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In vitro Induction of Axillary Turion Production in *Hydrilla Verticillata* with the Potential for Short-Term Storage via Alginate Encapsulation

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ABSTRACT

Hydrilla verticillata is an aquatic macrophyte that has attracted increasing attention due to its potential applications in ecological management and pharmaceutical research. To advance these applications, cultivating this plant using efficient large-scale operations under controlled conditions and germplasm storage technology, such as plant tissue culturing, are much more desirable than harvesting it from nature, which also has the potential to cause environmental damage. However, information regarding the tissue culture of this submerged vascular plant remains limited. Therefore, the present study aimed to establish an efficient in vitro stock culture system that could subsequently be utilized to investigate the effects of in vitro culture conditions, as well as the potential for synthetic seed production and short-term storage. In vitro cultivation of *H. verticillata* was successfully achieved using basal liquid Murashige and Skoog (MS) medium. The addition of either benzyladenine or paclobutrazol to the medium resulted in reduced shoot elongation and decreased lateral branching. Notably, when explants cultured in basal liquid MS basal medium were transferred to semi-solid MS medium, the development of axillary turions was observed in 55% of the jars, compared to 0% in those that remained submerged in the liquid medium, suggesting that water stress, reduced direct availability of nutrients and improved aeration conditions may stimulate axillary turion formation in vitro. Furthermore, these axillary turions were found to be a suitable explant source for synthetic seed production and enhanced plant survival during low-temperature storage compared with synthetic seeds derived from shoot tissues. The findings of this study provide a useful approach for *H. verticillata* germplasm conservation and facilitate the transportation of plant materials.

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1. Introduction

Hydrilla verticillata (L.f.) Royle, commonly called hydrilla or water thyme, is a perennially submerged vascular plant belonging to the family Hydrocharitaceae, typically inhabiting freshwater ponds and lakes. As a rapidly proliferating aquatic macrophyte, this species has garnered considerable attention for its ecological applications, particularly its capacity to accumulate heavy metals and metalloids from contaminated aquatic environments, positioning it as a viable candidate for phytoremediation strategies [1, 2]. Beyond its role in water quality restoration, *H. verticillata* has demonstrated utility as a soil amendment, contributing to improvements across the physical, chemical, and biological dimensions of soil health. Additionally, *H. verticillata* is a fast-growing, high biomass accumulating aquatic plant that is rich in protein and nitrogen, could be of economic importance as it may be an excellent, nutrient-dense biomass for making compost or producing biogas [3]. Notably, bioactive extracts derived from this plant have been reported to exert pronounced anti-adipogenic effects, primarily through the suppression of intracellular lipid accumulation and the inhibition of lipase activity. *H. verticillata* is also rich in some secondary metabolites with potent antioxidant and antimicrobial properties which are of pharmaceutical interest. These findings collectively suggest that *H. verticillata* represents a promising natural resource for the development of therapeutic agents targeting obesity and its associated metabolic complications [4–6]. To advance these large-scale practical applications, technology for large-scale cultivation such as plant tissue culture biotechnology under controlled conditions would be required.

Within its life cycle, *H. verticillata* reproduces predominantly through vegetative means, employing three principal mechanisms: axillary turion, subterranean tuber formation, and stem fragmentation [7, 8]. Both turions and tubers function as dormant vegetative propagules, yet they originate from distinct locations on the plant. Axillary turions arise from the axils of leaves or lateral branches along submerged stems, whereas subterranean tubers develop at the terminal nodes of belowground rhizomes. Morphologically, the two propagule types differ considerably in size; tubers are notably larger, typically measuring 4-15 mm in length and ranging in coloration from off-white to nearly black, while turions present as compact, green shoots, generally not exceeding 3-12 mm in length. During the course of natural asexual propagation, physical detachment of these vegetative propagules from the parent plant is a prerequisite for subsequent growth and establishment. Subterranean tubers are released following the senescence and degradation of the rhizome, whereas axillary turions undergo a more structured separation process, involving the formation of a distinct abscission zone prior to their detachment and deposition onto the substrate, where they subsequently germinate and develop into independent plants [9, 10].

Plant tissue culture represents a core biotechnological approach that facilitates the rapid, aseptic, and large-scale production of clonal plants, particularly for applications in agriculture and biodiversity conservation. This method overcomes several limitations associated with conventional propagation techniques, such as lengthy multiplication cycles, low propagation efficiency, vulnerability to pathogens and insect pests, and dependence on seasonal conditions [11–13]. Under in vitro conditions, plants grown in liquid culture may encounter physiological stress when transferred to a semi-solid medium. Consequently, examining plant responses following subculture onto different media under such conditions is of considerable

interest. Investigations of stress responses in tissue-cultured plants provide a valuable platform for identifying genotypes with enhanced tolerance to abiotic stresses, including drought and salinity, as well as for promoting the development of disease resistance in a controlled, sterile environment [14, 15].

Benzyladenine (BA) is one of the most widely used plant hormones for micropropagation. It helps plants grow new shoots rapidly from tiny hidden buds. Therefore, it is interesting to study its effect on growth and branching of *H. verticillata*. Also, *H. verticillata* has been previously cultured in basal MS medium supplemented with 0.25 and 0.5 mg/L paclobutrazol (PBZ) under in vitro monoculture or polyculture conditions [16]. In this experiment, the PBZ concentration was increased to 4 mg/L to investigate its effects on growth and branching of this plant.

In vitro short-term storage constitutes a practical application of plant tissue culture for maintaining diverse types of plant germplasm, including shoots, shoot tips, nodal segments, axillary buds, and embryos, over a limited period without the need for frequent subculturing at reduced temperatures. This active conservation approach supports the preservation of plant materials while simultaneously lowering operational costs related to media components, laboratory space, and manpower, and minimizes the risk of microbial contamination, thereby facilitating safer germplasm exchange [17–19]. Moreover, advances in storage technologies have highlighted the alginate encapsulation method used in synthetic seed production as an effective strategy for the short-term maintenance of plant propagules under sterile conditions [20–22].

To date, explants from a wide range of plant species have been utilized for synthetic seed production; however, reports involving aquatic plants remain rare. Only a few species have been documented, including *Rotala malabarica* using shoot tip explants, and *Cryptocoryne lutea* together with *Rotala rotundifolia* employing nodal segments [23–25]. In light of the limited data and scarce evidence regarding the tissue culture of *H. verticillata*, and the feasibility for producing synthetic seeds from in vitro-derived axillary turions for short-term storage, the present study was designed to fill these knowledge gaps. Specifically, this research investigated the effects of pH, BA, PBZ, and change in culture conditions from liquid to semi-solid medium on the in vitro cultivation of *H. verticillata*, which could serve as a reliable source of axillary turion explants for synthetic seed production intended for short-term preservation.

2. Materials and Methods

2.1 Plant materials and surface sterilization

Healthy and robust *H. verticillata* plants were randomly chosen from the nursery at the Faculty of Applied Science, King Mongkut's University of Technology North Bangkok. Twenty shoots, each approximately 1.5 cm in length, were excised and thoroughly washed under clean tap water five times each for 10 min (i.e., a total of 50 min). During the second and third washes, 10% (v/v) St. Andrews Vegetable and Fruit Washing Liquid (Lion Company, Thailand) was added to aid in removing debris. The shoots were then surface-sterilized by immersion in a 15% (v/v) Clorox® solution (containing 5.25% sodium hypochlorite) with 3-4 drops of Tween 20 for 2 min. Following this, they were rinsed with sterile distilled water for 3 min, submerged again in the 15% (v/v) Clorox® solution for 2 min, and rinsed once more with sterile distilled water for 3 min. This sterilization step was repeated one final time by soaking the explants in the 15% (v/v) Clorox® solution for 2 min, followed by three successive rinses with sterile distilled water, each lasting 3 min. Finally, the disinfected explants were transferred into basal Murashige and Skoog (MS) liquid medium [26] to establish the stock plants for further culture and experiments. Using this surface sterilization protocol, it was possible to obtain 100% survival of the explants without any

contamination. All the cultures in this study, if not specified, were kept in a growth room at a constant temperature of 25±2°C for 16 h per day, with 8 h of darkness in between, under regulated light conditions (20.87 μmol/m^2/s). Also, the size of all tissue culture containers used in this research was 5 cm diameter × 8.5 cm height, each containing 40 mL MS medium.

2.2 Effects of varying pH on growth and branching of in vitro cultured *H. verticillata*

The effects of varying pH levels on the in vitro cultivation of *H. verticillata* were investigated using the following methods. Shoot explants approximately 1.5 cm in length were excised from 3-week-old aseptically grown *H. verticillata* mother plants and subsequently introduced into basal MS liquid medium adjusted to one of four pH levels (2, 4, 5.7, and 7). There were 10 replicates in each pH treatment with a single explant cultured in a container. All cultures were maintained under the growth conditions outlined in Section 2.1 for 4 weeks, and shoot elongation and axillary branch development were then recorded.

2.3 Effects of BA or PBZ on growth and branching of in vitro cultured *H. verticillata*

Shoot and nodal segments of about 1.5 cm in length were excised from 3-week-old *H. verticillata* stock plants that had been established under aseptic conditions to evaluate the influence of BA or PBZ on in vitro growth. The explants were cultured in basal MS liquid medium supplemented with BA at concentrations of 2, 4, or 6 mg/L, or PBZ at concentrations of 0.5, 1, 2, or 4 mg/L. Explants maintained in hormone-free liquid MS medium served as the control treatment. There were 10 replicates in each treatment with a single explant placed in each culture vessel. Following 4 weeks of incubation under the conditions described in Section 2.1, axillary shoot proliferation and shoot length were recorded.

2.4 Effects of change in culture conditions on axillary turion production of in vitro cultured *H. verticillata*

Shoots approximately 1.5 cm in length were excised from 3-week-old *H. verticillata* stock plants and transferred into basal liquid MS medium or placed vertically (Fig. 1) on semi-solid basal MS medium gelled with 0.8% (w/v) agar. Each culture container had one explant, with 20 replicates in each treatment. The cultures were then kept in a growth room for 25 days under the conditions outlined in Section 2.1 in order to observe the effects of culture conditions on the growth of *H. verticillata* cultured in vitro.



Fig. 1. *H. verticillata* shoot placed vertically on basal semi-solid MS medium.

2.5 Production of synthetic seed from shoot and axillary turion of in vitro cultured *H. verticillata*

Synthetic seed production followed a modified protocol originally described by Sukthavornthum et al. [27]. Briefly, shoots or axillary turions approximately 1.5 cm in length were obtained from axenic cultures of *H. verticillata* and individually immersed in a 3% (w/v) sodium alginate solution prepared in basal liquid MS medium. Each explant was then carefully aspirated using a 1,000 μL sterile pipette equipped with a micropipette tip trimmed to an internal diameter of 7 mm, and subsequently dispensed dropwise into a cooled 2% (w/v) $CaCl_2 \cdot 2H_2O$ solution which was

also prepared in basal liquid MS medium. The resulting encapsulated beads were subjected to gentle agitation for 30 min to ensure complete polymerization, after which the synthetic seeds were washed three times with sterile distilled water before subsequent storage treatments.

2.6 In vitro short-term storage of shoot and axillary turion synthetic seeds of *H. verticillata*

Synthetic seeds derived from *H. verticillata* axillary turions were stored at temperatures of 4°C and 25°C for a duration of 7 days. In contrast, synthetic seeds originating from *H. verticillata* shoots were stored solely at 25°C for the same period, serving as the control group. Subsequently, the alginate-encapsulated axillary turions and shoots were cultured on a semi-solid basal MS medium gelled with 0.8% (w/v) agar for 21 days. A single encapsulated bead was placed in a culture vessel (5 × 8.5 cm^2) in the dark, with 15 replicates in each treatment. Following the incubation period, germination rates and total germination counts were recorded. A synthetic seed was considered germinated when there was growth that protruded through the artificial coat encapsulating the shoot or axillary turion.

2.7 Statistical analysis

In this research, the experiments were conducted using a completely randomized design (CRD). Data on explant length, number of branches, and germination rates were subjected to analysis of variance (ANOVA) at a 95% confidence level. When significant differences were detected, mean comparisons were performed using Duncan's multiple range test (DMRT) at the same significance level. For the analysis of data in Table 4, an independent samples t-test for the number of jars containing turions and number of turions produced was carried out. All statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS).

3. Results and Discussion

3.1 Stock plant establishment

After surface sterilization and culturing of *H. verticillata* shoot explants in basal liquid MS medium, all the explants initially appeared pale whitish but gradually developed a green coloration within the first week of culture (Figs. 2a and 2b). Vigorous shoot elongation was observed during the second week, followed by pronounced shoot proliferation in the third week (Figs. 2c and 2d), providing a suitable source of material for subsequent experiments. These observations demonstrate that the surface sterilization procedure for *H. verticillata* shoots was effective, despite the temporary loss of green pigmentation immediately after the disinfection process (Fig. 2a).

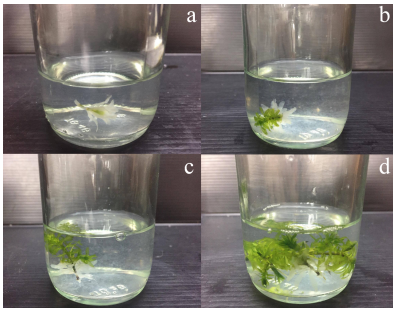


Fig. 2. Growth characteristics of *H. verticillata* shoot after surface disinfestation in basal liquid MS medium for 3 weeks (a = week 0, b = week 1, c = week 2, and d = week 3).

Surface sterilization is a prerequisite for obtaining axenic explants for in vitro culture. Many aquatic plant species may require different surface disinfection methods to achieve surface sterilization of their explants. For example, shoot tips of *Bucephalandra* sp. 'Wavy Dark Green' treated with 0.05-0.2% mercuric chloride alone for 10 min was effective in achieving 100% survival and no contamination [28]. The survival rate of nodal stem explants of *Nymphoides coronata* was high after decontamination with 75% ethanol for 30 s and 2% S206 fungicide for 60 min [29]. Shoot explants of *Anubias barteri* var. *nana* were effectively surface sterilized using 4% Clorox® for 10 min and 2%

Clorox® for 5 min [30]. In this experiment, surface sterilization with 3 rounds of 2-min treatment with 15% Clorox® was sufficient to disinfect excised *H. verticillata* shoots.

3.2 Effects of pH levels on growth and branching of in vitro cultured *H. verticillata*

During the cultivation of *H. verticillata* shoot explants in basal liquid MS medium across a range of pH levels, severely acidic conditions (pH 2) proved detrimental, causing the tissue to bleach to a pale white coloration with no discernible elongation or axillary branching by the fourth week of culture (Table 1, Fig. 3). As the medium pH was progressively raised, both shoot development and lateral branching became increasingly vigorous (Table 1). Optimal performance was recorded at pH 5.7, where the highest shoot length and number of branches were consistently achieved; consequently, this pH was adopted as the standard condition for all subsequent experiments. In contrast, when *H. verticillata* plants were grown in a hydroponic system supplemented with 10% Hoagland's solution, acidic pH values (5.5 and 6.5) resulted in markedly poor growth [31] – an outcome that stands in apparent contradiction to the in vitro findings. This discrepancy is likely attributable to differences in plant length, the physicochemical composition of the respective growth media, and the distinct environmental conditions inherent to each culture system.

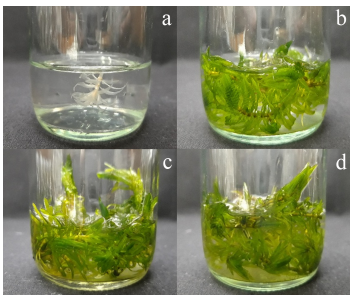


Fig. 3. Growth characteristics of *H. verticillata* shoot in basal liquid MS media with different pH after 4 weeks of culture (a = pH 2, b = pH 4, c = pH 5.7, and d = pH 7).

Table 1. Effects of varying pH of basal liquid MS medium on *H. verticillata* shoot growth and branching number after 4 weeks of in vitro culture.

pH of medium	Shoot length (cm)	Number of branching
2	1.50±0.00^{c}	0.00±0.00^{b}
4	6.40±0.31^{b}	7.90±0.72^{a}
5.7	8.62±0.42^{a}	8.80±0.39^{a}
7	8.43±0.67^{a}	8.70±0.76^{a}

Note: Data are from 10 replications. Values in each column are mean±S.E., and different letters after a value indicate the difference of means in each column.

3.3 Effects of BA or PBZ on growth and branching of in vitro *H. verticillata*

After supplementation of the culture medium with either BA or PBZ, neither plant growth regulator produced a notable enhancement in shoot elongation or axillary branch formation (Tables 2 and 3). In addition, nodal explants cultured in the liquid medium showed no signs of organogenic development, and all explants gradually turned brown by the end of the experimental period (Fig. 4). These observations suggest that basal liquid MS medium alone without any exogenous plant growth regulator is adequate for the in vitro culture of *H. verticillata* shoot, while nodal segments appear unsuitable as initial explants for this propagation system. Regarding the influence of BA and PBZ on shoot elongation, treatments containing different concentrations of BA, as well as PBZ at 0.5 and 1 mg/L, resulted in comparable shoot growth. In contrast, higher PBZ levels (2 and 4 mg/L) markedly reduced shoot elongation and also suppressed the formation of lateral branches in *H. verticillata* (Tables 2 and 3). Interestingly, plants growing from shoot explants maintained their green coloration and successfully developed branches at a PBZ concentration of 0.5 mg/L, while explants exposed to other concentrations eventually showed browning, despite initial growth (Fig. 4).

Table 2. Effects of BA or PBZ on growth of vitro *H. verticillata* at week 4.

Treatments	Length (cm)	
	Shoot explant	Nodal explant
MS basal	7.33±0.48^{a}	1.50±0.00^{e}
MS+2 mg/L BA	4.96±0.12^{bc}	1.50±0.00^{e}
MS+4 mg/L BA	4.57±0.08^{c}	1.50±0.00^{e}
MS+6 mg/L BA	4.20±0.17^{c}	1.50±0.00^{e}
MS+0.5 mg/L PBZ	5.50±0.52^{b}	1.50±0.00^{e}
MS+1 mg/L PBZ	4.92±0.77^{bc}	1.50±0.00^{e}
MS+2 mg/L PBZ	2.85±0.24^{d}	1.50±0.00^{e}
MS+4 mg/L PBZ	2.51±0.11^{d}	1.50±0.00^{e}

Note: Data are from 10 replications. Values are mean±S.E., and different letters after a value indicate the difference of means.

Table 3. Effects of BA or PBZ on branching number of in vitro *H. verticillata* at week 4.

Treatments	Number of branching	
	Shoot explant	Nodal explant
MS basal	9.70±0.47^{a}	0.00±0.00^{f}
MS+2 mg/L BA	8.50±0.48^{b}	0.00±0.00^{f}
MS+4 mg/L BA	6.80±0.33^{c}	0.00±0.00^{f}
MS+6 mg/L BA	6.60±0.45^{c}	0.00±0.00^{f}
MS+0.5 mg/L PBZ	5.30±0.75^{d}	0.00±0.00^{f}
MS+1 mg/L PBZ	1.00±0.33^{e}	0.00±0.00^{f}
MS+2 mg/L PBZ	0.00±0.00^{f}	0.00±0.00^{f}
MS+4 mg/L PBZ	0.00±0.00^{f}	0.00±0.00^{f}

Note: Data are from 10 replications. Values are mean±S.E., and different letters after a value indicate the difference of means.

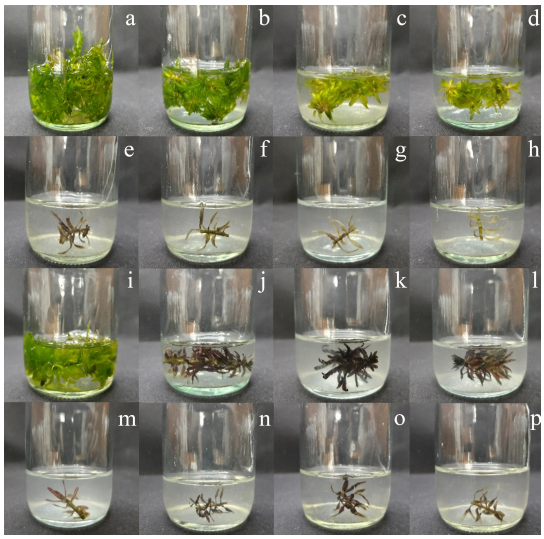


Fig. 4. Growth characteristics of *H. verticillata* shoot (a to d and i to l) and nodal (e to h and m to p) explants in basal liquid MS media (a and e), and MS liquid media supplemented with various concentrations of BA (b and f = 2 mg/L, c and g = 4 mg/L, and d and h = 6 mg/L) or PBZ (i and m = 0.5 mg/L, j and n = 1 mg/L, k and o = 2 mg/L, and l and p = 4 mg/L) at week 4.

Benzyladenine is widely employed in the tissue culture of aquatic plant species, primarily to stimulate shoot organogenesis from various explant types. Numerous studies have demonstrated that BA, when applied as a sole cytokinin source, is sufficient to achieve optimal shoot proliferation under in vitro conditions. For instance, twin scale explants of *Crinum malabaricum* produced the greatest number of shoots when cultured on MS medium containing 2.0 mg/L BA without additional growth regulators, while shoot tip explants of *Anubias barteri* var. *nana* exhibited highest shoot induction on MS medium augmented with 3 mg/L BA [32, 33]. Similarly, apical bud explants of *Bucephalandra* sp. 'Wavy Dark Green' responded most favorably to MS medium fortified with 5 mg/L BA [28]. In contrast, the present study revealed a different response pattern; although BA successfully promoted axillary bud break (Table 3), the greatest degree of axillary branching was paradoxically observed on hormone-free MS medium, suggesting that *H. verticillata* may possess sufficient endogenous cytokinin levels to support autonomous shoot development without exogenous supplementation.

Paclobutrazol, a member of the triazole class of plant growth regulators, exerts its primary action by blocking the gibberellin biosynthesis pathway, thereby constraining internode elongation and fostering a more compact plant architecture. Under sterile in vitro conditions, PBZ acts as a growth-suppressing agent that moderates overly vigorous development, alleviates the frequency of subculture intervals, and contributes to the orderly regulation of morphogenetic trajectories. Despite these well-recognized attributes, its application to aquatic plants within axenic culture systems remains largely unexplored in the literature [34].

In one of the few documented cases, Tauk et al. [35] demonstrated that specific PBZ concentrations could meaningfully alter the morphological characteristics of water hyssop (*Bacopa monnieri*) plantlets propagated in vitro. Notably, single-node explants cultured on MS medium supplemented with 2.5 mg/L PBZ exhibited the most favorable developmental outcomes, including maximum root elongation, superior shoot extension, and the greatest leaf number recorded across treatments. In a separate investigation involving three aquatic species–coontail (*Ceratophyllum demersum*), *H. verticillata*, and giant duckweed (*Spirodela polyrhiza*)–markedly divergent growth responses to PBZ were observed under both monoculture and polyculture in vitro regimes. Coontail was found to be particularly susceptible, with growth suppression evident at PBZ concentrations as low as 0.25-0.5 mg/L. While PBZ generally demonstrated limited efficacy in restraining *H. verticillata* under monoculture conditions, an application of 0.5 mg/L was sufficient to counteract the competitive dominance exerted by giant duckweed over *H. verticillata* in a mixed-culture setting [16]. Nevertheless, in this experiment, PBZ concentrations exceeding 1 mg/L induced visible browning of plantlet tissue (Fig. 4), an observation suggesting its potential utility as a chemical suppression tool for managing *H. verticillata* proliferation.

3.4 Effects of culture conditions on axillary turion production of in vitro cultured *H. verticillata* shoots

Following the culture of shoot explants in either basal liquid MS medium or semi-solid MS medium, distinct differences in growth responses were observed. Explants cultured in liquid medium exhibited greater shoot elongation; however, axillary turion formation was not detected under this condition (Fig. 5a). In contrast, more than half of the explants cultured on semi-solid MS medium developed axillary turions (Table 4, Fig. 5). This suggests that a change in the form of the physical culture matrix alone might be sufficient to trigger axillary turion induction in the absence of plant growth regulators, although this remains to be investigated further. When compared with observations from greenhouse-grown plants, *H. verticillata* typically initiated turions after approximately 76 days under a 11 h photoperiod [36]. Interestingly, axillary turion development in the in vitro system was detected much earlier, appearing within only 25 days of culture on semi-solid MS medium (Figs. 5b-5d). Furthermore, the turions produced in vitro were noticeably larger than those formed under natural conditions. While naturally occurring turions generally measured around 3-12 mm in length [10], those produced in vitro ranged between 5 to 17.5 mm.

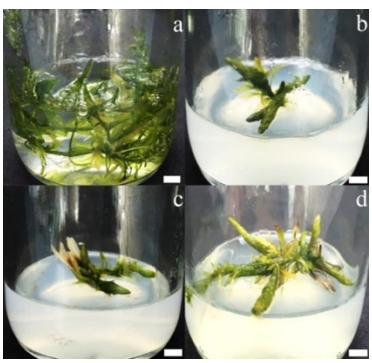


Fig. 5. *H. verticillata* shoot grown (a) in basal liquid MS media and (b-d) on basal semi-solid MS medium for 25 days. Scale bar = 5 mm.

Table 4. Effects of culture conditions on axillary turion production of in vitro cultured *H. verticillata* shoots after 25 days.

	Types of basal medium	
	Liquid medium	Semi-solid medium
No. of jars contained turions	0[b]	11[a]
% of jars contained turions	0	55
No. of turion production	0[b]	22[a]
Average no. of turion/jar	0	1.1

Note: Data are from 20 replications. Different letters after a value indicate the statistical difference.

In *H. verticillata*, the axillary turion acts as a stress-tolerant storage structure that serves as a persistent meristem reservoir and a specialized dormant vegetative organ. It would be worthwhile in future studies to carry out some histological, physiological biochemical investigations to examine the regeneration capacities of the axillary turions and also the types and quantities of food reserves in these structures that would sustain the conversion of the turions after return to favorable growth conditions. It would also necessary to examine the biochemical changes that might be associated with the dormant state and active conversion phases of the axillary turions. Comparable to a seed bank, this structure allows the plant to survive unfavorable conditions and later regenerate new growth axes once the environment becomes favorable [10, 37]. Examination of our results revealed an intriguing pattern: axillary turions were formed exclusively on semi-solid MS medium, whereas no such structures were observed in liquid MS medium. A plausible explanation may relate to the physiological stress experienced when explants were cultured on a semi-solid substrate. Compared with a liquid system, the semi-solid medium likely imposes a degree of water or nutrient availability limitation or physiological stress. This condition may arise because explants must adjust to reduced water availability and altered osmotic conditions, which can restrict water uptake relative to a fully submerged environment. In liquid medium, explants were continuously surrounded by water and nutrients, therefore had unrestricted access to them. Conversely, in semi-solid medium, water and nutrients were retained within the gel matrix, making them less readily available for absorption. In addition, there would be altered access to aeration when shoot explants were placed vertically on agar-based medium compared to submerging in liquid medium, but it is not known how altered aeration might be linked to axillary turion induction. Nonetheless, under these comparatively unfavorable or altered culture conditions, *H. verticillata* appeared to initiate axillary turion formation as an adaptive response. The development of these structures under water-limited or other altered culture conditions may represent a key survival strategy, enhancing the plant's persistence and resilience when cultured on semi-solid medium compared with liquid culture systems.

Water stress and osmotic stress share a common consequence–disruption of cellular water homeostasis–yet they arise through fundamentally distinct mechanisms. Water stress, most commonly triggered by drought conditions, results from an overall reduction in water availability at the cellular level. In contrast, osmotic stress originates from differential solute concentrations across cellular membranes, driving rapid water flux either into or out of cells in an attempt to restore equilibrium [38, 39]. Considerable research has now documented the effects of in vitro water stress across a diverse range of plant species. In potato, nodal explants subjected to progressively higher agar concentrations in MS medium exhibited marked reductions in plantlet height, foliar dry mass, root volume, and root dry mass, with the most severe growth suppression observed at 14 g/L agar [40]. Similarly, increasing concentrations of polyethylene glycol (PEG 1000) from 0 to 12% (w/v) in MS medium–used as an osmotic agent to simulate water deficit–progressively diminished shoot elongation, shoot fresh weight, leaf number, and root number in sunflower shoot tip

explants [41]. Beyond these growth parameters, moisture deficit imposed through pre-culture desiccation–by exposing flax (*Linum usitatissimum* L.) hypocotyl explants to laminar airflow for 30 min prior to culture initiation–has also been shown to compromise shoot organogenesis [42]. Nevertheless, the present study provides the first empirical evidence that this form of stress may serve as an effective stimulus for axillary turion activation and proliferation in *H. verticillata*. However, changing from a liquid medium to semi-solid medium might also involve changes in other conditions such as relative aeration supply and access to different nutrients. These are less studied compared to water or osmotic stress under in vitro conditions. While the primary focus of the present study was not to identify exactly the factors involved in the induction of axillary turion formation in vitro, future studies could be designed to investigate the factors involved, for example, by artificially elevating the oxygen dissolved in the liquid medium to see if axillary turion could be induced or not.

3.5 Production of synthetic seed from shoot and axillary turion of in vitro *H. verticillata* and short-term storage

Synthetic seed technology for submerged aquatic macrophytes, including *Rotala malabarica*, *Cryptocoryne lutea*, and *Rotala rotundifolia*, has been established through the encapsulation of shoot tips or nodal segments within a hydrogel-based protective matrix [24, 25]. This approach mimics the functional role of natural seeds in plant propagation, offering a viable alternative for the multiplication and short-term conservation of aquatic species for which conventional propagation remains technically difficult. In the present study, synthetic seeds derived from shoot apices and axillary turion of *H. verticillata* were successfully fabricated by embedding these vegetative explants in a calcium-alginate matrix (Fig. 6). Upon completion of the designated storage periods, the encapsulated propagules were transferred onto basal semi-solid MS medium to assess conversion potential.

Synthetic seeds originating from shoot apices of *H. verticillata* exhibited no conversion, as the enclosed shoots progressively developed necrotic browning and tissue deterioration within seven days of culture initiation. This response likely reflects that the shoots of *H. verticillata* did not store well at 25 °C and might be sensitive to the encapsulation treatment, while the axillary turions were not affected by the encapsulation treatment (Table 5). Furthermore, axillary turion-derived synthetic seeds stored at 4°C showed germination rates comparable to those kept at 25°C, with no statistically significant difference (Table 5, Fig. 7). This indicated that neither temperature condition hindered the development of axillary turions or their subsequent conversion. Notably, regenerated plantlets arising from axillary turion synthetic seeds spontaneously produced secondary axillary turions during culture on basal semi-solid MS medium (Fig. 7), a phenomenon attributable to the culture conditions which appeared to redirect developmental pathways toward axillary turion proliferation. Interestingly, the axillary turions formed in vitro did not seem to be dormant or require any exogenous plant growth regulators for regeneration after storage.

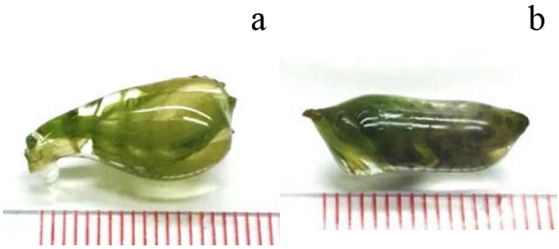


Fig. 6. Synthetic seed produced from (a) shoot and (b) axillary turion of *H. verticillata* cultivated in vitro. Red lines on the ruler represent 1 mm intervals.

Table 5. Germination of *H. verticillata* from shoot or axillary turion synthetic seed on day 21 after short-term storage in vitro.

Treatments	Number of germination	Germination percentage
SSS 25°C	0	0.00±0.00[b]
ATSS 4°C	7	46.67±12.05[a]
ATSS 25°C	10	66.67±17.21[a]

Note: Data are from 15 replications. Values are mean±S.E., and different letters after a value indicate the difference of means. SSS 25°C = shoot-derived synthetic seeds stored at 25°C, ATSS 4°C = axillary turion-derived synthetic seeds stored at 4°C, and ATSS 25°C = axillary turion-derived synthetic seeds stored at stored at 25°C.

Short-term preservation of alginate-encapsulated propagules from various plant species is strongly influenced by storage temperature. In many reports, storage at 4 °C has been shown to be considerably more effective than storage at 25 °C in maintaining high germination rates. For instance, gametophyte-derived synthetic seeds of eastern bracken (*Pteridium aquilinum* var. *latiusculum*) were successfully maintained under cold storage at 4 °C for short periods, and storage for up to 7 days had only minimal effects on sporophyte development, providing an adequate time window for transportation. After 7 days of storage at 4, 25, and 35 °C, the regeneration frequencies of gametophytes were 100%, 64.2%, and 1.2%, respectively [43]. Similarly, blackberry synthetic seeds stored at 4 °C exhibited satisfactory regeneration (above 60%) when cultured on an agarized substrate, whereas those stored at 25 °C showed a marked decline in regeneration, with rates of approximately 10% [44]. In contrast, encapsulated nodal explants of *Stevia rebaudiana* stored at 10 °C for 7 days prior to culture on MS agar medium at 25 °C for 4 weeks displayed germination rates ranging from 62.22% to 93.33% across treatments. Moreover, when storage temperatures of 4, 10, and 25 °C were compared, no significant differences in germination percentages were observed [45], which was comparable to the current investigation on axillary turion-derived synthetic seeds. Conversely, encapsulated shoot segments of *H. verticillata* subjected to 25 °C storage conditions failed to produce any germination response. This differential behavior indicates the possibility that shoot segments are more sensitive to the encapsulation treatment, and is worthy of further investigation. From a practical perspective, retaining axillary turion-derived synthetic seeds at 25 °C may offer advantages by reducing electricity consumption as low-temperature storage during transport and other cold-chain logistics would not be required, leading to a reduction in overall energy costs, an important consideration for large-scale propagation and germplasm handling.

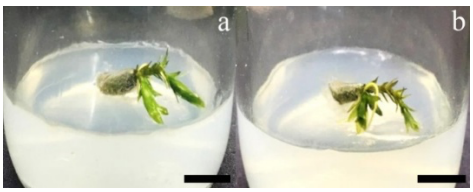


Fig. 7. Germination of *H. verticillata* from axillary turion synthetic seed after storage at (a) 4 and (b) 25 °C for 7 days and placed on basal semi-solid MS medium for 21 days. Scale bar = 10 mm.

4. Conclusion

For the establishment of in vitro stock plants of *H. verticillata* intended for subsequent applications, liquid MS medium devoid of plant growth regulators proved to be the most suitable formulation. Supplementation of the medium with either BA or PBZ yielded inferior results compared to the hormone-free basal medium. Notably, stress imposed by altered water, nutrient and aeration availability associated with the culture condition using the semi-solid medium was found to stimulate axillary turion development in *H. verticillata*–a phenomenon that, to the best of our knowledge, has not previously been reported. This observation is particularly significant, as the in vitro-derived axillary turions serve as a reliable explant source for synthetic seed production, while also offering a practical approach to *H. verticillata* germplasm conservation and its transfer through short-term in vitro storage. However, for long-term germplasm banking and

commercial distribution, future studies are required to investigate more comprehensively the optimal duration beyond 7 days (used in the present study) for the storage of the encapsulated axillary turions while maintaining high germination efficiency. In addition, it would be of great interest to determine the endogenous hormonal status of the shoots and axillary turions to better understand their growth on plant growth regulator-free culture medium.

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