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Establishment of Micropropagation Protocol for Double Petunia “Pink Diamond” from Leaf Tissues

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Abstract

Petunias are ornamental plants of high economic interest. A micropropagation protocol from leaf tissues of *Petunia hybrid* cv. ‘Pink Diamond’ was established in this study. Murashige and Skoog (MS) medium supplemented with 1 mg L^{-1} BA and 30 g L^{-1} sucrose was the most suitable for callogenesis and shoot regeneration from leaf explants. “Pink Diamond” cultured on this medium showed the highest callus induction rate (94.44%), the maximum shoot regeneration percentage (83.33%), the highest multiplication rate (15.28 shoots/explant), and the best quality of shoots. For the growth of shoot tip-derived shoots, MS medium fortified with 40 g L^{-1} sucrose and 10% coconut water was the optimal medium, achieving the highest shoot length (7.78cm) and the largest number of leaves (16.89 leaves/shoot). Meanwhile, MS medium containing 40 g L^{-1} sucrose and 15% coconut water was optimal for the growth of node-derived shoots, resulting in the largest leaf size (1.88cm in length and 0.75 cm in width) and the highest number of leaves (10.83 leaves/shoot). MS medium supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} AC was the most suitable for rooting, achieving the highest shoot length (3.22cm), the largest number of leaves (7.06 leaves/plantlet), and the highest number of roots (16 roots/plantlet). The *in vitro* plantlets transplanted to 100% peat moss were successfully acclimatized under poly-house conditions, with an 88.39% survival rate. These plantlets reached 8.16 cm in plant height and 15.35 leaves per plant after four weeks of being transferred.

Keywords

Micropropagation, leaf tissue, *Petunia hybrida*

Introduction

The garden petunia (*Petunia hybrida*), belonging to the Solanaceae family, is a popular and wide spread garden ornamental. Petunia plants flower profusely, presenting excellent coverage in patio containers, hanging baskets, window boxes, and landscapes. This plant is an annual or perennial herb, 0.3-1m in height, with

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diverse flower shapes and colors (Pham Hoang Ho, 2003). Garden petunias are derived from hybridizations between *P. axillaris* and one or more species in the *P. integrifolia* clade (Segatto *et al.*, 2014). Based on flower form, petunias are divided into three groups (single, double, and ruffled). Petunias are economically important due to their horticultural value in the global market (Vakili *et al.*, 2019; Farooq *et al.*, 2021). In the U.S. and Europe, petunias consistently rank among the top selling annual bedding plants. In 2020, petunias ranked first among annual bedding plants in the U.S., with a wholesale value of $159.8 million (USDA, 2021). In the Vietnam floriculture market, the demand for petunias has the potential to increase (Pham Thi Huyen Trang *et al.*, 2024). Therefore, it is necessary to improve the quality and yield of breeding sources for petunias.

Petunias are propagated by traditional methods through seeds or stem cuttings. However, these approaches have limitations in terms of being slow processes and having high susceptibility to infection. Therefore, high expectations have been raised for micropropagation in order to solve the main problems of traditional propagation methods as micropropagation produces a large number of disease-free plantlets in a short period of time, leading to reduced production costs without compromising quality. Furthermore, the *in vitro* offspring are genetically identical to the mother plant, helping to maintain the stability of plantlet quality.

Tissue culture techniques were started applying to petunias in the late 1960's (Izhar & Zelcer, 1984). It was generally found that an entire plant could be regenerated from explants. A variety of explants have been used for *in vitro* shoot regeneration of petunias including anthers (Martineau *et al.*, 1981), seeds (Borovaya & Boginskaya, 2022), axillary buds (Mehri *et al.*, 2018), shoot tips (Farooq *et al.*, 2021), nodal segments (Farooq *et al.*, 2021; Pham Thi Huyen Trang *et al.*, 2024), and leaf explants (Reuveni & Evenor, 2007; Abu-Qaoud, 2012, Natalija *et al.*, 2015; Vakili *et al.*, 2019; Farooq *et al.*, 2021; Pham Thi Huyen Trang *et al.*, 2024). To date, different cultivars of petunia have been investigated in order to optimize suitable *in vitro* regeneration and propagation protocols for each of them (Natalija *et al.*, 2015; Mehri *et al.*, 2018; Vakili *et al.*, 2019; Farooq *et al.*, 2021; Borovaya & Boginskaya, 2022, Pham Thi Huyen Trang *et al.*, 2024). Besides growth regulators, several supplements have also been found to contribute to improved *in vitro* propagation efficiency and *in vitro* plantlet quality. Some supplements have been commonly used in tissue culture in general and petunia tissue culture in particular such as sucrose (Ndagijimana *et al.*, 2014; Pham Thi Huyen Trang *et al.*, 2024) coconut water (Yong *et al.*, 2009; *Mu et al.*, 2024; Pham Thi Huyen Trang *et al.*, 2024), and activated carbon (Thomas, 2008, Pham Thi Huyen Trang *et al.*, 2024).

Although numerous studies have focused on the *in vitro* propagation of single petunia cultivars, research on that of double cultivars remains limited. Recently, the double petunia cultivar "Pink Diamond" has been introduced to Vietnam and gained popularity in the market. Therefore, our goal is to develop an *in vitro* propagation protocol for the "Pink Diamond" cultivar using leaf tissue to meet market demand.

Materials and Methods

Plant materials

Double petunia cultivar "Pink Diamond" (*Petunia hybrida* Hort. ex Vilm. - Andr. cv. 'Pink Diamond') was used in this study. The F1 plants were collected in Da Lat, Lam Dong and grown at the Department of Botany, Faculty of Agronomy, Vietnam National University of Agriculture in Hanoi, Vietnam.

Methods

Culture media and conditions

All culture media were adjusted to pH 5.8 and autoclaved at 121ºC for 20 minutes under 1.1 atm. *In vitro* experiments were conducted at 25 ± 1ºC, 65-70% humidity, with a 10h light/14h dark cycle and irradiance of 34.73 µmol/m²/s using LED tubes (Rang Dong T8 22w/1.2m, Vietnam). For acclimatization, plantlets were grown in a poly-house under natural light at 20-25ºC, 70-75% humidity, with periodic irrigation.

Experimental design and measurements of parameters

All *in vitro* experiments were assigned to a randomized complete design (RCD) with three replications. Each replication included ten culture vessels with six explants per vessel. Three culture vessels (18 explants) per formula were randomly observed.

The *ex vitro* experiment was arranged in a random, non-repeated sequence. The fixed 20 plantlets were randomly selected for measurement.

* *Callus initiation and shoot regeneration*

Healthy mature leaves of the double petunia were surface sterilized by 0.1% $HgCl_2$ for 10 minutes (Farooq *et al.*, 2021) followed by three rinses with sterile distilled water. Sterilized leaves were placed onto Murashige & Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 30 g L^{-1} sucrose and 6.5 g L^{-1} agar, pH 5.8. After one week of culture, green and disease-free leaves were cut into 0.5 x 0.5cm dimension segments and placed again on MS medium supplemented with 30 g L^{-1} sucrose, 6.5 g L^{-1} agar, pH 5.8, and BA (6-Benzylaminopurine) or α-NAA at different concentrations (0, 0.5, 1, 1.5, and 2 mg L^{-1}) for callus initiation and shoot regeneration. All culture vessels were maintained in dark conditions for one week, and were later incubated in a light culture room. Data were recorded after six weeks of culture including rate of callus initiation (%), rate of shoot regeneration (%), and callus or shoot morphology (color, size, and vitrification, etc.).

* *Shoot growth*

In vitro shoot tips (1.5-1.7cm with 4-5 leaves) or nodes (0.5-0.7cm) regenerated from leaf tissues of "Pink Diamond" were cultured in MS medium with sucrose concentrations of 20, 30, 40, and 50 g L^{-1}. The best medium was selected for further experiments. Shoot tips or nodes were then cultured in the chosen medium with coconut water concentrations of 0%, 5%, 10%, 15%, and 20%. After four weeks, parameters such as shoot diameter, shoot length, leaf length, leaf width, and number of leaves were recorded.

* *Root induction*

In vitro 2-cm-long shoot tips were cultured on MS medium with 30 g L^{-1} sucrose, 6.5 g L^{-1} agar, pH 5.8, and varying concentrations of α-NAA (0, 0.1, 0.3, 0.5 mg L^{-1}) or activated charcoal (AC) (0.1, 0.3, 0.5, 1 g L^{-1}) for root induction. After 10 days, data on shoot diameter, shoot length, leaf length, leaf width, number of leaves, root length, and number of roots were recorded.

To assess the adaptability of *in vitro* plantlets, the best-rooted plantlets were removed from the culture medium, washed to remove agar, and transferred into a 112-cell foam tray (49cm x 28.5cm x 4.3cm) with 100% peat moss. Survival rate, plant height, and number of leaves were evaluated at 1, 2, and 4 weeks post-transfer.

Statistical analysis

Data were statistically analyzed using ANOVA by IRRISTAT 5.0. Duncan's test was used to indicate means with significant effect at $P \leq 0.05$.

Results and Discussion

Callus initiation and shoot regeneration

Effects of BA and α-NAA on callus initiation and shoot regeneration from leaf tissues of "Pink Diamond"

For efficient shoot regeneration, a two-step culture protocol has been widely used, in which explants initiated callus formation, and then the calli induced shoot generation. The abilities of callus initiation and shoot regeneration in the culture medium are mainly controlled by the levels of phytohormones. Cytokinins and auxins are the most common phytohormones studied and used in relation to callus induction and shoot regeneration. In this study, α-NAA (auxin) and BA (cytokinin) were separately used to evaluate their effects on callus induction and shoot regeneration from leaf tissue in "Pink Diamond".

The data in **Table 1** and **Figure 1** show that leaf explants of "Pink Diamond" cultured on phytohormone-free medium were not able to induce calli or regenerate shoots. This result was similar to studies performed by Kaviani &

Kazemi (2017) and Tawfik *et al.* (2019). It is clear that phytohormones play a key role in the callogenesis and organogenesis of petunia explants cultured in *in vitro* conditions.

Media containing a range of 0.5-2.0 mg L^{-1} α-NAA promoted callus and root induction from leaf segments however the calli could not regenerate shoots (**Table 1**, **Figure 1**). According to Subotic *et al.* (2008) and Abu-Qaoud (2012), *in vitro* regeneration is mostly regulated by the balance and the interaction between the exogenous hormones in the medium and those endogenously produced by the explants. Therefore, elevating the endogenous auxin concentration with exogenous applications might have an inhibitory effect on shoot regeneration.

In contrast, leaf explants cultured on media supplemented with a range of 0.5-2.0 mg L^{-1} BA induced both callus initiation and shoot regeneration (**Table 1**, **Figure 1**). These results were similar to the earlier findings of Mehri *et al.* (2018) in that BA individually promoted the induction of shoots, nodes, and leaves in petunia cultivar "Opera Supreme Pink Morn". This induction was done without the presence of α-NAA (Mehri *et al.*, 2018). However, leaf explants cultured on 0.5 mg L^{-1} BA medium exhibited low quality regenerated shoots, which were weak and small with dark green leaves. When the BA concentration increased to 1.0 mg L^{-1}, the culture medium reached the optimal level for callus formation and shoot regeneration with the highest multiplication rate (15.28 shoots/explant) and the best quality of shoots (**Table 1**, **Figure 1**). Similar results were obtained in the study of Kaviani & Kazemi (2017), who found that the most suitable BA concentration for callus induction and shoot regeneration from leaf explants of petunia was 1.0 mg L^{-1}. Increasing the BA concentration from 1.0 to 2.0 mg L^{-1} reduced the callus initiation rate, shoot regeneration rate, multiplication rate, and shoot quality (**Table 1**, **Figure 1**). A similar result was reported by Tawfik *et al.* (2019) who found that lower concentrations of BA led to a higher regeneration percentage, which declined with increases in the concentration. These results were in agreement with the report of Rout *et al.* (2006) who reported that cytokinin alone in the culture medium induced shoot formation. However, high concentrations of cytokinins inhibited shoot formation *in vitro* from leaf explants in some ornamental plants (Rout *et al.*, 2006).

Thus, the most suitable culture medium for callus initiation and shoot regeneration of "Pink Diamond" from leaf tissues was MS supplemented with 1.0 mg L^{-1} BA and 30 g L^{-1} sucrose. Meanwhile, Natalija *et al.* (2015) reported that suitable shoot regeneration media were MS containing 0.8 mg L^{-1} BA and 0.1 mg

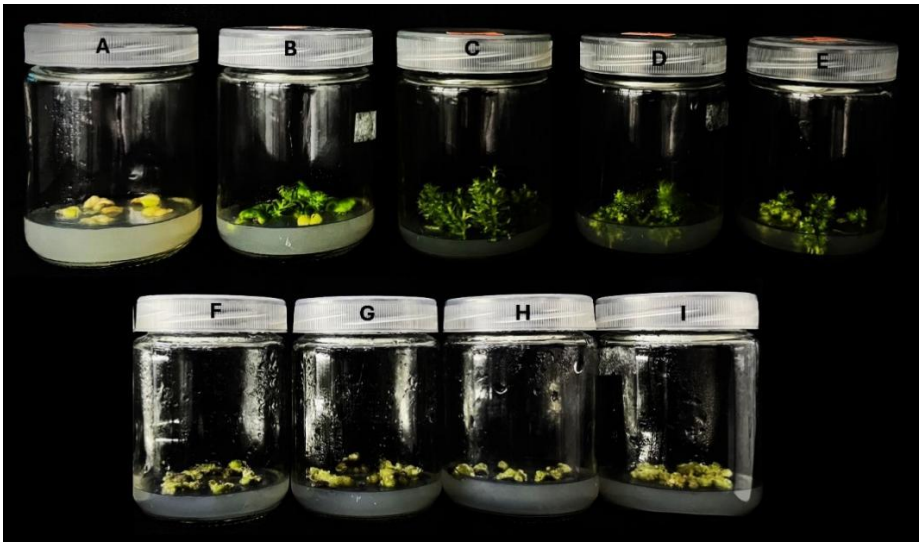

Figure 1. Effect of growth regulation on callus initiation and shoot regeneration from leaf tissues of "Pink Diamond"
(A. MS; B. MS + 0.5 mg L^{-1} BA; C. 1.0 mg L^{-1} BA; D. 1.5 mg L^{-1} BA; E. 2.0 mg L^{-1} BA, F. 0.5 mg L^{-1} α-NAA; G. 1.0 mg L^{-1} α-NAA; H. 1.5 mg L^{-1} α-NAA, I. 2.0 mg L^{-1} α-NAA)

Table 1. Effects of plant growth regulators on callus initiation and shoot regeneration from leaf tissues of "Pink Diamond"

α-NAA concentration (mg L^{-1})	BA concentration (mg L^{-1})	Rate of callus initiation (%)	Rate of shoot regeneration (%)	Multiplication rate (shoots/explant)	Visual observations
0	0	0.00^{d}	0.00^{c}	0.00^{e}	-
0	0.5	88.89^{ab}	83.33^{a}	3.33^{d}	++
0	1.0	94.44^{a}	83.33^{a}	15.28^{a}	+++
0	1.5	88.89^{ab}	77.78^{a}	6.17^{b}	+
0	2.0	83.33^{ab}	66.67^{b}	4.06^{c}	+
0.5	0	50.00^{c}	0	0	
1.0	0	77.78^{b}	0	0	
1.5	0	83.33^{ab}	0	0	White, green callus; root formation
2.0	0	88.89^{ab}	0	0	

Note: "-" = No response; "+" = Weak and small shoots, light green leaves; "++" = Weak and small shoots, dark green leaves; "+++" = Strong and big shoots, dark green leaves. *Different letters in each column indicate significant differences (Duncan's test; P ≤0.05).*

L^{-1} α-NAA for petunia cultivars "Purple Velvet" and "Ramblin Nu Blue" and MS containing 0.4 mg L^{-1} BA and 0.1 mg L^{-1} α-NAA for petunia cultivar "Touha". In addition, the study of Abu-Qaoud (2012) on petunia "Mix Color" revealed that the most suitable medium for shoot regeneration from leaf explant was MS containing 2 mg L^{-1} BA. Our findings agree with Skvirsky *et al.* (1984) and Natalija *et al.* (2015) that the optimal levels of growth regulators strongly vary depending on the requirements of different genotypes.

Shoot growth

In the shoot regeneration stage, most of the leaf calli produced dense shoot clusters containing numerous microshoots, which were small, thin, and weak. Therefore, it was necessary to improve the microshoot quality by undergoing a shoot growth stage. In this stage, a hormone-free culture medium was used and the effects of several media factors (sucrose and coconut water) on shoot growth were evaluated.

Effect of sucrose on shoot growth of "Pink Diamond"

Shoots regenerated from leaf tissues included both apical shoots and nodal segments, which both contain meristems. Therefore, these materials were used during the shoot growth stage to improve the efficiency of *in vitro* propagation.

Sucrose is considered as a crucial carbon and energy source required by organic compound synthesis, cell division, and biomass production of plant cells and tissues. In tissue culture, the sucrose level is one of the factors controlling the growth of *in vitro* shoots (Ndagijimana *et al.*, 2014). In order to determine a medium suitable for the shoot growth of "Pink Diamond", two kinds of explants, *in vitro* shoot tips and nodal segments, were cultured on MS medium supplemented with different sucrose concentrations.

The data in **Table 2** declare the effect of different sucrose concentrations on shoot growth. It was observed that shoots derived from both *in vitro* shoot tips and nodes grew weakly on low sucrose concentration media (20-30 g L^{-1}). Indeed, *in vitro* shoot tip-derived shoots cultured on 20 g L^{-1} of sucrose medium had the shortest shoot length and the fewest number of leaves, which were statistically different. Gould *et al.* (1981) supposed that a low concentration of sucrose could retard cell development by causing a cessation of the cell cycle when nutrients were limited. The decline in viability suffered by cells in the low sucrose medium was also due to sucrose starvation.

The results in **Table 2** also demonstrate that most of the growth indicators of the shoots cultured on the 40 g L^{-1} sucrose formula reached

the highest values among the formulas. Indeed, the shoots derived from both shoot tips and nodes grown in the 40 g L^{-1} sucrose medium reached the highest shoot lengths, the longest leaf lengths, and the highest number of leaves. These differences were statistically significant. It could be conjectured that 40 g L^{-1} of sucrose might induce optimal osmotic potential in the culture that facilitates *in vitro* growth of shoots. This present investigation was consistent with the findings of Pham Thi Huyen Trang *et al.* (2024), in which 40 g L^{-1} sucrose was the most suitable for the growth of *in vitro* Purple Petunia shoots.

However, when the sucrose concentration was increased to 50 g L^{-1}, most of the shoot growth parameters decreased (**Table 2**). Tawfik *et al.* (2019) suggested that using a sucrose concentration over 40 g L^{-1} might cause adverse effects on shoot growth. It was explained that the sucrose osmotic contribution had an inverse relationship with sucrose concentration and caused an initial increase followed by reductions in the values of the assessed growth indicators. These reductions can be caused by an excessive osmotic contribution or by toxicity of the carbohydrate source at high concentrations (de Paiva Neto & Otoni, 2003). In other words, the osmotic level in the high sucrose concentration medium may be inhibitory to shoot growth.

These results clearly indicated that 40 g L^{-1} sucrose was suitable to induce the growth of shoots derived from both *in vitro* shoot tips and nodes. Nevertheless, the results of the micropropagation study on petunia "Bravo" (Farooq *et al.*, 2021) revealed that the most suitable medium for shoot growth was MS supplemented with 30 g L^{-1} sucrose. It was suggested that the optimum sucrose concentration for shoot growth may vary among genotypes.

Effect of coconut water on shoot growth of "Pink Diamond"

Coconut water (CW) is widely used in the plant tissue culture industry thanks to growth-regulating properties such as cytokinin-type activity (Yong *et al.*, 2009). Some of the significant and useful components in CW include being comprised of sugar (sucrose, glucose, fructose), vitamins, minerals, amino acids, auxin, and cytokinins (Aguilar *et al.*, 2009; Yong *et al.*, 2009), which support cell division and elongation (Yong *et al.*, 2009; Mu *et al.*, 2024). In order to determine the impacts of CW on the shoot growth of "Pink Diamond", this experiment was carried out.

The data in **Table 3** indicated that shoots derived from both shoot tips and nodes grew weakly on CW-free medium. Indeed, both shoot tip-derived shoots and node-derived shoots cultured on MS medium without CW had the shortest shoot lengths and the fewest numbers of leaves, which were statistically different.

Furthermore, when the CW concentration increased to 10-15%, shoots derived from both materials reached their maximum growth. Shoot tip-derived shoots cultured on a medium with a 10% coconut water concentration reached the statistical highest shoot length and number of

Table 2. Effect of sucrose on shoot growth of double Petunia "Pink Diamond" after four weeks of culture

Explants	Sucrose concentration (g L^{-1})	Shoot diameter (cm)	Shoot length (cm)	Leaf length (cm)	Leaf width (cm)	Number of leaves (leaves/shoot)
Shoot tip	20	0.10^{b}	3.14^{d}	1.67^{c}	0.66^{b}	6.50^{d}
	30	0.11^{a}	4.08^{c}	1.73^{b}	0.69^{b}	8.83^{c}
	40	0.11^{a}	5.25^{a}	1.86^{a}	0.74^{a}	11.39^{a}
	50	0.10^{b}	4.96^{b}	1.72^{bc}	0.69^{b}	10.22^{b}
Node	20	0.10^{a}	2.19^{b}	1.54^{b}	0.61^{a}	4.56^{b}
	30	0.10^{a}	2.35^{b}	1.55^{b}	0.62^{a}	5.11^{b}
	40	0.10^{a}	3.69^{a}	1.65^{a}	0.66^{a}	8.06^{a}
	50	0.10^{a}	2.22^{b}	1.55^{b}	0.61^{a}	4.61^{b}

Note: Different letters in each column indicate significant differences (Duncan's test; P ≤0.05).

leaves. Meanwhile, node-derived shoots grown in a medium treated with a 15% coconut water concentration achieved the optimal level of shoot growth, leaf size, and number of leaves (**Table 3)**. This might be attributed to the presence of phytohormones in the CW, which were highly sufficient in promoting shoot elongation and leaf cell production (Yong *et al.*, 2009; Mu *et al.*, 2024).

Nevertheless, when the CW concentration was increased to 20%, most of the shoot growth indicators declined (**Table 3**). The reason might be that the application of a high CW concentration led to an increase in the level of sucrose and cytokinin contained in the CW, which caused the inhibition of shoot growth (Rout *et al.*, 2006; Yong *et al.*, 2009; Tawik *et al.*, 2019; Mu *et al.*, 2024).

Pham Thi Huyen Trang *et al.* (2024) found that CW had no effect on the shoot growth of "Purple Petunia". It was explained that different genotypes have unique requirements for growth, and their optimal culture medium may vary (Mu *et al.*, 2024).

In conclusion, the results of the study in the shoot nursing stage showed that the suitable growth medium for shoots derived from shoot tips of double petunia "Pink Diamond" was MS supplemented with 40 g L^{-1} sucrose and 10% CW, while the suitable growth medium for shoots derived from nodal segments was MS containing 40 g L^{-1} sucrose and 15% CW.

Root induction in regenerated shoots

Effects of α-NAA and AC on the growth and root induction of "Pink Diamond"

The rooting ability of *in vitro* propagated shoots is critical for adapting plants to *ex vitro* conditions because the *in vitro* plantlets with a strong root system have a high survival rate and fast growth during the acclimatization stage. Bellamine *et al.* (1998) reported that auxin plays a primary role in root formation of *in vitro* shoots in micropropagation. Additionally, activated charcoal can also be added to culture media due to its rooting induction ability and irreversible adsorption of inhibitory compounds in the culture medium, such as toxic metabolites, phenolic exudation, and brown exudate accumulation (Thomas, 2008).

In this study, α-NAA and AC were separately used to evaluate their effects on the growth and root formation of "Pink Diamond" *in vitro* shoots. The results after ten days of culture are shown in **Table 4** and **Figure 2**.

The data in **Table 4** reveal that MS medium without α-NAA and AC induced root formation with 2.08cm in root length and 12.06 roots per plantlet. Similar results were obtained for a double-flowered *P. hybrida* cultivar and "Purple

Table 3. Effect of coconut water on shoot growth of "Pink Diamond" after four weeks of culture

Explants	Coconut water concentration (%)	Shoot diameter (cm)	Shoot length (cm)	Leaf length (cm)	Leaf width (cm)	Number of leaves (leaves/shoot)
Shoot tip	0	0.11^{a}	5.23^{d}	1.85^{b}	0.73^{b}	11.33^{d}
	5	0.11^{a}	5.88^{c}	1.92^{ab}	0.76^{ab}	12.72^{c}
	10	0.11^{a}	7.78^{a}	1.95^{a}	0.78^{a}	16.89^{a}
	15	0.11^{a}	7.51^{a}	1.93^{a}	0.77^{ab}	15.67^{b}
	20	0.10^{b}	6.62^{b}	1.85^{b}	0.74^{ab}	13.56^{c}
Node	0	0.10^{a}	3.66^{b}	1.66^{d}	0.66^{c}	7.94^{c}
	5	0.10^{a}	3.87^{b}	1.71^{cd}	0.68^{c}	8.33^{c}
	10	0.10^{a}	4.54^{a}	1.85^{ab}	0.74^{ab}	9.78^{b}
	15	0.10^{a}	4.98^{a}	1.88^{a}	0.75^{a}	10.83^{a}
	20	0.10^{a}	4.46^{a}	1.77^{bc}	0.70^{bc}	9.44^{b}

Note: Different letters in each column indicate significant differences (Duncan's test; P ≤0.05).

Petunia" when it was grown on hormone-free MS medium (Borovaya *et al.*, 2022; Pham Thi Huyen Trang *et al.*, 2024). The rooting of shoots cultured on MS media without hormones and AC indicated that the concentration of endogenous auxin might be sufficient to stimulate root formation in the shoots.

By contrast, in media with added α-NAA in the range of 0.1-0.5 mg L^{-1}, all the shoots could not induce the formation of roots (**Table 4**, **Figure 2**), and callus formation was observed at the shoot base. Similarly, Pham Thi Huyen Trang *et al.* (2024) noted that α-NAA added to media at levels from 0.3 to 0.5 mg L^{-1} led to inhibited rooting of "Purple Petunia" shoots. It might be explained that the combination of exogenously supplied and tissue-produced α-NAA led to a high α-NAA concentration in the culture media, which inhibited root formation. The study of Yan *et al.* (2014) on the rooting ability of *Hemarthria compressa* also showed that, at low concentrations, α-NAA promoted the formation of plant roots but inhibited rooting at high concentrations.

Data in **Table 4** and **Figure 2** indicate that a 0.1 g L^{-1} AC medium induced most of the highest shoot growth indicators among the tested media. However, increasing the AC concentration from 0.1 to 1 g L^{-1} led to a dramatic decrease in shoot length, number of leaves, root length, and number of roots. According to Thomas (2008) and Martineau *et al.* (1981), apart from its inhibitory compound adsorption ability, AC has the ability to release naturally present substances that promote shoot growth and root formation. Nevertheless, a high level of AC might absorb significant quantities of endogenous α-NAA, substances, needed hormones, vitamins, and metal ions (Thomas, 2008) leading to the reduction of shoot growth and root formation.

It was clear that the lowest AC level (0.1 g L^{-1}) medium exerted the best results with respect to shoot length, number of leaves, and number of roots, which were statistically different (**Table 4, Figure 2**). This result was similar to a study performed by Pham Thi Huyen Trang *et al.* (2024) who found that MS medium supplied with 0.1 g L^{-1} AC was the most suitable for the rooting of Purple Petunia regenerated shoots with 12.83 roots/plantlet and 2.28cm in root length. Nevertheless, the study of Kag *et al.* (2012) on a single petunia cultivar revealed that half strength MS salt medium supplemented with 1 mg L^{-1} IBA and 2 g L^{-1} AC showed the highest root induction rate and the maximum number of roots. It might be explained that genotypic specificity of a particular cultivar resulted in variations for optimum AC concentration.

Acclimatization is the final stage of micropropagation, crucial for transferring plantlets from *in vitro* to *ex vitro* environments. It helps plantlets adapt to the natural environment, with their survival and adaptability depending on both their quality and environmental conditions. To assess their adaptability, plantlets from the best rooting medium were grown in the nursery. Growth indicators of the *in vitro* plantlets are shown in **Table 5**.

Based on the data in **Table 5**, "Pink Diamond" plantlets transferred to a 100% peat moss substrate were successfully acclimatized to the conditions in the poly-house, with a 100% of survival rate after one week of transfer. After four weeks of transfer, 85% survivability of the plantlets was achieved upon hardening with 8.16cm in plant height and 15.35cm leaves per plantlet (**Table 5**). These results were similar to the finding of Habas *et al.* (2019) in that the regenerated shoots with well-developed roots of *P. hybrida* were successfully acclimatized and established in pots containing sterilized peat moss with 70% survival rates. A high survival acclimatization of the *in vitro*-grown plantlets was proven by the validity of their development into healthy plants after being transferred to the *ex vitro* conditions (Habas *et al.*, 2019).

Conclusions

An *in vitro* propagation protocol for *P. hybrid* cv. 'Pink Diamond' was established using sterilized mature leaves (0.1% $HgCl_2$ for 10min). Leaf segments (0.25cm²) were cultured on MS medium with 1.0 mg L^{-1} BA and 30 g L^{-1} sucrose for callus initiation and shoot regeneration, achieving a callus induction rate of 94.44%, shoot regeneration of 83.33%, and a multiplication rate of 15.28 shoots/explant. *In*

vitro shoot tips were transferred to MS medium with 40 g L^{-1} sucrose and 10% CW, while nodal segments were cultured with 40 g L^{-1} sucrose and 15% CW for shoot growth. The shoots were then cultured on MS medium with 30 g L^{-1} sucrose and 0.1 g L^{-1} AC for root formation. The rooted plantlets, grown on a 100% peat moss substrate, adapted well to poly-house conditions with an 88.39% survival rate, 8.16cm height, and 15.35 leaves/plant after four weeks. The process is summarized in **Figure 3**. The plants propagated using this protocol were of good quality and exhibited high survival rates when planted in the nursery. In the future, this protocol can be applied for the large-scale production of "Pink Diamond" plantlets for commercial purposes.

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Table 4. Effects of α-NAA and AC on the growth and root induction of "Pink Diamond" after ten days of culture

α-NAA concentration (mg L^{-1})	AC concentration (g L^{-1})	Shoot diameter (cm)	Shoot length (cm)	Leaf length (cm)	Leaf width (cm)	Number of leaves (leaves/ plantlet)	Root length (cm)	Number of roots (roots/ plantlet)
0	0	0.11^{a}	2.81^{b}	1.72^{ab}	0.68^{bc}	6.11^{c}	2.08^{a}	12.06^{b}
0.1	0	0.11^{a}	2.24^{c}	1.64^{cd}	0.65^{c}	4.83^{d}	0	0
0.3	0	0.11^{a}	2.17^{c}	1.61^{de}	0.64^{c}	4.67^{d}	0	0
0.5	0	0.11^{a}	2.12^{c}	1.58^{e}	0.63^{c}	4.61^{d}	0	0
0	0.1	0.11^{a}	3.22^{a}	1.77^{a}	0.75^{a}	7.06^{a}	1.58^{b}	16.00^{a}
0	0.3	0.11^{a}	3.01^{b}	1.76^{a}	0.73^{ab}	6.56^{b}	2.11^{a}	12.22^{b}
0	0.5	0.11^{a}	2.99^{b}	1.69^{bc}	0.67^{bc}	6.50^{bc}	2.06^{a}	11.61^{bc}
0	1	0.10^{b}	2.91^{b}	1.66^{cd}	0.63^{c}	6.33^{bc}	1.29^{c}	11.06^{c}

Note: Different letters in each column indicate significant differences (Duncan's test; P ≤0.05).

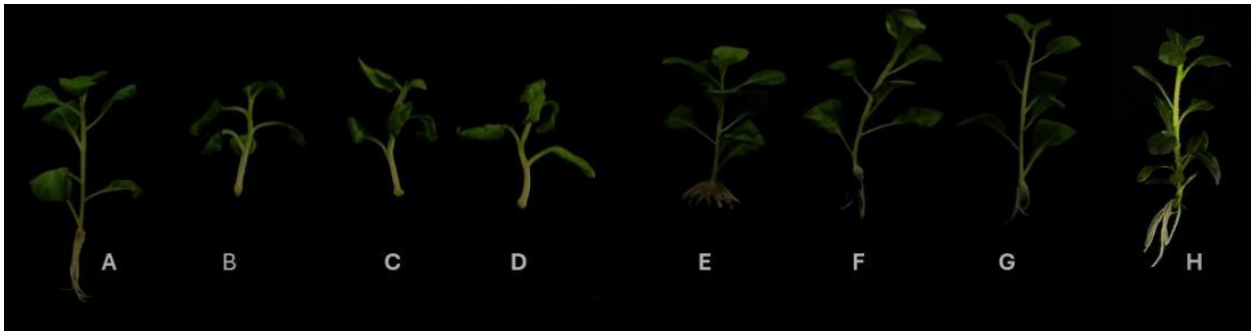

Figure 2. Effects of α-NAA and AC on the growth and root induction of "Pink Diamond"
(A. MS; B. MS + 0.1 mg L^{-1} α-NAA; C. MS + 0.3 mg L^{-1} α-NAA; D. MS + 0.5 mg L^{-1} α-NAA; E. MS + 0.1 g L^{-1} AC; F. MS + 0.3 g L^{-1} AC; G. MS + 0.5 g L^{-1} AC; H. MS + 1,0 g L^{-1} AC)

Table 5. Acclimatization and growth of "Pink Diamond" plantlets in poly-house conditions

Periods	Survival rate (%)	Plant height (cm)	Number of leaves (leaves/plantlet)
One week after transferring	100	3.66 ± 0.25	8.05 ± 0.89
Two weeks after transferring	88.39	5.08 ± 0.37	10.35 ± 1.14
Four weeks after transferring	88.39	8.16 ± 0.35	15.35 ± 1.04

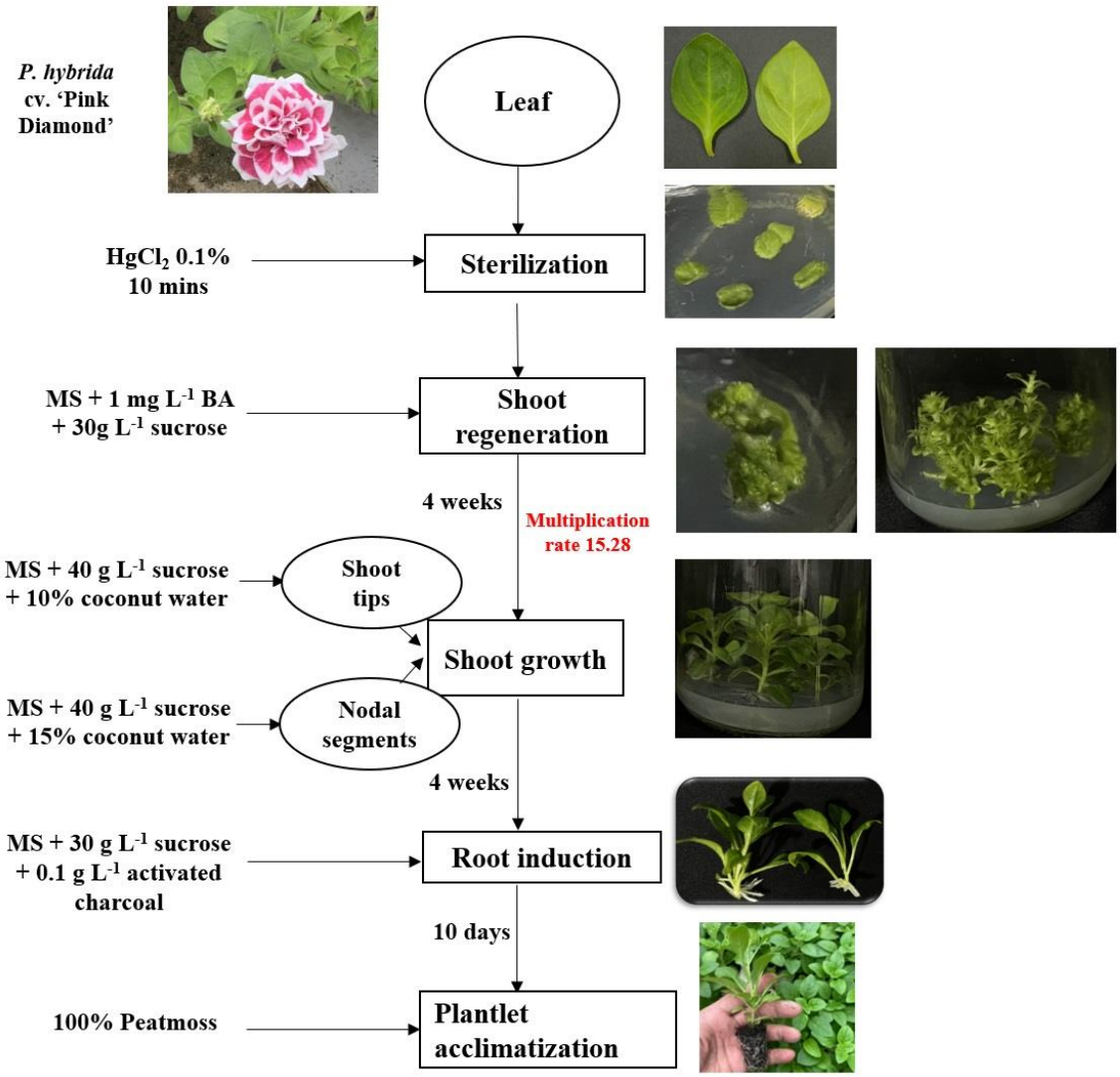

Figure 3. *In vitro* propagation protocol of *Petunia hybrida* cv. 'Pink Diamond'

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