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In vitro production of huperzine A, a promising drug candidate for Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is growing in impact on human health. With no known cure, AD is one of the most expensive diseases in the world to treat. Huperzine A (HupA), a anti-AD drug candidate from the traditional Chinese medicine *Qian Ceng Ta* (*Huperzia serrata*), has been shown to be a powerful and selective inhibitor of acetylcholinesterase and has attracted widespread attention because of its unique pharmacological activities and low toxicity. As a result, HupA is becoming an important lead compound for drugs to treat AD. HupA is obtained naturally from very limited and slowly growing natural resources, members of the Huperziaceae. Unfortunately, the content of HupA is very low in the raw plant material. This has led to strong interest in developing sources of HupA. We have developed a method to propagate in vitro tissues of *Phlegmariurus squarrosus*, a member of the Huperziaceae, that produce high levels of HupA. The in vitro propagated tissues produce even higher levels of HupA than the natural plant, and may represent an excellent source for HupA.

1. Introduction

Alzheimer's disease (AD) is a neurologically degenerative disorder that affects more than 20 million people worldwide including 4.5 million Americans. This number is expected to double every 20 years (Sloane et al., 2002; Wimo et al., 2007) as world populations continue to age. AD is now considered to be the third major cause of death in developed countries, after cardiovascular disease and cancer (Jiang et al., 2003). The total worldwide cost of dementia care (AD is the most common form of dementia) is estimated to be US$315.4 billion annually, and 77% of these costs occurred in the world's more developed regions (Wimo et al., 2007). The course of AD often takes a decade or more to progress, bringing with it a severe load to patients, families and society. There is currently no known cure for AD. However, several drugs for treatment of AD symptoms have been approved by the US Food and Drug Administration (FDA) in recent years, all of which are acetylcholinesterase inhibitors (AChEIs), including tacrine (trade name: Cognex), donepezil (trade name: Aricept), rivastigmine (trade name: Exelon), and galanthamine (trade name: Reminyl) (Fig. 1). Unlike the other three FDA-approved AChEIs, galanthamine is a natural plant alkaloid, produced by *Galanthus nivalis* L. and related plants (Amaryllidaceae family) (Heinrich and Lee Teoh, 2004). A fifth compound with great promise for treatment of AD symptoms is the natural product huperzine A (HupA), a Lycopodium alkaloid derived from a traditional Chinese medicine, *Qian Ceng Ta* (*Huperzia serrata* (Thunb. ex Murray) Trev.; Fig. 2A) (Liu et al., 1986a, b).

HupA has attracted considerable attention worldwide because of its unique chemical structure, its AChEI activity, its memory-enhancing effects observed in both animal and clinic trials, and its low toxicity (Ma and Gang, 2004; Ma et al., 2007). HupA was approved in the 1990s in China as a drug to treat AD and is marketed in the USA as a dietary supplement (as powdered *H. serrata* in tablet or capsule format). Animal and clinical trials in China have shown that HupA is as effective as the drugs currently on the market for treatment of AD symptoms, but safer in terms of side effects (Ma and Gang, 2004; Wang et al., 2006). A new compound, ZT-1 (Fig. 1), appears to have a similar pharmacological profile to HupA but possesses more selective inhibition of AChE as well as even less toxicity than Hup A. ZT-1 was identified in a screen from hundreds derivatives of HupA by the Zhu group at the Shanghai Institute of Materia Medica (SIMM), Chinese Academy of Sciences. Experimental data demonstrated that ZT-1 possesses better anti-AChE activity than the other FDA approved cholinesterase inhibitors (Fig. 1) (Capancioni et al., 2006; Csajka et al., 2006; Ma et al., 2007; Orgogozo et al., 2006; Scalfaro et al., 2003, 2006; Zangara, 2003; Zangara et al., 2004, 2006). ZT-1 is being developed as a new anti-AD drug; and phase II clinical trials have been completed in both China and Europe (Ma et al., 2007).

H. serrata, a club moss and the original source of HupA, actually possesses a very low content of HupA (*ca.* 0.007%), has very limited distribution, and grows very slowly. It takes at least 15 years from spore germination through the gametophyte stage to finally reach

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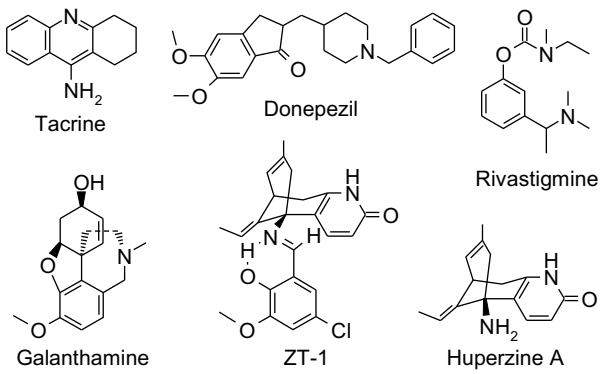

Fig. 1. Some FDA approved drugs used to treat Alzheimer's disease and the chemical structures of huperzine A (HupA) and ZT-1 (a HupA derivative).

the mature sporophyte stage (Fig. 2A), the tissue harvested for medicinal use (Ma and Gang, 2004). While some other species in the Huperziaceae produce larger amounts of HupA, these species are even more difficult to obtain and are much rarer in nature than *H. serrata*, making them even less desirable candidates as natural sources for HupA. So far, no successful commercial cultivation has been reported for *H. serrata* or other species in the Huperziaceae. Because of this, there is keen interest in developing alternative methods to produce HupA. The total synthesis of HupA has been reported (Qian and Ji, 1989; Xia and Kozikowski, 1989), and Kozikowski's group has optimized methods to synthesize HupA on a scale that could be industrialized (A. Kozikowski, personal communication). Nevertheless, wild populations of these plants will continue to be threatened and may soon become extinct if current harvest practices are not curtailed (Ma and Gang, 2004; Ma et al., 2005, 2006, 2007).

Because of these trends, in vitro propagation of plant material that produces HupA has attracted widespread interest in recent years. Unfortunately, efforts in this area have met only limited success. Several species of *Lycopodium* s. l., such as *L. clavatum*, *L. cernnum*, *L. annotinum*, *L. complanatum*, and *L. selago* were studied by Freeburg as targets for in vitro propagation in the 1950s and 1960s (Freeberg and Wetmore, 1957, 1967). Gametophyte growth of *L. lucidulum* and *H. selago*, spore germination of four species of *Huperzia* (*H. lucidula*, *H. crassa*, *H. cumingii*, and *H. saururus*) and three species of *Lycopodium* (*L. digitatum*, *L. obscurum*, and *L. clavatum*) in axenic cultures were reported by Whittier (Whittier, 1998; Whittier and Storchova, 2007; Whittier and Webster, 1986). A histological analysis of indirect somatic embryogenesis of *L. inundata* was reported more recently by (Atmane et al. (2000)). And, Waegel reported a fractional disinfection procedure and the effect of the growth regulators NAA (naphthaleneacetic acid) and BA-6 (benzyladenine) on *H. lucidulum* explants in vitro (Dwyer and Waegel, 2004; Waegel, 2001). Only one report, by Szypulaa et al.(2005), measured HupA content in the in vitro maintained plants (Szypulaa et al., 2005).

We evaluated a great variety of methods to realize our aim to establish in vitro propagation of *H. serrata* or taxonomically related species that contain HupA. Only plants from the genera *Huperzia* and *Phlegmariurus* produce HupA (Ma et al., 1998, 2005). As described in this manuscript, we succeeded in developing a method for the in vitro propagation of *Phlegmariurus squarrosus* (Forst.) Löve et Löve. Prothalli and sporophytes were successfully produced from in vitro cultures of *Ph. squarrosus* after 6 months to 1 year of culture. These cultured plants are now growing well and produce large amounts of HupA compared to wild-grown plants, providing an invaluable resource for production of HupA that is both renewable and natural.

2. Results and discussion

2.1. In vitro propagation of Phlegmariurus squarrosus

It is extremely difficult to get sterilized explants and to propagate HupA source plants in vitro. One of us, Ma, tried a great variety of methods to realize the in vitro propagation of *H. serrata* and taxonomically related species that contain HupA in the laboratory of Professor Dr. E. Wellmann (Freiburg, Germany). Fortunately, we were able to initiate propagation of *Ph. squarrosus* (Forst.) Löve et Löve by treating spores with acid and other reagents (patent has been applied for in Germany by Wellmann and Ma) that eventually led to their germination. As described in Section 3, the calli and prothalli used for this investigation were originally from these germinating spores (Fig. 2C–E). When the calli and prothalli were large enough to handle, they were transferred to M2 medium (see Section 3). They were then transferred from time-to-time, when their size warranted change, to M3 and M4 media for tests to optimize growth regulator concentrations and root formation or to M5 and M6 media for liquid culture.

Different conditions for growth were evaluated by combining plant growth regulators, such as IAA, NAA, ZT, GA3, and CM, together in M3 test medium (see Table 1). Our results showed that when the temperature was 25 °C, prothallus cultures grew better at pH 5 or pH 5.5 than they did at pH 5.8, and when the pH was 5.8 they grew faster at 20 °C or 22 °C. This result indicates that lower temperatures (~20–22 °C) and pH (5–5.5) should be used for *Ph. squarrosus* prothallus in vitro cultures. Meanwhile, NAA (0.5 mg/L) and ZT (0.5 mg/L) were found to be more efficacious compared to the control and IAA (0.5 mg/L) at inducing growth in culture.

M4 medium was used for prothallus root induction and sporophyte (plantlet) formation. Prothallus cultures in M4 medium were maintained in a 25 °C incubator with a photoperiod of 12 h of white light (15 wm^2). Chlorophyll was produced in the outermost cells of the prothallus after 3 months. These outermost cells appeared to be slightly green and tiny green gemmae differentiated from the top of the prothallus branch. Four months later, a green sporophyte (plantlet) with roots had developed as shown in Fig. 2C. Prothallus also differentiated into sporophytes when prothallus cultures were placed in darkness. These sporophytes, however, did not produce chlorophyll.

M5 and M6 media were used for callus and prothallus liquid culture. So far, this approach has produced fast growing callus and prothallus compared with cultures on M2 solid medium and the intact plant growing in nature, which requires more than 15 years from spore germination to mature sporophyte development (see Fig. 2A, Table 2).

2.2. Production of HupA in in vitro propagated Phlegmariurus squarrosus

In order for these in vitro propagated *Ph. squarrosus* plants to be of value as a feed stock or as a starting point for industrial scale production of HupA, they must produce this compound in significant amount. Thus, we were very interested in determining the content of HupA in the tissue of these propagated plants as compared to plant material collected from the wild. We developed an LC-PDA-MS/MS analytical method to determine HupA content, and analyzed metabolite extracts from the in vitro propagated plants and from wild collected plants.

Resolution of HupA from other components in the extracts was achieved using conditions described in Section 3. Identification of HupA in the samples was achieved by comparing retention time, UV spectra, MS spectra and MS/MS spectra against a HupA

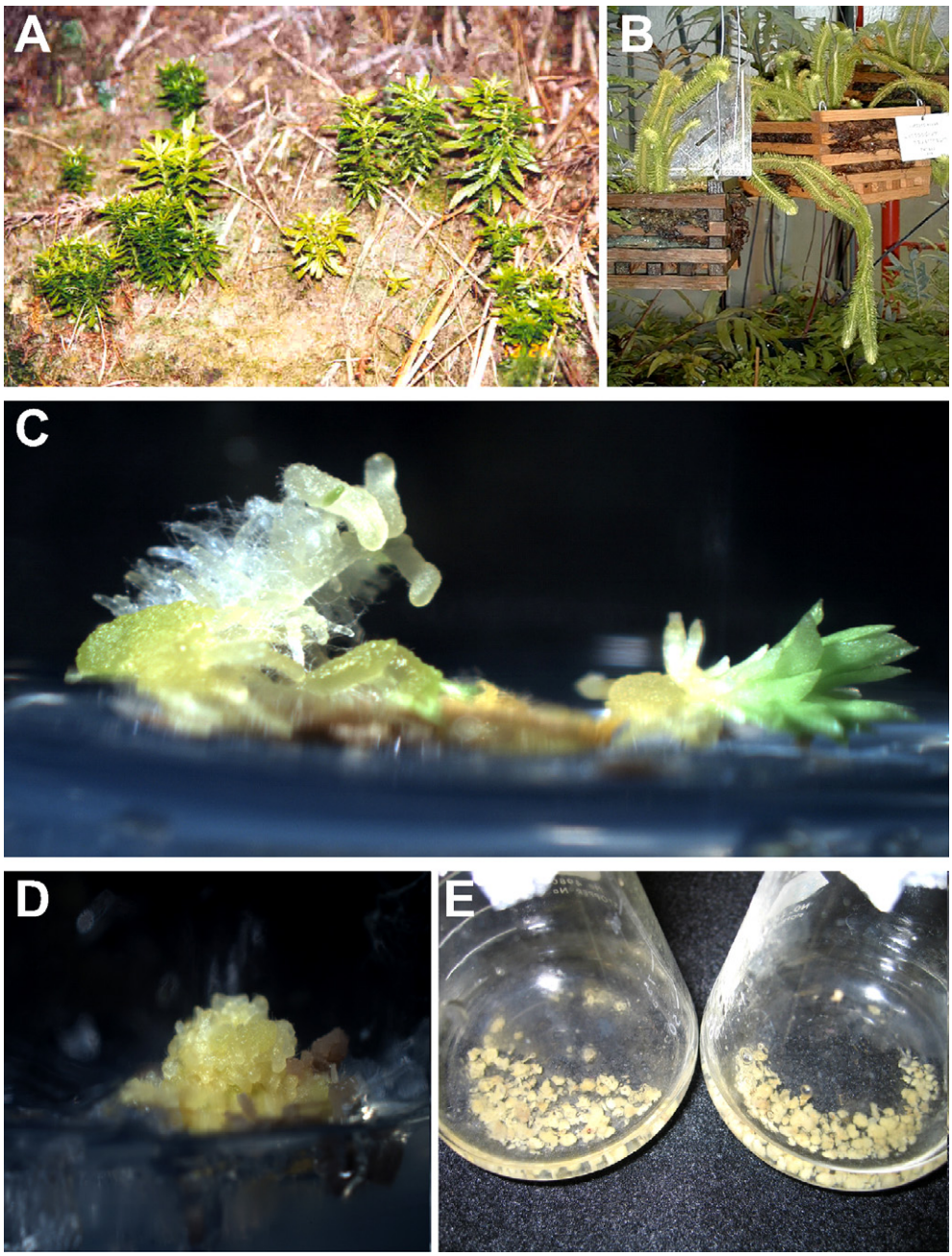

Fig. 2. Pictures of (A) *Huperzia serrata* growing in the wild. Note that these plants are over 15 years old (at least) and yet reach a height of less than 10 cm; (B) *Phlegmariurus squarrosus* growing in the greenhouse of Universität Freiburg; (C) *Ph. squarrosus* in vitro propagated prothalli and sporophyte; (D) *Ph. squarrosus* in vitro propagated callus and (E) *Ph. squarrosus* in vitro rapid callus liquid cultures. All photos taken by the authors.

Table 1
Growth of *Phlegmariurus squarrosus* prothallus on M3 control and test media*

Group	Mean growth (mg ± SE)					*N*
	Temperature (°C) (pH 5.8)			pH (25 °C)		
	20	22	25	5.0	5.5	
Control	3.33 ± 0.40	3.30 ± 0.44	2.87 ± 0.35	4.13 ± 0.25	4.07 ± 0.42	3
IAA (0.5 mg/L)	4.33 ± 0.25	4.27 ± 0.47	3.70 ± 0.56	5.60 ± 0.27	5.57 ± 0.40	3
NAA (0.5 mg/L)	5.93 ± 0.45	5.87 ± 0.35	4.73 ± 0.35	6.83 ± 0.71	6.53 ± 0.80	3
ZT (0.5 mL/L)	6.80 ± 0.36	6.60 ± 0.36	5.80 ± 0.40	7.43 ± 0.55	7.33 ± 0.45	3

Measurements were made before and after a 2-week-growth period, then means and standard error (SE) were calculated.
* All mean growth values are fresh weight, all media contained GA3 (0.25 mg/L) + CM (20 mL/L), all cultures were grown in the dark, and all initial fresh weights were 20 mg.

Table 2
Mean growth of *Phlegmariurus squarrosus* callus and prothallus in vitro cultures after 3 weeks on media containing 2,4-D (10 mg/L), 2,4-D (5 mg/L) and 2,4-D (5 mg/L) + NAA (0.5 mg/L), respectively, and HupA content from intact plant and these tissue cultures*

Samples	Mean growth (mg ± SE)*	HupA (μg/g ± SE)**	N
Intact plant (in greenhouse)	N/A	24.12 ± 0.11	3
callus (solid medium, M2)	7.47 ± 0.70	434.73 ± 0.2	3
callus (liquid medium, M5)	12.8 ± 0.70	675.69 ± 0.29	3
prothallus (solid medium, M2)	4.57 ± 0.86	312.46 ± 0.24	3
prothallus (liquid medium, M6)	9.53 ± 0.38	546.07 ± 0.18	3

* All tissue cultures were grown in the dark, 25 °C, and all initial fresh weights were 20 mg.
** All material weights used for calculation are dried weight.

standard. As shown in Fig. 3, this method is able to detect and verify the presence of HupA in tissues from wild collected plants and from in vitro propagated plants. The UV and MS/MS spectra of the major alkaloid peak in the extracts of the in vitro propagated tissues matched those of an authentic HupA standard (see Fig. 3). Furthermore, we found, to our surprise and delight, that the HupA content of *Ph. squarrosus* propagated on solid media (Table 2) was comparable to that from plants collected from nature in Xishuangbanna, Yunnan, China (378.83 ± 0.33 μg g^{-1}) (Ma et al., 2005) and significantly higher than from the parent plant maintained in the greenhouse (Table 2). Similar findings have been observed in other plant culture systems (Misawa, 1994), such as for the production of ginsenosides by *Panax ginseng* (Ushiyama, 1991), of rosmarinic acid by *Coleus blumei* (Ulbrich et al., 1985), of shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), of diosgenin by *Dioscorea* (Tal et al., 1984), and of ubiquinone-10 by *Nicotiana tabacum* (Ikeda et al., 1976). Because HupA-producing plants are now threatened with extinction in nature, having a source of the plant material that produces the compounds at comparable or higher levels than the native plants will enable production of HupA from a natural source (i.e., non-chemically synthesized, which is very desirable to a large percentage of the population) while protecting native populations (Ma and Gang, 2004; Ma et al., 2005, 2006, 2007).

2.3. Biosynthetic intermediates to HupA identified in in vitro propagated *Ph. squarrosus* callus and prothalli

In addition to HupA, other Lycopodium alkaloids were identified in extracts from in vitro propagated *Ph. squarrosus* tissues. These included huperzine B, des-*N*-methyl-β-obscurine, α-obscurine and β-obscurine (Fig. 4), among others. The presence of these compounds in these tissues supports the proposed pathway for the biosynthesis of HupA (Ma and Gang, 2004), see Fig. 5. Thus, it appears that the in vitro propagated tissues are ideal models to study the biosynthetic pathways of HupA and related Lycopodium alkaloids.

In conclusion, we succeeded in the in vitro propagation of a HupA source plant, *Ph. squarrosus*, in regeneration of prothallus, sporophyte, and callus cultures, both on solid and liquid media. These plant tissues are now growing very well, and will provide an invaluable resource for production of HupA.

3. Experimental

3.1. Plant material

The original plant material *Ph. squarrosus* was from a greenhouse at the Universität Freiburg, Germany (Fig. 2B). Dried plant material and different types of in vitro micropropagated cultures of *Ph. squarrosus* on solid media were provided by Prof. Dr. E. Wellmann (Universität Freiburg). These in vitro propagated materials are used as "seeds" for the experiments performed in this investigation.

3.2. Chemicals and standard compounds

Gamborg's B-5 basal medium with minimal organics (B_5), Phytagel, GA3 (gibberellic acid-3), IAA (indole-3-acetic acid), NAA (naphthaleneacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), AA (AA (ascorbic acid), ZT (zeatin riboside, trans isomer), and CM (coconut

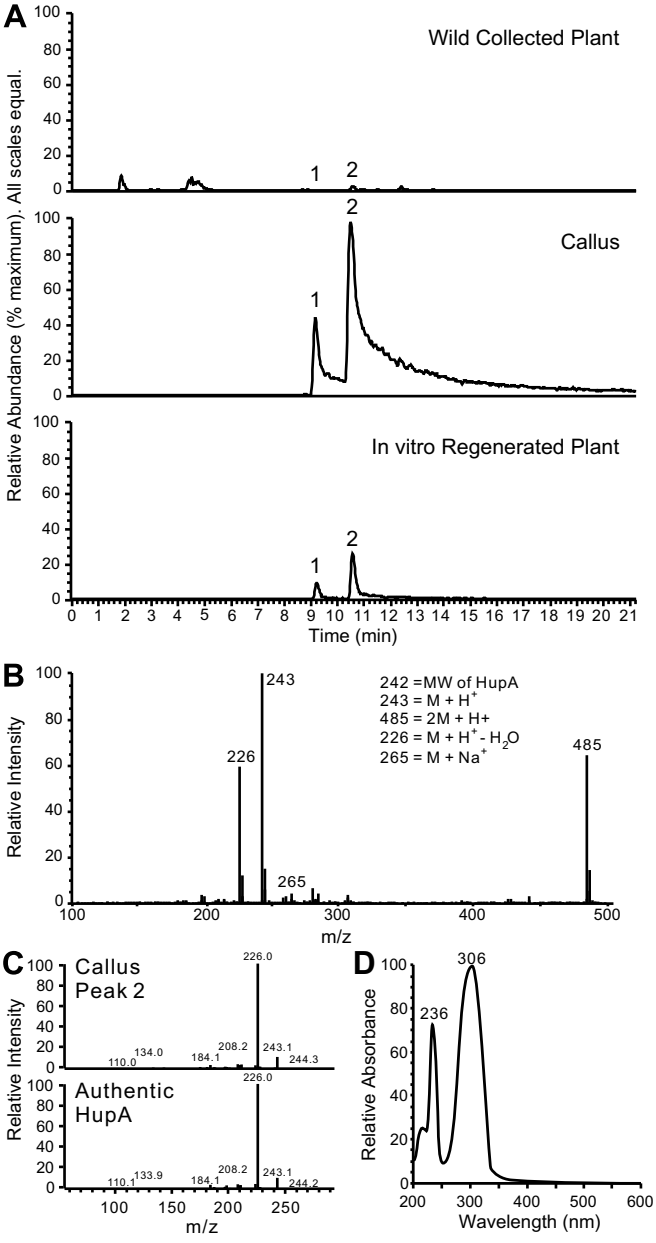

Fig. 3. LC-MS/MS analysis of extracts from wild collected and in vitro propagated *Ph. squarrosus* plants demonstrate that the propagated plants produce high levels of HupA. Equivalent sample masses, based on dried leaf weight, were injected into the HPLC. (A) Selected ion chromatograms for the M+1 peak (*m*/*z* 243) of HupA. (B) Mass spectrum of the largest peak (2) in chromatograms. (C) MS/MS spectrum of largest peak (2) in chromatograms compared to MS/MS spectrum of authentic HupA. (D) UV Spectrum of peak 2. This spectrum matches that of an authentic standard of HupA.

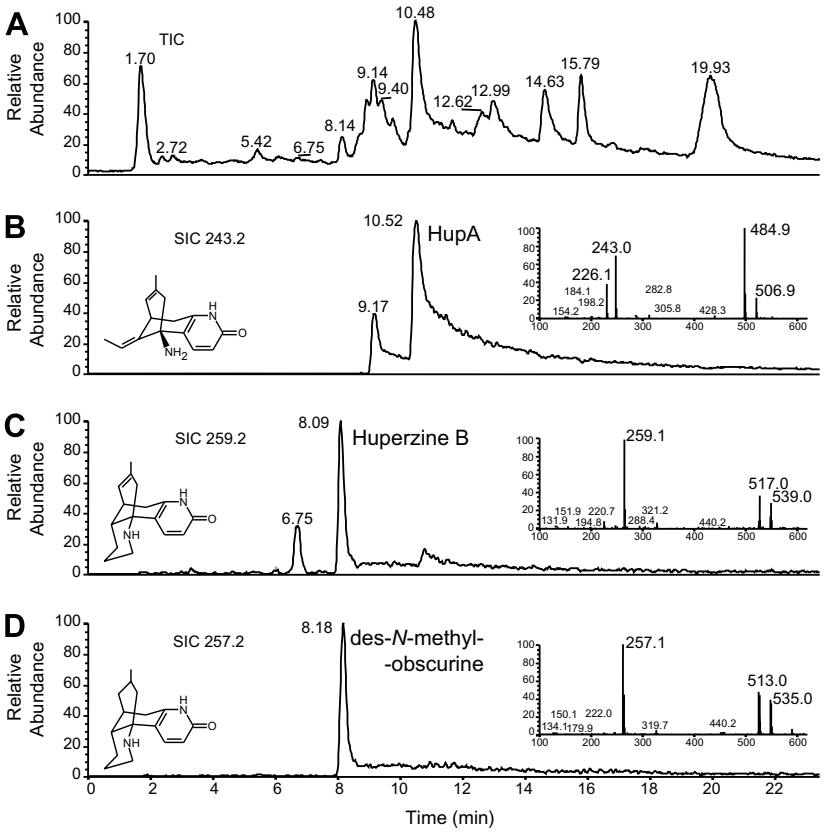

Fig. 4. LC-MS analysis of major Lycopodium alkaloids produced by *Ph. squarrosus* callus supports the proposed biosynthetic pathway outlined in Fig. 5. (A) Total ion chromatogram (TIC) of callus derived extract, analyzed as described for Fig. 3. (B) Selected ion chromatogram (SIC) for M+1 (=243.2) of HupA and corresponding mass spectrum for HupA peak in callus extract at 10.52 min. Peak assignments are given in Fig. 3B. (C) SIC for M+1 (=259.2) of Huperzine B and corresponding mass spectrum for Huperzine B peak at 8.09 min. Peak assignments: 259.1 = $M+H^+$; 517.0 = 2 $M+H^+$; 539.0 = 2 $M+Na^+$. (D) SIC for M+1 (=257.2) of des-*N*-methyl-β-obscurine and corresponding mass spectrum for des-*N*-methyl-β-obscurine peak at 8.18 min. Peak assignments: 257.1 = $M+H^+$; 513.0 = 2 $M+H^+$; 535.0 = 2 $M+Na^+$. α-Obscurine and β-obscurine were also found in this extract (data not shown).

milk) were purchased from Sigma (St Louis, MO, USA). Authentic HupA and huperzine B used as standard compounds were gifts from Prof. Zhu (SIMM, Chinese Academy of Sciences, China).

3.3. Tissue culture media preparation

Medium for spore germination (M1): half strength B_5 containing sucrose (1%) and Phytagel (0.8%), pH 5.8. Medium for calli and prothalli growing (M2): B_5 containing sucrose (2%), 2,4-D (10 mg/L), and Phytagel (0.8%), pH 5.8. Medium for test regulators (M3): B_5 containing sucrose (1%) and phytagel (0.5%) with different concentrations of IAA, NAA, GA3, and ZT with CM (20 mL/L), pH 5.0–6.0. This medium without growth regulators (basal M3 medium) served as control. Medium for root induction (M4): three-quarter strength B_5 containing sucrose (1%), NAA (0.5 mg/L), AA (100 mg/L) and phytagel (0.5%), pH 5.5. Medium for calli liquid culture (M5): B_5 containing sucrose (2%) with 2,4-D (5 mg/L), pH 5.8. Medium for prothalli liquid culture (M6): B_5 containing sucrose (2%) with 2,4-D (5 mg/L) and NAA (0.5 mg/L), pH 5.8.

3.4. Explant sterilization and in vitro propagation

The in vitro initiated cultures of *Ph. squarrosus* provided by E. Wellmann were first placed on M1 medium. When the resulting prothalli or calli were large enough to handle, they were transferred to M2 medium and then transferred from time-to-time as their increase in size warranted change to larger tubes and fresh medium or transferred into liquid medium for suspension culture initiation.

Production of prothalli cultures was attempted under different temperatures and photoperiods by combining GA3, ZT, IAA, NAA, and CM together in M3 medium (see Table 1). Induction of root formation in prothalli cultures was attempted in M4 medium. Rapid callus and prothallus liquid culture was performed at 25 °C in the dark on a rotory shaker at 140 rpm in 25, 50, 100, or 150 mL flasks containing 5, 10, 20, or 30 mL of M5 medium with varying pH and concentrations of 2,4-D. The flask size and volume of M5 medium depended on the size of the inoculating callus. The medium was changed every 2 weeks. The inoculum size was about 0.1, 0.2, 0.4, and 0.6 g fresh weight for 5, 10, 20, and 30 mL M5 medium, respectively. These experiments were repeated in triplicate.

3.5. Standard preparation

Stock solutions of HupA standard were prepared at a concentration of 1 mg/mL. Given the expected HupA concentration of the samples to be analyzed, a six-point calibration curve was prepared using diluted standard HupA at concentrations ranging from 0.01 μg/mL to 1 μg/mL. Samples were diluted as needed to fall within this calibration curve range.

3.6. LC-ESI-MS/MS analysis of HupA content of in vitro propagated tissues

Extracts of naturally and in vitro propagated specimens were obtained using the tartaric acid method previously described (Ma et al., 2005), but scaled down for small sample sizes. Briefly, 20 mg freeze dried tissue powder was extracted three times in a 10 mL vial by

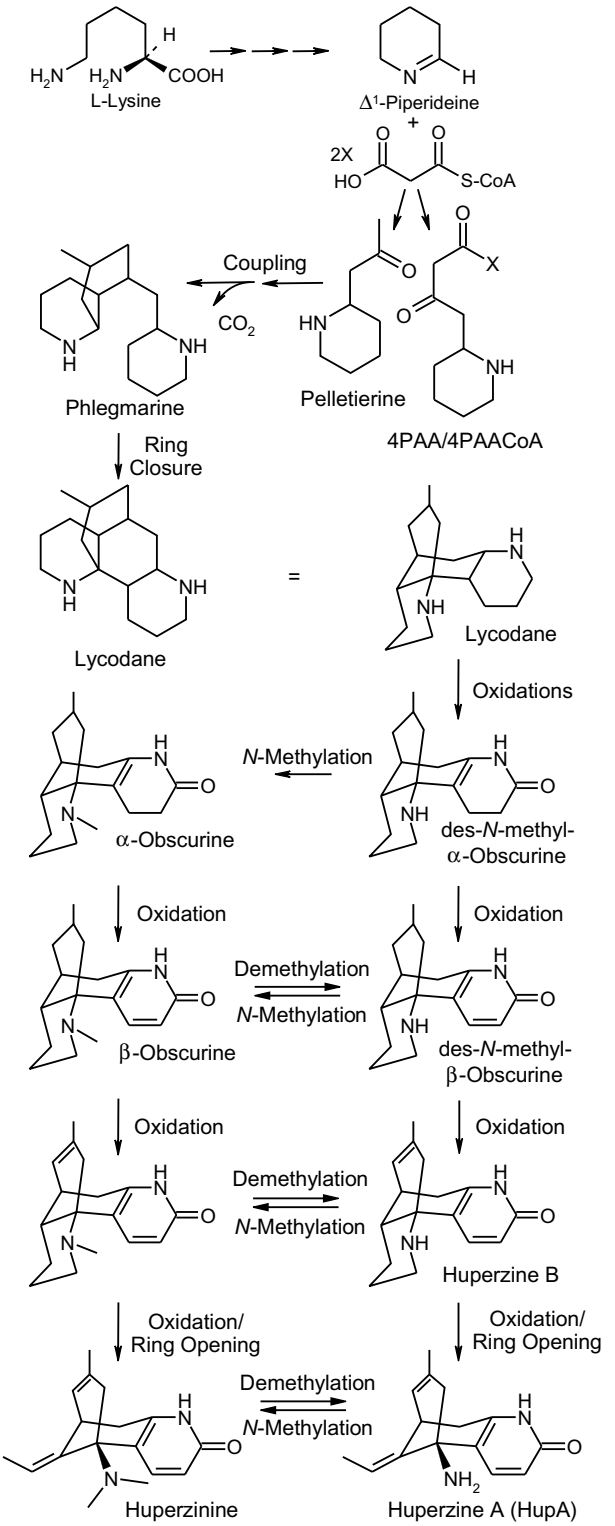

Fig. 5. Proposed biosynthetic pathway to huperzine A and other related lycopodium alkaloids in members of the Huperziaceae.

adding 5 mL of 2% tartaric acid and sonicating for 30 min in a water sonication bath. After filtration over Whatman #1 paper, concentration, extraction with $CHCl_3$ (discarded), adjustment to pH > 10 with aqueous ammonia, and extraction five times with $CHCl_3$, the combined $CHCl_3$ extracts were filtered over Whatman #3 paper, evaporated to dryness under dry N_2, dissolved in 1 mL of HPLC grade methanol, filtered through a 0.45-μm Millipore (Polytetrafluoroethylene filter (PTFE, 0.45 μm)) filter, and subjected to analysis by LC-ESI-MS/MS analysis coupled to photodiode array detection. Triplicate extractions were performed for each tissue type. Statistical analysis was performed using Microsoft Office Excel 2003.

Instrument: ThermoFinnigan Surveyor HPLC system with MS pump and LCQ Advantage mass spectrometer equipped with a Discovery® HS C18 column, 2.1 × 150 mm, i.d., 3 μm, with guard column (Supelco, Bellefonte, PA, USA), a photodiode array (PDA) detector, and an electrospray ion source. The elution conditions were as follows: flow rate 0.25 mL/min; column temperature, 40 °C; injection volume, 5 μL. The solvent system used was isocratic: methanol:buffer, 35:65 v/v (buffer: 0.08 M ammonium acetate–acetic acid buffer, pH = 6.0). The acquisition parameters for MS were: positive and negative mode; drying N_2 temperature, 350 °C, 10 L/min; nebulizer pressure 60 psi; HV capillary 4500 V; HV end plate offset −500 V; current capillary 65.9 nA (positive mode), 62.3 nA (negative mode); current end plate 1482.7 nA (positive mode), 1378.7 nA (negative mode); RF amplitude capillary exit 99.3 V (positive mode), −99.3 V (negative mode); skimmer 40.0 V (positive mode), −40.0 V (negative mode); Mass range measured: 50–900 *m*/*z*. Xcalibur (version 1.4) system was used as instrument control and data processing platform. The standard curve used for HupA content determination was produced using the linear least-squares regression equation derived from the peak area in the MS TIC. Identification of these peaks was achieved by comparing retention times and spectra against known standards.

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