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In vitro protocol optimization for micropropagation of elite Lemmon verbena (*Aloysia triphylla*)

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The family *Verbenaceae* includes 36 genera and 1035 species. Among them lemon verbena (*Aloysia triphylla*) is known to have high medicinal value. Therefore, development of fast and new *in vitro* micropropagation protocol will have a high importance in lemon verbena mass-propagation. This research study targeted to develop rapid *in vitro* micropropagation protocol for lemon verbena. Up to 88% of clean survived plantlets were obtained after treating the nodal explants with 0.5% berekina (NaClO) for 10 min. Shoot initiation and multiplication was achieved using node as explant planted on MS medium supplemented with different strength of 6-benzyladeninepourine (BAP) and kinetin (Kin) individually and in combination. Plants were put on root induction ½ strength MS medium fortified with different strength of indole-3-butyric acid (IBA) alone. The best treatment for shoot initiation was 6-benzyladeninepourine (1.5 mg/L) with 84.1% of initiation. The best treatment for shoot multiplication was 6-benzyladeninepourine (2.0 mg/L) with 9.23 shoots per explant. Best rooting (100%) and maximum root number per shoot (14.4) were found at 1.0 mg/L indole-3-butyric acid (IBA). The longest root (3.1 cm) was achieved without supplementing the media with plant growth regulators. The plantlets were hardened and acclimatized in fully automated greenhouse and survival percentage was greater than 70% planting on a combination of sterilized river sand, top forest soil and animal manure in a 1:2:1 (v/v/v) ratio. This *in vitro* micropropagation protocol can be used instead of conventional propagation techniques, as a fast and economically cheap method to propagate a wide range of similar plants.

Key words: Acclimatization, auxin, cytokinin, micropropagation, lemon verbena.

INTRODUCTION

Lemon verbena (*Aloysia triphylla* L.) is a perennial shrub that belongs to the family *Verbenaceae* (Gomes et al., 2006; Rotman and Mulgura, 1999). It has got its name due to the fact that it has whorls of three (tri) leaves (phylla) at each node. Lemon verbena is locally known as Lominat (Beemnet et al., 2013) and is native to South American countries; Argentina, Paraguay, Brazil, Uruguay, Chile, Bolivia and Peru (Carnat et al., 1999; Vogel et al., 1999; Armada and Barra, 1992; Botta, 1979).

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The leaves of lemon verbena are the most economical part of the plant that can be used to add a lemony taste in salads, tea, milk, ice creams and jellies (Hanna et al*.,* 2011; Beemnet et al*.,* 2010). It makes one of the best beverage teas, especially when blended with mint (Hanna et al*.,* 2011). Likewise, the essential oil obtained through distillation of the leaves is used in fragrance industries, food flavoring industries, soft drink industries and folk medicine. Traditionally, it is used for treatments of spasms, cold and fever as folk remedy (Carnat et al*.,* 2004), asthma, flatulence, colic, diarrhoea, indigestion, insomnia and anxiety (Durat and Chritina, 2005; van Hellemon, 1986; Newal et al*.,* 1996; Cowan, 1999; Graca et al*.,* 1996).

Essential oil of lemon verbena has anti-oxidant, anti-bacterial and anti-fungal properties (Hanna et al*.,* 2011) as well as being used in tea and tinctures (Bilia et al*.,* 2008; Cowan, 1999). Due to its diverse uses and applications, lemon verbena has got open and large market potential for herbal preparation and extraction of essential oils (Beemnet et al*.,* 2010). Despite its high importance in food, pharmaceutical, soft drink and stimulant-processing industries introduced to the country long time ago, continuous interest of producers and investors for its production and application in Ethiopia (EIAR, 2009) and the presence of different agro-ecological conditions in the country (NMSA, 1996; Andargachew, 2007), there exist limited information on the propagation, processing and utilization technologies in Ethiopia. Thus, lack of such information is the major problem to exploit the potential of the plant (Beemnet et al*.,* 2013).

Lemon verbena is best propagated by cutting, taken in summer by keeping it in a shade and well-watered conditions, which otherwise will wilt readily. It is highly susceptible to pests like that of spider mites and white flies and also requires long time to grow into full plantlets. Since it is difficult to obtain seeds of *A. triphilla* owing to Ethiopian climate, *in vitro* micropropagation of this plant to produce high quantity of genetically homozygous planting material will be a better solution.

MATERIALS AND METHODS

This *in vitro* protocol optimization for micropropagation of lemon verbena research was conducted in the Plant Tissue Culture Laboratory of National Agricultural Biotechnology Research Center at Ethiopian Institute of Agricultural Research from September 2014 to June 2015.

Media composition

The nutrient medium used for growth of lemon verbena was semi solid (Murashige and Skoog, 1962) which contains macro, micro elements and vitamins. To prepare the nutrient medium we used, individually prepared stock solutions were mixed with the required concentrations of plant growth regulators and sucrose (3%). The solution was mixed completely using magnetic stirrer. The pH of the media was adjusted at 5.75 using 0.1 N NaOH or 0.1 N HCl before the addition of 0.4% agar, and then boiled until the agar melts completely. Around 10 mL (for test tubes) and 50 mL (for Jars) of the media were added in each culture test tubes (150 mm long and 25 mm diameter) and culture jar (250 mL). The test tubes and culture jars containing the nutrient medium were plugged tightly with non-absorbent cotton and autoclavable lids prior to sterilization at 121°C with 0.15 KPa pressure for 20 min.

Mother plant establishment

The mother/donor plants, brought from Wondo Genet Agricultural Research Center, were further established by cutting in greenhouse at the National Agricultural Biotechnology Research Center, Holetta, Ethiopia (Figure 1).

Surface sterilization

Healthy and young shoots (3 to 4 cm length), having axillary buds (3^{rd}, 4^{th} and 5^{th} nodes; from top tip), were taken from lemon verbena plant by excising with clean scissor/surgical blade and taken as explant. Young parts (juvenile plants) were used as they give good response to shoot initiation and multiplication than explant sources from old/adult forms (Naghmouchi et al*.,* 2008). The shoots were washed with clean water three to five times followed by liquid soap for 15 min with continuous agitation to remove contaminants from the surface, and sterilized with 70% ethanol for 30 s and then in NaClO (0.5% and 1% w/v) containing two drops of 'Tween 20' per 45 mL solution for 5, 10 and 15 min respectively and thoroughly washed with sterile double-distilled water. They were aseptically cultured for four weeks in 40-ml glass tubes containing 10 ml of semi-solid Murashige and Skoog (MS) (1962) medium. Number of clean and survived plants was recorded and percentage of contaminated plants was computed.

Shoot initiation

Fully cleaned nodal explants were planted in test tubes containing MS (Murashige and Skoog) (1962) nutrient medium supplemented with 3% sucrose, 0.4% agar (Agar-Agar, Type I) and different level of BAP (0.5, 1.0, 1.5, 2, 2.5, 3, 3.5 and 4 mg/L) alone. Medium without the addition of hormone was used as control. For each shoot induction treatment, 15 glass tubes were ordered randomly in completely randomized design (CRD) in three (3) replications. All culture tubes were properly sealed with non-absorbent cotton and parafilm and placed in the growth room at standard conditions (25 ± 1°C and 16/8 h light/dark and relative humidity (RH) of 70 to 80%). After four weeks of culturing, number of explants initiated was recorded and shoot initiation percentage was computed.

Shoot multiplication

Fully initiated shoots were placed on a medium without plant growth hormone for two weeks. Cleanly initiated 1.5 to 2.5 cm long shoots with a number of nodes were cut at both ends and planted vertically in 250-ml culture jars with 50-ml nutrient medium fortified with 3% sucrose, 0.4% agar and different concentration of BAP (0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L) alone and in combination with 0.5, 1.0, 1.5 and 2.0 mg/L Kn. MS medium without plant growth hormones was used as control. For each treatment, 15 jars (five shoots per jar) were cultured randomly in CRD with five replications. All plants were allowed to grow for one and half month, thereafter number of shoots per explant was recorded.

Rooting of shoots

Multiplied shoots were planted on ½ strength MS medium fortified

Figure 1. Greenhouse established mother/donor plants of Lemon verbena (*Aloysia triphylla*).

Table 1. The effect of different concentrations of Berekina (NaClO) and length of exposure time on survival percentage.

Number of treatments	Berekina (NaClO) concentration (%)	Time of exposure (min)	Clean and Survived plants (%)	Contaminated plants (%)
1	0.5	5	82.4[b]	17.38[a]
2	0.5	10	87.9[a]	10[b]
3	0.5	15	74.8[c]	8.04[d]
4	1	5	78.5[c]	9.04[c]
5	1	10	63.4[d]	6.0[e]
6	1	15	40.0[e]	4.1[f]
CV			4.12	6.89

*Means followed by the same small letters are not different according to Tukey's test at 5% of probability. *CV: Coefficient of variation.

with 3% sucrose, 0.4% agar and different concentrations of IBA (0, 0.5, 1.0, 2.0 and 3.0 mg/L). Nutrient medium without plant growth hormones were used as control. For each treatment, 15 jars, each with five plantlets, were cultured randomly in CRD with five replications. Percentage of shoots with root, number of roots per shoot, and average root length (cm) were recorded after the shoots were planted on the root induction media for a month.

Acclimatization

Shoots with well-developed roots were transferred and planted on a seedling tray having a mixture of sterilized river sand, top forest soil and animal manure in a 1:2:1 (v/v/v) ratio and taken to fully automated greenhouse for acclimatization. Plants were put in greenhouse and covered by polyethylene sheets and red cheese cloth for two weeks, in order to decrease light intensity and maintaining the moisture. They were watered 2 to 3 times per day using plastic spray bottle. After two weeks days, percentage of plantlets that were successfully acclimatized was recorded and successfully acclimatized plants were transferred to pot.

RESULTS AND DISCUSSION

Surface sterilization

From the six sterilization treatments, treatment 2 (0.5% berekina for 10 min) resulted in 87.9% of clean and survived plants and 10% of contamination. The second good result comes from 0.5% berekina for 5 min resulting in 87.4% clean and survived plants and 17.38% of contamination. Also, treatment 6 results in 40% of clean and survived plants (Table 1). Disinfection procedures showed high efficiency in preventing fungal and bacterial contamination (Figure 2). The use of 0.5% commercial bleach (berekina) in surface sterilization produced more than 75% of the cultures which are free from bacterial and fungal contaminations during the *in vitro* initiation of nodal cuttings (Braga et al*.,* 2011).

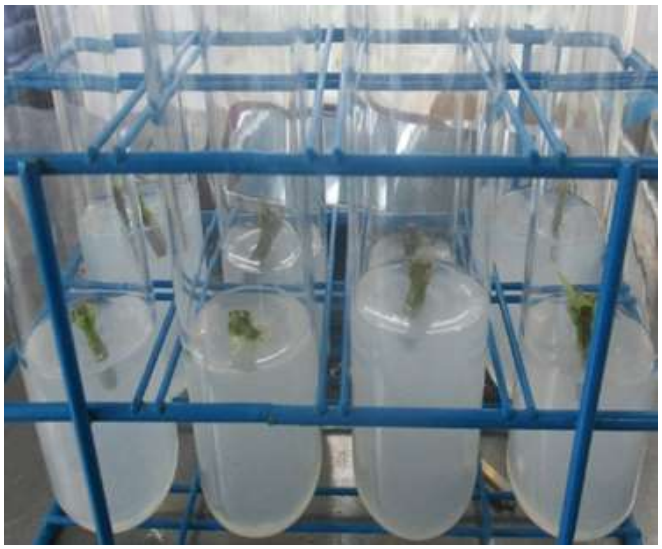

Figure 2. Clean survived plants after sterilization treatment.

Table 2. Effect of different concentration of BAP on shoot initiation.

Number of treatments	BAP (mg/L)	Initiated plants (%)
1	0	73.3[c]
2	1	77.6[b]
3	1.5	84.1[a]
4	2	86.7[a]
5	2.5	66.7[d]
6	3	60.3[e]
7	3.5	50.3[f]
8	4	46.7[g]
CV		4.06

*****Means with the same letter in the same column are non-significant at 5% significance level. *CV: Coefficient of variation.

Shoot initiation

Analysis of the CV indicated that supplement of different concentration of BAP alone had highly significant effect on time taken for shoots to initiate and percentage of usable shoots initiation (Table 2). On most of the treatments, explants started shoot initiation after one week of culture. There was shoot initiation in all treatments tested including the control treatment, medium without plant growth hormone (73.3%) indicating that lemon verbena has enough endogenous hormones for shoot induction. Nevertheless, length and number of usable shoots initiated differed with different treatments (Figure 3). Maximum percentage of shoot initiation were achieved on a media supplemented with 2 mg/L BAP alone and 86.7%, followed by 1.5 mg/L giving 84.1% of initiation. The shoot initiation percentage was increased up to 2 mg/L; and thereafter showed dramatic decrease in initiation along with increases in BAP concentration. The kind of explant used greatly influenced shoot induction and subsequent multiplication of the initiated shoots. Broadly speaking, the regeneration or shoot initiation frequencies were higher with nodal explants in micro propagation of *A. polystachya* (Burdyn et al*.,* 2006).

Shoot multiplication

Analysis of CV showed that all the treatments have highly remarkable effect on mean number of shoots, length of shoots, and mean number of leaves during shoot multiplication (Table 3). The concentration of BAP alone was highly remarkable on resulting good shoot multiplication in comparison to BAP and Kin combination. Multiplication results indicated that the maximum number of shoots (7.42 and 9.23) per explant was achieved on media containing 3 and 2 mg/L BAP respectively (Table 3). There was no remarkable inutility on the number of shoots between those two treatments. These result is in agreement with lemon verbena (*Lippia citriodora*) micro propagation, in which the maximum number of shoots was found on a media supplemented with 3 mg/L BAP in combination with 0.1 mg/L IBA (Oladzad et al*.,* 2012).

Combination of lower cytokinin with higher auxin showed an average of 2.0 shoots per explant and also revealed that IAA and Kn has effect on multiple shoot proliferation (Mosavi, 2012). BAP combined with NAA produced around five shoots per nodal explant in *Verbena litoralis* while applying NAA only decreased shoot multiplication, bolstering the effect of cytokinins for shoot multiplication (Braga et al*.,* 2011). The addition of 0.23 µM IAA to MS media for *L. alba* micropropagation significantly decreased shoot induction, number of shoots multiplied per explant and number of nodes per shoot, as compared to MS media without plant growth hormones (Tavares et al*.,* 2004). Generally, significant rate of shoot multiplications were found in the addition of higher concentrations of BAP alone. Similar results were found for *Lippia junelliana* (Juliani et al*.,* 1999), *L. alba* (Gupta et al*.,* 2001) and *L. filifolia* (Peixoto et al*.,* 2006).

Root induction

Anticipation of CV showed that various strength of IBA alone had highly considerable effect on root percentage, number of roots per shoot and length of roots (Table 4). The root induction results in Table 4 reveals that addition of 1.0 mg/L IBA on ½ strength MS medium resulted to 100% rooting, highest number of roots per shoot (14.4) and a root length of 1.8 cm (Figure 4). Earlier studies on lemon verbena (*L. citriodora*) indicated that root induction on MS media supplemented with 0.5 mg/L IBA resulted in high rate of root induction and root numbers per shoot (Oladzad et al*.,* 2012).

The effect of IBA on rooting of many plants has been

Figure 3. Initiated lemon verbena plants after two weeks of culture.

Table 3. The effect of different concentrations and combinations of BAP and KN on shoot multiplication.

Number of treatments	BAP (mg/L)	Kin (mg/L)	Shoot number/explant
1	0	0	2.5[g]
2	0.5	0	4.38[d]
3	1	0	6.29[b]
4	2	0	9.23[a]
5	3	0	7.42[a]
6	4	0	5.64[c]
7	2	0.5	3.58[e]
8	2	1	3.27[e]
9	2	1.5	2.48[f]
10	2	2	2.42[f]
CV			8.99

*Means with the same letter in the same column are non-significant at 5% significance level. *CV, Coefficient of variation.

Table 4. Effect of IBA on root induction of micro shoots of Lemmon verbena after 30 days of culture.

Number of treatments	IBA (mg/L)	Shoots with roots (%)	Number of Roots/shoots	Root length (cm)
1	0	96.71	4.76[d]	3.10[a]
2	0.5	90	11.95[b]	2.53[b]
3	1	100	14.4[a]	1.82[c]
4	2	93.3	9.6[c]	1.19[d]
5	3	93.3	9.27[c]	0.64[e]
CV				

*Means with the same letter in the same column are non-significant at 5% significance level. *CV: Coefficient of variation.

Figure 4. Multiplied lemon verbena plants after 4 weeks of culture.

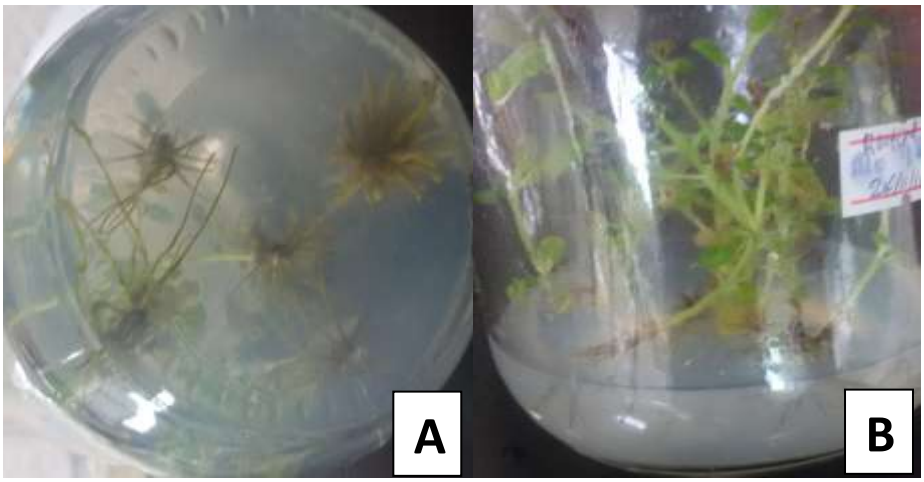

Figure 5. Root induction after 4 weeks from planting on rooting media supplemented (A) with 1.0 mg/L IBA and (B) without IBA.

reported and showed its effectiveness in comparison with NAA (Benelli et al*.,* 2001; Tanimoto, 2005; Ansar et al*.,* 2009). This could be due to slow movement and delayed degradation of IBA as compared to IAA and NAA. Various concentrations of IBA may also induce rooting by increased internal freely available IBA or may synergistically modify the action of endogenous synthesis of IAA (Krieken et al*.,* 1993).

In *A. polystachya, in vitro* root induction of multiplied shoots was found without the addition of plant growth regulators (Sansberro and Mroginski, 1995). Our research output merely indicated that addition of low concentration of IBA to well-developed shoots increased the root induction process (Figure 5A and B). This low IBA concentration also does not stimulate callus formation at shoot base, which is an advantage, since it could acts as a physical barrier to nutrient and water movement (Thorpe et al*.,* 1991; De Klerk, 2002).

Acclimatization

Well-developed plants with good roots were taken from the culture jars and washed with warm water to blow over agar adhering to roots and residue of nutrient media to decrease further adulteration. It was then moved onto seedling tray filled with a collection of heat sterilized river sand, top forest soil and animal manure in a 1:2:1 (v/v/v)

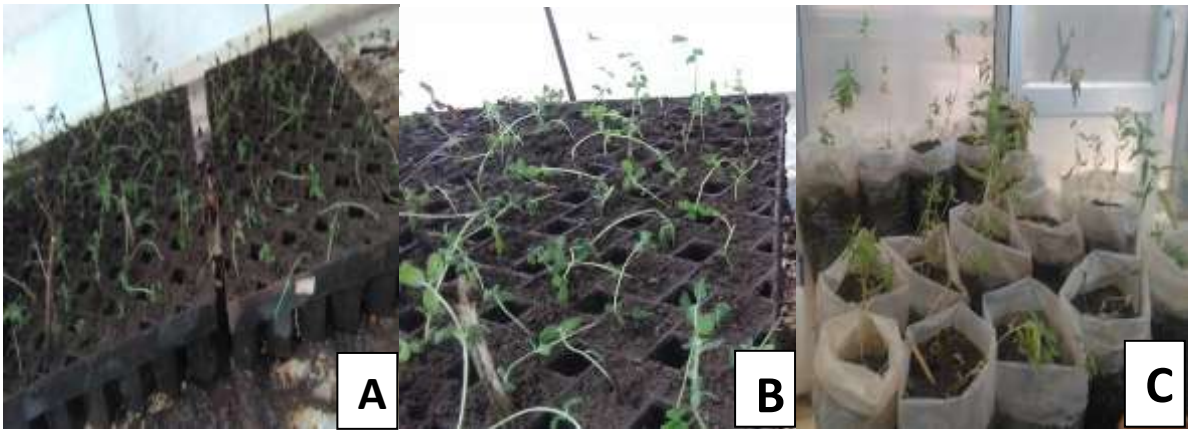

Figure 6. Combination of river sand, top forest soil and animal manure resulting in more than 70% survival and acclimatization in greenhouse (A) during acclimatization (B) after week of acclimatization, and (C) after month of acclimatization.

ratio and placed in fully automated glasshouse for further growth. After two weeks, survival rate greater than 70% was recorded (Figure 6). In acclimatization of apple at the same glasshouse, 65.70% survival rate was reported which is in line with previous finding (Demsachew, 2011).

Deepa et al*.* (2011) also indicated that vigorous growth and 70% survival rate after well rooted plants were planted to seedling trays filled with a mixture of sterilized ocean sand, soil and vermiculate in a 2:1:1 (v/v/v) ratio. According to Oladzad et al*.* (2012), high rate of acclimatization were achieved on soils composing a mixture of vermiculite, perlite and soil.

Conclusion

This research describes an efficient procedure for *in vitro* micropropagation and a successful hardening of lemon verbena. The protocol presented here for direct shoot initiation from nodal explants and consequent plant mass propagation will increase the ditch propagation of this crucial medicinal plant. This protocol will also have an impact on cryopreservation and genetic studies aimed at improving the essential oil composition of its extracts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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