



Effects of culture methods and plant growth regulators on *in vitro* propagation of *Brassavola nodosa* (L.) Lindl. hybrid

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Received: 29 March 2022 / Accepted: 14 May 2022 / Published online: 21 July 2022 / Editor: Jianxin Chen
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Abstract

Brassavola nodosa (L.) Lindl. is an epiphytic orchid with great potential for the ornamental plant industry. The lack of information on propagation and production techniques limits the development of large-scale commercial production. Furthermore, this species is experiencing a reduction in population due to habitat destruction, the impact of climate change, and over-collection from native habitats. This study aimed at developing an efficient protocol for micropropagation of *B. nodosa*, which could be valuable towards the large-scale commercial production as well as for conservation of this species. Six different concentrations of plant growth regulators (BA or IBA, with or without adenine sulfate) were evaluated in modified Murashige and Skoog (MS) medium for shoot multiplication. In addition, two concentrations of either NAA or IBA were evaluated for rooting. Explants were cultured under three different culture media conditions: semi-solid medium, liquid medium (partial immersion), and liquid medium (complete immersion). Results indicate that *B. nodosa* could be successfully micropropagated in liquid culture with partial immersion. The modified MS medium supplemented with 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate resulted in higher multiplication rates. Rooting was obtained using either 0.5 mg L⁻¹ NAA or 1.0 mg L⁻¹ IBA with no significant differences between both rooting treatments. Plantlets achieved 100% *ex vitro* survival after 30-d acclimatization.

Keywords *Brassavola nodosa* · *In vitro* culture · Plant growth regulator · Orchid · Commercial propagation · Liquid culture

Introduction

The Orchidaceae is a family of flowering plants with unique diversity and beauty placing them among the top commercialized cut flower or potted flowering plants in the international market (Vendrame 2018). With about 30,000 to 35,000 estimated known species within over 850 genera and over 160,000 hybrids developed, they represent about 10% of all flowering plants (Roberts and Dixon 2008; Vendrame and Khoddamzadeh 2017; Vendrame 2018). Their specialized floral morphology, structure, and physiological properties have always fascinated people making them very popular worldwide (Arditti 1992). Nevertheless, the Orchidaceae features a high proportion of threatened species due

to the destruction of natural habitat, the impact of climate change, and over-collection from natural habitats (Swarts and Dixon 2009; Reed *et al.* 2011; Mengarda *et al.* 2017). Among threatened orchid species, *Brassavola nodosa* (L.) Lindl. is an epiphytic orchid found from Mexico through Central America to Venezuela and Peru (Jones 1975; Mata-Rosas and Lastre-Puertos 2015). Their inflorescence is very fragrant at night from whence it gained its common name as the “Lady of the Night.” Some characteristics of this plant, such as fragrant flowers, low maintenance, year-round blooming, and unique flower shape, make it an excellent candidate for the commercial ornamental industry. However, large-scale commercial production is limited due to its recalcitrant propagation nature. *Brassavola* orchids propagate through branching, pseudobulbs, and seeds. Their natural germination is very low, and propagation by branching or pseudobulbs is time-consuming (Mengarda *et al.* 2017).

Micropropagation or *in vitro* propagation could be a feasible alternative for the propagation of *B. nodosa* (Chugh *et al.* 2009; Phillips and Garda 2019), thus allowing mass rapid multiplication of plants for commercial use (Kumar

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and Reddy 2011; Bhoite and Palshikar 2014). In addition, *in vitro* culture allowed the production of pathogen-free orchids and germplasm storage, and is indispensable to breeding and genetic improvement programs (Bhoite and Palshikar 2014). A number of protocols have been developed for the micropropagation of orchids, 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; Pierik 1997; Tisserat and Jones 1999; Kalimuthu *et al.* 2006; Chugh *et al.* 2009; Neumann *et al.* 2009; Alam *et al.* 2010; Khoddamzadeh *et al.* 2011; Paek *et al.* 2011; Panwar *et al.* 2012; Zeng *et al.* 2014; Divakaran *et al.* 2015; Teixeira da Silva *et al.* 2015; Rodrigues *et al.* 2015; 2014; Vendrame and Khoddamzadeh 2017).

Although orchid micropropagation has shown spectacular development in recent years, some hard to propagate genera, such as *Brassavola*, still remain a challenge. The few studies available on the genus *Brassavola* include aspects of *in vitro* germination of *Brassavola perrinii* hybrid (Chiapim *et al.* 2012) and *B. tuberculata* (Soares *et al.* 2020; Sousa *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*. In addition, no published reports are available on *in vitro* establishment and propagation of *B. nodosa*. Most recently, some studies have showed improved efficiency of orchid micropropagation in liquid culture (Young *et al.* 2000; Leyva-Ovalle *et al.* 2020). Liquid culture systems may offer some advantages over semi-solid media in *in vitro* systems improving plant growth and development and providing higher rates of multiplication and the potential of reduction in manipulation and labor costs *via* automation (Ziv 2000). The use of liquid cultures also allows better uniformization and distribution of nutrients, including easy renewal of media, the ability to use larger containers, and reduced number of subcultures needed, (Etienne and Berthouly 2002).

Therefore, the objective of this study was to evaluate different *in vitro* culture systems by comparing liquid and semi-solid media under different concentrations of plant growth regulators (PGRs) for *in vitro* propagation of *Brassavola nodosa*. Rooting of *in vitro* shoots and acclimatization of *in vitro*-derived plantlets were also evaluated.

Materials and Methods

Plant Material *Brassavola nodosa* ‘Remar’ x ‘Mas Mejor’ hybrid seeds were surface sterilized using 70% ethanol (Decon Labs, Inc., King of Prussia, PA) for 1 min followed by 0.8% sodium hypochlorite (NaClO, Thermo Fisher Scientific, Hampton, NH) for 5 min. Subsequently, seeds were rinsed three times

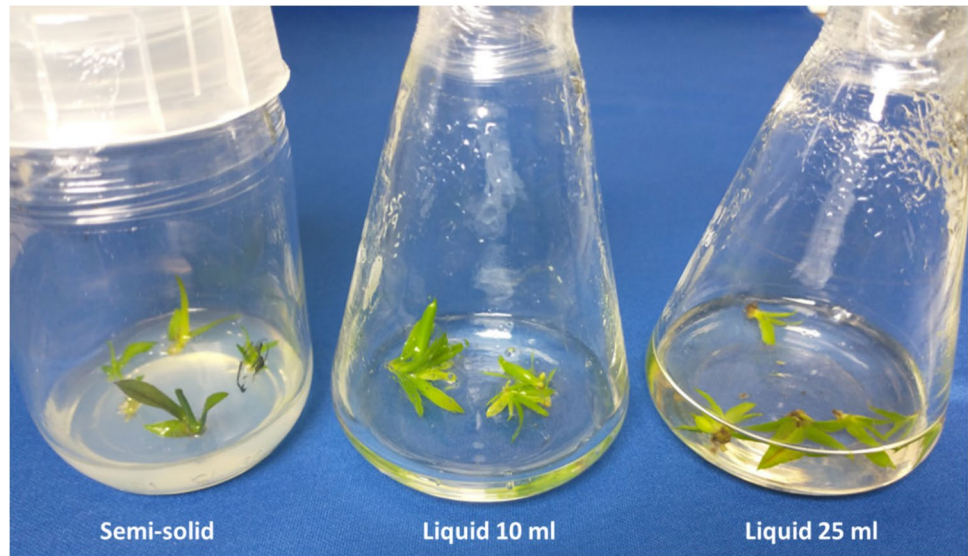
in sterile water and germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962, Phytotechnology Laboratories, Lenexa, KS) supplemented with 15 g L⁻¹ sucrose (Thermo Fisher Scientific) and 7 g L⁻¹ agar (Thermo Fisher Scientific). The pH of the medium was adjusted to 5.7 before autoclaving at 121 °C and 15 lbs pressure for 20 min. The medium was dispensed in 100-mm × 15-mm disposable Petri dishes (Thermo Fisher Scientific). After 60 d, when seeds germinated and protocorms developed 1 to 2 leaves and roots, seedlings were transferred to MS medium supplemented with 30 g L⁻¹ sucrose and 7 g L⁻¹ agar in RA40 Microboxes (Sac O₂, Deinze, Belgium).

Shoot Multiplication Individual shoot tips (0.3 to 0.5 cm) from 1-yr-old *in vitro* seedlings were used as explants for shoot multiplication. Shoots were cultured on modified MS medium supplemented with 2 mg L⁻¹ glycine (Thermo Fisher Scientific), 0.1 mg L⁻¹ NAA (naphthaleneacetic acid, Thermo Fisher Scientific), 10% coconut water (Phytotechnology Laboratories), and 30 g L⁻¹ sucrose. The pH of the medium was adjusted to 5.2 before autoclaving at 121 °C and 15 lbs pressure for 20 min. Six different concentrations and combinations of PGRs (Thermo Fisher Scientific) were evaluated, as follows:

- T1—1.0 mg L⁻¹ BA (6-benzylaminopurine)
- T2—1.0 mg L⁻¹ BA + 30.0 mg L⁻¹ adenine sulfate
- T3—2.0 mg L⁻¹ BA + 30.0 mg L⁻¹ adenine sulfate
- T4—1.0 mg L⁻¹ IBA (indole-3-butyric acid)
- T5—1.0 mg L⁻¹ IBA + 30.0 mg L⁻¹ adenine sulfate
- T6—2.0 mg L⁻¹ IBA + 30.0 mg L⁻¹ adenine sulfate

Shoots were cultured on these treatments under three different culture methods: (1) in 220-mL baby-food jars (Phytotechnology Laboratories) containing 25 mL semi-solid medium (7 g L⁻¹ agar), (2) in 125-mL Erlenmeyer flasks (Thermo Fisher Scientific) containing 10 mL liquid medium (partial immersion), and (3) in 125-mL Erlenmeyer flasks containing 25 mL liquid medium (complete immersion) (Fig. 1). Flasks containing liquid media were placed on an orbital shaker (New Brunswick Scientific, Edison, NJ) at 90 rpm. The experiment was comprised of 6 replicates (flasks) per treatment with each replicate (flask) containing 5 explants. Explants were selected randomly before being placed in the flasks under each treatment. The cultures were maintained under controlled environmental conditions under a 16-h photoperiod at light intensity of 50 μmol m⁻² s⁻¹ and temperature of 26 ± 4 °C. Shoot multiplication was calculated by the number of shoots forming per initial explant, and shoot survival was estimated by the number of shoots that continued growth and development after multiplication. These parameters were evaluated 45 d after culture establishment for 30 shoots per treatment. The entire experiment was repeated.

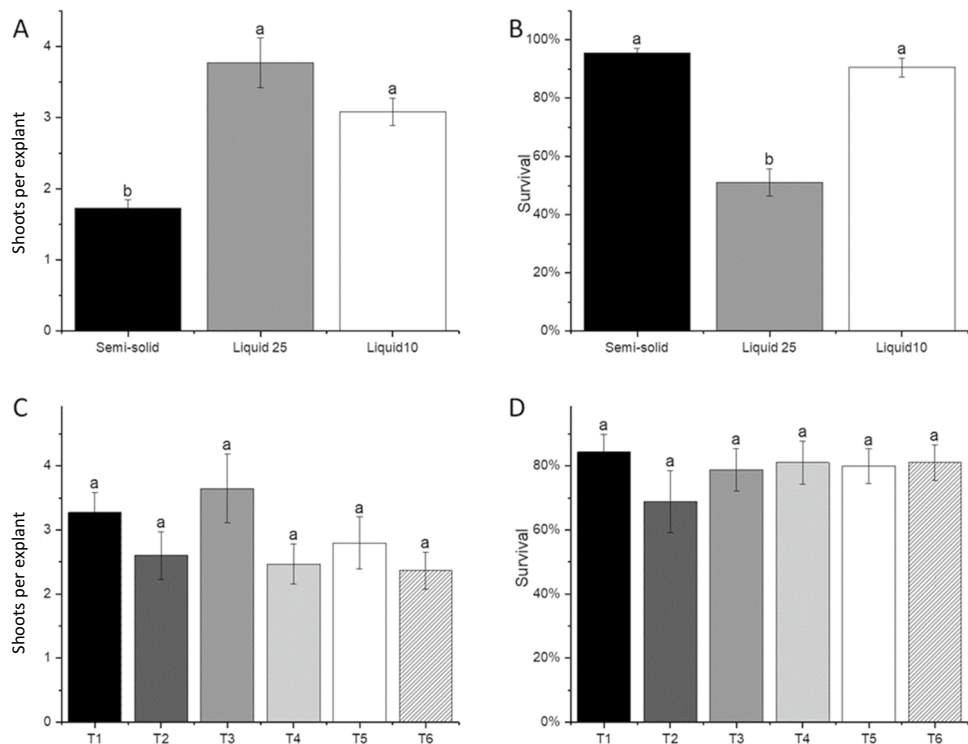
Figure 1. Shoot explants of *Brassavola nodosa* L. hybrid cultured *in vitro* on modified Murashige and Skoog medium with different concentrations of plant growth regulators in 175-mL baby food jars with 25 mL semi-solid medium (7 g L⁻¹ agar), in 125-mL Erlenmeyer flasks with 10 mL liquid medium, and in 125-mL Erlenmeyer flasks with 25 mL liquid medium.



Rooting Adventitious shoots that developed from shoot multiplication were collected and transferred to two different rooting media: (1) MS basal medium supplemented with 30 g L⁻¹ sucrose, 7 g L⁻¹ agar, and 0.5 mg L⁻¹ NAA; (2) MS basal medium supplemented with 30 g L⁻¹ sucrose, 7 g L⁻¹ agar, and 1.0 mg L⁻¹ IBA. The pH of the medium was adjusted to 5.2 before autoclaving at 121 °C at 15 lbs pressure for 20 min. Shoots were maintained in rooting media for 60 d, after which they were transferred to PGR-free MS

medium for 30 d before being transferred to the greenhouse for acclimatization. The experiment comprised of 3 replicates per treatment with each replicate containing 5 explants. Cultures were maintained under controlled environment with a 12-h photoperiod at light intensity 50 μmol m⁻² s⁻¹ provided by fluorescent lights (GE Lighting F96T8/XL/SPP35, East Cleveland, OH) and temperature of 26 ± 4 °C. Shoot multiplication, shoot survival, and number of roots per shoot were evaluated 60 d after culture establishment. Growth and

Figure 2. *Brassavola nodosa* L. shoot multiplication rate and shoot survival after 45 d. Multiplication rate and survival of explants cultured on 25 mL semi-solid medium, 25 mL liquid medium, and 10 mL liquid (A, B). Shoot multiplication rate and shoot survival among the different treatments (C, D). Treatments are T1: 1.0 mg L⁻¹ BA; T2: 1.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T3: 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T4: 1.0 mg L⁻¹ IBA; T5: 1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate; and T6: 2.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate. Bars indicate mean ± SE. Different letters indicate significant differences by Tukey's test at $P \leq 0.05$.



development parameters, including shoot length, root length, number of roots, number of leaves per shoot, and fresh and dry weight, were evaluated 30 d after explants were cultured on PGR-free medium. Dry weight was determined by oven-drying plantlets at 70 °C until they reached constant weight. A total of 30 shoots were used for evaluations of growth and development parameters.

Acclimatization Ten rooted plants were selected from each treatment for acclimatization. Rooted shoots were transferred to 40-cell plastic trays with soilless media containing orchid bark (Sequoia Bark Sales, Reedley, CA). Plants were maintained in a greenhouse with a mist system running for 20 s every 30 min. Plants were fertilized weekly with 300 ppm of 11–35–15 (N-P₂O₅-K₂O) orchid fertilizer (Better-Gro® Orchid Better-Bloom®, Arcadia, FL). The *ex vitro* plant survival was evaluated for all treatments after 30 d.

Experimental Design and Statistical Analysis A completely randomized experimental design was applied for all experiments, where random seedlings were selected and grouped for each treatment. Data were collected and submitted to

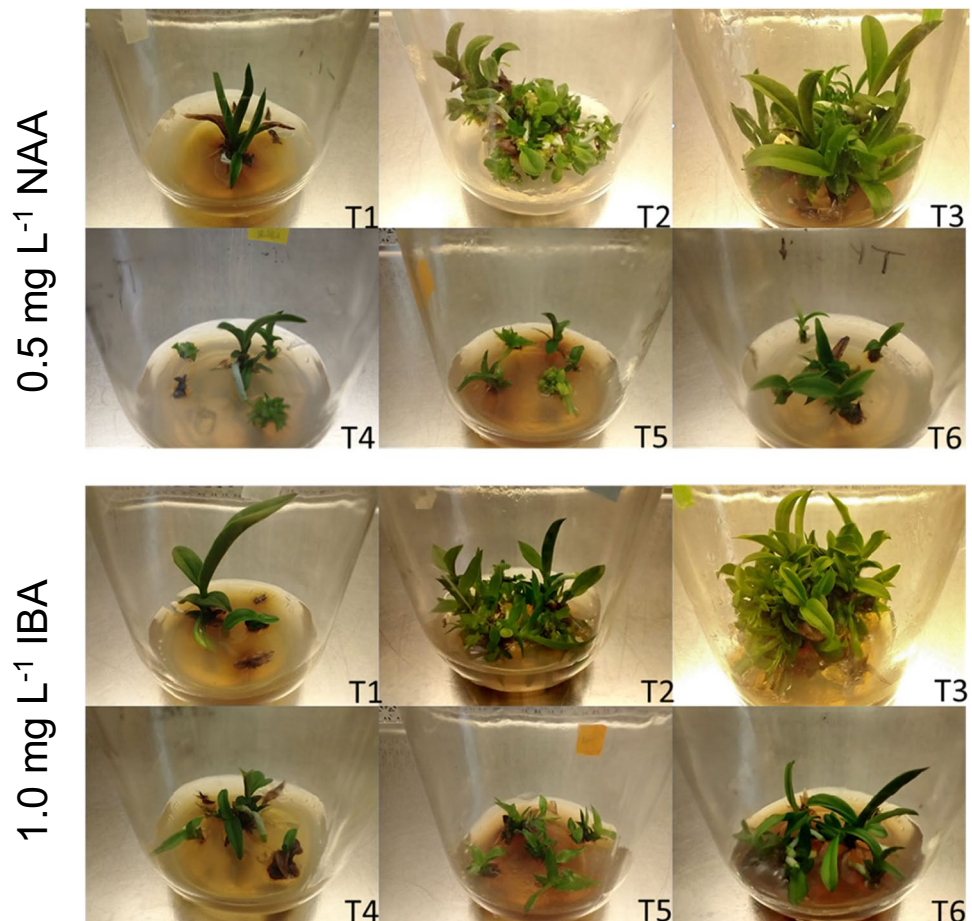
analysis of variance (ANOVA) using the OriginPro® 2021b software (OriginLab, Northampton, MA). Tukey's *post hoc* multiple comparison adjustment ($\alpha=0.05$) was used for all pairwise mean comparisons.

Results

Seed Germination Seed germination was observed within the first 30 d after sowing in Petri dishes, whereas seeds showed enlargement into round and green structures, namely protocorms. After 60 d of sowing, germinated seeds (protocorms) developed 1 to 2 leaflets and root primordia. After transferring germinated seedlings into a large container, the RA40 Microbox allowed for continued growth and development. Growth of seedlings was uniform with shoot size varying from 0.3 to 0.5 cm, which was adequate for subsequent *in vitro* multiplication studies.

Shoot Multiplication The different culture methods showed significant differences in shoot multiplication ($P < 0.001$)

Figure 3. Shoot explants of *Brassavola nodosa* L. hybrid *in vitro* cultures transferred from 25 mL liquid medium to rooting treatments on Murashige and Skoog medium containing either 0.5 mg L⁻¹ NAA (naphthaleneacetic acid) or 1.0 mg L⁻¹ IBA (indole-3-butyric acid) for 60 d. Treatments are T1: 1.0 mg L⁻¹ BA (6-benzylaminopurine); T2: 1.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T3: 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T4: 1.0 mg L⁻¹ IBA; T5: 1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate; and T6: 2.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate.



and survival ($P < 0.001$) (Fig. 2A, B). However, the different concentrations of PGRs did not show significant differences in shoot multiplication ($P < 0.132$) and survival ($P < 0.681$) after 45 d in culture (Fig. 2C, D).

The mean multiplication rate of explants cultured in 25 mL semi-solid medium, 25 mL liquid medium, and 10 mL liquid medium for all PGR treatments were 1.7, 3.8, and 3.1 shoots per explant, respectively. The multiplication rate of explants cultured in 25 mL semi-solid medium was significantly lower than that of explants cultured in 25 mL and 10 mL liquid media (Fig. 2A).

The average survival of explants cultured in 25 mL semi-solid medium was 95.5%, followed by 90.1% in 10 mL liquid medium, and 51.1% in 25 mL liquid medium. Explants cultured in 25 mL liquid medium had significantly lower

survival compared to those cultured in 25 mL semi-solid medium and 10 mL liquid medium ($P < 0.001$) (Fig. 2B).

Phenolic browning was observed in all three media. Dead leaves and shoots appeared in 25 mL liquid medium and in 10 mL liquid medium but rarely in 25 mL semi-solid medium. Although explants cultured in 25 mL liquid medium were larger than those in other treatments, some had a glassy appearance and low lignification.

Rooting Shoots transferred from the multiplication treatments to the rooting media continued to produce additional shoots with 5.6 shoots per explant on 25 mL liquid medium significantly higher than 2.4 shoots per explant on 25 mL semi-solid medium and 2.6 shoots per explant on 10 mL liquid medium ($P < 0.001$) (Figs. 3 and 4A). However, some

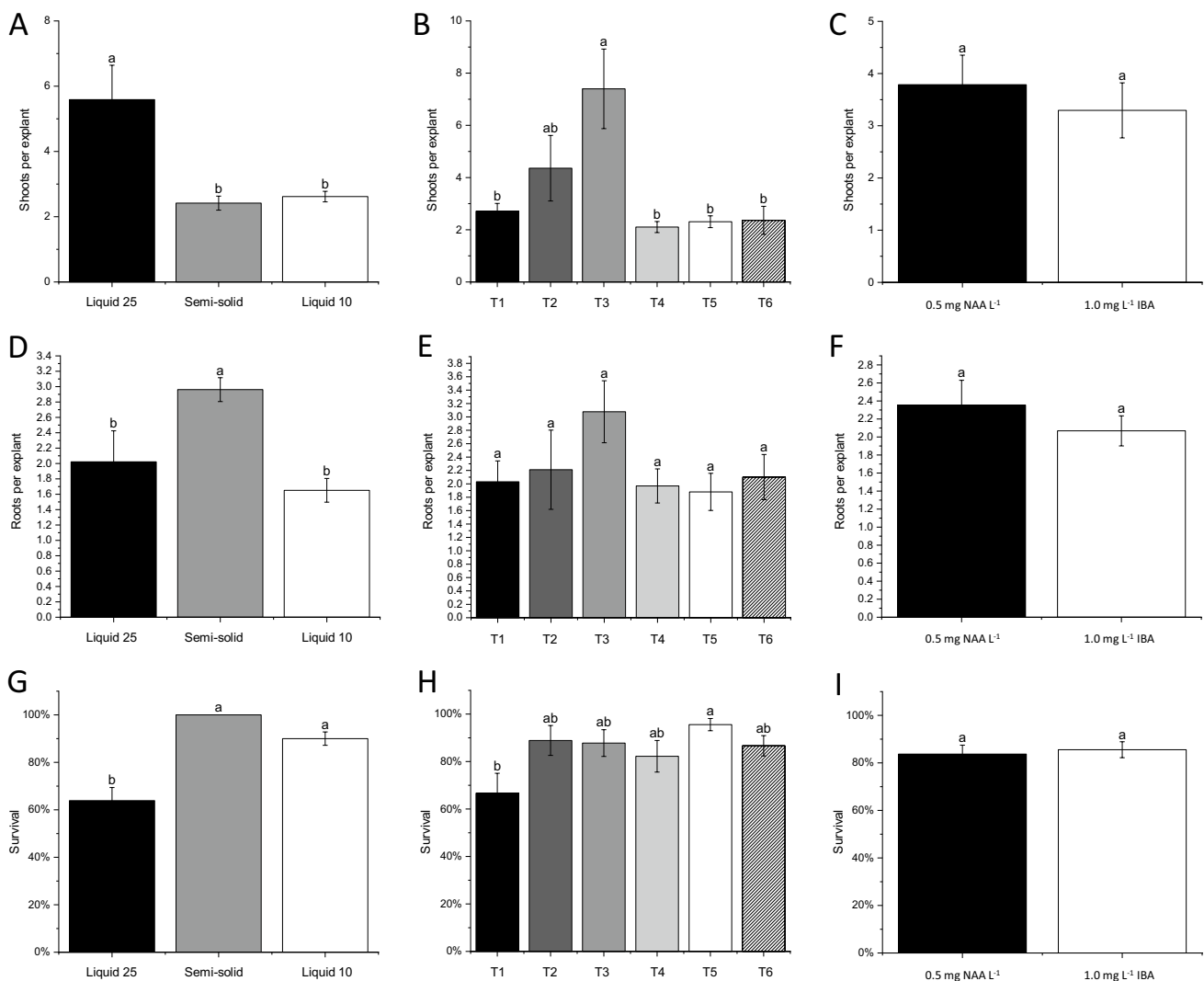


Figure 4. *Brassavola nodosa* L. shoot multiplication rate (A, B, C), rooting rate (D, E, F), and shoot survival (G, H, I), after explants transferred from multiplication to rooting that cultured on Murashige and Skoog medium containing either 0.5 mg L⁻¹ NAA or 1.0 mg L⁻¹ IBA for 60 d. Treatments are T1: 1.0 mg L⁻¹ BA; T2: 1.0 mg L⁻¹ BA

and 30.0 mg L⁻¹ adenine sulfate; T3: 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T4: 1.0 mg L⁻¹ IBA; T5: 1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate; and T6: 2.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate. Bars indicate mean \pm SE. Different letters indicate significant differences by Tukey's test at $P \leq 0.05$.

shoots on 25 mL liquid medium did not survive on rooting medium. The survival of shoots on 25 mL liquid medium (64%) was significantly lower than that on 25 mL semi-solid medium (100%) and that on 10 mL liquid medium (90%) ($P < 0.001$) (Figs. 3 and 4G). Shoots on 25 mL semi-solid medium had an average of 3.0 roots per shoot, which was greater than other treatments ($P < 0.001$) (Fig. 4D).

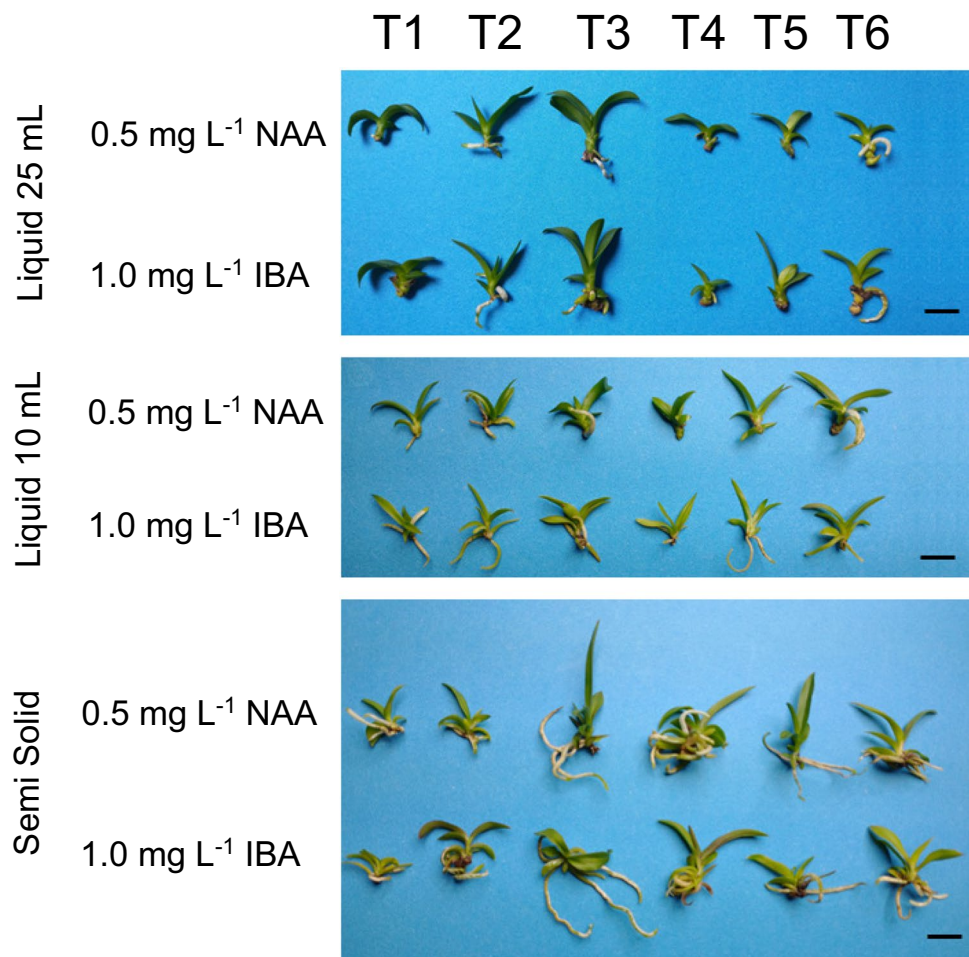
The different concentrations of PGRs influenced survival ($P < 0.001$) and shoot multiplication ($P < 0.001$) but did not affect rooting ($P < 0.115$) (Figs. 3 and 4B, E, H). Treatment 2 (1.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate) showed significantly higher multiplication rates (4.4 to 2.7 shoots per explant, respectively) and survival (89 to 67%, respectively) as compared to treatment 1 containing only 1.0 mg L⁻¹ BA (Figs. 3 and 4B, H). Treatment 3 (2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate) showed higher multiplication (7.4 shoots per explant), but similar survival (88%) compared to treatment 2 (Figs. 3 and 4B, H). Treatment 5 (1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate) showed the highest survival (96%) among all treatments (Fig. 4H). When the IBA concentration increased to 2.0 mg L⁻¹ in treatment 6, survival decreased to 87% (Fig. 5H). The

two rooting media containing either 0.5 mg L⁻¹ NAA or 1.0 mg L⁻¹ IBA did not show significant differences in shoot multiplication ($P < 0.526$), rooting ($P < 0.263$), or survival ($P < 0.715$) (Figs. 3 and 4C, F, I).

PGR-Free Medium After the rooting stage, shoots were transferred to PGR-free medium for 30 d. Results showed that there were no significant differences among all treatments (culture condition ($P < 0.064$), treatments ($P < 0.149$), and rooting hormone ($P < 0.604$)) for shoot length (Figs. 5 and 6A, B, C). Plants on semi-solid medium had the highest number of leaves (5.7 leaves) followed by plants on 10 mL (5.3 leaves) and 25 mL (4.7 leaves) liquid media, respectively ($P < 0.001$) (Fig. 6D). Treatments 4 and 5 showed the highest leaf number (5.6 to 5.8 leaves) among all treatments ($P < 0.001$) (Fig. 6E). The type of rooting media had no effect on leaf number ($P < 0.065$) (Fig. 6F).

The highest root number ($P < 0.001$) and root length ($P < 0.001$) were observed in plants on semi-solid medium (1.83 cm) followed by the plants on 10 mL (1.19 cm) and 25 mL (0.75 cm) liquid media, respectively (Figs. 5 and 7A, D). Treatment 3 showed the highest root length ($P < 0.021$)

Figure 5. Shoot explants of *Brassavola nodosa* L. hybrid from 6 treatments established in 25 mL semi-solid medium, 10 mL liquid medium, and 25 mL liquid medium for 45 d, followed by rooting in Murashige and Skoog medium with 1.0 mg L⁻¹ IBA (indole-3-butyric acid) or 0.5 mg L⁻¹ NAA (naphthaleneacetic acid) medium for 60 d and then culturing on PGR (plant growth regulator)- free medium for 30 d. Treatments are Treatments are T1: 1.0 mg L⁻¹ BA (6-benzylaminopurine); T2: 1.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T3: 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T4: 1.0 mg L⁻¹ IBA; T5: 1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate; T6: 2.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate. Scale bar = 1 cm.



(1.7 cm), but there were no significant differences on root number ($P < 0.071$) among all treatments (Fig. 7B, E). The rooting media had no effect on root length ($P < 0.607$) or root number ($P < 0.160$) (Figs. 6 and 7C, F).

Plants on semi-solid medium had the highest fresh weight ($P < 0.008$) (0.202 g) and dry weight ($P < 0.001$) (0.023 g) as compared to other media (Fig. 8A, D). Plants on 25 mL liquid medium had higher fresh weight (0.159 g) than plants on 10 mL liquid medium (0.124 g). However, dry weight was not significantly different (Fig. 8A, D). The different treatments and rooting media had no effect on fresh ($P < 0.243$) or dry weight ($P < 0.338$) (Fig. 8B, C, E, F).

Acclimatization Treatments 2 and 3 on 25 mL liquid medium showed continuous shoot multiplication on PGR-free medium, and shoots were used for acclimatization experiments. The rooting media that previously used supplemented with either 1.0 mg L^{-1} IBA or 0.5 mg L^{-1} NAA did not affect survival. Both treatments showed 100% survival after 30 d under acclimatization. New roots were developed from the shoots. No morphological variations were observed in the plants.

Discussion

Although the use of liquid culture systems, including temporary immersion bioreactors, has been developed many years ago and used in the development of various protocols for *in vitro* propagation, they have very limited use in commercial propagation of the most economically significant species. The use of liquid media for micropropagation of orchids has been reported, although it is limited to a few species and hybrids of *Phalaenopsis* (Park *et al.* 2000; Young *et al.* 2000; Kuo *et al.* 2005; Nge *et al.* 2006). The risk of hyperhydricity (vitrification) seems to be one of the reasons why this system is not widely used at the commercial level. This study showed similar results to those previously published. The multiplication rate of explants cultured in 25 mL liquid medium and 10 mL liquid medium was significantly higher than that of explants in 25 mL semi-solid medium. However, the survival of explants in 25 mL liquid medium was significantly lower as compared to other treatments. The explants in 25 mL liquid medium had enlarged, thick stems and leaves. Some of them had glassy appearance, which are typical symptoms of hyperhydricity caused by excessive

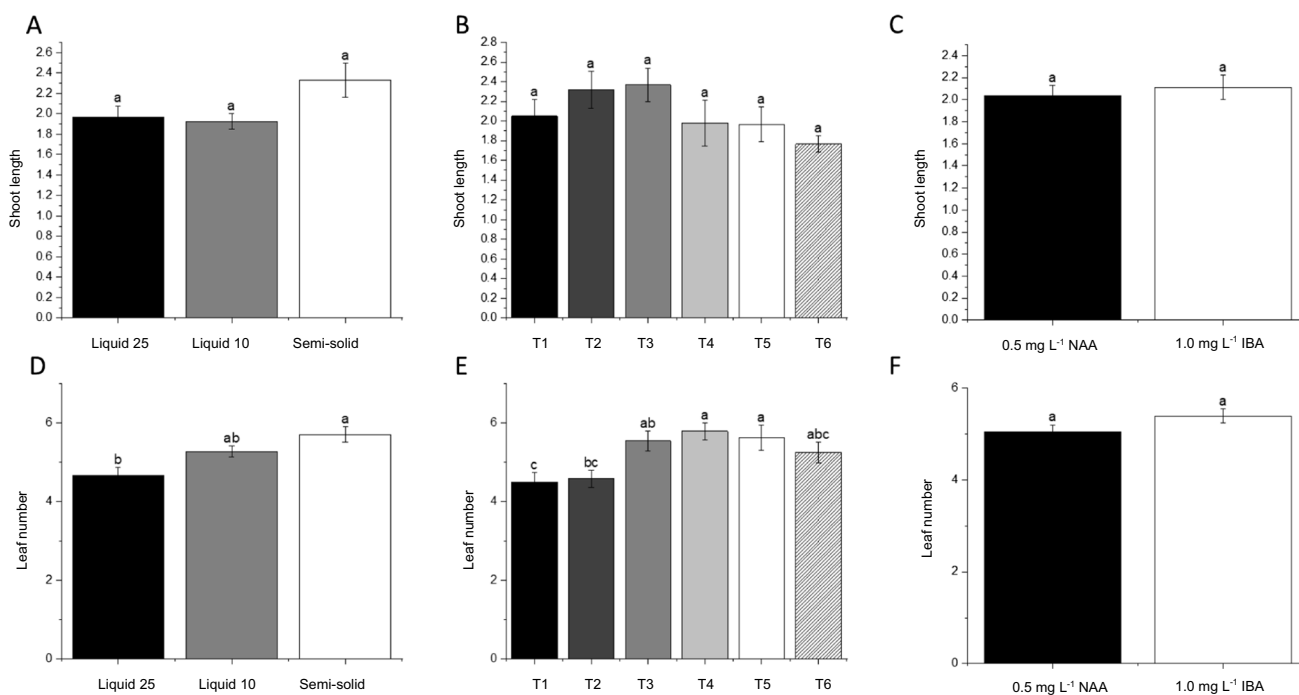


Figure 6. *Brassavola nodosa* L. shoot length (A, B, C) and leaves number (D, E, F) after plants transferred from rooting media (Murashige and Skoog medium containing either 0.5 mg L^{-1} NAA; naphthaleneacetic acid) or 1.0 mg L^{-1} IBA; indole-3-butyric acid) to PGR (plant growth regulator)-free medium for 30 d. Treatments are T1: 1.0 mg L^{-1} BA (6-benzylaminopurine); T2: 1.0 mg L^{-1} BA

and 30.0 mg L^{-1} adenine sulfate; T3: 2.0 mg L^{-1} BA and 30.0 mg L^{-1} adenine sulfate; T4: 1.0 mg L^{-1} IBA; T5: 1.0 mg L^{-1} IBA and 30.0 mg L^{-1} adenine sulfate; and T6: 2.0 mg L^{-1} IBA and 30.0 mg L^{-1} adenine sulfate. Bars indicate mean \pm SE. Different letters indicate significant differences by Tukey's test at $P \leq 0.05$.

water uptake. Hyperhydricity is a physiological malformation that results in excessive hydration, low lignification, poorly developed vascular bundles, impaired stomatal function, and reduced mechanical strength of tissue culture-generated plants (Kevers *et al.* 2004). This could explain why the survival of explants in 25 mL liquid medium was low. One interesting aspect is that explants cultured in 10 mL liquid medium with partial immersion in the medium showed a higher survival and higher multiplication rates than explants in 25 mL semi-solid medium with complete immersion. In addition, those explants on 10 mL liquid medium did not show any symptoms of hyperhydricity. This indicates that *B. nodosa* could be successfully propagated in liquid culture with partial immersion. It also shows that this species has the potential for being propagated in temporary immersion system (TIS) bioreactors. The application of TIS bioreactors for propagation of orchids has been reported for many genera such as *Phalaenopsis* (Hempfling and Preil 2005), *Anoectochilus* (Wu *et al.* 2007), *Guarianthe* (Leyva-Ovalle *et al.* 2020), and *Cattleya* (Ekmekçigil *et al.* 2019). These results warrant further investigation on the potential of TIS bioreactors for the micropropagation of *B. nodosa*.

The PGRs in the early stages of culture can have effects on growth and development in later stages described as carry-over effects. These carry-over effects have been

reported previously (Phillips 1987; Bairu *et al.* 2008; Kane 2011; Amoo *et al.* 2013). The results in this study showed that explants transferred from the multiplication treatments to the rooting media continued producing shoots. Explants on 25 mL liquid medium produced significantly higher amounts of shoots as compared to other treatments. This indicates a carry-over effect from PGRs used during multiplication. In fact, shoot explants from 25 mL liquid medium were continuously multiplying shoots after being transferred to PGR-free medium. This is characteristic of habituation, the autotrophic *in vitro* growth in cultures that had previously required auxin or cytokinin for *in vitro* growth and multiplication (Pischke *et al.* 2006). Habituation is caused by either DNA modification or mutation (Meins 1989; Limanton-Grevet *et al.* 2000; Pischke *et al.* 2006). Therefore, the study of genetic fidelity of explants needs to be considered. However, because habituation is reversible, increasing subculturing frequency may minimize its effects on *in vitro* cultures.

Among the PGRs used in micropropagation, BA is the most frequently used cytokinin for plant differentiation and regeneration (Giatti *et al.* 2007; Van Staden *et al.* 2008; Phillips and Garda 2019). It plays an important role in shoot multiplication. Treatment 3, which had 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate, showed higher multiplication

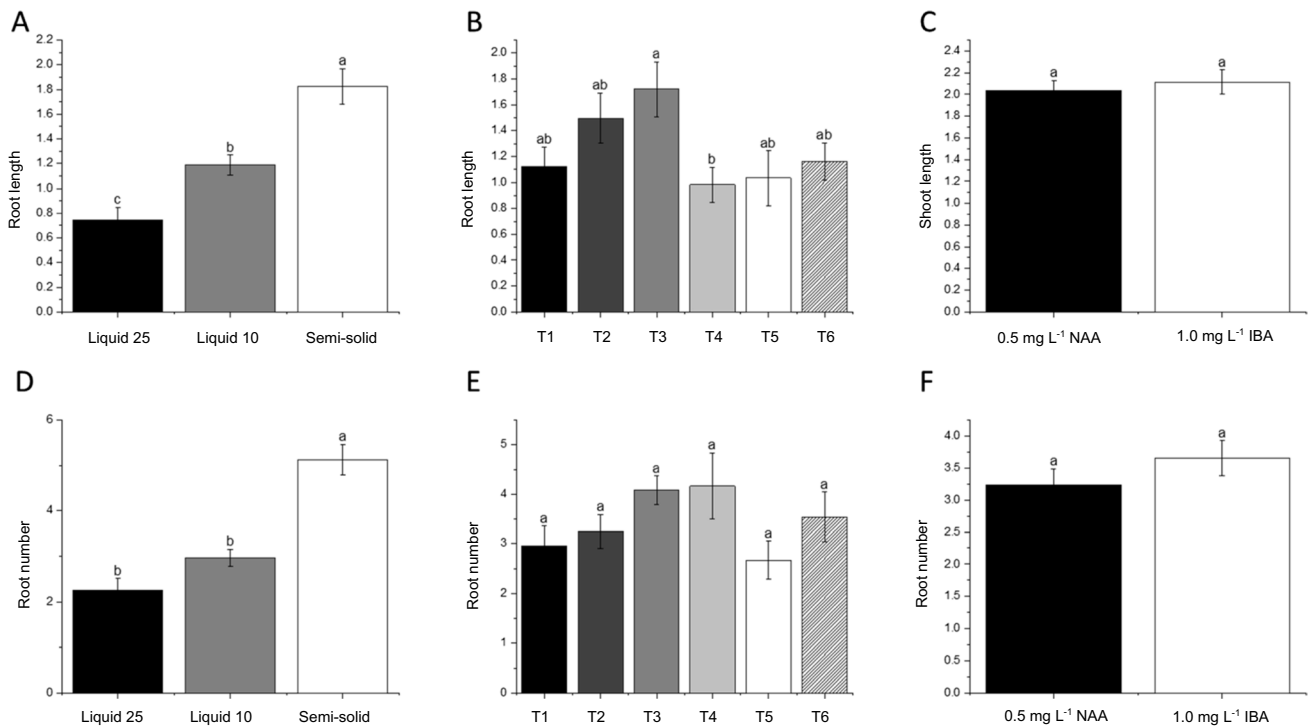


Figure 7. *Brassavola nodosa* L. root length (A, B, C) and root number (D, E, F) after plants transferred from rooting media (Murashige and Skoog medium containing either 0.5 mg L⁻¹ NAA; naphthaleneacetic acid or 1.0 mg L⁻¹ IBA; indole-3-butyric acid) to PGR (plant growth regulator)-free medium for 30 d. Treatments are T1: 1.0 mg L⁻¹ BA; T2: 1.0 mg L⁻¹ BA (6-benzylaminopurine)

and 30.0 mg L⁻¹ adenine sulfate; T3: 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T4: 1.0 mg L⁻¹ IBA; T5: 1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate; and T6: 2.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate. Bars indicate mean \pm SE. Different letters indicate significant differences by Tukey's test at $P \leq 0.05$.

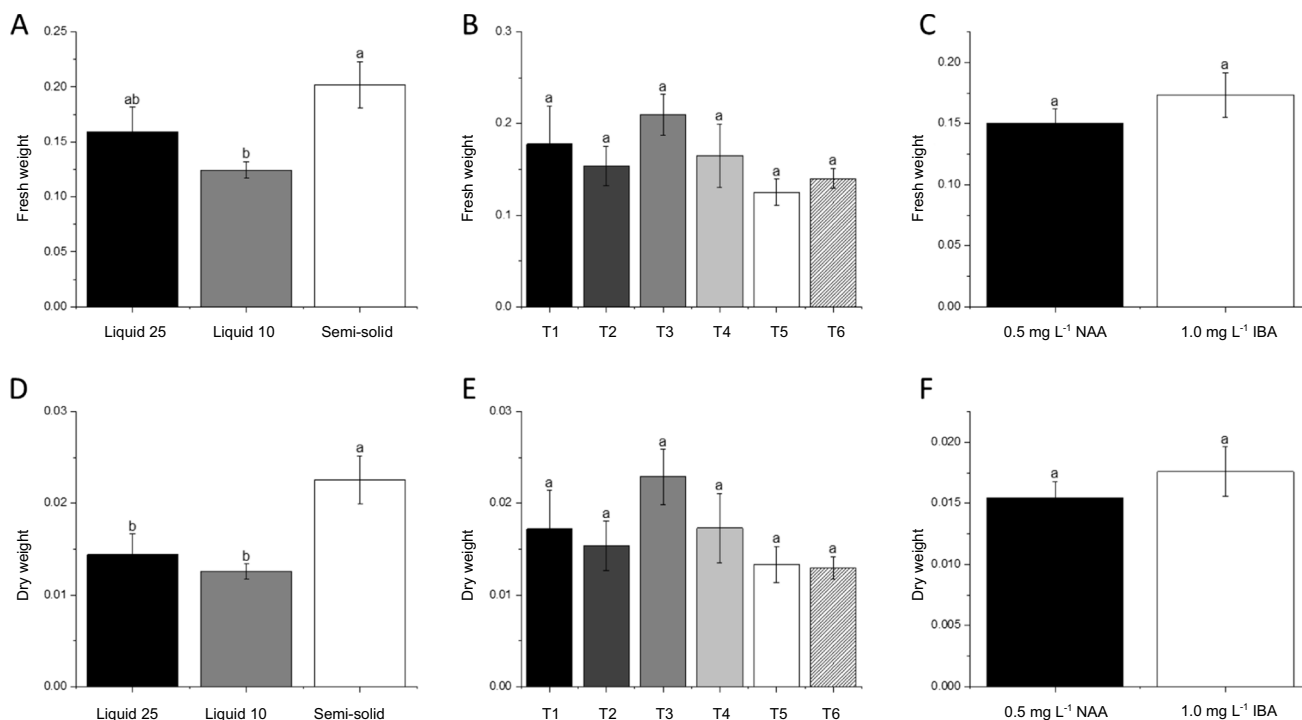


Figure 8. *Brassavola nodosa* L. fresh weigh (A, B, C) and dry weight (D, E, F) after plants transferred from rooting media (Murashige and Skoog medium containing either 0.5 mg L⁻¹ NAA; naphthaleneacetic acid or 1.0 mg L⁻¹ IBA; indole-3-butyric acid) to PGR (plant growth regulator)-free medium for 30 d. Treatments are T1: 1.0 mg L⁻¹ BA (6-benzylaminopurine); T2: 1.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine

sulfate; T3: 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate; T4: 1.0 mg L⁻¹ IBA; T5: 1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate; and T6: 2.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate. Bars indicate mean \pm SE. Different letters indicate significant differences by Tukey's test at $P \leq 0.05$.

rates but similar survival compared to treatment 2, which had 1.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate. This indicates that a higher concentration of BA can increase multiplication rates but not survival. Treatment 2, which had 1.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate, showed significantly higher multiplication and survival as compared to treatment 1, which had only 1.0 mg L⁻¹ BA. This indicates that adenine sulfate could improve survival and multiplication rates during micropropagation of *B. nodosa* orchids. Culture media containing adenine sulfate in addition to kinetin is conducive to shoot formation, as reported in classic studies with *Nicotiana tabacum* (Scott *et al.* 1964; Thorpe and Murashige 1968). The improvement of plant health and multiplication rate by adenine sulfate has been reported previously (George *et al.* 2008). Treatment 5 containing 1.0 mg L⁻¹ IBA and 30.0 mg L⁻¹ adenine sulfate showed the highest survival (96%) among all treatments. Paek *et al.* (1987) showed that adding adenine sulfate to a medium containing IBA resulted in increased shoot weight, darker green leaves, and healthier cultures in Chinese cabbage as compared to those without adenine sulfate. The evaluation of PGRs' effects on bud induction in *Cattleya walkeriana*, a species closely related to *B. nodosa*, showed

that IBA could also induce shoots (Prizão *et al.* 2012). In this study, IBA had a positive effect on shoot multiplication, as compared to BA. However, increased IBA concentrations could have a negative impact on plant development (Morgan *et al.* 1997), as shown in this study when we increased IBA from 1.0 to 2.0 mg L⁻¹.

B. nodosa as an epiphytic orchid is more dependent on its roots for growth and development as compared to other species. Thus, rooting is key to its successful adaptation to the *ex vitro* environment. This study showed that two rooting media containing either 0.5 mg L⁻¹ NAA or 1.0 mg L⁻¹ IBA had similar effects on rooting of *in vitro* shoots. The rooting of explants transferred from liquid culture media was inferior as compared to explants from semi-solid medium. This is likely because the roots of epiphytic orchids require aeration (Mengarda *et al.* 2017). However, plantlets from all treatments showed 100% survival after 30 d in acclimatization which was enhanced by prior transfer of cultures to PGR-free media. Similar results have been previously reported for *in vitro*-derived plantlets of date palm cultured on PGR-free media showing higher survival compared to plants on media containing PGRs (Mazri and Meziani 2013).

Conclusions

This study demonstrated the development of a successful protocol for the micropropagation of *B. nodosa*, which adds value towards the commercial production and conservation of this species. The results indicate that *B. nodosa* could be successfully micropropagated in liquid culture with partial immersion, thus preventing hyperhydricity and demonstrating that it has the potential for micropropagation in temporary immersion bioreactors. The medium supplemented with 2.0 mg L⁻¹ BA and 30.0 mg L⁻¹ adenine sulfate resulted in higher multiplication rates and survival. Although no abnormalities were observed, the carry-over effects and habituation need to be further investigated to prevent potential genetic variation.

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