ACADEMIA JOURNAL OF BIOLOGY 2024, 46(4): 47–56
DOI: 10.15625/2615-9023/21150

A STUDY ON REGENERATION OF MEDICINAL PLANT *Polygonatum punctatum* Royle ex Kunth VIA CALLUS INDUCTION FROM LEAVES AND PETIOLES

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Received 16 July 2024; accepted 13 December 2024

ABSTRACT

Polygonatum punctatum Royle ex Kunth is a rare medicinal plant species in Vietnam, with rhizomes used as a tonic for kidney yang deficiency, for treating joint pain, and for pain relief. Overexploitation of natural medicinal plants has driven the need for propagation techniques to meet increasing demand, compensate for habitat degradation, and conserve rare species. In this study, tissue culture techniques were applied to investigate the effects of different growth regulators on the *in vitro* regeneration of *P. punctatum*. The results showed that sterilizing young leaf and petiole samples with 0.1% $HgCl_2$ for 6 minutes achieved optimal effectiveness. The MS medium supplemented with 1.5 mg/L BA and 0.5 mg/L 2,4-D was identified as the most suitable for inducing callus formation from young leaves and petioles. The MS medium supplemented with 2.0 mg/L BA and 0.2 mg/L NAA yielded the best results for shoot regeneration, while the 1/2 MS medium supplemented with 1.0 mg/L NAA was appropriate for root formation. The *in vitro* propagation results of *P. punctatum* contribute to the conservation and sustainable development of this valuable medicinal plant and aim towards the rapid multiplication of healthy plantlets for commercialization.

Keywords: Callus, conservation, *in vitro*, medicinal, *Polygonatum punctatum.*

Citation: Dang Thi Tham, Nguyen Thi Thanh Hang, Tran Thai Vinh, Quach Van Hoi, Vu Kim Cong, Nong Van Duy, 2024. A study on regeneration of medicinal plant *Polygonatum punctatum* Royle ex Kunth via callus induction from leaves and petioles. *Academia Journal of Biology*, 46(4): 47–56. https://doi.org/10.15625/2615-9023/21150

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INTRODUCTION

Polygonatum punctatum Royle ex Kunth, belonging to the Asparagaceae family, is a perennial herbaceous plant that epiphytically grows on woody stems or rocks. *P. punctatum* is distributed in India, Nepal, Bhutan, and China. In Vietnam, it is naturally found in provinces such as Lao Cai, Yen Bai, Kon Tum, and Lam Dong. *P. punctatum* represents a rare genetic resource in Vietnam, with its rhizome being utilized for medicinal purposes such as tonifying kidney yang, treating joint pain, and reducing discomfort. Due to the increasing demand for medicinal herbs and the challenging nature of its cultivation, coupled with habitat loss and alteration due to human activities, *P. punctatum* faces a high risk of extinction in the wild. It has been listed in the Vietnam Red Data Book Part II- Plants and assessed as an endangered species (EN) (Ban et al., 2007). Currently, species within the *Polygonatum* genus are threatened and at high risk of extinction, emphasizing the importance of focused research on propagation for the conservation and sustainable development of rare medicinal resources. The application of plant tissue culture in micropropagation is a method that can generate homogeneous, high-quality seedlings in large quantities within a short period. Previous studies on tissue culture of species in the genus *Polygonatum* have primarily focused on using rhizomes or seeds as the initial explant materials. Specifically, the use of rhizomes as the starting material in *in vitro* propagation studies of *Polygonatum cyrtonema* has demonstrated high rates of callus induction and differentiation (Zhao et al., 2009; Mo et al., 2018; Xi et al., 2022). A tissue culture system for *Polygonatum verticillatum* has also been established using various explant sources, including stem discs, hypocotyl segments, leaves from seedlings germinated from seeds, and rhizome bud segments (Bisht et al., 2012; Tiwari & Chaturvedi, 2018; Tiwari & Chaturvedi, 2024). Similarly, rhizomes have also been utilized in propagation studies of *Polygonatum odoratum* and *Polygonatum stenophyllum* (Kim et al., 2014; Park et al., 2018). Additionally, Hong et al. (2017) and Xuyen et al. (2021) have used rhizomes as the starting material in propagation studies of *Polygonatum kingianum*. Notably, *Polygonatum* species with nutrient-rich rhizomes and shoots typically grow in microbe-dense soil environments, which presents challenges in surface sterilization when using rhizomes as explants (Yang et al., 2024). Seed-based propagation has also been explored; Qadir et al. (2020) applied *in vitro* seed germination in the propagation of *P. verticillatum*. Meanwhile, *Polygonatum macranthum* was propagated *in vitro* from immature seeds with a developed seed coat after 9 weeks of culture on 1/2 MS medium, achieving a germination rate of 30% (Lekamge et al., 2020). Although traditional seed-based propagation is feasible, studies indicate that germination rates of seeds from *Polygonatum* species are often slow (Kim et al., 2014; Tiwari & Chaturvedi, 2024). Additionally, limitations in seed quantity and seedling heterogeneity further restrict large-scale propagation. Recently, a significant breakthrough has been achieved with the successful establishment of an indirect somatic embryogenesis system using immature seeds of *P. cyrtonema* as explants (Yang et al., 2024). Practical studies have shown that leaves and petioles of *Polygonatum* exhibit greater abundance compared to other plant parts. Establishing an *in vitro* regeneration protocol using these materials not only facilitates collection but also ensures minimal impact on the growth of the parent plant. The callus culture technique using leaves and petioles has been widely applied in micropropagation for various plant species (Hien et al., 2014; Khiem et al., 2022; Bansal et al., 2024). However, no reports have yet documented *in vitro* seedling regeneration from *ex vitro* young leaves and petioles of *P. punctatum*. To date, there have been no reports on *in vitro* propagation and regeneration of *P. punctatum* seedlings. Therefore, conducting research on regenerating *P. punctatum* through tissue culture from leaf and petiole samples is essential and meaningful, contributing to conservation efforts and improving propagation efficiency for the large-scale production of Vietnam's rare medicinal species.

MATERIALS AND METHODS

Materials

The initial sample source utilized in this study consisted of juvenile leaves and petioles of *P. punctatum ex vitro*, approximately 2 months old, displaying vigorous growth, and cultivated without pest or disease infestations at the nursery of the Plant Resources Department, Tay Nguyen Institute for Scientific Research.

Methods

Medium and culture condition

The explants were cultured on 1/2 MS or MS medium (Murashige & Skoog, 1962) in glass vessels (V = 250 mL) with 30 mL culture solution, supplemented with plant growth regulators with concentrations depending on the experiment. The medium was adjusted to pH 5.8 and autoclaved at 121 ^{o}C, 1 atm for 25 minutes (Bing et al., 2023).

Condition of culture room: The explants were cultured in a culture room at a temperature of 25 ± 2 ºC, average humidity of 55–60%, under the fluorescent lamp with a photoperiod of 16 h per day and light intensity of 35 µmol/m^2/s (Bing et al., 2023).

The effect of sterilization duration on the viability rate of explants

Juvenile leaves and petioles of *P. punctatum* were collected and washed under running water. Subsequently, they were immersed in a 0.01% soap solution for 15 minutes and then rinsed again under running water for at least 30 minutes. Next, the samples were transferred to a sterile laminar flow hood, initially sterilized with 70^{o} alcohol for 30 seconds, and then rinsed three times with sterile distilled water. They were further surface sterilized with a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution (Xi et al., 2022) with two drops of Tween 20 per 100 ml of solution for 3, 6, 10, 15, or 20 minutes. The samples were then rinsed three times with sterile distilled water to remove all traces of $HgCl_2$ and dried on sterile filter paper discs. After sterilization, two types of explants were tested and cut to size: leaf explants (approximately 0.5 cm in width and 0.5 cm in length) and petiole explants (approximately 1 cm in length). All explants were cultured on MS medium in glass vessels (V = 250 mL) with 30 mL culture solution, supplemented with 30 g/L sucrose and 8 g/L agar. The leaf explants were placed with the abaxial side in contact with the medium. After 15 days of culture, parameters related to the effectiveness of explant sterilization were recorded.

Effect of BA and 2,4-D on callus induction

The leaf and petiole explants that survived and were free from contamination following the most effective sterilization procedure were cultured on MS medium (Tiwari & Chaturvedi, 2018) in glass vessels (V = 250 mL), containing 30 mL of culture solution, supplemented with various concentrations of 6-benzylaminopurine (BA) (0; 0.2; 0.5; 1.0; 1.5; 2.0 mg/L) and 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.2; 0.5; 1.0 mg/L) depending on the experiment. Each experiment consisted of 3 replicates, with each replicate comprising 10 culture vessels, and each vessel containing 3 leaf explants or 3 petiole explants. The callus induction rate was assessed using destructive measurements after 60 days of culture.

Effect of BA and NAA on shoots regeneration from callus

The *in vitro* shoots (approximately 0.5 cm) derived from callus were cultured on basal MS medium (Xi et al., 2022), supplemented with varying concentrations of BA (0; 1.0; 1.5; 2.0 mg/L) and naphthaleneacetic acid (NAA) (0; 0.2; 0.5 mg/L), depending on the experimental conditions. Six treatments were conducted, with each treatment replicated three times. Each treatment consisted of 10 glass vessels (V = 250 mL), each containing 30 mL of culture medium and 3 shoots per vessel. The number of the new shoots (shoots/explant) and shoot length (cm) were taken using destructive measurements after 60 days of culture.

Effect of IBA, IAA and NAA on root regeneration of shoots in vitro

For the root induction experiments, *in vitro* shoots approximately 3 cm in length were

transferred to 1/2 MS medium (Xi et al., 2022), supplemented with varying concentrations of indoleacetic acid (IAA) (0.5, 1.0, 1.5 mg/L), indole-3-butyric acid (IBA) (0.5, 1.0, 1.5 mg/L), and NAA (0.5, 1.0, 1.5 mg/L). Nine treatments were carried out, with each treatment replicated three times. Each treatment included 10 culture vessels (V = 250 mL), each containing 30 mL of culture solution with three shoots per vessel. The effects of the culture media on root formation were evaluated based on root length (cm), number of roots, and rooting percentage after 60 days of culture.

Statistical analysis

The results were analyzed using one-way Analysis of Variance (ANOVA) for each experiment. Significant differences between means were determined using Duncan's Multiple Range Test (DMRT) at a significance level of $p \leq 0.05$, performed in SPSS 25.0 software (Duncan, 1995).

RESULTS AND DISCUSSION

The effect of sterilization duration on the viability rate of explants

After 15 days of culture, the results indicated that the sterilization efficacy of $HgCl_2$ on the cultured samples depended on the sterilization duration (Table 1). The highest contamination rates for young leaf and petiole samples were observed with a 3-minute sterilization duration, at 56.67% and 60%, respectively. Increasing the sterilization time to 6 minutes yielded the best sterilization efficiency, with contamination rates reduced to 23.33% for young leaf samples and 20% for petiole samples, with no sample mortality (Table 1). The contamination rate significantly decreased when the sterilization time was increased to 10 and 15 minutes; however, the mortality rate of the samples also increased significantly in these treatments. When the sterilization time was increased to 20 minutes, microorganisms were completely eliminated (no contamination recorded); however, the samples died or became necrotic. Longer $HgCl_2$ treatment times resulted in higher sterilization efficacy, reducing the contamination rate but increasing the likelihood of tissue necrosis. Zhao et al. (2009) sterilized rhizome samples of *P. cyrtonema* using 0.1% $HgCl_2$ for 8 minutes. Bisht et al. (2012) sterilized young rhizome samples of *P. verticillatum* using 0.1% $HgCl_2$ for 3–6 minutes. Mo et al. (2018) used 0.13% $HgCl_2$ for 4–6 minutes to sterilize seed samples of *P. cyrtonema*. Qadir et al. (2020) sterilized seed samples of *P. verticillatum* using 0.01% $HgCl_2$ for 15 minutes. Thus, the concentration and duration of $HgCl_2$ treatment vary depending on the plant species and type of explant used. In this study, sterilizing young leaf and petiole samples of *P. punctatum* with 0.1% $HgCl_2$ for 6 minutes yielded the best results.

Table 1. Effect of sterilization duration on the viability rate of explants

Sterilization duration min	Young leaf		Petiole	
	Infection rate (%)	Death rate (%)	Infection rate (%)	Death rate (%)
3	56.67[a]	0	60.00[a]	0
6	23.33[b]	0	20.00[b]	0
10	16.67[b]	23.33[c]	16.67[b]	26.67[b]
15	3.33[c]	43.33[b]	6.67[c]	33.33[b]
20	0	66.67[a]	0	63.33[a]
ANOVA	*	*	*	*

Note: *: Significant at $p \leq 0.05$.

Effect of BA and 2,4-D on callus induction

The results in Table 2 indicate that, after 60 days, all explants cultured on media without BA and 2,4-D underwent oxidation and showed no growth. Minimal callus formation was observed at a concentration of 0.2 mg/L BA, possibly due to insufficient BA in the

medium to initiate callus formation. Increasing BA concentration from 0.5 to 1.5 mg/L resulted in higher rates of callus formation. When 1.5 mg/L BA was added individually to the culture medium, a significantly high induction rate of callus was achieved (63.33% for young leaf explants; 60% for petiole explants). There was a notable difference in callus induction between MS medium supplemented with BA alone (13.33–63.33% for young leaf explants; 6.67–60% for petiole explants) and BA + 2,4-D (70–83.33% for young leaf explants; 60–73.33% for petiole explants). The combination of BA + 2,4-D induced more callus compared to BA alone. The highest callus induction rate was observed when 1.5 mg/L BA was combined with 0.5 mg/L 2,4-D in the culture medium, reaching 83.33% for young leaf explants and 73.33% for petiole explants (Figs. 1b, c). Increasing the concentration of 2,4-D to 1.0 mg/L, combined with 1.5 mg/L BA, decreased the frequency of callus formation to 76.67% for young leaf explants and 63.33% for petiole explants. Particularly, these explants were pale yellow, soft, and unsuitable for shoot development. Auxin stimulates cell division in plant tissues by limiting organ formation and plays a crucial role in callus induction, while cytokinin supports callus by promoting cell division and cell expansion (Hu et al., 2019). Previous studies have demonstrated that 2,4-D and BA promote callus development and growth. Yan et al. (2009) used a combination of 2,4-D with BA to induce callus from basal explants of *Allium chinense*. Luciani et al. (2006) reported that the combination of 2,4-D and BA yielded the best callus formation in various garlic explants. Nasrin et al. (2017) indicated that supplementation with BA together with 2,4-D was optimal for callus induction in *Allium hirtifolium*. Additionally, Zhao et al. (2009) studied callus induction from rhizome explants of *P. cyrtonema*, achieving a high callus formation rate (72.5%) on MS medium supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L BA. Park et al. (2018) reported that MS medium supplemented with 0.5 mg/L 2,4-D resulted in 87% callus formation in 3-year-old bulbs of *P. stenophyllum*. Yang et al. (2024) reported that when using immature embryos of the species *P. cyrtonema* as explants, the MS medium supplemented with 1 mg/L BA and 0.5 mg/L 2,4-D exhibited the best callus formation capability. To date, no studies have been published on the addition of BA and 2,4-D to culture media for callus induction from leaf and petiole explants in species of the genus *Polygonatum*. This could represent a new direction in *in vitro* propagation research for these valuable medicinal plant species.

Table 2. Effects of BA and 2,4-D on callus induction

BA (mg/L)	2,4-D (mg/L)	Leaf explant	Petiole explant
		Callus induction rate (%)	Callus induction rate(%)
0	0	0	0
0.2	0	13.33^{d}	6.67^{e}
0.5	0	30.00^{c}	23.33^{d}
1.0	0	43.33^{c}	36.67^{c}
1.5	0	63.33^{b}	60.00^{b}
2.0	0	33.67^{c}	43.33^{c}
1.5	0.2	70.00^{ab}	60.00^{b}
1.5	0.5	83.33^{a}	73.33^{a}
1.5	1.0	76.67^{ab}	63.33^{b}
ANOVA		*	*

Note: *: Significant at $p \leq 0.05$.

Effect of BA and NAA on the *in vitro* shoots regeneration

The shoot regeneration process from the callus of *P. punctatum* under the influence of

different concentrations of BA combined with NAA after 60 days of culture is reflected through the indicators in Table 3.

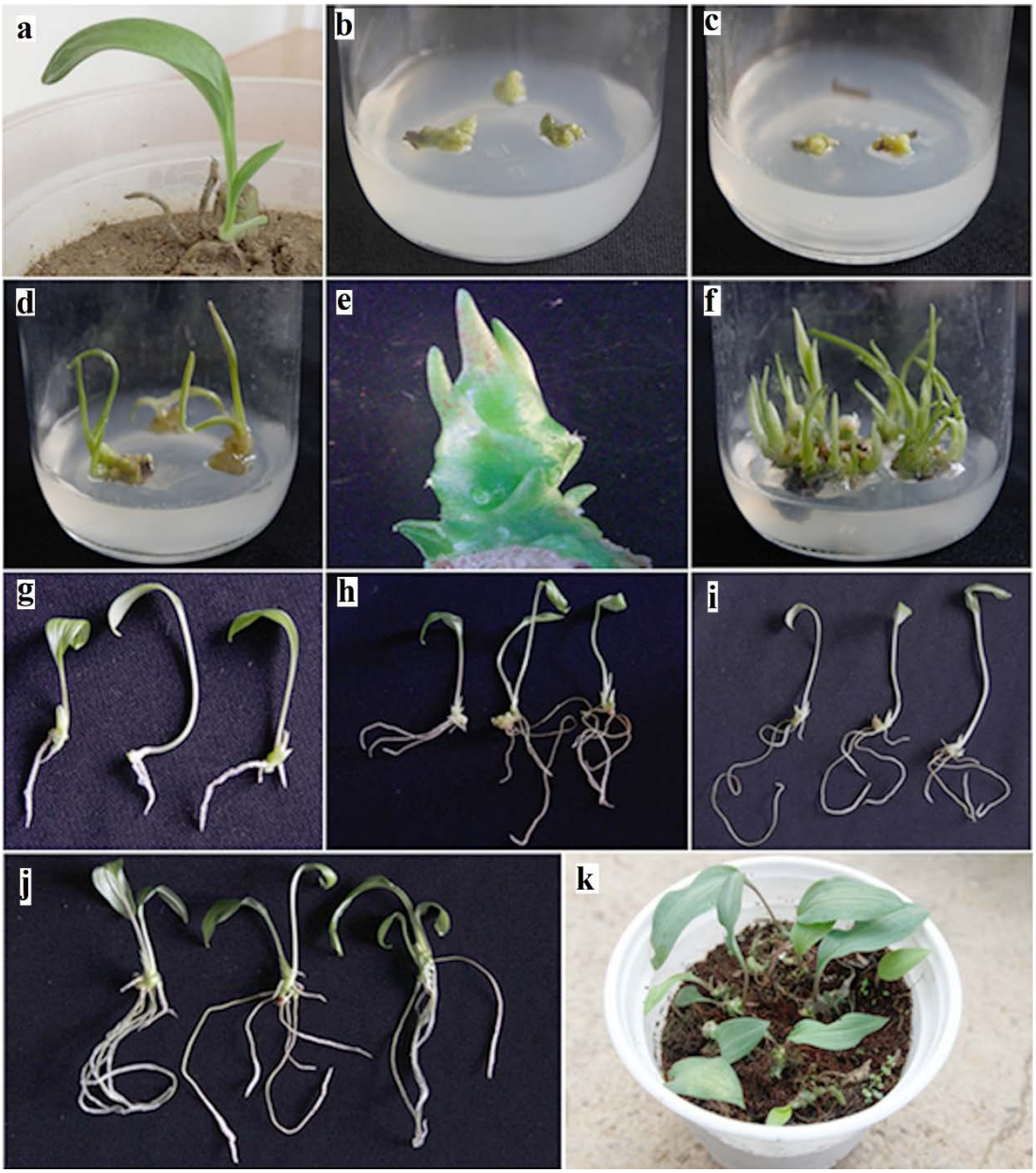

Figure 1. In vitro growth of *Polygonatum punctatum* (a) The *ex vitro* seedlings have grown from rhizomes approximately 2 months old; (b) Callus formed from young leaves in the medium MS + 1.5 mg/L BA + 0.5 mg/L 2,4-D; (c) Callus formed from petioles in the medium MS + 1.5 mg/L BA + 0.5 mg/L 2,4-D; (d) Control treatment in the experiment for shoot regeneration; (e, f) shoot regeneration from callus in the medium MS + 2 mg/L BA + 0.2 mg/L NAA; (g) Root formation in the control treatment; (h) Root formation in the medium 1/2 MS + 1 mg/L IBA; (i) Root formation in the medium ½MS + 1 mg/L IAA; (J) Root formation in the medium 1/2 MS + 1 mg/L NAA; (k) Seedlings were transplanted to the garden after 3 months

Table 3. Effect of BA and NAA on the *in vitro* shoots regeneration

BA (mg/L)	NAA (mg/L)	No. of shoots/explant	Shoots length (cm)
0	0	1.33^{f}	2.41^{d}
1.0	0.2	6.27^{d}	2.62^{c}
1.5	0.2	8.93^{b}	2.81^{b}
2.0	0.2	11.70^{a}	3.05^{a}
1.0	0.5	7.60^{c}	2.78^{bc}
1.5	0.5	5.10^{e}	2.72^{bc}
2.0	0.5	4.50^{e}	2.83^{b}
ANOVA		*	*

Note: *: Significant at $p \leq 0.05$.

Statistical analysis of the results indicates that the culture medium has a significant impact on both the number and length of shoots ($p = 0.05$). According to Table 3, after 60 days of culture, the medium supplemented with BA combined with NAA showed better shoot formation and growth compared to the control medium (Fig. 1d). The presence of appropriate concentrations of NAA and BA in the culture medium stimulated the callus to differentiate into embryos and develop into shoots after 30 days of subculture (Fig. 1e). The MS medium supplemented with 2.0 mg/L BA combined with 0.2 mg/L NAA was identified as optimal for shoot regeneration (Fig. 1f). The positive effect of the combination of cytokinin and auxin is explained through the enhanced RNA synthesis. This combination strongly stimulates the RNA synthesis process, which is associated with the emergence of the initial protoplasm, thereby facilitating shoot bud formation. The successful combination of BA and NAA in promoting shoot multiplication in plants of the genus *Polygonatum* has been reported in numerous studies (Bisht et al., 2012; Mo et al., 2018). Increasing the auxin concentration to 0.5 mg/L NAA inhibits shoot bud differentiation, possibly due to the endogenous auxin levels being sufficient to interact with the added cytokinin to achieve the necessary balance for shoot bud induction. The MS medium supplemented with 2.0 mg/L BA combined with 0.2 mg/L NAA recorded the highest number of shoots (11.7 shoots/explant) with an average length of 3.05 cm (Table 3, Fig. 1f). Similar results for shoot regeneration were reported by Mo et al. (2018) in *P. cyrtonema*. According to the study by Bisht et al. (2012), *P. verticillatum* cultured on MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L NAA achieved the highest number of shoots (8.6 shoots/explant), while our experiment achieved up to 11.7 shoots/explant.

Effects of auxins on the *in vitro* root formation

In general, most studies related to the *in vitro* rooting of *Polygonatum* shoots use 1/2 MS medium supplemented with IBA, IAA, and NAA (Zhao et al., 2009; Bisht et al., 2012; Park et al., 2018; Mo et al., 2018; Qadir et al., 2020). The results in Table 4 show that roots still appear in the medium without growth regulators (Fig. 1g). This indicates that endogenous auxin is formed in the shoots and moves downward to induce root formation, consistent with the findings of Park et al. (2018). However, when growth regulators are added to the culture medium, the rooting time is shortened, and the rooting rate increases. In treatments supplemented with IBA (0.5; 1.0; 1.5 mg/L), the rooting rates were 53.33%; 73.33%; 63.33%, respectively. Among them, the addition of 1.0 mg/L IBA resulted in the highest number of roots, reaching 3.53 roots/shoot, with a root length of 3.37 cm and 73.33% of shoots rooting; however, the roots were thin and weak (Fig. 1h). Treatments supplemented with IAA had lower rooting rates, number of roots, and root lengths compared to IBA and NAA at the same concentrations, indicating that IAA is not suitable for the *in vitro* rooting of *P. punctatum* (Fig. 1i). Similar results were obtained by Bisht

et al. (2012) for *P. verticillatum*. Treatments supplemented with NAA showed better root regeneration compared to those supplemented with IBA. The culture medium supplemented with 1.0 mg/L NAA yielded the best results in terms of growth parameters (100% shoot rooting, 4.73 roots/shoot, root length 2.8 cm), with strong and uniformly developed roots compared to other treatments (Figs. 1g–j).

Increasing the NAA concentration to 1.5 mg/L inhibited root formation, reducing the number of roots. High concentrations of auxin induce cell division in plant tissues to form callus, thereby inhibiting root formation (Vinh et al., 2021). Similar results for root regeneration in 1/2 MS medium supplemented with 1.0 mg/L NAA for *P. verticillatum* shoots *in vitro* were reported by Bisht et al. (2012). Mo et al. (2018) reported that the best root formation and development in *P. cyrtonema* occurred in ½MS medium supplemented with 1.0 mg/L IBA combined with 0.1 mg/L NAA. Zhao et al. (2009) reported that the roots of *P. cyrtonema* easily formed in 1/2 MS medium supplemented with 0.5 mg/L NAA. The concentration of NAA used in *in vitro* propagation varies among species; some species are suitable for low concentrations while others are suitable for high concentrations. Thus, 1/2 MS medium supplemented with 1.0 mg/L NAA is suitable for the *in vitro* root regeneration of *P. punctatum* shoots. The survival rate of transplanted seedlings was 100% three months after planting in the nursery (Fig. 1k).

Table 4. Effects of auxins on the *in vitro* root formation

Auxins	Concentration (mg/L)	No. of roots/shoot	Root length (cm)	Rooting percentage (%)
0	0	0.6^{f}	0.97^{f}	33.33
IBA	0.5	2.33^{d}	2.33^{d}	53.33
	1.0	3.53^{b}	3.37^{b}	73.33
	1.5	3.07^{c}	3.69^{a}	63.33
IAA	0.5	1.60^{e}	2.21^{d}	43.33
	1.0	2.83^{c}	2.80^{c}	56.67
	1.5	2.93^{c}	2.91^{c}	53.33
NAA	0.5	3.73^{b}	2.95^{c}	96.67
	1.0	4.73^{a}	2.80^{c}	100.00
	1.5	2.87^{c}	1.65^{e}	76.67
ANOVA		*	*	*

Note: *: Significant at $p \leq 0.05$.

CONCLUSION

In the *in vitro* propagation study of *P. punctatum*, sterilizing young leaf and petiole samples with 0.1% $HgCl_2$ for 6 minutes proved to be the most effective method. The MS medium supplemented with 1.5 mg/L BA combined with 0.5 mg/L 2,4-D was identified as the most suitable for inducing callus from young leaves and petioles. The MS medium supplemented with 2.0 mg/L BA and 0.2 mg/L NAA showed the best results in shoot regeneration; meanwhile, 1/2 MS medium supplemented with 1.0 mg/L NAA was suitable for the root formation stage.

Acknowledgements: The authors would like to thank the Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology for annual scientific research funding.

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