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INFLUENCE OF ELICITATION ON *GLAUCIUM FLAVUM* CULTURES IN IN VITRO CONDITIONS

Iva Doycheva[#], Stefan Philipov[*], Marina Stanilova

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Abstract

Glaucium flavum Crantz is a medicinal plant species which contains high variety of isoquinoline alkaloids, the main of which is the alkaloid glaucine, possessing antitussive activity. In vitro cultivation was investigated as an alternative and sustainable approach for plant cultivation of this species which is characterized by decreasing distribution. The biotechnological production of secondary metabolites could be improved by the addition of elicitors such as jasmonic acid, methyl jasmonate and yeast extract to the artificial medium. The seed germination of *G. flavum* was over 60% in all examined media supplemented with the abovementioned elicitors. They did not stimulate higher alkaloid production but the plants of some of the investigated in vitro cultures had similar percentage of the crude alkaloid mixture, about 3%, to the native plant population from which they had derived. The quantity of the alkaloids varied but their content in the studied samples remained similar.

Key words: glaucine, jasmonates, yeast extract

Introduction. *Glaucium flavum* Crantz is a medicinal plant species with antitussive, anti-inflammatory, antibacterial, antimycotic and antitumour activities [1–3]. The species contains high variety of isoquinoline alkaloids, the main of which is the alkaloid glaucine, which has strong antitussive properties.

[#]Corresponding author.

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The area of distribution of the species is decreasing because of the anthropogenic influence. In vitro cultivation of medicinal plants and secondary metabolites production are sustainable approaches and they would be significant especially in the case of rare and threatened plant species. In vitro cultivation of medicinal plants could be investigated as an alternative resource of alkaloids instead of traditional methods such as field cultivation or collection from natural habitats, where the latter is forbidden according to the Bulgarian legislation concerning *G. flavum*.

There are a lot of studies on alkaloid content in plants from *G. flavum* collected from natural habitats but phytochemical studies on in vitro cultivated plants are very limited.

Plant cells, tissue and organ cultures have the same capacity to produce valuable secondary metabolites as their parent plant does in the wild. Elicitation is one of the most effective techniques currently used for improving the biotechnological production of secondary metabolites [4].

The biosynthesis of the valuable compounds in the artificial culture media could be enhanced by the addition of external molecules called elicitors. Among them, jasmonic acid (JA) and methyl jasmonate (MeJ) are widely used. Jasmonates, which include jasmonic acid and its derivatives, are plant hormones that control plant defences against biotic and abiotic stress factors [5]. In contrast to that, their use as stimulants of seed germination is controversial and depends on the species.

The yeast extract (YE) is an inducer which is becoming more popular as biostimulant of plant growth and development and biosynthesis of secondary metabolites. It is natural, nutritious, low-cost and safe substance, whose use increases the vegetative growth and yield of many vegetables [6].

The influence of these three elicitors on *G. flavum* needs to be further investigated. That is why, the objective of our work was to investigate the effects of JA, MeJA and YE on the alkaloid production of in vitro cultures and seed germination of the species.

Materials and methods. ***Seed origin.*** Seeds were picked from plants grown in the natural locality of *G. flavum* near the town of Pomorie (N 42.58634, E 27.63191). Plants were identified by Dr. Iva Doycheva. The seeds were presoaked in distilled water for 24 h before sterilization.

Seed sterilization and germination. At first sterilization of the seeds started with soaking in 70%, ethanol (1–2 min) and then they were sterilized in two steps. In the first step, seeds were immersed in 0.1% $HgCl_2$ (2 min) and rinsed once with sterile distilled water. In the second step they were sterilized for 10 min with commercial bleach (chlorine $< 2.5\%$) half diluted with sterile distilled water and then rinsed three times with sterile distilled water.

The sterilized seeds were sown on Gamborg B5 medium as a control and on B5 supplemented with elicitors in different concentrations – JA (0.5 mg/l;

1 mg/l), MeJ (0.5 mg/l; 1 mg/l), YE (1 g/l; 3 g/l). Seeds were cultivated in dark at $12 \pm 2\,^{\circ}$C for two weeks and after that at $23 \pm 2\,^{\circ}$C with photoperiod of 16/8 h light/dark.

In vitro cultivation medium. The basal medium was B5 supplemented with 20 g/l sucrose, solidified with 6.5 g/l Plant agar (Duchefa, NL), pH was adjusted to 5.75 and the medium was autoclaved at 121 °C for 20 min. JA and MeJ were filter sterilized and supplemented in the autoclaved basal medium.

One of the cultures was obtained from sterilized seeds set on B5 medium (control) and on B5 supplemented with elicitors in different concentrations – JA (0.5 mg/l; 1 mg/l), MeJ (0.5 mg/l; 1 mg/l), YE (1 g/l; 3 g/l). These cultures were marked as GfS. The other part of the cultures were obtained by root excision of two-month seedlings (composed of hypocotyls and cotyledons) which were cultivated on B5 medium as a control and on a B5 supplemented with JA (0.5 mg/l) or MeJ (0.5 mg/l). These cultures were marked as GfE. The cultures were cultivated at $23 \pm 2\,^{\circ}$C with photoperiod of 16/8 h light/dark. Every variant had two repetitions with 15–25 plants in number.

Plant material for a phytochemical research. All in vitro plants used for phytochemical research were cultivated for two months on the cultivation media. Basal B5 medium was used as a control, and the other plant samples were obtained from the cultures cultivated on B5 supplemented with different elicitors: 0.5 mg/l JA; 0.5 mg/l MeJ (cultures obtained from forward developing of the germinated seeds) and 1.0 mg/l JA; 1.0 mg/l MeJ; 1.0 mg/l YE (cultures obtained from seedling explants). Elicitor concentration of 1.0 mg/l was chosen for the cultures deriving from seedling explants because the use of this concentration in our preliminary experiments resulted in higher germination percentage with MeJ and YE rather than the concentration of 0.5 mg/l.

The reference sample (R-Pm) was obtained from plants in flowering stage which had been picked from the natural locality of the species near Pomorie.

Gas chromatography – mass spectrometry (GC-MS). GC-MS: Hewlett Packard 6890/MSD5973 instrument, HP-5 MS column (30 m × 0.25 mm × 0.25 µm). The temperature programme was 50 to 300 °C at 4 °C min^{-1} and 10 min hold at 300 °C. Injector temperature was 280 °C. The flow rate of carrier gas (He) was 0.8 ml min^{-1}. The compounds were identified by comparison of their mass spectra with spectral library Wiley 275.

Chromatography. Short column chromatography (SCC): neutra alumina 90 (Merck, act. II-III, 70–230 mesh). Thin layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60 F_{254}, Merck), using petroleum/$CHCl_3$/Me_2CO/MeOH in the ratio 4/4/1/1 (v/v/v/v) as solvent systems. Visualization for TLC: Dragendorff's reagent. The identification of the alkaloids – thaliporphine, isoboldine, bracteoline, dehydroglaucine, 7-oxoglaucine, corunine, protopine, allocryptopine – in the TLC study was made by a comparison with authentic samples [7–10].

Alkaloid extraction. All samples from the aerial parts of *G. flavum* were developed in the same way to give a crude alkaloid mixture (CAM). The air-dried ground plant material (Table 2) was extracted five times with 2 ml of 95% ethanol for 5 min in an ultrasonic bath at room temperature. The combined extracts were dried on anhydrous Na_2SO_4 and evaporated under reduced pressure to dryness. Then 2 ml of 3% hydrochloric acid was added to the residue and the mixture thus obtained was kept for 3 min in an ultrasonic bath at room temperature. After filtration, the solution was mixed with neutra alumina and subjected to a SCC on alumina, eluted with dichloromethane saturated with ammonium hydroxide. The evaporation of eluate under reduced pressure to dryness gave the crude mixture of alkaloids.

Statistical analysis. Statistical significance of the tested media was evaluated using Mann–Whitney U test ($p = 0.05$).

Results. ***Seed germination.*** A high percentage of seed germination was observed in almost all examined medium variants. Only the seed germination in the medium supplemented with 3 g/l yeast extract was lower than 80%. Seed germination was slightly higher in the medium variants with lower concentration of two of the used elicitors – JA and YE, whereas in variants containing MeJ the highest percentage was observed when MeJ was used in the highest concentration (Table 1).

T a b l e 1

Seed germination in the media with different composition

Variant	Control	0.5 mg/l JA	1 mg/l JA	0.5 mg/l MeJ	1 mg/l MeJ	1 g/l YE	3 g/l YE
Germination percentage ±SD	93% ±4.45	96% ±5.66	90% ±14.14	80% ±16.97	96% ±5.66	89% ±4.82	66% ±20.34

No statistical differences were observed between the tested medium variants

Phytochemical studies. No qualitative differences were established by thin layer chromatography of CAM of GfS and GfE, where in sample GfE a larger amount of alkaloids was biosynthesized. Among the GfS samples, the MeJ supplemented medium (GfS-2) along with the control medium (GfS-K) had the highest CAM percentages – 1.78% and 1.81%, respectively. Low percentages of alkaloids were obtained from the plants cultivated in the other two types of medium supplemented with JA (GfS-1) and yeast extract (GfS-3) – 0.92% and 0.88%, respectively (Table 2).

All GfE samples had identical CAM percentages in the dry plant material and only in MeJ supplemented medium (GfE-2) the CAM percentage (2.92%) was slightly higher than the control (GfE–K) and JA supplemented medium (GfE-1) – 2.38% and 2.45%, respectively. Moreover, the CAM percentage of the plant sample

Table 2

Dry weight of the studied samples and CAM percentages in them

Abbreviation	Medium type	Dry weight of the plant material (g) with SD	CAM percentage in the dry plant material (%)
R-Pm	–	11.02	3.03
GfE-K	B5, explants	0.0714 ± 0.002	2.38
GfE-1	B5 + 0.5 JA, explants	0.2446 ± 0.003	2.45
GfE-2	B5 + 0.5 MeJ, explants	0.1848 ± 0.003	2.92
GfS-K	B5, seeds	0.0884 ± 0.011	1.81
GfS-1	B5 + 1.0 JA, seeds	0.1088 ± 0.006	0.92
GfS-2	B5 + 1.0 MeJ, seeds	0.1181 ± 0.011	1.78
GfS-3	B5 + 1.0 YE, seeds	0.2513 ± 0.003	0.88

from Pomorie was 3.0%, which was similar to the percentages of the GfE samples (Table 2). The alkaloids in the GfE samples were firstly identified through thin layer-chromatographic comparison, which revealed that all three samples had identical alkaloid composition. That is why further experiments were carried out with one of these samples – GfE-1.

The thin layer-chromatographic comparisons of GfE-1 with genuine samples of the following alkaloids – thaliporphine, isoboldine, bracteoline, dehydroglaucine, 7-oxoglaucine, corunine, protopine, allocryptopine – revealed that GfE-1 did not contain the alkaloids isoboldine, bracteoline, and allocryptopine.

GC-MS comparison of the alkaloid content of GfE-1 sample with R-Pm showed that in vitro cultures contained higher quantity of the alkaloids thaliporphine and protopine and lower quantity of the alkaloids dehydroglaucine and glaucine. Moreover, GfE-1 contained the alkaloid 7-oxoglaucine whereas R-Pm did not (Table 3).

Discussion. JA and its derivates are involved in the process of plant growth, defence, wounding, pollen and embryo development, seed dormancy and germination. Their influence on seed germination varies among plant species. JA and MeJ influence on seed germination is very controversial and unpredictable. They inhibited the seed germination in some species and promoted it in others [11–14]. In contrast to this, the influence of yeast extract was more pronounced in some species probably because of its hormone content and richness of different amino acids, minerals and vitamins, for example sugar beet seed germination was promoted when seeds were soaked in yeast [15]. The results obtained for the influence of the applied elicitors on seed germination of *G. flavum* revealed that elicitors did not stimulate the germination under the provided temperature and light conditions. Only the yeast extract at its higher concentration provoked slight decrease

Table 3

Alkaloids identified in the in vitro sample GfE-2 and the natural population R-Pm presented as a percentage of total ion current[a] in GC-MS

Alkaloid	RT	GfE-2	R-Pm
Thaliporphine	38	4	1
Glaucine	39	69	76
Protopine	42	9	4
Dehydroglaucine	50	10	14
7-Oxoglaucine	46	1	N/A
Corrunine	44	1	1

[a]The area of GC-MS peaks depends not only on the concentration of the corresponding compounds but also on the intensity of their mass spectral fragmentation, so the data given in the table are not a true quantification but can be used for comparison between the samples which is the objective of this work.

in the germination percentage. However, this result was not statistically significant.

In vitro organ cultures have the potential to be used for investigation of biosynthesis and production of alkaloids and other metabolites, as well as for the propagation of valuable species and genotypes. To the best of our knowledge, this is the first study on alkaloid content of in vitro germinated and cultivated plants of *G. flavum*. The alkaloid content in wild-grown *G. flavum* plants was found to be highest in their flowering and fruiting stages [16–18]. The content of glaucine in the calli from *G. flavum* was reported to be not nearly as high as its content in the plants taken from the natural populations [19].

The percentages of the alkaloid mixture and that of glaucine alone in the samples of the in vitro explants were close to those of the reference sample, which was obtained from plants in flowering stage and had been developed in the natural environment of the species. These close results between in vitro plants and wild plants suggested similarities in the conditions and plant response. The lower values of CAM in the samples from the in vitro plants grown with roots were probably due to the lower level of stress to which these plants had been exposed. In comparison, the explant cultures had been subjected to an additional stress factor in the form of tissue wounding.

The obtained results suggest that further research needs to be done in order to improve the in vitro propagation of this recalcitrant species, and subsequently obtain more plant material and find out which conditions could enhance the alkaloid biosynthesis in in vitro cultures compared to the wild-growing *G. flavum*.

Conclusion. The results of seed germination experiments showed that the JA, MeJ and yeast extract supplementation did not improve the germination of

the seeds. The elicitors did not stimulate higher alkaloid production but the in vitro cultivated plants of *G. flavum* had similar productivity of alkaloids to that of the population from which they had derived. The results showed that the quantity of alkaloids may vary but the qualitative composition remained similar in the studied samples.

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Institute of Biodiversity and Ecosystem Research
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 23
1113 Sofia, Bulgaria
e-mails: idoycheva@gmail.com
marina.stanilova@gmail.com
iva.doycheva@iber.bas.bg
marina.stanilova@iber.bas.bg

*Institute of Organic Chemistry
with Centre of Phytochemistry
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 9
1113 Sofia, Bulgaria
e-mail: stefan.philipov@orgchm.bas.bg