

An efficient high-frequency direct organogenesis protocol of *Mimosa pudica* suitable for mass multiplication and transformation experiments

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Short Report

Keywords: Apical meristems, Cytokinin, In-vitro rooting, Mass multiplication, Phyto hormones

Posted Date: August 7th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3171313/v1>

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Abstract

Mimosa pudica is a creeping perennial, flowering medicinal plant belonging to the family Mimosaceae. The plants have been well documented for their various valuable properties such as antiseptic, antimicrobial, antiviral, antimalarial, hyperglycemic, diuretic, anti-inflammatory, antihepatotoxic, anti-cancer and immune boosters. Due to the occurrence of various phytochemicals, the plant has been extensively used in AYUSH for the treatment of various ailments. Due to the immense medicinal properties of the plant and high exploitation, may lead to a high risk of drastic decreases in its population from natural habitats. Hence conservation of the plant is the utmost requirement by using plant tissue cultures protocols. In this investigation, we have developed a direct organogenesis protocol for the plant using apical meristem as the source of explant. From the tested 12 hormonal combinations; MPO4 (Kn 2.0 + BAP 1.0 + 2,4 D- 0.2 mg/lit) was able to generate a significantly higher number of multiple shoots (44.33/explant) and the lowest multiple shooting (0.67/explant) was noticed in MPO12 media. Out of 15 media combinations, the MPR10 (NAA 1.0 mg/lit) combination was the best for *in-vitro* rooting and was able to induce a significantly higher percentage (72.67% explants show rooting induction) of adventitious rootings, followed by MPR3 (IBA 1.5 mg/lit) media (62.67% 72.67% explants shows rooting). In this investigation, the rooted plants were successfully hardened to the field. The developed direct organogenesis protocol using apical meristem as an explant is the first time report for the mentioned plant and may be used for mass multiplication as well as plantlet production after genetic transformations.

Introduction

Mimosa pudica L. (sensitive plant/ touch-me-not) is a creeping perennial, flowering medicinal plant belonging to the family Mimosaceae. The species is native to the Caribbean and Central America, but it is now a pantropical weed and is found in the various regions of the United States, Asia, Australia and Africa (Fosberg 1980; Magda et al. 2006). The plant has rich in useful phytochemicals and studies on *M. pudica* have revealed the presence of alkaloids, non-protein amino acid (mimosine), flavonoids, C-glycosides, sterols, terpenoids, tannins, and fatty acids (Genest et al. 2008). Due to the occurrence of various phytochemicals in various parts of the plant; the plant has been extensively used in AYUSH (Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homeopathy) for the treatment of toothaches, cold & flu, jaundice, asthma, diarrhea, dysentery, piles, insomnia, conjunctivitis, cut wounds & glandular swellings, liver detoxification, rabies, tuberculosis, etc. (Hasan et al. 2010; Ramesh et al. 2013). The plants have been well documented for their various valuable properties such as antiseptic, antimicrobial, antiviral, antimalarial, hyperglycemic, diuretic, anti-inflammatory, antihepatotoxic, anti-cancer and also effective immune boosters (Varnika et al. 2012; Muhammad et al. 2016; Mandal et al. 2022). Hence, in the UK the plant has gained the Royal Horticultural Society's Award of Garden Merit (AGM Plants 2017).

Due to the immense medicinal properties of the plant along with the world's moving towards herbal medicine, proportionate use of the said plant may lead to a high risk of drastic decreases in its population from natural habitats. Hence conservation of the plant is the utmost requirement for the present scenario,

either in-situ or ex-situ. Plant tissue culture is the most popular method of ex-situ conservation strategy, which is user-friendly, low-cost; requires less space, energy and advanced tools. A few attempts have been tried to develop a tissue culture protocol for *M. pudica* using nodal explants and shoot tips as explants (Gharyal and Maheshwari 1982; Hassan et al. 2010; Ramesh et al. 2013, Bianchetti et al. 2017) and hardened the plantlets as well. All the described protocols are similar to shoot multiplication methods and direct organogenesis protocol for the plant has been not reported so far. Moreover, the plant contains many valuable compounds, and near future, it may require the genetic engineering experiments such as transformations to enhance the secondary metabolites production or any other gene manipulation of the desired trait/s. In the transformation experiments, the apical meristem is the best sample where the foreign gene manipulation became easier (Martignago et al. 2020). Hence the present investigation has aimed to develop an efficient high-frequency direct organogenesis protocol of *M. pudica* using apical meristems as explant which will be appropriate for mass multiplication and transformation experiments as well.

Material and Methods

Explant preparation

Two types of explants *i.e.*, apical meristems of *in-vitro* seed germinated and apical meristems of natural habituated plants were used for the development of direct organogenesis protocol. Dry viable seeds of *M. pudica* were used for *in-vitro* germination. The seeds were thoroughly washed under running tap water with liquid detergent (tween 20) and immersed in 70% ethanol for 30 seconds. Then after the seeds were transferred into the Laminar Air Flow cabinet for complete surface sterilization with 0.1% HgCl₂ for 5 minutes and then rinsed with three consecutive changes of sterile distilled water. The sterilized seeds were processed for germination in MS media. Apical meristems of the germinated plants were collected after 6 weeks of seed culture. Apical meristems from the young healthy plants were collected from natural habitats and the surface sterilization procedure of the apical meristems was done according to the protocol of Boruah (2020).

Media preparation

For culturing the sterilized seeds for germination, half-strength MS media (Himedia) without any hormone was used. For direct organogenesis and rooting experiments, MS media supplemented with the required amount of cytokinin (Kn and BAP; Duchefa make) and Auxin (IAA, NAA, IBA, 2,4-D; Duchefa make) were prepared. Details of the hormone concentration used for the direct organogenesis and *in-vitro* rooting experiments were shown in Tables 1 and Table 2. All media were supplemented with 30 g/l sucrose, 7 g/l agar and the pH of the medium was adjusted to 5.7 with the help of 1N HCl/ 1N NaOH (Murashige and Skoog, 1962). The media was melted in a microwave to completely dissolve agar, then approximately 20 ml of liquid media was dispensed into 55 ml culture tubes and 100 ml of media into 250 ml flasks. After solidifying the media, the tubes and flasks were fitted with cotton plugs followed by

autoclaving in the autoclave at 121°C, 15 psi for 20 mins. In this investigation, 12 and 15 different media combinations were used for direct organogenesis and rooting experiments, respectively (Tables 1 & 2).

Table 1
Effect of phytohormones on multiples shooting of *M. pudica*

Sl No	Media code	Kn (mg/ lit)	BAP (mg/ lit)	2,4-D (mg/ lit)	Average no shoots/ explant
1.	MP01	0.5	1	0.2	3.33 ^{de}
2.	MP02	1	1	0.2	3.67 ^{de}
3.	MP03	1.5	1	0.2	12.00 ^c
4.	MP04	2	1	0.2	44.33 ^a
5.	MP05	2.5	1	0.2	16.33 ^b
6.	MP06	3	1	0.2	1.00 ^f
7.	MP07	1	0.5	0.2	4.00 ^{de}
8.	MP08	1	1	0.2	4.33 ^d
9.	MP09	1	1.5	0.2	10.00 ^c
10.	MP010	1	2	0.2	4.00 ^{de}
11.	MP011	1	2.5	0.2	2.00 ^{ef}
12.	MP012	1	3	0.2	0.67 ^f
CV %					9.72
CD					2.33
Means in columns for each index, followed by the same letter, are not significantly different at the p < 0.05 level according to Duncan's Multiple Range Test (DMRT).					

Table 2
Effect of phytohormones on rooting of *M. pudica*

Sl. No.	Media code	IBA (mg/ lit)	IAA (mg/ lit)	NAA (mg/ lit)	% of shoots showing rooting
1.	MPR1	0.5	0	0	12.67 ^g
2.	MPR2	1	0	0	43.00 ^d
3.	MPR3	1.5	0	0	62.67 ^b
4.	MPR4	2	0	0	6.00 ^h
5.	MPR5	0	0.5	0	14.00 ^g
6.	MPR6	0	1	0	26.00 ^e
7.	MPR7	0	1.5	0	4.33 ^h
8.	MPR8	0	2	0	5.33 ^h
9.	MPR9	0	0	0.5	21.33 ^f
10.	MPR10	0	0	1	72.67 ^a
11.	MPR11	0	0	1.5	52.00 ^c
12.	MPR12	0	0	2	15.33 ^g
13.	MPR13	0.5	0.5	0.5	5.67 ^h
14.	MPR14	1	1	1	3.33 ^h
15.	MPR15	1.5	1.5	1.5	0.00 ⁱ
CV %					8.26
CD					3.16
Means in columns for each index, followed by the same letter, are not significantly different at the p < 0.05 level according to Duncan's Multiple Range Test (DMRT).					

Aseptic inoculation

The sterilized seeds were cultured on pre-sterilized Murashige and Skoog (MS) basal medium (without hormones) in the laminar airflow cabinet. All the cultures were kept in the culture room with 16-hour photoperiod (16-hour light and 8-hour dark) under 3000 lux light intensity at 25 ± 2°C with 70 to 80% humidity. After three weeks of inoculation, *in-vitro* seed germination was observed. The sterilized tender shoot tips (both *in-vitro* and *in-vivo*) were directly cut with the help of a scalpel and sterilized blade under

aseptic conditions and inoculated on MS media containing different hormone combinations for direct organogenesis. For rooting experiments, fully developed shoots with three to four internodes were cut from the mother bunch of multiple shoots and cultured in a rooting medium. Subcultures of the pre-cultures were maintained regularly at 4–6 week intervals/ as and when necessary.

Hardening

The rooted plantlets were taken out from the culture tubes, washed to remove agar gel adhered to the roots and transplanted to plastic pots with soil, cocopeat & compost (1:1:1) for hardening. The plantlets were kept in a greenhouse for 6–8 weeks at 70–80% relative humidity, $30 \pm 5^\circ \text{C}$ temperature for acclimatization with required nutrition. Established plants were transplanted in earthen pots under natural conditions and then transferred into the field and the survival rate was recorded.

Statistical Analysis

The experiment had 20 explants in each treatment with three replications and arranged in a Completely Randomized Design (CRD). The explants were maintained for 4 to 6 weeks and observations on the percentage of shoots and root induction were recorded by using the following equations.

$$\text{Shoot induction percentage} = \frac{\text{Number of explants showing shoot induction}}{\text{Total number of explants used for culture}} \times 100$$

$$\text{Root induction percentage} = \frac{\text{Number of explants showing root induction}}{\text{Total number of explants used for culture}} \times 100$$

Recorded data were statistically analyzed by Duncan's multiple range test (DMRT) using WASP - Web Agri Stat Package 2.0 (ccari.icar.gov.in) to test the significant differences between the designed treatments and the experiments.

Result and Discussion

Plant tissue culture is an important technique used in modern plant science and agriculture for the production of healthy disease-free, genetically identical plants, preserving valuable germplasm, creating new plant varieties through genetic engineering and secondary metabolites production (Moraes et al. 2021). In this investigation, direct organogenesis protocol from apical meristems of *M. pudica* has been optimized. In the current investigation, 100% *in-vitro* seed germination was observed in half strength MS medium. This is due to the viability of seeds being high and the provided *in-vitro* condition being favorable for the seeds used for the germination experiments. The results of the germination data are in agreement with the previous workers while working on the same or related plants (Chauhan and Johnson 2009; Li et al. 2022).

Multiple shooting induction was observed from the cultured apical meristems after three weeks of culture in all the tested hormonal combinations (Table 1). From the tested 12 combinations; MPO4 (Kn 2.0 + BAP 1.0 + 2,4 D- 0.2 mg/lit) was able to generate a significantly higher number of multiple (44.33/ explant) shoots followed by MPO5 media (Kn 2.5 + BAP 1.0 + 2,4 D- 0.2 mg/lit) (16.33 multiple shoots/explant)

and lowest multiple shooting (0.67/explant) was noticed in MPO12 media (Kn 2.5 + BAP 1.0 + 2,4 D- 0.2 mg/lit). The moderately higher concentration of two cytokines along with a lower concentration of auxin combinations of the MPO4 media was found to be best for the regeneration and dedifferentiation of the apical meristems in multiple shootings. It was also observed that media combinations having a lower or higher concentration of Kn and BAP in comparison to the MPO4 media combination resulted in a significantly lower number of multiple shootings (Table 1, Fig. 1a to f). Similar hormonal combinations with the best results were also observed in different plant species of previous reports, such as Hassan et al. (2010) in *Mimosa pudica* L., Singh et al. (2014) in *Psophocarpus tetragonolobus* (L.) DC., Bianchetti et al. (2017) in *Mimosa pudica* L., Joshi et al. (2021) in *Corchorus capsularis* Salisb, Pieracci et al. (2022) in *Artemisia caerulescens* L. and Custódio et al. (2023) in Halophyte Species.

Rooting induction was observed in 14 different combinations out of 15 combinations tested. Out of the 14 combinations, MPR10 (NAA 1.0 mg/lit) combination is the best combination for *in-vitro* rooting. The media MPR10 able to induce a significantly higher percentage (72.67% explants shows rooting induction) of adventitious rootings, followed by MPR3 (IBA 1.5 mg/lit) media (62.67% explants shows rooting) and the lowest percentage (0) was observed in MPR15 media (IBA-1.5 + IAA-1.5 + NAA 1.5 mg/lit) (Table 2, Fig. 2a to e). Results of the study indicated that single auxins either NAA or IBA were found to be the best hormone for *in-vitro* rooting of *M. pudica*. The obtained results are in agreement with the earlier reports of Hassan et al. (2010) in *Mimosa pudica* L., Shekhawat and Manokari (2015) in *Artemisia absinthium* L., Sharma et al. (2017) in *Bauhinia racemose* Lam. and Osman et al. (2023) in *Lycium barbarum* L. It was also observed that mixed hormonal combinations did not result in any significant rooting induction (Table 2) and these results contrast with Hassan et al. (2010) while working on the same plant species, where they reported BAP + NAA + IAA producing good rootings. Among the tested auxins NAA was the best rooting hormone for the plant which could be used as *ex-vitro* adventitious rooting of the plants where plant cutting is the chief planting material. NAA is also reported as the best rooting hormone which induces adventitious roots in *in-vitro*, *in-vivo* as well as *ex-vitro* conditions in various plant species (Yan et al. 2014; Zhao et al. 2022). In the hardening experiments, about 90% of the transplanted plants of *M. pudica* were survived in the provided greenhouse conditions. After four weeks, the plants were transferred to an open place and gradually acclimated to outdoor conditions, where 70 percent plants survived. The success of adventitious rooting formation in micropropagation protocols is estimated by the success of the acclimatization stage since some species are able to establish in *ex-vitro* conditions even presenting poor *in-vitro* rooting (Sharma et al. 2017; Jafari et al. 2020; Souza et al. 2022). Hence the protocol developed by this investigation is an efficient micro-propagation protocol with profuse multiple shooting and effective rooting with a high percentage of acclimatization success in the naturally field conditions.

The present investigation successfully established an efficient direct organogenesis protocol for *Mimosa pudica* using shoot apical meristem as sources of explant. The protocol has a higher number of healthy plantlets per explant with well-organized rooting and high success of acclimatization in the field. Due to the utilization of apical meristems as explants, all the plants generated from the protocol will be viral disease resistance plants. The protocol is also well suitable for regeneration of planting material after

transformation experiments. As per our knowledge, this is the first time report on an efficient direct organogenesis protocol of *M pudica* which will be suitable for mass multiplication, the protocol should allow the development of transformation protocol for *M. pudica* and have special reference to other plants of the same and related species with regeneration problems.

Declarations

Acknowledgments

The authors are thankful to Dr. Father Jomon Thommana (Director), Dr. Yvonne Fernandes (Principal), CHRIST college, Rajkot for providing the necessary facilities for conducting the research work and for their encouragement throughout the investigation.

Author contribution

KR carried out the investigation, sample collection, methodology; AK conceptualization of the investigation, supervision, writing of original draft; JJ methodology, data curation and analysis; AKB supervision, analyzed the results, correction and editing of the final draft.

Conflict of interest: The authors declare that they have no conflict of interest.

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Figures

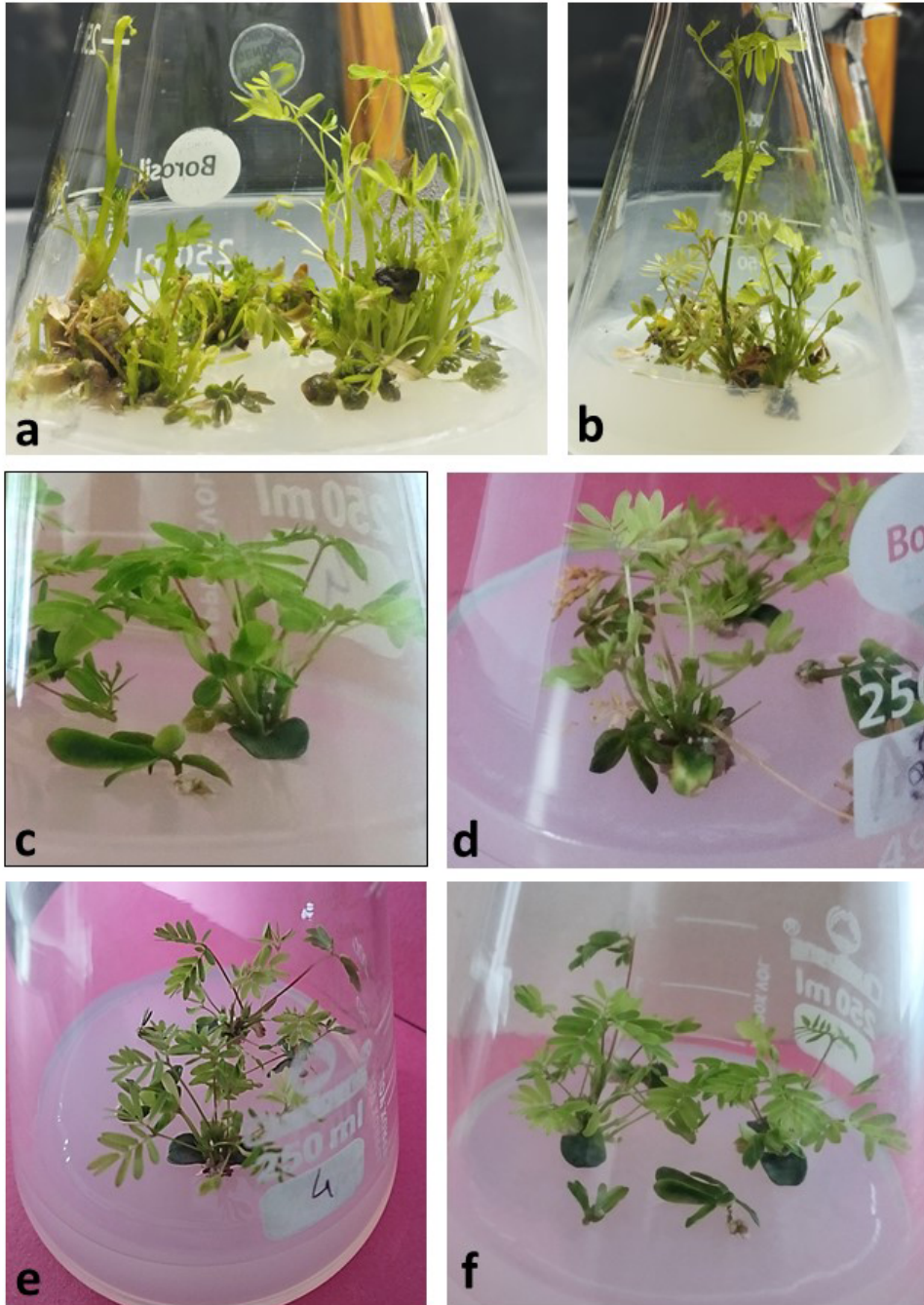


Fig. 1

Figure 1

In-vitro regeneration of *Mimosa pudica* L. from apical meristems. (a) & (b)- profuse multiples shootings generated from apical meristems; (c) to (f): multiple shootings were separated from the mother bunch for establishment of shots for rooting

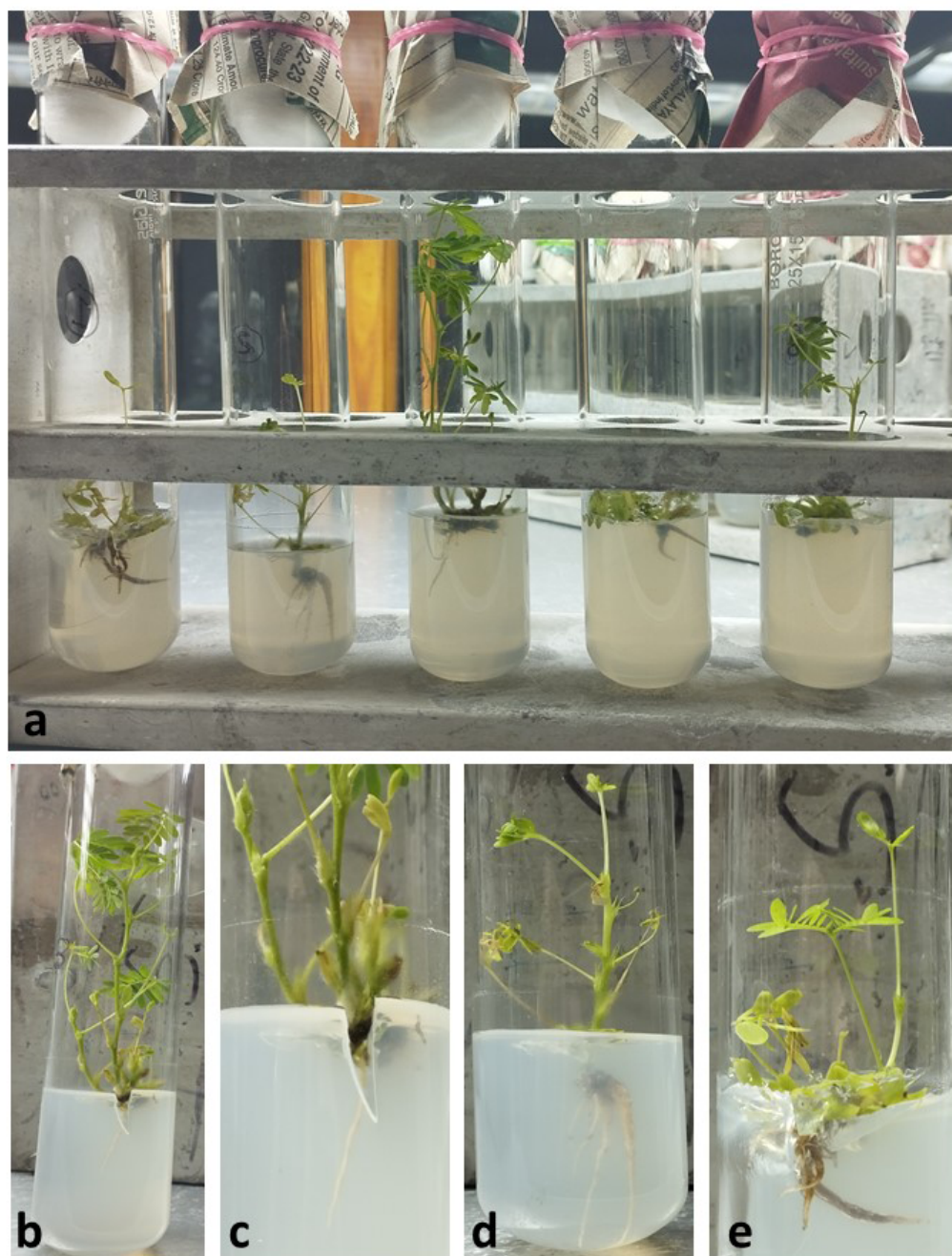


Fig. 2

Figure 2

In-vitro adventitious root generation of *Mimosa pudica* L. from the established shoots. (a) to (e): shoots showing rooting initiation and well developed root elongation.