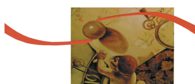

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# Micropropagation protocol for *Syngonanthus elegans* (Bong.) Ruhland: an ornamental species

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**ABSTRACT.** The research aimed to establish a protocol of micropropagation of *Syngonanthus elegans*. Seed germination in WPM media containing 0, 25, 50, 75 and 100% salt concentrations was evaluated. Establishment of plantlets in WPM and MS media, with 50 or 100% salt concentrations, was studied. The addition of 0, 5, 10, 15, 20, 25 and 30 g $L^{-1}$ of sucrose in WPM media was evaluated. The effects of TDZ (0.0; 0.5; 1.0; 2.0 and 4.0 mg $L^{-1}$) and ANA (0.0; 0.5 and 1.0 mg $L^{-1}$) in all possible combinations were investigated. To test acclimatization, sand, Plantmax$^{TM}$ or vermiculite substrates were evaluated by direct acclimatization and pre-acclimatization. The germination of *S. elegans* seeds was not influenced by salt concentration in the culture media, but the germination speed was affected. The WPM medium with 17 g $L^{-1}$ of sucrose is recommended for establishment of plantlets. Callus formation occurs in presence of 0.5 or 1.0 mg $L^{-1}$ of NAA, in absence of TDZ, or when same concentrations of NAA and TDZ were added. Sprouts were most frequently produced when 0.5+0.5 mg $L^{-1}$ or 1.0+1.0 mg $L^{-1}$ of NAA and TDZ were used. The *S. elegans* plants had survival rate was 25.6% after acclimatization.

**Keywords:** floriculture, *in vitro* cultivation, multiplication, acclimatization.

## Protocolo de micropropagação de *Syngonanthus elegans* (Bong.) Ruhland: uma espécie ornamental

**RESUMO.** Este estudo objetivou estabelecer um protocolo de micropropagação de *Syngonanthus elegans*. A germinação das sementes em meios WPM contendo 0, 25, 50, 75 e 100% da concentração de sal foi avaliada. O estabelecimento de plântulas em meio WPM ou MS, diluídos a 50 ou 100% foi estudado. A adição de 0, 5, 10, 15, 20, 25 e 30 g $L^{-1}$ de sacarose no meio WPM foi avaliada. Os efeitos de TDZ (0,0, 0,5, 1,0, 2,0 e 4,0 mg $L^{-1}$) e ANA (0,0, 0,5 e 1,0 mg $L^{-1}$) em todas as combinações possíveis foram investigadas. Os substratos areia, vermiculita ou Plantmax$^{TM}$ foram avaliados na aclimatação direta ou pré-aclimatação de plantas. A germinação de sementes de *S. elegans* não foi influenciada pela concentração de sal, mas a velocidade de germinação foi afetada. O meio WPM com 17 g $L^{-1}$ de sacarose é recomendado para o estabelecimento de plântulas. Formação de calo ocorre em presença de 0,5 ou 1,0 mg $L^{-1}$ de ANA, na ausência de TDZ, ou quando mesmas concentrações de ANA e TDZ foram adicionados. Brotos foram mais frequentemente produzidos quando 0,5+0,5 mg $L^{-1}$ ou 1,0+1,0 mg $L^{-1}$ de ANA e TDZ foram utilizados. As plantas tiveram porcentagem de sobrevivência de 25,6% após a climatização.

**Palavras-chave:** floricultura, cultivo *in vitro*, multiplicação, aclimatização.

## Introduction

*Syngonanthus elegans* (Bong.) Ruhland, belonging to the botanical family Eriocaulaceae, is an ornamental species native to the *Espinhaço* Mountains, specifically in the region of Diamantina, Minas Gerais State, Brazil (NUNES et al., 2008a; LANDGRAF; PAIVA, 2009). The flowers are an important commercial product and are sold as cut flowers (NERI; PAIVA, 2012). Because most of these flowers sold are obtained by collecting, this caused a drastic decrease in the natural populations has occurred and consequently resulted in the inclusion of *S. elegans* on the list of endangered plants (BRASIL, 2012; NUNES et al., 2008b). One of the most effective strategies for avoiding the collection of native plants is domestication, which enables the production of plants and the maintenance of germplasm banks outside of natural areas. Domestication also permits the development of sustainable cultivation processes that allow for a better quality of life for the workers and their families that depend on floral production (NERI et al., 2005; NUNES et al., 2008b; SIMÕES et al., 2007).

Studies on the micropropagation of *S. mucugensis*, another important species in the Eriocaulaceae family, have focused on exploring aspects of seed germination and plant growth (PAIXÃO-SANTOS et al., 2003; SILVA et al., 2005a and b; PAIXÃO-SANTOS et al., 2006). The establishment of specific micropropagation protocols has allowed for the domestication and propagation of these plants. Paixão-Santos et al. (2008) reported that callus regeneration is a viable alternative for the multiplication of *Syngonanthus*, as long as somaclonal variations do not reach high percentages. But more research is necessary to consolidate knowledge of this technique.

Both Murashige and Skoog (MS) (MURASHIGE; SKOOG, 1962) and Woody Plant Medium (WPM) (LLOYD; McCOWN, 1980) media, appropriately diluted, have been used to in vitro production of many species. Studies of *S. mucugensis* showed that both *in vitro* germination and seedling development are inhibited by high solute concentrations in culture media (PAIXÃO-SANTOS et al., 2003; SILVA et al., 2005a; PAIXÃO-SANTOS et al., 2006). In addition to their sensitivity to different salt concentrations in the culture media, plants of the genus *Syngonanthus* show sensitivity to variations in concentrations of other components, such as growth regulators and sucrose, that can also decrease plant growth (LIMA-BRITO et al., 2011a and b).

Acclimatization is a critical step in plant micropropagation, and there may be high mortality; thus, pre-acclimatization has been employed to reduce the effects caused by the environmental changes between *in vitro* and natural conditions to favor plant survival and growth (NASCIMENTO et al., 2008). Due to the economic importance and risk of extinction of *S. elegans*, the aim of this study was to establish a protocol for its micropropagation. This research is important for the micropropagation of this species because it is the first protocol for *in vitro* plant establishment that will facilitate both the production of seedlings and the conservation of genetic resources.

## Material and methods

Capitula of *S. elegans* were manually collected in fields of naturally occurring specimens located in Diamantina, state of Minas Gerais, Brazil. These flowers were dried in the shade at 24°C for 7 days. The seeds were stored in permeable Kraft paper bags at 10°C for 60 days.

### Germination of seeds in culture media with different salt concentrations

Plots containing 50 seeds each were used. The seeds were sterilized with 70% alcohol for 1.0 minute followed by immersion in 2.5% sodium hypochlorite for 10 minutes, and they were then washed three times with distilled water. As treatments were used WPM medium with 25%, 50%, 75% and 100% salt, and a control using deionized water, all supplemented with 15 g $L^{-1}$ sucrose and solidified with 8 g $L^{-1}$ agar. The pH of the WPM media was adjusted to 5.8 before autoclaving at 1.5 atm, 120°C for 20 minutes. The test for seed germination was conducted with five repetitions per treatment. Flasks were maintained in a growth room for 30 days with irradiance of 43 μmol $m^{-2}$ $s^{-1}$, a photoperiod of 16 hours and a temperature of 25 ± 2°C. After 30 days of inoculation, the germination percentage was calculated. During this period, the number of germinated seeds was counted daily, and the germination speed index (GSI) was calculated according to Maguire (1962). The data were submitted to a polynomial regression.

### Establishment of plantlets in culture media with different salt concentrations

MS and WPM media, with either 50% or 100% salt concentrations, supplemented with 15 g $L^{-1}$ sucrose and 8 g $L^{-1}$ agar, were tested. The pH of the media was adjusted to 5.8 before autoclaving at 1.5 atm, 120°C for 20 minutes. Seedlings 1 cm in length were inoculated and maintained for 30 days in a growth room with irradiance of 43 μmol $m^{-2}$ $s^{-1}$, a photoperiod of 16 hours and a temperature of 25 ± 2°C. Analyses to determine the number of normal leaves, number of chlorotic leaves, shoot length and fresh seedling mass were performed. The experimental design was completely randomized using a factorial design 2 (culture media) x 2 (salt concentrations), with four replicates and three tubes per plot, and one explant per tube. The data were submitted to an analysis of variance, and the averages were compared using the Tukey test at 5% probability.

### Development of plantlets in culture media with different sucrose concentrations

Based on the observations of the establishment of plantlets in the previous tests, we found that WPM was the most suitable medium for investigating the optimal concentrations of sucrose for developing seedlings. Thus, seedlings 1 cm in length were inoculated in WPM medium supplemented with 0, 5, 10, 15, 20, 25 and 30 g $L^{-1}$ sucrose and solidified with 8 g $L^{-1}$ agar, with a pH of 5.8 that had been autoclaved for 20 minutes at 1.5 atm at a temperature of 120°C. The inoculated seedlings were kept in a growth room for 30 days with irradiance of 43 μmol $m^{-2}$ $s^{-1}$, a photoperiod of 16 hours and a temperature of 25 ± 2°C. After 30

days of inoculation, analyses to determine the number of leaves, shoot length, root number and fresh mass of seedlings were performed. The experiment used a completely randomized design with six replicates and three tubes per plot, with one seedling per tube. The data were submitted to a polynomial regression.

### Plantlet multiplication in culture media with TDZ and ANA

Seedlings containing leaves and roots were grown on WPM medium, used as explants, and then inoculated in WPM medium supplemented with 0.0, 0.5, 1.0, 2.0 and 4.0 mg $L^{-1}$ thidiazuron (TDZ) and 0.0, 0.5 and 1.0 mg $L^{-1}$ naphthalene acetic acid (NAA), in all possible combinations, with 15 g $L^{-1}$ sucrose. The medium was solidified with 8 g $L^{-1}$ of agar, and the pH was adjusted to 5.8 before autoclaving. After inoculation, the plantlets were maintained in a growth room with irradiance of 43 µmol $m^{-2}$ $s^{-1}$, a photoperiod of 16 hours and a temperature of 25 ± 2°C. The experimental design was completely randomized using a factorial design 5 (TDZ) x 3 (NAA) in five replicates with three tubes per plot and one explant per tube. After 30 days, the percentage of explants with callus induction and the number of shoots were determined. The data were analyzed using an analysis of variance, and the averages were compared using the Scott-Knott test at 5% probability.

### Acclimatization of plantlets in different substrates

Plantlets used for the acclimatization process had approximately 16 leaves, were 3 cm in length and were obtained from seed germination. Sand, Plantmax™ and vermiculite were evaluated as substrates for the acclimatization of micropropagated plantlets that either had or had not been submitted to pre-acclimatization.

For pre-acclimatizing the plantlets, the culture medium was diluted with distilled water, and the seedlings were transferred to 250 mL flasks containing one of the substrates: sand, Plantmax™ or vermiculite. All substrates were moistened with a WPM salt solution. Plants were maintained in a growth room with irradiance of 43 µmol $m^{-2}$ $s^{-1}$, a photoperiod of 16h and a temperature of 25 ± 2°C; the flasks was sealed for three days and then opened, thus maintaining the same conditions for five days.

Pre-acclimatized or recently acclimatized seedlings were transferred to 12.5 cm tall polyethylene tubes with a diameter of 3 cm containing sand, Plantmax™ or vermiculite and covered with transparent plastic bags to maintain humidity in the environment. The polyethylene tubes were maintained in a growth room with irradiance of 67 µm $m^{-2}$ $s^{-1}$, a photoperiod of 16 hours and a temperature of 25 ± 2°C. The bags were increasingly perforated weekly until their complete removal thirty days after the beginning of the acclimatization process. The design was completely randomized using a factorial design of 2 (types of acclimatization) x 3 (substrates) with 15 replications. The plant survival rate was analyzed after 30 days.

Direct acclimatization was performed by removing the plants from the tubes and transferring them immediately into plastic pots filled with the various substrates without the use of pre-acclimatization.

Statistical analyses was performed using the methodology of generalized linear models (GLMs) with the statistical package R (R DEVELOPMENT CORE TEAM, 2010). The presence or absence of live seedlings showed a binomial distribution; therefore, the logistic link function was used as a linear predictor.

## Results and discussion

The salt concentration in the culture media had no influence on seed germination in *S. elegans*, with germination values reaching 63% (Figure 1); this value can be considered high for this species. However, a tendency towards a decrease in the germination percentage with an increase in the salt concentration of the culture medium was observed.

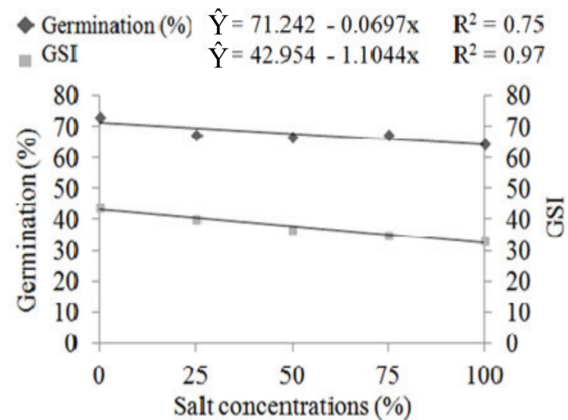


**Figure 1.** Germination percentage and germination speed index (GSI) of *S. elegans* seeds cultivated at various WPM salt concentrations.

Silva et al. (2005b) reported a high percentage (85%) of *in vitro* germination of *S. mucugensis* seeds on MS medium. *In vitro* germination is a better technique for comparison with other techniques commonly found in the literature that allow for a maximum germination of 35%, using germitest paper as a substrate (NUNES et al., 2008a). These results confirm the superiority of micropropagation for obtaining seedlings when compared with traditional methods of germination.

The highest germination speed index (GSI) was observed in media consisting of water alone or in the presence of 25% WPM salts, confirming the sensitivity of the seeds to media with high salt concentrations (Figure 1). Paixão-Santos et al. (2003), working with *S. mucugensis* seeds, observed that higher solute concentrations caused slower seed germination, as the highest germination rate was 22.3, which was considered low when compared with the rate obtained in the current study using *S. elegans*.

On average, 15.8 and 5.9 leaves of *S. elegans* were formed in undiluted WPM and MS culture media, respectively (Table 1). There was no difference between the number of leaves and the number of chlorotic leaves that this species grew in medium diluted to 50%; however, the original composition of MS medium inhibited the growth of leaves in seedlings (Table 1).

**Table 1.** Number of leaves, number of chlorotic leaves, shoot length (cm) and fresh mass (mg) of micropropagated *S. elegans* seedlings at different salt concentrations in MS and WPM media[(1)].

| | Leaves | | Chlorotic leaves | | Shoot length | | Fresh mass | |
|---|---|---|---|---|---|---|---|---|
| Concentration | MS | WPM | MS | WPM | MS | WPM | MS | WPM |
| 50% | 11.1 Aa | 12.6 Ba | 1.1 Ba | 0.8 Aa | 2.3 Aa | 2.7 Ba | 0.06 Ab | 0.09 Aa |
| 100% | 5.9 Bb | 15.8 Aa | 5.2 Aa | 0.1 Ab | 1.6 Bb | 3.3 Aa | 0.01 Bb | 0.11 Aa |

[(1)]Capital letters represent the same culture media and lowercase letters represent the same salt concentration (within each level), and these do not differ ($p < 0.05$) according to the Tukey test.

Seedlings grown in undiluted MS medium had an average of 5.2 chlorotic leaves, and leaf damage was caused by high concentrations of the culture medium. However, in WPM medium, no more than one leaf showed these symptoms. The development of leaf chlorosis was initiated 15 days after inoculation of the seedlings, and it later evolved into necrosis (Figure 2).

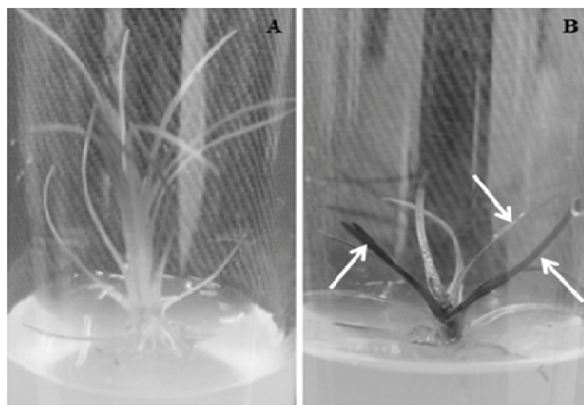


**Figure 2.** Normal development of *S. elegans* seedlings cultivated on undiluted WPM medium (A) and seedlings grown on MS medium showing complete leaf necrosis (B). Both images were obtained at thirty days after inoculation.

As shown above for *S. elegans*, *S. mucugensis* plantlets showed inhibited formation of leaves when grown in the original MS medium and grew best in media with a 50 % salt concentration (PAIXÃO-SANTOS et al., 2006). Ivanova and Staden (2009) reported that the addition of nitrogen sources to culture media is essential for the regeneration and development of shoots in *Aloe polyphylla*. However, high $NH_4^+$ concentrations cause a reduction in the shoot number and length. Low $NH_4^+$ levels in WPM medium, when compared with MS medium, stimulated the normal development of *S. elegans* plantlets, demonstrated by toxicity symptoms only being observed in micropropagated plants grown on media with higher ammonia concentrations.

The fresh mass of *S. elegans* seedlings was affected by the salt concentration of the culture media, and a higher fresh mass was observed in seedlings grown on WPM medium, regardless of salt concentration; in contrast, the fresh mass was reduced in seedlings grown on MS medium (Table 1). As shown above in this study with *S. elegans*, *S. mucugensis* plants showed inhibited leaf formation when grown in complete MS medium and grew best under conditions with salt concentrations above 50% in MS medium (PAIXÃO-SANTOS et al., 2006).

Complete MS medium was demonstrated to be unsuitable for *in vitro* production of *S. mucugensis,* as when grown in MS medium at 50% or 33% of the original concentration, these seedlings had a longer shoot length and higher biomass (PAIXÃO-SANTOS et al., 2006). *Syngonanthus* sp. is naturally adapted to low-fertility sandy soils, which explains the lower demand for nutrients for the proper development of these plants. Consequently, it can be grown in diluted nutrient media (NUNES et al., 2008b).

The number of leaves formed in *S. elegans* increased with the addition of sucrose to the culture medium. *S. elegans* produced the highest number of leaves when grown in a culture medium with 23 g $L^{-1}$ of sucrose added (Figure 3).

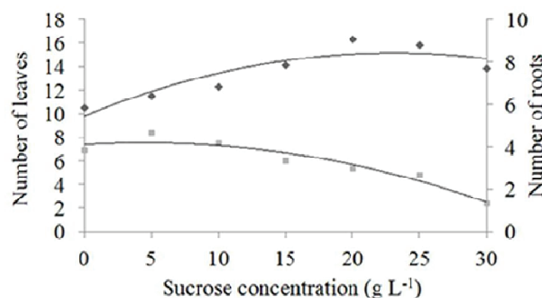


**Figure 3.** Number of leaves and roots in *S. elegans* seedlings grown in WPM medium supplemented with different sucrose concentrations.

Increasing sucrose concentrations inhibited seedling rooting, and the highest number of roots was observed using culture medium with 5 g $L^{-1}$ of added sucrose. The use of 30 g $L^{-1}$ of sucrose caused rooting inhibition, with 1.3 roots occurring per plant (Figure 3).

The fresh mass weight was affected by sucrose concentrations (Figure 4) and increased as sucrose concentrations increased.

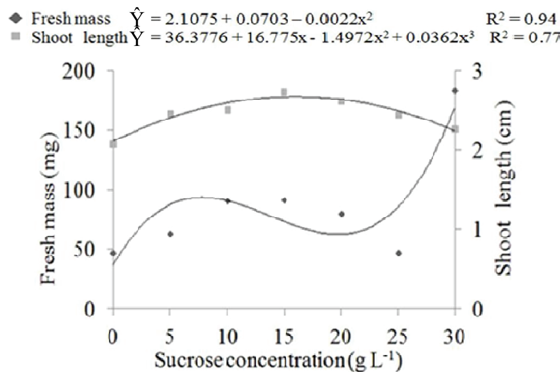


**Figure 4.** Fresh mass and shoot length of *S. elegans* plantlets grown in WPM medium supplemented with different sucrose concentrations.

Eighteen days after transplantation, the seedlings inoculated in the medium containing 30 g $L^{-1}$ of sucrose showed morphological changes in the neck region (cell proliferation), resulting from osmotic stress. This stress, caused by high sucrose concentrations, promoted the accumulation of cell mass in the neck region of the seedlings, which was the main factor contributing to the increased fresh mass production in the seedlings (Figure 5).

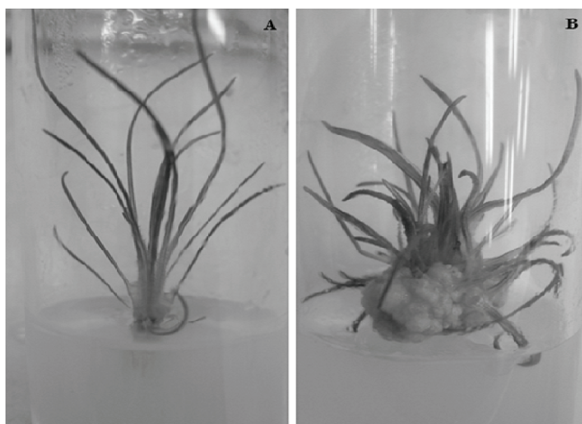


**Figure 5.** *S. elegans* seedlings with normal development in medium supplemented with 15 g $L^{-1}$ sucrose (A) and callus formation in medium supplemented with 30 g $L^{-1}$ sucrose (B) 30 days after transplantation.

High sucrose concentration influenced both competence and cell differentiation, inhibiting root formation in *S. elegans*. Similar to the result presented above to root formation of *S. elegans*, the root formation process of *S. mucugensis* seedlings was also affected by carbohydrate concentrations above 10 mg $L^{-1}$ in the culture medium (SILVA et al., 2005a). Lima-Brito et al. (2011a) reported that high concentrations of carbohydrates inhibit the *in vitro* growth of *S. mucugensis*. These results are useful when applied to other steps in the micropropagation of *Syngonanthus* sp., principally in the multiplication of plants where the roots are used for callus induction and plant acclimatization.

The addition of sucrose to the medium should take into account issues related to the osmotic potential of both the seedlings and the medium. *S. mucugensis* seedlings experience better growth when they are micropropagated in media supplemented with 15 g $L^{-1}$ of sucrose (PAIXÃO-SANTOS et al., 2003, SILVA et al., 2005a).

Media supplementation with various TDZ and NAA concentrations was effective for callus induction. However, callus formation only occurred on roots (Figure 6). Media supplemented with 0.5 mg $L^{-1}$ or 1.0 mg $L^{-1}$ of NAA, both in the absence of TDZ, were effective in inducing callus formation in *S. elegans*. Callus induction in this species does not require the presence of cytokinin TDZ, but rather the addition of NAA. The combinations of 0.5 + 0.5 mg $L^{-1}$, 1.0 +1.0 mg $L^{-1}$ and 4.0 + 1.0 mg $L^{-1}$ of TDZ and NAA, respectively, were also effective for callus induction.

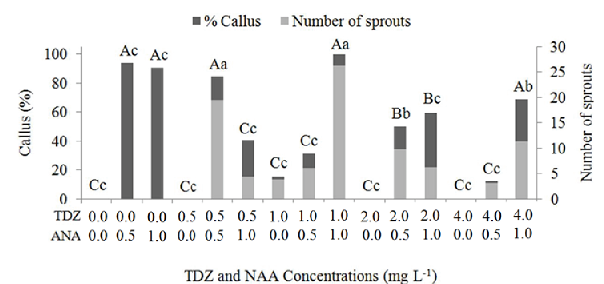


**Figure 6.** Percentage of explants with callus induction and number of sprouts in *S. elegans* in culture medium supplemented with TDZ and NAA. Averages followed by the same capital letter for the induced explants percentage and lowercase letter for callus percentages do not differ using the Scott-Knott test at 5% probability.

Shoot formation was observed from the callus that had formed in the explants after 30 days. The isolated addition of TDZ and NAA did not directly cause shoot formation. However, the presence of 1.0 mg $L^{-1}$ TDZ in the culture medium was effective

in inducing sprouts in *S. elegans*. Higher numbers of shoots were observed when a TDZ/NAA ratio of 1.0 was used.

The exposure of stem and leaf explants of *S. mucugensis* to 2.68 μM of NAA without adding BAP resulted in callus formation without shoot regeneration (LIMA-BRITO et al., 2011b). These authors observed an unorganized callogenic tissue surface, with meristemoidal organization appearing as areas of green dots that corresponded to bud formation. Similar results were observed in the current study.

There was no difference observed with the use of different substrates or with pre-acclimatization in the *S. elegans* acclimatization process (Table 2).

**Table 2.** 'Deviance' analysis of the effects of substrates and acclimatization type on *S. elegans* seedlings.

| Source of Variation | Degrees of freedom | Mean Square | p-Value |
|---|---|---|---|
| Substrate (S) | 2 | 3.245 | 0.1974 |
| Acclimatization (A) | 1 | 1.525 | 0.2168 |
| S x A | 2 | 0.207 | 0.9015 |
| Error | 84 | 97.327 | |

On average, after 30 days, only 25.6% of the *S. elegans* plants survived the acclimatization process. Therefore, the methods tested were not efficient. *Syngonanthus* sp. plants are adapted to sandy soils with physical and chemical compositions that are significantly different from those of commercial substrates. Such differences may have contributed to the low proportion of plants surviving after 30 days of acclimatization (NUNES et al., 2008c). Other factors might be considered as critical in establishing plants, such as the need to interact with microorganisms or other plant species, in addition to the physical and chemical properties of the soil. Transferring the seedlings to tubes was a critical step in the process because *Syngonanthus* sp. seedlings have low resistance to transplantation shock, as reported by Paixão-Santos et al. (2003). According to these authors, plants of the genus *Syngonanthus* are extremely sensitive to changes in the composition of cultural substrates. No success was obtained in producing plants on commercial substrates, and their cultivation was only possible in areas of natural occurrence.

The present study obtained on an average survival rate of 25.6% of plants after acclimatization in all substrates tested. Although the percentage of acclimatization of these plants has been relatively low, this technique remains promising for the preservation of these plants because of their commercial importance and their risk of extinction. Moreover, this is the first report of the acclimatization of these plants, and it will provide support for future studies of the micropropagation of *S. elegans*.

## Conclusion

The germination of *S. elegans* seeds was not influenced by salt concentration in the culture media, but the germination speed was inversely proportional to salt concentration.

The original WPM medium with 17 g $L^{-1}$ of sucrose added is recommended for establishment of plantlets.

Callus formation occurs in presence of 0.5 or 1.0 mg $L^{-1}$ of NAA, in absence of TDZ, or when the same concentrations of NAA and TDZ were added. Sprouts were most frequently produced when 0.5+0.5 mg $L^{-1}$ or 1.0+1.0 mg $L^{-1}$ of NAA and TDZ were used.

The *S. elegans* plants had survival rate was 25.6% after acclimatization.

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