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# Revisiting *In Vitro* Micropropagation Protocols of *Mimosa pudica* for Enhanced Seed Germination, Shoot Multiplication, and Root Initiation

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## ABSTRACT

*Mimosa pudica* is a medicinal plant worthy of therapeutic properties. It is often overlooked as one of the weed species, and the potential was underappreciated despite its abundance in nature, particularly in tropical climate countries. Considering the aptitude of this species, the micropropagation protocol of *M. pudica* was revisited and enhanced. The seed surface sterilization and germination were assessed, followed by shoot multiplication rate and root initiation efficiency. Seeds of *M. pudica* were best surface sterilized with 35% of Clorox and recorded the highest germination rate at 65.55% in media of three-quarter strength Murashige and Skoog (MS) combined with 30 g of sucrose. The explants were harvested and subcultured into multiplication media fortified with 6-benzylaminopurine, kinetin, and coconut water. The highest number of new shoots and leaves were obtained at 3 shoots and 3.27 cm in media of MS combined with 17.6 µm kinetin. Half-strength MS media devoid of hormone was recommended for root induction, resulting in the 5 healthy roots at 2.06 cm after 4 weeks of culture. Overall, the developed

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micropropagation protocol of *M. pudica* could facilitate its large-scale cultivation, indicating its potential as a medicinal crop for the extraction of bioactive compounds.

*Keywords*: Medicinal weed, micropropagation, *Mimosa pudica*

## INTRODUCTION

*Mimosa pudica* is a medicinal plant known for its sensitive trait in which its leaves fold inward and droop as a form of defensive response. In previous reports, *M. pudica* has been demonstrated to possess promising antioxidant and antidiabetic properties (Baharuddin et al., 2021). The significant amount of phenols and flavonoids in *M. pudica* extract, especially when extracted using optimal solvent extraction techniques, has positioned it as the next valuable medicinal plant species to be regenerated *in vitro*.

Among many advantages of *in vitro*-cultured plants is the ability to synthesize and accumulate many of the same important bioactive compounds as the parent plant in nature (Baskaran et al., 2014). It also enables biosynthetic pathways to be manipulated to boost the synthesis and accumulation of compounds of interest (Dias et al., 2016). The most significant aspect is the capacity to produce a continuous, sustainable, affordable, and viable synthesis of natural substances within a controlled microenvironment (Anand, 2010). However, studies on the protocols of *in vitro* micropropagation of *M. pudica* are limited (Hassan et al., 2010; Ramesh et al., 2013). In general, the medium composition is one of the many factors that influence the success of an *in vitro* micropropagation protocol. The result from the most recent micropropagation study of *M. pudica* by Bianchetti et al. (2017) in the germination part was not reproducible in this present study. To investigate this issue, other germination media components not addressed in the previous study, such as the type and strength of the basal medium and sucrose concentration, were assessed to establish a reproducible *in vitro* germination media for *M. pudica* seeds. Also, an organic additive was tested in the shoot multiplication test, and 2,4-dichlorophenoxyacetic acid (2,4-D) was used in the rooting part of this experiment to fill in the gap in the previous studies.

Therefore, the present study aimed to develop reliable micropropagation protocols for the *in vitro* seed germination, shoot multiplication, and root initiation of *M. pudica*. Through a series of experiments, the effects of media composition were determined: (1) effects of basal medium type, basal medium strengths, and sucrose concentration in response to seed germination, (2) effects of different types of cytokinins and organic additive in response to the shoot multiplication, and (3) effects of Murashige and Skoog (MS) basal medium strength and 2,4-D in response to root initiation of *M. pudica* plantlets.

## MATERIALS AND METHODS

### Surface Sterilization, Seed Germination, and Culture Initiation

Seeds of *M. pudica* were obtained from a single source from Beijing, China, to

ensure uniformity of origin. The seeds were put in a conical flask and washed under running tap water, followed by a Decon 90 (Decon, United Kingdom) wash. The seeds were treated with fungicide (Ancom Thiram 80, Malaysia) containing several drops of Tween-20 (Merck Schuchardt OHG, Germany) for 30 min on a rotary shaker before being rinsed with distilled water. Next, the seeds were transferred to a laminar airflow chamber (BIOBASE, BBS-V800, China) and soaked in 70% ethanol (Systerm Chemicals, Malaysia) for 1 min. Subsequently, the seeds were treated with three different treatments of 35, 70, and 100% Clorox© (contains 5% sodium hypochlorite, NaOCl (Systerm Chemicals, Malaysia) as an active ingredient) for 15 min (Table 1). In each treatment, 100 seeds were used with three replicates. The surface-sterilized seeds were rinsed with sterile distilled water five times before culturing them into media containing MS basal medium. The rate of contamination and germination was recorded after 14 days of culture using equations 1 and 2 (Ahmadi et al., 2012). The appropriate concentration of Clorox© was selected for the subsequent seed germination experiment.

Table 1

Mimosa pudica *seed sterilization treatment steps*

| Treatment no. | Sterilizing agents with exposure time | | | |
|---|---|---|---|---|
| | Step 1 (30 min) | Step 2 (30 min) | Step 3 (1 min) | Step 4 (15 min) |
| 1 | Decon 90 + Teepol | Thiram + Tween 20 | 70% ethanol | 35% Clorox |
| 2 | | | | 70% Clorox |
| 3 | | | | 100% Clorox |

Equation 1:
Contamination percentage (%) = (Number of seeds with microbial growth/Total number of seeds) × 100%

Equation 2:
Germination percentage (%) = (Number of grown seeds/Total number of seeds) × 100%

To assess the effects of media composition on seed germination of *M. pudica*, the surface-sterilized seeds were cultured into two different basal mediums (Murashige and Skoog, MS and Gamborg, B5) at different basal medium strengths (half, three-quarter, and full) with different sucrose (MSM Holdings Berhad, Malaysia) concentrations (15, 30, 45 g/L). The media used were adjusted to a pH of 5.7 using sodium hydroxide (NaOH) (Systerm Chemicals, Malaysia) or hydrochloric acid (HCl) (Systerm Chemicals, Malaysia) and solidified with 3% agar (Gelrite, Sigma Aldrich, USA) before autoclaving at 121°C and 103 kPa for 20 min. The flasks were then sealed with parafilm M® (Bemis Company, USA), and the cultures were incubated in a culture room at 25 ± 2°C, illuminated by white fluorescent light at an intensity of 3,000 lux (OSRAM Licht AG, Germany),

with a photoperiod of 16 hr light and 8 hr darkness. The same condition of the culture room was used for subsequent *in vitro* experiments. Cultures were checked routinely for any contamination. The seed germination percentage, number of shoots, and shoot length were recorded on the 30$^{th}$ day of culture.

### Shoot Multiplication of *M. pudica*

To determine the effects of different types of cytokinin, i.e., 6-benzylaminopurine (BAP) and kinetin (KIN), and coconut water (CW) to shoot multiplication of *M. pudica*, sterile nodal segments of 2.0 – 3.0 cm obtained from culture initiation with decapitated leaves were vertically cultured on MS containing 3% sucrose and 3 g/L of agar with varying concentration of BAP and KIN (2.2, 4.4, 8.8, 17.6, and 35.2 µM) and CW (10, 20, 30, 40, and 50% v/v). CW was acquired from fresh coconuts sold by a local vendor. MS was the control group without adding plant growth hormone or organic additives. The number of new shoots, shoot length, and morphology were visually assessed and recorded after 30 days of culture.

### Root Initiation of *M. pudica*

To evaluate the effects of MS basal medium strength and auxin concentration, which was 2,4-D to root initiation of *M. pudica*, sterile plantlet clusters of about 4 cm having at least 4 shoots were excised and cultured into rooting media treatment with different MS strength (half and full) and varying 2,4-D concentrations (5.33 and 10.66 µM). Days to root emergence, number of roots, root length, and morphology were recorded after 30 days of culture.

### Statistical Analysis

All the experimental data were given as mean ± standard error of the mean and submitted to analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using the Tukey post hoc test ($p$<0.05) using the statistical software program SPSS (version 23).

## RESULTS AND DISCUSSION

### Effect of Different Sterilization Treatments on Contamination and Seed Germination Rate

Surface sterilization of seeds is a crucial step in plant tissue culture since it is necessary not only to disinfect seeds from seed-borne microorganisms but also to ensure they can grow into healthy plantlets. Failure to optimize this step will result in microorganisms competing for nutrients with the growing explants (Tiwari et al., 2012). Furthermore, this procedure is required to eliminate or destroy contaminants while preserving the biological activity of the explants (Felek et al., 2015).

Clorox is a commercial bleach commonly used in sterilization treatments with 5% NaOCl as its active ingredient in an undiluted solution. In the control group (0% Clorox), contamination recorded was 100%, observed in each culture, and the germination rate was 9.67% (Figures 1A and 1B). Furthermore, the 35% Clorox treatment indicated 13.66 and 41.33% contamination and germination rates,

respectively. Contrariwise, the 70% Clorox treatment resulted in a marginally lower contamination rate of 12.33% with a significantly reduced seed germination rate of 27.33% ($p$<0.05). In 100% Clorox, zero contamination was observed, resulting in extremely few germinated seeds. The morphology and survival of explants are known to be affected by sterilizing agent dosages and exposure time. Explants must be disinfected for a specific amount of time and at a specific concentration, as overdosing might result in plant tissue death (Oyebanji et al., 2009). Even though there was no contamination, 100% Clorox was shown to be ineffective for seed surface sterilization of *M. pudina* since it dried out the seeds (Figure 1C) and impaired their propensity to germinate.

The same pattern was observed in the surface sterilization of *Zea mays* seeds, where the combination of 70% ethanol and 10% NaOCl was agreed to be the most effective treatment in eliminating seeds-associated microbes. It resulted in the highest number of germinated seeds with the lowest contamination rate, as compared to the treatments with 10% hydrogen peroxide ($H_2O_2$) or 10% sodium hypochloride (NaOCl) (Davoudpour et al., 2020). Similarly, using 4% NaOCl to surface-sterilize the seeds of *Althaea officinalis* showed the lowest contamination rate compared to using mercuric chloride. The germination percentage recorded was also comparable to the treatment that resulted in the highest germination rate (Younesikelaki et al., 2016).

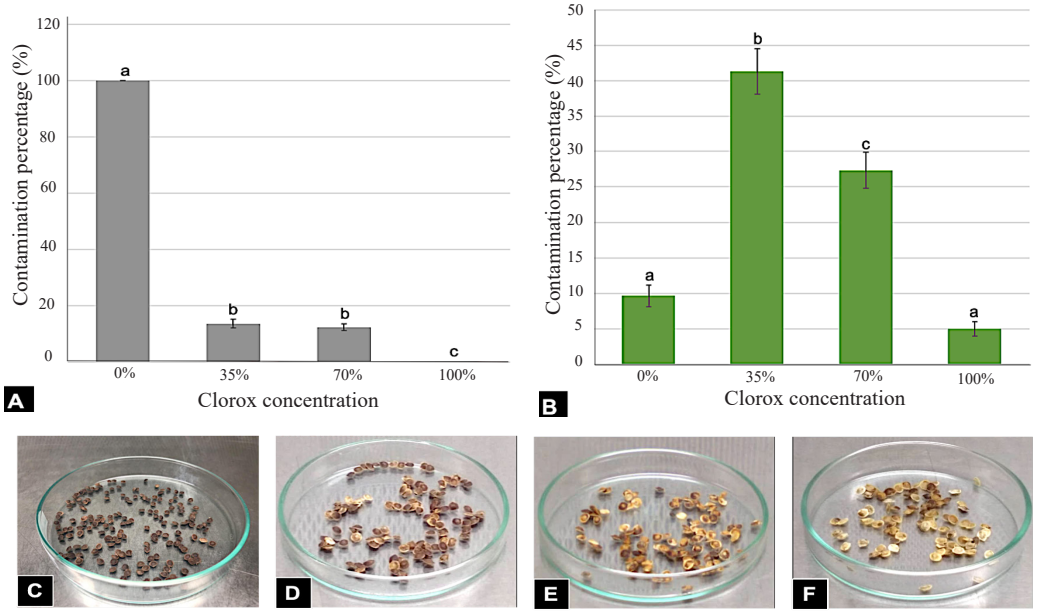


*Figure 1*. A) Contamination rate of *Mimosa pudica* after different sterilization treatments; B) Germination rate of *M. pudica* after different sterilization treatments. Values are expressed as mean ± standard error (n = 3). Different letters indicate statistically significant differences between factors (one-way ANOVA + Tukey post-hoc test at $p$<0.05); C-F) Seeds of *M. pudica* after surface sterilization with different concentrations of Clorox ranging from 0, 35, 70, and 100%, respectively

### Seed Germination of *M. pudica* Seeds and Plantlets Development in Response to Different Medium Composition

Seed germination was recorded starting from day five after inoculation in germination media as the hypocotyls emerged (Figure 2A). Following that, the dicotyledons appeared (Figure 2B), the first true leaves were formed (Figure 2C), and the shoot was established (Figure 2D). The most recent micropropagation study of *M. pudica* by Bianchetti et al. (2017) incorporated a full-strength MS basal medium and recorded 87% germination. In this study, other germination media parameters, such as type and strength of the basal medium and sucrose concentration, need to be assessed to establish a reproducible *in vitro* germination media for *M. pudica* seeds, which were not addressed in the previous study.

Therefore, different media compositions were incorporated in the basal medium, MS and B5, with varied concentrations (i.e., ½, ¾, and full), including sucrose with concentrations ranging from 15, 30, to 45 g/L. The overall results (Table 2) demonstrated that the ½ MS + 15 g/L sucrose recorded the highest percentage of germinated seeds (68.85%), the ¾ MS + 30 g/L sucrose recorded the highest number of new shoots (3.22 cm), and full MS + 15 g/L sucrose recorded the longest shoot length (3.78 cm). In contrast, the full B5 + 45 g/L sucrose recorded the lowest values for all three responses. Therefore, based on the results, the ¾ MS + 30 g/L sucrose was selected as germination media for plantlet production for the subsequent study shoot multiplication study.

All three factors demonstrated statistically significant differences ($p$<0.05) in seed germination percentage. However, only the basal medium type was found significant to the number of new shoots

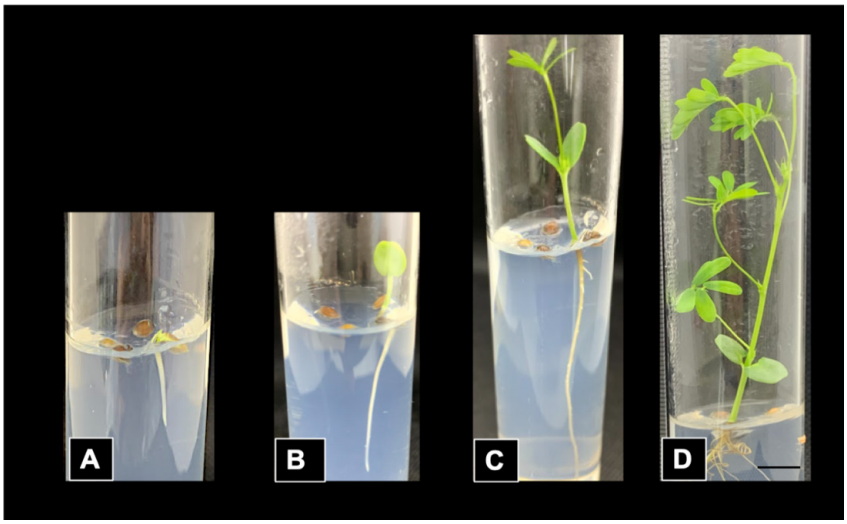


*Figure 2*. Culture initiation of *Mimosa pudica* seed. A) Emergence of hypocotyls; B) Emergence of dicotyledons; C) Formation of first true leaves; D) Formation of shoots

*Note*. Bar = 1.0 cm

formed and the shoot length. In terms of interactions between the factors, overall interactions were significant to germinated seeds percentage. Nevertheless, the factors' interactions had no notable effect on the number of shoots. The same applies to the length of the shoot, except that basal medium strength and sucrose were the only significant interaction factors (Table 3).

Table 2

*The effects of the type and concentration of the basal medium and the sucrose concentration in the germination media on the percentage of seed germination, number of shoots, and shoot length of* M. pudica

| Basal medium | Basal medium strength | Sucrose concentration (g/L) | Germinated seeds (%) | Number of shoots | Shoot length (cm) |
|---|---|---|---|---|---|
| - | Control | - | $50.00 \pm 5.6^{b,c}$ | $2.78 \pm 0^{a}$ | $1.18 \pm 0.2^{b}$ |
| MS | 1/2 | 15 | $68.85 \pm 4.5^{a}$ | $2.22 \pm 1.4^{a}$ | $3.09 \pm 1.3^{a}$ |
| | | 30 | $52.20 \pm 1.1^{a,b,c}$ | $2.11 \pm 0.7^{a}$ | $2.86 \pm 1.3^{a,b}$ |
| | | 45 | $36.70 \pm 1.1^{c,d}$ | $2.11 \pm 0.7^{a}$ | $3.36 \pm 0.7^{a,b}$ |
| | 3/4 | 15 | $47.75 \pm 5.6^{c}$ | $2.61 \pm 0.7^{a}$ | $2.88 \pm 0.8^{a,b}$ |
| | | 30 | $65.55 \pm 3.3^{a,b}$ | $3.22 \pm 1.4^{a}$ | $3.38 \pm 0.6^{a}$ |
| | | 45 | $47.80 \pm 1.1^{c}$ | $2.33 \pm 0.7^{a}$ | $3.68 \pm 1.1^{a,b}$ |
| | Full (1) | 15 | $44.45 \pm 2.3^{c,d}$ | $3.11 \pm 1.4^{a}$ | $3.78 \pm 0.2^{a,b}$ |
| | | 30 | $40.00 \pm 2.2^{c,d}$ | $2.67 \pm 0.7^{a}$ | $3.16 \pm 0.4^{a,b}$ |
| | | 45 | $38.9 \pm 3.3^{c,d}$ | $2.11 \pm 0.7^{a}$ | $3.04 \pm 0.9^{a}$ |
| B5 | 1/2 | 15 | $44.4 \pm 2.2^{b,c}$ | $2.44 \pm 0.8^{a}$ | $2.90 \pm 0.6^{a,b}$ |
| | | 30 | $36.67 \pm 5.6^{c,d}$ | $2.11 \pm 0.7^{a}$ | $3.19 \pm 0.4^{a,b}$ |
| | | 45 | $42.22 \pm 0^{d}$ | $1.78 \pm 0.8^{a}$ | $1.92 \pm 0.7^{a,b}$ |
| | 3/4 | 15 | $28.89 \pm 2.2^{d}$ | $1.67 \pm 0.6^{a}$ | $2.10 \pm 0.8^{a,b}$ |
| | | 30 | $27.78 \pm 1.1^{c,d}$ | $1.44 \pm 0.8^{a}$ | $2.58 \pm 0.9^{a,b}$ |
| | | 45 | $43.33 \pm 1.1^{c,d}$ | $2.22 \pm 1.3^{a}$ | $3.38 \pm 1.1^{a}$ |
| | Full (1) | 15 | $51.11 \pm 2.2^{c,d}$ | $2.67 \pm 1.2^{a}$ | $3.04 \pm 0.6^{a,b}$ |
| | | 30 | $42.22 \pm 2.2^{c,d}$ | $2.11 \pm 1.1^{a}$ | $2.59 \pm 1.1^{a,b}$ |
| | | 45 | $27.78 \pm 1.1^{c,d}$ | $1.44 \pm 1.3^{a}$ | $2.08 \pm 1.4^{a,b}$ |

*Note*. Data were collected and expressed as the mean ± standard error after 30 days of culture of three replicates, in which each replicate contains three explants. The data were tested for statistical differences by three-way ANOVA followed by the Tukey post hoc test. The criterion for significance was set at $p<0.05$. Means that do not share a letter are significantly different

MS = Murashige and Skoog
B5 = Gamborg B5

Table 3

*The* p *value of interactions between factors affecting seed germination of* M. pudica

| Factor's interaction | Response | *p* value |
|---|---|---|
| Basal medium type * Basal medium strength | Germinated seeds | 0.000* |
| Basal medium type * Sucrose | | 0.003* |
| Basal medium strength * Sucrose | | 0.000* |
| Basal medium type * Basal medium strength * Sucrose | | 0.009* |
| Basal medium type * Basal medium strength | Number of shoots | 0.219 |
| Basal medium type * Sucrose | | 0.697 |
| Basal medium strength * Sucrose | | 0.458 |
| Basal medium type * Basal medium strength * Sucrose | | 0.557 |
| Basal medium type * Basal medium strength | Length of shoots | 0.787 |
| Basal medium type * Sucrose | | 0.495 |
| Basal medium strength * Sucrose | | 0.021* |
| Basal medium type * Basal medium strength * Sucrose | | 0.368 |

*Note*. Value with * is significant ($p<0.05$)

MS and B5 have their properties and potential effects on *in vitro* plantlets. Generally, the most notable difference between MS and B5 media is the decreased ammonium/nitrate ($NH_4^+$ / $NO_3^-$) ratio in the B5 medium (Russowski et al., 2006). The saturated nutritional content of full-strength MS might be inappropriate for the small, immature seeds of *M. pudica*, which hinders germination and healthy growth of the plantlets. On the other hand, the low inorganic nutrients of B5 (e.g., calcium and magnesium) might cause nutrient deficiency, thus compromising the germination and growth of seedlings (Li & Zhang, 2018). MS contains high inorganic salts (e.g., nitrate, potassium, and ammonium), facilitating plant nutrient uptake and development (Koné et al., 2015). This study supports evidence from previous observations on *Dendrobium hookerianum* seeds (Paul et al., 2012), where the highest percentage of germination and plantlets growth was recorded in those of MS medium. Similar to the case of embryo germination of *Juglans regia*, the MS medium demonstrated outstanding proliferation from the embryonic axis compared to the other media (Sánchez-Zamora et al., 2006). According to these data, it can be generally inferred that MS basal medium is more suitable than B5 for micropropagation of *M. pudica*.

Regarding the MS basal medium strength, the results confirmed that high MS strength was unnecessary to improve overall seed germination. This observation might be attributed to the increased quantity of a certain nutritional salt, which inhibits the plant's ability to absorb other mineral nutrients. The ½ MS resulted in the highest

germination percentage, whereas full MS resulted in a 24% lower germination rate. The finding is inconsistent with those observed on *Vanilla planifolia* by Jing (2016), in which seed germination performed better in a full-strength MS. Nevertheless, fewer shoots were recorded in the same media at 2.22 shoots per explant and a reduced shoot length at 3.09 cm. This result indicated that a low macro- and micronutrient concentration in the culture media might promote germination but not plantlet development. No significant difference was detected between the different strengths of MS in response to the number of new shoots of each plantlet and the shoot length, which exhibited a similar trend to a study on *Vigna subterranea* by Koné et al. (2015). Thus, the ¾ strength or 3.3 g/L of MS is sufficient for germinating *M. pudica* seeds.

Plantlets grown in culture vessels are called semi-autotrophic plants since their leaves may not acquire photosynthetic competency due to a lack of carbon dioxide (Hazarika, 2003). Therefore, a carbohydrate source like sucrose is commonly added to provide a sufficient supply of carbon for *in vitro* multiplication and growth of plant cells, tissues, organs, and entire plantlets. Carbon sources are also required as osmotic agents in the culture medium, which can alter cell physiology, proliferation, and differentiation (Gibson, 2000). Different carbohydrate sources can affect the development of plantlets in different ways. Improper type and concentration selection could cause morphogenesis to be delayed and physiological diseases like vitrification to develop. Sucrose has been widely used as a carbon source in most studies involving *in vitro* shoot induction and development, root induction, callogenesis, embryogenesis, and regeneration (Yaseen et al., 2012). Sucrose is a significant carbon and energy source in plant tissue culture since it is the most frequent carbohydrate in phloem sap and regulates many development stages (Zahara et al., 2016). The high solubility in water, electrical neutrality, and lack of inhibitory effect on most metabolic processes are all linked to sucrose's favorable effects on explant growth *in vitro* (Liu et al., 2006). In this study, sucrose concentration was found significant to the percentage of germinated seeds of *M. pudica*, although it did not significantly affect the plantlets' development in terms of the number of new shoots and length. The highest seed germination percentage was observed from the ½ MS + 15 g/L of sucrose, while higher sucrose concentrations up to 45 g/L resulted in a lower germination rate. At high sucrose levels, seed germination and plantlet development were stunted, implying that the seeds were either subjected to osmotic stress at greater molarities or that sucrose hydrolysis products produced during autoclaving hampered their growth and development (Johnson et al., 2011).

Koné et al. (2015) reported that the plant height and biomass of *Bambara* groundnuts were increased when the sucrose content was increased from 1 to 3%. However, there was no significant variation in the number of leaves and root length among the different

sucrose concentrations examined. Studies on *Ruscus hypoglossum* and *Cornus alba* also found similar results (Ilczuk & Jacygrad, 2016). A high sucrose concentration also hindered orchid plantlets' growth, particularly the shoot and root numbers (Wotavová-Novotná et al., 2007). In *Drosera intermedia*, maximum shoot length and shoot number were reported on liquid medium containing 10 and 20 g/L sucrose, respectively (Rejthar et al., 2014). On the contrary, *Abelmoschus esculentus* did not thrive in a medium containing 10 and 15 g/L sucrose, where the explants displayed shoot tip necrosis and could not produce leaves. This finding indicated that lowering sucrose content influenced cellular division directly owing to a lack of carbon supply and energy, resulting in poor shoot regeneration and growth (Woldeyes et al., 2021). The contrasting difference is due to the variation in genotype, growth regulators, and culture conditions. In the case of *M. pudica* seeds, 3% sucrose concentration in the basal medium, equivalent to 30 g/L, is sufficient for normal plant growth.

## Shoot Multiplication of *M. pudica* in Response to Different Plant Growth Hormone and Organic Additives at Different Concentrations

Nodal segments of *M. pudica* obtained from germinated seeds cultured in germination media were inoculated in media of MS basal medium supplemented with different concentrations of exogenous cytokinin hormones (i.e., BAP and KIN) and organic additive CW. The results revealed that plant growth hormone or additive types and the interaction with the concentration had statistically significant differences ($p<0.05$) to shoot multiplication, determined by shoot number and shoot length. However, the difference in plant growth hormone or additive concentration alone was significant only to shoot number but not to the shoot length. Based on Table 4, media supplemented with 35.2 µM KIN exhibited the highest shoot number formed (4.87), which was two-fold higher as compared to MSO (control). MSO is Murashige and Skoog only with no plant growth regulator added. All concentrations of CW treatments and 17.6 µM and 35.2 µM BAP treatments recorded the lowest shoot number of below 2. Treatments with 2.2 µM BAP, 35.2 µM BAP and 35.2 µM KIN produced more than four shoots per explant. While all CW treatments yielded the longest shoot length of over 3 cm, they produced a relatively small number of shoots. The shortest shoot of 0.32 cm was exhibited by 35.2 µM BAP.

Leaves production and basal callus formation were visually assessed and scored as – (absent), + (low), ++ (moderate), and +++ (high). MSO (control) and 17.6 µM BAP produced moderate leaves (++). A low concentration of BAP resulted in shoots with leaves, but leaves were not produced at a concentration of 17.6 µM and above. As for KIN, at 17.6 µM, leaves produced were twice as produced in BAP and coconut water, but at other concentrations, leaves were minimal (+) or absent (-). CW, at all concentrations, consistently produced

Table 4

*Effects of types of plant growth regulators (PGR), additive, and their concentrations on shoot multiplication of* M. pudica

| Plant growth regulator | Concentration | Number of shoots | Shoot length (cm) | Leaves | Initiated callus |
|---|---|---|---|---|---|
| MSO (Control) | - | $2.33 \pm 1.4^{a,b,c,d,e}$ | $2.89 \pm 2.0^{a,b,c}$ | ++ | - |
| BAP (µM) | 2.2 | $4.07 \pm 0.96^{a,b}$ | $3.06 \pm 0.4^{a,b,c}$ | + | ++ |
| | 4.4 | $2.87 \pm 1.6^{b,c,d}$ | $2.17 \pm 1.2^{b,c}$ | + | ++ |
| | 8.8 | $3.8 \pm 2.3^{a,b}$ | $3.2 \pm 1.4^{a,b,c}$ | + | - |
| | 17.6 | $1.67 \pm 1.0^{d,e}$ | $2.07 \pm 0.9^{c}$ | - | - |
| | 35.2 | $0.07 \pm 0.3^{e}$ | $0.32 \pm 1.2^{d}$ | - | - |
| KIN (µM) | 2.2 | $4.0 \pm 1.1^{a,b}$ | $3.18 \pm 0.9^{a,b,c}$ | + | + |
| | 4.4 | $3.53 \pm 1.3^{a,b,c}$ | $3.09 \pm 1.0^{a,b,c}$ | - | ++ |
| | 8.8 | $3.93 \pm 2.1^{a,b}$ | $2.79 \pm 1.0^{a,b,c}$ | + | - |
| | 17.6 | $3.6 \pm 2.3^{a,b,c}$ | $3.27 \pm 1.0^{a,b,c}$ | ++ | + |
| | 35.2 | $4.87 \pm 1.2^{a}$ | $3.65 \pm 0.9^{a,b}$ | - | + |
| CW (%) | 10% | $1.27 \pm 0.7^{d,e}$ | $3.43 \pm 1.1^{a,b,c}$ | + | - |
| | 20% | $1.27 \pm 1.0^{d,e}$ | $3.8 \pm 1.8^{a}$ | + | - |
| | 30% | $1.73 \pm 0.8^{d,e}$ | $3.98 \pm 1.5^{a}$ | + | - |
| | 40% | $1.93 \pm 0.7^{c,d}$ | $3.1 \pm 1.6^{a,b,c}$ | + | - |
| | 50% | $1.67 \pm 0.6^{d,e}$ | $4.2 \pm 0.8^{a}$ | + | - |

*Note*. Data were collected and expressed as the mean ± standard error after 30 days of culture. The data were tested for statistical differences by two-way ANOVA followed by the Tukey post hoc test. The criterion for significance was set at $p<0.05$. Means that do not share a letter are significantly different. Leaves and basal callus rating: – (absent), + (low), ++ (moderate), and +++ (high)

MSO = Murashige and Skoog without plant growth regulator
BAP = 6-benzylaminopurine
KIN = kinetin
CW = coconut water

shoots with a small number of leaves. Basal callus formation was observed in plantlets treated with 2.2 - 4.4 µM BAP (Figure 3C) and all concentrations of KIN except for one at 8.8 µM while MSO (control) and CW exhibited zero basal callus. The basal callus is a common issue in most plants during shoot induction; however, the severity varies by species. It was most likely caused by a disruption in endogenous hormones in the tissue, as a common physiological stress reaction. In several cases, callus is formed due to the accumulation of endogenous auxin at the wounded parts of the plants (Fehér, 2019). Above all, MS basal medium fortified with 17.6 µM KIN (Figure 3A) was selected as the best media for shoot multiplication of *M. pudica* due to the satisfactory shoot number and shoot length, a moderate amount of leaves with low basal callus formation.

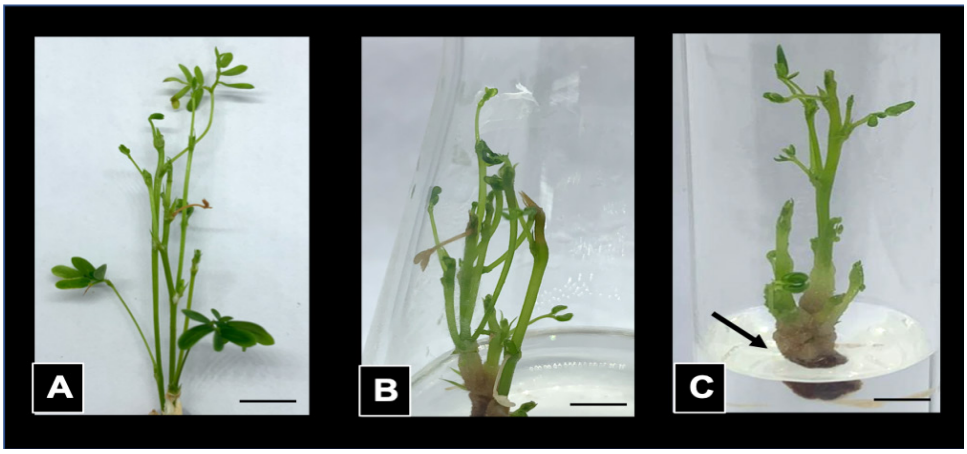


*Figure 3*. Shoot multiplication of *Mimosa pudica*. A) Shoot multiplication in media fortified with 17.6 µM KIN; B) Shoot multiplication in media fortified with 35.2 µM KIN; C) Shoot multiplication in media fortified with 4.4 µM BAP, the arrow pointing to formed basal callus

*Note*. Bar = 1.0 cm
KIN = kinetin
BAP = 6-benzylaminopurine

Consistent with the previous observation by Bianchetti et al. (2017), the highest number of shoots recorded in 5 µM BAP was 5 shoots/explant, which was just marginally higher than those in the current study (4.87 shoots/explant in 35.2 µM KIN). It was also reported that increasing BAP concentration hindered shoot number and elongation, with plantlets reaching an average height of 2.5 cm, which was lower than the shoot length achieved in this study, which was over 3 cm. On the contrary, they found that supplementing *M. pudica* shoots with KIN did not significantly increase their proliferation with no significant differences compared to the control. In addition, supplementing with 1-naphthaleneacetic acid (NAA) and BAP did not affect shoot elongation, demonstrating a negative relationship between multiplication rate and shoot length.

The cytokinin signaling system involves coordinating three types of proteins: histidine kinase receptors for signal perception, histidine phosphotransfer proteins for the signal relay, and response regulators for signal output (Keshishian & Rashotte, 2015). When the signal is relayed to the nucleus, it will be conveyed to two types of gene expression regulators that govern the plant's developmental stages. The signal receptor perceives different types of cytokinin differently, which explains the differences in plant response even though it comes from the same group of PGR (Schepetilnikov & Ryabova, 2017). Plant growth hormone supplementation in cultures causes the transcription of specific genes that modulate each stage of plant growth. When the groups of genes responsible for a particular plant growth process are identified, the media culture can

be better optimized, promoting the defined hormones with their effective concentration to influence gene expression throughout the development process (Jiang & Asami, 2018).

Cytokinin is commonly used in the induction and proliferation stages of explants. Aside from PGR, organic additives like CW, apple juice, tomato juice, and peptone may help plant tissues grow and develop (Gupta, 2016; Kaur & Bhutani, 2016; Prando et al., 2014). CW contains sugars, amino acids, vitamins, enzymes, and organic acids, as well as endogenous cytokinin that promotes explant development and regeneration by stimulating cell division (Ge et al., 2005). When plant portions are excised into smaller sizes, the production of cytokinin in plants is restricted owing to the damage (Gallavotti, 2013). Therefore, in the present study, cytokinin was added to the basal media to stimulate shoot induction, cell division, and cell differentiation. In the case of *M. pudica* plantlets, this study revealed that KIN was the most suitable cytokinin option for its shoot multiplication and proliferation.

On the contrary, previous research on *Justicia gendarussa* found that supplementing BAP in MS basal medium resulted in the highest percentage of shoot induction, the number of shoots, and shoot length compared to KIN and thidiazuron (TDZ) (Janarthanam et al., 2011). The same finding was observed in *Clinacanthus nutans*, in which 12 µM BAP was more effective than the other cytokinins in the early stages of shoot induction (Haida et al., 2020). Moreover, comparable research on *Clitoria ternatea* and *Pisum sativum*, which belong to the same family as *M. pudica*, reported that the proportion of shoot regeneration and the number of shoots increased when BAP was incorporated into the culture media (Das et al., 2014; Mukhtar et al., 2010).

According to this study, CW supplementation in *M. pudica* culture media did not have a significant effect on the number of shoots, but it did have a significant effect on shoot length. It accords with a previous report (Buah & Agu-Asare, 2014), which revealed that CW treatment only exhibited 25 shoots on the Cavendish banana plant compared to 37 shoots by BAP. Nevertheless, the banana plantlet's height was 3 cm longer in CW treatment compared to BAP. Conversely, other species like *Pomacentrus amboinensis* thrived in a medium fortified with CW in terms of root elongation, leaf size, and plantlet elongation (Utami & Hariyanto, 2019). The same trend was observed in plantlets of *Renanthera imschootiana*, in which 10% (v/v) of CW supplementation in ¼ MS enhanced the growth of the protocorms (Wu et al., 2014). The same pattern was seen in *in vitro* cultivated olive embryos, where CW supplementation enhanced the number of leaves but did not affect shoot length or other features (de Souza et al., 2013). The discrepancies in plantlet responses could be related to the CW treatment's reaction being relatively species-specific (Villa et al., 2010).

Leaves are the most critical organ to develop to initiate rooting. A classical study

of *in vitro* rooting on avocado cuttings revealed that the number of leaves preserved on the cuttings was linked with the number of root plantlets. The role of leaves in ensuring the continuation of photosynthesis has resulted in a build-up of carbohydrates at the cuttings' base (Reuveni & Raviv, 1980). In *Plectranthus scutellarioides*, longer roots were produced at stem cuttings with apical leaves, as compared to leafless stem cuttings. The correlation between the presence of leaves and rooting performance was due to the leaves providing reserves, hormones, and co-factors that stems alone lack sufficient quantity (Belniaki et al., 2018). The same behavior was observed in bitter melon cuttings, where leaf-on cuttings had a major effect on roots, with rooting remaining extremely modest even when the incubation time was increased to 10 days. While bitter melon failed to root on leafless cuttings even with auxin hormone treatment, sweet potato cuttings can root easily without leaves (Malik et al., 2012). A moderate 17.6 µM KIN supplementation was deemed the most suitable shoot multiplication media for *M. pudica*. After that, it was used for the next *in vitro* rooting study.

## Rooting Initiation of *M. pudica* in Response to Different MS Strength and Auxin Hormone, 2,4-D Concentrations

The effect of MS basal medium strength and supplementation of exogenous auxin hormone 2,4-D on *in vitro* rooting of *M. pudica* was evaluated. Based on Table 5, only two treatments, ½ and full MS were shown to respond with rooting, both of which were devoid of 2,4-D addition. Other treatments supplemented with 2,4-D resulted in no rooting response, and gall formation was observed at the basal part (Figure 4B). The emergence of roots in plantlets in ½ MS media was recorded after 5.93 days; for full MS, they took 7.27 days to root. In addition, the number of roots produced in ½ MS was higher (5.13) as compared to full MS (1.47). However, both treatments exhibited the same root length of 2.06 cm.

Table 5

*Effects of Murashige and Skoog (MS) strength and 2,4-dichlorophenoxyacetic acid* (*2,4-D) concentration on* in vitro *rooting of* M. pudica

| MS strength | 2,4-D (µM) | Days to root | Number of roots | Length of root (cm) | Morphology of root |
|---|---|---|---|---|---|
| ½ | - | 5.93 ± 3.28[a] | 5.13 ± 2.61[a] | 2.06 ± 1.41[a] | Thin, long |
| ½ | 5.33 | - | 0 | 0 | Gall formed |
| ½ | 10.66 | - | 0 | 0 | Gall formed |
| Full | - | 7.27 ± 2.87[a] | 1.47 ± 0.83[b] | 2.06 ± 1.41[a] | Thick, rotten |
| Full | 5.33 | - | 0 | 0 | Gall formed |
| Full | 10.66 | - | 0 | 0 | Gall formed |

*Note*. Data were expressed as the mean ± standard error of fifteen replicates. The data were tested for statistical differences by two-way ANOVA followed by the Tukey post hoc test. The criterion for significance was set at $p<0.05$. Means that do not share a letter are significantly different

The roots of *M. pudica* in ½ MS treatment appeared healthy, thin, and long (Figure 4A), while in full MS, the single root that emerged were thick, and some were observed rotten (Figure 4 C). It may be inferred that adding 2,4-D did not help the rooting initiation of *M. pudica*; hence PGR incorporation was unnecessary. Additionally, ½ MS is sufficient for rooting since full MS resulted in low rooting efficacy, with only 1–2 thick and rotting roots emerging, compared to a higher number of healthy roots in ½ MS.

These results reflect those of Bianchetti et al. (2017), who found that no significant difference was observed between control and NAA-fortified treatments of *M. pudica*, indicating that this PGR did not promote rooting regardless of the concentration. Since there has been little research on 2,4-D as a source of auxin in *M. pudica* rooting, it was examined in this study.

Even when auxins are present in culture media, excessive salt concentrations in basal medium might hamper root development. Therefore, it is necessary to reduce macro- and micronutrient concentrations to half their normal levels during the rooting phase of most plant species (Woldeyes et al., 2021). Similar observations were recorded in *Ruscus hypoglossum* (Dahab et al., 2005), *Drosera intermedia* (Rejthar et al., 2014), and *Abelmoschus esculentus* (Rizwan et al., 2018) in which ½ strength MS media produced the highest root number and length. However, at a much lower concentration of MS (¼ MS strength), the least weak root development and proliferation were recorded in *Abelmoschus esculentus* (0.70 root number and 0.54 cm root length).


*Figure 4*. Root initiation of *Mimosa pudica*. A) Plantlet in rooting media fortified with ½ MS void of 2,4-D; B) Plantlet in rooting media fortified with ½ MS + 1.0 mg/L 2,4-D, an arrow pointing to rotten gall formed; C) Plantlet in rooting media fortified with full MS void of 2,4-D

*Note*. Bar = 1.0 cm
MS = Murashige and Skoog
2,4-D = 2,4-dichlorophenoxyacetic acid

When 2,4-D is administered at effective concentrations to dicotyledonous plants, it will be absorbed by the roots, stems, and leaves and translocated to the plant's meristems (Munro et al., 1992). As a result, the roots thicken and become stunted, stem phloem and xylem tissue disintegrate, and leaf development ceases, followed by stem curling, leaf withering, and eventually plant death (Grossmann, 2009; Song, 2013). The molecular mode of action of auxin is administered by the auxin receptor residing at the plasma membrane, which involves an amino acid permease-like protein that mediates auxin inflow (Swarup et al., 2008), efflux carriers that mediate auxin outflow and ATP-binding cassette, which serve as auxin transporters (Petrásek et al., 2006). The structure or size of their auxin binding pockets on the receptor, which is regulated by its aromatic ring size, may explain the difference in the effectiveness of various auxin types on the plants (Calderon-Villalobos et al., 2010).

According to observations in vine rootstock cuttings, 2,4-D did not stimulate the formation of adventitious roots or sprouting. However, it did reduce mortality, attributed to the stimulation of ethylene biosynthesis, which promoted senescence in various tissues (Tofanelli et al., 2014). The rooting of *Lippia* species cuttings treated for 24 hr with IBA, NAA, or 2,4-D showed that 2,4-D did not promote the formation of adventitious roots and that 100% of the treated cuttings died (Pimenta et al., 2007), which recorded similar response to this study. Despite this, 2,4-D is a key synthetic plant regulator, acting similarly to auxins in promoting lateral roots and preventing root stretching (Simon & Petrášek, 2011). On the one hand, 2,4-D might produce harmful metabolic and biochemical alterations in nucleic acids, cell wall flexibility, and the function of the RNA-polymerase enzyme causing excessive cell proliferation in tissues, in stem epinasty and phloem disruption (Tofanelli et al., 2014). It can be deduced that *M. pudica* can root without supplementing exogenous auxins such as IBA, NAA, and 2,4-D.

## CONCLUSION

This study outlined a procedure for seed germination, shoot multiplication, and root initiation of *in vitro* micropropagation of the undervalued medicinal plant, *M. pudica*. The best basal medium for seed germination and culture initiation was ¾ MS supplemented with 30 g/L of sucrose. MS fortified with 35.2 µM of kinetin was the most satisfactory treatment for shoot multiplication. Roots of *in vitro M. pudica* were effectively initiated in ½ strength of MS devoid of any plant hormone. The impact of other main media components not explored in this study could improve the culture medium. Further research is warranted to gauge the adaptability of the *in vitro* plantlets to the environment. Overall, *in vitro* culture is a sustainable alternative to harvesting wild plants for their medicinal properties and an appealing approach that may provide sufficient preliminary materials for the large-scale cultivation of medicinal plants.

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## REFERENCES

Ahmadi, E., Nasr, S. M. H., Jalilvand, H., & Savadkoohi, S. K. (2012). Contamination control of microbe *Ziziphus spina* [christti] seed *in vitro* culture. *Trees*, *26*, 1299-1304. https://doi.org/10.1007/s00468-012-0705-8

Anand, S. (2010). Various approaches for secondary metabolite production through plant tissue culture. *Pharmacia*, *1*(1), 1–7.

Baharuddin, N. S., Roslan, M. A. M., Bawzer, M. A. M., Mohamad Azzeme, A., Rahman, Z. A., Khayat, M. E., Rahman, N. A. A., & Sobri, Z. M. (2021). Response surface optimization of extraction conditions and *in vitro* antioxidant and antidiabetic evaluation of an under-valued medicinal weed, *Mimosa pudica*. *Plants*, *10*(8), 1692. https://doi.org/10.3390/plants10081692

Baskaran, P., Moyo, N., & Staden, J. V. (2014). *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. *South African Journal of Botany*, *90*, 74–79. https://doi.org/10.1016/j.sajb.2013.10.005

Belniaki, A. C., Rabel, L. A. D. N., Gomes, E. N., & Zuffellato-Ribas, K. C. (2018). Does the presence of leaves on coleus stem cuttings influence their rooting? *Ornamental Horticulture*, *24*(3), 206–210. https://doi.org/10.14295/oh.v24i3.1204

Bianchetti, R. E., de Resende, C., F., Pacheco, V. S., Dornellas, F. F., de Oliveira, A. M. S., Freitas, J. C. E., & Peixoto, P. H. P. (2017). An improved protocol for *in vitro* propagation of the medicinal plant *Mimosa pudica* L. *African Journal of Biotechnology*, *16*(9), 418–428. https://doi.org/10.5897/AJB2016.15831

Buah, J. N., & Agu-Asare, P. (2014). Coconut water from fresh and dry fruits as an alternative to BAP in the *in vitro* culture of dwarf cavendish banana. *Journal of Biological Science*, *14*(8), 521-526. https://doi.org/10.3923/jbs.2014.521.526

Calderon-Villalobos, L. I., Tan, X., Zheng, N., & Estelle, M. (2010). Auxin perception - Structural insights. *Cold Spring Harbor Perspectives in Biology*, *2*(7), a005546. https://doi.org/10.1101/cshperspect.a005546

Dahab, A., Habib, A. M. A., Hosni, Y. A., & Gabr, A. M. M. (2005). Effect of MS-salt strength, sucrose and IBA concentration and acclimatization media on *Ruscus hypoglossum* L. micropropagation. *Arab Journal of Biotechnology*, *8*(1), 141–154.

Das, A., Kumar, S., Nandeesha, P., Yadav, I. S., Saini, J., Chaturvedi, S. K., & Datta, S. (2014). An efficient in vitro regeneration system of fieldpea (*Pisum sativum* L.) via shoot organogenesis. *Journal of Plant Biochemistry and Biotechnology*, *23*, 184–189. https://doi.org/10.1007/s13562-013-0200-3

Davoudpour, Y., Schmidt, M., Calabrese, F., Richnow, H. H., & Musat, N. (2020). High resolution microscopy to evaluate the efficiency of surface sterilization of *Zea mays* seeds. *PLOS One*, *15*(11), e0242247. https://doi.org/10.1371/journal.pone.0242247

de Souza, R. A. V., Braga, F. T., Setotaw, T. A., Neto, J. V., de Azevedo, P. H., de Azevedo, V. H., & de Almeida Cançado, G. M. (2013). Effect of coconut water on growth of olive embryos cultured *in vitro*. *Ciência Rural*, *43*(2), 290–296. https://doi.org/10.1590/S0103-84782013000200016

Dias, M. I., Sousa, M. J., Alves, R. C., & Ferreira, C. F. R. (2016). Exploring plant tissue culture to improve the production of phenolic compounds: A review. *Industrial Crops and Products*, *82*, 9-22. https://doi.org/10.1016/j.indcrop.2015.12.016

Fehér, A. (2019). Callus, dedifferentiation, totipotency, somatic embryogenesis: What these terms mean in the era of molecular plant biology? *Frontiers in Plant Science*, *10*, 536. https://doi.org/10.3389/fpls.2019.00536

Felek, W., Mekibib, F., & Admassu, B. (2015). Optimization of explants surface sterilization condition for field grown peach (*Prunus persica* L. Batsch. Cv. Garnem) intended for *in vitro* culture. *African Journal of Biotechnology*, *14*(8), 657–660. https://doi.org/10.5897/ajb2014.14266

Gallavotti, A. (2013). The role of auxin in shaping shoot architecture. *Journal of Experimental Botany*, *64*(9), 2593–2608. https://doi.org/10.1093/jxb/ert141

Ge, L., Yong, J. W. H., Goh, N. K., Chia, L. S., Tan, S. N., & Ong, E. S. (2005). Identification of kinetin and kinetin riboside in coconut (*Cocos nucifera* L.) water using a combined approach of liquid chromatography-tandem mass spectrometry, high performance liquid chromatography and capillary electrophoresis. *Journal of Chromatography*, *829*(1-2), 26–34. https://doi.org/10.1016/j.jchromb.2005.09.026

Gibson, S. I. (2000). Plant sugar-response pathways. Part of a complex regulatory web. *Plant Physiology*, *124*(4), 1532–1539. https://doi.org/10.1104/pp.124.4.1532

Grossmann, K. (2009). Auxin herbicides: Current status of mechanism and mode of action. *Pest Management Science*, *66*(2), 113-120. https://doi.org/10.1002/ps.1860

Gupta, A. (2016). Asymbiotic seed germination in orchids: Role of organic additives. *International Advanced Research Journal in Science, Engineering and Technology*, *3*(5), 143–147. https://doi.org/10.17148/IARJSET.2016.3530

Haida, Z., Nakasha, J. J., & Hakiman, M. (2020). *In vitro* responses of plant growth factors on growth, yield, phenolics content and antioxidant activities of *Clinacanthus nutans* (Sabah snake grass). *Plants*, *9*(8), 1030. https://doi.org/10.3390/plants9081030

Hassan, A. K. M. S., Sultana, R., Jahan, M. A. A., & Khatun, R. (2010). *In vitro* mass propagation of *Mimosa pudica* L., using shoot tip and nodal explants. *Bangladesh Journal of Scientific and Industrial Research*, *45*(2), 95–100. https://doi.org/10.3329/bjsir.v45i2.5704

Hazarika, B. N. (2003). Acclimatization of tissue-cultured plants. *Current Science*, *85*(12), 1704–1712. https://doi.org/10.17660/ActaHortic.1992.314.13

Ilczuk, A., & Jacygrad, E. (2016). *In vitro* propagation and assessment of genetic stability of acclimated plantlets of *Cornus alba* L. using RAPD and ISSR markers. *In Vitro Cellular and Developmental Biology - Plant*, *52*, 379–390. https://doi.org/10.1007/s11627-016-9781-6

Janarthanam, B., Gayathri, B., & Sumathi, E. (2011). A rapid, high frequency regeneration of *Justicia gendarussa* Burm.f. *Bangladesh Journal of Scientific and Industrial Research*, *46*(2), 201–204. https://doi.org/10.3329/bjsir.v46i2.4388

Jiang, K., & Asami, T. (2018). Chemical regulators of plant hormones and their applications in basic research and agriculture. *Bioscience, Biotechnology, and Biochemistry*, *82*(8), 1265-1300. https://doi.org/10.1080/09168451.2018.1462693

Jing, G. F. (2016). *Effects of selected additives for growth efficiency on in vitro grown* Vanilla planifolia *Andrews* [Master's thesis, Universiti Sains Malaysia]. Repository@USM. http://eprints.usm.my/31559/1/GOH_FOONG_JING_24.pdf

Johnson, T. R., Kane, M. E., & Pérez, H. E. (2011). Examining the interaction of light, nutrients and carbohydrates on seed germination and early seedling development of *Bletia purpurea* (Orchidaceae). *Plant Growth Regulator*, *63*, 89–99. https://doi.org/10.1007/s10725-010-9516-3

Kaur, S., & Bhutani, K. K. (2016). Asymbiotic seed germination and multiplication of an endangered orchid – *Paphiopedilum venustum* (Wall. ex Sims.). *Acta Societatis Botanicorum Poloniae*, *85*(2), 3494. https://doi.org/10.5586/asbp.3494

Keshishian, E. A., & Rashotte, A. M. (2015). Plant cytokinin signalling. *Essays in Biochemistry*, *58*, 13–27. https://doi.org/10.1042/bse0580013

Koné, M., Koné, T., Silué, N., Soumahoro, A. B., & Kouakou, T. H. (2015). *In vitro* seeds germination and seedling growth of bambara groundnut (*Vigna subterranea* (L.) Verdc. (Fabaceae)). *The Scientific World Journal*, *2015*, 595073. https://doi.org/10.1155/2015/595073

Li, H., & Zhang, D. (2018). *In vitro* seed germination of *Kalmia latifolia* L. hybrids: A means for improving germination and speeding up breeding cycle. *American Society for Horticultural Science*, *53*(4), 535–540. https://doi.org/10.21273/HORTSCI12829-17

Liu, T.-H. A., Lin, J.-J., & Wu, R.-Y. (2006). The effects of using trehalose as a carbon source on the proliferation of *Phalaenopsis* and *Doritaenopsis* protocorm-like-bodies. *Plant Cell, Tissue and Organ Culture*, *86*, 125–129. https://doi.org/10.1007/s11240-006-9092-4

Malik, N. S. A., Perez, J. L., & Kunta, M. (2012). The effect of leaf presence on the rooting of stem cutting of bitter melon and on changes in polyamine levels. *Agricultural Sciences*, *3*(7), 936–940. https://doi.org/10.4236/as.2012.37114

Mukhtar, S., Anis, M., & Ahmad, N. (2010). *In vitro* optimization of phytohormones on micropropagation in butterfly pea (*Clitoria ternatea* L.). *Journal of Herbs, Spices and Medicinal Plants*, *16*(2), 98–105. https://doi.org/10.1080/10496475.2010.499310

Munro, I. C., Carlo, G. L., Orr, J. C., Sund, K. G., Wilson, R. M., Kennepohl, E., Lynch, B. S., & Jablinske, M. (1992). A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. *Journal of the American College of Toxicology*, *11*(5), 559–664. https://doi.org/10.3109/10915819209141893

Oyebanji, O. B., Nweke, O., Odebunmi, O., Galadima, N. B., Idris, M. S., Nnodi, U. N., Afolabi, A. S., & Ogbadu, G. H. (2009). Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. *African Journal of Biotechnology*, *8*(20), 5395–5399. https://doi.org/10.5897/AJB09.923

Paul, S., Kumaria, S., & Tandon, P. (2012). An effective nutrient medium for asymbiotic seed germination and large-scale *in vitro* regeneration of *Dendrobium hookerianum*, a threatened orchid of northeast India. *Annals of Botany PLANTS*, *2012*, plr032. https://doi.org/10.1093/aobpla/plr032

Petrásek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertová, D., Wiśniewska, J., Tadele, Z., Kubeš, M., Covanová, M., Dhonukshe, P., Skůpa, P., Benková, E., Perry, L., Křeček, P., Lee, O. R., Fink, G. R., Geisler, M., Murphy, A. S., … Friml, J. (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science*, *312*(5775), 914–918. https://doi.org/10.1126/science.1123542

Pimenta, M. R., Fernandes, L. S., Pereira, U. J., Garcia, L. S., Leal, S. R., Leitão, S. G., Salimena, F. R. G., Viccini, L. F., & Peixoto, P. H. P. (2007). Flowering, germination and rooting of cuttings of *Lippia* L. (Verbenaceae). *Revista Brasileira de Botânica*, *30*(2), 211–220. https://doi.org/10.1590/S0100-84042007000200006

Prando, M. A. S., Chiavazza, P., Faggio, A., & Contessa, C. (2014). Effect of coconut water and growth regulator supplements on *in vitro* propagation of *Corylus avellana* L. *Scientia Horticulturae*, *171*, 91–94. https://doi.org/10.1016/j.scienta.2014.03.052

Ramesh, S., Chandran, C., & Venkatesan, G. (2013). *In vitro* clonal propagation of vulnerable medicinal plant, *Mimosa pudica* L. *International Journal of Current Research and Development*, *2*(1), 18–30.

Rejthar, J., Viehmannova, I., Cepkova, P., Fernández, E., & Milella, L. (2014). *In vitro* propagation of *Drosera intermedia* as influenced by cytokinins, pH, sucrose and nutrient concentration. *Emirates Journal of Food and Agriculture*, *26*(6), 558–564. https://doi.org/10.9755/ejfa.v26i6.18022

Reuveni, O., & Raviv, M. (1980). Importance of leaf retention to rooting of avocado cuttings. *Journal of the American Society for Horticultural Science*, *106*(2), 127–130. https://doi.org/10.21273/JASHS.106.2.127

Rizwan, H. M., Irshad, M., He, B. Z., Debnath, B., Mitra, S., Li, M., Lu, X. C., Sun, Y. T., & Qiu, D. L. (2018). Silver nitrate ($AgNO_3$) boosted high-frequency multiple shoot regeneration from cotyledonary node explants of okra (*Abelmoschus esculentus* L.). *Applied Ecology and Environmental Research*, *16*(3), 3421–3435. https://doi.org/10.15666/aeer/1603

Russowski, D., Maurmann, N., Rech, S. B., & Fett-Neto, A. G. (2006). Role of light and medium composition on growth and valepotriate contents in *Valeriana glechomifolia* whole plant liquid cultures. *Plant Cell, Tissue and Organ Culture*, *86*, 211–218. https://doi.org/10.1007/s11240-006-9109-z

Sánchez-Zamora, M. Á., Cos-Terrer, J., Frutos-Tomás, D., & García-López, R. (2006). Embryo germination and proliferation *in vitro* of *Juglans regia* L. *Scientia Horticulturae*, *108*(3), 317–321. https://doi.org/10.1016/j.scienta.2006.01.041

Schepetilnikov, M., & Ryabova, L. A. (2017). Auxin signaling in regulation of plant translation reinitiation. *Frontiers in Plant Science*, *8*, 1014. https://doi.org/10.3389/fpls.2017.01014

Simon, S., & Petrášek, J. (2011). Why plants need more than one type of auxin. *Plant Science*, *180*(3), 454–460. https://doi.org/10.1016/j.plantsci.2010.12.007

Song, Y. (2013). Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *Journal of Integrative Plant Biology*, *56*(2), 106–113. https://doi.org/10.1111/jipb.12131

Swarup, K., Benková, E., Swarup, R., Casimiro, I., Péret, B., Yang, Y., Parry, G., Nielsen, E., Smet, I. D., Vanneste, S., Levesque, M. P., Carrier, D., James, N., Calvo, V., Ljung, K., Kramer, E., Roberts, R., Graham, N., Marillonnet, S., … Bennett, M. J. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. *Nature Cell Biology*, *10*, 946–954. https://doi.org/10.1038/ncb1754

Tiwari, S., Tiwari, R., & Singh, A. (2012). Impact of cypermethrin on fingerlings of common edible carp (*Labeo rohita*). *The Scientific World Journal*, *2012*, 291395. https://doi.org/10.1100/2012/291395

Tofanelli, M. B. D., Freitas, P. L., & Pereira, G. E. (2014). 2,4-dichlorophenoxyacetic acid as an alternative auxin for rooting of vine rootstock cuttings. *Revista Brasileira de Fruticultura*, *36*(3), 664–672. https://doi.org/10.1590/0100-2945-266/13

Utami, E. S. W., & Hariyanto, S. (2019). *In vitro* seed germination and seedling development of a rare Indonesian native orchid *Phalaenopsis amboinensis* J.J.Sm. *Scientifica*, *2019*, 8105138. https://doi.org/10.1155/2019/8105138

Villa, F., Pasqual, M., & de Faria Freitas, G. (2010). Optimization of a protocol for the micropropagation of olive tree cv. Ascolano 315. *Revista Ceres*, *57*(4), 530–534. https://doi.org/10.1590/S0034-737X2010000400015

Woldeyes, G. G., Senbeta, T. F., Adugna, A. Y., & Abegaz, A. W. (2021). The effect of MS strength, pH and sucrose concentrations on *in vitro* propagation of okra (*Abelmoschus esculentus* L.) from shoot tip explants. *Research Square*. https://doi.org/10.21203/rs.3.rs-153475/v1

Wotavová-Novotná, K., Vejsadová, H., & Kindlmann, P. (2007). Effects of sugars and growth regulators on *in vitro* growth of *Dactylorhiza* species. *Biologia Plantarum*, *51*, 198–200. https://doi.org/10.1007/s10535-007-0040-x

Wu, K., Zeng, S., Lin, D., da Silva, J. A. T., Bu, Z., Zhang, J., & Duan, J. (2014). *In vitro* propagation and reintroduction of the endangered *Renanthera imschootiana* Rolfe. *PLOS One*, *9*(10), e0110033. https://doi.org/10.1371/journal.pone.0110033

Yaseen, M., Ahmad, T., Sablok, G., Standardi, A., & Hafiz, I. A. (2012). Review: Role of carbon sources for *in vitro* plant growth and development. *Molecular Biology Reports*, *40*, 2837-2849. https://doi.org/10.1007/s11033-012-2299-z

Younesikelaki, F. S., Ebrahimzadeh, M. H., Desfardi, M. K., Banala, M., Marka, R., & Nanna, R. S. (2016). Optimization of seed surface sterilization method and *in vitro* seed germination in *Althaea officinalis* (L.) - An important medicinal herb. *Indian Journal of Science and Technology*, *9*(28), 1-6. https://doi.org/10.17485/ijst/2016/v9i28/90896

Zahara, M., Datta, A., & Boonkorkaew, P. (2016). Effects of sucrose, carrot juice and culture media on growth and net $CO_2$ exchange rate in *Phalaenopsis* hybrid 'Pink'. *Scientia Horticulturae*, *205*, 17–24. https://doi.org/10.1016/j.scienta.2016.04.011