ACADEMIA JOURNAL OF BIOLOGY 2022, 44(1): 145–153
DOI: 10.15625/2615-9023/16207

IN VITRO SHOOT MULTIPLICATION AND THE CONTENT OF HUPERZINE A IN *IN VITRO* CULTURED *Huperzia serrata* (Thunb. Ex Murray) Trevis PLANTS

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Received 25 June 2021; accepted 18 March 2022

ABSTRACT

Huperzia serrata (Thunb. Ex Murray) Trevis belonging to the Lycopodiaceae family contains the main active ingredient Huperzine A (HupA), which is effective in the treatment of dementia. However, *H. serrata* in nature contains a low content of HupA. Additionally, this plant grows very slowly and is overexploited, thereby leading to the risk of extinction. In this study, we used the *in vitro* shoot of *H. serrata* to induce shoot multiplication by tissue culture method and quantify the content of HupA in the *in vitro* cultured plant by High-Performance Liquid Chromatography (HPLC). The suitable nutrient medium for inducing shoot multiplication was 1/4 MS medium supplemented with 1 mg/L of kinetin. In this medium, the shoot multiplication rate was as high as 77.33%, the number of shoots/shoot multiplication was 6.02, and the shoot height was 1.61 cm. Based on the HPLC method, the content of HupA in the *in vitro* cultured plant was 300 µg/g. The content of HupA in the cultured *H. serrata* in Vietnam is reported for the first time in our study. This study shows *in vitro* shoot multiplication is a promising method for propagating *H. serrata* and can produce the cultured *H. serrata* with a high content of HupA. These findings contribute to conserving and preserving the gene source of *H. serrata* species, and at the same time, enhancing the content of HupA in the cultured *H. serrata*.

Keywords: HPLC, *Huperzia serrata*, Huperzine A, shoot multiplication, tissue culture.

Citation: Ho Thi Huong, Le Thi Lan Anh, Nguyen Duc Thanh, Ngo Thi Thuy Linh, Ton That Huu Dat, Le Thi Bich Thuy, 2022. *In vitro* shoot multiplication and the content of huperzine a in *in vitro* cultured *Huperzia serrata* (Thunb. Ex Murray) Trevis plants. *Academia Journal of Biology*, 44(1): 145–153. https://doi.org/10.15625/2615-9023/16207

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INTRODUCTION

Huperzia serrata (Thunb. Ex Murray) Trevis is a herbaceous species belonging to family Lycopodiaceae is distributed widely but concentrated in the Eastern, Southern and Southeastern areas of Asia, Oceania and Central America (Wang et al., 2011). The main active ingredient of *H. serrata* is Huperzine A (HupA), which is effective in treating memory disorders, especially Alzheimer's disease in the elderly (Ferreira et al., 2016). HupA was first discovered in 1948 and approved for Alzheimer's disease treatment in the 1990s in China and marketed in the US as a dietary supplement (Ma et al., 2007). To date, HupA is increasingly used in the production of drugs and medicinal products.

HupA can be chemically synthesized, however, studies show that racemic mixtures are less effective in inhibiting the acetylcholinesterase (AChE) enzyme than natural plant-derived HupA (Ding, 2014). Consequently, commercial HupA is mainly extracted from natural *H. serrata*. However, the content of HupA in natural *H. serrata* is very low. The previous studies report that the content of HupA in natural *H. serrata* is less than 0.02% fresh weight and 0.0047–0.025% dry weight (Bialer et al., 2010; Ha et al., 2011). Furthermore, the content of HupA in natural *H. serrata* is varied considerably, depending on the collecting seasons, growing regions, plant organs, collecting process as well as extraction method (Ma et al., 2005; Ha et al., 2011). Therefore, large quantities of *H. serrata* are required in order to extract enough quantity of HupA for medicinal uses. The over-exploitation of *H. serrata* has drastically reduced the wild *H. serrata* population in the globe (Ma et al., 2006). Furthermore, the plant grows very slowly and normally requires 15–20 years of growth from spore germination to maturity (Ma & Gang, 2008). Hence, it is necessary to find sustainable solutions for the exploitation and conservation of wild *H. serrata*.

The regeneration and cultivation of natural *H. serrata* are very difficult. Several efforts of propagation of *H. serrata* by tissue culture have been reported (Yang et al., 2008; Li et al., 2009; Zhou et al., 2009; Ma et al., 2015). However, there have been no studies on the content of HupA in the *in vitro* cultured *H. serrata*. Interestingly, Ma et al. (2008) report that the content of HupA in the *in vitro* cultured *Phlegmariurus squarrosus*, a member of the Huperziaceae, is higher than that in the natural plant. Furthermore, Lim et al. (2010) also indicate that the content of HupA is very different in tissue types, in which the shoot tips of *H. serrata* contain the highest content of HupA. In this study, we applied successfully the *in vitro* shoot multiplication method to propagate *H. serrata* with a high content of HupA, while also contributing to the conservation and preservation of the gene source of *H. serrata* species in Vietnam.

MATERIALS AND METHODS

Materials

The culture materials used in the present study were healthy 5-year old *H. serrata* with uniform sizes (plant height: 23–25 cm). The plant samples were collected at Lam Ha natural forest, Da Lat, Lam Dong province.

HupA (≥ 98% purity) was provided by Sigma-Aldrich (USA). Other chemicals were provided by Agilent (USA), Merck (Germany), Sigma- Aldrich (USA).

Methods

Sterilization and preparation of in vitro shoots of H. serrate

The shoot tips of *H. serrata* (2–3 cm) were disinfected by 70% C_2H_5OH for 30 s, followed by 0.1% $HgCl_2$ for 5 min and 20% Javen for 7 min (Le Thi Lan Anh et al., 2019b). These shoot tips were cultured in the 1/4 MS medium supplemented with 20 g/L glucose, 1 mg/L kinetin, 8 g/L agar and pH 5.7–5.8 for 60 days. The experimental samples were cultured in the room at a temperature of 22 ± 2 ^oC and illuminated by white fluorescent light with a light intensity of 22.2 $\mu mol/m^2/s$ for 16 hours/day.

These *in vitro* shoots were then used for investigating the influence of mineral media and growth stimulants (i.e., BA and kinetin) on shooting and shoot multiplication of *H. serrata*.

Influence of the different concentrations of mineral nutrient on the in vitro shooting of H. serrate

The shoots of *H. serrata* were cultured in different mineral media, including MS, 1/2 MS, 1/4 MS, 1/6 MS (Murashige & Skoog, 1962), WPM (Lloyd & McCownb, 1980), and B5 (Gamborg et al., 1980) in order to determine suitable mineral medium for the shooting of *H. serrata*. The media were supplemented with 20 g/L glucose, 8 g/L agar, and pH 5.7–5.8.

Culture conditions were as above described. The growth of the shoots was monitored for 60 culture days. Thirty shoots were used for each treatment and the treatments were performed in triplicates.

Influence of BA and kinetin on the shoot multiplication of H. serrate

The shoots of *H. serrata* were cultured on 1/4 MS medium supplemented with BA or kinetin at different concentrations (0.1, 0.5, 1.0 and 1.5 mg/L) to evaluate the influence of BA and kinetin on shoot multiplication of *H. serrata*.

Culture conditions were as above described. The shoot multiplication and growth of *H. serrata* were monitored for 120 culture days. One hundred shoots were used for each treatment and treatments were performed in triplicates. Shoot multiplication rate (%) = number of cultured samples inducing shoot multiplication × 100/number of cultured samples. Number of shoots/shoot multiplication = number of shoots in shoot multiplications/number of shoot multiplications.

Determination of the HupA content in the cultured H. serrata by HPLC

The content of HupA in the *in vitro* cultured *H. serrata* was determined by HPLC as the described protocol by Vu Thi Ngoc et al. (2016). In brief, whole *in vitro* cultured shoot was extracted with MeOH, and then the extract was filtered through a membrane with 0.45 µm pore size before analysis by LC/MS system connected to the software Aligent OpenLAB Control Panel. The amount of the extract injected into the system was 1 µL at a flow rate was 0.7 mL/min. Nitrogen gas was injected at a flow rate of 5.0 L/min, spraying pressure at 60 psi, and drying temperature at 250 ^{o}C. ESI selective mass spectrometry fragmentation in positive mode with molecular ion peak was selected at 243.0 $[M+H]^+$ of HupA. Mobile phase using solvent system ACN/H_2O (20 mM ammonium acetate, pH=4,0) with the concentration gradient from 10/90–100/0 (v/v). The signal of HupA in the extract was detected according to the retention time (Rt) of HupA standard on the LC system (Rt 11.4–11.7 min) and MS system (11.5–11.8 min). The content of HupA in the extract was determined according to the HupA standard curve established by software Chemstation using peak area at UV 310 nm at Rt 11.4–11.7 min.

Data analyses

Experiments were designed according to randomized blocks and performed in triplicates. The experimental data was presented by the mean values of the three replications. The statistical analyses were calculated by IRRISTAT v.5.0. Values of $P < 0.05$ indicated significant differences.

RESULTS AND DISCUSSION

Influence of the mineral media on the shooting of *H. serrate*

In vitro shoots of *H. serrata* were cultured on 6 different mineral media. The shooting of *H. serrata* after 60 culture days was shown in Table 1.

As shown in Table 1, after 60 culture days, shoots of *H. serrata* could not grow on WPM and B5 media, whereas shoots of *H. serrata* could grow on MS, 1/2 MS, 1/4 MS, 1/6 MS. Of these, the most suitable mineral medium for the shooting of *H. serrata* was the 1/4 MS medium. With 1/4 MS medium, the

shoot height, leave length and leave with of *H. serrata* were recorded as 2.19 cm, 0.94 cm and 0.24 cm, respectively, the number of leaves was 7.33. Also, the stems of *H. serrata* were large and strong, and the leaves were dark green (Fig. 1). The shoots of *H. serrata* developed 3 to 4 new leaves each month and the shoot height increased from 3.5 mm to 4 mm each month. Based on the experimental results, the 1/4 MS medium was chosen as the basic mineral medium for *in vitro* culture of *H. serrata* in further experiments.

Table 1. Influence of the mineral media on the shootis of *H. serrata*

Mineral media	Shoot height (cm)	Number of leaves	Leaf size		Characterization of shoots
			Length (cm)	Width (cm)	
MS	1.70^{d}	4.67^{b}	0.75^{c}	0.20^{c}	Shoots developed slowly and had many deformed shoots; the stem and leaves were yellow-green; the leaves were curled
1/2MS	1.94^{b}	6.33^{a}	0.86^{b}	0.23^{b}	Shoots developed moderately; stems and leaves were green
1/4MS	2.19^{a}	7.33^{a}	0.94^{a}	0.24^{a}	Shoots developed well; the stems were large and strong, and leaves were dark green
1/6MS	1.78^{c}	5.00^{b}	0.73^{d}	0.19^{d}	Shoots developed slowly and had many deformed shoots; the stem and leaves were yellow-green; the leaves were curled.
WPM	1.54^{f}	2.33^{c}	0.64^{f}	0.18^{d}	Shoots were not developed and died; leaves were yellow
B5	1.57^{e}	2.67^{c}	0.69^{e}	0.18^{d}	Shoots were not developed and died; leaves were yellow
5% LSD	0.012	1.033	0.0103	0.0079	

Note: The different letters in a column indicated significant differences at $P < 0.05$.

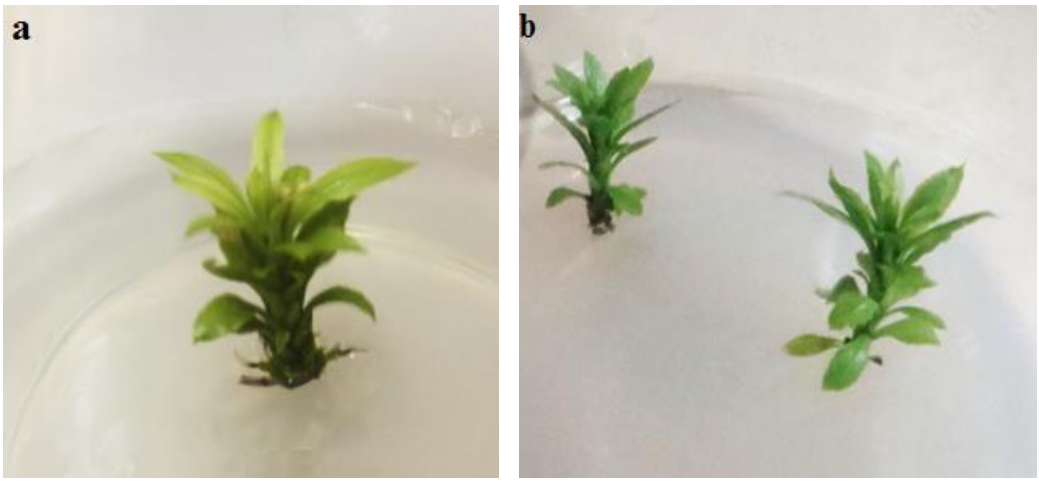

Figure 1. H. serrata shoots cultured in the 1/4 MS medium after 60 culture days (**a**) and after 90 culture days (**b**)

Influence of BA and kinetin on the shoot multiplication of *H. serrata*

The growth stimulants (e.g., BA, kinetin, BAP, IBA) are effective in shooting and rooting of *H. serrata* (Zhou et al., 2009). In this study, we evaluated the influence of two growth stimulants (i.e., BA, kinetin) on the shoot multiplication of *H. serrata*. The influence of BA and kinetin on the shoot multiplication of *H. serrata* after 120 culture days was shown in Table 2 and Table 3.

Table 2. Influence of BA on multiple shoot induction of *H. serrata*

Growth stimulant	Concentration of BA (mg/L)	Number of samples appear shoot/100 culture samples	Number of shoots/shoot multiplication	Shoot height (cm)	Characterization of shoots
Control	0	36.33^{d}	3.34^{d}	0.91^{e}	The stem and leaves were green and grew slowly
BA	0.1	41.33^{c}	4.06^{c}	1.21^{d}	The stem and leaves were green and grew slowly
BA	0.5	75.67^{b}	5.65^{a}	1.60^{a}	The stem and leaves were dark green and grew rapidly
BA	1.0	76.67^{ab}	5.20^{b}	1.58^{b}	The stem and leaves were small and thin
BA	1.5	77.67^{a}	5.12^{b}	1.44^{c}	The stem and leaves were small and thin
5% LSD		1.031	0.099	0.018	

Note: The different letters in a column indicated significant differences at $P < 0.05$.

Table 3. Influence of kinetin on the shoot multiplication of *H. serrata*

Growth stimulant	Concentration of kinetin (mg/L)	Number of samples appear shoot/100 culture samples	Number of shoots/shoot multiplication	Shoot height (cm)	Characterization of shoots
Control	0	36.33^{e}	3.34^{e}	0.91^{e}	The stem and leaves were green and grew slowly
Kinetin	0.1	39.33^{d}	3.54^{d}	1.21^{d}	The stem and leaves were green and grew slowly
Kinetin	0.5	55.67^{c}	4.91^{c}	1.37^{c}	The stem and leaves were green and grew slowly
Kinetin	1.0	77.33^{b}	6.02^{a}	1.61^{a}	The stem and leaves were dark green and grew rapidly
Kinetin	1.5	79.67^{a}	5.78^{b}	1.52^{b}	The stem and leaves were green
5% LSD		1.458	0.055	0.019	

Note: The different letters in a column indicated significant differences at $P < 0.05$.

The study results showed that in the medium without BA or kinetin supplementation, the shoot multiplication rate of *H. serrata*, the number of shoots/shoot multiplication, and the shoot height were low with the values of 36.33%, 3.34 and 0.91 cm, respectively (Table 2 and Table 3). When BA or kinetin was added separately to the medium at a concentration of 0.1 mg/L, the growth of *H. serrata* increased slightly compared to the control. In the case of adding BA to the medium at a concentration of 0.5 mg/L, the growth of *H. serrata* increased significantly, such as the shoot multiplication rate reached 75.67%, the number of shoots/shoot multiplication reached 5.65, the shoot height reached 1.60 cm, the leaves were dark green, and the stems grew rapidly. At higher concentrations of BA, the growth of *H. serrata* tended to decrease. For kinetin, the growth of *H. serrata* was increased when the concentration of kinetin in the medium was increased and reached the highest growth at a concentration of 1.0 mg/L. At this concentration, the shoot multiplication rate reached 77.33%, the number of shoots/shoot multiplication reached 6.02, the shoot height reached 1.61 cm, the stems and leaf grew well and had dark green color. When the concentration of kinetin was increased to 1.5 mg/L, the growth of *H. serrata* was decreased. Thus, the supplement of BA at a concentration of 0.5 mg/L or kinetin at a concentration of 1.0 mg/L into medium supported the shoot multiplication of *H. serrata*.

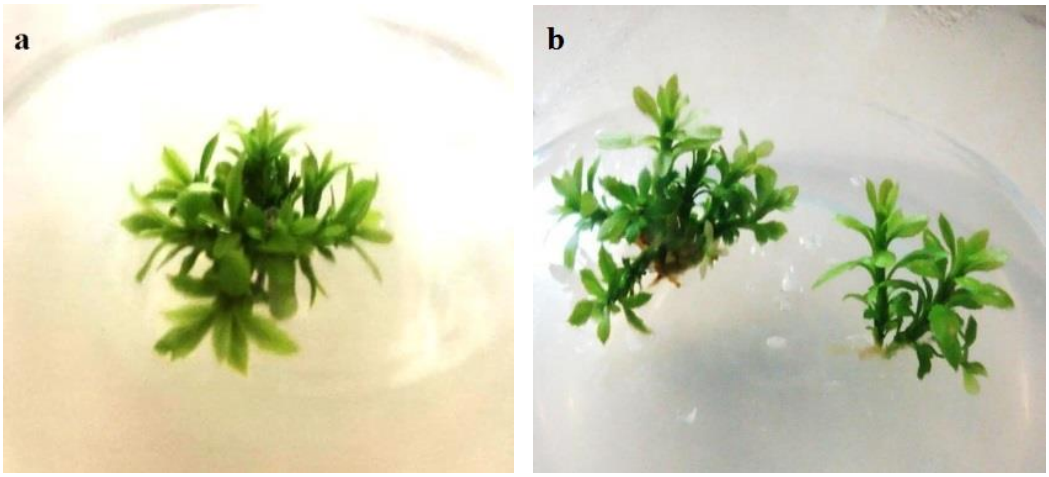

Figure 2. H. serrata cultured in the medium 1/4 MS supplemented with 1.0 mg/L kinetin after 90 culture days (**a**) and after 120 culture days (**b**)

The influence of BA and kinetin at suitable concentrations on the growth of *H. serrata* was also indicated in Table 2 and Table 3. The growth parameters such as the shoot multiplication rate and the shoot height of *H. serrata* cultured in the medium supplemented with 0.5 mg/L BA or 1.0 mg/L kinetin were not statistically different; however, the number of shoots/shoot multiplication of *H. serrata* cultured in the medium supplemented with 1.0 mg/L kinetin was higher significantly than that cultured in the medium supplemented with 0.5 mg/L BA. Therefore, the medium 1/4 MS supplemented with 1.0 mg/L kinetin was used for further experiments. The growth of *H. serrata* in the medium 1/4 MS supplemented with 1.0 mg/L kinetin after 90 culture days and 120 culture days was shown in Figure 2. Apart from BA and kinetin, other phytohormones have promoted the growth of the cultured *H. serrata* and other species of the genus *Huperzia*. Zhou et al. (2019) reported that the medium supplemented with 0.05 µmol/L IBA, 1.4 µmol/L KT, and 2 mg/L pyridoxine induced callus of the culture *H. serrata*. Yang

et al. (2021) reported that the best induction medium for the cultured explants of *H. serrata* was the Schenk and Hildebrandt (SH) medium supplemented with 0.5 mg/L NAA and 0.1 mg/L 2,4-Dichlorophenoxyacetic acids. Phan Xuan Binh Minh et al. (2018) reported that the medium MS supplemented with 0.3 mg/L BAP and 0.01 mg/L IBA induced the shoot growth of the cultured *H. serrata*. Le Thi Lan Anh et al. (2019b) reported that the medium ¼ MS supplemented with 1.0 mg/L IBA or 1.0 mg/L NAA promoted the root growth of cultured *H. serrata* shoots.

Determination of the content of HupA in the cultured *H. serrata* by HPLC

The content of HupA in the cultured *H. serrata* in our study was 300 µg/g dry weight (Fig. 3), which is higher than 3 times compared to the content of HupA in leaves of natural *H. serrata* (75.4–92.5 µg/g) (Vu Thi Ngoc et al., 2016). The content of HupA in the cultured *H. serrata* in our study has been also higher than compared to the content of HupA in natural *H. serrata* from previous studies. For example, the content of HupA in whole natural *H. serrata* collected in Da Lat (104.231 µg/g), Kon Tum (181.644 µg/g) (Chuong et al., 2016), in whole natural *H. serrata* in China (46.35–182.55 µg/g) (Ma et al., 2005). The higher HupA content in the *in vitro* cultured plants than natural counterparts has also been reported in species of the family Lycopodiaceae. Szypula et al. (2005) reported that the content of HupA in the cultured *H. selago* (3,330 µg/g) was 2.5 times higher than that in the natural *H. selago* (1270 µg/g), and Ma & Giang (2008) also reported that the content of HupA in the cultured *P. squarrosus* was higher than that in the natural *P. squarrosus*. The higher HupA content in the *in vitro* cultured *H. serrata* than natural counterparts could be explained by the fact that the *in vitro* cultured *H. serrata* samples were derived from the shoot tips of *H. serrata*. The shoot tips of this plant have been studied and reported to contain the highest levels of HupA compared to other parts of the plant (Lim et al., 2010). The successful *in vitro* propagation of *H. serrata* by the tissue culture method will contribute to enhancing the commercial value of this plant in the production of the active ingredient HupA for medicinal uses.

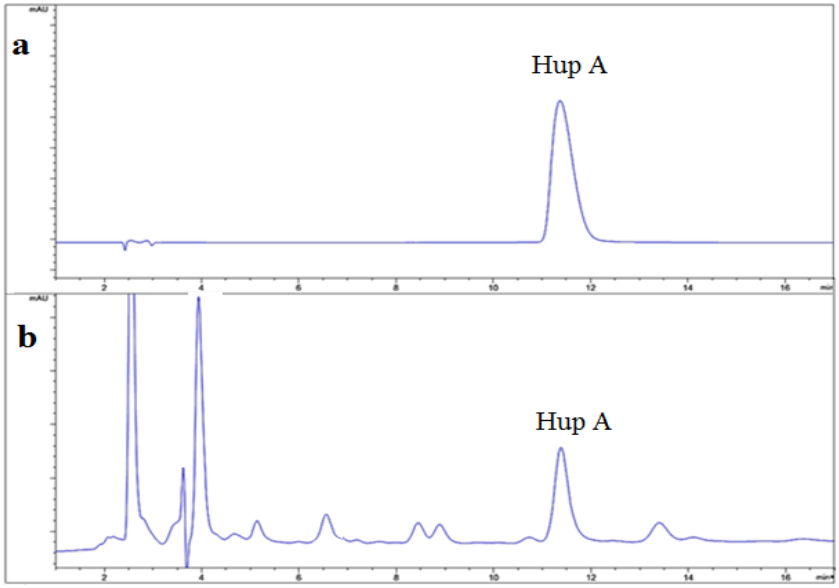

Figure 3. The HPLC chromatograms (at UV 310 nm) of HupA in the standard sample (**a**) and the cultured *H. serrata* (**b**)

CONCLUSION

The *H. serrata* collected was successfully propagated using the *in vitro* tissue culture method. The suitable nutrient medium for shoot multiplication of *H. serrata* was the 1/4 MS supplemented with 1 mg/L kinetin. The content of HupA in the *in vitro* cultured *H. serrata* was able to go up to 300 µg/g, which is higher than the content of HupA obtained from natural *H. serrata* samples reported from previous studies. Our research contributes to conserving *H. serrata* as well as enhancing the commercial value of this plant in the production of the active ingredient HupA.

Acknowledgements: The work was funded by the program on conservation and sustainable use of genetic resources from the Ministry of Science and Technology under the project "Exploitation and development of genetic resources of *Huperzia serrata Huperzia serrata* (Thunb. Ex Murray) Trevis in Sa Pa and Da Lat".

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