

THE EFFECT OF SALICYLIC ACID AS AN ELICITOR FOR  
GALANTHAMINE PRODUCTION IN *HIPPEASTRUM*  
*PAPILIO* (RAVENNA) VAN SCHEEPEN HYDROPONIC  
CULTURES

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**Abstract**

*Hippeastrum papilio* (Ravenna) Van Scheepen plant (*H. papilio*), endemic to South America, is an ornamental plant, a potential new source for industrial production of galanthamine (GAL). GAL is a natural compound, used for the cognitive treatment of Alzheimer's disease. In order to enhance the GAL and haemanthamine (HAEM) accumulation in *H. papilio*, elicitation with salicylic acid (SA) was tested in one-year-old hydroponic cultures. Three different concentrations of SA were applied (10, 50, and 100  $\mu\text{M/L}$ ), at various time points (0, 6, 12, 24, 48, 96, and 148 h). Gas chromatography-mass spectrometry analyses were performed to determine the alkaloid levels in leaves. The quantity of GAL and HAEM through the tested time points varied. Treatment with 100  $\mu\text{M/L}$  SA for 148 h enhanced GAL biosynthesis by 19% compared to the initial level. A similar trend was observed for HAEM accumulation. The hydroponic system is suitable for *H. papilio* cultivation.

**Key words:** alkaloids, elicitation, galanthamine, *Hippeastrum papilio*, hydroponic cultures, salicylic acid

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**1. Introduction.** The Amaryllidaceae alkaloids (AAs) are secondary metabolites with a wide range of biological and pharmacological activities, such as antitumour, antiviral, antibacterial, anti-acetylcholinesterase (anti-AChE) [1, 2]. Among all AAs, galanthamine is yet the most successfully exploited AA, acetylcholinesterase (AChE) inhibitor, approved by the U.S. Food and Drug Administration (FDA) for cognitive treatment of Alzheimer's disease (AD) [1]. AD remains the most common form of dementia, affecting more than 50 million of the elderly population worldwide, and is expected to triple the number by 2050 [3]. Since the pathogenesis of AD is not completely elucidated, the treatment remains symptomatic, based on medicinal agents, some of them of plant origin, namely galanthamine [1].

Although the synthetic formation of galanthamine has been achieved, plants remain the main sources for its industrial production. Different Amaryllidaceae species have been utilized, such as *Narcissus pseudonarcissus* L. cv bulbs. Carlton (containing about 2.2–3.3 mg/g DW) [4] in Central and West Europe, leaves of snowflake (*Leucojum aestivum* L., galanthamine content ranges from traces to about 5.0 mg/g DW) [5] in East Europe, and bulbs of red-tubed lily (*Lycoris radiata* (L'Hér.) Herb., (galanthamine content from 0.2 to 5.0 mg/g DW) [6] in China. *Leucojum aestivum* L. is currently categorized as endangered and placed in the Red Book of Bulgaria. Its utilization is under regulation since it is gathered from nature, and its leaf biomass is used for galanthamine production [7]. On the other hand, *Hippeastrum papilio* (Ravenna) Van Scheepen (*H. papilio*) is an ornamental plant endemic to South America and is considered a potential new source for galanthamine production. Compared to the plants currently used, it is an evergreen and large-sized plant with superior galanthamine content in bulbs and leaves. Previous studies have shown a content of 5.7 mg/g DW of galanthamine (Fig. 1A) in dry leaves from *H. papilio* [8]. Haemanthamine was also found in *H. papilio* (Fig. 1B) [8] which has received considerable attention since it shows antitumour properties inducing apoptosis in various tumour cells at micromolar concentrations [9].

Various approaches have been applied in order to enhance the biosynthesis of galanthamine in plants. Our previous studies found that galanthamine accu-

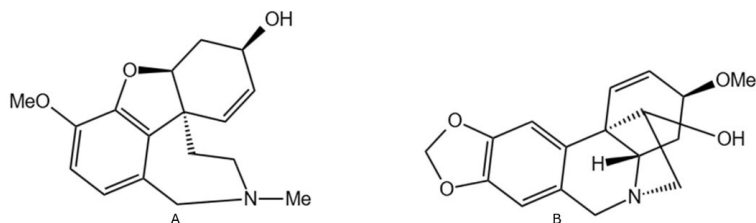


Fig. 1. Structures of galanthamine (A) and haemanthamine (B)

mulation and the growth dynamics of *H. papilio* are age- and organ-dependent processes. Moreover, fertilization in hydroponic cultures was found to be an interesting strategy to elevate the accumulation of galanthamine in *H. papilio* [8].

Elicitation, a term used to describe the activation of the plant defence system that stimulates secondary metabolite production, is a common strategy to enhance the accumulation of bioactive molecules in plants [10]. The elicitors can be of biotic and abiotic (physical or chemical) origin, or naturally occurring substances, such as phytohormones (salicylic acid (SA), jasmonates, among others). SA is a phenolic compound that plays a role in the defence system against pathogens and many other physiological processes such as photosynthesis, transpiration, stomatal regulation, biosynthesis of chlorophylls and proteins, enzyme activities, and nutrient uptake [11]. Even though it naturally occurs in plants, when applied exogenously, SA exerts various effects on the physiological, metabolomic, and transcriptomic levels in plants, via activating genes involved in a cascade of reactions [12]. Several studies reported a positive effect of SA on secondary metabolite production [10, 11] and alkaloid accumulation, particularly [13, 14]. PTAK et al. [15] reported that SA promoted galanthamine and lycorine biosynthesis in *L. aestivum* ‘Gravity Giant’ plants grown in a bioreactor. While the effect of elicitation on galanthamine accumulation in in vitro systems has been investigated [16, 17], there are no studies performed on Amaryllidaceae plants as hydroponic cultures. The effect of SA elicitation is a complex process related to different factors, such as concentration, time exposure, plant species, and type of treatment. The only way to find the best outcome dose and treatment time is empirically.

As part of our ongoing research on *H. papilio* hydroponic cultures as a potential industrial source of economically important molecules, here we report the preliminary evaluation of SA (relatively cheap and accessible for industrial use) as an elicitor for galanthamine and haemanthamine accumulation. As the physiological status and conditions for in vitro and hydroponic cultivation are different, the aim was to answer the questions: 1) can SA affect galanthamine biosynthesis in hydroponic cultures, 2) which concentration of SA is the most effective, 3) which time point of action has the best outcome in *H. papilio* hydroponic cultures. To the best of our knowledge, this is the first report analysing the effect of exogenous SA application on alkaloid accumulation in hydroponic *H. papilio* cultures.

**2. Material and methods. 2.1. Plant material and hydroponic culture.** One-year-old plants of *Hippeastrum papilio* (Ravenna) Van Scheepen, provided by Ludwig and Co BV, the Netherlands (<https://www.ludwigandco.nl>) were grown at the greenhouse of the Institute of Biodiversity and Ecosystem Research (Bulgarian Academy of Sciences) as a hydroponic culture from April until November. *H. papilio* was grown on a flood and drain hydroponic system in plastic crates (40 × 60 cm) with clay pebbles (Fig. 2). The plants were grown with a basic nutrient solution, described in the recipe formulations proposed by KROGGEL and KUBOTA [18] for optimized greenhouse production of tomatoes. The pH of



Fig. 2. Cultivation of *H. papilio* on flood and drain hydroponic system

the nutrient solution was maintained below 6.5 while its electroconductivity was maintained at  $1500 \pm 300 \text{ mS m}^{-1}$ . The nutrient solution was circulated for 15 min at intervals of 4 hours. Five plants per concentration and at each time point were randomly selected for the present study during the autumn of the year of cultivation.

**2.2. Elicitation.** Five plants were collected as a control before the elicitor (SA) was added (0 h). SA at 10, 50, and 100  $\mu\text{M/L}$  concentrations were added to the nutrient solution. Afterwards, at each time point – 6, 12, 24, 48, 96, and 148 h five plants per concentration were collected for subsequent phytochemical analysis.

**2.3. Phytochemical analysis.** **2.3.1. Sample preparation.** The leaves from each individual were cut, dried at  $60^\circ\text{C}$ , and then powdered. The sample preparation was as described in [8]: 100 mg of dried plant material was macerated in screw-top Eppendorf tubes (2 mL of volume) with 1 mL methanol adjusted to pH 8 with 25% ammonia and 50  $\mu\text{L}$  of codeine as internal standard. After 24 h of extraction at room temperature, the samples were centrifuged at 6000 rpm for 1 min. Then, about 700  $\mu\text{L}$  of plant extract was transferred into another Eppendorf tube and dried overnight. Afterwards, 500  $\mu\text{L}$  of 2% sulphuric acid in distilled water was added, followed by vortexing, and the neutral compounds were eliminated by triplicate extraction (vortexing) with 500  $\mu\text{L}$  chloroform. The purified mixtures were basified with 60  $\mu\text{L}$  25% ammonia and the alkaloids were extracted in triplicate with 400  $\mu\text{L}$  chloroform. For the elimination of unwanted moisture, each chloroform fraction was carried out through sodium sulphate and collected in a glass vial. The organic solvent was evaporated, and to the dry extract was added 50  $\mu\text{L}$  of *N,O*-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) and 50  $\mu\text{L}$  of pyridine for 2 h at  $50^\circ\text{C}$ . After cooling, 300  $\mu\text{L}$  of chloroform was added to the derivatized fractions and analysed by gas chromatography-mass spectrometry (GC-MS).

**2.3.2. Gas chromatography-mass spectrometry.** The GC-MS chromatograms and spectra were recorded on a Thermo Scientific Focus GC coupled with a Thermo Scientific DSQ II mass detector (Thermo Fisher Scientific Inc., Waltham, MA, USA) operating in EI mode at 70 eV. A DB-5MS column (30 m  $\times$  0.25 mm  $\times$  0.25 mm) was used. The temperature programme was: 100–180 °C at 15 °C  $\times$  min<sup>-1</sup>, 180–300 °C at 5 °C  $\times$  min<sup>-1</sup>, and 10 min hold at 300 °C. The injector temperature was 250 °C. The flow rate of the carrier gas (Helium) was 0.8 mL  $\times$  min<sup>-1</sup>. 1 mL of the derivatized samples was injected at a split ratio of 1:10.

**2.3.3. Quantification of galanthamine.** A calibration curve for galanthamine quantification was prepared using eight concentrations (20, 50, 100, 200, 400, 600, 800, and 1200  $\mu$ g/mL) of galanthamine and 50  $\mu$ g of codeine as internal standard (IS), which were analysed as TMS derivatives. The ratios of the peak areas of selected (base) ions in the selected ion monitoring (SIM) mode of galanthamine TMS ( $m/z$  at 216) versus those of codeine ( $m/z$  at 178) were plotted against the corresponding concentration of galanthamine to obtain the calibration curve. Galanthamine content was expressed as milligrams per gram of the sample's dry weight (DW). Haemanthamine content was calculated as a galanthamine equivalent in the samples using the base ion at  $m/z$  181 for haemanthamine TMS-derivative [8].

**2.4. Statistical analysis.** Statistical analysis for significant differences in GAL content was performed with Minitab Statistical Software v. 19 (Minitab LLC, State College, PA, USA) (free trial) using a general linear model followed by Tukey's test at a significant level of  $p < 0.05$ . The data values are expressed as mean  $\pm$  standard deviation ( $n = 5$ ).

**3. Results and discussion.** GC-MS analyses revealed the effect of three different concentrations of SA as an elicitor (10, 50, and 100  $\mu$ M/L) at various time points (6, 12, 24, 48, 96, and 148 h) on galanthamine and haemanthamine (Fig. 1) accumulation (Fig. 3), in hydroponic *H. papilio* cultures (Fig. 2). As seen in Fig. 3, applying 10  $\mu$ M/L caused a peak in the GAL content during 6 h, followed by a decrease during 12 h, and a return to a relatively stable level until the last analysed time point (148 h). On the other hand, applying 50  $\mu$ M/L SA in the nutritional medium decreased GAL content during 12 h, followed by an increase reaching a peak at 96 h and again reduced at the last time point (148 h). The highest applied concentration of SA (100  $\mu$ M/L) was found to be the most effective for galanthamine elicitation resulting in a 19% increase from the initial one (from  $4.2 \pm 0.8$  to  $5.0 \pm 0.7$  mg/g DW) (Fig. 3A). A similar trend was observed regarding the HAEM accumulation (from  $1.4 \pm 0.3$  to  $1.7 \pm 0.3$  mg/g DW) (Fig. 3B). Even though the differences were not significant, there is a trend showing that the exogenous application of SA to the nutritional solution had a positive effect on galanthamine and haemanthamine accumulation in *H. papilio* hydroponic cultures.

The data showed that the effect of the SA on the GAL and HAEM accu-

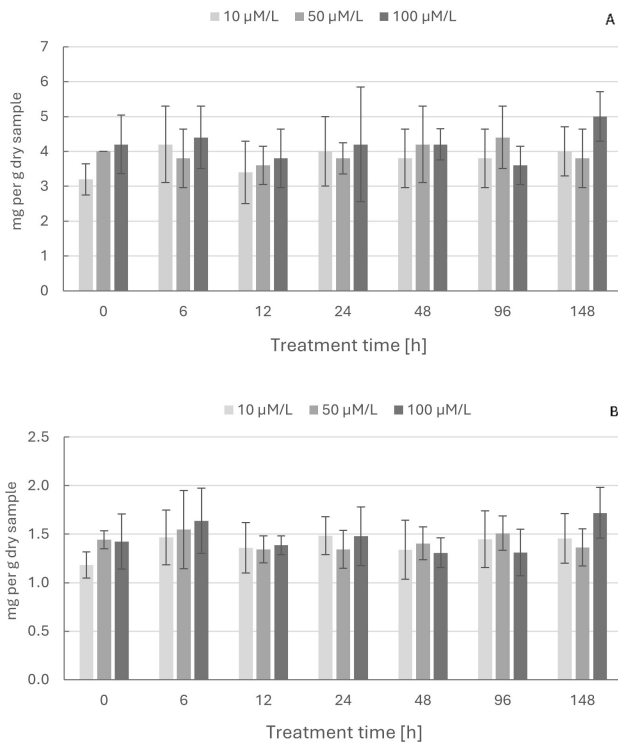


Fig. 3. Quantity of galanthamine (A) and haemanthamine (B) expressed as mg/g DW sample in leaves of hydroponically grown *H. papilio* plants treated with three different concentrations of salicylic acid (10, 50, and 100 μM/L) at various time points (0, 6, 12, 24, 48, 96, 148 h). Data are shown as mean  $\pm$  standard deviation ( $n = 5$ )

mulation in *H. papilio* is related to its concentration and time of application. Previous studies in Amaryllidaceae species showed ambiguous effects of SA as an elicitor. While Ptak et al. [15] reported that the highest galanthamine content in *Leucojum aestivum* 'Gravity Giant' grown in a bioreactor was observed when plants were treated with 5 μM SA for 10 h, another scientific team [16] found that applying SA arrests the GAL accumulation in *Lycoris chinensis* seedlings. Other researchers [17] also reported that SA failed while other elicitors enhanced the GAL biosynthesis in *Leucojum aestivum* shoots grown in vitro in a temporary immersion system.

SA elicitation causes a cascade of reactions that activates the plant defence system leading to the production of secondary metabolites [10]. However, the mechanism of elicitation is a complex process, based mostly on the type of elicitor, but also on the plant species, time of action, the condition of the plant, and type

of cultivation, among others. Studies performed by other authors have shown that different kinds of SA treatments, such as seed soaking or added to the medium in a hydroponic system, induced different physiological and biochemical effects in wheat plants, moreover, the effect of SA was organ-, and age-related [12].

Various studies have shown that the application of SA as an elicitor activates genes encoding key enzymes involved in the secondary metabolism in plants [12,19] like phenylalanine ammonia-lyase (PAL) [12]. PAL is involved in flavonoid and alkaloid metabolism, catalysing the reaction of deamination of phenylalanine to produce *trans*-cinnamic acid – the starting point in the AAs biosynthesis pathway [20]. GONDOR et al. [12] found that in leaves of hydroponically grown wheat plants, PAL was activated right after SA application and when removed from the nutrition medium, was back to the initial level. Moreover, PAL was upregulated in older plants, unlike in fully developed seedlings, while SA decreased the PAL activity in roots.

**4. Conclusion.** The present results indicated that elicitation with SA does affect the galanthamine biosynthesis in *H. papilio* hydroponic cultures. Even though the concentrations of 10 and 50  $\mu\text{M/L}$  SA did not induce significant GAL accumulation at the end of the experiment, the highest level of GAL was observed when treated with 100  $\mu\text{M/L}$  SA during 148 h. As the in vitro and hydroponic systems are different in many aspects of culture conditions and the physiological state of the plants, it is of interest to continue with the elicitation experiments of valuable secondary metabolites, such as galanthamine and haemanthamine in *H. papilio* hydroponic cultures, optimizing further the time and concentration of SA and other elicitors, on the one hand, and to elucidate the mechanism of action, including the genes involved in the process and the effect of elicitors on other primary and secondary metabolites, on the other.

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