

GROWTH ACCELERATION AND GALANTHAMINE CONTENT OF *HIPPEASTRUM PAPILIO* PLANTS GROWN ON HYDROPONIC SYSTEMS

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<https://doi.org/10.55251/jmbfs.9247>

ARTICLE INFO

Received 25. 7. 2022

Revised 10. 4. 2024

Accepted 19. 4. 2024

Published 1. 6. 2024

Regular article

OPEN ACCESS

ABSTRACT

Hippeastrum papilio (Ravenna) Van Scheepen (Amaryllidaceae) is an endangered endemic species from the rainforests of Brazil. It is not only an attractive ornamental plant, but the species with the highest content of the valuable alkaloid galanthamine used for treatment of Alzheimer's disease. The rapid propagation of the plants is crucial for biomass production as an alternative plant raw material for industrial galanthamine extraction. The aim of the present study was to accelerate the growth of *H. papilio* bulbs using two different hydroponic systems and to evaluate their effectiveness, as well as to determine the galanthamine content in the leaves. Differences in seedlings' growth on Flood & Drain hydroponic system, on Cutting board hydroponic system, and in soil substrate as a control were significant ($P < 0.001$, Anova single factor). Best results were obtained on the Cutting board hydroponic system where plant weight increased an average of 59.1 ± 24.0 times while the lowest growth was in the control, with an average weight increase of 11.1 ± 4.7 times. One month after adaptation of plants to soil substrate they were analysed by GC-MS. Galanthamine was the main alkaloid in the leaves of plants from all variants, and its content varied between 0.66% and 0.86%, which was commensurable with that of the native plants. Feasibility of soilless cultivation of *in vitro* propagated *H. papilio* bulblets was also proven.

Keywords: butterfly amaryllis, soilless cultivation, ornamental plants, Amaryllidaceae alkaloids

INTRODUCTION

Hippeastrum papilio (Ravenna) Van Scheepen (Amaryllidaceae) is an endangered endemic species from the rainforests of Brazil. It was considered extinct, but in 1990 was rediscovered with about 50 individuals (BFG, 2015). The interest toward this species is due to both its attractive flowers which gave it the name 'butterfly amaryllis' and its alkaloid content, especially galanthamine which is used for treatment of numerous neuromuscular disorders and diseases of the nervous system including the Alzheimer's syndrome (Marco and Carreiras, 2006). *H. papilio* is the species with the highest content of galanthamine, approximately 0.6-0.8% in the dry weight of the leaf samples (Berkov *et al.*, unpublished results) while in other species of industrial importance it is between 0.1 and 0.4% (Poulev *et al.*, 1993; Cherkasov and Tolkachev, 2002; Gussev *et al.*, 2007). *H. papilio* plants contain also other Amaryllidaceae alkaloids including 11 β -hydroxygalanthamine, all of them expressing acetylcholinesterase inhibitory activity (de Andrade *et al.*, 2011). These characteristics define butterfly amaryllis as a perspective ornamental and medicinal plant. The rapid propagation of this species, and especially of galanthamine-rich genotypes, is crucial for its introduction as an alternative plant raw material for industrial galanthamine extraction. Beautiful hybrid cultivars with ornamental value have been created and multiplied (Meerow, 2014). However, to keep the high galanthamine content of the plants, it is important to propagate the native genotypes. The method of *in vitro* clonal propagation has been successfully applied for rapid multiplication of other species from the same genus (Zayed *et al.*, 2011). Our first trials showed that this method was suitable for *H. papilio* as well, but the growth of *in vitro* obtained bulblets was very slow during their conventional soil acclimation. It could be accelerated by soilless technologies, which main advantages are rapid plant growth and development, independence from seasons and soil type, low water consumption, less place needed, absence of weeds and pests (Mugundhan *et al.*, 2011; Texier, 2013). While conventional production of some bulbous species is time and labour consuming, soilless cultivation was reported as a successful alternative for large-scale production of *Polygonum tuberosum*, *Crocus sativus*, *Lilium* hybrids, and *Tulipa* cultivars, using different hydroponic systems (Souret and Weathers, 2000; Miller, 2002; Moraghebi and Mohebbi, 2011; Asker, 2015). Owing to its numerous advantages, and its suitability for any plant species, hydroponics is considered as the future of farming (Mugundhan *et al.*, 2011). Seeds would be the most suitable starting material for testing the effect of hydroponic technologies on *H. papilio* bulblet and plant growth. The aim of the present study was to accelerate the growth of *H. papilio* plants using two different hydroponic systems and to compare their

effectiveness. In addition, alkaloid composition and galanthamine contents of plants grown by soilless technologies and in soil substrate were determined.

MATERIAL AND METHODS

Soilless cultivation

Several *H. papilio* bulbs were provided by Berbee Beheer B.V, bulb grower from the Netherlands. The bulbs were potted in a soil substrate and successfully developed into flowering and fruiting plants. The seeds were sown in the soil and regularly watered. Sixty 2-month old seedlings (average weight 0.60 ± 0.34 g) were evenly distributed in 3 variants, 20 plants each, and putted in meshy pots with clay pebbles on Cutting board hydroponic system (GHE), perlite on Flood & Drain hydroponic system, and terrine with soil substrate (Light mix, Biobizz®) as a control, all of them in a room phytotron under 23 ± 2 °C, air humidity of $56 \pm 18\%$ and mixed daily and artificial light, 16/8 h photoperiod. In both hydroponic variants the nutrient solution was the same, consisting of distilled and tap water (3:1), supplemented with Flora Micro, Flora Grow, and Flora Bloom (GHE) (in 1:1:1 ratio) in quantities required to reach the optimum electrical conductivity (EC) which was found to vary between 0.40 and 0.98 mS/cm depending on the phenophase of plants development, the pH was maintained between 5.5 and 6.5. The solution in the Cutting board (CB) hydroponic system was constantly agitated by supplemental aeration from a small aquarium pump and airstones, while the grow tray with perlite of the Flood & Drain (F&D) hydroponic system was flooded with the solution 15 min every 6 h using a pump placed into the reservoir. The control plants were watered regularly, and nourished with the same nutrient solution once every two weeks. After 16 weeks all bulbs from the three variants were lifted and their weight and diameter were measured. Then plants were transferred to pots with soil substrate and leaved in the phytotron for 2-month adaptation.

In parallel, 33 *in vitro* obtained well-shaped and rooted bulblets (average weight 1.94 ± 1.34 g) were grown on the F&D hydroponic system for 16 weeks. The initial and final weights of each bulb as well as their initial and final diameters were measured. Due to the considerable differences in the initial sizes of seed-derived and *in vitro* multiplied bulbs, their growth was compared by introducing a growth index (GI) and the increase in weight and diameter of the bulbs was calculated by the formulas $GIW = (FW-IW)/IW$ and $GID = (FD-ID)/ID$, where FW and IW are the final and initial bulb weights, and FD and ID are the final and initial bulb diameters.

In vitro propagation

Intact plants of *H. papilio* were introduced *in vitro* after Zayed et al. (2011). Bulbs of *H. papilio* were carefully washed with tap water and then outer scales and roots were removed. After that, they were sterilized with 70% EtOH for 30 s and 0.1% HgCl₂ for 6 min. Further, the bulbs were rinsed with distilled sterile water, then cut to twin-scales after (Hussey, 1982) and placed in Petri dishes with solid MS medium (Murashige and Skoog, 1962) supplemented with plant growth regulators: 2 mg/L Benzylaminopurine (BAP) and 0.15 mg/L α-Naphthaleneacetic acid (NAA), and 30 g/L sucrose, and solidified with 6.5 g/L Plant agar (Duchefa, NL). When the new bulblets appeared, the twin scales were transferred in glass jars on MS medium free of plant growth regulators, and supplemented with 1g/L active charcoal. Cultures were maintained at 22 ± 2 °C and 16/8 h light photoperiod. Sub-cultivations were performed every 4 months, and *in vitro* bulblets reaching 6-8 mm in diameter were cut in four parts and cultivated as described above to repeat the propagation procedure. Four months after the last cutting, the new bulblets with well-developed roots were transferred to the F&D hydroponic system.

Statistical data analysis

Comparison of the growth of the seed-derived plants in the three variants was done by Excel ANOVA single factor (Tables 1 & 2). The differences between the growth indexes concerning the weight and the diameter of seed-derived and *in vitro* multiplied plants (GI_w and GI_d) were also estimated by Excel ANOVA single factor (Tables 4 & 5).

Phytochemical analyses

Aggregated samples consisting of leaves from different plants of one and the same variant (about 20 g FW, one aggregate sample per variant) were prepared for analysis.

Extraction of alkaloids

Leaf samples were dried at 60 °C and powdered; 50 mg of dried plant material was macerated in screw-top Eppendorf tubes (1.5 mL of volume) with 1 mL of methanol adjusted to pH 8 with 25% of ammonia and containing 50 µg of codeine as an internal standard (IS). After 2 h of extraction at room temperature assisted by an ultrasonic bath for 15 min every 30 min, the samples were centrifuged and 500 µL aliquots were transferred to other Eppendorf tubes. Then, 500 µL of 2% sulfuric acid in distilled water was added and the neutral compounds were eliminated by duplicate extraction (vortexing) with 500 µL chloroform. The mixtures were basified with 200 µL 25% ammonia and the alkaloids extracted in triplicate with 500 µL chloroform. The organic solvent was evaporated and the dry extract dissolved in 300 µL chloroform for further GC–MS analysis.

Instrumental analysis

The GC–MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ II mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m × 0.25 mm × 0.25 m) was used. The temperature program was: 100–180 °C at 15 °C/min, 1 min hold at 180 °C and 180–300 °C at 5 °C/min and 1 min hold at 300 °C. Injector temperature was 280 °C. The flow rate of carrier gas (Helium) was 0.8 mL/min. The split ratio was 1:10. The galanthamine content and alkaloid identification was determined as described in Berkov et al. (2011).

RESULTS AND DISCUSSION

Soilless cultivation

Seedlings' growth acceleration on the two hydroponic systems

In all the three variants, seed-derived plants developed vigour root system and new leaves (Fig. 1).

Best results were obtained on the CB hydroponic system where all plants survived and their average weight and bulb diameter were 27.2 ± 16.1 g and 20.8 ± 4.2 mm, respectively, i.e. plant weight increased an average of 59.1 ± 24.0 times and bulb diameter 4.0 ± 0.8 times (Fig. 2; Tables 1 & 2). Plant growth was medium on the F&D hydroponic system, with 85% survival rate of plants, an average weight of 15.4 ± 9.2 g, and an average bulb diameter of 15.8 ± 3.4 mm. Growth was lowest in the control variant, with an average weight of 7.9 ± 3.3 g, and an average bulb diameter of 13.1 ± 2.3 mm, while 95% of the plants survived. Differences in plants' growth between the 3 variants were significant (P<0.001, Anova single factor) (Tables 1 & 2). Variation within variants was due to the non-uniform size of the seedlings. Significant increase of corms' growth in term of dry weight was noticed for saffron in aeroponics and hydroponics compared to the growth in soil (Souret, 2000).

Growth acceleration in both hydroponic systems, compared to the control variant, was due to the optimal balance of the nutrient salts in the solution, and their easily digestible ionic form, which are common advantages of the soilless cultivation

(Mugundhan et al., 2011; Texier, 2013). Besides, in the Cutting board hydroponic system, the roots were submerged in the solution with constant active aeration, ensuring its oxygen enrichment, which contributed to the quick plant growth. Furthermore, the high oxygen level prevented microorganism contamination of the solution. Similar effect of growth and flowering acceleration was reported for the Asiatic hybrid lily cv. "Blackout" cultivated on another type of hydroponic system named "nutrient film technique" where plant roots were able to uptake optimal amounts of nutrients, water, and oxygen owing to the oxygen-rich thin film of nutrient solution (Asker, 2015). During the adaptation stage of 2 months, new leaves appeared in all variants.

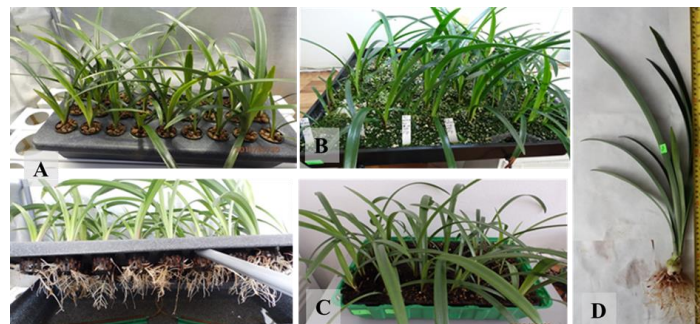


Figure 1 Plants after 16 weeks of cultivation in the 3 tested variants: A) CB hydroponic system – leaves and roots; B) F&D hydroponic system; C) Control with soil substrate; D) Plant ready to be transferred to soil substrate for further adaptation

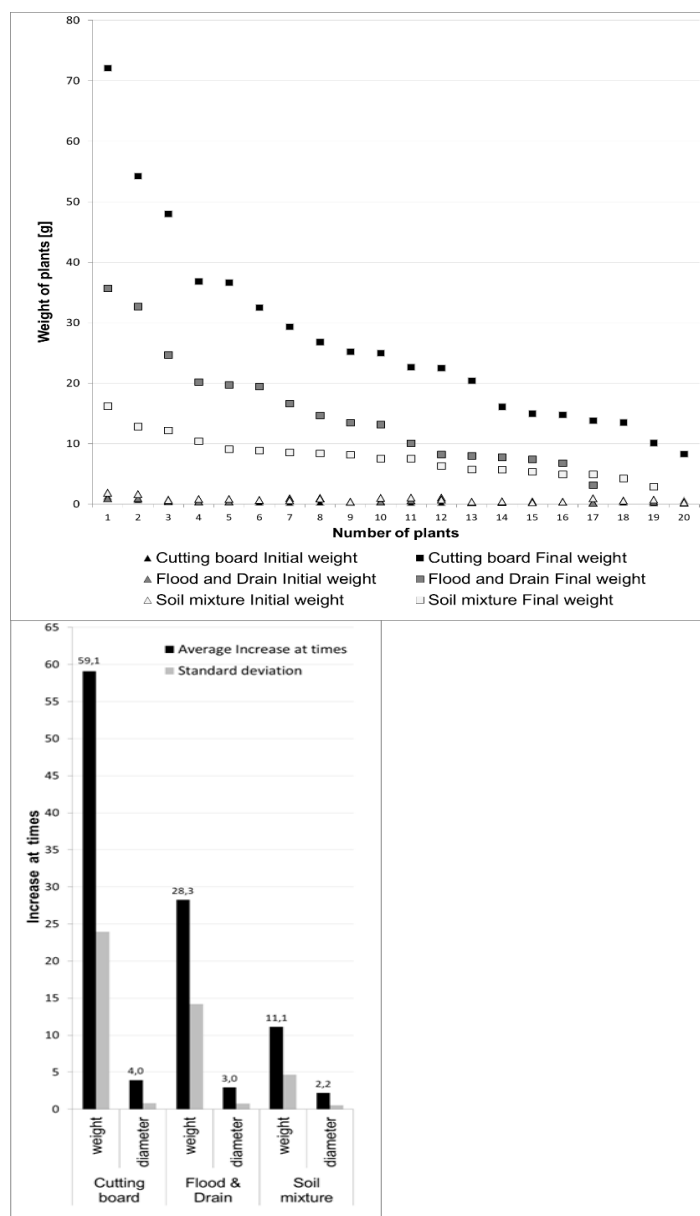


Figure 2 Growth of seedlings for 16 weeks: A) Initial and final plants' weight in the 3 variants; B) Growth increase at times

Table 1 Comparison of the increase of the bulb weight in the three variants

SUMMARY						
Groups	Count	Sum	Average	Variance		
Cutting board	20	79,03333	3,951667	0,683629		
Flood and Drain	17	50,18571	2,952101	0,570105		
Control	19	42,11548	2,216604	0,304067		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	29,61431	2	14,80716	28,4507	4,04E-09	3,171626
Within Groups	27,58383	53	0,52045			
Total	57,19814	55				

Table 2 Comparison of the increase of the bulb diameter in the three variants

SUMMARY						
Groups	Count	Sum	Average	Variance		
Cutting board	20	1182,089	59,10444	574,2779		
Flood and Drain	17	480,2816	28,25186	201,4541		
Control	19	211,0439	11,10758	21,73135		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	23106,85	2	11553,43	42,15501	1,11E-11	3,171626
Within Groups	14525,71	53	274,07			
Total	37632,56	55				

Growth of seedlings and in vitro bulblets on F&D

Initial and final values of plants' weight and bulbs' diameter of seedlings and in vitro multiplied bulblets are shown on Table 3.

The growth rate of in vitro bulblets on the F&D hydroponic system was lower than that of seed-derived plants on the same system and for the same duration, P < 0.001 for both GI_w and GI_D (Tables 4 & 5), which was most probably due to their more fine roots. On the other hand, in vitro bulblets successfully adapted to the F&D hydroponic system, 90.9% of them surviving and developing more vigour roots. Despite the smaller final size of the plants obtained from in vitro bulblets for 16 weeks, in vitro multiplication of *H. papilio* needs to be applied owing to the limited

number of the available seeds of this endangered and protected species. Using in vitro propagation as a first step will allow the rapid increase of the number of bulblets which could be further grown using hydroponic systems. Plants obtained by this procedure are expected to take some more time to reach flowering stage, but all of them easily adapted to soil substrate and developed new leaves. Applying of CB hydroponic system will most probably increase the growth rate of the in vitro bulblets of *H. papilio*. The new bulbs appear between 30 and 45 days after initial twin-scaling and achieve a size (> 6-8 mm) suitable for new cutting after 4 months.

Table 3 Main parameters of in vitro obtained and seed-derived plants grown on the F&D hydroponic system for 16 weeks

Plants	In vitro obtained plants		Seed-derived plants	
	Weight [g]	Bulb diameter [mm]	Weight [g]	Bulb diameter [mm]
Initial values	1.94 ± 1.34	8.62 ± 2.09	0.58 ± 0.29	5.59 ± 1.46
Final values	5.35 ± 3.30	11.40 ± 2.30	15.39 ± 9.17	15.76 ± 3.36

Table 4 Growth rates of in vitro obtained and seed-derived plants, presented by their weight growth indexes (GI_w)

SUMMARY						
Groups	Count	Sum	Average	Variance		
GI _w seed-derived	17	463,2816	27,25186	201,4541		
GI _w in vitro obtained	30	64,41876	2,147292	4,22048		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6838,768	1	6838,768	91,98323	1,89E-12	4,056612
Within Groups	3345,659	45	74,34798			
Total	10184,43	46				

Table 5 Growth rates of in vitro obtained and seed-derived plants, presented by their diameter growth indexes (GI_D)

SUMMARY						
Groups	Count	Sum	Average	Variance		
GI _D seed-derived	17	33,18571	1,952101	0,570105		
GI _D in vitro obtained	30	12,04495	0,401498	0,264631		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	26,08996	1	26,08996	69,90056	1,04E-10	4,056612
Within Groups	16,79597	45	0,373244			
Total	42,88593	46				

Alkaloid composition and galanthamine content

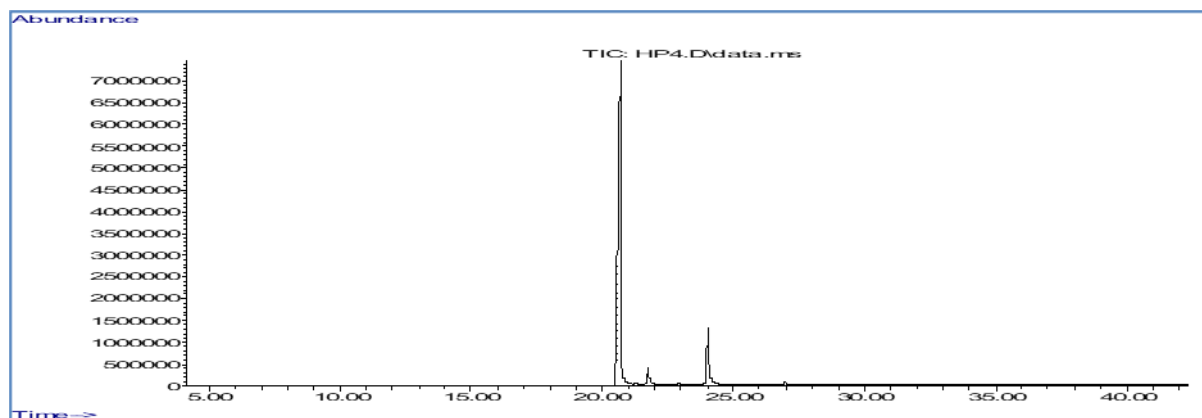
The alkaloid composition of the aggregated samples of the three variants is presented on Table 6. A total of 8 alkaloids were detected in all of the three variants (Table 6). Galanthamine was the main alkaloid in the leaves of all leaf samples, about 90% (Fig. 3). Haemanthamine and narwedine were in relatively high content similarly to previously reported results (Berkov et al., 2019).

The content of galanthamine in the leaves of the plants grown in soil substrate was 0.86% in the dry weight, while these percentages were 0.66% and 0.72% for the CB and the F&D hydroponic systems, respectively. The galanthamine contents of all the three tested variants were commensurable with those of the native plants.

The related Amaryllidaceae alkaloids remained in very low concentrations. The results indicate that plants maintain their ability to biosynthesize high levels of galanthamine in soilless systems. Similar concentrations of the bioactive compounds were also reported for saffron grown hydroponically, aeroponically, and in soil (Souret, 2000). Taking in account that the biomass accumulation in the variant of the CB hydroponic system was 5 times higher than in the control variant (Fig. 2-B), the application of soilless cultivation would considerably shorten the period required to reach flowering stage, i.e. for the use of plants for cut flowers or for production of galanthamine.

Table 6 Alkaloid composition in the leaves of plants grown on the two hydroponic systems and the control, determined by GC-MS, and expressed as a percent of the total alkaloid mixture

Alkaloids in the leaves	Retention time (min)	Control (soil) (%)	CB hydroponics (%)	F&D hydroponics (%)
Apogalanthamine	19.65	0.2	trace	0.1
Galanthamine	20.89	93.5	88.2	91.0
Narwedine	22.00	2.3	3.4	1.2
9-O-Demethyllycosinine	22.14	0.5	0.5	1.8
11 β -Hydroxygalanthamine	23.16	0.2	0.4	1.4
Haemanthamine	24.35	3.2	6.8	4.4
Papilline	27.16	0.1	0.6	0.1
3-O-Demethyl-3-O-(3-hydroxybutanoyl)-haemanthamine	29.20	trace	0.1	trace

**Figure 3** GC-MS chromatogram of alkaloid fraction from leaves of *H. papilio*

CONCLUSION

The growth of *H. papilio* plants was significantly enhanced by means of soilless cultivation, compared to the control terrine with soil substrate. Cutting board hydroponic systems being the best variant. The galanthamine content in the leaves remained commensurable with that of the native plants. All experimental plants will be further analysed at individual level in order to select those with the highest galanthamine concentrations. The selected genotypes will be used for the production of uniform galanthamine-rich plants in two steps: *in vitro* rapid bulblets multiplication, followed by hydroponic cultivation for plant growth acceleration. This novel procedure, combining *in vitro* clonal propagation and soilless cultivation seems to be very suitable for slow-growing bulbous plants.

Acknowledgments: This work was partially supported by the Bulgarian Ministry of Education and Science under the National Research Programme “Young scientists and postdoctoral students” approved by DCM # 577 / 17.08.2018.

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