MICROPROPAGATION OF *Syngonanthus elegantulus**

Micropropagação de *Syngonanthus elegantulus**

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ABSTRACT

The species *Syngonanthus elegantulus* Ruhland is an important native plant used as cut flowers, but extractive exploration has caused the endangered status of this plant. Therefore, the objective of this study was to establish a protocol of the micropropagation of *S. elegantulus*. To this end, seed germination was tested in media containing different concentrations of WPM salts. For plantlet establishment, MS and WPM media were evaluated with 50 and 100% of the salt concentrations. Different the sucrose levels in the WPM medium were also evaluated. For multiplication, the plantlets were inoculated in WPM media with 0.0, 0.5, 1.0, 2.0 and 4.0 mg L^{-1} TDZ and 0.0, 0.5 and 1.0 mg L^{-1} NAA in all possible combinations. The pre-acclimatization effect was tested in the plantlets using the following substrates: sand, Plantmax™ and vermiculite. The concentration of the medium did not influence the percentage of germination, but the germination speed index was inversely proportional to the nutrient addition to the culture medium. The best medium for plantlet establishment was WPM at the original salt concentration plus 17 g L^{-1} sucrose. The addition of 0.5 and 1.0 mg L^{-1} NAA is recommended for callus induction. The highest number of shoots was obtained in the presence of 1.0 mg L^{-1} TDZ or the combination of 1.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA. *S. elegantulus* presented higher survival when acclimatized directly in sand.

Index terms: *In vitro* cultivation, floriculture, conservation, endangered, plant.

RESUMO

A espécie *Syngonanthus elegantulus* Ruhland é uma importante planta nativa usada como flor de corte, mas sua exploração extrativista tem causado risco de extinção. Assim, neste estudo, objetivou-se estabelecer um protocolo de micropropagação de *S. elegantulus*. Para isso, a germinação das sementes foi testada em meio WPM, contendo diferentes concentrações de sais. Para o estabelecimento de plântulas, os meios MS e WPM foram avaliados com 50% e 100% de concentração de sais. Diferentes níveis de sacarose em meio WPM também foram avaliados. Para a multiplicação de plântulas, foram inoculadas em meio WPM adicionado de 0,0, 0,5, 1,0, 2,0 e 4,0 mg L^{-1} de TDZ e 0,0, 0,5, 1,0 mg L^{-1} de NAA em todas as combinações possíveis. O efeito da pré-aclimatização de plântulas foi testado nos substratos areia, plantmax™ e vermiculite. A concentração do meio não influenciou a porcentagem de germinação, mas o índice de velocidade de germinação foi inversamente proporcional à adição de nutrientes. O melhor meio para o estabelecimento de plântulas foi o WPM, na concentração de sais originais, adicionado a 17 g L^{-1} de sacarose. Para a indução de calo, a adição de 0,5 e 1,0 mg L^{-1} de NAA foi recomendada. O maior número de brotos foi obtido na presença de 1,0 mg L^{-1} de TDZ ou combinando 1,0 mg L^{-1} de TDZ com 0,5 mg de L^{-1} de ANA. *S. elegantulus* apresentaram maior sobrevivência quando aclimatadas diretamente na areia.

Termos para indexação: Cultivo *in vitro*, floricultura, conservação, planta em extinção.

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INTRODUCTION

In the rupestrian area of Serra do Espinhaço, Minas Gerais State, approximately 379 native species of the Eriocaulaceae family are found, with approximately 18% belonging to *Syngonanthus* (NUNES et al. 2008a; COSTA; TROVÓ; SANO, 2008). *Syngonanthus elegantulus* Ruhland is one of the most exploited native species for ornamental purposes because the plants produce long flower stems with a small terminal capitulum composed of white bracts (COSTA; TROVÓ; SANO, 2008). The flower stems of this genus retain their ornamental characteristics after long storage periods without use of preservation solutions or temperature control.

The flowers of genera *Syngonanthus* are important for marketing as cut flowers in the state of Minas Gerais and are used as decoration or for handicraft purposes (LANDGRAF; PAIVA, 2009a; NÉRI; PAIVA, 2012). Furthermore, most of the flowers are exported to European countries, the USA and Japan (NERI; PAIVA; BORÉM, 2005; LANDGRAF; PAIVA, 2009b). However, the constant extractive exploitation of these plants has

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contributed to the risk of their extinction, and they are listed as endangered plants (COSTA; TROVÓ; SANO, 2008).

In Brazil, techniques, including tissue culture, have been used for the propagation of native plants that are rare or endangered. Although studies have been conducted to establish culture for *Syngonanthus mucugensis* (PAIXÃO-SANTOS et al., 2003; SILVA et al., 2005a; PAIXÃO-SANTOS et al., 2006; PAIXÃO-SANTOS et al., 2008; LIMA-BRITO et al., 2011a; LIMA-BRITO et al., 2011b), such efforts have not been attempted to date for *S. elegantulus*.

It is known that *S. mucugensis* plantlets are sensitive to salt concentration variations in culture medium, and high concentrations of solutes inhibit seed germination and plantlet growth (PAIXÃO-SANTOS et al., 2003; SILVA et al., 2005a, LIMA-BRITO et al., 2011a). Other studies for the micropropagation of *S. mucugensis* have show that, for the mass production of plants *in vitro* using MS culture medium (MURASHIGE; SKOOG, 1962), it is necessary to include such cytokinins as 6-benzilaminapurine (BAP), whereas the production of friable calli are obtained in the presence of auxins, such as alpha-naphthyl acetic acid (PAIXÃO-SANTOS et al., 2008; LIMA-BRITO et al., 2011b).

Because Brazilian research on the micropropagation of species of genus *Syngonanthus* has been restricted to *S. mucugensis*, with no report on the micropropagation of *S. elegantulus*, the objective of this work was to determine the characteristics of *S. elegantulus* seed germination, establishment, *in vitro* multiplication and acclimatization.

MATERIAL AND METHODS

Floral stems of *Syngonanthus elegantulus* were manually collected in areas of natural occurrence in the rupestrian areas of Serra do Espinhaço, Diamantina-MG. After collection, the capitula were dried under shade at ambient temperature for seven days and then stored for further studies.

Salt concentrations in culture media for *in vitro* seed germination

Lots of 50 *S. elegantulus* seeds were used to evaluate germination. The seeds were sterilized with 70% alcohol for one minute, followed by immersion in sodium hypochlorite (1% active chlorine) for 10 minutes and three washes in distilled water. The seeds were placed on the WPM culture medium of Lloyd and McCown (1980) at salt concentrations of 25, 50, 75 and 100%, with a control consisting of deionized water. The media included 15 g L^{-1} sucrose and were solidified with 8 g L^{-1} agar; the pH was adjusted to 5.8 before autoclaving under a pressure of 1.5 atm and temperature of 120° C for 20 minutes. The flasks were incubated in a growth chamber for 30 days under an irradiance of 43 μmol $m^{-2}s^{-1}$ and a 16 h photoperiod at 25 ± 2° C, and the germination percentage was then calculated. During this 30 day period, the number of germinated seeds was recorded daily, and the germination speed index (GSI) was calculated according to the following equation: $GSI = G_1/N_1 + G_2/N_2 + ... + G_n/N_n$, in which G_1, G_2 and G_n are the number of seeds germinated in the first, second and up to the last count and N_1, N_2, N_n are the number of inoculation days for the first, second and up to the last count (MAGUIRE, 1962). The data were subjected to polynomial regression with the aid of the Sisvar statistical software (FERREIRA, 2011).

Culture medium and salt concentration for plantlet establishment

To evaluate the *in vitro* development of *S. elegantulus* on different culture media, 1.0 cm plantlets were used. MS and WPM media were tested with 50 and 100% of the salt concentrations, with both including 15 g L^{-1} sucrose and solidified with 8 g L^{-1} agar; the pH was adjusted to 5.8 before autoclaving at a pressure of 1.5 atm and temperature of 120° C for 20 minutes. The inoculated plantlets were maintained for 30 days in a growth chamber under an irradiance of 43 μmol $m^{-2}s^{-1}$ and a 16 h photoperiod at 25±2° C. The experimental design was completely randomized in a 2x2 factorial (two culture media and two salt concentrations), with four replicates, three tubes per plot and one explant per tube. The number of leaves, number of chlorotic leaves, shoot length and fresh mass weight of the plantlets were evaluated. The data were subjected to an analysis of variance, and the means were compared by the Tukey test at a 5% probability.

Influence of sucrose level on the *in vitro* development of plantlets

To evaluate the effect of sucrose on the *in vitro* development of *S. elegantulus*, plantlets 1.0 cm in length were inoculated in WPM medium supplemented with 0, 5, 10, 15, 20, 25 or 30 g L^{-1} sucrose and solidified with 6 g L^{-1} agar; the pH was adjusted to 5.8 before autoclaving for 20 minutes at a pressure of 1.5 atm and temperature of 120° C. The inoculated plantlets were incubated for 30 days in a growth chamber under an irradiance of 43 μmol m^{-2} s^{-1} and a 16 h photoperiod at 25±2° C. The experimental design was completely randomized, with six replicates, three tubes

per plot and one seedling per tube. The number of leaves, shoot length, number of roots and fresh mass weight of the plantlets were evaluated. The data were subjected to polynomial regression with the aid of the Sisvar statistical software (FERREIRA, 2011).

Effect of the cytokinin thidiazuron (TDZ) and auxin 1-naphthaleneacetic acid (NAA) on plantlet multiplication

Whole plantlets were used as the explants and inoculated on WPM medium supplemented with the cytokinin TDZ (0.0, 0.5, 1.0, 2.0 or 4.0 mg L^{-1}) and the auxin NAA (0.0, 0.5 or 1.0 mg L^{-1}) in all possible combinations. The media included 15 g L^{-1} sucrose and were solidified with 8 g L^{-1} agar. The pH was adjusted to 5.8 prior to autoclaving. The material was maintained in a growth chamber under an irradiance of 43 µmol $m^{-2}s^{-1}$ and a 16 h photoperiod at 25±2° C. The experimental design was completely randomized in a factorial 5 (TDZ concentration) x 3 (NAA concentration), with five replicates, three tubes per plot and one explant per tube. After 30 days, the percentage of explants showing callus induction and the number of sprouts were assessed. The data were subjected to an analysis of variance and the Scott-Knott test (5% probability).

Pre-acclimatization of plantlets on different substrates

The plantlets used in the acclimatization process had approximately 16 leaves and were 3 cm in length. The effects of sand, vermiculite and Plantmax™ substrates on the acclimatization of the micropropagated plantlets, subjected or not to a pre-acclimatization period, were studied. The pre-acclimatization of the plantlets consisted of removing them from the culture medium, washing the roots in distilled water and then transferring them to glass flasks of 250 mL containing 50 ml sand, vermiculite or Plantmax™, all moistened with 15 ml of WPM medium liquid. The flasks were incubated in a growth chamber under an irradiance of 43 µmol $m^{-2}s^{-1}$ and a 16 h photoperiod at 25±2° C and remained sealed for three days; thereafter, the flasks were opened and maintained under the same conditions for five additional days. After this period, the plantlets were transferred to tubes filled with the different substrates. Direct acclimatization was performed by removing the plantlets from the test tubes and transferring them to tubes filled with the substrates without pre-acclimatization. The tubes with the plantlets were covered with transparent plastic bags to maintain the relative humidity and were perforated each week until their complete removal at 30 days after the acclimatization process. The samples were incubated in a growth chamber under an irradiance of 67 µm $m^{-2}s^{-1}$ and a 16 h photoperiod at 25±2° C.

The experiment design was completely randomized in a 2x3 factorial (acclimatization forms x substrate), with 15 replicates. The proportion of live plantlets after 30 days was evaluated. A statistical analysis was performed using the generalized linear model (GLM) methodology with the R statistical package. The presence or absence of live plantlets had a binomial distribution, thus the logistic link function was used as a linear predictor statistic.

RESULTS AND DISCUSSION

There was a slight tendency of a reduction in seeds germination when the salt concentration was increased, but the concentration was not limiting for the *in vitro* germination of *S. elegantulus* seeds (Figure 1). However, the medium concentration did affect the physiological performance of the seeds because those seeds inoculated in media with low salt concentrations begin the germination process more rapidly, stabilizing at 20 days after inoculation. The seeds inoculated on media containing salt concentrations of 50%, 75% and 100% stabilized germination by 25 days after inoculation.

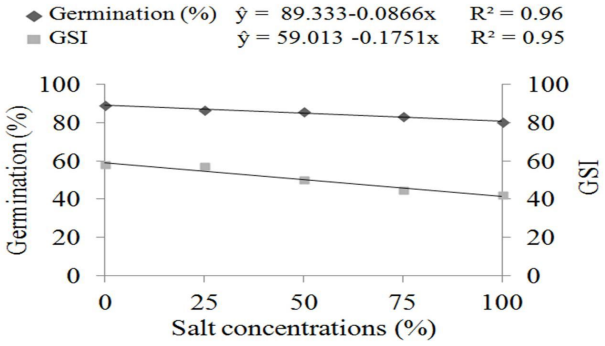

Figure 1 – Germination percentage and germination speed index (GSI) of *S. elegantulus* seeds at different salt concentrations in WPM medium.

Traditional tests using paper as a substrate found that *S. elegantulus* seeds reached a maximum germination of only 36.5% (OLIVEIRA; GARCIA, 2005). Working with *S. elegans*, Nunes et al. (2008a) obtained 35% germination using seeds of the highest physiological quality.

A higher germination speed index was observed in the medium consisting of only water or in the presence of 25% salts in WPM medium (Figure 1). The germination

speed index was significantly lower for the seeds germinated in media with 75% and 100% salt concentration, corresponding to 44 and 42, respectively. Paixão-Santos et al. (2003) found that the germination of *S. mucugensis* is inhibited by high salt concentrations.

The germination speed index can be used to determine the sensitivity of seeds to salt concentrations and to determine the germination conditions that allow the expression of the greatest physiological potential of seeds (BRASIL, 2009). Silva et al. (2005b) reported that the salt concentration and the addition of growth regulators and carbohydrate sources determine the *in vitro* cultivation of *S. mucugensis*. Paixão-Santos et al. (2003) found that the germination of *S. mucugensis* was possible only in a culture medium without the addition of salts, with the germination speed index being 22 after 30 days. When compared with the traditional paper substrate techniques presented by Oliveira and Garcia (2005) and Nunes et al. (2008a), the micropropagation technique utilized in our study was more effective to increase the germination speed index of *Syngonanthus* sp.

The effect of the salt concentration of the culture medium on plantlet development is shown in table 1.

A larger number of leaves was formed in WPM when compared with the MS medium at full strength (100%). There was no difference in the formation of leaves when the plantlets were cultured at the different concentrations of WPM medium, but the different salt concentrations in the MS medium did influence leaf formation, which was reduced when the medium was 100% of salt concentrations.

The *S. elegantulus* plantlets grown on MS medium with 50% of the salt concentration had a low number of leaves with toxicity symptoms, as characterized by the presence of chlorotic leaves, whereas the original concentration caused damage to the plantlets *in vitro* (Table 1). Leaf chlorosis was observed from the 15th day after plantlet inoculation (Figure 2), with the occurrence of spots that progressed to total necrosis of the leaves. The symptoms were more pronounced in the plantlets propagated in complete MS medium (100%) due to the high salt concentration in this medium. Such symptoms were not evident in the plantlets grown in WPM.

Similar to the observations in *S. mucugensis*, leaf formation during the establishment of *S. elegantulus* seedlings was inhibited when the plantlets were cultured in full-strength MS medium (PAIXÃO-SANTOS et al., 2006). These authors observed that the complete MS medium was inadequate for the *in vitro* production of *S. mucugensis* because higher shoot lengths and biomass were obtained when these plantlets were grown in MS medium at 50% or 33% of the original concentration.

The height of the plantlets did not change when they were grown in 50% and 100% WPM; however, differences were observed in the plantlets grown MS media with 100% of the salt concentration (Table 1). The fresh mass weight of the *S. elegantulus* plantlets was influenced by the salt concentration, being higher in the plantlets grown on WPM medium (50 and 100%) and lower in the plantlets grown on MS medium.

WPM medium is more frequently recommended for woody plants, whereas MS medium is often used for herbaceous plants; however, although *S. elegantulus* is herbaceous, its plantlets developed best in the WPM medium. The WPM medium contains higher levels of PO_4^{3-}, SO_4^{2-} and Mg^{2+} but lower NO_3^- and NH_4^+ levels in relation to the media used for herbaceous plants (ANTONIDAKI-GIATROMANOLAKI et al., 2010). According Nunes et al. (2008b), *S. elegantulus* is adapted to low-fertility sandy soils, which explain the better performance in WPM medium due to the lower demand of nutrients for adequate development.

Table 1 – Number of leaves, chlorotic leaves, plantlet height and fresh mass of *S. elegantulus* grown at different salt concentrations in MS and WPM culture media.

	Number of leaves		Chlorotic leaves		Plantlet height (cm)		Fresh mass (g)	
Concentration	MS	WPM	MS	WPM	MS	WPM	MS	WPM
50%	13.6 Ab*	17.0 Aa	2.4 Ba	0.3 Aa	2.85 Aa	3.14 Aa	0.08 Ab	0.11 Aa
100%	9.4 Bb	17.5 Aa	7.0 Aa	0.7 Ab	1.94 Bb	3.50Aa	0.04 Bb	0.11 Aa

*The same capital letters for the salt concentrations and lowercase letters for the culture media do not differ (P <0.05) by Tukey's test.

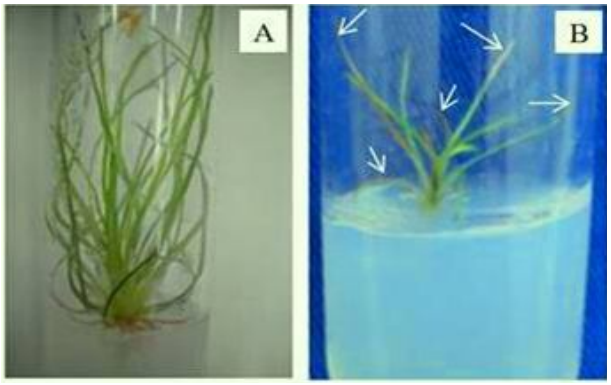

Figure 2 – Visual characteristics of *S. elegantulus* plantlets grown on WPM (A) and MS (B) media.

The number of leaves formed by *S. elegantulus* tended to increase with the addition of sucrose to the culture medium: the plantlets presented approximately fifteen leaves when grown in media with 30 g L^{-1} sucrose (Figure 3). However, increasing the sucrose concentration inhibited the rooting of the plantlets, and a maximum of 3 roots was observed without any sucrose in the culture medium, whereas only 1.2 roots were formed with the addition of 30 g L^{-1}.

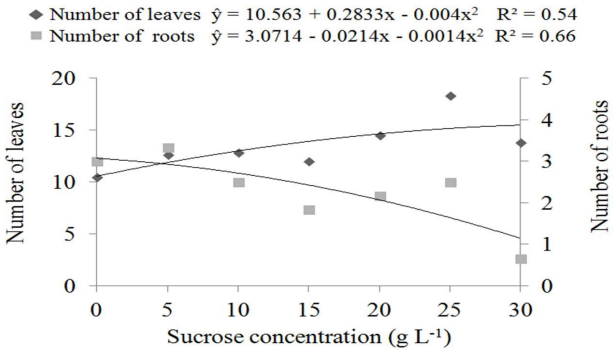

Figure 3 – Number of leaves and roots in *S. elegantulus* plants cultivated on WPM medium supplemented with different sucrose concentrations.

The highest proportion of leaves and fresh mass of *S. mucugensis* plantlets were obtained when micropropagated in media with 15 g L^{-1} sucrose (PAIXÃO-SANTOS et al., 2003, Silva et al., 2005a). As observed in this study, low sucrose levels stimulated the production of roots in *S. mucugensis* (PAIXÃO-SANTOS et al., 2005). Although the addition of sucrose in nutrient medium is a common practice for tissue culture, the aspects related to the plant osmotic potential of the culture medium must be considered (LIMA-BRITO et al., 2011a). These authors observed that the osmotic effect caused by high concentrations of sucrose, sorbitol and mannitol, between 40 and 60 g L^{-1}, inhibited root development.

The fresh mass production was affected by an increase in the sucrose concentration and was higher in the plantlets grown on the culture medium supplemented with 30 g L^{-1} sucrose (Figure 4). Plantlets with the greatest length were obtained in culture media with 17 g L^{-1} sucrose.

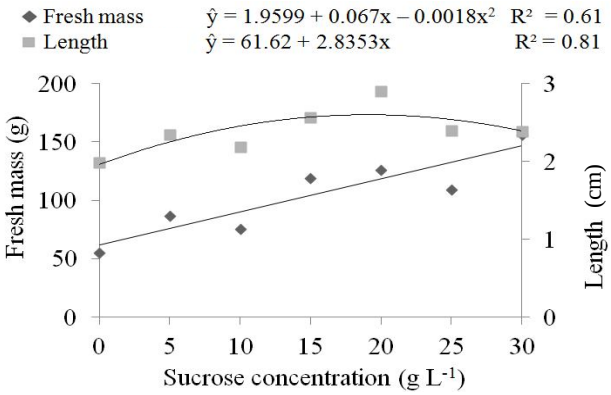

Figure 4 – Fresh mass and plant length of *S. elegantulus* plantlets cultivated on WPM medium supplemented with different sucrose concentrations.

The results presented in this work agree with Silva et al. (2005a), who observed that the addition of 15 to 20 g per L^{-1} sucrose promoted the highest fresh mass production in micropropagated *S. mucugensis* plantlets. Similarly, working with the same species, Lima-Brito et al. (2011a) achieved longer plantlets when 15 g L^{-1} sucrose was added to the culture media, reporting that the adjustment of the carbohydrate level in the culture medium is important for the establishment of plantlets and the subculturing frequency.

The use of different TDZ and NAA concentrations was effective for callus induction, which was not formed in absence of growth regulators (Figure 5). Callus induction occurred exclusively in the roots, with oxidation and the subsequent death of the shoot. The use of media containing 0.5 mg L^{-1} or 1.0 mg L^{-1} NAA was more efficient for callus formation in *S. elegantulus*; similarly, media with 1.0 mg L^{-1} or 2.0 mg L^{-1} TDZ resulted in the formation of callus. Callus formation was also efficient in the media with 1.0 mg L^{-1} NAA in combination with 0.5 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA plus 1.0 mg L^{-1} TDZ.

Lima-Brito et al. (2011b) observed that the exposure of *S. mucugensis* stem and leaf explants to 0.5 mg L^{-1} NAA resulted in the formation of non-friable calli, with a white coloring and spongy texture and no shoot regeneration. However, the induction of callus on leaf explants can occur by adding any growth regulator to the culture medium, as observed by Paixão-Santos et al. (2006). However, in the present work no callus was observed on the *S. elegantulus* leaf explants and was only observed on the root explants.

Thirty days after the inoculation of the explants, it was observed that, regardless of the concentration, the callus tissue induced with media including TDZ showed a green coloration, which did not occur in the absence of this regulator. After 60 days of callus induction, the green callus tissue differentiated, forming sprouts. The induction of new plants occurred with the use of 1.0 mg L^{-1} TDZ or 1.0 mg L^{-1} TDZ combined with 0.5 mg L^{-1} NAA (Figure 5). Lima-Brito (2011b) related that, in the unorganized surface of *S. mucugensis* callogenic tissue, organization of the meristem was observed with the subsequent appearance of green areas dots corresponding to sprout formation, which then differentiated into shoots. For *S. elegantulus*, we obtained the best shoots formation using a cytokinin / auxin ratio equal to one.

Acclimatization was affected by the substrate and also by the pre-acclimatization process, with the best acclimatization occurring in the plants acclimatized on sand for which a proportion of 0.74 of the plantlets survived. Lower proportions of 0.43 and 0.29 were obtained with plants acclimatized on Plantimax™ and vermiculite, respectively (Figure 6A).

Analyzing the pre-acclimation process, it was observed that, after 30 days, direct acclimatization resulted in a proportion of 0.66 surviving plantlets compared to 0.35 for the pre-acclimatized plants (Figure 6B). The acclimatization process is a critical step in the micropropagation of *S. elegantulus* and is a determining factor in plant survival.

Paixão-Santos et al. (2003) reported that the propagation of *S. mucugensis* plantlets in substrates different from those found in the natural environment was not possible. The high proportion of acclimatization observed for *S. elegans* in sand is because this species is adapted to sandy soils, such as those in its natural areas of occurrence (NUNES et al. 2008c). Paixão-Santos et al. (2003) studied *S. mucugensis* and observed that the survival rate of this species is normally very low when subjected to transplantation, yet there are no reports on acclimatization in these plants.

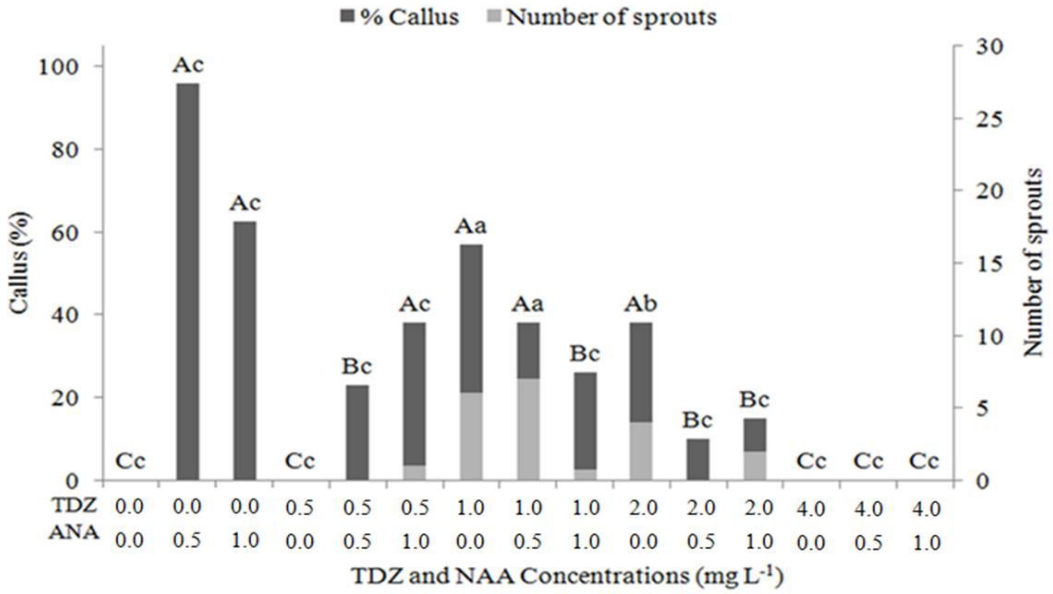

Figure 5 – Percentage of induced calli and number of shoots formed for *S. elegantulus* explants with the addition of TDZ and NAA to the culture medium. The means followed by same capital letters for the percentage of induced explants and small letters for the callus percentage did not differ by the Scott-Knott test at 5% probability.

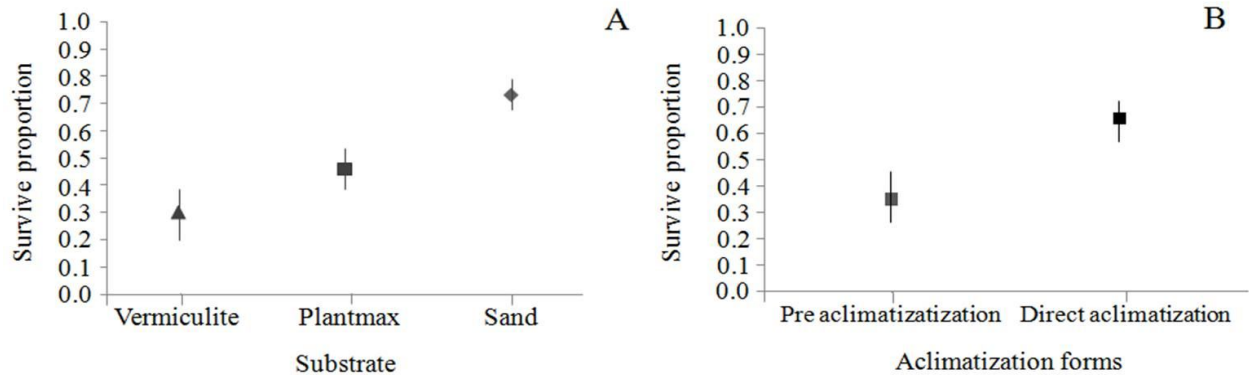

Figure 6 – Proportion of surviving of *S. elegantulus* plants acclimatized on different substrates.

This report describes an unpublished protocol for the micropropagation of *S. elegantulus*, providing information that may enable the domestication of this species and the establishment and maintenance of germplasm banks and allow the *in vitro* production of plantlets for commercial cultivation.

CONCLUSIONS

The culture medium concentration does not influence the percent germination of *Syngonanthus elegantulus* seeds, but the germination speed index is inversely proportional to the culture medium salt concentration.

The best culture medium for the establishment of *S. elegantulus* is WPM medium with 100% salt concentration and 17 g L^{-1} sucrose.

The best concentration for callus induction in *S. elegantulus* is 0.5 mg L^{-1} NAA and the absence of TDZ. The highest number of shoots is obtained in media with 1.0 mg L^{-1} TDZ or 1.0 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA.

The plants showed a higher survival rate when acclimatized directly in sand.

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