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An improved protocol for *in vitro* propagation of the medicinal plant *Mimosa pudica* L.

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This work aimed to develop a protocol for the *in vitro* establishment, multiplication, rooting and *ex vitro* acclimatization of *Mimosa pudica* L., a species used in folk medicine and with pharmacological activity. Aseptic cultures were established from seeds inoculated in MS medium, without growth regulators, followed by an *in vitro* stabilization phase in culture medium supplemented with 2.22 µM BAP. The cultures were transferred to MS medium supplemented with different cytokinins, combined or not with an auxin, aiming its large-scale propagation. The culture medium supplemented with 5 µM BAP plus 0.5 µM NAA provided the highest multiplication rate and top quality plantlets. The combination of 0.6 µM TDZ plus 0.05 µM NAA resulted in higher multiplication rates than in response to combination of BAP plus NAA, although the subsequent maintenance of the cultures in a medium without growth regulators has resulted in low regenerative response. *In vitro* rooting of micro-cuttings was high even in the absence of auxins. Over 90% of plantlets transferred to the greenhouse survived after the acclimatization phase. Acclimatized plants presented normal vegetative and reproductive development. The procedures established in the present study allow a massive production of *M. pudica* plants for further pharmacological studies.

Key words: Biodiversity conservation, *ex vitro* acclimatization, *in vitro* rooting, micropropagation, *Mimosa pudica.*

INTRODUCTION

Mimosa pudica L., popularly known in Brazil as *dormideira*, *sensitiva*, *mimosa*, and *maria-fecha-a-porta*, is a perennial and native species of Tropical America, which also can be found in Tanzania, South Asia, South-East Asia and many Pacific Islands having near-frequent rainfall (Patra et al., 2016). Mimosa plants present

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herbaceous habit, with the presence of thorns throughout the stem. The growth is annual and, despite presenting erect stem when young, they become creeper plants at the adult phase. The leaf sensitiveness to mechanical, electrical or thermal stimuli is a feature of this species, evidenced by the fast closure of leaflets when disturbed (Jensen et al., 2011). *M. pudica* is used in folk medicine, mainly in Asian countries, whereas its extracts are attributed diuretic, astringent and antispasmodic activities. Its roots and leaves are used in hemorrhoids and fistula treatments and against convulsions (Hassan et al., 2010). *In vitro* and *in vivo* analysis of flavonoids and phenolic compounds of leaves, stems, and seeds of the *M. pudica* have found high antioxidant, antimicrobial, anticancer and antidiabetic activities, justifying its use in different diseases treatments (Doss et al., 2011; Kaur et al., 2011; Zhang et al., 2011; Joseph et al., 2013; Gunawardhana et al., 2015; Tunna et al., 2015; Jose et al., 2016; Muhammad et al., 2016; Patro et al., 2016).

Mimosa is the main genus of legumes associated to β-rhizobia, bacteria related to soil fertility increase, due to its N_2 fixing action (Elliott et al., 2007; Reis Jr. et al., 2010). Being a nutrient cycle promoter, their species are used in recovery of natural and degraded ecosystems (Camargo-Ricalde et al., 2004). On the other hand, *M. pudica* is described as a highly invasive pantropical weed, having a negative impact on natural and agricultural ecological systems (Klonowska et al., 2017). Although presenting fast and easy propagation in nature, the seeds of *M. pudica* are subject to biotic and abiotic agents, which affect the germination success (Jensen et al., 2011).

Considering its pharmacological and medicinal properties and its ecological relevance, there are, relatively, few studies involving large-scale micropropagation of *M. pudica* (Ramakrishna et al., 2009; Hassan et al., 2010; Ramesh et al., 2013). Micropropagation allows the production of genetically uniform plants in reduced space and in controlled conditions of temperature, photoperiod and luminosity (Parveen and Shahzad, 2011; Varshney and Anis, 2012). Studies related to *in vitro* cultivation of the *M. pudica* are very important, especially because it is a non-domesticated species and an environment facilitator, allowing the comprehension of specific biochemical and physiological mechanisms and the use of the characters that make it a medicinal species, as well as for its control as a weed.

The present study aimed to establish an efficient protocol for large-scale propagation of aseptic cultures of *M. pudica*, assessing the effects of different plant growth regulators on *in vitro* multiplication and rooting phases, as well as on acclimatization to *ex vitro* conditions. Plants produced through tissue culture can be used in pharmacological and ecophysiological researches, avoiding the need to collect specimens in a natural environment, in which genetic variability plays a fundamental role.

MATERIALS AND METHODS

Plant material and establishment of *in vitro* micropropagation procedure

In vitro establishment of aseptic cultures of *M. pudica* was carried out by seeds harvested from dry and mature fruits, from plants developed in the natural environment. After collection, the seeds were subjected to surface asepsis by rinsing in tap water for 60 min. Then, the seeds were immersed in ethanol 70% (v/v) for 30 s and, later, in a bleach solution with 2% of active chlorine, diluted at 30% (v/v) for 15 min. Finally, the seeds were washed in distilled and autoclaved water. After asepsis, the seeds were inoculated in MS basal medium (Murashige and Skoog, 1962), without growth regulators, in a laminar flow hood. The success of *in vitro* establishment was estimated 30 days after inoculation, considering the percentages of seeds contamination and total germination. In order to obtain stabilized cultures, 60 days after *in vitro* establishment, nodal segments (2-3 cm) from seedlings were transferred to MS culture medium supplemented with α-naphthalene acetic acid (NAA: 0.107 μM) or 6-benzylaminopurine (BAP: 2.22 μM), besides the control group (without growth regulators). After 60 days, the *in vitro* cultures were evaluated regarding the number of shoots per explants, height of shoots, and percentage of rooted plantlets.

In vitro multiplication

Aiming to eliminate the residual effects of previous culture media, after *in vitro* stabilization phase, the explants were transferred and kept for 30 days on MS medium without growth regulators. Then, nodal segments (2-3 cm) were obtained from plantlets maintained in this condition and transferred to MS culture medium supplemented with BAP, kinetin (KIN), adenine sulphate (AS), diphenylurea (DFU) or thidiazuron (TDZ), at 0, 2.5, 5 or 7.5 μM, totaling 16 treatments. The cultures were kept in these culture media for 30 days and evaluated regarding the number of shoots and roots per explant, height of shoots, and callus development. Root quality was also evaluated with scores, ranging from 1 to 5 given by three independent evaluators, with 5 corresponding to the root system that presents the best development, and 1 for explants that did not present root development.

After we found that BAP was the cytokinin that provided the highest *in vitro* multiplication rates, new assays were carried out aiming to further increase culture proliferation. Nodal segments (2-3 cm) obtained from explants aseptically established in MS medium without growth regulators, were inoculated in MS medium supplemented with BAP (0, 5 or 7.5 μM) and NAA (0, 0.05, 0.25 or 0.5 μM), in all possible combinations, totaling 12 treatments. The cultures were evaluated after 45 days regarding percentage of explants presenting three or more shoots, number and height of shoots.

Aiming to obtain elongated shoots, nodal segments (2-3 cm) from the best treatment in the previous assay (5 μM BAP plus 0.5 μM NAA) were inoculated in MS medium supplemented with gibberellic acid (GA_3: 0, 0.28, 1.44, 2.89 or 4.53 μM), in presence or absence of 5 μM BAP, totaling 10 treatments. After 45 days, the cultures were evaluated regarding number and height of shoots and the number of roots per explant.

Considering the results found in the assay with the different cytokinins, another set of explants, previously kept in MS medium without growth regulators, was transferred to MS medium supplemented with TDZ (0.6, 0.9 or 1.2 μM) plus NAA (0, 0.05, 0.25

or 0.5 µM), in all possible combinations, totaling 12 treatments. After 45 days, the cultures were evaluated considering the number of shoots higher than 0.5 cm. As the new shoots, in this assay, did not show suitable elongation, the explant clusters (rosettes) produced (0.5 ± 0.1 cm) were transferred from MS culture media with 0.6 µM TDZ, singly or combined with NAA, to MS culture media without growth regulators or to MS culture media supplemented with 0.25 µM NAA. After 45 days, the cultures were evaluated considering the percentage of regenerated shoots and height of shoots.

In vitro rooting

Aiming to stimulate the rooting of micro-cuttings, nodal segments from plantlets (2-3 cm, excluded the apical part) were transferred to MS culture media supplemented with NAA, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0.1, 0.2, 0.3 or 0.4 µM, besides the control). After 40 and 60 days of inoculation, the cultures were evaluated regarding the number of roots and the length of the largest root.

In vitro culture conditions

At all stages of *in vitro* culture, the plantlets were kept in 2.5 × 15 cm test tubes. The MS culture media was supplemented with MS vitamins, sucrose (30 g L^{-1}), and agar (7 g L^{-1}). The culture media pH was adjusted to 5.7 ± 0.1 before autoclaving, carried out for 20 min at 120°C and 1 atm of pressure. The test tubes were capped with autoclaving polyethylene closures, and sealed with PVC film (Vitaspenser, Goodyear, 15 µm). The cultures were kept in a growth room under controlled conditions of temperature (26 ± 1°C), photoperiod (16 h) and luminosity (40 µmols m^{-2} s^{-1}).

Ex vitro acclimatization

After 60 days of cultivation in rooting medium, the plantlets were removed from test tubes and their roots were washed in running tap water to remove culture media debris. Later, the plantlets were transplanted to polystyrene trays with 128 cells, filled with commercial substrate Plantmax Hortaliças HT®. The trays were covered and wrapped with transparent plastic, remaining for 20 days in shadowed environment. After this period, the trays were transferred to a greenhouse covered with transparent plastic and Sombrite® 70%, and maintained under programmed micro sprinkler system undertaken for 5 min twice a day. After 40 days, the plants were transferred to pots with a mixture of soil/washed sand/cattle manure at the proportion of 3:2:1 (v/v/v). The efficiency of acclimatization procedures was evaluated taking in account the plantlets final survival percentage.

Statistical analysis

All experiments were conducted in a completely randomized design, with five replicates, except for cases that percentages were compared, in which ten repetitions per treatment were used. Linear or polynomial regression in accordance with residual requirements in assays related to multiplications and rooting phases was employed. In some of the experiments, counting data were normalized by the equation $\sqrt{x+0.5}$ and the results submitted to analysis of variance (ANOVA). The obtained means were compared through Scott-Knott test at 5% probability, using SAEG software (version 9.1).

RESULTS

In vitro establishment and stabilization

In this work, the *in vitro* disinfection procedures of *M. pudica* seeds were very effective, with less than 2% of microbial contamination and high germination rates (87%). Figure 1a presents features of *M. pudica* plantlets, 15 days after *in vitro* germination in MS medium without growth regulators.

In stabilization phase of *M. pudica* cultures, the multiplication rate was 4.5 shoots/explant in response to 2.22 µM BAP; the best result among the three treatments used. Micro-cuttings rooting in this condition was around 40% less than that found in plantlets kept in culture medium with 0.107 µM NAA. In contrast, the supplementation of NAA to the culture medium did not significantly affect the number of shoots, rooting percentage or shoots height, with results similar to the control, without BAP. Although BAP has stimulated shoot multiplication, a negative correlation was observed between elongation and culture propagation rates (Figure 2a).

In vitro multiplication

At the *in vitro* multiplication phase, the supplementation of 5 µM BAP to MS medium resulted in the production of approximately 5 shoots/explant (Figure 2b), the highest multiplication rate among all cytokinins used. Nevertheless, at 7.5 µM BAP, an inhibition was observed in shoots production. *M. pudica* plantlets kept *in vitro* in the presence of cytokinins are shown in Figure 1b. The increase in BAP concentration resulted in inhibition of shoots elongation, with plantlets reaching, in average, 2.5 cm height in culture media supplemented with 5 µM BAP (Figure 2c). In this condition, plantlets showed poor root quality (Figure 1b 4-6 and Figure 2d).

In contrast to what was observed in relation to BAP, the supplementation of KIN did not significantly stimulate *M. pudica* shoots proliferation (Figure 1b: 7-9). The development of more than one shoot/explant was observed only in response to KIN higher than 5 µM. However, there were no differences compared to the control. In relation to TDZ, the concentrations evaluated initially (2.5, 5 and 7.5 µM) did not promote significantly shoot proliferation, besides having promoted an excessive callus proliferation (Figure 1b 10-12).

In this study, the best shoot multiplication was found when 5 µM BAP was associated to 0.5 µM NAA, with 90% of explants presenting three or more shoots (Table 1). Nevertheless, in the higher BAP concentration, the number of shoots reduced. Additionally, NAA exclusion of culture media resulted in impaired morphogenetic response of the *M. pudica* cultures. As it does not affect significantly the number of shoots (Table 2), the

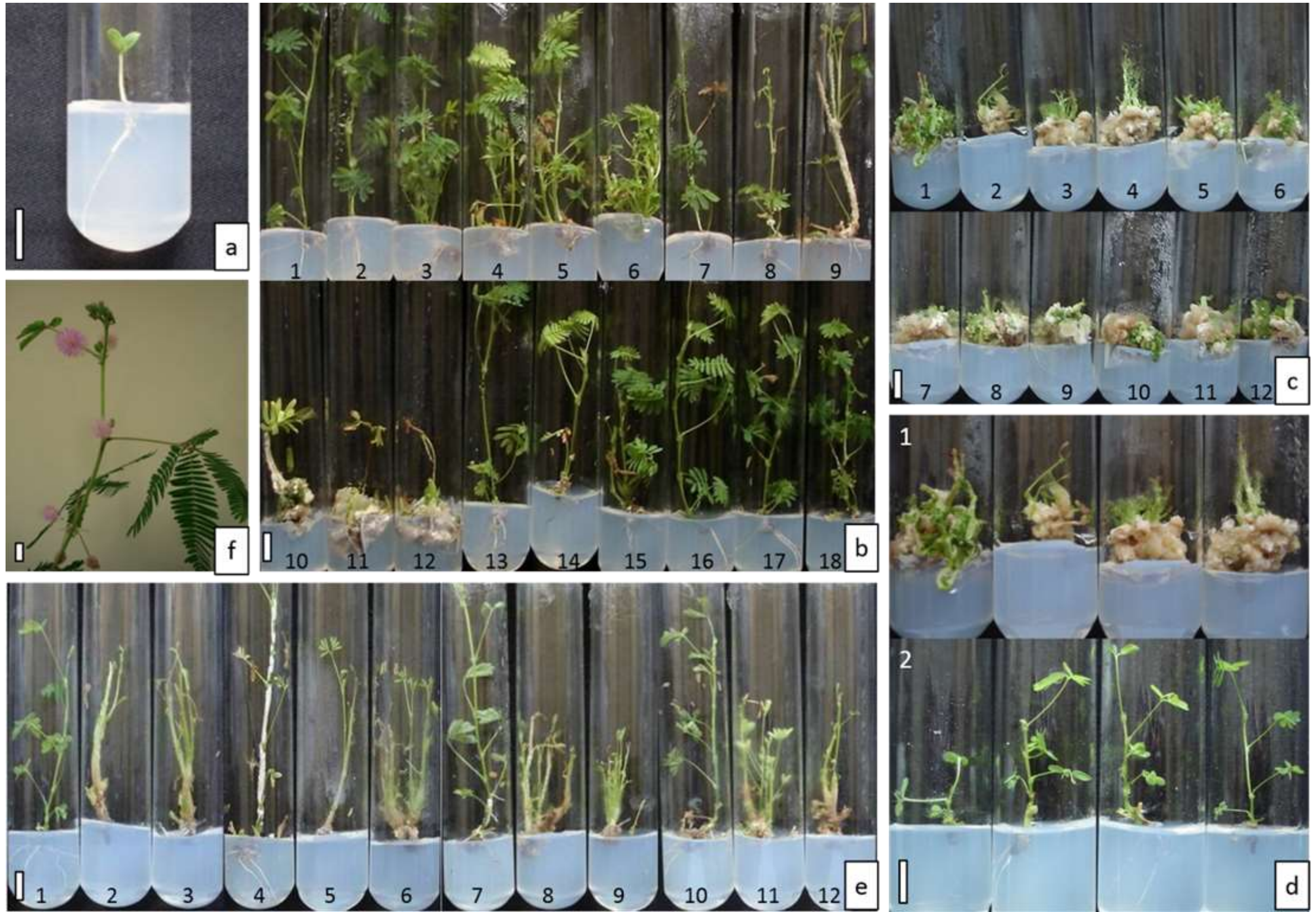

Figure 1. Different stages of micropropagation and acclimatization of *M. pudica* plants: **(a)** *In vitro* stabilization stage. **(b)** *In vitro* multiplication stage in response to different cytokinins: 1, 2, and 3 - control; 4 - BAP 2.5 µM; 5 - BAP 5 µM; 6 - BAP 7.5 µM; 7 - KIN 2.5 µM; 8 - KIN 5 µM; 9 - KIN 7.5 µM; 10 - TDZ 2.5 µM; 11 - TDZ 5 µM; 12 - TDZ 7.5 µM; 13 - DFU 2.5 µM; 14 - DFU 5 µM; 15 - DFU 7.5 µM; 16 - SA 2.5 µM; 17 - SA 5 µM; 18 - SA 7.5 µM. **(c)** *In vitro* multiplication stage in response to TDZ and to NAA: 1 - TDZ 0.6 µM; 2 - TDZ 0.6 µM + NAA 0.05 µM; 3 - TDZ 0.6 µM + NAA 0.25 µM; 4 - TDZ 0.6 µM + NAA 0.5 µM; 5 - TDZ 0.9 µM; 6 - TDZ 0.9 µM + NAA 0.05 µM; 7 - TDZ 0.9 µM + NAA 0.25 µM; 8 - TDZ 0.9 µM + NAA 0.5 µM; 9 - TDZ 1.2 µM; 10 - TDZ 1.2 µM + NAA 0.05 µM; 11 - TDZ 1.2 µM + NAA 0.25 µM; 12 - TDZ 1.2 µM + NAA 0.5 µM. **(d)** *M. pudica* plantlets in presence of TDZ 0.6 µM (1) and 45 days after transfer to MS medium (2). **(e)** *In vitro* multiplication stage in response to BAP and NAA: 1 - control; 2 - BAP 5 µM; 3 - BAP 7.5 µM; 4 - NAA 0.05 µM; 5 - NAA 0.05 µM + BAP 5 µM; 6 - NAA 0.05 µM + BAP 7.5 µM; 7 - NAA 0.25 µM; 8 - NAA 0.25 µM + BAP 5 µM; 9 - NAA 0.25 µM + BAP 7.5 µM; 10 - NAA 0.5 µM; 11 - NAA 0.5 µM + BAP 5 µM; 12 - NAA 0.5 µM + BAP 7.5 µM. **(f)** Details of reproductive development in acclimatized plants of *M. pudica.* Scale-bar: 1 cm.

supplementation of NAA to the culture medium increased the number of plantlets responding to treatments (Table 1). The combination of 5 µM BAP with 0.5 µM NAA resulted in production of more vigorous explants, without callus development or hyperhydricity symptoms (Figure 1e).

When shoots height was analyzed (Table 3), negative effects of BAP supplementation were observed, regardless of its concentration. Positive effects of NAA supplementation were only observed in media without BAP. The joint supplementation of NAA and BAP did not influence significantly shoots elongation, showing negative correlation between the multiplication rate and shoots length.

The use of GA_3 was ineffective to stimulate *M. pudica* plantlets elongation and multiplication. Although GA_3 supplementation has not promoted significant increases in adventitious roots proliferation when compared to control, in the concentration of 0.28 µM roots formation was observed in some plantlets even in presence of BAP (data not shown).

Due to the hyperhydricity and callus development at the micro-cuttings basis in response to TDZ doses used in the previous essay (2.5, 5 and 7.5 µM), a second investigation was undertaken with lower concentrations (0.6, 0.9, and 1.2 µM), in combination with NAA (0, 0.05,

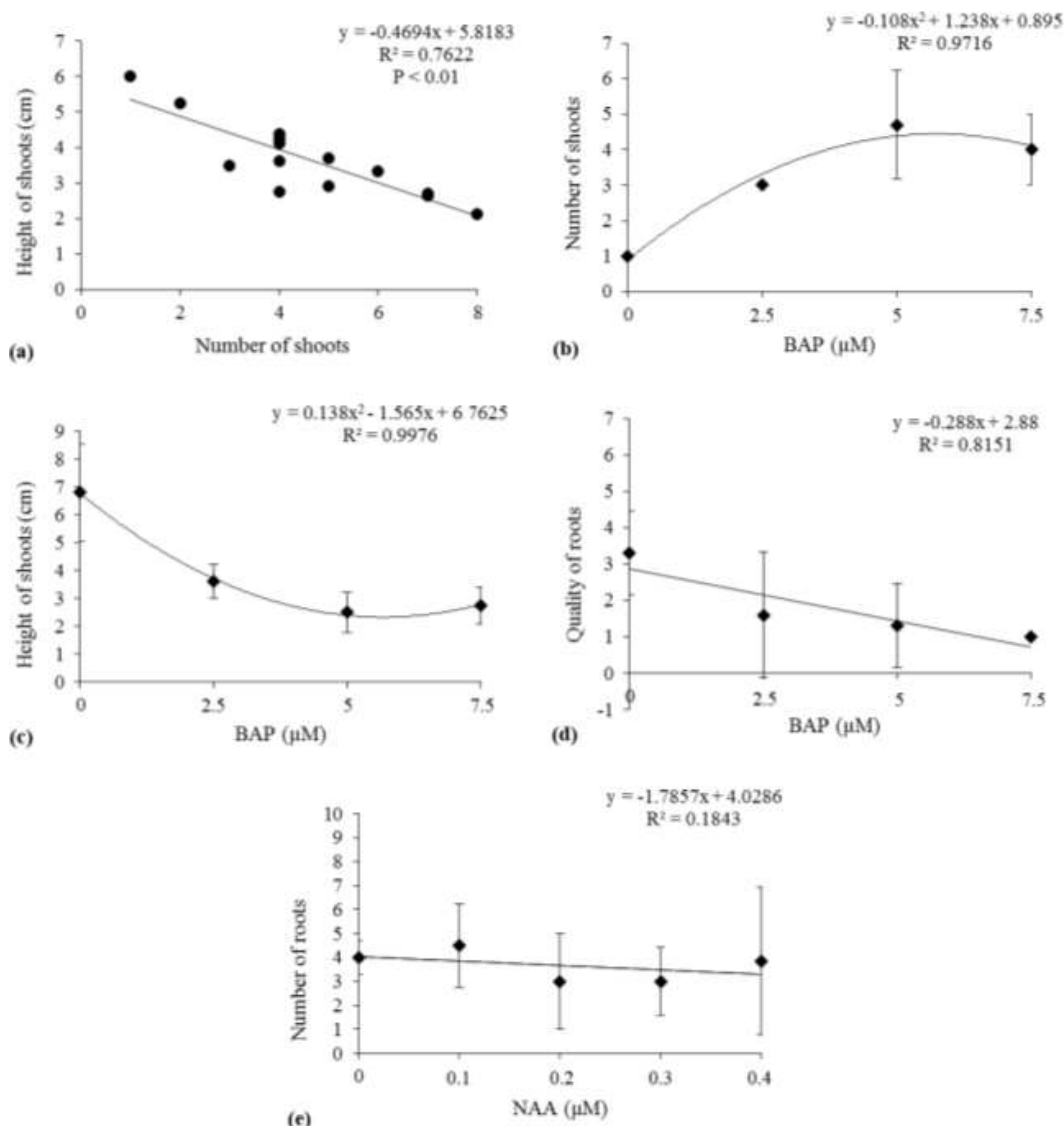

Figure 2. Relation between height and number of shoots in *M. pudica* explants cultivated for 60 days in media with 2.22 µM BAP, in the stabilization phase **(a)**, relation between BAP concentration and the number of shoots **(b),** height of shoots **(c)**, and roots quality **(d)** in the multiplication phase, 30 days after *in vitro* cultivation, and relationbetween increasing NAA concentration and number of roots in the rooting phase, after 60 days of *in vitro* cultivation **(e)**. Bars = mean standard error. n = 5.

0.25, and 0.5 µM). Although callus development and hyperhydricity had been observed in response to 0.6 µM TDZ, approximately ten shoots higher than 0.5 cm were obtained in culture media without NAA. The shoots obtained in response to 0.6 µM TDZ were qualitatively better than shoots produced in response to higher concentrations, due to less severe symptoms of hyperhydricity (Table 4 and Figure 1c).

In our study, the supply of NAA to the culture medium resulted in significant reduction in the number of shoots

Table 1. Percentage of *M. pudica* explants that presented three or more shoots in each treatment 45 days after beginning of experiments.

Treatment	% of explants with three or more shoots
MS without growth regulator	NR
BAP 5 μM	45
BAP 7.5 μM	50
NAA 0.05 μM	NR
BAP 5 μM + NAA 0.05 μM	50
BAP 7.5 μM + NAA 0.05 μM	40
NAA 0.25 μM	NR
BAP 5 μM + NAA 0.25 μM	70
BAP 7.5 μM + NAA 0.25 μM	60
NAA 0.5 μM	NR
BAP 5 μM + NAA 0.5 μM	90
BAP 7.5 μM + NAA 0.5 μM	40

NR = No response to treatment.

Table 2. Number of shoots in *M. pudica* explants cultivated *in vitro* for 45 days in response to different BAP and NAA concentrations.

BAP (μM)	NAA (μM)			
	0	0.05	0.25	0.5
0	1^{Ab}	1^{Ab}	1^{Ab}	1^{Ab}
5.0	3.6^{Aa}	3^{Aa}	4.3^{Aa}	5.3^{Aa}
7.5	4^{Aa}	4.6^{Aa}	5^{Aa}	2^{Aa}

Upper and lower case letters denote significant differences in each line and each column respectively, attested by the Scott-Knott test (P < 0.05).

Table 3. Height of shoots (cm) in *M. pudica* explants cultivated *in vitro* for 45 days in response to different BAP and NAA concentrations.

BAP (μM)	NAA (μM)			
	0	0.05	0.25	0.5
0	3.83^{Ba}	6.83^{Aa}	8^{Aa}	8.2^{Aa}
5.0	2.8^{Aa}	3.83^{Ab}	2.3^{Ab}	2.5^{Ab}
7.5	2.75^{Aa}	2.25^{Ab}	2^{Ab}	1.75^{Bb}

Upper and lower case letters denote significant differences in each line and each column respectively, attested by the Scott-Knott test (P < 0.05).

Table 4. Number of shoots higher than 0.5 cm in *M. pudica* explants cultivated *in vitro* for 45 days in response to different TDZ and NAA combinations.

TDZ (μM)	NAA (μM)			
	0	0.05	0.25	0.5
0.6	10^{Aa}	7^{Ba}	6^{Ba}	4^{Ba}
0.9	3.67^{Ab}	2.3^{Ab}	1.67^{Ab}	1.3^{Ab}
1.2	$0.67^{A}c$	$0^{A}c$	0.3^{Ab}	1^{Ab}

Upper and lower case letters denote significant differences in each line and each column respectively, attested by the Scott-Knott test (P < 0.05).

Table 5. Regeneration rate of *M. pudica* feasible explants after 45 days of *in vitro* cultivation. Arrows represent transfer to a new culture medium.

Treatment	**Regeneration rate** (%)
TDZ 0.6 μM → MS medium	20
TDZ 0.6 μM → NAA 0.25 μM	0
TDZ 0.6 μM + NAA 0.05 μM → MS medium	40
TDZ 0.6 μM + NAA 0.05 μM → NAA 0.25 μM	0
TDZ 0.6 μM + NAA 0.25 μM → MS medium	0
TDZ 0.6 μM + NAA 0.25 μM → NAA 0.25 μM	10
TDZ 0.6 μM + NAA 0.5 μM → MS medium	30
TDZ 0.6 μM + NAA 0.5 μM → NAA 0.25 μM	0

higher than 0.5 cm only in treatments containing 0.6 μM TDZ (Table 4 and Figure 1c). In spite of promoting shoots proliferation, TDZ does not stimulate elongation, resulting in shoot in the rosette feature (Figure 1c). The subdivided rosettes transferred to MS culture media without growth regulators presented better regeneration than those transferred to culture media having 0.25 μM NAA (Table 5 and Figure 1d). In this study, the best regeneration of cultures derivate from rosettes explants was observed in

treatments that culture medium was supplemented with 0.6 µM TDZ combined with NAA at 0.05 or 0.5 µM. The plantlets from rosettes explants regenerated in MS medium showed normal morphology (Figure 1d).

In vitro rooting

The analysis related to number of roots did not evidence significant differences between control and treatments in which NAA was added, showing that regardless of concentration, this growth regulator did not increase shoots rooting (Figure 2e). Also, differences were not found when the largest root length was evaluated, although the treatment with 0.4 µM NAA resulted in more vigorous and longer roots in the end of the first 40 days of *in vitro* cultivation. However, these differences vanished after 60 days. No significant difference was also observed in response to IBA between control and treatments regarding the number and elongation of adventitious roots in micro-cuttings of *M. pudica* (data not shown).

Despite higher IAA concentrations (0.4 µM) in the first days of cultivation promoted the production of branched and elongated roots, no significant difference was found between this treatment and the control after 60 days of *in vitro* cultivation. Additionally, IAA concentration between 0.1 and 0.3 µM inhibited adventitious roots development (data not shown).

Ex vitro acclimatization

Plantlets from *in vitro* cultivation developed very well in *ex vitro* conditions, with average survival of over 90%. 20 days after the beginning of the acclimatization phase, plants were already in conditions to be transferred to the greenhouse, presenting suitable vegetative development (around 15 cm height). After 40 days at the greenhouse, period in which all plantlets survived, they were transferred to pots under natural conditions. Acclimatized plants of *M. pudica* presented normal vegetative and reproductive development (Figure 1f). After 60 days of acclimatization, they presented blossoming, showing that the reproductive development was not affected by procedures of the *in vitro* cultivation.

DISCUSSION

An efficient establishment of *M. pudica in vitro* cultures

Although the *Mimosa* species produced large amounts of seeds, they are generally preyed and lose their viability when exposed to extreme conditions (Camargo-Ricalde et al., 2004). The *in vitro* establishment of *M. pudica* cultures from seeds was very successful, with only 2% microbial contamination and 87% germination. For *Carya illinoinensis* (Wangenh.) K. Koch, similar disinfection procedure showed contamination level of 5% after one week of establishment, reaching 18 to 20% after three weeks (Renukdas et al., 2010). According to George and Sherrington (1984), 10% is the acceptable contamination threshold in the *in vitro* establishment phase, which shows the efficiency of disinfection procedures used in this work.

The multiplication rate achieved in stabilization phase in response to BAP (4.5 shoots/explant) was similar to the results observed for *Uraria picta* (Jacq.) Desv. ex DC. (Anand et al., 1998) and for *Albizia falcataria* (L.) Fosberg (Widiyanto et al., 2008), two other Fabaceae. The rooting in this condition was approximately 40% less than in culture medium supplemented with 0.107 µM NAA, which can be attributed to the inhibitory effects of BAP on the rhizogenesis. Besides, a negative correlation was observed between elongation and cultures propagation, similar to that found for *in vitro* cultures of *Salvadora persica* L. (Phulwaria et al., 2011) and *Albizia falcataria* (L.) Fosberg (Widiyanto et al., 2008).

The use of BAP and NAA combination, essential for *M. pudica* shoots multiplication

In the multiplication phase, the inhibitory effect observed in the shoot propagation at 7.5 µM BAP shows that for the *M. pudica* cultures, high BAP concentration may be harmful to shoot proliferation. In studies related to *in vitro* cultivation, BAP is one of the most important cytokinins in shoot induction (Janarthanam and Seshadri, 2008). Positive effects of BAP supplementation at 5 µM were also reported in the multiplication phase for *Artemisia vulgaris* L. (Sujatha and Kumari, 2007) and *Acacia mangium* (Shahinozzaman et al., 2012) cultured *in vitro*.

While cytokinins are widely used in the morphogenesis and in *in vitro* proliferation phase, other substances have been employed in some researches. Ramakrishna et al. (2009) reported promising results for *in vitro* proliferation of *M. pudica* in response to the addition of melatonin and serotonin to the culture media, producing 15 and 22 shoots/explants, respectively. Although that analysis had been carried out after two months, twice the evaluated period in our work, those results suggest an alternative for *in vitro* shoots induction, since those substances may be used alone or combined with cytokinins and/or auxins, aiming to increase the effectiveness of the micropropagation process.

The increase in BAP concentration inhibited the shoots elongation and produced plants with reduced root quality, which, however, is not a limiting factor at this *in vitro* cultivation phase. The results found in this study related to BAP effects on *M. pudica* culture proliferation are typical of the effects of cytokinins in *in vitro* morpho-

genesis control. As predicted by the classic morphogenesis model suggested by Skoog and Miller (1957), beyond a certain concentration, the cytokinins promote an unbalancing in the hormonal metabolism. The results from this study show that *M. pudica* explants are adversely affected by excessive concentrations of BAP that, however, differs from the findings for *Justicia gendarussa* Burm. f. (Thomas and Yoichiro, 2010) and *Acacia tortilis* subsp. *raddiana* (Savi) Brenan (Nandwani, 1995), species that showed high multiplication rates in response to BAP concentrations that caused inhibition in *M. pudica* shoot proliferation. Banu et al. (2014) obtained a high proliferation rate (about 19 shoots/seed) after *in vitro* cultivation of *M. pudica* in media with approximately 8.9 µM (2 mg L^{-1}). They used synthetic seeds produced both with shoot tips and nodes, demonstrating that this technique can be effectively used to propagate this species. However, the production of synthetic seed can raise the cost of procedures and the time required for propagation.

The results obtained with the supplementation of KIN, wherein no differences were found among the control and other treatments, suggest that it may be required higher levels of this cytokinin to stimulate shoots proliferation. According to Shiva et al. (1994), cytokinins relative effectiveness in shoots induction follows the BAP > KIN > zeatin > AS order. Studying plant of *Psoralea corylifolia* L., a leguminous used in Indian and Chinese medicine, Baskaran and Jayabalan (2008) also verified low effectiveness of the KIN in promoting *in vitro* proliferation. This same result was found by Banu et al. (2014), after germination of synthetic seeds of *M. pudica*.

Several studies used TDZ as cytokinin to stimulate *in vitro* cultures proliferation (Rolli et al., 2012). In our assessment, however, TDZ did not stimulate *M. pudica* shoots proliferation. These results contradict, in certain extent, the expectations related to the beneficial effects of the phenylurea derivatives in promoting the organogenesis and shoots proliferation, compared to cytokinins derived from adenine, such as BAP and KIN (Khurana-Kaul et al., 2010). TDZ, in addition to acting as synthetic cytokinin, also promotes the overexpression of natural cytokinins, and therefore is successfully used in shoots proliferation in concentrations below 1 µM (Varshney and Anis, 2012). TDZ effects seem to be related to its influence in the synthesis of *IPT* genes, stimulating the natural production of zeatin, in addition to promote upstream regulation of the genes related to specific responses to cytokinins, resulting in cell division increase and, often in the stimulus to callus development (Taiz and Zeiger, 2010), an unwanted morphogenetic response observed in this work.

The use of NAA in association with BAP is widely reported in the literature. In this study, the shoots multiplication increased when these growth regulators were combined. Differences were observed for the *Acacia mangium* Willd for which the supplementation of 0.5 µM NAA to the culture medium for multiple shoots induction (4 µM BAP) was harmful, leading to the reduction of shoots proliferation (Shahinozzaman et al., 2012). For *M. pudica* cultures, Hassan et al. (2010) found high multiplication rates when 6.6 µM BAP and 2.6 µM NAA were combined, although the response percentages have been smaller. Also, studying *M. pudica* micropropagation, Ramesh et al. (2013) achieved an increase in number of shoots/explant using simultaneously IAA and NAA in culture media supplemented with BAP. The isolated supply of BAP in culture media promoted a less intense proliferative response, showing that these growth regulator effects are related to the balance between auxins and cytokinins, which is crucial to break the apical dominance and for the synthesis and activation of cyclin-dependent kinases (CDKs), enzymes related to the cell division (Taiz and Zeiger, 2010).

Despite the use of gibberellins be reported for the promotion of *in vitro* shoot elongation (Chen et al., 2008) and low doses of GA_3 associated to BAP affect positively the *in vitro* rooting formation and elongation (Magyar-Tábori et al., 2010), for *M. pudica* the GA_3 was ineffective in this process. Nevertheless, root formation was observed in culture media supplemented with 0.28 µM GA_3 even in the presence of BAP. This result was corroborated by Žiauka and Kuusienė (2010), who observed complete inhibition in root formation when *Populus tremula* L. nodal segments were treated with high doses of paclobutrazol, an antagonist molecule of gibberellin synthesis, indicating that GAs, in low concentrations, may become beneficial to root development. Apparently, reduced doses of GA_3, when associated with BAP, increase the effectiveness of *in vitro* propagation processes, particularly in explants elongation and root formation phases (Pati et al., 2006; Magyar-Tábori et al., 2010). However, in this study, the beneficial effects of gibberellins were not observed for *M. pudica*.

Although TDZ stimulates hyperhydricity more intensively than BAP and KIN (Kadota and Niimi, 2003), in the present study, 0.6 µM TDZ stimulated the proliferation of greater number of elongated shoots than in presence of other cytokinins. Low concentrations of TDZ were effective in inducing multiple *in vitro* shoots in peanuts cultures (Joshi et al., 2008). In *Lens culinaris* Medik., 0.5 µM TDZ also stimulate *in vitro* shoots formation (Chhabra et al., 2008). However, in response to 2.5 µM TDZ, they noticed complete inhibition in the shoots proliferation.

There was a decrease in the number of shoots higher than 0.5 cm in response to addition of NAA to the culture medium supplemented with 0.6 µM TDZ. According to Yucesan et al. (2007), high TDZ concentrations might reduce the shoots proliferation, regardless of the presence of auxins. According to Murthy et al. (1996), the ineffectiveness of auxins in stimulating shoots

proliferation may be attributed to TDZ effects on the modulation of its endogenous concentrations, suggesting that TDZ stimulates the *de novo* synthesis of natural auxins, dismissing its inclusion in the culture medium.

According to Ahmad et al. (2006), the transference of shoots cluster to a culture media without cytokinins reverse the rosette feature and stimulates the shoots elongation. A successful regeneration of the subdivided rosettes from TDZ was observed when they were transferred to MS culture media without growth regulators, showing that subcultivation in these conditions is crucial for later development of these explants (Khalafalla and Hattori, 1999; Joshi et al., 2008). Although Prathanturarug et al. (2005) has suggest that a second treatment with TDZ may increase culture multiplication, the results from our work do not corroborate with this possibility, since explants regeneration in culture media supplemented with high doses of TDZ was very low. The plantlets regenerated in MS medium presented normal morphology and similarity of plants not subjected to TDZ treatment, a result also found by Siddique and Anis (2007) in *Ocimum basilicum* L. cultures. Although the cultivation in culture media supplemented with 0.6 µM TDZ produced ten shoots higher than 0.5 cm, a number twice as high as that obtained with the best treatment using 5 µM BAP plus 0.5 µM NAA, the percentage of explants regeneration in subcultures was very low (20%). In fact, any treatment with TDZ, supplemented or not with NAA, did not reach 50% of regeneration. These results confirm the use of BAP as the best cytokinins among the tested for multiplication of *M. pudica*.

Auxin type and concentration effect on *in vitro* rooting process in *M. pudica*

The induction, initiation, and expression are the major phases of adventitious rhizogenesis (Kose et al., 2011). The auxins are important in all stages, presenting a crucial role in stimulating the pericycle cells determination (Hartmann et al., 1990). The majority of studies employing auxins are species dependent (Gürel and Wren, 1995; Parveen and Shahzad. 2011). Nevertheless, in our study, the supplementation with NAA did not increase shoots rooting. The same response was observed for IBA, with no significant differences between control and other treatments. Nevertheless, studying *Acacia tortilis* subsp. *raddiana* (Savi) Brenan micropropagation, Nandwani (1995) found the best rooting responses in medium supplemented with 14.7 µM IBA, suggesting that, for woody species, it may be necessary the addition of auxins at high concentrations. Despite that IBA has been successfully used in studies related to promotion of *in vitro* rooting (Shahinozzaman et al., 2012), it is more effective when employed to stimulate new rooting formation in cuttings (Hartmann et al., 1990; Ahmad et al., 2006).

In contrast to what was observed in relation to the NAA and IBA, some effects on rooting micro-cutting of *M. pudica* were observed in response to the IAA. In the first days of cultivation, the higher IAA concentration (0.4 µM) promoted the formation of branched and elongated roots. However, no significant differences between this dose and the control were found after 60 days of *in vitro* cultivation. IAA concentration ranging from 0.1 and 0.3 µM inhibited adventitious roots formation. The IAA also was the most efficient auxin for rhyzogenesis in *Cichorium intybus* L., although in a concentration tenfold lower than used in this study (Yucesan et al., 2007). It is known that in excessive concentrations, the IAA may affect the metabolism of endogenous auxin, promoting the activation of the *GH3* genes, encoding proteins involved in auxin homeostasis, making them inactive (Ludwig-Müller, 2011). The IAA endogenous level, in response to IAA doses up to 0.3 µM, was probably changed due to inactivation of this molecule by conjugation, which results in lower free contents of auxins in the bioactive form and, consequently, reducing adventitious roots formation. Nevertheless, at concentrations of 0.4 µM, despite possible activation of the *GH3* genes has occurred, IAA content was probably not fully conjugated, resulting in levels of auxins close to those found in the control.

The results from this study showed that, for *M. pudica in vitro* cultures, both type and concentration of auxins added to culture medium did not interfere significantly on *in vitro* rooting. The requirement of adventitious rooting formation in micropropagation protocols is estimated by success of the acclimatization stage, since some species are able to establish in *ex vitro* conditions even presenting poor *in vitro* rooting (Meiners et al., 2007; Parveen et al., 2010).

The procedures of acclimatization

The results found in this study show that *M. pudica* is a species of easy acclimatization, with survival of over 90% and with excellent vegetative development after transference to the greenhouse. The results found in our study show that the adventitious rooting developed at *in vitro* phase under stimulus of auxins was irrelevant, since both rooted and non-rooted micro-cuttings of *M. pudica* presented high survival rate to acclimatization. Moreover, the success in the acclimatization phase found in the present study was better than those found in other studies with *M. pudica* (Hassan et al., 2010; Ramesh et al., 2013). The plantlets mortality observed during the *in vitro* rooting stage was higher than in acclimatization phase. This result suggests that cultures of *M. pudica* exhibit high sensitivity to the ethylene generated *in vitro*, due to auxins added in the culture medium.

After 40 days in greenhouse conditions, period in which

all plantlets survived, they were transferred to pots under field conditions. *M. pudica* acclimatized plants presented normal vegetative and reproductive development (Figure 1f). After 60 days of acclimatization, the plants presented blossoming, showing that the reproductive development had not been affected by procedures of the *in vitro* cultivation.

Conclusion

The culture medium supplemented with 5 µM BAP plus 0.5 µM NAA is recommended for high multiplication rates and for providing top quality plantlets. *In vitro* rooting of micro-cuttings is high for *M. pudica*, even in the absence of auxins. Over 90% of plantlets transferred to the greenhouse can survive after the acclimatization phase. The procedures established in the present study allow a massive production of *M. pudica* plants for further pharmacological and ecophysiological studies on a species which has ecological, ornamental and medicinal importance.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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