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EFFECT OF DIFFERENT SURFACE STERILIZING AGENTS AND GROWTH MEDIA ON *IN VITRO* SEED GERMINATION OF *LIPPIA NODIFLORA* L.

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Lippia nodiflora is a fast-growing, small creeping perennial herb that belongs to the family Verbenaceae. *L. nodiflora* is used for ethnomedicinal and pharmacological applications like adenopathy, chronic indolent ulcers, knee joint pain, cold, fever, anticancer, antidiabetic, and gastrointestinal disorder. This study was aimed to optimize the *in vitro* seed germination protocol to address**.** Seeds were sterilized with different surface sterilizing agents at different time period. Sterilized seeds were inoculated on different strengths of MS basal medium with various levels of sucrose concentrations. By comparison of measured characteristic results, we found that the maximum percentage of seed germination was recorded as 85.26 ± 0.04 on ½ strength MS basal medium supplemented with 1% (w/v) sucrose when the seeds were surface sterilized with 0.1% $HgCl_2$ for 5 minutes. We optimized the seed surface sterilization protocol and culture condition for the first time that may be useful for regeneration, transformation, and conservation studies.

Keywords: *In vitro, Lippia nodiflora*, MS media, Seed germination

Lippia nodiflora (Phyla nodiflora) is a small creeping herb that belongs to the family Verbenaceae, which is fast-growing. It is widely distributed throughout the world, particularly in South Africa and Central America (Terblanché and Kornelius 1996). The plant is also distributed throughout India and found in the warmer parts of Telangana, Karnataka, Kerala, and Andhra Pradesh, and in some places of Rajasthan, Tamilnadu, West Bengal, and Uttar Pradesh (Sharma and Singh 2013). *L. nodiflora* is grown in maritime areas close to the rivers, marshy places like ponds, paddy fields, and wet places (Sharma 2018). When competing with other species, *L. nodiflora* can grow up to a height of 20 - 30 cm, and its life form is set to be a runner plant (Amir *et al.* 2011). The leaves are rounded (10-20 mm long and 3-7 mm wide), whole or bluntly serrated at the tip, narrow towards the petiole at the leaf base, and appear in pairs at stem nodes. Leaves can be somewhat canescent, which means they have a grayish-green appearance due to a thin hair covering on their surface, as shown in Fig1(A). *L. nodiflora* contains scanty roots that possess various ethnobotanical and medical applications in adenopathy, chronic indolent ulcers (Kirtikar and Basu 1975). In Ayurvedic, Unani, and Siddha systems, the plant extract is utilized for knee joint discomfort, diarrhea, cold, fever, pneumonia, aphrodisiac, urinary, and gastrointestinal diseases (Khare 2007, Narendra *et al*. 2012, Quattrocchi 2012). Several pharmacological activities of *L. nodiflora* have been documented, including anti-inflammatory, antipyretic, analgesic, antitussive, antidiabetic, and antimelanogenesis actions (Balakrishnan *et al.* 2010, Balamurugan and Ignacimuthu 2011, Forestieri *et al.* 1996, Yen *et al.* 2012). Halleridone and Hallerone bio-active compounds serve as anticancer, antitumor, antimalarial activities (Ravikanth *et al.* 2000). *L. nodiflora* contains antioxidant and cytotoxic activities, which have been reported (Shukla *et al.* 2009). Methanolic extraction of aerial plant parts led to the isolation of a triterpenoid compound known as lippiacin, which serves as an anticancer activity (Siddiqui *et al.* 2007). Aerial parts of *L. nodiflora* possess anodyne, antibacterial, diuretic, refrigerant, and febrifuge agents (Bhaskar *et al.* 2009). Plant

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Figure 1(A-D): Marphology of *Lippia nodiflora* L. **A.** Complete plant, **B.** Inflorescence of plant, **C.** Fruits of the plants, **D.** Seeds of the plants.

extracts have been used to cure multiple skin disorders and hair afflications (Abbasi *et al.* 2010). Leaves infusion and tender stalks are given to children suffering from indigestion and to women after delivery (Chopra *et al.* 1956). Several phytochemical compounds were reported in the plant species, such as flavonoids (Barua *et al.* 1969), Flavones glycosides (Nair *et al.* 1973), alkaloids, terpenoids, essential oils, resin (Elakovich and Stevens 1985), and steroids (Balamurugan *et al.* 2011). The incidence of contamination in plant tissue cultures by various pathogens such as bacteria and fungi decreases productivity and disrupts the cultivation; hence an efficient protocol of surface sterilizing the explants is crucial (Sen *et al.* 2013). The plant propagation using the seeds is easy, reliable, and fast compared with other parts of the plants. Seed germination is a prerequisite for the large-scale cultivation of any important ethano-medicinal plants. Where, an effective seed germination protocol leads to a successful plant regeneration (Sirikonda *et al.* 2020, Kudikala *et al.* 2020). Invivo seed germination of *Lippia nodiflora* has become a hurdle for mass cultivation and plant development. Seed germination success rate depends on various internal factors like seed viability, seed coat, and external factors like light, temperature, etc. (Park *et al.* 2011). Studies on other species like *Lippia turbinata* and *Lippia integrifolia* show that seed germination is light-dependent and temperature-independent, and also the efficiency is increased when GA_3 is added (Galíndez *et al.* 2017). Most of the reports are limited with studies on physical characters such as temperature and light, but not on the surface sterilants and nutritional requirements for germination. So far, no reports are available on seed germination of *Lippia nodiflora*. Hence we have undertaken this study and successfully optimized sterilizing agents and media for in vitro seed germination in *Lippia nodiflora.*

MATERIAL AND METHODS

Plant material: The plant material of *Lippia nodiflora* was collected from the pond area near the university college of Engineering, Kakatiya University, Warangal. Collected plants were maintained in the garden of the medicinal arboretum in the Department of Biotechnology, Kakatiya University Warangal, Telangana. This plant material was authenticated by Professor.V. S. Raju (Taxonomist). A voucher specimen (KUW 20200107) is deposited in the Herbarium of the Department of Botany, Kakatiya University, Warangal, Telangana.

Seed collection: Mature seeds of *L. nodiflora* were collected from the well-grown plants from the medicinal garden of the Department of Biotechnology Kakatiya University Warangal, Telangana. Collected seeds were stored in the bottle at room temperature for further use.

Surface sterilization: Collected seeds were washed under running tap water to remove the ruminants present on the surface of the seeds. These seeds were soaked in sterile distilled water for one hour. Discarded the water and then surface sterilized with two different sterilizing agents 0.1% (w/v) $HgCl_2$ and 2% (w/v) sodium hypochlorite with different duration of time (3min, 5min, and 7 min) followed by rinsing with sterile distilled water for 3-4 times to remove the traces of sterilizing agent. Sterilized seeds were inoculated on different strengths of MS basal, ½ MS basal, liquid MS, ½ liquid MS, and DW (Distilled water). All the above treatments were repeated thrice, and the cultured seeds were observed for germination.

Germination media: Solid MS (Murashige and Skoog 1962) medium and liquid MS medium with different concentrations of sucrose were tried for invitro seed germination. Seeds were inoculated on different strengths of MS basal medium with 2% sucrose and ½ strength MS basal with 1% sucrose. Similarly, seeds were also inoculated on full strength MS liquid medium with 2% sucrose and ½ strength MS liquid medium with 1% sucrose and distilled water as control. Paper boat (Whatman no.1 filter paper) was used as supporting material to hold the seeds cultured on liquid MS, ½ liquid MS, and distilled water. The pH of the culture media was adjusted to 5.7 before sterilization.

Culture conditions: All the culture media were autoclaved at 121°C for 15 -20 minutes. All the inoculated culture vessels were incubated at 25 ± 2°C in the culture room under 16 light/8dark hours photoperiod with a light intensity of 3000 lux using the white fluorescent tube.

Data analysis: Data were collected based on the percentage of in vitro seed germination, the mean number of days for germination, and the average length of the seedlings. Recorded data were analyzed by using SPSS software (version 20). The collected data were computed and subjected to Analysis of Variance (ANOVA), and means of comparative analysis was made by (DMRT) Duncan's multiple range tests ($P \leq 0.05$) to determine the most suitable sterilizing agent and growth media for the germination of seeds
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RESULTS AND DISCUSSION

Surface sterilization is a prerequisite technique to propagate the plants from germ-free conditions. Various parameters and combinations studied under the seed germination are 1) Sterilizing agents ($HgCl_2$ and NaOCl), 2) Duration of treatment (3, 5, and 7 min), 3) Different strengths of the medium (Full strength MS medium with 2% sucrose, half-strength MS medium with 1% sucrose, liquid MS with 2% sucrose, half strengths with 1% sucrose) and 4) Distilled water is used as a control with all the media. Among all the different combinations studied, seeds treated with 0.1% $HgCl_2$ for 5 min followed by

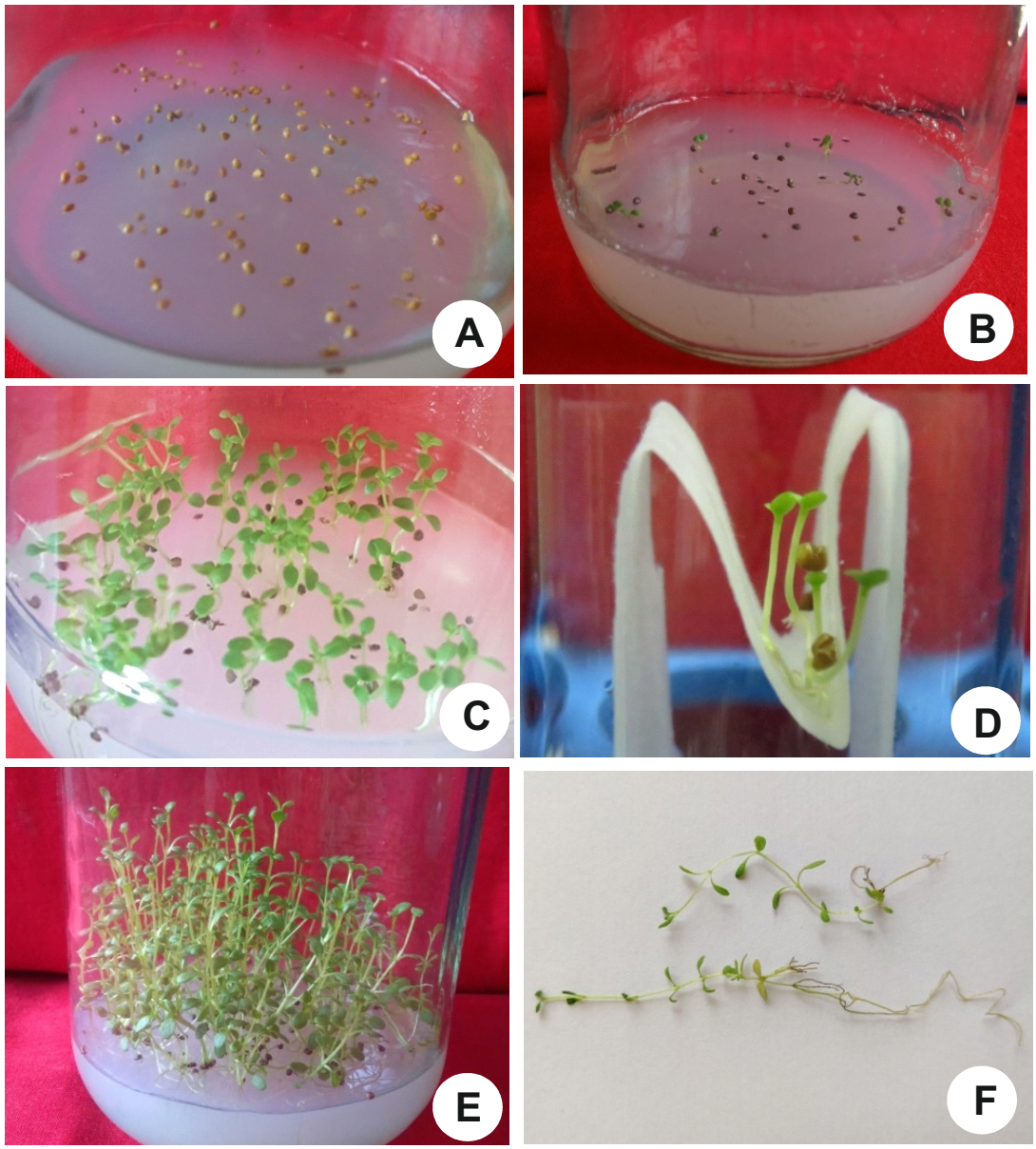

Figure 2 (A-F): *In vitro* seed germination of *Lippia nodiflora* L. **A.** Seed inoculation on MS media, **B.** Initiation of germination, **C.** Seed germination after 15 days of incubation, **D.** Seed germination on the paper boat using liquid MS, **E.** Complete plant growth after 30 days of incubation, **F.** Rooting of seedlings.

culturing on ½ strength MS medium with 1% sucrose showed promising results than other combinations. These results show an 85.26 mean percentage of germination in 8.26 mean number of days for germination and an 8.76 mean length of the seedling (as shown in Table. 1 and Fig. 2). Our results clearly concordance with (Kiani *et al.* 2017), validating the above combinations for better seed germination. Seed sterilization with NaOCl (2% w/v) for 5 minutes duration has shown a maximum germination percentage (73.21%) compared with 3 min and 7 min treatment of sterilization. When sucrose concentration increases to 2% in the medium, significant changes are observed in reducing the germination percentage and seedling growth. DW as control has shown a lower rate of germination when compared with MS media. Sucrose plays a crucial role in most plants as an energy source and acts as a

Table 1: Effect of different surface sterilization methods and growth media on *in vitro* seed germination of *Lippia nodiflora L.*

Sterilizing agent	Time interval	Diff. strength of MS media	Mean % of germination ± (SE)	No of days for germination ± (SE)	Average length of seedlings (cms) ± (SE)
$HgCl_2$	3 min	MS basal	59.42 ± 0.13^{m}	14.22 ± 0.06^{a}	4.25 ± 0.07^{lm}
		½ MS basal	65.26 ± 0.09^{h}	12.29 ± 0.13^{c}	5.31 ± 0.10^{i}
		Liquid MS	54.27 ± 0.10^{p}	13.29 ± 0.02^{b}	4.16 ± 0.03^{m}
		½ liquid MS	60.11 ± 0.06^{l}	11.26 ± 0.09^{d}	5.41 ± 0.16^{hi}
		Distilled water	55.34 ± 0.11^{o}	13.33 ± 0.05^{b}	3.83 ± 0.04^{n}
	5 min	MS basal	78.30 ± 0.12^{b}	10.22 ± 0.06^{e}	7.47 ± 0.16^{b}
		½ MS basal	85.26 ± 0.04^{a}	8.26 ± 0.09^{g}	8.76 ± 0.06^{a}
		Liquid MS	69.31 ± 0.06^{f}	12.26 ± 0.15^{c}	6.93 ± 0.02^{d}
		½ liquid MS	76.39 ± 0.10^{c}	10.34 ± 0.13^{e}	7.23 ± 0.06^{c}
		Distilled water	60.17 ± 0.06^{l}	10.24 ± 0.07^{e}	5.16 ± 0.04^{i}
	7 min	MS basal	70.30 ± 0.13^{e}	12.24 ± 0.06^{c}	5.94 ± 0.02^{g}
		½ MS basal	73.19 ± 0.04^{d}	10.34 ± 0.10^{e}	6.65 ± 0.07^{e}
		Liquid MS	63.27 ± 0.09^{j}	13.12 ± 0.01^{b}	5.87 ± 0.05^{g}
		½ liquid MS	69.45 ± 0.16^{f}	11.16 ± 0.03^{d}	6.19 ± 0.04^{f}
		Distilled water	58.38 ± 0.20^{n}	12.22 ± 0.06^{c}	4.49 ± 0.15^{kl}
NaOCl	3 min	MS basal	58.34 ± 0.06^{n}	14.15 ± 0.03^{a}	3.85 ± 0.07^{n}
		½ MS basal	61.26 ± 0.09^{k}	12.40 ± 0.22^{c}	4.77 ± 0.11^{j}
		Liquid MS	47.40 ± 0.23^{s}	13.19 ± 0.04^{b}	4.27 ± 0.06^{lm}
		½ liquid MS	51.44 ± 0.12^{r}	12.31 ± 0.13^{c}	4.26 ± 0.05^{kl}
		Distilled water	47.50 ± 0.20^{s}	11.90 ± 0.03^{d}	3.21 ± 0.03^{o}
	5 min	MS basal	66.22 ± 0.05^{g}	10.34 ± 0.12^{e}	6.64 ± 0.06^{e}
		½ MS basal	73.21 ± 0.07^{d}	9.23 ± 0.10^{f}	7.19 ± 0.04^{c}
		Liquid MS	58.18 ± 0.03^{n}	12.23 ± 0.06^{c}	5.62 ± 0.09^{h}
		½ liquid MS	64.26 ± 0.14^{i}	11.17 ± 0.04^{d}	6.21 ± 0.02^{f}
		Distilled water	55.23 ± 0.10^{o}	10.30 ± 0.13^{e}	5.19 ± 0.04^{i}
	7 min	MS basal	60.15 ± 0.03^{l}	11.23 ± 0.06^{d}	4.61 ± 0.15^{jk}
		½ MS basal	66.32 ± 0.06^{g}	10.21 ± 0.03^{e}	5.19 ± 0.03^{i}
		Liquid MS	55.44 ± 0.06^{o}	12.22 ± 0.11^{c}	4.79 ± 0.05^{j}
		½ liquid MS	59.16 ± 0.03^{m}	11.23 ± 0.01^{d}	5.37 ± 0.08^{i}
		Distilled water	52.46 ± 0.17^{q}	10.23 ± 0.04^{e}	4.25 ± 0.01^{lm}

signaling molecule for genes involved in photosynthesis, respiration, development, and metabolism in higher plants (Xu *et al.* 2010). In our study, we observed that a greater concentration of sucrose on MS medium decreases seed germination, the number of days to germination, and the length of seedlings, as shown in Table 1. Similar types of results were observed in different plant species like *Catharanthus roseus* (Ramandi *et al*. 2019), *Arabidopsis* (Gibson 2005), *Althaea officinalis* (Younesikelaki *et al.* 2016) where concentration of sucrose in culture medium effect on seed germination and seedling growth.

CONCLUSION

The effects of different seed surface sterilization methods and growth media composition on seed germination of *Lippia nodiflora* L. is well studied. Results suggest that using 0.1% $HgCl_2$ for 5 minutes duration is more efficient in surface sterilizing the seeds. Among the various culture conditions studied, half-strength MS media with 1% sucrose have yielded promising results with 85% of seed germination and a mean seedling length of 8.76 ± 0.06 and 8.26 mean number of days to germinate. This is the first report of seed germination in *Lippia nodiflora* L. Being medicinally sound for its immense number of bio-active compounds, mass propagation of plant and large-scale production of the compounds is needed to meet the industrial demands. This study paves the path for further research on complete *in vitro* plant regeneration and genetic transformation, thereby exploiting the plant commercially.

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