

Micropropagation of *Piper hancei*, A *Phytophthora*, and nematode-resistant *Piper* species

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ABSTRACT

Piper hancei, a wild species in the genus *Piper*, has been widely used in Chinese herbal medicinal prescriptions for the treatment of rheumatism, asthma, and arthritic conditions. It is resistant to pathogenic species of *Phytophthora* and nematodes. This study developed a rapid method for micropropagation of this species, aimed at mass-producing plantlets to serve as rootstocks in the production of disease-resistant pepper. The most effective sterilization method for the stem segment was shaking with a 2% Ridomil Gold (Syngenta) fungicide solution for 30 minutes, followed by a 60 minutes treatment of 2% Antisuper 80wp (Greenfarm) bactericide solution, and then with 10% NaClO for 10 minutes, achieving infection-free survival rate of 37.78%. The optimal medium for shoot regeneration from stem segments was Murashige and Skoog (MS) medium supplemented with 1.0 mg/l 6-Benzylaminopurine (BAP), yielding 1.83 shoots/sample. The medium for *in vitro* shoot multiplication was basal MS supplemented with 1.0 mg/l BAP and 2.0 mg/l kinetin, producing 4.87 shoots/cluster, with an average shoot height of 1.9 cm. Root formation was most suitable in MS medium supplemented with 1.5 mg/l indole butyric acid, yielding 7.43 roots/shoot, with a root length of 1.97 cm and a shoot height of 3.41 cm. *Piper hancei* plantlets were successfully transplanted to the nursery with a survival rate of 80.55% after 6 weeks, averaging 4.49 leaves and 5.64 cm in height.

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), commonly referred to as the "king" of spices, is extensively used worldwide [1]. Currently, approximately 70 countries cultivate black pepper, with India maintaining the largest cultivation area of 195,000 hectares across its territory, followed by Indonesia with 116,000 hectares, and Brazil with 45,000 hectares. In Vietnam, black pepper cultivation began in the 17th century in regions such as Phu Quoc district, Kien Giang province. By 2022, the total black pepper cultivation area in Vietnam will reach 119,600 hectares, with the Central Highlands region accounting for 60% (71,760 hectares), making it the largest black pepper production region in the country [2]. However, the annual yield of black pepper is significantly impacted by various diseases, particularly root rot caused by *Phytophthora capsici*, a pathogenic oomycete [3,4]. The widespread presence of this disease in many regions has made black pepper cultivation increasingly challenging [3–6]. Chemical plant protection measures have achieved only partial success, there is a growing demand for spices free from chemical residues. Currently, all available commercial pepper varieties are susceptible to this disease, and continuous efforts over the past 2 decades have only identified a few resistant to *P. capsici* [5,7,8].

One of the strategies to enhance black pepper resistance against foot rot disease is by grafting, for instance, using *Piper colubrinum* as a disease-resistant rootstock [9].

Currently, the rootstock source of disease resistance is wild pepper species, such as *P. colubrinum* [9], *P. hancei* [10], or *P. flaviflorum* [11]. Moreover, black pepper is a perennial plant, and conventional breeding programs face numerous difficulties due to its slow growth rate. Therefore, the development of new alternative strategies is essential to identify and produce disease-resistant pepper varieties that meet market demands without chemical residues. In previous reports, we successfully selected *Piper hancei* varieties resistant to diseases caused by *Phytophthora capsici* and *Meloidogyne incognita* [10,12]. *Piper hancei* belongs to the genus *Piper* of the family *Piperaceae* and is mainly distributed in southwest China. Its aerial parts have been widely used in Chinese herbal medicinal prescriptions for the treatment of rheumatism, asthma, and arthritic conditions [13–15]. *P. hancei* growth habit was climbing and had a polymorphic branching type with the production of many runners. *P. hancei* produced multiple runner shoots and lots of adventitious roots. Pubescence on the stem was absent. Leaf lamina shapes were ovate-elliptic with acrodromous venation. The flower spike was erect type. The spike shape was cylindrical. *P. hancei* produced only pistillate flowers. The fruit shape was not round [12].

Biotechnological tools like tissue culture are important for the selection, multiplication, and conservation of medicinal plant genotypes

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[16,17]. *In vitro* regeneration plays a great role in the production of high-quality and disease-free plantlets for crop programs [18–20]. However, the study on *in vitro* propagation of *Piper hancei* was not well-studied. This study describes the *in vitro* propagation process of *Piper hancei*, aimed at mass-producing plantlets to serve as rootstocks in the production of disease-resistant pepper.

2. MATERIALS AND METHODS

2.1. Media and Cultural Conditions

The *Piper hancei* variety, known for its resistance to *Phytophthora capsici* and *Meloidogyne incognita*, have been cultivated at the Institute of Biotechnology, Hue University. The plants, around 2.5 m tall and over 2 years old, robust and disease-free, were selected as initial materials. Healthy stem segments with large leaves, containing 6–7 nodes from the entire plant, were used for sterilizing in sample inoculation. The culture medium used was MS basal medium [21] containing 3.0% sucrose and 0.8% agar, supplemented with different concentrations of plant growth regulators (PGRs). The pH of the medium was adjusted to 5.8. The medium was autoclaved at 121°C, 1 atm for 15 minutes. Experiments were conducted under conditions of 25°C, light intensity of 2,000–3,000 lux, and a photoperiod of 10–12 hours/day [18].

2.2. Explant Sterilization

Explants from the second to fifth nodes counting from the apex of pepper vines cut into 3 cm segments for sterilization. These segments were initially shaken in 1% dilution of Lifebouy soap for 15 minutes. Next, samples were treated in a 2% solution of Ridomil Gold fungicide (Syngenta) for 30 minutes, followed by immersion in the bactericide Antisuper 80wp (2%, Greenfarm) for 60 minutes. After each treatment, the explants were thoroughly rinsed with sterile distilled water [18]. The sterilized segments were transferred to autoclaved Duran bottles within a laminar flow hood. The explants were further treated by shaking in 70% ethanol for 60 seconds, followed by rinsing in sterile distilled water. Additional sterilization with 10% NaClO for 6–12 minutes, followed by rinses in sterile distilled water (5–6 times) [22]. Finally, the explants were cut into 2 cm lengths containing dormant buds and cultured on MS medium supplemented with 3.0% sucrose, 0.8% agar, and 0.5 mg/l [6-Benzylaminopurine (BAP)] [18]. The efficacy of sterilization after 3 weeks of culture was evaluated through the proportions of explants that were non-contaminated, contaminated, and dead.

2.3. Effects of PGRs on *In Vitro* Shoot Regeneration

In vitro stem explants were cultured on a basal MS medium containing 0.8% agar and 3% sucrose, supplemented with either BAP or kinetin (KIN) (1–2 mg/l) [23], or a combination of BAP (1 mg/l) with 0.1–0.5 mg/l indole butyric acid (IBA) to evaluate the shoot regeneration ability [24]. After four weeks of culture, the following parameters were recorded: percentage of explants forming shoots, shoot numbers per explant, and average shoot height (cm).

2.4. Effects of PGRs on *In Vitro* Shoot Multiplication

To evaluate rapid shoot multiplication, *in vitro* shoots were cultured on a basal MS medium containing 0.8% agar and 3% sucrose, supplemented with BAP at concentrations of 1.0–1.5 mg/l in combination with KIN at concentrations of 0.5–2.5 mg/l [23] and IBA at 0.5 mg/l [25]. After eight weeks of culture, the following parameters were recorded: number of shoots per explant and average shoot height (cm).

2.5. *In vitro* Root Formation

To investigate the root formation and growth potential of *in vitro* shoots, shoots with an average height of 2 cm, 2–4 leaves, and robust growth were individually separated and cultured on MS basal medium containing 0.8% agar and 3% sucrose, supplemented with IBA (0–2.0 mg/l) [23]. The results were evaluated after a 4-week culture period based on several criteria: percentage of explants forming roots, root number per shoot, root length (cm), and average height of shoots (cm).

2.6. Transplantation

The nursery, covering an area of 200 m², was equipped with a 50% shade net and utilizes natural light and temperature. *In vitro* healthy plantlets (3–5 leaves, 3–4 cm in height, and 6–11 roots) were selected for transplantation into the nursery. The *in vitro* culture bags containing the plants were acclimatized to external conditions over a period of 3–5 days. Subsequently, the bags were opened and left undisturbed for 1–2 days to allow the plants to adjust to the natural environment [26]. Post acclimatization, the *in vitro* plants were removed from their culture medium. The roots were thoroughly washed to remove any adhering medium, and then soaked in a 1% Ridomil Gold (Syngenta) solution for 5 minutes. The treated plants were then transplanted into seedling trays filled with a mixture of fertilizer, sand, and soil in a ratio of 1:1:1. To facilitate acclimatization, the plants were covered with transparent polyethylene bags for 2 weeks. The polyethylene covers were gradually removed over a period of 2 weeks. The plants were then transferred to a greenhouse for four weeks as described by Hussain *et al.* [26]. After 2–6 weeks of transplantation, the growth of the *in vitro* plantlets was evaluated by measuring survival rate (%), leaf number/plantlet, and plant height (from the soil surface to the shoot tip). The experiments were conducted in a nursery with ambient temperatures ranging from 28°C–32°C.

2.7. Statistical Analysis

Each experiment was repeated 3 times, and 30 samples were observed in each condition. The data were analyzed in SPSS 19.0 software, using Duncan's test ($p \leq 0.05$).

3. RESULTS

3.1. Effect of Sterilization Time

To enhance the sterilization efficiency of the explants, we conducted preliminary sterilization using commercially available fungicides and bactericides, followed by treatment with 10% NaClO. The sterilization results after three weeks of culture indicated a significant influence of the sterilization time with 10% NaClO on the survival rate, infected rate, and death rate of the explants. When the explants were sterilized for 6 minutes, the number of survival rate was the lowest for uninfected explants, at 4.45%, while the infected rate was very high, at 91.1%. Increasing the sterilization duration from 6 minutes to 8–10 minutes resulted in an increase in the number of surviving samples from 4.45% to 37.78%, and a significant reduction in the infected rate from 91.1% to 51.11%. However, extended sterilize/exposure times to 12 minutes did not significantly change the infected rate, but led to a considerable increase in the death rate (11.11%–24.45%) and reduced the survival rate (Table 1). Hence, the 10% NaClO treatment in 12 minutes duration has been found to be excessively long and caused significant damage to the stem nodes while failing to completely eliminate all microbial sources present in samples. In summary, treating samples with 10% NaClO for 10 minutes yielded the best sterilization results, achieving 37.78% of infection-free survival rates.

3.2. Effect of PGRs on Shoot Regeneration

Nodal segments were initially sterilized before being cultured on MS basal medium containing 3.0% sucrose and 0.8% agar and supplemented with different concentrations of BAP or KIN or both combined to assess the shoot regeneration capacity. After 2 weeks of cultivation, reproductive buds were formed on the nodes in certain culture media. The regeneration rates of shoots varied across different MS media by the fourth week. The MS medium without PGRs supplementation resulted in the lowest regeneration rate at 70%, while all PGR-supplemented media achieved 100% shoot regeneration (Table 2; Fig. 1).

The results illustrate that the MS medium supplemented with 1 mg/l BAP yielded the highest shoot regeneration efficiency, producing robust shoots with a light green color and an average of 1.83 shoots per explant. However, increasing the BAP concentration to 2 mg/l resulted in a decrease in shoot regeneration efficiency, with an average of 1.2 shoots per explant, and the growth of these shoots was slower. In media supplemented solely with KIN and those supplemented with a combination of BAP and IBA, the average number of shoots obtained was nearly equivalent (1.03–1.27 shoots/explant).

3.3. The Effects of PGRs on Shoot Multiplication

BAP stimulates *in vitro* shoot formation, while KIN prolongs shoot and promotes leaf development. Therefore, we combined these 2 regulators, either alone or in combination with IBA, to investigate the potential for shoot multiplication, aiming to find the optimal conditions for high-quality shoot production. The results were evaluated after 8 weeks of culture.

In comparison to natural shoot regeneration from node explants, *in vitro* shoot multiplied relatively slowly. Thus, shoot multiplication efficiency was calculated after 8 weeks of cultivation. The data revealed that the number of shoots increased with a medium supplemented with 1 mg/l BAP and increasing KIN concentrations from 0.5 to 2 mg/l, but

declined when KIN increased to 2.5 mg/l. In a medium with 1.5 mg/l BAP and KIN ranging from 0.5 to 1.5 mg/l, the shoot number increased, then decreased when KIN was further increased to 2–2.5 mg/l. Shoots formed in BAP and KIN-supplemented media displayed vigorous growth, with large size and dark green foliage (Table 3; Fig. 2).

Among all experimental media, MS medium supplemented with 1 mg/l BAP and 2 mg/l KIN, and MS medium supplemented with 1.5 mg/l BAP and 1.0 mg/l KIN, yielded the highest and comparable shoot numbers, at 4.87 and 4.77 shoots/explant, respectively. However, shoots grown in MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l KIN exhibited stronger growth and larger size than those in the other medium. Therefore, MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l KIN was selected as the optimal medium for shoot multiplication process.

3.4. Root Formation

In this study, the effect of IBA for *in vitro* rooting was investigated, and the results after 4 weeks of cultivation were summarized (Table 4). Shoots cultured on IBA-free MS medium showed no rooting ability, with slow growth, underdeveloped leaves with yellowing, and an average shoot height of 2.36 cm.

On the MS media supplemented with 0.5–2 mg/l IBA, all shoots achieved a rooting percentage of 100%. However, the rooting intensity and shoot development varied with different IBA concentrations. The medium containing 0.5 mg/l IBA yielded the lowest average number of roots per shoot (3.13) with a root length of 1.14 cm and no root hairs. Despite slow growth and pale green leaves, the presence of a developed root system enhanced better growth compared to non-IBA supplemented media, with an average shoot height of 2.8 cm. Increasing the IBA concentration to 1.0 mg/l resulted in an increase in root formation with

Table 1. Effect of 10% NaClO sterilization duration on living sample viability.

Time (minutes)	Infection-free survival rates (%)	Infected rate (%)	Death rate (%)
6	4.45 ^c	91.10 ^a	4.45 ^b
8	11.11 ^c	77.78 ^a	11.11 ^b
10	37.78 ^a	51.11 ^b	11.11 ^b
12	20.00 ^b	55.55 ^b	24.45 ^a

Different letters (a,b,c) in the same column indicate statistically significant differences at $p \leq 0.05$ (Duncan's test).

Table 2. Effect of PGRs on shoot regeneration after 4 weeks.

PGRs			Regeneration rate (%)	Number of shoots (Shoots/explant)	Shoot height (cm)
BAP	KIN	IBA			
-	-	-	70.00	0.70 ^d	0.65 ^c
1	-	-	100	1.83 ^a	1.79 ^{ab}
2	-	-	100	1.20 ^b	1.33 ^c
-	1	-	100	1.03 ^b	1.52 ^b
-	2	-	100	1.13 ^{bc}	1.85 ^a
1	-	0.1	100	1.27 ^b	1.37 ^c
1	-	0.5	100	1.10 ^{bc}	1.35 ^c

Different letters (a,b,c) in the same column indicate statistically significant differences at $p \leq 0.05$ (Duncan's test).



Figure 1. Regenerated shoots from stem node explants on MS medium supplemented with 1.0 mg/l BAP after 4 weeks.

Table 3. Effect of PGRs on shoot multiplication after 8 weeks.

Medium	PGRs (mg/l)			Number of shoots/explant	Shoot height (cm)
	BAP	KIN	IBA		
N1	1	0.5	-	2.83 ^{efg}	1.60 ^{cd}
N2	1	1	-	3.40 ^{cd}	1.71 ^{bc}
N3	1	1.5	-	4.27 ^b	1.56 ^{cde}
N4	1	2	-	4.87 ^a	1.90 ^{ab}
N5	1	2.5	-	2.77 ^{fg}	1.43 ^{def}
N6	1.5	0.5	-	3.27 ^{cde}	1.53 ^{cde}
N7	1.5	1	-	4.77 ^a	1.52 ^{cde}
N8	1.5	1.5	-	3.70 ^c	1.98 ^a
N9	1.5	2	-	2.97 ^{def}	1.73 ^{bc}
N10	1.5	2.5	-	2.47 ^{gh}	1.40 ^{def}
N11	1	0.5	0.5	3.03 ^{def}	1.36 ^{ef}
N12	1	1	0.5	4.30 ^b	1.54 ^{cde}
N13	1	1.5	0.5	3.57 ^c	1.71 ^{bc}
N14	1	2.0	0.5	3.43 ^{cd}	1.46 ^{def}
N15	1	2.5	0.5	2.13 ^h	1.29 ^f

Different letters (a–f) in the same column indicate statistically significant differences at $p \leq 0.05$ (Duncan's test).

an average of 4.57 roots/shoot and a root length of 1.42 cm, without root hairs. Shoots exhibited slow growth, reaching an average height of 2.87 cm, with larger green leaves. Further increases in IBA to 1.5–2.0 mg/l significantly enhanced the rooting ability, the highest root numbers ranged from 7.03 to 7.43 roots/shoot (non-significant difference between both media), characterized by abundant root hairs. However, shoots on 1.5 mg/l IBA exhibited better growth and root development compared to those supplemented with 2.0 mg/l IBA. In 1.5 mg/l IBA-supplemented medium, roots formed early and rapidly, averaging 1.97 cm in length and 3.41 cm in height, whereas shoot formation on 2.0 mg/l IBA was slower, with an average root length of 1.35 cm and shoot height of 3.09 cm (Fig. 3). IBA concentrations beyond 1.5 mg/l may inhibit shoot and

Table 4. Effect of PGRs on rooting ability of *in vitro* pepper shoots.

IBA (mg/l)	Rooting rate (%)	Number of root (Roots/shoot)	Root length (cm)	Shoot height (cm)
0	0	0	0 ^d	2.36 ^d
0.5	100	3.13 ^c	1.14 ^c	2.80 ^c
1.0	100	4.57 ^b	1.42 ^b	2.87 ^c
1.5	100	7.43 ^a	1.97 ^a	3.41 ^a
2.0	100	7.03 ^a	1.35 ^b	3.09 ^b

Different letters (a–d) in the same column indicate statistically significant differences at $p \leq 0.05$ (Duncan's test).

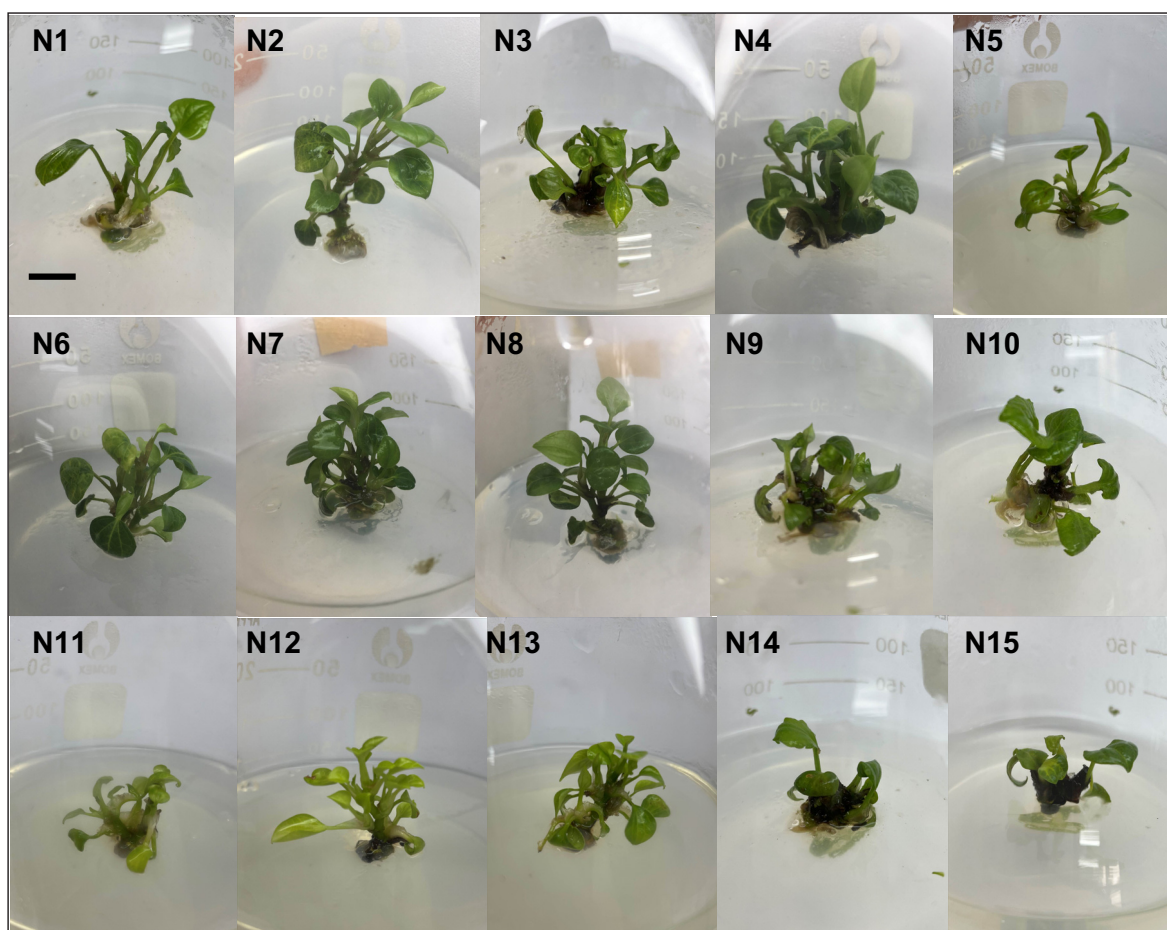


Figure 2. Formation of shoot clusters on different media after 8 weeks (bar = 1 cm). Among all experimental media, MS medium supplemented with 1 mg/l BAP and 2 mg/l KIN, and MS medium supplemented with 1.5 mg/l BAP and 1.0 mg/l KIN, yielded the highest and comparable shoot number, at 4.87 and 4.77 shoots/explant, respectively. However, shoots grown in MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l KIN exhibited stronger growth and larger size than those in the other medium. Therefore, MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l KIN was selected as the optimal medium for shoot multiplication process.

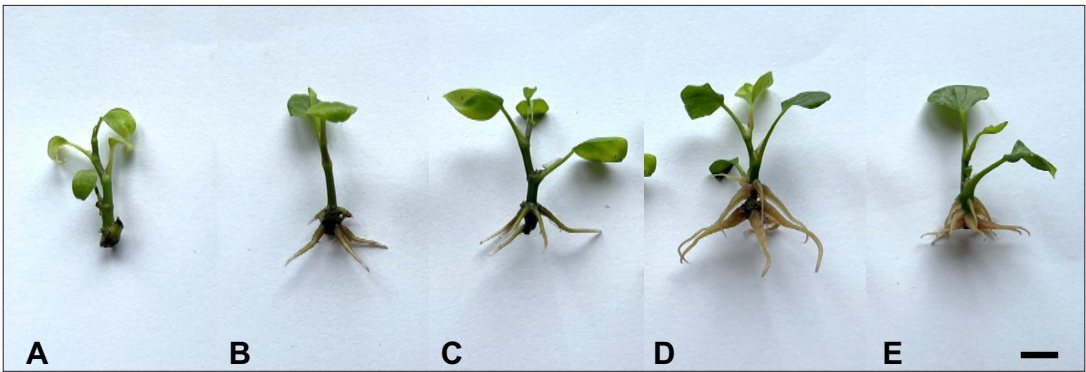


Figure 3. *In vitro* shoots after 4 weeks of culture on MS medium supplemented with different concentrations of IBA. (A) 0 mg/l IBA; (B) 0.5 mg/l IBA; (C) 1.0 mg/l IBA; (D) 1.5 mg/l IBA; (E) 2.0 mg/l IBA. (bar = 1 cm).



Figure 4. The growth of *in vitro* plantlets after 2–6 weeks in nursery.

root formation, so, the higher IBA concentrations were not investigated. The optimal medium for rooting *in vitro* pepper shoots was found to be MS medium supplemented with 0.8% agar, 3% sucrose, and 1.5 mg/l IBA.

3.5. Acclimatization and Growth of *In Vitro* Plantlets in Nursery

In vitro plantlets were transplanted to nursery beds containing a mixture of soil, sand, and organic fertilizer in a ratio of 1:1:1. Due to the high susceptibility of *in vitro* plants to dehydration and mortality upon transfer, transparent plastic sheets were used to cover the trays during the initial 2 weeks, ensuring adequate ventilation while minimizing water loss. In the last 3–5 days of the second week, the plastic coverings were gradually removed to help the plants acclimate to natural growing conditions. Assessment of plant survival and growth parameters including survival rate, number of leaves/plantlet, and plant height were conducted after 2 and 4 weeks of transplantation (Table 5).

In the first 2 weeks, the plantlets exhibited minimal growth, primarily adapting to external conditions and developing a root system suitable to the new substrate. The survival rate during this period was 86.11%. Some leaves showed slight yellowing and underdevelopment, with an average of 3.39 leaves/plantlet and a plant height of 3.15 cm. These plantlets were exposed to natural environmental conditions after 2 weeks. Beginning from 2 to 4 weeks, the plantlets adapted well to the external environment, although some plants did not survive, reducing the survival rate to 80.55% after 4 weeks. Survived plantlets displayed

Table 5. The growth of *in vitro* plantlets in a nursery.

Time of growth (weeks)	Survival rate (%)	Number of leaves (leaves/plant)	Plant height (cm)
2	86.11	3.39	3.15
4	80.55	4.31	3.99
6	80.55	4.49	5.64

vigorous growth, with shoot height increasing from 3.15 to 3.99 cm. Most plantlets produced new leaves, with older leaves turning a dark green and robust, while new leaves were light green; some older leaves yellowed and fell off, at around 4.31 leaves/plantlet. By week 6, the plantlets had fully adapted to the external conditions, and no further plant losses occurred between weeks 4 and 6. The plants grew strong, with large robust leaves and an average height of 5.64 cm and 4.49 leaves/plantlet (Fig. 4).

4. DISCUSSION

Traditional methods typically involve pepper propagation through cutting, which can reduce the yield and quality of pepper due to fungal, bacterial, or viral infections. Additionally, plants propagated by cutting increased genetic uniformity but lack uniformity in both morphology and quality [27–29]. With *in vitro* plantlets, disease-free propagation is ensured, and vigorously grown plantlets can be selected before being transferred to the field [30,31]. In the *Piperaceae* family, the *in vitro* propagation of *Piper nigrum* was well-studied [24,29,32], however,

lack of publications on *P. hancei* micropropagation. Some studies have demonstrated that BAP alone or the combination of BAP and KIN yields the best shoot multiplication results for pepper [33–35]. Betel vine (*Piper betle* L.) produced the highest number of shoots on Woody Plant Medium (WPM) medium supplemented with 3.0 mg/l BAP and 1.0 mg/l KIN [36]. Long pepper (*Piper longum*) showed the best shoot formation on MS medium supplemented with 8.9 M BAP and 4.64 M KIN [37]. However, in this study, shoots grown in KIN-supplemented media exhibited faster growth, larger leaves, and greater height compared to those grown in media with a BAP and IBA combination. In comparison to the medium without IBA, the medium supplemented with IBA showed a reduction in the number of shoots formed.

The combination of BAP with KIN and IBA indicated that IBA negatively affected shoot multiplication and growth, resulting in slow, weak shoots with small, pale green leaves. Callus formation was observed at the shoot base, with the outer part being soft, pale yellow, and the firm inner part. High KIN concentrations (1.5–2.5 mg/l) caused the leaf petioles near the shoot base to swell and form soft white callus that later turned black. These results differ from previously published research in root formation of *in vitro* shoots in various species within the genus *Piper*. Divakaran *et al.* [36] reported that *Piper betle* L. developed a robust root system and grew consistently on WPM medium without PGR supplementation. Similarly, *in vitro* shoots of *Piper aduncum* and *Piper hispidinervum* showed optimal root formation on MS medium without PGR supplementation [38]. *Piper auritum* shoots successfully rooted (100%) on ½ MS medium supplemented with 2 mg/l IAA [39]. Strong root development from shoots of *P. longum* was achieved on MS medium supplemented with 2.46 µM IBA [37]. These findings indicated that root development from *in vitro* shoots varies significantly among different species within the *Piper* genus.

IBA, a synthetic auxin, is known for its role in stimulating root formation from *in vitro* shoots of various plant species, including black pepper [18,33,34]. In this study, the effect of IBA concentration for *in vitro* rooting was investigated, and the optimal medium was found to be 1.5 mg/l IBA. In this medium, roots formed early and rapidly, averaging 1.97 cm in length and 3.41 cm in height. Previous studies on *in vitro* propagation of some species within the genus *Piper* have demonstrated high survival rates and vigorous growth when tissue-cultured plantlets were transferred to the nursery [26,40]. *P. betle* L. was successfully propagated by tissue culture methods, reaching an 80% survival rate when planted in soil [36]. Anand and Srinivasa Rao [41] reported a survival rate of 98% when transplanted *in vitro* *P. barberi* plantlets into the nursery, with a subsequent survival rate of 75% in the field [41]. In this study, the survival ratio value of 80.55% after 6 weeks, similar to other reports.

5. CONCLUSION

We have succeeded in developing a procedure to propagate *Piper hancei* *in vitro*. Shoots were regenerated from the stem on basal MS medium supplemented with 1.0 mg/l BAP after 4 weeks of culture (1.83 shoots/sample). The MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l KIN was optimal for shoot multiplication (4.87 shoots/cluster) whereas 1.5 mg/l IBA addition was optimal for rooting (7.43 roots/shoot). The plantlets were successfully transplanted to the nursery with a survival rate of 80.55% after 6 weeks, averaging 4.49 leaves and 5.64 cm in height. The plantlets can be served as rootstocks in the production of disease-resistant pepper.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

The data are available from the corresponding author upon appropriate request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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