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SCIENTIFIC ARTICLE

Micropropagation of *Trichopilia suavis* Lindl. & Paxton

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Abstract

Trichopilia suavis is one of the important representatives of the genus *Trichopilia*, which can be used as a potted plant for the interior design. also, its beautiful inflorescences are added unusual element to bouquet compositions and actively used in the production of perfumes. This study was carried out in order to develop and improve micropropagation method for mass clonal production of *T. suavis* through protocorm culture. Half-strength Murashige and Skoog culture medium (½) has been modified by adding different organic components and growth regulators. The protocorms obtained from seeds germinated by asymbiotic method were used as explants. The results showed that the optimal treatment for formation of new protocorms (7.50 ± 0.7 units per protocorm) was obtained by culture on ½ MS medium with 0.5 mg L^{-1} of 6-Benzylaminopurine (6-BAP) and 100 mL L^{-1} of coconut water. At the stage of rooting, the highest number and length of roots (7.0 ± 0.45 units per plantlet, 2.64 ± 0.18 cm) were obtained through subsequent cultivation of plantlets on ½ MS medium with 0.5 mg L^{-1} indolyl-3-butyric acid (IBA), 1.0 g L^{-1} charcoal with the addition of 50 g L^{-1} of banana puree. The obtained seedlings were successfully adapted in a substrate consisting of bark, perlite and peat in a ratio of 1:1:1.

Keywords: Orchid, protocorm, growth regulator, organic additive, *in vitro.*

Resumo

Micropropagação de *Trichopilia suavis* Lindl. & Paxton

Trichopilia suavis Lindl. & Paxton é um dos representantes mais importante do gênero *Trichopilia*, que pode ser usado como vaso de plantas para decoração de interiores. além disso, suas belas inflorescências são um elemento incomum adicionado às composições de buquês e usado ativamente na produção de perfumes. Este estudo foi realizado com o objetivo de desenvolver e aprimorar o método de micropropagação para produção clonal massal de *T. suavis* através do cultivo de protocormos. O meio de cultura de MS (½) foi modificado pela adição de diferentes componentes orgânicos e reguladores de crescimento. Os protocormos obtidos de sementes germinadas por método assimbiótico foram utilizados como explantes. Os resultados mostraram que no tratamento com ½ MS com 0,5 mg L^{-1} de 6-Benzilaminopurina (6-BAP) e 100 mL L^{-1} de água de coco foi considerado ótimo para formação de novos protocormos (7,50 ± 0,7 unidades por protocormo). Na fase de enraizamento, o maior número e comprimento de raízes (7,0 ± 0,45 unidades por plântula, 2,64 ± 0,18 cm) foram obtidos através o cultivo subsequente de plântulas em meio ½ MS com 0,5 mg L^{-1} de ácido indolil-3-butírico (IBA), 1,0 g L^{-1} de carvão com adição de 50 g L^{-1} de purê de banana. As mudas obtidas foram aclimatadas com sucesso em um substrato composto por casca, perlita e turfa na proporção de 1:1:1.

Palavras-chave: Orquídea, protocormo, regulador de crescimento, aditivo orgânico, *in vitro*.

Introduction

The genus *Trichopilia* belongs to the orchid family (Orchidaceae) and has more than 40 species widespread from Central America in the North to Brazil in the South (Perrot and Reinke, 2020). The name of this genus derives from the Greek words trichos (hair) and pilos (felt) (Umnova, 2023). In recent years molecular genetic studies show that this genus forms an isolated group within the subtribe Oncidiinae (Williams, 2001). In original habitat, these plants grow in humid mossy tropical forests between 1100 and 1700 meters above sea level (Bogarín et al., 2014).

Trichopilia is certainly one of the most charming genera of orchids in Central America. The plant is not difficult to grow indoors, so it can be used in phytodesign,

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and their wonderful inflorescences are added to bouquet compositions. It is also used for the production of perfumes (Ryan, 2001; Everett, 1980). The flowers are often fragrant, with a variety of colors, their morphology is generally similar to that of *Cattleya* (Morales, 2002).

One of the most beautiful members of the genus *Trichopilia* is *Trichopilia suavis* Lindl. & Paxton. Each inflorescence carries from two to five large fragrant flowers with a diameter up to 10 cm. The petals and sepals are white or creamy-white, the lip is funnel-shaped, white with a transition to pink (Frowine, 2005). It is found in Costa Rica, Panama and Colombia. Its natural habitat is tropical or subtropical moist forests. Natively, climate data suggest that moderate or cool temperatures are preferred for this plant, so it should be kept in a fairly moist state most times of the year, on a well-drained substrate. It easily tolerates cooler nights in winter, and a decrease in temperature may not be required to start flowering (Perrot and Reinke, 2020).

This species is considered one of the rare and endangered even in its original habitat (Hagsater et al., 1996). At the same time, *T. suavis* is used as bright object for landscape gardening. As other orchid species Seeds are characterized by an insufficient supply of lipids and cannot develop in the absence of symbiosis with the mycelium of specific fungi (Jolman et al., 2022). As well as it is traditionally propagated by dividing pseudobulbs in the period after flowering and currently represents a certain rarity. Clonal micropropagation is the only key to solving such problems for obtaining this plant in terms of commercial purposes (Warner, 1882; Zdenek, 2006).

The method of asymbiotic orchid seed germination was developed in the early 90s of the XIX century by Lewis Knudson. His method was an important theoretical and technological innovation that played an important role in the development of modern biotechnology (Yam and Arditti, 2009). Recently, clonal micropropagation is effectively utilized to obtain plants of *Trichopilia suavis*. The germination of orchid seeds under *in vitro* conditions can contribute to an increase in the effectiveness of conservation and propagation programs due to their high germination rate, which is usually more than 70% for epiphytic orchids, as opposed to be less than 5% for *ex vitro* conditions. The culture media used for asymbiotic germination of seeds vary depending on the species, and the most common media used for clonal micropropagation of orchids are Murashige and Skoog 1962 (MS), Knudson C 1946 (KC) e Woody Plant Medium (WPM) (Reddy, 2016; Koene et al., 2020; Castillo-Pérez et al., 2021).

Regeneration and propagation of some species under *in vitro* conditions are still difficult due to the high degree of callus formation, and the release of a large number of phenolic compounds, which causes culture medium browning**,** and the development of necrosis on explants, which finally lead to tissue/plant death (Molnár et al., 2011).

In some cases, culture media can be optimized by using various additives that stimulate the growth and development of explants. Many researchers have reported that organic additives have a positive effect on the propagation formation, and development of regenerates of many subtropical and tropical orchid species. Various types of organic additives have been used in orchid clonal micropropagation to promote the growth of the plants, including coconut water, banana puree, potato homogenate, etc. (Hussien et al., 2023; Samala and Thipwong, 2023).

Organic additives contain not only carbohydrates, but also other nutrients such as amino acids, lipids, many minerals and other substances that play an essential role in stimulating the plant growth (Molnár et al., 2011). In our investigation three organic extracts were used (coconut water, potato puree and banana puree). Coconut water contain many phytohormones such as IAA, kinetin, and zeatin. These components unlike synthetic cytokinins facilitate plant cell proliferation without increasing unwanted mutations that may occur due to their toxicity at higher concentrations (Akhiriana et al.,2019). Banana extract contains carbohydrates, proteins, fats and a lot of vitamins especially vitamin C, which improve regeneration response in plants (Apensa and Mastuti,2018). Potato puree is a proven source of quality minerals, carbohydrates such as starch and essential amino acid, that can enhance protocorm like bodies proliferation in many orchid species (Aung et al., 2022).

In recent years several protocols have been developed for propagation of many orchid species by using growth regulators and organic additives. However, there are still some species that have not been studied before, such as *T. suavis*. Therefore, after a comprehensive bibliographic review the aim of this study was to determine the plant growth regulators (6-BAP, IBA) and different organic additives (coconut water, banana puree and potato puree) effects on reproduction, formation and development of seedlings to create an effective protocol for commercial production of *T. suavis*

Materials and Methods

Plant material

The research was carried out in the Laboratory of Plant Biotechnology of the Tsytsin Main Botanical Garden of the Russian Academy of Sciences in 2021-2022. Traditional biotechnological techniques and methods developed in the Laboratory of Plant Biotechnology were used to carry out the experiments (Molkanova et al., 2018).

Germination and Multiplication and in vitro morphogenesis of plbs

The seeds were germinated in glass jars on hormone-free half strength MS nutrient medium with the addition of 1.0 g L^{-1} of charcoal, 30 g L^{-1} of saccharose and 7.0 g L^{-1} of agar. Culture was inducted in a culture room of the Laboratory of Plant Cellular Biotechnology of Central Botanical Garden of the National Academy of Sciences of Belarus at 25 ± 2 °C under 16/8 (light /dark light condition) with light intensity of 3,000 lux. Germinated protocorms (1.0 mm to 2.0 mm diameter) were taken to the Laboratory of Plant Biotechnology of the Tsytsin Main Botanical Garden of the Russian Academy to continue study.

In the multiplication phase, protocorms were sub-cultured in half-strength modified MS (Murashige and Skoog, 1962) as basal medium, containing 1.0 g L^{-1} of charcoal (Serva, Germany), 20 g L^{-1} of sucrose and 7.0 g L^{-1} of agar (Roeper, Germany) with the addition of 6-benzylaminopurin (6-BAP) (Sigma, USA) at different concentrations (0.5, 0.8, 1.0 and 2.0 mg L^{-1}) and organic additives (20 g L^{-1} of potato puree, 100 mL L^{-1} of coconut water). Before autoclaving, the pH of the culture medium was adjusted to 5.8 using KOH solution. The culture media were sterilized in a WAC-60 autoclave (Daihan Scientific, South Korea) under pressure (P = 101 kPa) at 120 °C for 20 minutes.

All cultures at this stage were incubated in a Phytotron at a temperature of 25 ± 2 °C, 70% ± 5% relative humidity with a light intensity of 1500 to 2000 Lux, and a photoperiod of 16-h.

After 70-90 days of cultivation, the following parameters were assessed: multiplication rate, number of leaves, the total number of plantlets leaves on both organic additives, number of roots, and root length. At this stage the experiment had 8 treatments. For each treatment it was obtained 3 independent replicates and each replicate contained approximately 10 individual protocorms.

In vitro rooting of plantlets

In the rooting phase, plantlets were transferred to ½ MS culture medium containing indolyl-3-butyric acid (further IBA) (Sigma, USA) at different concentrations (0.5 and 1.0 mg L^{-1}) with the addition of various organic additives (100 mL L^{-1} coconut water, 50 g L^{-1} banana puree). As a control, ½ MS culture medium without organic additives was used. At this stage, the following parameters were assessed: seedling length, number of roots, and root length. The experiment in this phase had 6 treatments with 3 replicates. Each replicate consisted of 10 plantlets.

Acclimatization of *T. suavis* plantlets

After rooting stage, only plantlets with optimal root development (more than 3 roots/plantlet) were removed from *in vitro* culture, washed under tap water. Subsequently, were planted in containers filled with 1:1:1 (v:v:v) peat: perlite: bark. Plants were kept in greenhouse conditions for further acclimatization. They were sprayed with water twice weekly. After 70 days the survival rate was determined.

Experimental design

The experiments were conducted using completely randomized design (CRD), and the analysis of experimental data was carried out in the Microsoft Office Excel 2016 and PAST 2.17c (Paleontological Statistics) programs using the methods of descriptive statistics, and two-way analysis of variance (ANOVA). Means were compared by Tukey multiple-range test (for features whose distribution corresponds to the normal distribution law at a level of $p < 0.05$ to determinate the significant difference between the experimental variants.

Results and Discussion

Clonal micropropagation of plants is a method of plant propagation conducted under aseptic conditions and controlled environment to produce plant clones. In this process, plant tissues or cells can proliferate and grow rapidly to produce progeny plants (Rai et al., 2022). Modification of the culture medium by adding growth regulators and organic additives can stimulate growth and produce commercial-scale *T. suavis* plants.

At the multiplication stage, it was observed that the addition of 6-BAP and organic additives to the ½ MS culture medium influenced the number of newly formed protocorms and their morphometric parameters (Figure 1).

The ½ MS culture medium containing 0.5 mg L^{-1} of 6-BAP and 100 mL L^{-1} of coconut water, showed a higher number of formed protocorms (7.50 ± 0.7 units) compared to other variants (Figure 2).

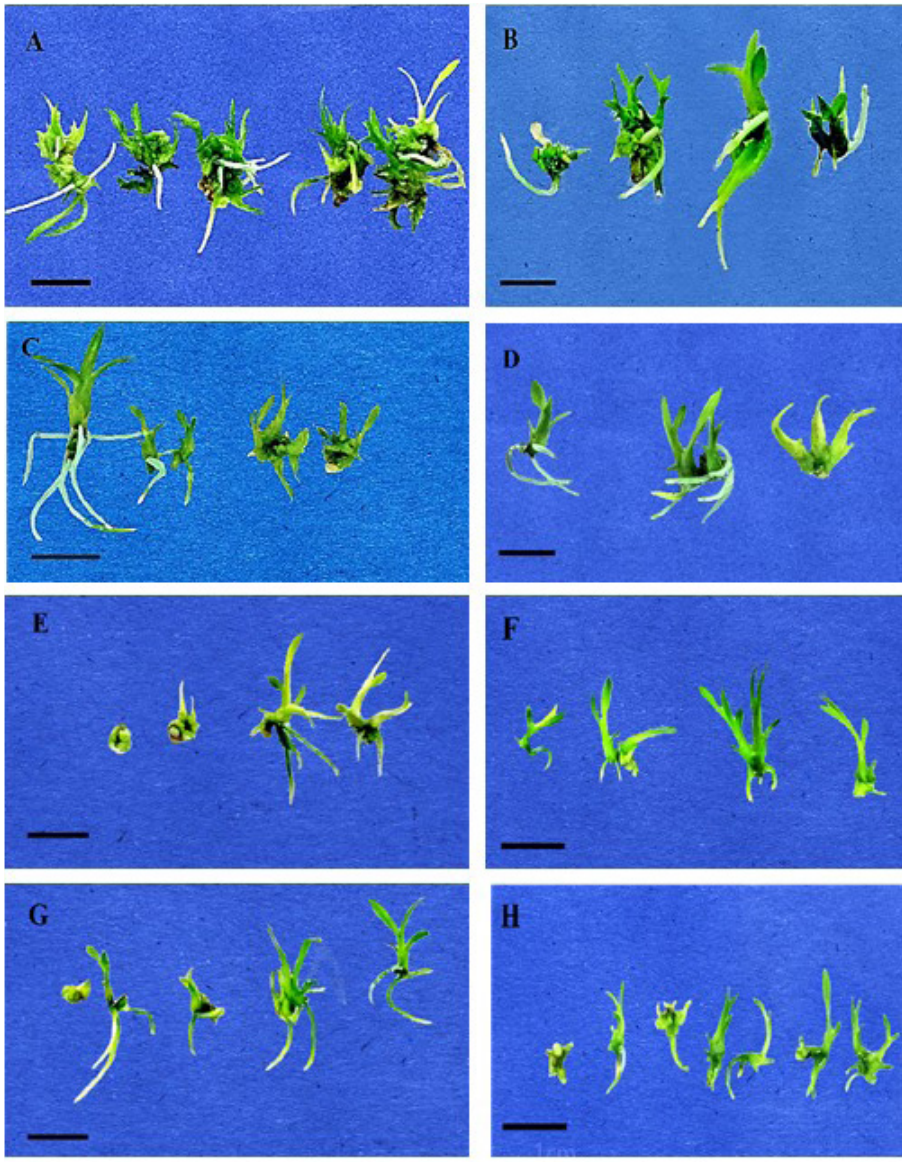

Figure 1. Influence of different culture medium on the morphometric parameters of *T. suavis* plantlets : A - 0.5 mg L^{-1} 6- BAP + coconut water, B - 0.8 mg L^{-1} 6-BAP + coconut water, C - 1.0 mg L^{-1} 6-BAP + coconut water, D - 2.0 mg L^{-1} 6- BAP +coconut water, E - 0.5 mg L^{-1} 6- BAP + potato puree, F- 0.8 mg L^{-1} 6- BAP + potato puree, G - 1.0 mg L^{-1} 6- BAP + potato puree, H - 2.0 mg L^{-1} 6- BAP + potato puree (bar = 1cm).

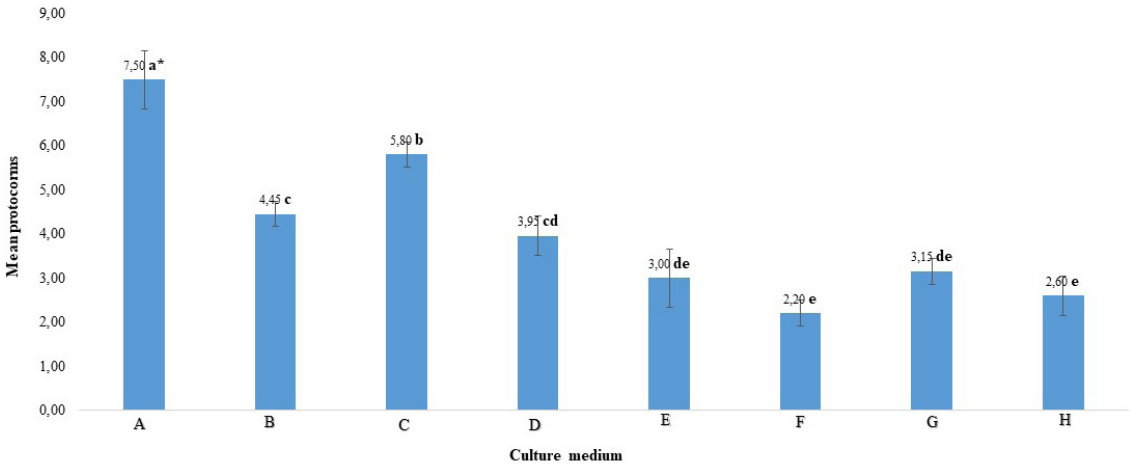

Figure 2. Influence of different culture medium: A - 0.5 mg L^{-1} 6- BAP + coconut water, B - 0.8 mg L^{-1} 6-BAP + coconut water, C - 1.0 mg L^{-1} 6-BAP + coconut water, D - 2.0 mg L^{-1} 6- BAP +coconut water, E - 0.5 mg L^{-1} 6- BAP + potato puree, F- 0.8 mg L^{-1} 6- BAP + potato puree, G - 1.0 mg L^{-1} 6- BAP + potato puree, H - 2.0 mg L^{-1} 6- BAP + potato puree on the number of newly formed protocorms after 90 days of cultivation (LSD_{05}1.80). *Values are mean ± SD, the letters "a", "b" and "c" denote groups that significantly differ from each other according to Tukey multiple-range test ($p \leq 0.05$).

In many studies, protocorms were used for propagation and production of seedlings for several genera of orchids such as *Phalaenopsis* and *Cymbidium* (Lo et al., 2022; Regmi et al., 2017). This can be explained by the fact that protocorm cells have the ability to divide, which makes them ideal explants in the process of clonal micropropagation and studying the morphology of growth and development of representatives of the orchid family (Chookoh et al., 2019; Yeung, 2017). It is known that the addition of organic additives contributes to an increase in the number of orchid protocorms (Sukma et al., 2019). Coconut water promotes growth and contributes to a higher reproduction rate. This may be due to its ability to induce cell division, therefore, to promote early differentiation of protocorms (Rohmah and Taratima, 2022). At the stage of micropropagation, the combination of different organic additives and 6-BAP had a different effect on leaf formation, root emergence and regenerate into complete plantlets (Table 1).

Table 1. Influence of 6-BAP concentration and organic additives on the morphometric parameters of *T. suavis* protocorms after 90 days in vitro culture.

6-BAP mg L^{-1}	Organic additives	N. leaves (Unit)	N. roots (Unit)	Root length (cm)
0.5	Coconut water	2.32 ± 0.47 bc *	1.45 ± 0.70 c	0.61 ± 0.12 c
0.8		3.04 ± 0.84 a	2.55 ± 0.95 abc	0.58 ± 0.09 c
1.0		2.56 ± 0.82 abc	1.50 ± 0.85 c	0.60 ± 0.15 c
2.0		2.88 ± 0.78 ab	1.70 ± 0.93 c	0.75 ± 0.08 bc
0.5	Potato puree	2.20 ± 0.70 bc	3.50 ± 1.57 a	1.18 ± 0.23 ab
0.8		2.44 ± 0.82 abc	3.45 ± 1.22 ab	1.53 ± 0.21 a
1.0		2.00 ± 0.99 c	1.85 ± 0.92 c	0.90 ± 0.11 bc
2.0		1.96 ± 1.05 c	2.05 ± 1.23 bc	0.89 ± 0.16 bc

* Values are mean ± SD, the letters "a", "b" and "c" denote groups that significantly differ from each other at $p \leq 0.05$ (Tukey multiple-range test)

Many studies have shown that 6-BAP not only stimulates propagation and proliferation of protocorms, but also plays an important role in plant regeneration (Chookoh et al., 2019).

In our study, it was noticed that low concentrations of 6-BAP had positive effect not only on the reproduction rate of protocorms, but also on the formation of leaves and the growth of plantlets (Table 1). This is consistent with the results of other studies that have shown that cytokinins play a role in stimulating the propagation of protocorms and the development of regenerates. It has also been reported that high concentrations of 6-BAP reduce the reproduction rate, although they can stimulate the formation of leaves and the development of regenerants, while low concentrations in our study showed positive morphometric parameters of seedlings compared with high concentrations.

The results showed that organic additives significantly contributed to the growth of protocorms and the formation of plantlets. However, the influence level of organic additives, as well as the concentration of 6-BAP and their interaction on the morphometric parameters of *T. suavis* seedlings were ranged.

It was found that, cultivation of protocorms on a culture medium with coconut water, the number of leaves was higher compared to a culture medium containing potato puree (Figure 3).

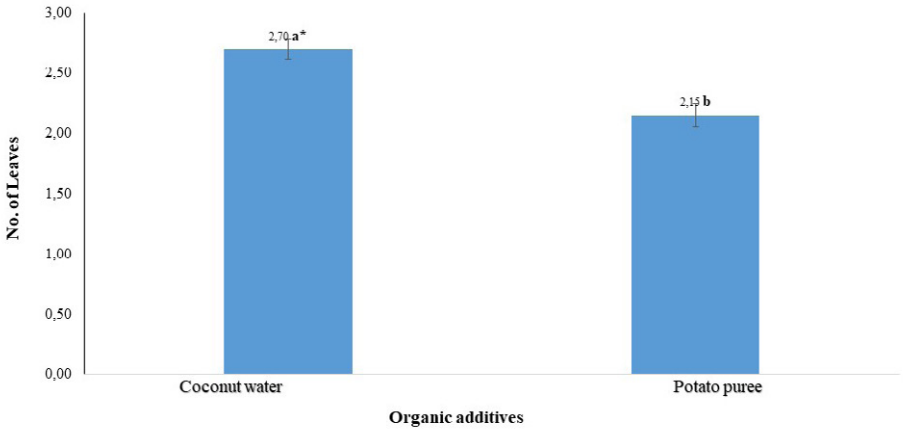

Figure 3. Influence of organic additives on the total number of plantlets leaves after 90 days of in vitro cultivation. *Values are mean ± SD, the letters "a", "b" and "c" denote groups that significantly differ from each other at $p \leq 0.05$ (Tukey multiple-range test)

Literature reports that, this may be due to compounds contained in coconut water, including plant hormones such as cytokinins, which stimulate leaf formation. This is consistent with the results of other studies, which confirmed the stimulated effect of coconut water on the formation of shoots and leaves of various orchid species such as *Phalaenopsis amabilis* (Salsabila et al., 2022). In our case, the culture medium containing potato puree had a positive effect on the spontaneous rhizogenesis compared to the culture medium with coconut water (Table 1). At the same time, the cultivation of protocorms on this medium contributed to the development of explants suitable for further rooting. This is consistent with the results of other studies, which reported a positive effect of potato puree on the number and length of roots of different epiphytic orchid species such as *Dendrobium anosmum* (Nguyen et al., 2020).

It is known that the culture medium at the stage of multiplication is not suitable enough for the development of root system of plantlets. it was previously revealed that in order to create a strong root system, shoots were cultured on a culture medium for rooting (Bhowmik and Rahman, 2020). At the rooting stage, the most efficient culture medium was ½ MS, containing 0.5 mg L^{-1} IBA with the addition of 50 g L^{-1} of banana puree. A more developed root system was formed on this medium: the number of roots was 7.0 ± 0.45 units per seedling, and the length of the roots 2.64 ± 0.18 cm. At the same time, the use of coconut water as organic additives also showed positive results compared to the control (Table 2, Figure 4).

In vitro grown plantlets acclimatization plays an important role in the rapid clonal propagation of different species of orchids. The suitable substrate for the acclimatization of epiphytic orchids should provide good root growth and aeration to obtain high survival rate and further development of plants (Sanghamitra et al., 2019).

Table 2. Indolyl-3-butyric acid and organic additives influence on root parameters of *T. suavis* plantlets at 180 days of *in vitro* cultivation.

IBA (mg L^{-1})	Organic additives	N.of roots (Unit)	Root length(cm)	Plantlet length (cm)
0.5	Control	2.95 ± 0.77 c *	0.94 ± 0.18 c	1.41 ± 0.19 d
	Coconut water	4.86 ± 0.95 b	2.13 ± 0.28 b	1.84 ± 0.13 c
	Banana puree	7.00 ± 0.73 a	2.64 ± 0.65 a	2.98 ± 0.20 a
1.0	Control	1.70 ± 0.27 d	0.65 ± 0.10 c	1.20 ± 0.11 d
	Coconut water	2.10 ± 0.22 cd	0.91 ± 0.23 c	1.30 ± 0.12 d
	Banana puree	4.40 ± 0.65 b	2.00 ± 0.18 b	2.25 ± 0.18 b

* Values are mean ± SD, the letters "a", "b" and "c" denote groups that significantly differ from each other at $p \leq 0.05$ (Tukey multiple-range test).

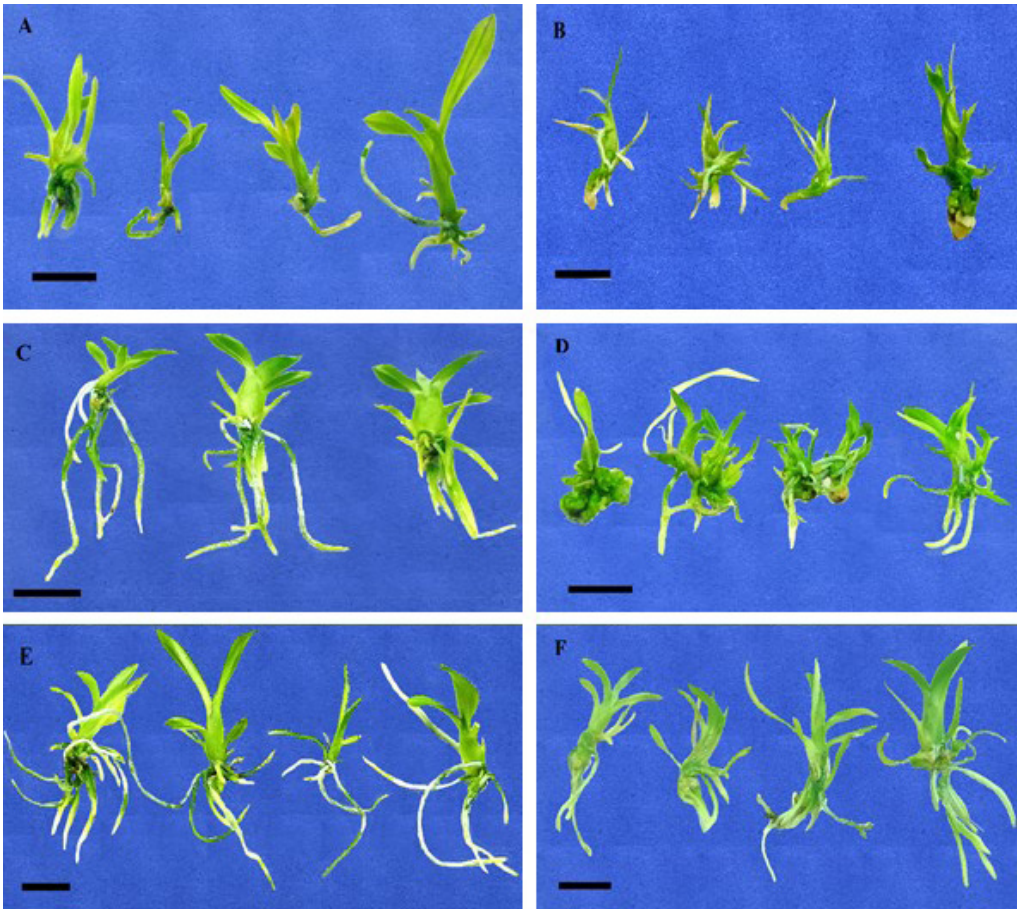

Figure 4. Effect of IBA concentrations and organic additives on *in vitro* rooting of *T. suavis* seedlings: A - 0.5 mg L^{-1} IBA; B - 1.0 mg L^{-1} IBA; C - 0.5 mg L^{-1} IBA + coconut water; D - 1.0 mg L^{-1} IBA + coconut water; E - 0.5 mg L^{-1} IBA + banana puree; F - 1.0 mg L^{-1} IBA + banana puree (bar = 1cm).

After the rooting phase, the seedlings were transferred to the greenhouse in containers filled with a mixture consisting of bark, peat and perlite in equal proportions. The survival rate of acclimatized plants reached 95-100%. It can be explained by the fact that this combination contributed to a good root aeration, and thus to the seedling development. These results show the effectiveness of the followed protocol for mass micropropagation of *T. suavis* (Figure 5).

Figure 5. Acclimatized seedlings of *T. suavis* after 70 days under greenhouse conditions.

Conclusions

In the present work, the stages of clonal micropropagation of *T. suavis* were developed for the first time. The results indicate that a large number of new formed protocorms could be obtained from ½ MS medium with 0.5 mg L^{-1} 6-BAP and 100 mL L^{-1} coconut water by culturing protocorms within 70 days. The ½ MS culture medium supplemented with 0.5 mg L^{-1} IBA and banana puree resulted in strong root system. The obtained seedlings successfully adapted to *ex vitro* conditions, they were characterized by high survival rate in a substrate consisting of bark, peat and perlite in a ratio of 1:1:1. This protocol adds value towards the commercial production and conservation of this species.

Author Contribution

MH: Conceived the study and planned the experiments; resources; writing- original draft preparation; designed the figures and wrote the manuscript with input from all authors and writing review and editing; **OM**: Conceived the study and planned the experiments; validation; visualization and formal analysis; **IM**: Visualization and formal analysis. All authors have read and agreed to the published version of the manuscript.

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