Improving propagation of the rare plant *Huperzia squarrosa* using cuttings and *in vitro* techniques

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ABSTRACT

The tassel fern, *Huperzia squarrosa*, is a rare and medicinally valuable plant known for containing Huperzine A. It propagates naturally through spores, rhizomes, cuttings, and clump division, but with a slow multiplication rate. This study aimed to optimize propagation conditions for *H. squarrosa* using stem cuttings and *in vitro* culture techniques to support its preservation and development. Apical and stem cuttings were treated with varying concentrations of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) before being planted in a substrate of coir dust, charcoal dust, and burnt rice husk (3:2:2). Apical cuttings treated with 1500 ppm IBA for 30 min showed the highest rooting success, identifying this method as optimal for propagation. Additionally, surface sterilization with a 40% bleach solution, followed by antibiotic treatment, achieved a 73.8% clean sample rate. *In vitro* culturing on ¼ MS (Murashige and Skoog) medium resulted in 70% survival and 55% rooting after 60 days. The highest callus formation rate (13.3%) was achieved with 0.01 mg/L IBA and 0.3 mg/L Kinetin, while the addition of 3 mg/L Glutamine did not significantly enhance callus induction. Ongoing research focuses on enhancing complete plant regeneration and improving the efficiency of *in vitro* propagation for *H. squarrosa*.

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1. Introduction

Huperzia squarrosa (G. Forst.) Trevis is a valuable ornamental and medicinal plant known for its therapeutic properties. In India, it is collected in winter, dried, and ground into powder for use as a supplement to improve memory and to treat sleep disorders and epilepsy (Yumkham & Singh, 2012). The primary active ingredient, huperzine A (HupA), is particularly effective in treating memory disorders such as Alzheimer's disease (Ferreira et al., 2016). Other alkaloids in *H. squarrosa*, such as Lycoposquarrosamin-A, Acetylaposerratinin, 8-α-hydroxyfawcettimin, 8-β-hydroxyfawcettimin, 8-β-acetoxyfawcettimin, acetyllycoposerramin-U, and lycoflexin N-oxide, are bioactive compounds with various biological activities. Though their specific functions are not fully understood, many *Huperzia* alkaloids are known for their neuroprotective effects, particularly through acetylcholinesterase inhibition, which may aid in treating neurodegenerative diseases like Alzheimer's (Katakawa et al., 2011).

Huperzia squarrosa, a valuable medicinal plant, is native to Vietnam and can be found in highland regions such as Lao Cai, Lam Dong, Tam Dao (Vinh Phuc), Nghe An, Tay Nguyen, and Quang Tri (Nguyen, 2020). This plant plays a significant role in traditional medicine due to its bioactive compound HupA, which has been associated with cognitive-enhancing and neuroprotective properties (Upadhyay et al., 2020). Despite its importance, *H. squarrosa* faces significant challenges in cultivation. The plant reproduces through unicellular spores encased in a thick, pale yellow spore wall, with germination occurring typically between 3 to 8 years post-release. Stem propagation involves laying apical sections of 5 - 15 cm long horizontally on a propagation medium and keeping them moist and warm until new growth emerges after 6 - 15 months. The method can be inconvenient due to the lengthy time required for new growth and the slow production of large specimens, which can take several years (Yumkham & Singh, 2011). Overexploitation, coupled with its slow development and limited natural reproduction rate, has led to a notable decline in this important genetic resource.

Plant tissue culture presents a viable solution for the large-scale propagation of *Huperzia* sp. while maintaining genetic uniformity. This technique not only aids in conserving this genetic resource but also facilitates the production of HupA for potential medical applications (Yang et al., 2021a). Despite its benefits, research on the micropropagation of *H. squarrosa* remains sparse. Recent studies, such as those by Tran et al. (2019), have demonstrated that MS (Murashige and Skoog) medium (Murashige and Skoog, 1962) supplemented with specific growth regulators can enhance regeneration rates and shoot multiplication. Additionally, ½ MS medium with indole-3-butyric acid (IBA) has been effective in promoting rooting.

Given the medicinal significance of *H. squarrosa* and the challenges associated with its propagation, developing efficient in vitro propagation methods is crucial for its conservation and sustainable cultivation. This study aims to address the gaps in current research by focusing on optimizing propagation techniques for *Huperzia squarrosa* using cuttings and in vitro culture methods, with the objective of enhancing callus formation and plant regeneration to support both conservation and medical application efforts.

2. Materials and Methods

2.1. Plant material

Huperzia squarrosa plants were collected from Tuyen Quang province and identified based on morphological characteristics described by Pham (1999). The plants were cultivated in the greenhouse at the Faculty of Biological Sciences and used as research materials.

2.2. Investigating propagation through cutting

This experiment investigates how two factors, including the type of cutting and the plant growth regulator (PGR) treatments, influence the propagation of *H. squarrosa* through cuttings. Healthy, pest- and disease-free *H. squarrosa* plants were cut into 8 cm segments with a bevelled end to maximize the exposed surface area with the substrate. Two types of cuttings were prepared: apical cuttings (V1) and stem cuttings (V2) taken 17 cm from the base (Figure 1a). The 10 cm-long cuttings were treated with different concentrations of Indole-3-Butyric Acid (IBA, Biobasic) at 500, 1000, and 1500 ppm for 30 min, and Naphthaleneacetic Acid (NAA, Biobasic) at 10, 20, and 30 ppm for 5 min. After treatment, the cuttings were planted in pots (7 x 7 cm) containing a substrate mixture of coconut fibre, shredded charcoal, and burnt rice husks in a 3:2:2 ratio. The pots were kept in a greenhouse with 70% light coverage and watered regularly.

The experiment was conducted three times, with each treatment including 10 apical cuttings (V1) or 30 stem cuttings (V2). After 60 days, the percentage of surviving explants, dead explants, rooted explants, and the morphological characteristics of the explants were recorded.

2.3. *In vitro* propagation of *Huperzia squarrosa*

Explant sterilization

A 3 cm shoot segment was initially washed under clean tap water, soaked in diluted soap solution for 20 min, and then rinsed under running water. The segment was soaked in a fungicide solution (Mancozeb, India) for 40 min, then briefly immersed in 70% ethanol for 30 sec. The segments were then treated with a Javel solution (NaOCl, 5%) at varying concentrations (20%, 30%, and 40% v/v) with 2 - 3 drops of Tween-80 for durations of 20, 30, and 40 min. Following this, explants were immersed in an antibiotic solution (ampicillin 2.5 mg/mL and tetracycline 2.5 mg/mL) for 30 min. Both ends of the shoots were trimmed to 2 cm, and the explants were cultured in MS medium supplemented with 2% (w/v) sucrose. The medium was adjusted to pH 5.8 and sterilized by autoclaving at 121°C and 1 atm for 20 min. The efficiency of sterilization was evaluated after 10 days of culture.

Influence of mineral salts on shoot growth

Huperzia squarrosa shoots were cut into 5 mm segments and cultured on MS or ¼ MS medium. Each treatment was performed in triplicate. After 8 weeks of culture, the percentage of survival rate (%), rooting rate (%), number of root (roots/explant), and morphology of shoot were recorded.

Effects of combinations of plant growth regulators and glutamine on callus formation

This experiment evaluated how specific combinations of IBA, kinetin, and glutamine influence callus formation in *Huperzia squarrosa*. Shoots were cut into 5 mm segments and cultured on ¼ MS medium, with or without IBA (0.01 or 0.015 mg/L), kinetin (0.3 mg/L, Biobasic), and glutamine (0.3 mg/L, Biobasic). Each treatment was replicated three times to ensure reliability.

After 8 weeks of culture, data were collected on the percentage of callus induction and the morphology of the callus.

***In vitro* culture conditions**

The *in vitro* culture conditions were maintained at a temperature of 25 ± 2°C under cool-white fluorescent lighting with a 16-h light/8-h darkness photoperiod, a light intensity of 2000 -3000 lux, and humidity levels of 60 - 70%.

2.4. Statistical analysis

All experiments were conducted using a completely randomized design (CRD). The collected data were analysed using one-way or multi-way analysis of variance (ANOVA) with Minitab 16. T-tests or Tukey's tests were used to compare mean values at a 5% significance level. Prior to analysis, data were transformed to ensure a standard normal distribution. Results are presented as M ± SD, where M is the mean and SD is the standard deviation.

3. Results and Discussion

3.1. Propagation for *Huperzia squarrosa* using cuttings

The results indicated a significant difference in survival rates across the treatments ($P < 0.05$) (Table 1). For V1 cuttings (apical cuttings), a 100% survival rate was observed in treatments B1 and B3, where cuttings were exposed to IBA at 500 ppm and 1500 ppm for 30 min, respectively. In contrast, the lowest survival rate for V1 cuttings was 20% in treatment B6, where NAA was applied at 30 ppm for 5 min. For V2 cuttings (stem cuttings), the highest survival rate of 100% was recorded in treatment B14, which did not involve any growth regulator. The lowest survival rate for V2 cuttings was observed in treatment B13 (NAA at 30 ppm for 5 min).

Overall, treatments involving NAA resulted in lower survival rates compared to those involving IBA. Specifically, V1 cuttings treated with IBA at concentrations of 500 ppm or 1500 ppm for 30 min achieved up to 100% survival, whereas V2 cuttings under the same IBA conditions had a maximum survival rate of 80%. In contrast, treatments with NAA for 5 min showed survival rates ranging from 20% to 90% for V1 cuttings and 43.3% to 63.7% for V2 cuttings, with survival rates decreasing as NAA concentrations increased from 10 to 30 ppm.

The rooting results demonstrated that V1 cuttings (apical cuttings) achieved superior rooting compared to V2 cuttings (stem cuttings). V1 cuttings reached a highest rooting rate of 100%, with the lowest at 20%, while V2 cuttings failed to form roots in all samples (Figure 1d, 1e). Treatment B3, involving V1 cuttings treated with 1500 ppm IBA for 30 min, yielded the best rooting performance. This treatment resulted in a 100% rooting rate, an average root length of 11.9 mm, and 6.1 roots per sample. The cuttings from this treatment remained green, elongated, and rooted after 2 months (Figure 1b, 1c). These results were significantly better than those from treatments using NAA at 10 ppm, which only achieved a 90% rooting rate, 3.4 roots per sample, and an average root length of 7.4 mm, with rooting performance declining at higher NAA concentrations.

Table 1. Effects of type of cutting and the plant growth regulator treatments on survival and mortality rates of *Huperzia squarrosa* cuttings after 2 months

Treatment	Cutting site	PGRs	Concentration (ppm)	Death rate (%)	Survival rate (%)	Rate of root induction (%)	Root length (cm)	No. of root (roots/ shoot)
B1	V1	IBA	500	0.00 ± 0.00^{i}	100.0 ± 0.00^{a}	100.0 ± 0.00^{a}	9.8 ± 0.4^{b}	5.6 ± 0.2^{a}
B2	V1	IBA	1000	20.0 ± 5.00^{f}	80.0 ± 5.00^{b}	80.0 ± 5.00^{b}	8.2 ± 0.2^{c}	4.2 ± 0.2^{b}
B3	V1	IBA	1500	0.0 ± 0.00^{i}	100.0 ± 0.00^{a}	100.0 ± 0.00^{a}	11.9 ± 0.1^{a}	6.1 ± 0.1^{a}
B4	V1	NAA	10	10.0 ± 5.00^{h}	90.0 ± 5.00^{b}	90.0 ± 5.00^{ab}	7.4 ± 0.2^{d}	3.4 ± 0.2^{c}
B5	V1	NAA	20	40.0 ± 5.00^{d}	60.0 ± 5.00^{c}	60.0 ± 5.00^{c}	6.9 ± 0.2^{e}	2.7 ± 0.2^{d}
B6	V1	NAA	30	80.0 ± 5.00^{a}	20.0 ± 5.00^{d}	20.0 ± 5.00^{d}	1.5 ± 0.2^{f}	0.7 ± 0.2^{e}
B7	V1	0	0	20.0 ± 5.00^{f}	80.0 ± 5.00^{b}	80.0 ± 5.00^{b}	9.6 ± 0.1^{b}	3.6 ± 0.2^{c}
B8	V2	IBA	500	10.0 ± 2.00^{h}	90.0 ± 2.00^{b}	0.0	0.0	0.0
B9	V2	IBA	1000	20.0 ± 2.00^{f}	80.0 ± 2.00^{b}	0.0	0.0	0.0
B10	V2	IBA	1500	13.3 ± 0.57^{g}	86.7 ± 0.57^{b}	0.0	0.0	0.0
B11	V2	NAA	10	36.7 ± 5.77^{e}	63.3 ± 5.77^{c}	0.0	0.0	0.0
B12	V2	NAA	20	46.7 ± 2.89^{c}	53.3 ± 2.89^{c}	0.0	0.0	0.0
B13	V2	NAA	30	56.7 ± 2.89^{b}	43.3 ± 2.89^{d}	0.0	0.0	0.0
B14	V2	0	0	0.00 ± 0.00^{i}	100 ± 0.00^{a}	0.0	0.0	0.0

In the same column, means with distinct letters indicate significant differences (Tukey HSD test, α=0.05). PGR: plant growth regulator; IBA: indole-3-butyric acid; NAA: naphthaleneacetic acid.

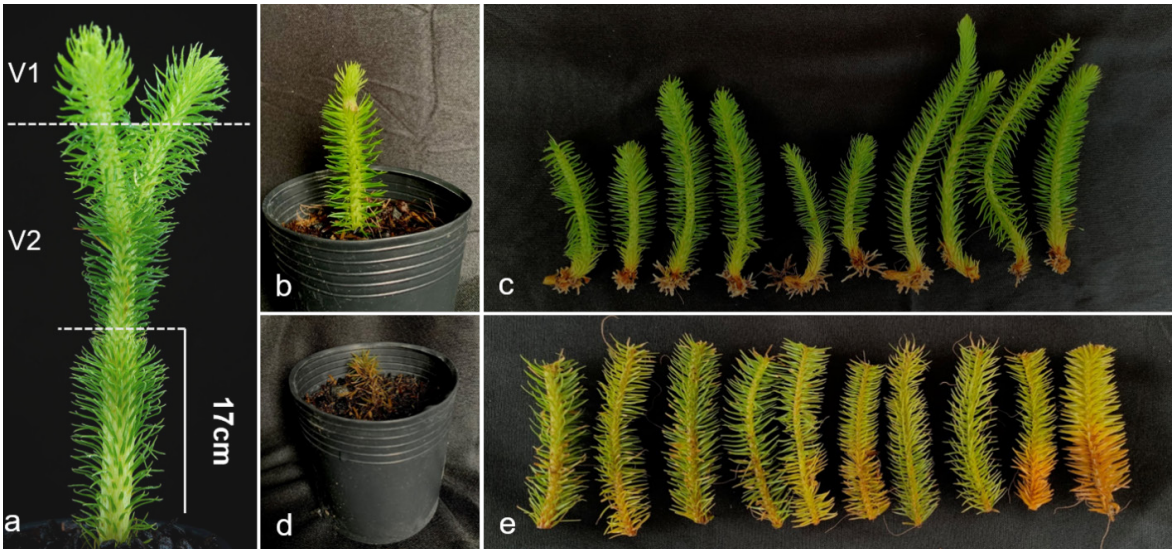

Figure 1. Stages of *Huperzia squarrosa* cuttings treated with IBA (indole-3-butyric acid). (a) Initial cuttings; (b) - (c) V1 cuttings (apical cuttings) treated with 1500 ppm IBA for 30 min, showing rooting and growth after 2 months; (d) - (e) V2 cuttings (stem cuttings) treated with 1500 ppm IBA for 30 min, showing no growth after 2 months.

Overall, treatments with NAA generally produced lower rooting rates and poorer root quality compared to IBA. Based on these findings, treatment B3 (V1 cuttings treated with 1500 ppm IBA for 30 min) was identified as the most effective method for propagating *Huperzia squarrosa* through cuttings. This conclusion aligns with the findings of Le et al. (2019), who demonstrated that treating 6 cm long *H. serrata* cuttings with 1000 ppm IBA for 30 min was effective for propagation. Previous studies also support the efficacy of IBA in root induction (Zhang et al., 2009; Zuo et al., 2010), whereas NAA has shown varying results for *H. serrata* (Long et al., 2014). The use of V1 cuttings (shoot segments) for propagation of *Huperzia squarrosa* represents a novel approach compared to earlier studies, contributing to the preservation of this species through asexual propagation.

3.2. In vitro propagation for Huperzia squarrosa

Effects of duration and concentration of javel solution on sterilization efficiency

The effects of different concentrations and durations of Javel solution on sterilization efficiency were evaluated by disinfecting 3 cm shoots with 5% sodium hypochlorite at concentrations of 20%, 30%, and 40% (v/v) for 20 - 40 min, with results monitored after 10 days (Table 2, Figure 2). A two-way ANOVA revealed that both the concentration of Javel (%) and the duration of exposure (min) significantly affected infection and disinfection rates, with a notable interaction between them ($P = 0.041$). Sterilization efficiency varied significantly among treatments ($P < 0.05$). The proportion of disinfected samples increased from 2.38% at 20% Javel for 20 min to 54.76% at 30% Javel for 40 min, indicating improved disinfection efficiency with higher Javel concentrations and longer

exposure times. However, when using 40% Javel, disinfection efficiency initially decreased from 67.70% at 20 min to 61.90% at 30 min, followed by an increase to 73.80% at 40 min. ANOVA results indicated that treatment duration did not significantly affect disinfection efficiency ($P > 0.05$), while increasing Javel concentration from 20% to 40% significantly improved sterilization outcomes ($P < 0.05$).

Table 2. Effects of Javel solution concentration and duration on surface sterilization efficiency of *Huperzia squarrosa* shoots after 10 days

Treatments	Concentration of Javel (%)	Duration (min)	Infection rate (%)	Disinfection rate (%)
A1	20	20	$97.62^{a} \pm 4.12$	$2.38^{c} \pm 4.13$
A2	20	30	$76.20^{ab} \pm 18.00$	$23.80^{bc} \pm 18.10$
A3	20	40	$71.43^{abc} \pm 14.29$	$28.57^{abc} \pm 14.30$
A4	30	20	$61.90^{bc} \pm 14.87$	$38.10^{ab} \pm 14.87$
A5	30	30	$54.76^{bc} \pm 10.91$	$45.24^{ab} \pm 10.91$
A6	30	40	$45.24^{bc} \pm 10.91$	$54.76^{ab} \pm 10.91$
A7	40	20	$33.30^{bc} \pm 23.00$	$67.70^{ab} \pm 23.00$
A8	40	30	$38.10^{bc} \pm 16.50$	$61.90^{ab} \pm 16.50$
A9	40	40	$26.20^{c} \pm 18.00$	$73.80^{a} \pm 18.00$
		F (Concentration)	12.35^{**}	15.23^{**}
		F (Duration)	9.54^{**}	10.67^{**}
		F (Concentration x Duration)	3.47^{*}	4.12^{*}

In the same column, means with distinct letters indicate significant differences (Tukey HSD test; (*) $P \leq 0.05$; (**) $P \leq 0.01$).

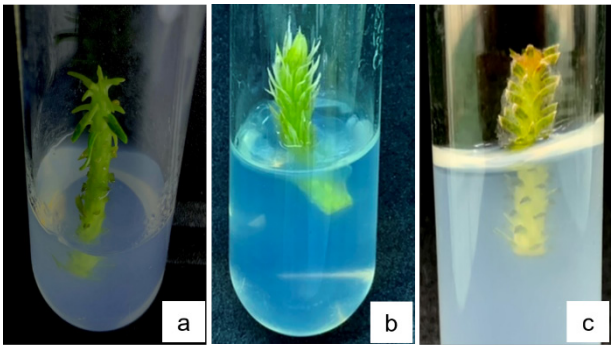

Figure 2. *Huperzia squarrosa* after sterilization.
(a) sterilized shoot; (b) - (c) bacterium and fungal-contaminated explants.

The lowest sterilization efficiency observed was 2.38% when using a 20% Javel solution for 20 min. In contrast, soaking shoot segments in a 40% Javel solution for 40 min achieved the highest sterilization rate of 73.80%. Thus, the most effective sterilization method in this study was treating the samples with 40% Javel for 40 min, followed by immersion in an antimicrobial solution (Ampicillin 2.5 mg/mL and Tetracycline 2.5 mg/mL) for 30 min.

Previous research on the *Huperzia* genus has used $HgCl_2$ for sterilizing sporangia and shoot tips, stem segments (Zhou et al., 2009), or combined $HgCl_2$ with H_2O_2 for shoot tip sterilization (Yang et al., 2008). While $HgCl_2$ is effective, it poses toxicity and handling challenges. In contrast, Javel has proven effective and safer for sterilization (Szypula et al., 2005). This study's approach of using 40% Javel for 40 min represents a novel and suitable method for sterilizing *Huperzia* cultures.

Influence of mineral salt on the growth of in vitro shoots

After 60 days of culture, significant differences between MS and ¼ MS mineral salt concentrations were observed ($P < 0.05$) in terms of survival rate, rooting rate, and shoot characteristics. Detailed data on these effects are presented in Table 3.

Table 3. Influence of mineral salt on the growth of *in vitro H. squarrosa* shoots

Mineral salt	Survival rate (%)	Rooting rate (%)	Number of root (roots/explant)	Morphological characters
MS	$15.00^a \pm 7.07$	$0.00^a \pm 0.00$	0.00	No growth, yellow leaves and death
¼ MS	$70.00^b \pm 14.10$	$55.00^b \pm 7.07$	2.09	Growing well, thick and strong stems, dark green leaves

In the same column, means with distinct letters indicate significant differences ($P \leq 0.05$). MS: Murashige & Skoog.

After 2 months of culture on ¼ MS mineral salt medium, shoots exhibited a rooting rate of 55% and a survival rate of 70%, with high-quality growth characterized by dark green stems and leaves (Figure 3.C1). In contrast, shoots cultured on MS mineral salt medium showed no growth, with explants gradually turning yellow and dying. Only 15% of the explants survived, and none developed roots (Figure 3.C2). Consequently, ¼ MS medium was selected as the optimal medium for the in vitro propagation of *Huperzia squarrosa*. These findings are consistent with previous studies indicating that low-nutrient mineral media are suitable for the micropropagation of species within the *Huperzia* genus (Szypuła et al., 2005; Yang et al., 2021b; Ho et al., 2022).

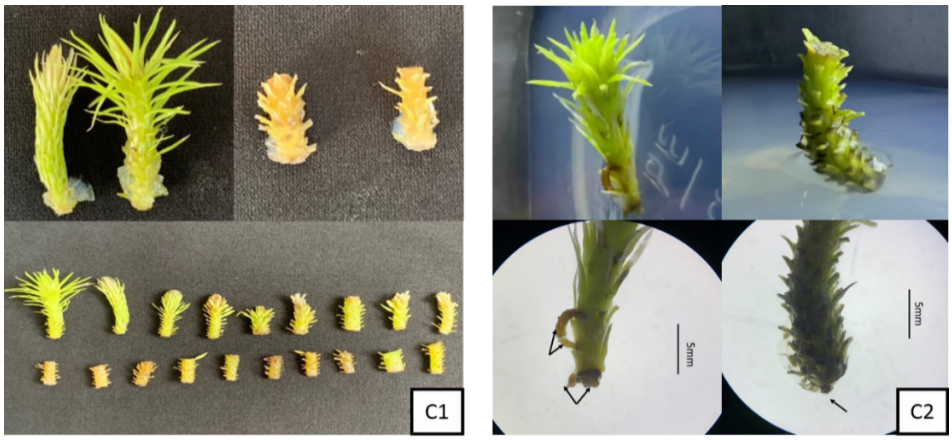

Figure 3. *Huperzia squarrosa* shoots after 60 days of culturing on MS (Murashige & Skoog) medium and ¼ MS mineral salt media. (C1) Shoots cultured on MS medium, showing no growth and yellowing; (C2) Shoots cultured on ¼ MS medium, displaying healthy growth and root development (arrows indicate root development).

Effects of plant growth regulators and glutamine concentrations on callus formation from in vitro shoot of Huperzia squarrosa

The results in Table 4 show that adding IBA significantly affects callus formation in *Huperzia squarrosa* shoots after two months of culture. The highest callus induction rate (13.3%) was achieved with Treatment S2, which included 0.01 mg/L IBA and 0.3 mg/L Kinetin. This suggests that this combination is the most effective for promoting callogenesis (Figure 4). Treatment S3, with 0.015 mg/L IBA and 0.3 mg/L Kinetin, had a slightly lower callus induction rate of 10.0%, similar to Treatment S5, which also included 3 mg/L Glutamine. The addition of Glutamine did not significantly improve callus formation compared to IBA and Kinetin alone. Treatment S4, with 0.01 mg/L IBA, 0.3 mg/L Kinetin, and 3 mg/L Glutamine, showed a lower callogenesis rate of 6.67%, indicating that this combination is less effective. Overall, the results highlight the influence of IBA on callogenesis and indicate potential interactions with other factors, although not all interactions are statistically significant. Further analysis might be necessary to explore these interactions in more detail.

Table 4. The percentage of callus induction after two months of culture

Treatment	IBA (mg/L)	Kinetin (mg/L)	Glutamine (mg/L)	Callogenesis rate (%)
S1	0	0	0	$0.00^{b} \pm 0.00$
S2	0.01	0.3	0	$13.3^{a} \pm 5.77$
S3	0.015	0.3	0	$10.0^{a} \pm 0.00$
S4	0.01	0.3	3	$6.67^{ab} \pm 5.77$
S5	0.015	0.3	3	$10.0^{a} \pm 0.00$
S6	0	0	3	$0.00^{b} \pm 0.00$
			CV (%)	3.33
			F-value	8.40^{**}

In the same column, means with distinct letters indicate significant differences (Tukey HSD test, α=0.01. IBA: indole-3-butyric acid.

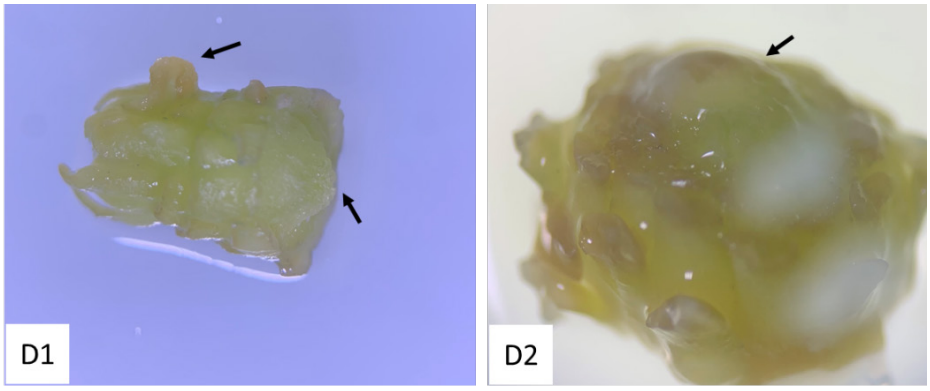

Figure 4. Callus induction after two months of culture. (D1) ¼ MS (Murashige & Skoog) medium supplemented with 0.01 mg/L IBA (indole-3-butyric acid) and 0.3 mg/L Kinetin, and (D2) ¼ MS medium supplemented with 0.015 mg/L IBA, 0.3 mg/L Kinetin, and 3 mg/L Glutamine. Arrows indicate callus development.

The callus formation process of *H. squarrosa* in this study only required a small amount of plant growth regulator, similar to the experiment conducted by Szypula et al. (2005). When 3 mg/L Glutamine was added to the culture medium, the callus formation rate for *H. squarrosa* decreased significantly to 6.67% in the S4 treatment and 10.0% in the S5 treatment (Figure 4). This result contrasts sharply with previous research on *H. serrata*, which reported a callus formation rate of 75.56% on ¼ MS medium supplemented with 0.015 mg/L IBA, 0.3 mg/L Kinetin, and 3 mg/L Glutamine (Le, 2021). This discrepancy could be due to genetic differences between *H. serrata* and *H. squarrosa*. Further research on the callogenesis of this medicinal plant is needed to optimize conditions for callus induction and growth, thereby enhancing the production of valuable medicinal metabolites. Understanding the effects of growth regulators, nutrient composition, and environmental factors can help improve the yield and quality of these bioactive compounds.

4. Conclusions

This study established effective methods for the propagation of *Huperzia squarrosa*. For cuttings, V1 shoot-tip segments treated with 1500 ppm IBA for 30 min achieved optimal rooting (100% rate, 6.1 roots per explant, 11.9 mm root length). *In vitro* conditions, the best sterilization method was 40% Javel for 40 min, followed by an antimicrobial solution, achieving a 73.8% disinfection rate. The ¼ MS medium supported shoot growth with a 70% survival rate and 55% rooting rate, and callus formation was highest with 0.01 mg/L IBA and 0.3 mg/L Kinetin. These methods provide a foundation for conserving *H. squarrosa*.

Conflict of interest

The authors declare no conflict of interest.

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