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Phytoregulators and Explant Size in the *in vitro* Culture of *Malva sylvestris*

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Abstract

In vitro propagation of plants makes it possible to accelerate the process of plant multiplication, the study of secondary metabolite production and the cultivation of biotrophic fungi. The objective of this work was to study the combination of indoleacetic acid (IAA) and benzylaminopurine (BAP) and explant sizes in *in vitro* multiplication of *M. sylvestris*. Five concentrations of BAP (0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) and two of IAA (0 and 0.5 mg L^{-1}) were used in explants of 4 to 9 mm and of 14 to 23 mm. Contaminated explants, oxidation, establishment, relative growth rate (RGR), sprouting, rooting and callus formation were evaluated. There was no interaction effect between BAP and IAA concentrations. At 28 days, explants were established at 32.76% and callus formation was 62.5% for explants associated with 0.0 mg L^{-1} of IAA. There was 32.14% establishment and 63.79% callus formation at 0.5 mg L^{-1} of IAA. Bacterial contamination at 28 days was 60.53%, twice as much the amount found at 14 days, suggesting that the explants presented endogenous contamination. It was found that explant size influences the subsequent meristematic development. The use of smaller explants (4 to 9 mm) allowed greater formation of calli and larger explants (14 to 23 mm) allowed greater formation of shoots. In conclusion, larger explants are preferable for production of *M. sylvestris in vitro* by organogenesis while smaller ones are preferable for embryogenesis.

Keywords: micropropagation, indoleacetic acid, benzylominopurine

1. Introduction

Mallow or malva-sylvestris (*Malva sylvestris* L.) is a plant of medicinal interest with antitussive, antiseptic, anti-inflammatory, anti-osteclastogenic and antioxidant effects (Brasil, 2011; Benso, Franchin, Massarioli, Paschoal, Alencar, Franco, & Rosalen, 2016). Medicinal plants are regularly used to heal humans and animals because they contain different metabolites of interest (Marouane, Soussi, Murat, Bezzine, & El Feki, 2011; Prudente, Sponchiado, Mendes, Soley, Cabrini, & Otuki, 2017). Among the compounds found in *M. sylvestris*, phenolics are the main components in the leaves (386.45 mg g^{-1} of methanolic extract), but other components with antioxidant action include flavonoids (210.81 mg g^{-1}) and carotenoids (0.19 mg g^{-1}), as well as high amounts of ascorbic acid in flowers (1.11 mg g^{-1}) (Barros, Carvalho, & Ferreira, 2010). Malvone A, 2-methyl-3-methoxy-5,6-dihydroxy-1,4-naphthoquinone, is a terpene present in flowers which, in addition to antimicrobial activity, can assist in the wound healing process (Pirbalouti, Yousefi, Nazari, Karimi, & Koohpayeh, 2009). The main sugars that compose mucilage are fructose, glucose, sucrose, trehalose and raffinose (Tomoda Gonda, Shimizu, & Yamada, 1989).

Genotype and plant part being used are important variables for chemical constitution of plants (Tabaraki, Yosefi, & Hossein, 2012). Khatami and Ghanati (2011) used *in vitro* cultivation of *M. negleta* for callus production to evaluate the effects of flavonoids, anthocyanins and tannins on UV ray absorption. Quantification of fatty acids in callus culture of plants of the genus *Malva* sp. showed that *M. sylvestris* and *M. parviflora* produce significant amounts of cyclic acids *e.g.*, cyclopropene and cycloprane, which form a biosynthetic system, whereas in other

mallows, the largest production occurs in young seeds (Yano, Nichols, Morris, & James, 1972).

The production system recommended for medicinal plants is based on good cultivation practices and genotypic identity that can meet market demands and provide safe access to products processed as phytotherapeutics (Embrapa, 2015).

Micropropagation is one of the options to qualify the production of seedlings and maintain their genotypic identity associated with the respective phytochemical descriptors (Rout, Samantaray, & Dasa, 2000). According to Kintzios (2002), explants of branches and petioles of *M. sylvestris* are responsive to *in vitro* growth in Murashige and Skoog (1962) (MS) medium, whereas leaves did not allow formation of viable propagules in their various treatments.

Previous research on *in vitro* micropropagation of *Malva sylvestris*, using benzylominopurine (BAP) and naphthaleneacetic acid (NAA), showed higher fresh mass, plant height and number of leaves at the concentrations of 2.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA but without rooting, however (Filter, Freitas, & Périco, 2014). These two growth regulators had been tested for *M. sylvestris* calli, and the best results were found in equimolar combinations of 9 μM, whereas higher concentrations negatively affected callogenesis (Kintzios, Katsouri, Peppes, & Koulocheri, 1998). In studies with *Althea rosea* (Malvaceae), Tyub, Kamili & Bhat (2016) found higher numbers of shoots after adding BAP and NAA, while rooting percentage was higher after use of IAA. In embryogenesis studies and *in vitro* establishment of *M. sylvestris*, Kintzios et al. (1998) found that there was production of callus and metabolites, including substances other than those found in *in vivo* tissues.

Vegetative propagation may also be useful in the phytosanitary management of *M. sylvestris*, because *Puccinia malvacearum* may survive in seeds, and infection may occur at the beginning of the budding of this fungus, which is the major cause of mallow leaves (Gavériaux, 2012). Kuvalekar and Gandhe (2010) found infection by *Uromyces hobsoni*, a fungus that causes rust, after *in vitro* establishment of *Jasminum officinale* var. *grandiflorum*. The occurrence of rust on M. sylvestris may compromise the quality of the plant and derived products, as well as of purity tests. Thus, leaves may present at most one (1) blister-like pustule of *P. malvacearum* teliospore per cm^2 to be considered to have good quality (Brazil, 2000). On the other hand, *in vitro* culture of *M. sylvestris* may be an important technique for cultivation of *P. malvacearum*, a biotrophic fungus which is dependent on living tissues for survival (Agrios, 2005). In addition to contributing to the phytosanitary aspect of seedlings, micropropagation can increase uniformity and large-scale production, thus preserving the standard that increases medicinal interest and use of the plant (Grattapaglia & Machado, 1998).

The goal of this work was to study the effect of combinations of plant growth regulators, indoleacetic acid (IAA) and benzylominopurine (BAP), and to verify the influence of plant growth regulators on the potential supply of calluses and buds for studies on Phytopathology, secondary metabolites and vegetative propagation of different sizes of explants associated with IAA in the introduction of *in vitro* explants of *M. sylvestris*.

2. Method

2.1 Producing Matrices for Propagation and General Study Conditions

Malva sylvestris plants were produced by planting seeds in 600 ml expanded polystyrene (EPS) pots with Agrinobre TMX substrate and keeping them in a greenhouse at a variable temperature for three months. The seeds were selected for planting, excluding those with signs of *P. malvacearum*.

The experiments of introduction and multiplication of mallow were carried out at the Plant Tissue Culture Laboratory, EPAGRI (Experimental Station of EPAGRI (Agricultural Research and Extension Service Agency of Santa Catarina State, Brazil). The experiments were set up in a growth chamber where the flasks were then exposed to a 16-hour photoperiod.

The MS medium (Murashige & Skoog, 1962) with all its salts and vitamins was used, and pH was adjusted to 5.8±0.05 before addition of the solidifier (6 g L^{-1} Merck agar). This medium was supplemented with 30 g L^{-1} of sucrose and plant growth regulators prior to autoclaving, as described in the experiment on combinations of plant growth regulators and different explants and IAA concentrations, in addition to the addition of 0.5 mg L^{-1} activated carbon. The medium was poured into test tubes (25 × 150 mm).

Plants with approximately 90 days of age were used as sources of explants in both experiments. To that end, herbaceous branches of mallow, containing between 30 and 60 centimeters, were excised, washed in running water containing neutral detergent and then washed three times in distilled water.

Asepsis of explants consisted of washing and immersion in sterile distilled water in the two experiments. After this procedure, the aseptic cells were cleaned in a laminar flow chamber, using 70% v/v ethanol for one minute, followed by immersion for 15 minutes in sodium hypochlorite solution (NaClO) containing 1.5% of active principle and Tween® 20 at 10 drops L^{-1}. The buds were then washed four times in sterile distilled water, and remained submerged in sterile distilled water until introduced into the medium. After introduction, the buds were kept in a growth chamber (temperature of 25±2 °C and photoperiod of 16 hours). The tubes were exposed to luminous intensity of 75 μmol m^{-2} s^{-1}, provided by white and cold fluorescent lamps.

2.2 Exp. 1 Combination of Phytoregulators in the in vitro Introduction of Mallow

Axial buds, containing 6 to 8 mm and 2 or 3 leaves (Figure 1), were used as explants in the *in vitro* introduction. The experiment was conducted in a completely randomized design in a 2 × 5 factorial arrangement, with the following factors: absence of IAA and IAA 0.5 mg L^{-1} combined with five (5) levels of BAP concentration. BAP concentrations were 0; 0.5; 1.0; 1.5 and 2.0 mg L^{-1} present in complete MS medium (10 mL $tube^{-1}$), totaling 10 treatments, with 15 replicates in each treatment. The replicates consisted of one (1) explant.

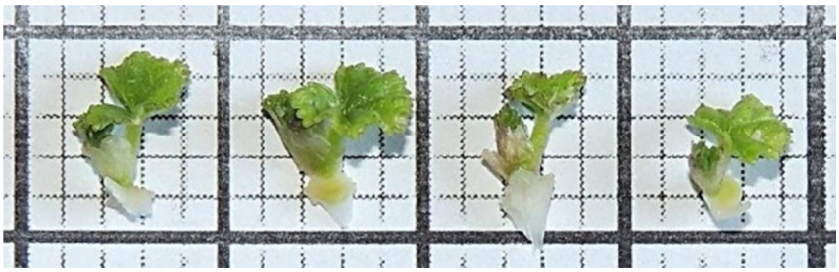

Figure 1. Axillary buds of *Malva sylvestris* used as explants in the *in vitro* introduction in Murashige and Skoog medium

Note. Smaller and larger squares correspond to 2 and 10 mm, respectively.

2.3 Exp. 2 Influence of Different Explant Sizes and IAA Concentrations in the Culture Medium on in vitro Introduction of Mallow

The experiment was conducted in a completely randomized design, and consisted of a 2 × 2 factorial arrangement according to the explant size in use and IAA concentration in the medium. Two concentrations of IAA (0 and 0.5 mg L^{-1}) and two explant sizes, small (4 to 9 mm) and large (14 to 23 mm), were analyzed, as shown in Figure 2. These explants were inserted into the MS medium, containing 2.5 mg L^{-1} of BAP (10 mL $tube^{-1}$), with 4 treatments in total, with a different number of replicates: for the small types with IAA 0.0 and 0.5 mg L^{-1}, 30 and 28 explants, respectively; for large types with IAA 0.0 and 0.5 mg L^{-1} 29 and 32 explants, respectively.

Figure 2. Axillary buds of *Malva sylvestris*. Small (<10mm) (A) and large, (> 10mm) (B), used as explants in the *in vitro* introduction of mallow (*Malva sylvestris*)

Note. Smaller and larger squares correspond to 2 and 10 mm, respectively.

2.4 Evaluations

In vitro evaluations of Experiment 1 were performed at 14 and 28 days for the explants. The evaluations included: count of explants showing bacterial and fungal contamination, rooting (%), oxidation (%) and relative growth rate (RGR) (mm mm d^{-1}). At the 28 days, number of sprouts and callus was recorded.

In Experiment 2, the evaluations performed at 14 days consisted of: number of explants with bacterial and fungal contamination, oxidation (%), establishment (%), RGR and number of shoots. At 28 days, the percentages of establishment, rooting, callus, RGR, discarding and number of shoots were evaluated.

Contamination by bacteria and fungi was evaluated according to the methodology described by Scherwinski-Pereira (2010). Explants that were partially or totally darkened were considered to be oxidized while the explants that were partially or totally greenish, in addition to having emitted sprouts or leaves, were considered to be established. The evaluation of adventitious root production was based on the methodology described by Grattapaglia and Machado (1998). RGR (equation 1) was calculated as described by Briggs, Kid and West (1920):

$$CR = \frac{(LnC_2 - LnC_1)}{(t2 - t1)} \quad (1)$$

where, Ln is the natural/Napierian logarithm of the length values obtained in the different evaluations, and C_2 and C_1 indicate growth (in millimeters) measured at t2 (final) and t1 (initial) times, respectively.

2.5 Data Analysis

The bacterial and fungal contamination, oxidation, rooting, establishment, disposal and callus were analyzed by using the Binomial model with the logit function. Deviance analysis was performed with the chi-square test. The number of shoots was analyzed using the Poisson model, with a superdispersion (quasipoisson) parameter, and deviance analysis was performed with the F-test. For RGR, normal distribution was used, and the Box-Cox transformation was performed in cases where the assumptions of the model were not met; in this case, normality was verified through the Shapiro-Wilk test and homoscedasticity, using the Bartlett test. Tukey's contrasts were used to compare treatment levels using the procedure for testing linear hypotheses described by Hothorn, Bretz, and Westfall (2008). All tests were performed at the 5% level of significance. The procedures were implemented in the R (R Core Team, 2017) environment.

3. Results and Discussion

Average establishment of *in vitro* explants at 28 days reached 32.76% for BAP concentrations associated with 0.0 mg L^{-1} of IAA and 32.14% when associated with 0.5 mg L^{-1} of IAA, without interaction between treatments (Table 1). In this period, calluses were formed (Figures 3C and D) in 62.50% of the explants at the different

concentrations of BAP and IAA 0.0 mg L^{-1} and in 63.79% of BAP and 0.5 mg L^{-1} IAA, respectively. High percentages of callogenesis were also found by Sié et al. (2010) in *Hibiscus sabdariffa* L., another species of the family Malvaceae, regardless of type of explant in use and concentrations of growth regulators in use.

Table 1. Establishment and growth of *Malva sylvestris* explants in *in vitro* culture in Murashige and Skoog environments, with different concentrations of plant growth regulators, 2017

IAA (mg L^{-1})	BAP (mg L^{-1})	EST (%)	CALLUS (%)	BUD (n)	RGR[1] (mm mm d^{-1})	
					14° day	28º day
0.0	0.0	50.00	87. 50	1.88	0.013	0.017
	0.5	36.36	63.64	1.73	0.013	0.018
	1.0	18.18	45.45	1.91	0.016	0.008
	1.5	30.77	53.85	2.54	0.021	0.015
	2.0	33.33	73.33	2.27	0.031	0.012
Mean		32.76 ^{ns}	63.79 ^{ns}	2.10 ^{ns}	0.018 b	0.014 ^{ns}
0.5	0.0	30.77	69.23	2.38	0.021	0.016
	0.5	36.36	45.45	2.55	0.022	0.015
	1.0	45.45	63.64	2.73	0.035	0.013
	1.5	30.00	70.00	2.20	0.028	0.011
	2.0	18.18	63.64	2.27	0.029	0.010
Mean		32.14^{ns}	62.50 ^{ns}	2.43 ^{ns}	0.027 a	0.013 ^{ns}

Note. Means followed by the same letter in the column of each variable do not differ significantly from each other by deviance analysis ($p \leq 0.05$); ns: non-significant; BAP: abscisic acid; IAA: indoleacetic acid; EST: establishment; BUD: budding; RGR: relative growth rate; 1 transformed variable (lambda = 0.3).

Number of shoots per explant ranged from 1.73 to 2.73 with different associations of plant growth regulators (Table 1). The RGR of the explants in the association of BAP with 0.5 mg L^{-1} of IAA was higher than that with 0.0 mg L^{-1} of IAA at 14 days, and there was no difference between the treatments performed at 28 days.

Contamination and oxidation were the major impact factors in the establishment of *in vitro* explants. Bacterial and fungal contamination may be endogenous or exogenous (Ray & Ali, 2017). In the case of *M. sylvestris,* bacterial contamination at 28 days (Table 2) was twice as much (60.53%) the rate found at 14 days (28.97%), suggesting that the explants had endogenous contamination.

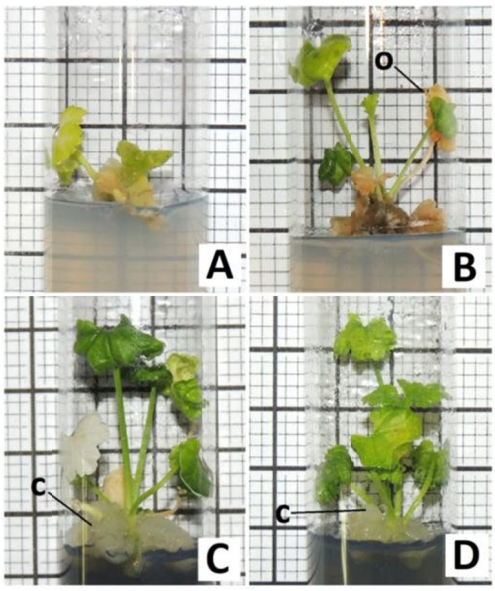

Figure 3. *Malva sylvestris* explants introduced *in vitro* in Murashige and Skoog (MS) medium supplemented with different concentrations of growth regulators, 2017

Note. Explant partially oxidized after 14 days *in vitro* (A); explants with some oxidized shoots (o) after 28 days *in vitro* (B); explants that produced calluses at the base after 28 days *in vitro* (C and D); o, oxidation; c, callus. Smaller and larger squares correspond to 2 and 10 mm, respectively.

Scherwinski-Pereira (2010) reported that *in vitro* cultures may develop late bacterial growth, as they often contain endophytic or fastidious microorganisms. The occurrence of endophytic microorganisms in medicinal plants has been reported in studies that were aimed at verifying whether or not there is a risk of toxin presence and the possibility of producing bioactive compounds from these microorganisms (Kaul, Gupta, Ahmed, & Dhar, 2012; Mussi-Dias, Araújo, Silveira, Rocabado, & Araújo, 2012).

There was no significant effect of the interaction between growth regulators IAA and BAP on bacterial, fungal and oxidation contamination (Table 2). Fungal contamination of 22.81% at 28 days was higher than on the first evaluation date and corresponded to the percentage of explant discard in this experiment. Mussi-Dias et al. (2012) evaluated fungal endophytic microbiota in eleven species of medicinal plants and found seven different species, and stressed the importance of their presence in the plant without expression of disease sypmtoms.

Another factor that can lead to loss of explants is explant oxidation (Figures 3A and 3B). Final oxidation percentage was 7.6 times higher than initial oxidation at 14 days (Table 2).

There was no rooting of the explants during the 28-day period after use of different concentrations of IAA and BAP.

Table 2. Bacterial and fungal contamination and oxidation of *Malva sylvestris* explants at 14 and 28 days of *in vitro* culture in Murashige and Skoog (MS) medium, at different concentrations of plant growth regulators, 2017

BAP (mg L^{-1})	IAA (mg L^{-1})					
	14 days BC (%)			28 days BC (%)		
	0.0	0.5	Mean	0.0	0.5	Mean
0	28.57	33.33	31.03	37.50	53.85	47.62
0.5	26.67	20.00	23.33	63.64	54.55	59.09
1.0	40.00	21.42	31.03	72.72	63.64	68.18
1.5	40.00	23.08	32.14	61.54	70.00	65.22
2.0	26.67	28.57	27.59	60.00	63.64	61.54
Mean	32.43^{ns}	25.35 ^{ns}	28.97 b	60.34 ^{ns}	60.71 ^{ns}	60.53 a
	14 days FC (%)			28 days FC (%)		
0	14.29	6.67	10.34	46.67	13.33	30.00
0.5	13.33	13.33	13.33	26.67	20.00	23.33
1.0	20.00	0.00	10.34	26.67	26.67	26.67
1.5	13.33	15.38	14.28	13.33	33.33	23.33
2.0	6.67	14.29	10.34	0.00	21.42	10.34
Mean	13.51 ^{ns}	9.86 ^{ns}	11.72 b	22.67 ^{ns}	22.97 ^{ns}	22.81 a
	14 days OX (%)			28 days OX (%)		
0	0.00	0.00	0.00	25.00	30.77	28.57
0.5	0.00	6.67	3.33	0.00	18.18	9.09
1.0	6.67	0.00	3.45	9.09	18.18	13.64
1.5	0.00	7.69	3.57	7.69	10.00	8.70
2.0	0.00	0.00	0.00	13.33	27.27	19.23
Mean	1.35 ^{ns}	2.82 ^{ns}	2.07 b	10.34 ^{ns}	21.43 ^{ns}	15.79 a

Note. Means followed by the same letter in the row of each variable do not differ significantly from each other ($p \leq 0.05$); ns: non-significant; ABA: abscisic acid; IAA: indoleacetic acid; BC: bacterial contamination; FC: fungal contamination; OX: oxidation.

In the experiment with different concentrations of IAA and explant sizes, budding of *M. sylvestris* was detected in the evaluation at 14 days (Figures 4A and 4C). At 28 days, there were completely formed leaves and different root branches (Figures 4B, 4D, 4E, and 4F).

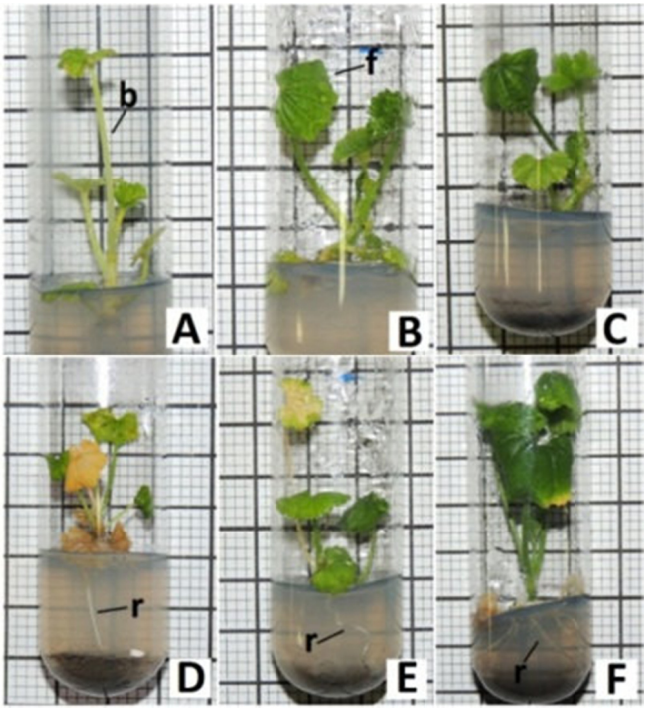

Figure 4. *Malva sylvestris* explants introduced *in vitro* in Murashige and Skoog (MS) medium supplemented with different concentrations of growth regulators

Note. After 14 days *in vitro* (A and C); after 28 days *in vitro* (B, D, E, F); b, shoot; f, fully formed leaves; r, roots. Smaller and larger squares correspond to 2 and 10 mm, respectively.

At 14 days of *in vitro* introduction, there was no significant interaction effect between IAA concentrations and different explant sizes for bacterial and fungal contamination. In this period, however, fungal contamination was found to be significantly higher in the larger explants (48.33%) than in the smaller ones (25.56%), regardless of IAA concentrations (Table 3).

According to Grattapaglia and Machado (1998), larger explants present a greater tendency to *in vitro* microbial contaminations because there is a greater amount of established *ex vitro* material; the larger the explant in use, the greater the tendency for disinfectant substances to permeate all regions of the explant, which will hence lead to *in vitro* contamination more easily (Scherwinski-Pereira, 2010).

There was no difference between explant oxidation and average establishment for either small or large explants (Table 3).

Table 3. Interference of IAA and different explant sizes for contamination, oxidation, establishment and growth of *Malva sylvestris* after 14 days of *in vitro* culture in Murashige and Skoog medium, 2017

Size (mm)	IAA (mg L^{-1})	BC (%)	FC (%)	OX (%)	EST (%)	BUD (n)	RGR[1] (mm $mm d^{-1}$)
4-9	0.0	43.33	16.67	13.33	76.67	3.03 a	0.021
	0.5	33.33	33.33	14.81	81.48	2.59 a	0.018
Average		38.60 ^{ns}	25.56 b	14.03 ^{ns}	78.95 ^{ns}	2.82	0.020 a
14-23	0.0	34.48	44.83	20.69	75.86	2.41 b	0.012
	0.5	22.58	51.61	22.58	83.87	3.13 a	0.010
Average		28.33 ^{ns}	48.33 a	21.67 ^{ns}	80.00 ^{ns}	2.78	0.011 b

Note. Means followed by the same letter in the column of each variable do not differ significantly from each other ($p \leq 0.05$); ns: non-significant ($p \leq 0.05$). SIZE: size; IAA: indoleacetic acid; BC: bacterial contamination; FC: fungal contamination; OX: oxidation; EST: establishment; BUD: budding; RGR: relative growth rate; 1 transformed variable (lambda = 0.3).

There was a significant effect of the interaction between IAA concentrations and explant size for the budding variable. The use of 0.5 mg L^{-1} of IAA favored an increase in the number of shoots per explant when segments of 14 to 23 mm were used.

Relative growth rate was higher for small explants (4 to 9 mm) than for large (14 to 23 mm) explants in this first evaluation period.

At 28 days, 55.74% of the large explants and 41.07% of the small explants were discarded. In the remaining explants, it was found that 100% and 86.11% of the larger and smaller ones, respectively, were established, with a rooting rate of 12.5% for the largest and 2.78% for the smaller ones (Table 4). Filter, Freitas and Périco (2014) reported success with BAP and NAA (naphthaleneacetic acid) for micropropagation of *M. sylvestris*, but without rooting of explants. In the present research, there was more callus formation when small explants (58.33%) were used in comparison to large explants (34.35%).

In our study, it was found that larger explants developed more efficiently *in vitro* than smaller explants. Number of shoots per explant was found to be higher when explants with 14 to 23 mm length were used in comparison to explants of 4 to 9 mm.

Table 4. Interference of phytoregulators and different sizes of explants in the establishment and growth of *Malva sylvestris* explants after 28 days of *in vitro* culture in Murashige and Skoog medium, 2017

Size (mm)	IAA (mg L^{-1})	DISC (%)	EST (%)	ROOT (%)	CALLUS (%)	BUD (n)	RGR[1] (mm mm d^{-1})
4-9	0.0	40.00	95.00	5.00	45.00	3.95	0.011 b
	0.5	42.30	75.00	0.00	75.00	3.98	0.016 a
Mean		41.07 ^{ns}	86.11^{ns}	2.78 ^{ns}	58.33 a	3.69 b	0.014
14-23	0.0	55.17	100.00	13.33	33.33	4.53	0.063 a
	0.5	56.25	100.00	11.76	35.29	4.53	0.030 a
Mean		55.74 ^{ns}	100.00	12.50 ^{ns}	34.38 b	4.53 a	0.045

Note. Means followed by the same letter in the column of each variable do not differ significantly from each other ($p \leq 0.05$); for the RGR variable the comparisons refer to the different levels of IAA in each size; ns: non-significant ($p \leq 0.05$); TAM: size; IAA: indoleacetic acid; DISC: discard; EST: establishment; ROOT: rooting; BUD: budding; RGR: relative growth rate; 1: transformed variable (lambda = 0.12).

For *in vitro* establishment of mallow, an interaction occurred ($p \leq 0.05$) between the IAA concentrations and the RGR values. It was found that IAA stimulated faster tissue growth in explants that were 4 to 9 mm in length, but the same effect did not occur in the larger explants.

Souza, Schuch, da Silva, Ferri, and Soares (2007) also found that larger explants of Brazilian cherry (*Eugenia uniflora*, Myrtaceae) presented better *in vitro* establishment and development results than smaller explants. According to Hartmann, Kester, and Davies (1990), larger explants usually present larger amounts of nutritional reserves, especially in the form of complex or easily assimilated carbohydrates, which can favor the greater or more rapid development in the establishment *in vitro*.

The present data showed that when aiming to develop the protocol of *in vitro* production of *M. sylvestris* by organogenesis, one should choose to introduce larger explants, because they produce a greater number of shoots more quickly. On the other hand, if one's objective is to develop the protocol by embryogenesis, one should use of smaller explants, since they develop 70% more calluses when compared to larger explants.

4. Conclusion

Micropropagation of *M. sylvestris* is viable and occurs the development of shoot and root of the plant.

Explant size influences the development of *M. sylvestris*. The use of explants of 4 to 9 mm allows greater formation of calluses and explants with a size ranging from 14 to 23 mm produce a greater number of shoots.

The addition of 0.5 mg L^{-1} IAA in the MS medium positively affects the plant growth rate of the small explants, which was similar to that of the large ones, at 28 days.

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