Establishment of *in vitro* protocol and impact of mycorrhization with phosphobacteria on micro propagated *Pogostemon mollis* Benth. (Lamiaceae)

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**ABSTRACT**

Plants and microbes compete for survival and their organized interactions play a vital role in adapting to stress environments. Microbes have a positive impact on transplanting performance of *in vitro* propagated plantlets. The present study aims at survival development of micropropagated *Pogostemon mollis* through microbial inoculation. The leaf and stem explants on MS medium with different concentrations and combinations of BAP, Kn, NAA, IAA and IBA used for the establishment of plantlet. The survival rate of plantlets was successfully increased from 71% to about 70.21% by the inoculation of *Rhizophagus fasciculatus* and *Bacillus megaterium*. This inoculation also clearly increases the growth, biomass and chlorophyll of *in vitro* derived platelets. The present protocol emphasizes the need of bio-hardening on micropropagated plants, particularly the mycorrhization along with phosphobacteria.

**1. Introduction**

The Lamiaceae includes aromatic plants that are being used in traditional medicine for various disorders. Particularly, *Pogostemon* species are used by tribes, mostly for its roots and leaves. The fresh root or poultice of the leaves is applied on the snake bite of Phursa snake (*Echis carinatus*) and other snake bites, is used for haemorrhage especially in uterine haemorrhage and leaf extract is used as an insect repellent. The plant extract is used for the treatment of food poisoning such as vomiting and stomach troubles, respiratory tract infection and pollen and nectar were the sources of Panagol honey in Maharashtra [1–3].

The essential oil is one of the naturally occurring vital materials used in the perfume industry and other therapies. The patchouli essential oil is uniquely derived from the *Pogostemon* species. The essential oil contains a large number of sesquiterpenes, subsequent to oxidative modifications. This may have anti-inflammatory, antimicrobial, antiprotozoal and antitumour properties [4]. *Pogostemon mollis* is one of the important medicinal plants and endemic to the Western Ghats [5]. The whole plant was used by the Irula tribes from the Western Ghats for the treatment of fever, cold, cough and they have collected from wild. Micropropagation techniques increase the scale, speed of production and yield of healthy plants. Consequently, the *in vitro* propagation has been widely applied to the product for a short time or any time of the year and ensuring varietal authenticity [6]. In the development of sustainable crop production practices, the use of microbial inoculants as a replacement for chemical fertilizers and pesticides is receiving attention. It is believed that delivery of microbial inoculants via micropropagation is one of the solutions to this problem [7]. The major problem of commercial micropropagation plant production is low survival and poor growth while shifting these plantlets to field conditions. Micropropagated plants have functionally less associated with rhizosphere microorganisms such as mycorrhizae [8].

Micropropagation techniques can produce pathogen-free plantlets but eliminate AM fungi. Although micropropagation is an established technique for the production of elite plants, owing to transient transplantation shock, plants require biological hardening before transplantation. For this reason, mycorrhizal technology can be applied to reduce transplantation shock during acclimatization, thus increasing plant survival and establishment rates [7,9]. Significantly, mycorrhizae influence survival and growth of micropropagated medicinal plants [10].

This problem can be obviated by combining the technique of micropropagation with mycorrhization during hardening for improved plant survival and growth [11]. An experiment was undertaken with the major objectives of assessing the effect of AM fungi on growth, the physiological status of micropropagated *P. mollis* and also the identified effective *Rhizophagus fasciculatus* and *Bacillus megaterium* or their suitability to be used as bio-hardening agents.
2. Materials and methods

2.1. Plant material, micropropagation procedures and plant acclimatization

Plantlets of *P. mollis* were propagated through *in vitro* cultures. The explants sources were surfaced disinfected with 70% ethanol for 3 min, soaked in 10% (v/v) NaOCl solution with a few drops of Tween 20 for 10 min., followed by three rinses with sterile distilled water. This step was repeated twice. The leaf and stem were cultured in the initiation medium. The cultures were grown under sterile conditions on a basal salt mixture and vitamin medium [12] supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The growth regulators used were BAP and Kn for the initiation and multiplication stage (1 mg L\(^{-1}\)) and IAA, NAA and IBA for the rooting stage (1 mg L\(^{-1}\)). All media were adjusted to pH 5.7 with 0.1 N NaOH or HCl and were autoclaved at 1.05 kg/cm\(^2\) and 121°C for 15 min. All cultures were incubated at 25 ± 1°C with a 16/8 h light/dark photoperiod illuminated by a cool white fluorescent light (50 µmol m\(^{-2}\) s\(^{-1}\)). The cultures were transferred to fresh media every 4 weeks until a sufficient number of plantlets were obtained.

2.2. Biological inoculation

After rooting and before acclimatization, 6-week-old uniform plantlets were selected from *in vitro* cultures and washed with tap water to remove residues of agar from the roots. Plantlets were then transplanted to individual 300 mL plastic pots containing a sterile substrate mixture. The *R. fasciculatus* spores were collected from the natural forest soil and multiplied by monoculture by using onion plant. The *B. megaterium* strain was collected from the Tamil Nadu Agricultural University, Coimbatore. It was inoculated with approximately 1000 spores before inserting the roots of the plantlets into the substrate with the bacterial strain. Non-inoculated plants received the same amount of bacterial strain without mycorrhizal spore and bacterial strain. Callus induction was observed on MS medium containing 

2.3. Growth conditions and harvest

After transplanting, the inoculated and non-inoculated plantlets were acclimatized in a controlled glasshouse with day–night temperatures of about 27 ± 1°C. Plantlets were maintained in 16 h photoperiod with cool white fluorescent lamps (50 µmol m\(^{-2}\) s\(^{-1}\)). The relative humidity (RH) was about of 70–80% at night and of 80–85% at day period. Distilled water was added daily to maintain moisture at 80% of field capacity. Plants were harvested at 30, 60 and 90 days after emergence. Plants were dug out with almost the entire root system. Roots and shoots were separated and their dry weights determined after recording their respective lengths and fresh weight.

2.4. Biomass of the experimental plant

The effects of bio-fertilizers (*R. fasciculatus* and *B. megaterium*) in the growth enhancement of micropropagated plant species *P. mollis* were assessed by shoot length, root length and number of leaves. Also the biomass assessment was estimated like dry weight estimation in shoot and root, ash biomass [13] and chlorophyll [14].

2.5. Statistical analysis

The data were analysed using statistical package for SPSS analysis software. The one-way ANOVAs were constructed using a factorial model and the means of the main factors were separated by Duncan’s Multiple Range test at 5% using GLM. Tabulate was used to compare the means and the standard deviations. The data for the inoculation (%) traits were seen as transformed to increase normality, although the untransformed data were used to calculate means.

3. Result

3.1 Callus culture

Callus induction was observed on MS medium containing a different concentration of BAP (0.25–2.0 mg L\(^{-1}\)) with 9–11 and 10–11 days of incubation of leaf and stem explants, respectively (Figures 1 and 2). There is a wide range of variation in the percentage of leaf callus (3.3 ± 0.1–94.17 ± 0.4) and stem callus (4.23 ± 0.37–92.23 ± 0.12) induction according to the concentration of hormone (Tables 1 and 2). In both explants, the morphology of the callus was green, pale green, pale yellow and brown. The basal media supplemented (3% sucrose) with BAP (0.5 mg L\(^{-1}\)) was found to be more suitable for the callus induction with 94.17 ± 0.14 and 92.23 ± 0.12 percentage from leaf and stem explants (Figure 1). All other concentrations of BAP were also effective but not at the level of the previous combination.

3.2 Shoot proliferation

The callus obtained from stem and leaf was transferred to the medium containing different concentrations of
BAP, Kn and IAA for shoot induction. The optimum concentration of BA (0.5 mg L\(^{-1}\)), Kn (0.25 mg L\(^{-1}\)) and IAA (0.02 mg L\(^{-1}\)) was proved to be the best for shoot survival (89.37 ± 0.03%) and shoot elongation (1.66 ± 0.01 cm for after 30 days and 3.263 ± 0.02 cm for after 60 days), the number of shoots (5.16 ± 0.03) also being higher in the combination of BAP (0.5 mg L\(^{-1}\)), Kn (0.25 mg L\(^{-1}\)) and IAA (0.02 mg L\(^{-1}\)) (Table 3; Figure 1). The callus along with initiated shoots was transferred to MS medium with the various concentrations of BAP (0.25–2.0 mg L\(^{-1}\)) for multiple shoot formation. The highest mean shoot proliferation was observed on MS medium containing BAP (0.5 mg L\(^{-1}\)) with 78.48 and 98.44 after 30 and 60 days, respectively (Table 4).

3.3. Root induction

The good growth shoots formed were excised and subjected to root induction. Among the three auxins (NAA, IAA and IBA) along with BAP in full and half strength MS medium, BAP (0.5 mg L\(^{-1}\)) and IAA (0.5 mg L\(^{-1}\)) in half strength MS medium proved best for the induction of roots. Here, the response percentage was 92.26 ± 0.03, mean number of roots/shoot was 5.03 ± 0.01 and mean root length was 2.24 ± 0.01 cm. This is

**Figure 1. In vitro regeneration of P. mollis from leaf and stem explant.**
Figure 2. Effect of bio-inoculums with hardening medium on in vitro developed plantlets of *P. mollis* and their AM fungal root colonization.

Table 1. Effect of BAP on initiation callus formation from leaf explants of *P. mollis* cultured in MS medium.

| S. no. | Plant growth regulator (mg L$^{-1}$) BAP | Days required for callus formation after inoculation | % of explants produced that callus | Callus colour | Callus amount |
|--------|----------------------------------------|----------------------------------------------------|-----------------------------------|---------------|--------------|
| 1      | 0.25                                   | 10–17                                              | 87.3 ± 0.1$^b$                    | Green         | ++           |
| 2      | 0.5                                    | 9–11                                               | 94.17 ± 0.14$^a$                  | Green         | +++          |
| 3      | 0.75                                   | 10–15                                              | 78.2 ± 0.1$^c$                    | Pale green    | ++           |
| 4      | 1.0                                    | 10–20                                              | 54.11 ± 0.08$^d$                  | Pale green    | ++           |
| 5      | 1.25                                   | 17–20                                              | 30.37 ± 0.45$^e$                  | Light green with pale yellow | +            |
| 6      | 1.5                                    | 14–22                                              | 19.7 ± 0.1$^f$                    | Pale yellow   | +            |
| 7      | 1.75                                   | 17–25                                              | 9.21 ± 0.017$^g$                  | Pale yellow   | +            |
| 8      | 2.0                                    | 22–30                                              | 3.3 ± 0.1$^h$                     | Brown and pale yellow | +            |

Note: Values are mean ± standard deviation (SD).

$^a$–$^h$ *P* < 0.05, designates a significant response among the treatment.

+: Poor; ++: Moderate; +++: Good.
followed by half strength MS medium containing BAP (0.5 mg L⁻¹) and IAA (0.25 mg L⁻¹) concentration with the response percentage of 83.55 ± 0.24, mean number of roots/shoot of 3.05 ± 0.02 and mean root length of 1.57 ± 0.02 cm. All other combinations also induced roots but not at the level of these two combinations (Table 5).

3.4. Hardening

The well-developed plantlets were transferred to a net pot containing different types of autoclaved hardening media under lower shade condition for hardening (Table 6). The plants were kept in high RH 90% for 1 week. After 1 week, they brought to lower RH (60%) for further hardening for another 2 weeks. Among the four different combinations of hardening medium, more survival percentage (66.21%) was observed in the forest soilsandvermicompost (1:1:1). The well-developed plants were transferred to a field with a survival rate of 79%. All plants had normal leaf development and no morphological variation was observed.

3.5. Inoculation effect on in vitro regenerated plantlets

The micropropagated plantlet was inoculated with R. fasciculatus, B. megaterium and both combined. The survival percentage was higher in T4 (79.21%) followed by T3 and T2 than the control (Table 7). The morphological parameters like length and dry weight of shoot and root were observed in 30, 60 and 90 days of the treated plants (Tables 7 and 8; Figure 2). Similarly, the green cover of leaf area also increases by the increasing number of leaves in dual inoculation such

| S. no. | Plant growth regulator (mg L⁻¹) | BAP | % of callus produced callus | No. of shoots/explant | Shoot length (cm) After 30 days | After 60 days |
|-------|---------------------------------|------|-----------------------------|------------------------|-------------------------------|---------------|
| 1     | 0.25                            | 0.25 | 0.25                         | 87.23 ± 0.15 a         | 3.04 ± 0.02 b                 | 1.25 ± 0.01 a |
| 2     | 0.5                             | 0.25 | 0.02                         | 89.37 ± 0.035 a        | 5.16 ± 0.036 a                | 1.66 ± 0.015 a|
| 3     | 0.75                            | 0.25 | 0.02                         | 76.22 ± 0.15 a         | 4.06 ± 0.014 b                | 1.156 ± 0.015 a|
| 4     | 1.0                             | 0.25 | 0.02                         | 65.41 ± 0.42 a         | 2.016 ± 0.02 b                | 1.1 ± 0.016 a |
| 5     | 1.25                            | 0.25 | 0.02                         | 38.38 ± 0.15 c         | 2.26 ± 0.04 a                 | 1.1 ± 0.02 a  |
| 6     | 1.5                             | 0.25 | 0.02                         | 26.88 ± 0.39 c         | 2.14 ± 0.051 a                | 1.09 ± 0.016 a|
| 7     | 1.75                            | 0.25 | 0.02                         | 15.43 ± 0.301 d        | 1.03 ± 0.011 b                | 1.0 ± 0.055 a |
| 8     | 2.0                             | 0.25 | 0.02                         | 6.43 ± 0.39 e          | 1.04 ± 0.036 f                | 0.96 ± 0.0152 a|

Table 3. Effect of growth regulators on shoot initiation from leaf and stem explants of P. mollis cultured in MS medium.

| S. no. | Plant growth regulators (mg L⁻¹) | BAP | Kn | IAA | % of callus produced shoots | No. of shoots/explant | Shoot length (cm) After 30 days | After 60 days |
|-------|---------------------------------|------|----|-----|-----------------------------|------------------------|-------------------------------|---------------|
| 1     | 0.25                            | 0.25 | 0.25 | 0.02 | 89.3 ± 0.15 b               | 65.19 ± 0.13 b         | 79.68 ± 0.11 c                |               |
| 2     | 0.5                             | 0.25 | 0.25 | 0.02 | 94.84 ± 0.04 a              | 78.48 ± 0.13 a         | 98.44 ± 0.015 a               |               |
| 3     | 0.75                            | 0.25 | 0.25 | 0.02 | 79.5 ± 0.065 c              | 34.54 ± 0.35 c         | 94.41 ± 0.27 a               |               |
| 4     | 1.0                             | 0.25 | 0.25 | 0.02 | 56.32 ± 0.041 d             | 20.18 ± 0.055 a        | 37.36 ± 0.14 c               |               |
| 5     | 1.25                            | 0.25 | 0.25 | 0.02 | 31.4 ± 0.38 c              | 15.46 ± 0.28 a         | 20.3 ± 0.03 a                |               |
| 6     | 1.5                             | 0.25 | 0.25 | 0.02 | 19.66 ± 0.083 f             | 4.38 ± 0.15 c          | 3.44 ± 0.37 a                |               |
| 7     | 1.75                            | 0.25 | 0.25 | 0.02 | 12.7 ± 0.27 f              | 4.14 ± 0.066 b         | 3.88 ± 0.088 b               |               |
| 8     | 2.0                             | 0.25 | 0.25 | 0.02 | 3.54 ± 0.11 b              | 3.74 ± 0.209 a         | 3.22 ± 0.047 a               |               |

Note: Values are mean ± standard deviation (SD). *P < 0.05, designates a significant response among the treatment.
Table 5. Effect of different concentrations of growth regulators on root number, rooting percentage and root length in in vitro shoots of *P. mollis*.

| S. no. | MS medium strength | BAP (mg L\(^{-1}\)) | IBA (mg L\(^{-1}\)) | IAA (mg L\(^{-1}\)) | NAA (mg L\(^{-1}\)) | Response % of root | Mean no. of roots/shoots | Mean root length (cm) |
|--------|-------------------|---------------------|---------------------|---------------------|---------------------|---------------------|------------------------|-----------------------|
| 1      | MS                | 0.5                 | 0.00                | 0.00                | 0.00                | 63.38 ± 0.14\(^a\) | 2.43 ± 0.06\(^d\) | 1.48 ± 0.03\(^d\) |
| 2      | MS                | 0.5                 | 0.00                | 0.00                | 0.00                | 81.55 ± 0.53\(^b\) | 3.49 ± 0.08\(^b\) | 1.95 ± 0.05\(^b\) |
| 3      | MS                | 0.5                 | 0.00                | 0.00                | 0.00                | 42.16 ± 0.04\(^d\) | 2.38 ± 0.03\(^a\) | 1.33 ± 0.03\(^d\) |
| 4      | MS                | 0.5                 | 0.00                | 0.00                | 0.00                | 35.89 ± 0.05\(^m\) | 1.22 ± 0.28\(^m\) | 0.97 ± 0.01\(^m\) |
| 5      | MS                | 0.5                 | 0.00                | 0.00                | 0.00                | 37.55 ± 0.09\(^k\) | 3.15 ± 0.03\(^m\) | 1.67 ± 0.01\(^m\) |
| 6      | MS                | 0.5                 | 0.25                | 0.00                | 0.00                | 25.68 ± 0.1\(^l\)   | 2.03 ± 0.02\(^m\) | 1.26 ± 0.02\(^m\) |
| 7      | MS                | 0.5                 | 0.50                | 0.00                | 0.00                | 32.21 ± 0.02\(^p\) | 1.41 ± 0.11\(^m\) | 1.17 ± 0.01\(^m\) |
| 8      | MS                | 0.5                 | 0.00                | 0.25                | 0.00                | 28.32 ± 0.04\(^m\) | 1.38 ± 0.03\(^m\) | 1.07 ± 0.03\(^m\) |
| 9      | MS                | 0.5                 | 0.00                | 0.00                | 0.75                | 18.24 ± 0.02\(^m\) | 1.23 ± 0.06\(^m\) | 0.86 ± 0.03\(^m\) |
| 10     | MS                | 0.5                 | 0.00                | 0.00                | 0.75                | 14.25 ± 0.02\(^m\) | 1.04 ± 0.04\(^m\) | 0.81 ± 0.02\(^m\) |
| 11     | MS 1/2            | 0.5                 | 0.25                | 0.00                | 0.00                | 57.74 ± 0.13\(^a\) | 3.11 ± 0.03\(^a\) | 1.68 ± 0.01\(^a\) |
| 12     | MS 1/2            | 0.5                 | 0.50                | 0.00                | 0.00                | 62.26 ± 0.15\(^a\) | 2.41 ± 0.11\(^a\) | 1.84 ± 0.01\(^a\) |
| 13     | MS 1/2            | 0.5                 | 0.75                | 0.00                | 0.00                | 44.86 ± 0.04\(^a\) | 2.25 ± 0.07\(^a\) | 1.57 ± 0.01\(^a\) |
| 14     | MS 1/2            | 0.5                 | 1.00                | 0.00                | 0.00                | 21.23 ± 0.06\(^a\) | 2.19 ± 0.02\(^a\) | 1.35 ± 0.01\(^a\) |
| 15     | MS 1/2            | 0.5                 | 0.25                | 0.00                | 0.00                | 83.55 ± 0.24\(^a\) | 3.06 ± 0.02\(^a\) | 1.57 ± 0.01\(^a\) |
| 16     | MS 1/2            | 0.5                 | 0.50                | 0.00                | 0.00                | 92.26 ± 0.03\(^a\) | 5.30 ± 0.01\(^a\) | 2.24 ± 0.01\(^a\) |
| 17     | MS 1/2            | 0.5                 | 0.75                | 0.00                | 0.00                | 68.41 ± 0.04\(^a\) | 4.03 ± 0.02\(^a\) | 1.67 ± 0.01\(^a\) |
| 18     | MS 1/2            | 0.5                 | 1.00                | 0.00                | 0.00                | 58.4 ± 0.3\(^a\)    | 2.06 ± 0.02\(^a\) | 1.24 ± 0.01\(^a\) |
| 19     | MS 1/2            | 0.5                 | 0.00                | 0.25                | 0.00                | 31.4 ± 0.2\(^a\)    | 2.04 ± 0.02\(^a\) | 1.21 ± 0.02\(^a\) |
| 20     | MS 1/2            | 0.5                 | 0.00                | 0.50                | 0.00                | 27.26 ± 0.15\(^a\) | 2.03 ± 0.02\(^a\) | 1.06 ± 0.03\(^m\) |
| 21     | MS 1/2            | 0.5                 | 0.00                | 0.00                | 0.75                | 18.36 ± 0.15\(^a\) | 1.05 ± 0.02\(^a\) | 0.97 ± 0.01\(^a\) |
| 22     | MS 1/2            | 0.5                 | 0.00                | 0.00                | 0.75                | 13.77 ± 0.015\(^m\) | 1.04 ± 0.02\(^m\) | 0.8 ± 0.015\(^m\) |

Note: Values are mean ± standard deviation (SD).

\(^a\) P < 0.05, designates a significant response among the treatment.

Table 6. Effect of different combinations of hardening medium on survivability of in vitro developed plantlets of *P. mollis*.

| Hardening medium composition (V/V) | No. of plantlets under hardening | No. of plantlets that survived | Percentage of survivability |
|----------------------------------|---------------------------------|-------------------------------|-----------------------------|
| Forest soil + sand (1:1)         | 25                             | 8.73 ± 0.165\(^d\)           | 34.93                       |
| Forest soil + sand + Vermicompost (1:1:1) | 25                         | 16.55 ± 0.196\(^e\)         | 66.21                       |
| Vermicompost + sand + forest soil + charcoal (1:1:1:1) | 25                   | 15.72 ± 0.165\(^d\)         | 62.89                       |
| Decomposed coir waste + Perlite + Vermicompost (1:1:1) | 25                   | 14.37 ± 0.176\(^f\)         | 58.30                       |

Note: Values are mean ± standard deviation (SD).

\(^a\) P < 0.05, designates a significant response among the treatment.
The shoot proliferation was satisfactory on MS medium supplemented with BAP (0.5 mg L\(^{-1}\)), Kn (0.25 mg L\(^{-1}\)) and IAA (0.02 mg L\(^{-1}\)). Though kinetin is less effective on multiple shoot regeneration, it played a role in increasing the length and strength of shoots. The combination was found to exhibit a minimum number of shoots with the highest shoot length. The efficacy of BAP either fortified individually or in combination with Kn has been demonstrated for the axillary bud proliferation in Mentha spicata and Lavendula viridis [19,20]. The above result clearly indicates that a combination of BA, KN and IAA is a better choice for the better morphogenetic response of *P. mollis*.

George et al. [21] reported that BAP is the most effective in enhancing shoot multiplication and triggering shoot elongation, and this was in agreement with Arya et al. [22] and Hembrom et al. [23] in Leptadenia reticulata and Pogostemon heyneanus. However, Mishra [24] found the best response to shooting induction, with maximum shoot elongation obtained by MS basal medium supplemented with 1.0 mg L\(^{-1}\) BAP and 0.5 mg L\(^{-1}\) IAA with 82% shoot bud proliferation in the *O. sanctum*. The findings of Bharati [25] and Sheena and Jothi [26] report that the higher concentration of BAP has resulted in decreased multiple shoot regeneration in Orthosiphon stamineus which is confirmed in this study but opposed by Kukreja et al. [27].

The high per cent frequency of rooting (92.26%) was achieved within 15 days of shoot implantation on half

### Table 7. Effect of different inoculums (mycorrhiza and phosphobacteria) with hardening medium on survivability and plant length of *P. mollis*.

| Type of culture | Survivability of plants | Shoot length (cm) | Root length (cm) |
|----------------|-------------------------|-------------------|-----------------|
|                | 30 days | 60 days | 90 days | 30 days | 60 days | 90 days |
| T1            | 71.82 ± 0.155\(^{a}\) | 8.603 ± 0.09\(^{a}\) | 14.68 ± 0.111\(^{a}\) | 18.52 ± 0.113\(^{a}\) | 4.653 ± 0.095\(^{a}\) | 6.76 ± 0.098\(^{a}\) | 7.783 ± 0.081\(^{a}\) |
| T2            | 73.80 ± 0.216\(^{a}\) | 8.243 ± 0.083\(^{a}\) | 13.83 ± 0.108\(^{a}\) | 18.55 ± 0.169\(^{a}\) | 3.26 ± 0.055\(^{a}\) | 5.843 ± 0.055\(^{a}\) | 7.24 ± 0.112\(^{a}\) |
| T3            | 77.57 ± 0.307\(^{a}\) | 9.43 ± 0.279\(^{a}\) | 16.32 ± 0.049\(^{a}\) | 20.223 ± 0.030\(^{a}\) | 6.266 ± 0.126\(^{a}\) | 7.86 ± 0.105\(^{a}\) | 8.88 ± 0.068\(^{a}\) |
| T4            | 79.21 ± 0.22\(^{a}\) | 10.01 ± 0.098\(^{a}\) | 17.36 ± 0.144\(^{a}\) | 22.39 ± 0.265\(^{a}\) | 6.816 ± 0.02\(^{a}\) | 8.176 ± 0.055\(^{a}\) | 9.14 ± 0.036\(^{a}\) |

Note: Values are mean ± standard deviation (SD).

\(^{a}\)–\(^{d}\) *P* < 0.05, designates a significant response among the treatment.

### Table 8. Effect of different inoculums (mycorrhiza and phosphobacteria) with hardening medium on shoot and root dry weight of *in vitro* developed plantlets of *P. mollis*.

| Type of culture | Shoot dry weight (g) | Root dry weight (g) |
|----------------|---------------------|---------------------|
|                | 30 days | 60 days | 90 days | 30 days | 60 days | 90 days |
| T1            | 0.9663 ± 0.012 | 1.8233 ± 0.128 | 2.2960 ± 0.056 | 0.6823 ± 0.007 | 0.7880 ± 0.004 | 0.9723 ± 0.018 |
| T2            | 1.2533 ± 0.018 | 1.9813 ± 0.004 | 2.5987 ± 0.022 | 0.7307 ± 0.012 | 0.8337 ± 0.009 | 1.1083 ± 0.114 |
| T3            | 1.8570 ± 0.024 | 2.0313 ± 0.015 | 2.9570 ± 0.023 | 0.7933 ± 0.004 | 0.8923 ± 0.005 | 1.6000 ± 0.063 |
| T4            | 1.9833 ± 0.06 | 2.4467 ± 0.111 | 3.2937 ± 0.071 | 0.8887 ± 0.008 | 1.0427 ± 0.01 | 1.9700 ± 0.01 |

### Table 9. Effect of different inoculums (mycorrhiza and phosphobacteria) with hardening medium on number of leaves and AM fungal root colonization of *in vitro* developed plantlets of *P. mollis*.

| Type of culture | No. of leaves | % of AMF root colonization |
|----------------|--------------|---------------------------|
|                | 30 days | 60 days | 90 days | 30 days | 60 days | 90 days |
| T1            | 10     | 24     | 38     | –      | –      | –      |
| T2            | 11     | 26     | 41     | –      | –      | –      |
| T3            | 14     | 37     | 61     | 52     | 61     | 78     |
| T4            | 15     | 39     | 68     | 54     | 58     | 65     |

### Table 10. Effect of different inoculums (mycorrhiza and phosphobacteria) with hardening medium on ash biomass of *in vitro* developed plantlets of *P. mollis*.

| Type of culture | Total ash value (%) | Water-soluble ash (%) | Acid in soluble ash (%) |
|----------------|---------------------|-----------------------|-------------------------|
|                | 30 days | 60 days | 90 days | 30 days | 60 days | 90 days | 30 days | 60 days | 90 days |
| T1            | 12.72   | 13.26   | 12.4   | 7.23   | 8.32   | 9.04   | 3.5     | 3.78    | 3.63 |
| T2            | 13.51   | 13.98   | 14.5   | 7.21   | 7.94   | 7.37   | 4.85    | 4.12    | 4.69 |
| T3            | 19.81   | 20.71   | 16.24  | 9.51   | 8.74   | 9.37   | 5.7     | 5.87    | 6.31 |
| T4            | 27.65   | 29.42   | 31.2   | 18.22  | 19.23  | 21.32  | 11.43   | 9.78    | 12.74 |

4. Discussion

In the present study, *in vitro* propagation strategies and the effect of bio-inoculants (*R. fasciculatus* and *B. megaterium*) were assessed in the micropropagated *P. mollis*. In tissue culture, cytokinins are very effective in promoting direct and indirect shoot regeneration. In this investigation, lower concentrations of cytokinins (BAP 0.25–0.5 mg L\(^{-1}\)) resulted in callus formation in leaf and stem explants. In contrast, the *Ocimum sanctum*’s (Lamiaceae) callus induction was obtained at the higher auxins concentration and it’s formed profuse callus [15]. But this study indicates that the increase of cytokinins beyond 0.5 mg L\(^{-1}\) resulted in a decrease in the number of shoot buds coupled with callus proliferation and sometimes affects the totipotency of viable cells, which was similar to with the findings of Chaudhari et al. [16], Ahmad et al. [17] and Nikam et al. [18].
strength MS medium. The successful application of half strength MS medium in the root-inducing experiment has also been documented in various in vitro protocols [28–31]. The results of Mishra [32] support the usage of auxins for rhizogenesis in Patchouli which is contrary to the present observation. The rooted shoots were successfully transplanted to a net pot containing forest soil, sand and vermicompost dust in the ratio of 1:1:1, which had a better survival percentage (66.21%). Similar observations are recorded by Misra [32] and Conde et al. [33].

In micropropagation systems, the weaning stage represents a developmental phase where plants are subjected to environmental stress due to poor root, shoot and cuticular development [34]. The plants are inoculated with appropriate symbiotic organisms in the transfer prior and there is a scope for improving plant survival [35]. Because of AM Fungal pivotal role in plant community ecology and multiple beneficial effects on the plant as well as soil health, they appear to be an essential component in successful growth and survival of the treated plants. *Glomus intraradices* and *Glomus deserticola* have been successfully used to improve acclimatization, survival and growth of micropropagated plants [36–38]. Several studies have demonstrated the benefit of mycorrhizal inoculation on survival and establishment of micropropagated plants [39,40]. Lata et al. [41] found the four AM fungal species increase the survival percentage ranging between 83% and 92% in *Echiacea pallida*. A similar result was also reported by Caro et al. [42] in *Tectona grandis* with 100% survival and increased root area.

The root–microbe contact can be positive to the plant like the association of mycorrhizal fungi and plant growth-promoting rhizobacteria. They act as plant growth advocates through stimulating plant growth, defence mechanisms against pathogens, solubilization of mineral as nutrients, production of plant growth-promoting substances and secretion of specific enzymes [43]. Phosphorus is the essential element for the growth of plants; the chemical speciation of phosphorus that plants could absorb is in the form of H\(_2\)PO\(_4^−\) and HPO\(_4^{2−}\), while most of the phosphorus exists in the insoluble state. Consequently, the liberated inorganic PO\(_4^{3−}\) plays an important role in the nutrition cycle of plants.

The production of organic acid by *B. megaterium* is one of the known mechanisms responsible for phosphate solubilization and it could act as a phosphate biofertilizer to improve plant growth [44]. But in this study, the T2 (*B. megaterium*) plant shows some growth enhancement than the control but not than other treated plants (T3 and T4). Here, the *R. fasciculatus*-treated plant shows significant growth than *B. megaterium*-treated plant, which may confirm the vital role of AM fungal. At the same time, the plants inoculated with *R. fasciculatus* and *B. megaterium* (T4) had higher growth and ash biomass and total chlorophyll content which correlated with the findings of Joshee et al., [45], Sharma et al. [46] and Yadav et al. [47].

Numerous investigators have reported on the mechanism of plant growth improvement caused by mycorrhizal. The growth improvement is mostly credited by uptake of diffusion-limited nutrients like P, Zn, Cu and so on from the soil. The other beneficial effects are their role in the biological control of root pathogens, hormone production, greater ability to withstand water stress and synergistic interaction with nitrogen fixers, P solubilizers and plant growth-promoting rhizomicroorganisms. The rate at which plant roots absorb phosphorus from the soil solution is much faster than the rate at which phosphorus moves in the soil solution by diffusion. This results in a phosphorus-depletion zone around the root. Here, AM fungi play the most important role because the external hyphae travel much beyond the P depletion zone, scavenge a large volume of soil and supply P to the plants [48]. These may be confirmed by the present study, AM fungal acting as significant biofertilizer than *B. megaterium*. Simultaneously, the present result revealed that both microbes were vitally important alone or in combination. The overall findings confirm that bio-hardening is the best for the micropropagated plantlet via reducing transplanting shock.

### 5. Conclusion

Currently, the biological approaches for improving crop production are gaining strong status in the agriculture and environment following an integrated plant nutrient management system. This may improve the growth and survivability of the plantlets due to an increase in nutrient uptake in the soil and adaptable ability. This technology may also be applied to improve the post-transplanting performance of some other medicinally important plants which require conservation efforts.

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**Table 11. Effect of different inoculums (mycorrhiza and phosphobacteria) with hardening medium on chlorophyll content of in vitro developed plantlets of *P. mollis.***

| Type of culture | 30 days Chlorophyll (a) (mg g\(^{-1}\) tissue) | 90 days Chlorophyll (a) (mg g\(^{-1}\) tissue) | 90 days Chlorophyll (b) (mg g\(^{-1}\) tissue) | Total chlorophyll (mg g\(^{-1}\) tissue) 30 days | Total chlorophyll (mg g\(^{-1}\) tissue) 60 days | Total chlorophyll (mg g\(^{-1}\) tissue) 90 days |
|----------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| T1             | 1.27 ± 0.01                     | 1.36 ± 0.01                     | 0.65 ± 0.01                     | 0.73 ± 0.00                     | 0.74 ± 0.00                     | 1.79 ± 0.01                     |
| T2             | 1.21 ± 0.01                     | 1.34 ± 0.02                     | 0.67 ± 0.01                     | 0.71 ± 0.00                     | 0.73 ± 0.00                     | 1.93 ± 0.01                     |
| T3             | 1.50 ± 0.08                     | 1.54 ± 0.01                     | 0.74 ± 0.01                     | 0.78 ± 0.00                     | 0.75 ± 0.00                     | 2.08 ± 0.03                     |
| T4             | 1.61 ± 0.01                     | 1.64 ± 0.03                     | 0.81 ± 0.00                     | 0.85 ± 0.01                     | 0.82 ± 0.01                     | 2.04 ± 0.03                     |
Disclosure statement

No potential conflict of interest was reported by the authors.

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