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Research Article

Medium-Term Preservation of *Trichopilia suavis* Lindl. and Paxton *in vitro* Using Slow-Growth Technology

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Abstract

Background and Objective: Medium-term *in vitro* storage using plant growth inhibitors under controlled conditions is a cost-effective method for preserving endangered plant species without frequent subculturing. This study aimed to optimize storage conditions for protocorm-like bodies (PLBs) of *Trichopilia suavis*, a valuable epiphytic orchid. **Materials and Methods:** The PLBs were cultured on half-strength Murashige and Skoog (1/2 MS) medium supplemented with varying concentrations of chlorocholine chloride (CCC) and paclobutrazol (PBZ). Cultures were incubated under two environmental conditions: A climate chamber (15$\pm$2°C, 1.7 Klux) and a culture room (24$\pm$2°C, 3.4 Klux). Data were analyzed using Analysis of Variance (ANOVA) and Duncan's Multiple Range Test ($p\leq0.05$). **Results:** The PLBs cultured on 1/2 MS medium with 0.2 mg/L CCC in the climate chamber achieved 87% survival after 18 months without subculturing, with 80% regenerating into plantlets. Maximum PLB proliferation occurred with 0.4 mg/L CCC in the culture room and 1.0 mg/L PBZ in the climate chamber. The best plantlet regrowth was observed on medium with 0.5 mg/L kinetin (KIN) and coconut water. Cultivation conditions also influenced root anatomy. Roots grown in the climate chamber exhibited large diameters, a thin velamen and a thick cortex with large parenchyma cells, while roots from the culture room had thicker velamen, a more developed stele and increased xylem diameter, showing starch storage during regrowth. **Conclusion:** This study provides an effective protocol for medium-term preservation of *T. suavis* using growth inhibitors and climate chamber incubation, ensuring reliable storage of this species.

Key words: Orchidaceae, chlorocholine chloride, paclobutrazol, culture conditions, root anatomy

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The biodiversity of plant communities plays a critical role in shaping the life and development of humanity. Human activities, such as active intervention in natural biocenoses and the unsustainable exploitation of natural resources, are causing the degradation of established ecosystems. This threatens the survival of numerous plant species, including various members of the Orchidaceae family, putting them at risk of extinction[1,2]. To address this issue, several countries have developed biodiversity conservation strategies[3].

Orchidaceae is one of the largest families of angiosperms, comprising over 28000 species[4]. The majority of orchids are found in the epiphytic communities of tropical forests[2]. However, the resources of tropical germplasm in their natural habitats are rapidly declining due to anthropogenic impact and climate change. As a result, the conservation of the natural genetic diversity of tropical species, particularly through the establishment of *ex situ* collections, has become a priority[5].

The genus *Trichopilia* belongs to the family Orchidaceae and includes more than 40 species, which are distributed from Central America to Brazil[6]. One of the most remarkable species within this genus is *T. suavis*. Its fragrant flowers, which can reach up to 10 cm in diameter, are arranged in inflorescences containing 2 to 5 blooms. The sepals and petals are white or creamy-white, while the lip is funnel-shaped, white with a pink gradient[7]. Unfortunately, this species is at risk of extinction in its natural habitat due to illegal harvesting[8].

There are various strategies for conserving genetic resources, especially in endangered species. Botanical gardens, in conjunction with *ex situ* field collections and seed banks, also propagate plant tissues and organs under controlled aseptic conditions[9]. Despite the notable advantages of this *in vitro* conservation method, only 10% of botanical gardens in the Russian Federation maintain *in vitro* germplasm collections[10]. The establishment of conservation protocols is critical for the preservation of rare plant species, ensuring genetic stability and long-term viability.

Germplasm conservation *in vitro* is categorized into short-term, medium-term and long-term storage methods. Short-term *in vitro* conservation usually extends from 1 to 3 months, during which plant tissues are cultivated under controlled conditions[11]. A highly effective clonal micropropagation protocol has been developed for *T. suavis*[12]. However, the frequent subculturing of explants not only increases the cost of the process but also heightens the risk of contamination and the occurrence of somaclonal variations[9,13].

Long-term storage, commonly known as cryopreservation, is used to extend the viability of living plant material for several years. This method involves the initial preparation of plant tissues or organs *in vitro*, followed by rapid cooling in liquid nitrogen at -196°C[14]. Research has demonstrated protocols that successfully preserve seeds, pollen and orchid callus tissue for over 30 years through cryopreservation[15]. However, this technique requires specialized, costly equipment, liquid nitrogen and the development of additional protocols to pre-treat tissues before freezing to ensure successful preservation[16,17]. A more economical alternative to short-term *in vitro* and long-term conservation, which addresses their limitations, is medium-term storage. This approach involves slowing down the metabolic activities of explant tissues by manipulating environmental factors such as temperature, light intensity and the addition of growth inhibitors or osmotic agents to the culture medium[18]. Under medium-term storage conditions, plant tissues and organs can remain viable for up to three years without the need for subculturing onto fresh culture media. Several studies have investigated the impact of light conditions, growth inhibitors and osmotic agents on the viability of PLBs and seedlings of epiphytic orchid species over periods ranging from 2 to 20 months[19-21].

Thus, medium-term preservation of explants at low temperatures emerges as an effective and cost-efficient method for conserving plant biodiversity. However, the scientific literature contains a limited number of studies examining the effects of temperature and growth inhibitors on the conservation, PLB regeneration and plantlet development of rare orchid species.

This study aims to evaluate the impact of varying concentrations of growth retardants on the growth and development of *T. suavis in vitro* under different cultivation conditions.

MATERIALS AND METHODS

Study area: The investigation was conducted at the Laboratory of Plant Biotechnology of the Main Botanical Garden, named after N.V. Tsitsin, Russian Academy of Sciences. The work was carried out between 2022 and 2024.

Plant materials: The single *T. suavis* PLBs, obtained by cultivation of protocorm on 1/2 MS medium supplemented with 0.5 mg/L of 6-benzylaminopurine (BA) and 100 mL/L of coconut water, served as the plant material. The medium used in all experiments consisted of half-strength Murashige and Skoog[22] (1/2 MS) supplemented with 20 g/L sucrose and 100 mg/L Myo-inositol. The culture medium and instruments were sterilized at 121°C and 15 psi for 20 min using a WAC-60 autoclave (Daihan Scientific, South Korea). Before autoclaving, the pH of the medium was adjusted to 5.5-5.8.

***In vitro* culture conditions and growth inhibitors in a culture medium to preserve PLBs:** The culture vessels with explants were incubated under two different conditions, in a culture room at 24±2°C with a light intensity of 3.4 Klux and a photoperiod of 16 hrs light and 8 hrs dark and in a climate chamber (Forma Scientific, USA) at 15±2°C with a light intensity of 1.7 Klux, also under a photoperiod of 16 hrs light and 8 hrs dark.

Explants were cultured on 1/2 MS supplemented with growth inhibitors in different concentrations: 0.2 and 0.4 mg/L of chlorocholine chloride (CCC) (Acros Organics, China); 1.0 and 2.0 mg/L of paclobutrazol (PBZ) (Green AgroLab, Russian Federation). A culture medium without the addition of growth inhibitors was used as a control.

Experimental design: The experiment was performed according to a complete randomized scheme (CRD) in 3 replicates with 30 explants in each treatment. Regenerants were assessed after 3, 9 and 18 months of cultivation. In each period, the following indicators were calculated: Survival rate (%), PLBs regeneration (%) and rhizogenesis (%). After 18 months of explant preservation, the following parameters were carried out: PLBs formation (%), plantlets height (cm), root length (cm) and the number of adventitious shoots and roots.

Regrowth stage: After 18 months of conservation, the formed plantlets and PLBs were transplanted onto a 1/2 MS medium supplemented with 0.7 g/L activated charcoal, various cytokinins at a concentration of 0.5 mg/L, both with and without the addition of coconut water (100 mL/L) (Table 1).

The culture vessels with explants were incubated in a culture room at 24±2°C, the photoperiod was 16/8 hrs (light/dark) and the light intensity was 3.4 Klux. After 3 months of cultivation, the following parameters were assessed: Percentage of new PLBs formation, plantlets height (cm), number of leaves, number of roots and root length (cm).

Root anatomy: Roots were collected from different conservation conditions and from the regrowth stage *in vitro*, then fixed in 70% ethanol. To examine the anatomical structure of the fixed roots, both permanent and temporary slides were prepared. Specimen sections, 60-80 µm thick, were cut using an MS-2 sliding microtome (Tochmedpribor, Kharkiv, Ukraine) with an OMT-2802E freezing stage (LLC 'KB TEKHOM', Yekaterinburg, Russia). For lignification zone detection, the specimens were stained with safranin and alcian blue[23]. The slides were examined under an Olympus CX41 light microscope (Olympus Corp., Tokyo, Japan) and photographs of the sections were captured using a Canon 7D Mark II digital camera (Canon Inc., Tokyo, Japan), attached to the microscope via an adapter. Morphometric measurements of the following parameters were taken: Root diameter (µm), velamen thickness (µm), exodermis thickness (µm), cortex thickness (µm), endodermis thickness (µm) and stele diameter (µm) using the ImageJ software (NIH, MD).

Statistical analysis: Statistical analysis of the obtained data was carried out using Microsoft Office Excel 2019 and SPSS Statistics 26 software packages. One-way Analysis of Variance (ANOVA) was performed to examine the differences between groups of variables and if significant, Duncan's Multiple Range Test at $p \leq 0.05$ was used for *post hoc* analysis. The tables and graphs present the Mean±Standard Deviation (SD) values.

Table 1: Combination of cytokinin and organic additives for *in vitro* regrowth

Organic additives	Cytokinin type	Treatments symbol
Coconut water	Control	A
	Kinetin (KIN)	B
	6-benzylaminopurine (BA)	C
	Thidiazuron (TDZ)	D
Without coconut water	Control	E
	Kinetin (KIN)	F
	6-benzylaminopurine (BA)	G
	Thidiazuron (TDZ)	H

RESULTS

The results demonstrated a high survival rate of *T. suavis* PLBs in all experimental variants at 24°C during the first 3 months of preservation, except for the culture media containing PBZ, where all PLBs turned brown due to tissue necrosis (Fig. 1). The PLBs survival rate decreased by 40% in the control group and by 30% when cultivated on culture media containing CCC throughout the experiment (Fig. 2).

During the entire period of *T. suavis* PLBs cultivation at a temperature of +15°C, the survival rate decreased slightly. The highest survival rate of *T. suavis* PLBs (87%) was observed on 1/2 MS medium with the addition of 0.2 mg/L CCC after 18 months of preservation. At the same time, a low survival rate was recorded on 1/2 MS media containing 1.0 and 2.0 mg/L PBZ (55 and 50%, respectively) compared to other treatments (Fig. 3).

The effects of the growth inhibitors and cultivation conditions on PLBs development and plantlet formation were assessed. After 9 months of cultivation at +24°C, PLBs regeneration into plantlets and their subsequent rooting were observed in all treatments. However, the PLBs cultured on 1/2 MS supplemented with 0.2 mg/L CCC recorded the highest percentages of both PLBs regeneration (93%) and rhizogenesis (97%) compared to the other treatments. The lowest percentage of PLBs regeneration (30%) was recorded on 1/2 MS medium containing 0.4 mg/L CCC. The control variant exhibited intermediate results, with 60% PLBs regeneration and 50% rhizogenesis (Fig. 4a). After 18 months, the percentage of PLBs that regenerated into plantlets increased significantly in the control variant (90%). However, this parameter did not show a significant change in the culture medium with the addition of either 0.2 or 0.4 mg/L CCC under culture room conditions (Fig. 4b).

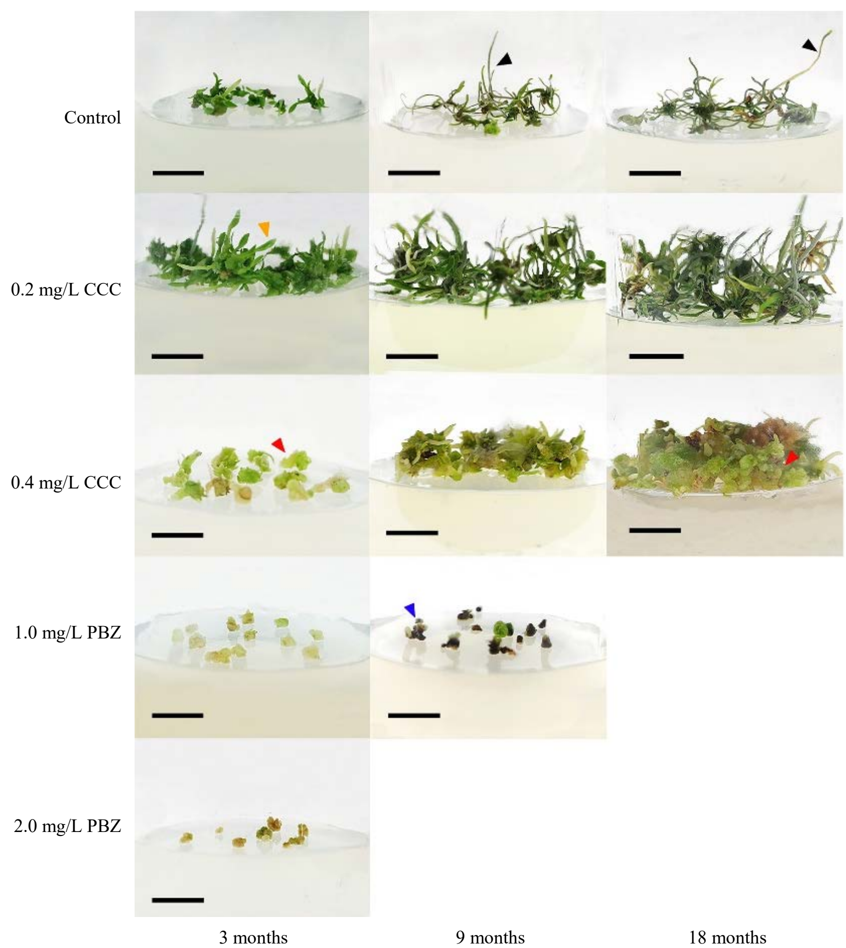

Fig. 1: Development of *T. suavis* PLBs during preservation on a culture medium with growth inhibitors under conditions of culture room *in vitro*

Black arrows: Root, Red arrows: PLBs proliferation, Orange arrows: Leaf formation, Blue arrows: Necrosis and Scale bars: 1 cm

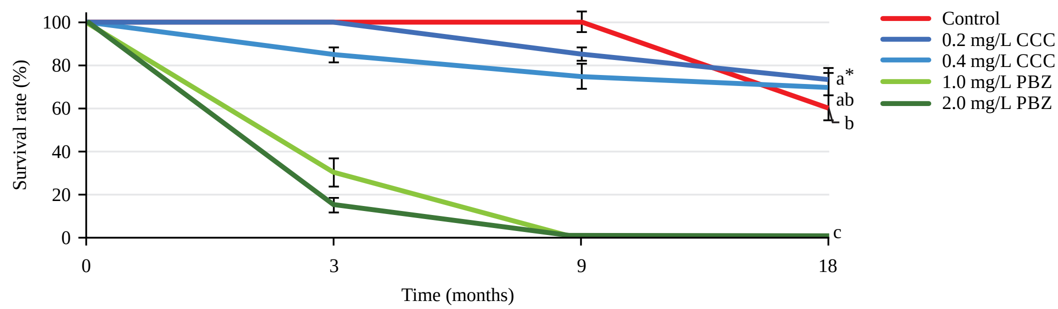

Fig. 2: Changes in the survival rate of *T. suavis* PLBs during preservation on a culture medium with growth inhibitors under conditions of the culture room *in vitro*

*Values are Mean±SD and the letters "a", "b" and "c" denote groups by Duncan's Multiple Range Test at the 5% level

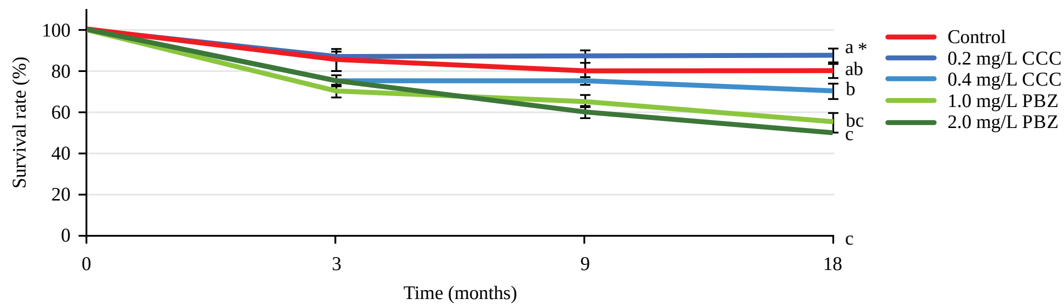

Fig. 3: Changes in the survival rate of *T. suavis* PLBs during preservation on a culture medium with growth inhibitors under conditions of climate chamber *in vitro*

*Values are Mean±SD and the letters "a", "b" and "c" denote groups by Duncan's Multiple Range Test at the 5% level

Table 2: Morphometric characteristics of *T. suavis* explants after 18 months of preservation under different conditions and on culture media with growth inhibitors

Cultivation conditions	Treatment	PLBs formation (%)	Plantlet height (cm)	Number of adventitious shoots/explant	Number of root/explant	Root length (cm)
15±2°C, illumination intensity of 1.7 Klux	1/2 MS	$30.00\pm5.00^{e*}$	0.78 ± 0.13^{b}	2.97 ± 0.32^{b}	2.50 ± 0.25^{a}	1.14 ± 0.05^{b}
	1/2 MS+0.2 mg/L CCC	40.00 ± 2.88^{de}	1.49 ± 0.03^{a}	4.50 ± 0.29^{a}	2.41 ± 0.13^{a}	0.90 ± 0.05^{b}
	1/2 MS+0.4 mg/L CCC	50.00 ± 5.77^{cd}	0.53 ± 0.09^{c}	2.39 ± 0.20^{c}	1.67 ± 0.17^{b}	0.56 ± 0.12^{c}
	1/2 MS+1.0 mg/L PBZ	66.67 ± 3.33^{b}	0.19 ± 0.03^{d}	0.99 ± 0.18^{ef}	0^{d}	0^{d}
	1/2 MS+2.0 mg/L PBZ	60.00 ± 5.70^{bc}	0.14 ± 0.04^{d}	0.55 ± 0.22^{fg}	1.11 ± 0.11^{c}	1.12 ± 0.19^{b}
24±2°C, illumination intensity of 3.4 Klux	1/2 MS	28.33 ± 3.14^{e}	0.53 ± 0.07^{c}	1.44 ± 0.11^{de}	2.56 ± 0.12^{a}	1.68 ± 0.17^{a}
	1/2 MS+0.2 mg/L CCC	35.00 ± 3.30^{e}	1.36 ± 0.04^{a}	1.92 ± 0.08^{cd}	2.19 ± 0.09^{a}	1.69 ± 0.09^{a}
	1/2 MS+0.4 mg/L CCC	86.67 ± 8.62^{a}	0.19 ± 0.05^{d}	1.33 ± 0.17^{de}	1.00 c	0.25 ± 0.01^{d}
	1/2 MS+1.0 mg/L PBZ	0^{f}	0^{d}	0^{g}	0^{d}	0^{d}
	1/2 MS+2.0 mg/L PBZ	0^{f}	0^{d}	0^{g}	0^{d}	0^{d}

*Values are Mean±SD and superscript letters denote groups by Duncan's Multiple Range Test at the 5% level

Preserving explants at a temperature of 15°C resulted in a slight decrease in the percentage of regenerated PLBs and a significant reduction in rhizogenesis, compared to those incubated at 24°C (Fig. 4-5). The PLBs cultivated in the control variant at both 9 and 18 months of preservation demonstrated the highest percentage of regenerated PLBs (90%) compared to the other treatments during the same preservation periods. Furthermore, when comparing plantlets after 9 and 18 months of preservation, an increase in root formation was noted on culture media supplemented with 0.2 mg/L CCC (43%) and 2.0 mg/L PBZ (47%) after 18 months (Fig. 5a-b).

At the end of the preservation process, differences were found in the percentage of explants that formed new PLBs, depending on cultivation conditions and growth inhibitors. Cultivation of PLBs at +24°C on 1/2 MS medium with 0.4 mg/L of CCC contributed to the maximum percentage of new PLBs formation (86%). In turn, explants on a culture medium with 1.0 mg/L PBZ (67%) were characterized by the highest percentage of newly formed PLBs at +15°C.

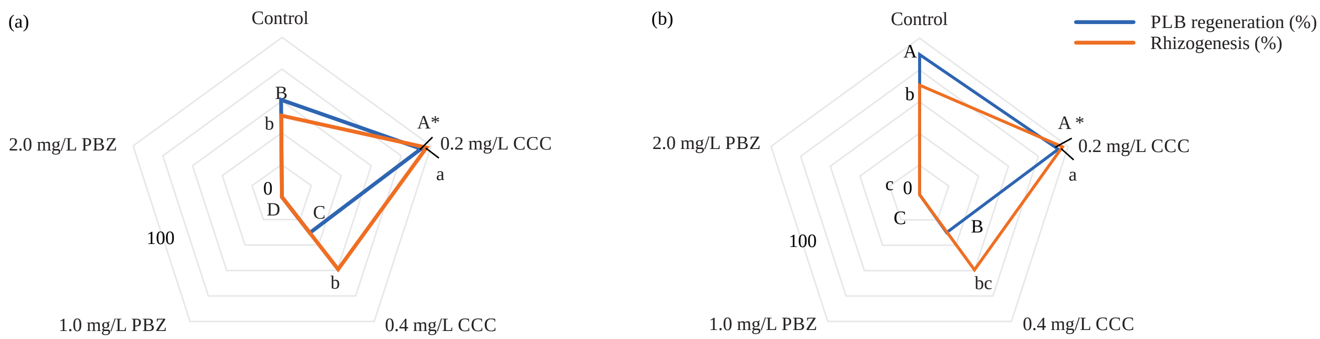

Fig. 4(a-b): Dynamics of PLBs regeneration and rhizogenesis of *T. suavis* plantlets during preservation at a temperature of +24°C on culture media with growth inhibitors, (a) 9 months and (b) 18 months

Capital letters indicate differences in PLBs regeneration; small letters indicate differences in rhizogenesis. *Values are Mean±SD and the letters "a", "b" and "c" denote groups by Duncan's Multiple Range Test at the 5% level

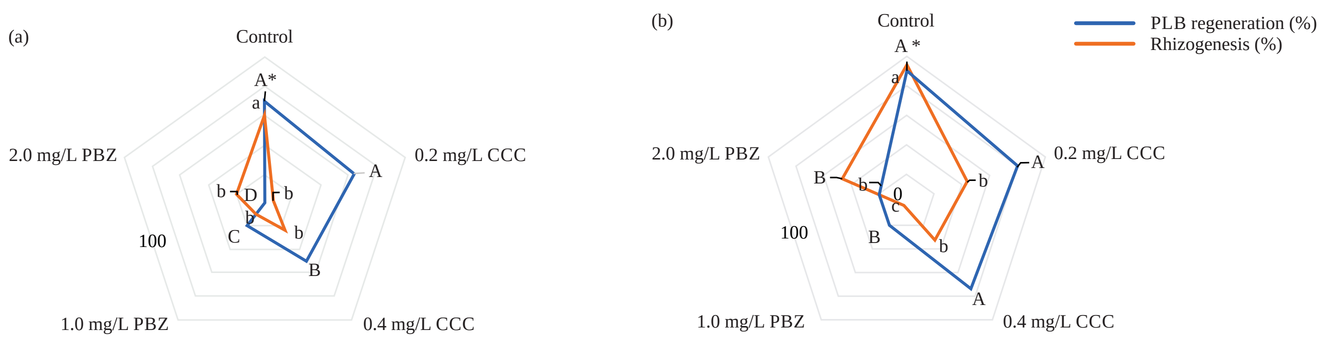

Fig. 5(a-b): Dynamics of PLBs regeneration and rhizogenesis of *T. suavis* plantlets during preservation at a temperature of +15°C on culture media with growth inhibitors

Capital letters indicate differences in PLBs regeneration; small letters indicate differences in rhizogenesis. *Values are Mean±SD and the letters "a", "b" and "c" denote groups by Duncan's Multiple Range Test at the 5% level

The plantlets formed at the end of preservation on various culture media and under different cultivation conditions exhibited differences in their morphometric parameters. After 18 months of preservation at 24°C, the plantlets in the control variant and those on 1/2 MS medium supplemented with 0.2 mg/L CCC showed the highest number and length of roots (Table 2). Additionally, it was observed that, after 18 months, leaf shrinkage occurred in the plantlets of the control variant (Fig. 2).

At the end of the preservation stage, PLBs cultivated on 1/2 MS medium supplemented with 0.2 mg/L CCC under growth chamber conditions produced plantlets with the maximum height (1.49±0.03 cm), as well as the highest number of adventitious shoots (4.5±0.29 units/explant) (Fig. 6).

The type of cytokinin and the addition of coconut water significantly influenced the regrowth of explants after 18 months of preservation. The plantlets formed during the preservation stage were able to continue growing during the regrowth stage (Fig. 7).

Plantlets grown on a medium supplemented with 0.5 mg/L KIN, with or without coconut water, demonstrated the maximum plantlet height (2.21±0.06 and 1.87±0.03 cm, respectively). Additionally, both the cytokinin type and the addition of coconut water had a significant effect on PLBs proliferation. The PLBs grown on a medium containing 0.5 mg/L BA recorded the highest proliferation rate (64%). Regarding rooting parameters, the cytokinin type significantly affected root development. The highest rooting percentage and number of roots were observed on the medium containing 0.5 mg/L KIN and 100 mL/L coconut water. Furthermore, the plantlets grown on the medium with kin, with or without coconut water, achieved the highest rooting percentage (70 and 50%, respectively) and the highest root length (1.78±0.13 and 1.59±0.09 cm, respectively) compared to other treatments (Table 3).

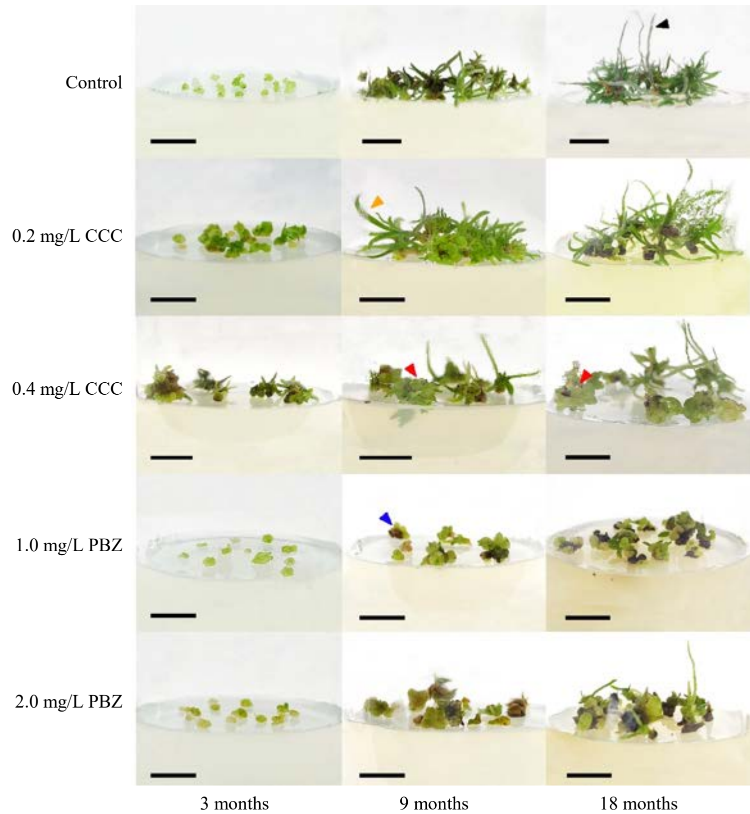

Fig. 6: Development of *T. suavis* PLBs during preservation on a culture medium with growth inhibitors under conditions of climate chamber *in vitro*

Black arrows: Root, Red arrows: PLBs proliferation, Orange arrows: Leaf formation, Blue arrows: Necrosis and Scale bars: 1 cm

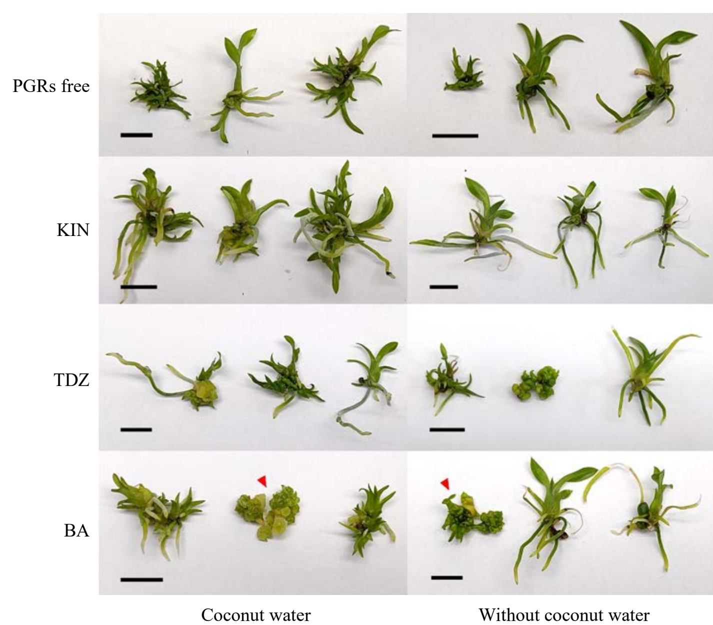

Fig. 7: Influence Plantlet development of *T. suavis* on nutrient medium supplemented with various cytokinins and coconut water after 90 days of *in vitro* culture

Red arrows: PLBs proliferation and Scale bars: 1 cm

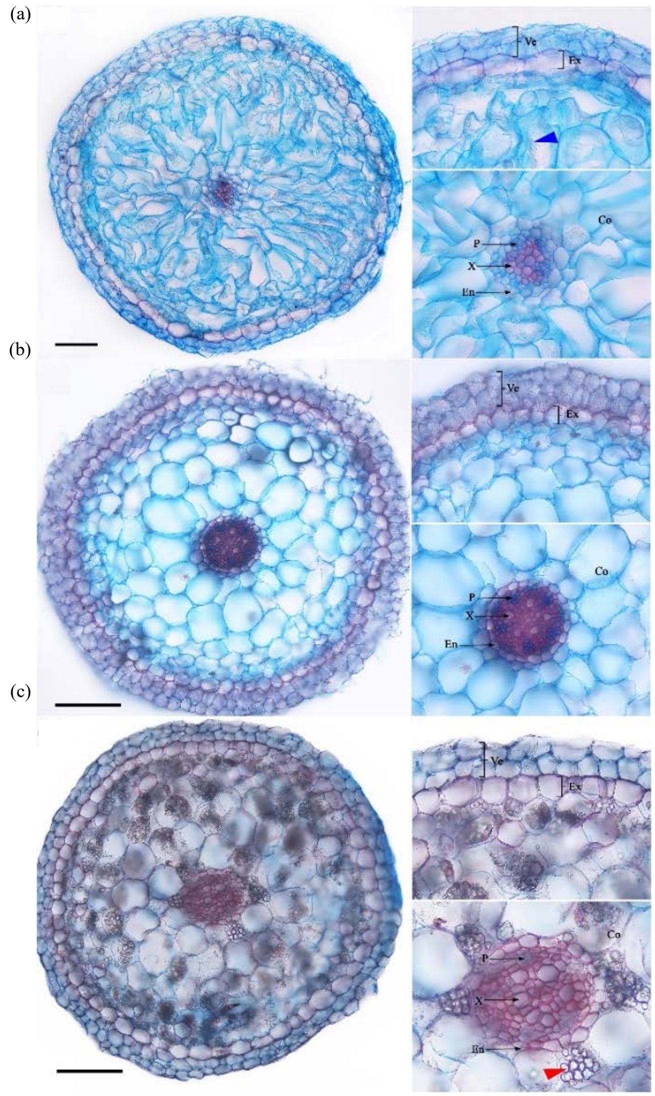

Fig. 8(a-c): Comparative root structure of *T. suavis* plantlets grown under different conditions *in vitro*, (a) Root cross-section under climate chamber conditions after 18 months of cultivation, (b) Root cross-section under culture conditions after 18 months of cultivation, (c) Root cross-section under culture conditions after 3 months of cultivation (regrowth stage)

Blue arrow: Cell nucleus, Red arrow: Cortex parenchymal cells filled with starch grains, Ve: Velamen, Ex: Exodermis, Co: Cortex parenchyma, En: Endodermis, P: Phloem, X: Xylem and Scale bars: 100 µm

The study demonstrated a significant difference in the anatomical structure of the *T. suavis* root grown under various conditions. Roots developed in a climate chamber exhibited a significantly greater diameter compared to those grown in a culture room. The velamen of these roots consisted of two layers of cells with a small thickness (24.73±1.15 µm) (Fig. 8a). The cortex was composed of enlarged cells with folded walls. The thickness of this layer was (444.84±4.94 µm) and constituted about 75% of the cross-section of the root. While the stele exhibited primary development and was of the triarch type. In contrast, roots grown at 24°C for 18 months had a smaller diameter (433.95±3.85 µm), cortex and a thicker velamen (32.62±2.09 µm) than those grown in the climate chamber (Fig. 8b). The cortex was composed of thin-walled parenchymatous cells, which were isodiametric and arranged in a regular pattern, unlike the irregular arrangement seen in roots grown under chamber conditions. Additionally, the stele exhibited a thicker diameter (76.15±2.37 µm), showing pentarch type with the presence of both phloem and protoxylem (Fig. 8c).

Table 3: Morphometric characteristics of *T. suavis* plantlets at regrowth stage after 90 days of cultivation

Treatment	PLBs proliferation (%)	Plantlets height (cm)	Rooting (%)	Number of root/explant	Root length (cm)
A	17.00±4.41^{c*}	1.54±0.05^{c}	26.00±3.33^{c}	2.00±0^{bc}	0.87±0.03^{cd}
B	5.00±3.33^{d}	1.87±0.03^{b}	50.00±5.77^{b}	2.50±0.29^{b}	1.59±0.09^{ab}
C	30.00±3.33^{b}	1.52±0.08^{c}	40.00±0^{b}	1.93±0.23^{bc}	1.03±0.14^{cd}
D	10.00±0^{cd}	1.34±0.06^{d}	10.00±0^{d}	1.60±0.21^{c}	0.79±0.02^{d}
E	20.00±5.77^{c}	1.50±0.04^{c}	15.00±3.33^{d}	1.70±0.21^{c}	0.79±0.02^{d}
F	10.00±5.77^{cd}	2.21±0.06^{a}	70.00±5.77^{a}	3.70±0.38^{a}	1.78±0.13^{a}
G	64.00±3.33^{a}	1.21±0.07^{d}	27.00±3.33^{c}	2.03±0.15^{bc}	1.08±0.07^{c}
H	13.00±3.33^{cd}	1.60±0.03^{c}	43.00±3.33^{d}	1.77±0.15^{bc}	0.79±0.07^{d}

*Values are Mean±SD and superscript letters denote groups by Duncan's Multiple Range Test at the 5% level

Table 4: Anatomical parameters of *T. suavis* roots under different cultivation conditions

	Cultivation conditions		
Root parameters (µm)	Climate chamber after 18 months of cultivation	Culture room after 18 months of cultivation	Regrowth stage
Root diameter	574.07±6.58^{a*}	433.95±3.85^{c}	449.24±1.08^{b}
Velamen thickness	24.73±1.15^{b}	32.62±2.09^{a}	27.91±0.68^{b}
Cortex thickness	444.84±4.94^{a}	277.79±5.93^{b}	292.10±4.46^{b}
Stele diameter	66.52±1.42^{b}	76.15±2.37^{a}	74.61±1.14^{a}
Xylem diameter	5.06±0.26^{c}	6.83±0.28^{b}	9.95±0.57^{a}

*Values are Mean±SD and superscript letters denote groups by Duncan's Multiple Range Test at the 5% level

During the regrowth stage, newly formed roots had an anatomical structure that was similar to that of roots grown after 18 months of preservation under culture room conditions, although starch grains were also present in the parenchymal cells. The stele also demonstrated pentarch style with secondary development, accompanied by an increase in the thickness of the root xylem (Table 4). The root parameters varied under different cultivation conditions. The highest root diameter (574.07 µm) and cortex thickness (444.84 µm) were observed in the climate chamber after 18 months. Velamen thickness was highest (32.62 µm) in the culture room, while the regrowth stage showed the maximum xylem diameter (9.95 µm). Significant variations ($p<0.05$) were recorded among treatments.

DISCUSSION

This study demonstrates the successful preservation of *T. suavis* for 18 months *in vitro* using PLBs as explants without the need for subculturing. The PLBs are identified as optimal explants, not only for artificial seed production and bioreactor research but also for *in vitro* preservation[18]. This is attributed to the fact that PLBs are somatic embryos, which possess high regenerative potential and enable the production of plants in large number[24]. After 9 months of preservation, PLBs cultivated in the culture room (24°C and 3.4 Kcal light intensity) showed active growth, which required them to be transplanted to a fresh culture medium. Plantlets regenerated from PLBs formed numerous aerial roots with a thicker velamen layer, which may serve as an adaptive strategy to maintain viability. In orchids, aerial roots can absorb moisture from the surrounding environment and effectively channel it into the root system. This process is facilitated by the velamen radicum, a specialized, porous tissue that covers the roots[25]. The velamen enables efficient water uptake by allowing moisture to be absorbed directly from the air in cultural vessels, thereby contributing to the plant's hydration and overall survival over 18 months. However, after 18 months, it was observed that the leaves of the plantlets began to dry out, leading to a significant decrease in the survival rate, especially in the control group.

Explants preserved in the climate chamber conditions (at a temperature of 15°C and light intensity of 1.7 Klux) exhibited a high survival rate for up to 18 months. Several studies have reported that medium-term preservation can be achieved *in vitro* by cultivating at low temperatures and light conditions, as these conditions lead to growth retardation[5,26]. For tropical species, which are typically sensitive to cold, a temperature range of 15-20°C is used, consistent with the results of our study[27]. The reduction in temperature induces physiological and biochemical changes that enhance survival, including the production of antifreeze proteins and the activation of cold shock proteins in the explant tissues[18].

Trichopilia suavis plantlets formed under low temperature and light conditions exhibited a bright green color, with no signs of leaf desiccation. This can be explained by the fact that low temperature prevents the growth of leaves but did not affect their water potential[28]. The roots displayed a thin velamen layer, a thick cortex consisting of enlarged cells with folded walls and a small stele diameter with primary developed xylem. This finding is consistent with the results reported by Wu *et al*.[29], which showed that in a rice variety more sensitive to cold stress, the diameter of xylem vascular bundles was reduced and the xylem cells were not well-defined. In the study conducted by Snider *et al*.[30], when cultivating three types of cotton at a lower temperature (20/15°C), the root cross-sectional area was found to be larger than that of the control group, which was grown at the optimal cultivation temperature (30/20°C).The increased size of the cortical cells helps reduce the metabolic costs of the roots[31]. Their irregular shapes and folded walls suggest a specialized role in enhancing nutrient and water uptake, increasing surface area and improving absorption, particularly under stress conditions. Additionally, larger nuclei were observed in the parenchymatous cells of the roots. This may be attributed to chromatin condensation in the nucleus resulting from the cooling of the explant tissue[32].

Growth inhibitors, such as PBZ and CCC, are commonly used for medium-term preservation *in vitro* of various plant species[33]. Paclobutrazol (PBZ) is a triazole compound that inhibits gibberellins (GAs) biosynthesis in plants, thereby slowing cell elongation, particularly in the shoot tip region[34]. This compound is commonly used to enhance the acclimatization of orchids, control growth and improve stress resistance. When added to the culture medium or substrate, it reduces transpiration, inhibits excessive growth and enhances resistance to environmental stressors such as drought and temperature fluctuations[35]. However, the effect of PBZ on *T. suavis* growth *in vitro* has not yet been studied. Additionally, it may be necessary to select PBZ concentrations individually, depending on the specific taxon being investigated. The use of PBZ at high concentrations may inhibit plant development and cause tissue necrosis. Our study found that the death of the PLBs within three months at 24°C can be attributed to the high concentration of PBZ in the culture medium. This finding is consistent with the result of another study, which found that an increase in PBZ concentration in a culture medium resulted in a decrease in the viability of *Grammatophyllum speciosum* Blume seedlings to 30% after one month of cultivation at 25°C[36]. Therefore, it is necessary to study the effect of low PBZ concentrations to preserve the viability of *T. suavis* PLBs. At the same time, under conditions of a climate chamber, explants cultured on a culture medium containing PBZ maintained viability, but lower rate than other variants under the same conditions.

The PLBs cultured on 1/2 MS media supplemented with PBZ did not exhibit significant regeneration. These findings are consistent with those observed in other orchid species, such as *Arundina graminifolia* (D.Don) Hochr. and *Dendrobium bicaudatum* Reinw. ex Lindl., where growth reduction, delayed development of vegetative organs and abnormal shoot morphology were observed compared to explants grown on a medium without any growth inhibitors[21,37]. This may be attributed to inhibition of gibberellin biosynthesis by blocking the formation of the enzyme, kaurene oxidase P450, leading to the cessation of apical growth[38].

In our study, we observed that the use of PBZ promoted the formation of new PLBs throughout the entire preservation period under climate chamber conditions. This finding is consistent with other studies, which have demonstrated that the cultivation of representatives of the *Cymbidium* genus on a medium containing 1.0 mg/L PBZ enhances the formation of new PLBs[39]. The ability of PBZ to enhance the regenerative capacity is associated with an increase in the content of endogenous cytokinins, which facilitates cell division and stimulating the propagation of PLBs[40].

Cycocel (2-chloroethyltrimethylammonium chloride), also known as chlorocholine chloride, is a plant growth regulator commonly used to inhibit plant growth. It exerts this effect by interfering with the activity of endogenous plant hormones, particularly by inhibiting the biosynthesis of GAs, which are responsible for promoting cell elongation and growth. As a result, Cycocel reduces excessive vegetative growth, promoting more compact and controlled plant development[41].

In our study, we observed that PLBs cultivated on a culture medium containing CCC at a temperature of +15°C, regenerated and produced bright green plantlets with a high survival rate after 18 months. This finding is consistent with previous studies that have reported the inhibitory effect of CCC on the vegetative growth of several plant species, such as *Moehringia jankae* Griseb. ex Janka and *Dendrobium* SW orchid cv. Sonia[42,43], without inducing morphological abnormalities in the plantlets. This can be explained by the fact that CCC enhances nutrient absorption, water balance and protein synthesis in developing organs and also plays a significant role in enhancing photosynthetic capacity and, therefore, the formation of plantlets over time compared to other growth inhibitors such as PBZ[44]. At a temperature of +24°C, it was observed that the PLBs cultivated on 1/2 MS medium supplemented with 0.4 mg/L of CCC significantly enhanced PLBs proliferation. Several studies have reported the effect of CCC on the formation of PLBs in certain species of epiphytic orchids[45]. For example, in *Phalaenopsis*, it was observed that the addition of CCC to the culture medium had an effect on the formation of PLBs (proliferating lateral buds), but an increase in the concentration of CCC led to a reduction in PLBs formation[44]. However, our results do not support these findings, as an increase in CCC concentration under culture room conditions led to the activation of PLBs proliferation and a decrease in differentiation. This may be attributed to the fact that CCC inhibits the growth of explants while simultaneously promoting the accumulation of carbohydrates within plant cells, which enhances PLBs mass and stimulates further cell division[43].

The successful preservation of *T. suavis* for 18 months was confirmed by the continued growth of the formed plantlets and the regeneration of new PLBs on the regrowth medium under culture room conditions. These results are consistent with several studies in which plantlets, such as those of apple and orchids, were preserved at low temperatures in the laboratory and successfully recovered after a long period[46,47]. Plantlets grown on a medium containing 0.5 mg/L KIN and coconut water showed better growth compared to other treatments. Roots formed during the regrowth stage exhibited an anatomical structure similar to that formed under culture room conditions during the preservation period. However, in the recovered plantlets, an increase in the volume of lignified tissue in the root stele and starch grains in the parenchymal cells was observed. These results are consistent with those of a previous study on *Vanda tessellata* (Roxb.) Hook. G. Don, which reported the presence of starch grains in the root cortex under *in vitro* conditions[48]. The presence of starch grains in the root is a distinctive feature and presumably contributes to its storage function[49].

In the cultivation protocol of *T. suavis* and many other orchid species, it was shown that the addition of cytokinins and coconut water plays a major role in the formation of plantlets without passing through the callus stage[12]. This is due to the biochemical composition of coconut water, which includes vitamins, natural plant hormones and sugars such as sorbitol and mannose, which contribute to the regulation of the biological pathway for cell wall formation and ion absorption, in addition to GAs, which contribute to the elongation of cell and the regulation of cambium activity in plant cells[50,51].

CONCLUSION

This study presents an efficient protocol for the medium-term conservation of *T. suavis* PLBs *in vitro*. The cultivation of PLBs on 1/2 MS medium supplemented with 0.2 mg/L CCC under climate chamber conditions (15°C and 1.7 Klux light intensity) was found to be effective, achieving an 87% survival rate, with 80% of the PLBs regenerating into healthy, desiccation-free plantlets after 18 months without subculturing. Additionally, the cultivation conditions had a significant effect on root anatomy, with roots under climate chamber conditions exhibiting larger diameters, thicker cortex and developed primary xylem. These findings offer a cost-effective strategy for the preservation of *T. suavis*, which could also be applied to the preservation of other endangered orchid species.

SIGNIFICANCE STATEMENT

Trichopilia suavis, a fragrant epiphytic orchid with high ornamental and commercial value, has not previously been studied for *in vitro* conservation. This study establishes the first effective medium-term storage protocol for its PLBs, using plant growth retardants and controlled environmental conditions. The optimized treatment, combining chlorocholine chloride (CCC) and specific temperature-light cultivation conditions, achieved high PLB survival and regeneration over 18 months without subculturing. Root anatomical adaptations under different cultivation conditions were also characterized. These findings provide a cost-effective and reliable method for conserving *T. suavis*, offering valuable insights that can support conservation efforts for other species within the genus *Trichopilia* and advance orchid conservation biotechnology.

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