Micropropagation of Brassavola nodosa (L.) Lindl. using SETIS™ bioreactor

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Abstract

Brassavola nodosa (L.) Lindl. is a tropical epiphytic orchid showing characteristics of interest for the ornamental nursery industry. However, problems with traditional propagation methods limit the development of a large-scale commercial production system. In addition, this species is considered endangered due to the reduction in population caused by habitat destruction, climate change and over-collection from native areas. The use of micropropagation has been investigated for this species, and the use of liquid *in vitro* systems showed potential for use of temporary immersion bioreactors for micropropagation of *B. nodosa*. This study evaluated the efficiency of the SETIS™ bioreactor system for the micropropagation of *B. nodosa* by adjusting parameters of immersion (frequency and duration) and by comparing it to conventional semi-solid culture systems. Results indicate that temporary immersion of *B. nodosa* explants with a frequency of 2 h and duration of 2 min returned the highest multiplication rates, with 4.6 shoots produced per explant compared with 2.8 shoots per explant in semi-solid agar-based systems. The use of bioreactors also promoted increased growth and development and *in vitro* rooting, therefore improving survival and facilitating acclimatization of *in vitro*-derived plantlets. This is the first study demonstrating a successful protocol for large-scale micropropagation of *B. nodosa* using SETIS™ bioreactors, which could have significant value and impact for the commercial production of this species as well as for conservation purposes.

Key Message

Micropropagation of *Brassavola nodosa* was achieved using SETIS temporary immersion bioreactors, showing good multiplication rates, increased growth and development and in vitro rooting, thus improving survival and acclimatization ex vitro.

Introduction

The expansion of the ornamental market in conjunction with the increased demand for flowering plants as well as lower production costs and advanced production techniques have placed orchids among the top flowering plants in the USA (Vendrame and Khoddamzadeh 2017). There is also an increased interest by growers and customers in new and improved orchid hybrids. *Brassavola nodosa* (L.) Lindl. is a tropical epiphytic orchid distributed from Mexico through Central and South America (Jones 1975; Mata-Rosas and Lastre-Puertos 2015) and produces very fragrant inflorescences rendering its common name as the “Lady of the Night”. In addition to flower fragrance, this species flowers throughout the year, has a unique flower shape, and requires low maintenance, making it desirable for the potted flower ornamental industry. However, the propagation of orchids in the genus Brassavola is difficult and limited to branching, pseudobulbs, or seeds. Seed germination in nature is very low while propagation by branching or pseudobulbs is a lengthy process (Mengarda et al. 2017). These factors limit commercial large-scale production. Furthermore, habitat destruction, the impact of climate change, and over-collection from native habitats has caused a reduction in population and distribution of these orchids (Swarts and Dixon 2009; Reed et al. 2011). Propagation and conservation strategies are required to guarantee population

survival in nature and to provide plant material for commercial production. Micropropagation provides a feasible alternative for rapid mass clonal propagation of true-to-type plants with commercial potential, and it has been responsible for the success of the orchid industry (Chugh et al. 2009; Kumar and Reddy 2011; Bhoite and Palshikar 2014; Phillips 2019).

Micropropagation of several orchid species and hybrids has been reported for various genera, including Anoectochillus, Arundina, Cymbidium, Dendrobium, Doritaenopsis, Phalaenopsis, Paphiopedilum, Vanda, and Vanilla, among many others (Griesbach 1986; Jones and Tisserat 1990; Arditti and Ernst 1993; Tokuhara and Mii 1993; Kyte and Kleyn 1996; Devi et al. 1997; Tisserat and Jones 1999; Kalimuthu et al. 2006; Wu et al. 2007, Chugh et al. 2009; Neumann et al. 2009; Alam et al. 2010; Paek et al. 2011; Panwar et al. 2012; Divakaran et al. 2015; Teixeira da Silva et al. 2015; Rodrigues et al. 2015; Zeng et al. 2015).

However, micropropagation of Brassavola orchids remains a challenge (Xu et al. 2022) due to the limited number of studies in the genus Brassavola, including *in vitro* germination of a *Brassavola perrinii* hybrid (Chiapim et al. 2012)d *tuberculata* (Soares et al. 2020), micropropagation of a Brassavola hybrid (Villa et al., 2014), and acclimatization of *in vitro* derived plantlets (Sousa et al. 2015) and *in vitro* shoot production (Mengarda et al. 2017) of *B. tuberculata*.

The use of liquid cultures could be a feasible approach to improve orchid micropropagation (Young et al. 2000; Wu et al. 2007; Murthy et al. 2018). Liquid culture systems offer advantages over traditional semi-solid agar-based systems in micropropagation including improved nutrient uptake and therefore enhanced plant growth and development, higher rates of multiplication, the potential of reduction of contamination, and reduced costs by automation of liquid systems (Ziv 2000). In addition, liquid cultures improve uniformity of culture conditions, such as easier renewal of media, the opportunity to use of larger containers, and the need for fewer frequent subcultures (Etienne and Berthouly 2002).

Higher multiplication rates in liquid media have been reported for many species (Douglas 1984; Bergervoet et al. 1989; Chu et al. 1993). However, the use of liquid culture systems is recent and not yet popular for the propagation of orchids. More recently, micropropagation of *B. nodosa* using liquid culture with partial immersion has been investigated and showed positive results creating an opportunity for the use of temporary immersion bioreactors (Xu et al. 2022).

Temporary immersion systems (TIS) are advanced techniques for micropropagation using liquid media in bioreactors and are based on periodic immersion of plant tissues into the liquid medium for a pre-determined period of time, with alternating periods of aeration. This allows increased nutrient uptake, thus making TIS an efficient system for large-scale micropropagation of plants (Georgiev et al. 2014; Ramírez-Mosqueda and Bello-Bello 2021). Various TIS bioreactor systems have been designed, including ebb-and-flow bioreactor (Tisserat and Vandercook 1985), RITA® (Teisson and Alvard 1995), and Twin Flask System (Escalona et al. 1999). More recently, a new bioreactor system has been developed (SETIS™, VERVIT, Belgium) and it has been used commercially for the micropropagation of several crops, including berries, sugarcane and orchids, among others. Compared to other systems, the SETIS™ bioreactor system allows for increased light irradiation, automation of cultivation systems, easy handling,

large culture medium capacity, and successful protocols have been reported for the micropropagation of banana and vanilla (Bello-Bello et al. 2019; Ramírez-Mosqueda and Bello-Bello 2021). These characteristics make the SETIS™ bioreactor an ideal system for commercial micropropagation of orchids.

Therefore, the objective of this study was to evaluate the efficiency of the SETIS™ bioreactor for the micropropagation of *B. nodosa*. Specific objectives included the development of an efficient micropropagation protocol using SETIS™ bioreactor by adjusting parameters of immersion (frequency and duration) and by comparing it to conventional semi-solid culture systems. The development of a large-scale micropropagation system for *B. nodosa* may aid in its commercialization and contribute for the conservation of the species.

Materials And Methods

Plant Material Seeds originated from a Brassavola hybrid, *Brassavola nodosa* 'Remar' x 'Mas Mejor' were selected for this study. Seeds were surface sterilized with 70% ethanol (Decon Labs, Inc., King of Prussia, PA) for 1 min followed by immersion in 0.8% sodium hypochlorite (NaClO, Thermo Fisher Scientific, Hampton, NH) with 2 drops of Tween 20 (Fisher Scientific, Pittsburg, PA) for 5 min. Subsequently, seeds were rinsed three times in sterile autoclaved water. Seeds germinated *in vitro* on half strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962, Phytotechnology Laboratories, Lenexa, KS) containing 15 g L^{-1} sucrose (Thermo Fisher Scientific) and 7 g L^{-1} agar (Thermo Fisher Scientific). The medium pH was adjusted to 5.7 and the medium was autoclaved at 121°C and 15 lbs pressure for 20 min. About 15 mL of medium was dispensed in 100 mm x 15 mm disposable Petri dishes (Thermo Fisher Scientific). Sixty days after seed germination, when protocorms had developed 1 to 2 leaves and roots, seedlings were transferred to 120 mm x 75 mm RA40 Microboxes (Sac O2, Deinze, Belgium) containing about 100 mL MS medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar.

Effect of Culture System on Shoot Multiplication Explants consisting of young shoots tips measuring between 0.3 and 0.5 cm were collected from 1-yr-old *in vitro* seedlings and used for shoot multiplication. Shoot tips were cultured on MS medium containing 2 mg L^{-1} glycine, 0.1 mg L^{-1} NAA (naphthalene acetic acid), 2.0 mg L^{-1} BA (benzyladenine), 30 mg L^{-1} adenine sulfate, 10% coconut water, and 30 g L^{-1} sucrose. The medium pH was adjusted to 5.2 and autoclaved at 121°C and 15 lbs pressure for 20 min.

Four different culture systems were used for *in vitro* culture of shoot tips: 1) in 160 mm x 100 mm TP1600 Microbox (Sac O2, Belgium) containing 500 ml semi-solid medium (7g L^{-1} agar) as a control, 2) in 4 L temporary immersion bioreactor (SETIS™, VERVIT, Belgium) with an immersion and aeration frequency of 2 h, 3) in 4 L temporary immersion bioreactor with an immersion and aeration frequency of 4 h, and 4) in 4L temporary immersion bioreactors with an immersion and aeration frequency of 8 h (Fig. 1). For all treatments duration of immersion and aeration was 2 min. The experiment was comprised of 3 replicates per treatment, each replicate containing 20 explants. The cultures were maintained under controlled environment conditions with a 12-hour photoperiod at light intensity of 50 µmol m^{-2} s^{-1} and temperature of 26°C. Shoot multiplication, shoot length, number of leaves per shoot, rooting percentage,

root number, root length, fresh weight, dry weight, chlorophyll relative content, stomata number, and stomata size were evaluated 60 days after culture establishment. The entire experiment was repeated.

Chlorophyll Relative Content Analysis Fully expanded leaves were selected from 5 plants per treatment for chlorophyll relative content analysis. Chlorophyll relative content was measured as SPAD value by placing the third leaf of each plantlet, counted from top downwards in a portable SPAD-502 chlorophyll meter (SPAD-502, Minolta Co., Ltd., Japan).

Stomatal Analysis Five plantlets were randomly selected from each treatment for stomata analyses. The middle of the third fully expanded leaf was sectioned. Leaf sections were prepared on glass slides on top of a thin layer of super glue previously applied. After the glue was completely dry, the leaves were covered by a piece of translucent tape and the cuticle bind to the tape carefully removed, leaving a leaf impression. The stomata on the leaf impressions were visualized under an optical Leica DMLB microscope (Leica microsystems, Buffalo, NY, USA), at 100x magnification. The stomata number on the abaxial and adaxial epidermis were counted within a diameter of 5 mm under the microscope from three different fields on the leaf surface. Results were expressed as means of counts per mm^2.

The length and width of stomata were measured by randomly choosing 3 stomata on the adaxial and abaxial surface of each leaf in three different locations. The slides were observed and photographed under a light Leica DMLB microscope (Leica microsystems, Buffalo, NY, USA), coupled to a SPOT 4.7 IDEA (SPOT Imaging, Sterling Heights, MI, USA) digital camera. The images and the size of stomata were analyzed using the microscope imaging software SPOT basic (SPOT Imaging, Sterling Heights, MI, USA).

Acclimatization Eighteen shoots were selected from each culture system for acclimatization. Shoots were treated with rooting hormone powder (TakeRoot® Rooting Hormone, Bridgeton, MO) containing 1.0 mg L^{-1} IBA. and transferred to 40-cell plastic trays with soilless media containing orchid bark (Sequoia Bark Sales, Reedley, CA). Plants were maintained in a greenhouse with mist system running for 20 seconds every 30 minutes. Plants were fertilized weekly with 300 ppm of 11-35-15 (% N-P_2O_5-K_2O) orchid fertilizer (Better-Gro® Orchid Better-Bloom®, Arcadia, FL). The *ex-vitro* plant survival, shoot length, number of leaves per shoot, rooting percentage, root number, root length, fresh weight, and dry weight were evaluated for all treatments after 60 days.

Statistical Analysis A completely randomized experimental design was used for all experiments. Data were collected and submitted to analysis of variance (ANOVA) using the OriginPro® 2021b software (OriginLab, Northampton, MA). Tukey's post hoc multiple comparison adjustment ($\alpha = 0.05$) was used for all pairwise comparisons of means.

Results

Evaluation of Different Culture Systems on Shoot Multiplication Significant differences were observed among the different culture systems for *in vitro* shoot multiplication ($P < 0.036$) (Fig. 2A). The highest number of shoots (4.6 shoots/explant) occurred in SETIS™ bioreactor under immersion and aeration

frequency of 2 h, followed by immersion and aeration frequency of 4 h (3.5 shoots/explant) and 8 h (3.5 shoots/explant). The control in semi-solid medium had a multiplication rate of 2.8 shoots/explant. For leaf number and shoot length, there were no significant differences among culture systems (Fig. 2B, 2C). Rooting percentage ($P < 0.001$) (73.3–80.0%) and root number ($P < 0.001$) (2.0 to 2.6 roots per explant) in bioreactors were significantly higher than the explants cultured in semi-solid, with 6.7% rooting percentage and 0.1 roots per explant (Fig. 2D, 2E). The longest root length ($P < 0.024$) was recorded in the SETIS™ bioreactor under 2 h immersion frequency (1.58 cm), followed by 4 h (1.01 cm) and 8 h (0.97 cm), but were not significantly different (Fig. 2F). However, the control in semi-solid medium showed a significantly reduced root length of 0.03 cm (Fig. 2F).

Chlorophyll relative content was higher in explants cultured in bioreactors with SPAD values of 39.4 under 2 h immersion frequency, 37.7 under 4 h immersion frequency, and 30.6 under 8 h immersion frequency, but they were not significantly different ($P < 0.001$). However, the control in semi-solid medium showed a significantly lower SPAD value of 26.5 ($P < 0.001$) (Fig. 3A). The largest fresh weigh ($P < 0.001$) was observed in bioreactors under 2 h immersion frequency (0.121 g), followed by 4 h (0.083 g), 8 h (0.079 g), and the control in semi-solid medium (0.060 g) (Fig. 3B). The largest dry weight ($P < 0.003$) was recorded in bioreactors under 2 h immersion frequency (0.012 g), followed by 4 h (0.009 g), 8 h (0.010 g), and the control in semi-solid medium (0.006 g) (Fig. 3C). There were no significant differences in fresh weight between immersion frequency of 8 h and the control. However, dry weight in bioreactors under 8 h immersion frequency was higher than the control (Fig. 3B, C).

Effects of Culture Systems on Stomata Formation The assessment of the effect of different culture systems on stomata formation showed that explants cultured in bioreactors under 2 h immersion frequency presented the highest adaxial ($P < 0.022$) (75.3) and abaxial ($P < 0.013$) (95.0) stomata number, followed by 58.7 adaxial stomata and 84.3 abaxial stomata under 4 h, 52.7 adaxial stomata and 61.7 abaxial stomata under 8 h, and 53.3 adaxial stomata and 60.0 abaxial stomata under control, respectively (Table 1). Significant longest adaxial stomata length ($P < 0.006$), adaxial stomata width ($P < 0.002$), and abaxial stomata length ($P < 0.008$) were observed for plants in bioreactors under 2 h immersion frequency compared to other treatments. Both 2 h and 4 h immersion frequency treatments resulted in largest size of abaxial stomata width ($P < 0.002$) (Table 1). Explants cultured under 2 h immersion frequency produced spherical, wide-open stomata distributed on both adaxial and abaxial leaf surfaces (Fig. 4). Under 4 h immersion frequency, stomata morphology was similar to those under 2 h immersion frequency, but with lower stomata number. Explants cultured under 8 h immersion frequency and in the control had mostly closed stomata and lower total stomata number on either adaxial or abaxial leaf surfaces (Fig. 4).

Table 1
Effect of different *in-vitro* culture systems on stomata formation of *Brassavola nodosa* after 60 days in culture.

Culture systems	AdSN	AbSN	AdSSL (µm)	AdSSW (µm)	AbSSL (µm)	AbSSW (µm)
Control	53.3 ± 4.6^{b}	60.0 ± 10.6^{b}	20.73 ± 1.7^{b}	18.0 ± 2.7^{b}	20.8 ± 1.9^{b}	16.1 ± 2.7^{b}
Bioreactor 2h	75.3 ± 10.0^{a}	95.0 ± 8.7^{a}	29.68 ± 1.3^{a}	23.7 ± 1.2^{a}	27.9 ± 2.0^{a}	22.1 ± 2.2^{a}
Bioreactor 4h	58.7 ± 9.1^{ab}	84.3 ± 8.0^{ab}	22.85 ± 0.4^{b}	21.2 ± 1.5^{ab}	25.0 ± 2.4^{ab}	22.6 ± 2.1^{a}
Bioreactor 8h	52.7 ± 5.7^{b}	61.7 ± 16.3^{b}	22.85 ± 2.1^{b}	17.9 ± 3.0^{b}	23.3 ± 3.0^{ab}	18.4 ± 0.9^{ab}

Numbers represent mean ± SE (standard error). Means with different letters are significantly different (Tukey, $p \leq 0.05$).

Control = Semi-solid medium; Temporary Immersion Bioreactor: 2 hours immersion/aeration frequency; 4 hours immersion/aeration frequency; 8 hours immersion/aeration frequency. Immersion duration was 2 minutes for all bioreactor treatments. AdSN = Adaxial Stomata Number; AbSN = Abaxial Stomata Number; AdSSL = Adaxial Stomata Length; AdSSW = Adaxial Stomata Width; AbSSL = Abaxial Stomata Length; AbSSW = Abaxial Stomata Width.

Acclimatization During the acclimatization stage, significant differences in plant development and survival were observed. Plantlets derived from bioreactors under 2 h immersion frequency had the highest survival ($P < 0.001$) (93.3%), followed by 4 h immersion frequency (66.7%), 8 h immersion frequency (33.3%), and the control (6.7%) (Table 2). Plantlets derived from 2 h and 4 h immersion frequency showed 100% rooting, while plantlets derived from 8 h immersion frequency showed 53.3% rooting ($P < 0.001$) (Table 2). The highest number of roots was observed in plantlets derived from 4 h immersion frequency with an average of 5.3 roots per plant, followed by 2h with 4.3 roots per plant, and 8 h with 1.7 roots per plant ($P < 0.002$) (Table 2). Highest fresh weight ($P < 0.029$), dry weight ($P < 0.025$), and root length ($P < 0.032$) were recorded in plants derived from 2 h immersion frequency (Table 2, Fig. 5). There were no significant differences in shoot length and leaf number among the different culture systems, except for the control (Table 2). New roots were developed in plantlets derived from bioreactors under 2 h and 4 h immersion frequency but were reduced under 8 h (Fig. 5). The control showed no root development and low survival, therefore preventing the collection of data for other parameters (Table 2, Fig. 5). No morphological variations were observed for plants grown from treatments under 2h, 4h and 8h immersion and they showed normal phenotype (Fig. 5).

Table 2
Effect of different *in-vitro* culture systems on plant development of *Brassavola nodosa* after 60 days acclimatization.

Culture systems	Shoot length (cm)	Leaf number	Fresh weight (g)	Dry weight (g)	Rooting percentage	Root number	Root length (cm)	Survival (%)
Control	NA	NA	NA	NA	NA	NA	NA	6.7 ± 3.3^{d}
Bioreactor 2h	2.2 ± 0.3^{a}	6.7 ± 1.3^{a}	0.33 ± 0.07^{a}	0.030 ± 0.006^{a}	100.0 ± 0^{a}	4.3 ± 0.3^{ab}	2.8 ± 0.4^{a}	93.3 ± 6.7^{a}
Bioreactor 4h	2.1 ± 0.1^{a}	4.0 ± 0.6^{a}	0.18 ± 0.05^{ab}	0.016 ± 0.003^{ab}	100.0 ± 0^{a}	5.3 ± 1.2^{a}	1.5 ± 0.4^{ab}	66.7 ± 6.7^{b}
Bioreactor 8h	1.7 ± 0.2^{a}	4.3 ± 0.7^{a}	0.10 ± 0.02^{b}	0.009 ± 0.002^{b}	53.3 ± 5.8^{b}	1.7 ± 0.9^{b}	0.7 ± 0.4^{b}	33.3 ± 3.3^{c}

Numbers represent mean ± SE (standard error). Means with different letters are significantly different (Tukey, $p \leq 0.05$).

Control = Semi-solid medium; Temporary Immersion Bioreactor: 2 hours immersion/aeration frequency; 4 hours immersion/aeration frequency; 8 hours immersion/aeration frequency. Immersion duration was 2 minutes for all bioreactor treatments. NA = data not available.

Discussion

Micropropagation of orchids using TIS bioreactors has been reported to result in higher multiplication rates, increased biomass production, and healthier plant growth development compared to conventional culture systems using semi-solid medium. (Young et al. 2000; Wu et al. 2007; Murthy et al. 2018; Leyva-Ovalle et al. 2020; Ramírez-Mosqueda and Bello-Bello 2021). In this study, *B. nodosa* propagated in SETIS™ bioreactors showed higher multiplication rates and increased fresh/dry weight as compared to those under semi-solid culture medium (Fig. 2 and Fig. 3). Multiplication rates of 4.6 shoots per explant were obtained for *B. nodosa* using the SETIS™ bioreactor within a period of 60 days. Results are similar to those by Bello-Bello et al. (2019) comparing different culture systems for *in vitro* shoot multiplication of banana, whereby the highest number of shoots (7.3 shoots per explant) was obtained in SETIS™ bioreactors. Similarly, Ramirez-Mosqueda (2021) reported highest shoot multiplication (11.4 shoots per explant) of vanilla in SETIS™ bioreactors. Leyva-Ovalle et al. (2020) also reported higher shoot multiplication (6 shoots per explant) for the orchid *Guarianthe skinneri* when using temporary immersion bioreactors for the micropropagation, although the bioreactors used were different from the SETIS™ system.

Specifically for the genus Brassavola, Mengarda et al. (2017), reported a 90-day period for the induction of 4 shoots per explant for *Brassavola tuberculate* micropropagated under semi-solid, agar-based medium, contrasting with our study whereby shoots were produced in a shorter period of time (60 d).

These results demonstrate the efficiency of micropropagation of orchid species in the genus *Brassavola* using TIS bioreactors. Shoot multiplication can be achieved in a shorter period of time, which can also result in higher multiplication rates. In addition, studies on optimization of culture conditions, including medium composition and adjustment of immersion parameters (frequency and duration) in bioreactors may assist with increased multiplication rates.

One interesting observation was that the explants in bioreactors generated roots during the multiplication stage. This represents time and cost savings during *in vitro* multiplication and is a factor that facilitated acclimatization of micropropagated plantlets in the greenhouse. Similarly, the presence of roots was observed during the micropropagation of the orchid *Guarianthe skinneri* in TIS bioreactors (Leyva-Ovalle et al. 2020). In contrast, *B. nodosa* explants cultured in semi-solid medium produced a reduced number of roots (Fig. 2), possibly because of cytokinins in the medium. Higher cytokinin concentration could reduce auxin biosynthesis and inhibit the growth of roots (Su et al. 2011). In addition, semi-solid cultures may require frequent subculturing to improve multiplication and rooting (Leyva-Ovalle et al. 2020), although in our study, the frequency of subculturing in *in vitro* cultures of *B. nodosa* was not addressed. However, the need for increased subculturing to improve rooting adds another element that increases both labor and cost in the micropropagation process, therefore making the TIS bioreactor system more efficient.

Immersion and aeration frequency and duration in TIS bioreactors play important roles in plant *in vitro* multiplication as well as in plant development, and the optimization of such parameters is important to prevent physiological disorders such as hyperhydricity, among others. In this study, immersion/aeration frequency of 2 h with duration of 2 min in SETIS™ bioreactors returned the highest number of shoots during the micropropagation of *B. nodosa*. Results reported in the literature for other orchid species showed variation in immersion/aeration frequency and duration parameters, respectively, such as for 3 h/10 min for *Phalaenopsis* (Hempfling and Preil 2005), 4 h/2 min for *Vanilla planifolia* (Ramírez-Mosqueda and Bello-Bello 2021), 6 h/3 min for *Bletilla striata* (Zhang et al. 2018), and 4 h/2 min for *Guarianthe skinneri* (Leyva-Ovalle et al. 2020). This clearly demonstrates that genotype plays a role in determining *in vitro* responses, and immersion and aeration parameters should be evaluated and adjusted accordingly for each species. Our study also shows that responses *in vitro* of *B. nodosa* are improved under high immersion and aeration frequencies and duration, such as 2 h/2 min.

Chlorophyll relative content was significantly higher for plantlets produced in TIS bioreactors as compared to those from semi-solid medium. The increase in chlorophyll relative content could be related to increase irradiance through the SETIS™ bioreactor vessels as compared to the limited irradiance provided to explants in semi-solid medium under microboxes. Irradiance is an important factor in physiological processes which affects the synthesis of chlorophyll (Mancilla-Álvarez et al. 2021). Increase in chlorophyll synthesis using temporary immersion has been reported for the micropropagation of *Anthurium andreanum* (Martínez-Estrada et al. 2019). In this study, the frequent aeration provided may have also contributed to increased chlorophyll relative content. Increased air exchange in temporary immersion systems has been reported to enhance photosynthetic activity (Aragón et al. 2010; Bello-Bello et al. 2019).

Stomata number, size, and functionality are affected by transpiration and availability of water (Larraburu et al. 2016). In this study, stomata morphology was similar for all treatments. However, parameters for stomata number and size were higher for plantlets in TIS bioreactors under 2 h immersion frequency followed by 4 h immersion frequency, 8 h immersion frequency, and semi-solid medium, respectively. The number of open stomata was higher under 2 h immersion frequency but similar to the other TIS treatments. The higher relative humidity (RH) provided under 2 h immersion frequency likely resulted in higher transpiration rates contributing for the increased stomata parameters. The oppositive effect was observed under semi-solid medium with lower stomata parameters, possibly as a result of reduced RH and increased air exchange caused by the filters in the lids of microboxes. It has been reported that high survival of plantlets during acclimatization is related to closed stomata and lower stomata numbers, which helps prevent dehydration (Ramirez-Mosqueda et al. 2019). However, in this study, plantlets that showed open stomata achieved highest survival during acclimatization. According to Joshi et al. (2006), although closed stomata are preferred for acclimatization of micropropagated plantlets *ex vitro*, high survival could still be obtained by gradually decreasing air humidity. This could explain the results reported for this study, as gradual reduction in RH was promoted during acclimatization.

The high survival (93%) observed for micropropagated plantlets derived from TIS bioreactors under 2 h immersion frequency as compared to other treatments indicates that these parameters could be successfully applied for micropropagation of *B. nodosa* orchids using the SETIS bioreactor system. Root formation during multiplication stages also contributed to the successful acclimatization and survival of plantlets and it could significantly increase the efficiency of propagation for this species.

Conclusions

This is the first study reporting the use of the SETIS™ temporary immersion bioreactor system for the micropropagation of *B. nodosa* orchids. We determined that *B. nodosa* plantlets micropropagated under TIS bioreactors had higher multiplication rates compared to conventional culture systems using semi-solid agar-based medium. Root formation during the multiplication phase improved the efficiency of micropropagation, thus reducing labor and cost. Our study demonstrated a successful protocol for large-scale micropropagation of *B. nodosa* using bioreactors, which could have significant value and impact for the commercial production of this species as well as for conservation purposes. The evaluation and adjustment of immersion parameters is warranted for future studies to improve the rate of shoot multiplication.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by JianJian Xu, and David Beleski. The first draft of the manuscript was written by Wagner Vendrame and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

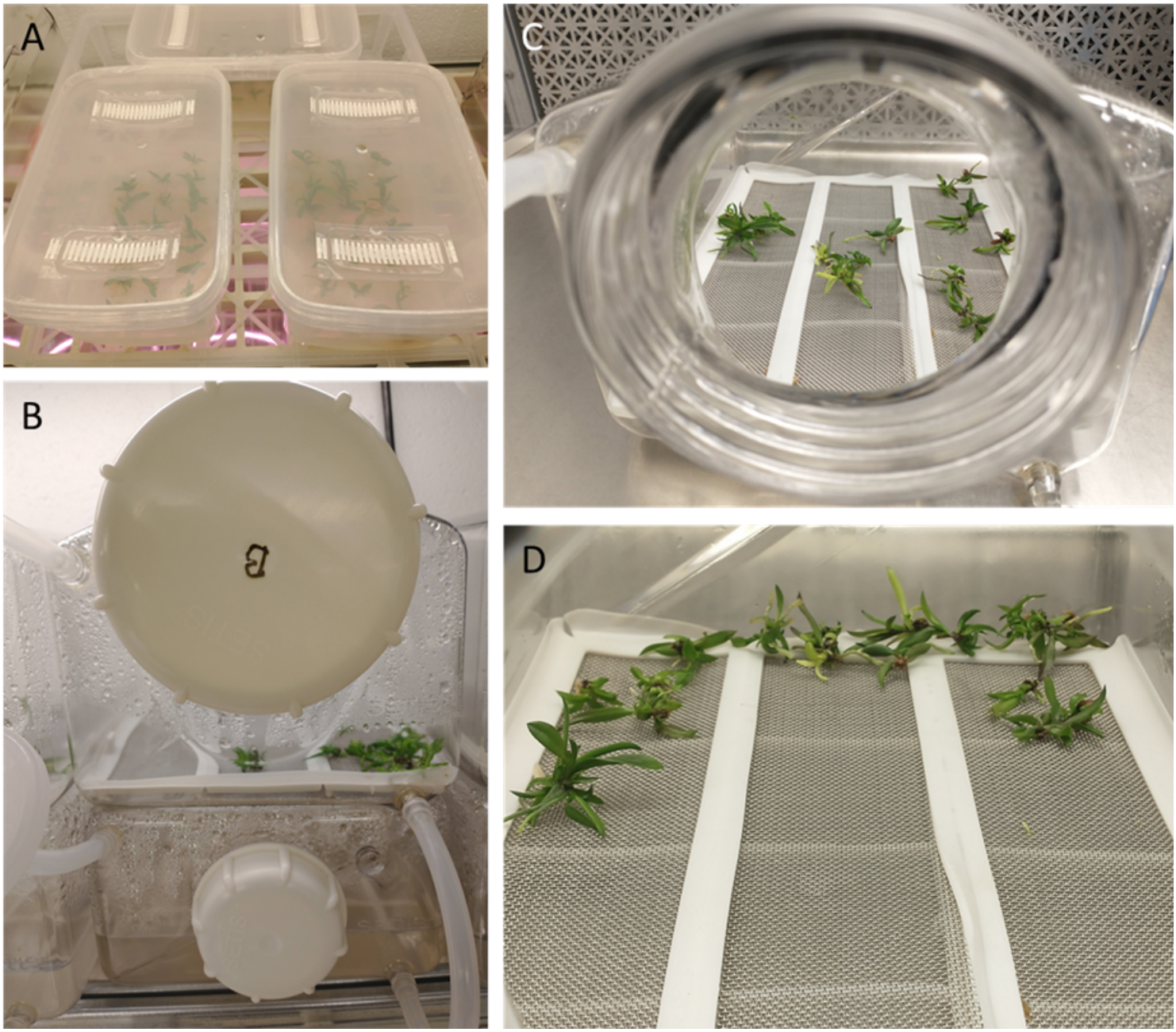

Figure 1

Micropropagation of *Brassavola nodosa* under different culture systems. Semi-solid medium in microboxes (A). Liquid medium in SETIS™ temporary immersion bioreactors (B). Detail of *B. nodosa in vitro* shoots inside the bioreactor vessel (C, D).

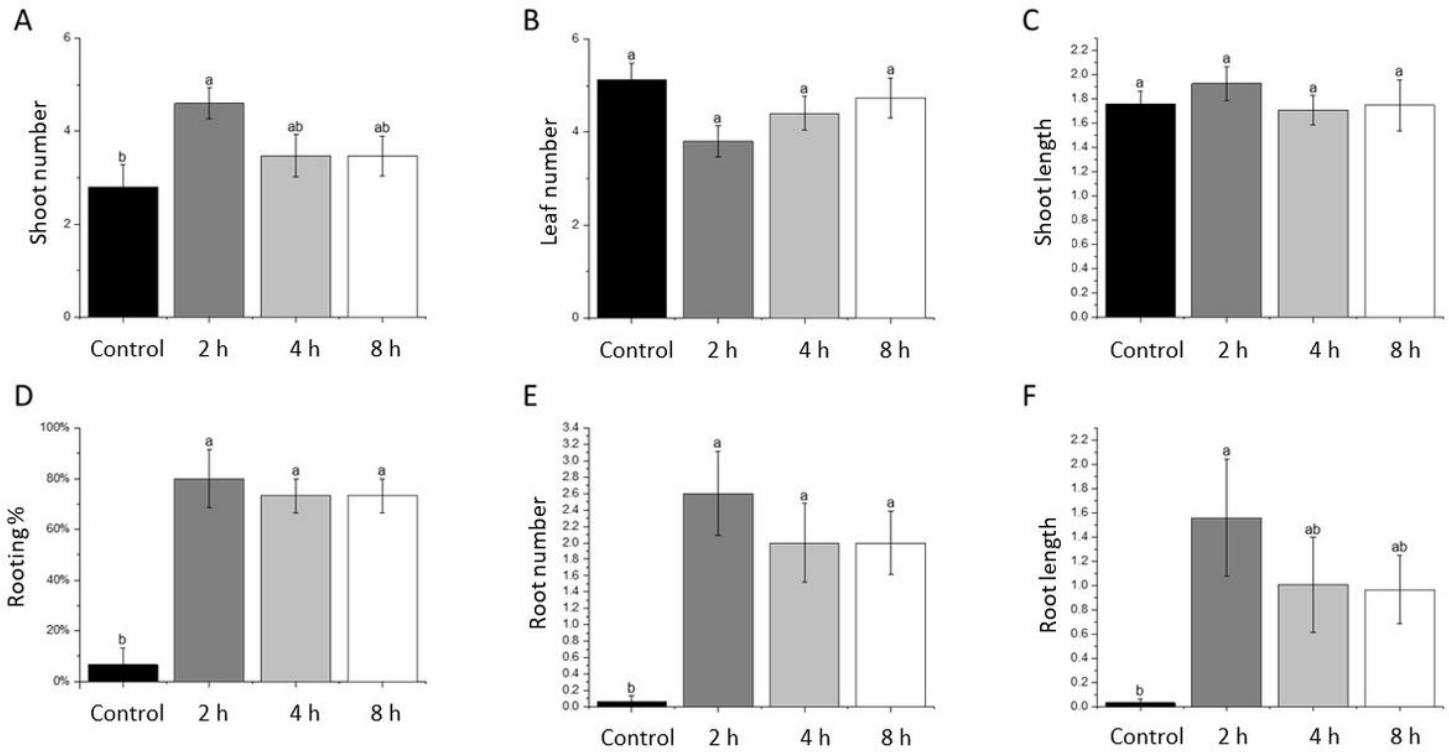

Figure 2

Effects of different *in vitro* culture systems on growth and development of *Brassavola nodosa* after 60 days in culture. A) Shoot multiplication (number of shoots per explant), B) Leaf number, C) Shoot length, D) Rooting percentage, E) Root number, and F) Root length. Control = Semi-solid medium; Temporary Immersion Bioreactor: 2 hours immersion/aeration frequency; 4 hours immersion/aeration frequency; 8 hours immersion/aeration frequency. Immersion duration was 2 minutes for all bioreactor treatments. Bars indicate mean ± SE. Different letters indicate significant differences by Tukey'stest at $p \leq 0.05$.

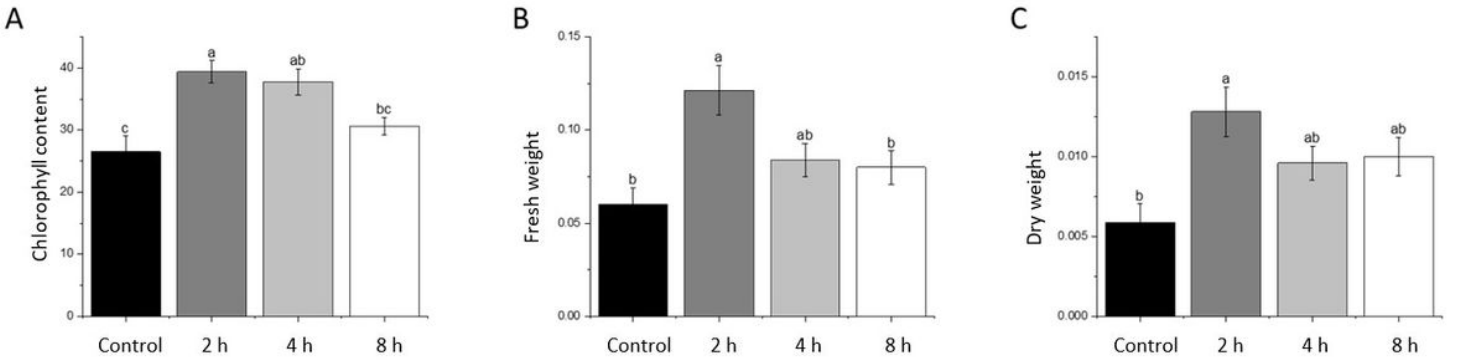

Figure 3

Effects of different *in-vitro* culture systems on chlorophyll content (A), and fresh (B) and dry (C) weight of *Brassavola nodosa* after 60 days in culture. Control = Semi-solid medium; Temporary Immersion Bioreactor: 2 hours immersion/aeration frequency; 4 hours immersion/aeration frequency; 8 hours

immersion/aeration frequency. Immersion duration was 2 minutes for all bioreactor treatments. Bars indicate mean ± SE. Different letters indicate significant differences by Tukey's test at $p \leq 0.05$.

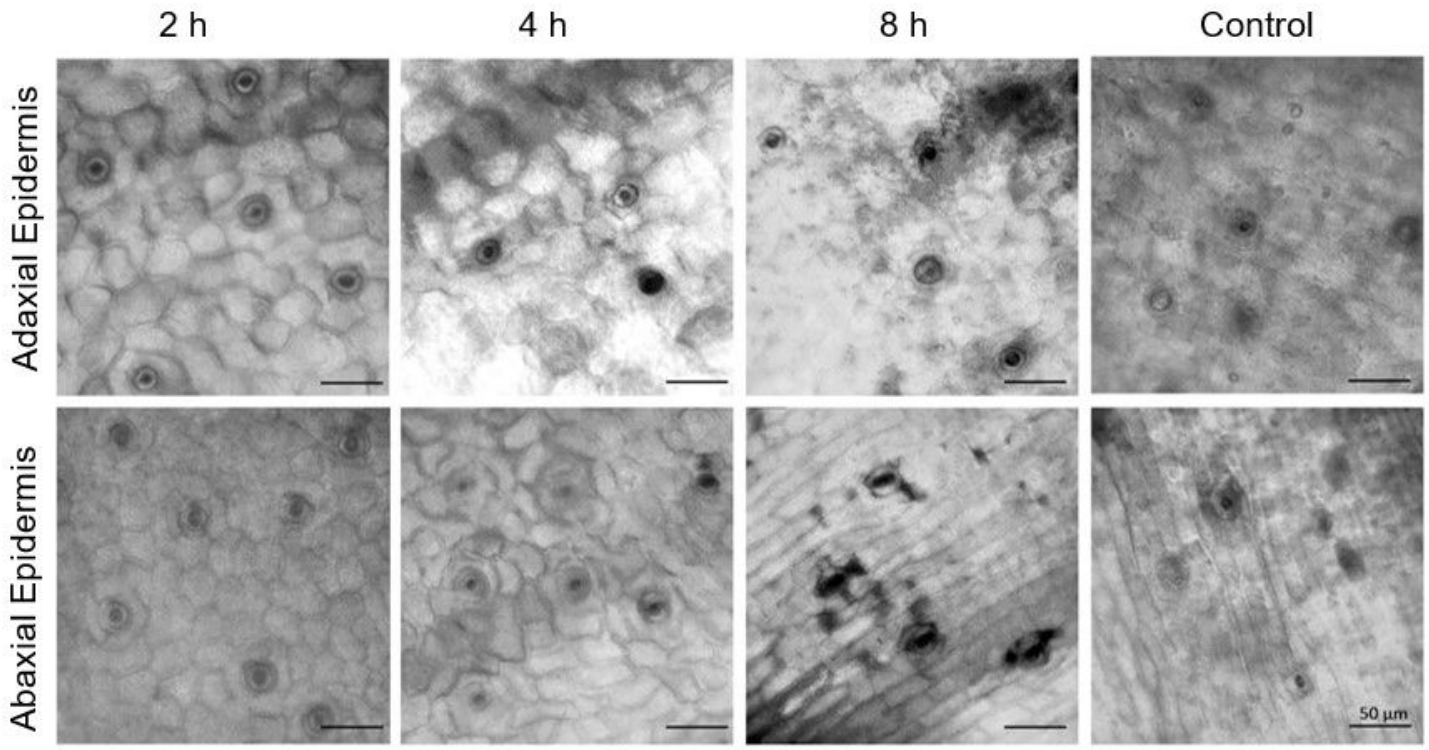

Figure 4

Stomatal prints of abaxial and adaxial leaf surfaces of *Brassavola nodosa* after 60 days in culture under different culture systems. Control = Semi-solid medium; Temporary Immersion Bioreactor: 2 hours immersion/aeration frequency; 4 hours immersion/aeration frequency; 8 hours immersion/aeration frequency. Immersion duration was 2 minutes for all bioreactor treatments. Bars = 50 µm.

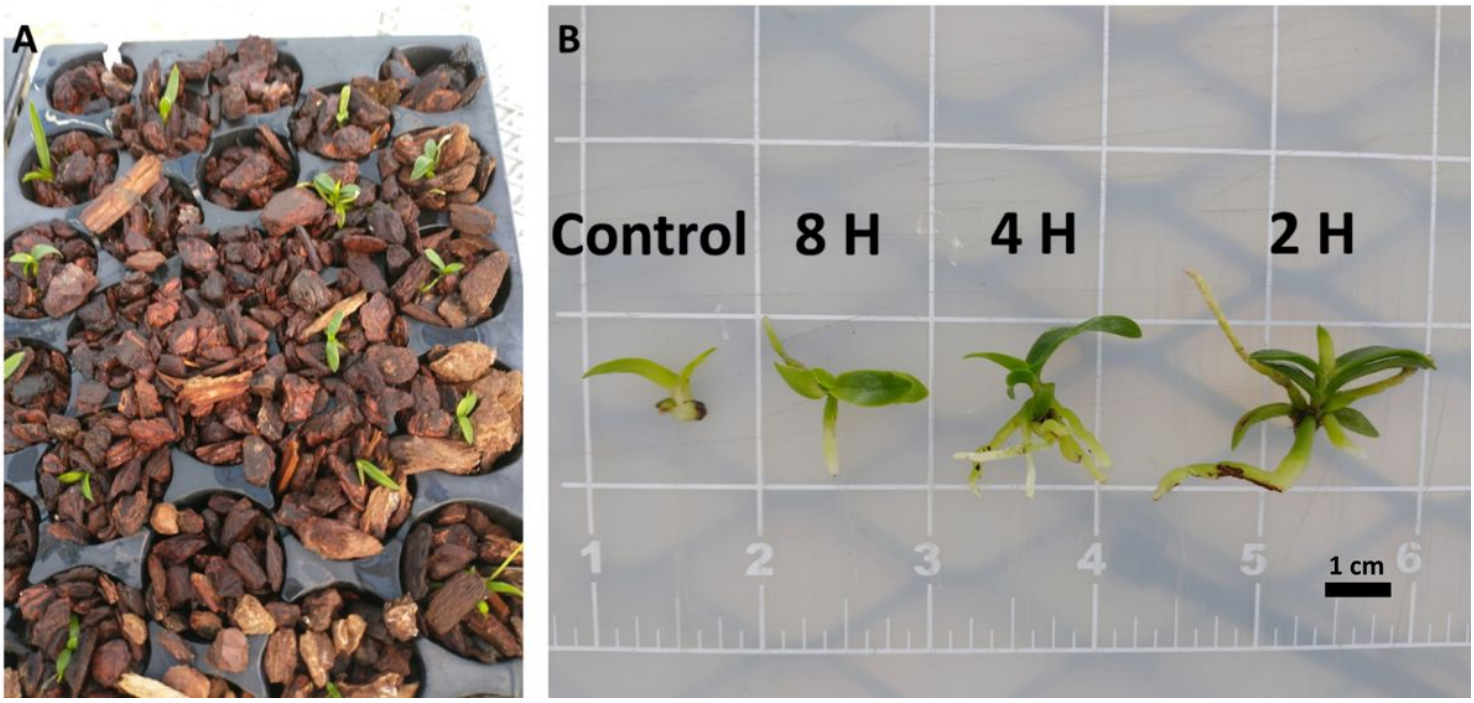

Figure 5

In vitro-derived plantlets after 60 days under acclimatization. A) Acclimatization of *in vitro*-derived *Brassavola nodosa* plants in greenhouse with mist system. B) Effect of culture systems on plant development during acclimatization: Control = Semi-solid medium; Temporary Immersion Bioreactor: 2 hours immersion/aeration frequency; 4 hours immersion/aeration frequency; 8 hours immersion/aeration frequency. Immersion duration was 2 minutes for all bioreactor treatments. Bars indicate mean ± SE. Scale bar = 1 cm.