.1% mercury chloride, 70% ethanol and Bavstin (5%). Water used in sterilization is double distilled water. All these activities were performed in laminar air flow and now the explants were ready for culturing. All the set of test culture tubes containing IBA and BAP are inoculated with shoot tips /stem explants. A special care to

maintain sterile/aseptic condition was taken. Most nutrient media are sterilized by using autoclave at 121^0C temperature with pressure of 15 psi for 15 minutes.

Culture conditions: The culture tubes were inoculated and were placed inside the tissue culture room. The relative humidity (60-75%) and temperature (25 ± 2^0C) were maintained. Light intensity of 1000 lux/sec and diurnal illumination of 16 hrs. light and 8 hrs. dark per day was maintained. After keeping the whole experiment in culture room, the progress in growth specifically for each different media was observed and interpreted on the basis of number of auxiliary branches and difference in size of regenerated leaflets and length of root grown.

Results and discussions

Almost all the cultures got initiated after five days of culturing. The results which are quite noticeable were taken on 28^{th} day of culturing. For interpretation of results, number of axillary branches and regenerated leaflets were counted. The average size of three different sized leaves was measured on three bases. i) Large sized- 1.7 cm or 17 mm, ii) Medium size- 1.2 cm or 12 mm iii) Small size- 0.6 cm or 6 mm.

Effect on callus proliferation in MS medium supplemented with IBA

The growth characteristics of stem/shoot tip explants of *Lavandula angustifolia* in IBA supplemented MS medium is shown in Table-1. The two large leaflets (17mm each) were appeared in 15.0mg/l concentration while one large leaflet (17mm) was appeared in 5.0mg/l concentration of IBA in MS medium. The seven medium sized leaflets (12mm) were appeared in all the test concentrations except blank/control set. The three small leaflets (6mm) were appeared in 1.0mg/l concentration and two small leaflets (6mm) were observed in 0.2mg/l concentration of IBA in MS medium. The total numbers of leaflets were appeared maximum (10) in 1.0mg/l concentration of IBA in MS medium followed by 15.0mg/l and 0.2mg/l concentrations (9 each) and 5.0mg/l concentration with 8 total numbers of leaflets. The maximum leaflets length or growth (118mm) was found at 15 mg/l of IBA concentration in MS medium. Whereas other concentrations of IBA like 0.2 mg/l, 1.0 mg/l, 5.0 mg/l of IBA showed total leaflets length/growth as 96mm, 102mm, and 101mm respectively. But no any leaf initiation or regeneration was reported in blank/control set. So 15 mg/l of IBA concentration was best for *in vitro* culturing of *L. angustifolia.*

Table 1. Growth characteristics of shoot tip explants of *Lavandula angustifolia* in IBA supplemented MS medium

Test Tubes	IBA conc./l in MS medium	Number of leaflets & size			Total number of leaflets	Total leaflets Length (mm)
		Large leaf (17mm)	Medium leaf (12mm)	Small leaf (6mm)		
Blank	0.0mg/l	No leaflets	0	0	0	0
1.	0.2mg/l	0	7	2	9	96
2.	1.0mg/l	0	7	3	10	102
3.	5.0mg/l	1	7	0	8	101
4.	15.0mg/l	2	7	0	9	118

Effect on callus proliferation in MS medium supplemented with BAP

The growth characteristics of stem/shoot tip explants of *Lavandula angustifolia* in BAP supplemented MS medium is shown in Table-2. Two large leaflets and one large leaflet (17mm) were appeared in 2.0mg/l and 4.0mg/l concentrations of BAP in MS medium respectively. But no any large leaflet was appeared at high concentrations i.e. 6.0mg/l, 8.0mg/l of BAP in MS medium and blank/control set. The eight medium sized leaflets (12mm) were appeared in 2.0mg/l, 4.0mg/l and 6.0mg/l concentrations of BAP in MS medium while seven medium sized leaflets (12mm) were appeared in 8.0mg/l concentration of BAP in MS medium except blank/control set in which no leaflet was observed. Only two small leaflets and one leaflet (6mm) were appeared in 2.0mg/l and 4.0mg/l concentrations of BAP in MS medium. The maximum leaflets length/growth (142mm) was observed at 2.0 mg/l concentration of BAP in MS medium. Whereas other concentrations i.e. 4.0 mg/l, 6.0 mg/l, 8.0 mg/l of BAP in MS medium showed total leaflets length/growth as 119mm, 96mm, 84mm respectively. But no any leaf initiation or regeneration was reported in blank/control set. Therefore, 2.0 mg/l concentration of BAP was best for *in vitro* culturing of *L. angustifolia.*

Multiplication of callus and further shoot proliferation was carried out in concentrations which were proved conducive for growth characteristics of explants. Therefore, 2.0mg/l of BAP + 15mg/l of IBA supplemented MS medium was used for multiplication of callus and shoot proliferation. The newly proliferated/regenerated shootlets were grown in MS medium, supplemented with 1mg/l IAA for rooting which was a suitable concentration as per the standard protocol already established in the laboratory.

Table 2. Growth characteristics of shoot tip explants of *Lavandula angustifolia* in BAP supplemented MS medium

Test Tubes	BAP conc./l in MS medium	Number of leaflets & size			Total number of leaves	Total leaf Length (mm)
		Large leaf (17mm)	Medium leaf (12mm)	Small leaf (6mm)		
Blank	0.0mg/l	No leaflets	0	0	0	0
1.	2.0mg/l	2	8	2	12*	142
2.	4.0mg/l	1	8	1	10	119
3.	6.0mg/l	0	8	0	8	96
4.	8.0mg/l	0	7	0	7	84

*Shoot initiation was reported after 28th day (4 weeks) of culturing.

The first significant use of soft plant tissue culture in ornamental plant was made during 1920s when orchid seeds were germinated under laboratory conditions (Knodson *et al.*, 1922). Micro-propagation generally involves four steps: initiation of culture, shoot multiplication, rooting of *in vitro* grown shoots and acclimatization. Many commercial ornamental plants are being propagated by *in vitro* culture on the culture medium containing auxins and cytokinines (Rout and Jain, 2004). Several different explants have been used for direct shoot formation. There was a report on four week old seedling of *Lavandula latifolia* leaf explants bud regeneration. When MS media supplemented with different concentration of auxin IAA or NAA with cytokinin BA, the bud regeneration was achieved in medium containing BA or BA/ Auxin (Calro *et al.*, 1989). Nodal segment from micro propagated plants were used to evaluate the effect of growth regulators on *in vitro* shoot proliferation and rooting of *Lavandula.* The multiplication rate was obtain by using MS media supplemented with 1.0mg/l of TDZ. Rooting and root growth is increased with increase in concentration of NAA (Echeverrigaray *et al.*, 2005). Multiple shoots formed in leaf derived callus of *Lavandula vera* using open culture system. The production of multiple shoots and formation of roots from the shoot were studied when callus were culture in medium supplemented $4.0x10^{-7}$ N-(2-chloro-4-pyridyl-N-phenyl urea) CPPU type cytokinin. Multiple shoots were obtained from greenish surface of callus. In root induction culture, shoot culture in open system were rooted and grew vigorously and high rate was in ½ MS media supplemented with 100mg/l of IAA (Tsuro *et al.*, 2001). Similarly, multiple shoots were observed on the greenish part of the callus in the present study.

In another study on somatic embryo in plant tissue culture of *Lavandula angustifolia*, it was promoted by using selected mineral salts concentration; carbon source and charcoal supplement the best conduction of embryo

differentiation of Lavander consist of basal media supplemented with 4% glucose and 0.1mg/l – 1.0mg/l of IBA or Zeatin together with 1.5gm/l of charcoal (Onisei *et al.*, 1999). There are also some research papers on *in vitro* culture of different medicinal aromatic shrubs for their multiplication and conservation. Rapid multiplication of *Jasminum* by *in vitro* culturing of nodal explants using auxiliary buds were cultured in MS media supplemented with 4.44 – 18.6 μM Kinetins alone or in combination with 0.53 – 16μM Naphthalene acetic acid. Roots were induced by adding 4.9μM Indole butyric acid (Bhattacharya and Bhattacharya, 2010). Somatic embryogenesis, adventitious bud formation and plant propagation of Jasminum was done for callus induction in dark, adventive embryo and/ or adventitious buds are formed in light. The accumulation of monoterpenes resulting from de novo synthesis has been demonstrated in tissue cultures of these oil bearing plants of *Jasminum* sp. (Banthorpe and Osborne, 1984). Similarly, in this study, shoot tip/stem explants when supplemented with 1mg/l IAA in MS medium was reported best and suitable concentration for rooting initiation in regenerated shootlets.

Establishment of *in vitro* mass propagation protocol in Rasna (*Vanda tessellata*) was investigated and the seeds of *Vanda tessellata* were aseptically grown on 0.8% (w/v) agar solidified MS media and PM basal media. Multiplication of protocorm along with simultaneous elongation of mini seedlings occurred on BAP (2.0mg/l) + IAA (1.0mg/l) and BAP (2.0.mg/l) + NAA (2.0mg/l) enriched in MS media. As the elongated seedling develop they are supplemented with MS media fortified with IAA (1.0mg/l) and 0.1% (w/v) was found to most efficient for induction of strong and stout root system (Bhadra *et al.*, 2005). Lavender is mainly propagated by seeds, cuttings, layering, tissue culture and division of roots. To ensure genetic uniformity propagation by seeds should not be used. In a germination study of lavender seeds, the effects of Giberellic acid, light/dark regimes, and pre-freezing were studied. It was determined that the Giberellic treatments, with or without pre-freezing, significantly increased the percentage of germination and accelerated overall germination rates (Aoyama, 1996). But mass propagation through tissue culture would be an alternate technique for conservation and production of plantlets at large scale where medicinal shrub like *L. angustifolia* is required at large quantity by pharmaceutical companies. In this standardization of protocol for *in vitro* micro-propagation of *L. angustifolia*, it has been observed that BAP is required at 2.00mg/l for callus production, shoot initiation and proliferation than 15mg/l of IBA. Therefore, BAP is required in less quantity for callus production, shoot initiation and proliferation than IBA in the protocol that can conserve both chemicals input and money also. For hardening and

acclimatization of *L. angustifolia* in the natural soil, the experiments are going on and the encouraging results have been observed.

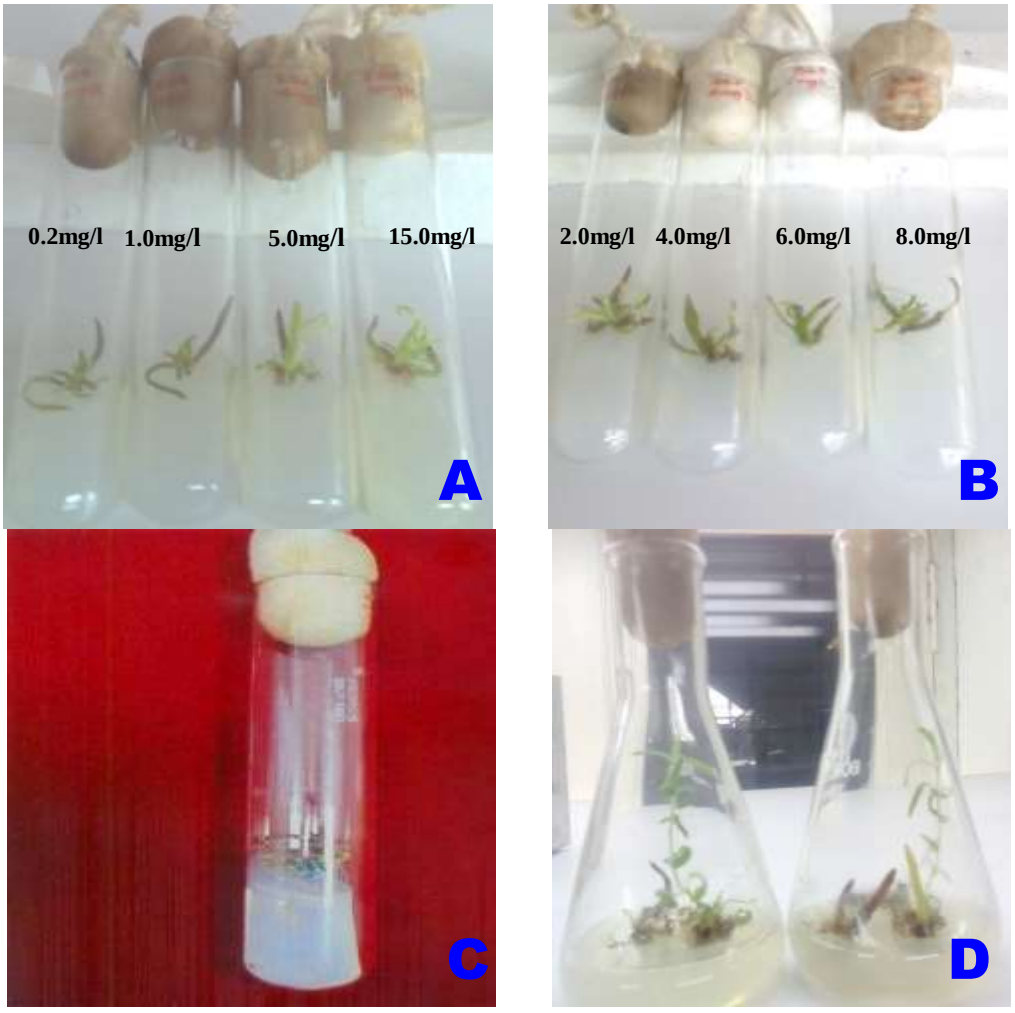

Fig. 1. A: Stem/shoot-tip explants grown in MS medium supplemented with IBA concentrations, **B**: Growth of stem/shoot-tip explants in MS medium supplemented with BAP concentrations, **C**: Shootlet/regenerant in MS medium supplemented with 1mg/l IAA for rooting, **D**: Multiplication of callus in to shoot proliferation in 2.0mg/l of BAP + 15mg/l of IBA supplemented MS medium

Conclusion

In vitro culturing is basic to the improvement of species and can be done at the cellular level. Plant tissue culture is used for production of plantlets and

their propagation to obtain the maximum growth by using better techniques to quantify various essential inputs so as to have maximum growth. Some medicinally useful plants can be a boon to mankind as useful plants like *Lavandula angustifolia* can be easily grown under artificial conditions. A proper and minimal supplementation of growth regulators can enhance their production and provide us sufficient biomass for production of medicinal extract and plants to spread generally all over.

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