Development of in vitro cultures from Astragalus thracicus Griseb and Astragalus aitosensis (Ivanisch.) and evaluation of their bioproduction potential.

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Development of *in vitro* cultures from *Astragalus thracicus* Griseb and *Astragalus aitosensis* (Ivanisch.) and evaluation of their bioproduction potential.

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Key words: *Astragalus* species; callus cultures; elicitation; flavonoid; stress-induction

Key message: Development of *in vitro* cultivation media preferences of two endangered *Astragalus* species, resulting in a developed micropropagation protocol. Measurement of characteristics of callus cultures and flavonoid content analysis after stress induction.

Abstract: Two endemic *Astragalus* species - *A. thracicus* and *A. aitosensis* were investigated to establish and develop their *in vitro* cultures. We managed to optimize the *in vitro* growth conditions, suitable for growing and maintaining stable and long-lasting callus cultures. Furthermore, we measured the behavior and flavonoid biosynthetic potential of callus cultures after stress induction with selected elicitors methyl jasmonate (MJ), salicylic acid (SA), and yeast extract (YE). In case of biotic stress, we concluded that growth index of callus cultures from *A. thracicus* decreased inversely proportional to the concentrations of elicitors applied, as SA showed the strongest growth inhibiting effect. The synthetic potential of the same callus cultures was evaluated based on HPLC analysis

of levels of three flavonol aglycons – kaempferol, quercetin, and methylquercetin. The highest amount of kaempferol (9.12 μg/g dry weight) and quercetin (5.72 μg/g dry weight) was detected when callus cultures were exposed to YE at a concentration of 50 mg/l in medium for 72 hours. For the synthesis of the third aglycon studied, methylquercetin, the best inductive conditions (equivalent to 4.69 μg/g dry weight) were measured in media supplemented with MJ at a concentration of 200 μmol/l for 72 hours. The fastest kaempferol synthesis was found in the medium supplemented with 1000 μmol/l SA at 24 hours, quantified as 7.61 μg of kaempferol per gram of dry weight. Based on all these experiments, the study resulted in a development of a micropropagation protocol.

Abbreviations: Indole-3-acetic acid (IAA), kinetin (6-Furfuryladenine or kin), 1-naphthylacetic acid (NAA), thidiazuron (TDZ), dry weight (DW), yeast extract (YE), methyl jasmonate (MJ), salicylic acid (SA)

Introduction: With more than 2200 taxonomically classified species, the genus *Astragalus* (Fabaceae) is one of the largest genera of dicotyledonous plants (Frodin 2004). *Astragalus* species are widespread mainly in the temperate regions of the northern hemisphere (Heywood et al. 1972), growing in arid and semiarid regions up to 1700 meters above sea level (Pistelli 2002). Nearly 133 species grow in Europe, of which 29, classified in 8 subgenera, are found in Bulgaria (Heywood et al. 1972; Tutin et al. 1972; Platikanov et al. 2005; Assyov et al. 2006). Several of them are endemic or protected with vulnerable, endangered or critically endangered status (Petrova and Vladimirov 2009).

Among the protected species, *Astragalus thracicus* (Griseb) is a tertiary relict, endemic to the Balkans, listed under the "vulnerable" status. It is a xeromorphic shrub with strong roots and hairy, densely branched stems (16-40 cm high), and leaves ending in a spine. Nowadays it is found in only three habitats in Bulgaria (around the towns of Sliven, Yambol, and Haskovo), but it also occurs in some limited areas in the Greek and Turkish regions of Thrace (Stoeva).

The second object of this research is *Astragalus aitosensis* (Ivan.). As an endemic relic plant in Bulgaria, it is also protected by the Bulgarian Biodiversity Act and is included in the Bulgarian Red List of Threatened Plants. *A. aitosensis* is a low, spiny, tussock-forming shrub with highly branched stems (30-50 cm high) (Apostolova). The plant grows on dry, stony sites (90-550 m altitude) with neutral to alkaline soils. Nowadays the plant is only found in the suburbs of the small Bulgarian town of Aytos (from which it takes its name).

The long history of use of *Astragalus* species as medicines, food, and cosmetics has led to considerable progress in its chemical investigation. In general, literature describes a high content of saponins and phenolics, which contribute to a variety of biological activities of their extracts (Ionkova et al. 2014; Bratkov et al. 2016). The phenolic compounds isolated from *Astragalus* are composed of a wide range of flavonoids. Many species of *Astragalus* naturally contain the widely distributed aglycones – kaempferol, quercetin and methylquercetine in their free and glycosidic forms (Bratkov et al. 2016; Vasilev et al. 2019, 2021, 2024).

The approach to grow plants in *in vitro* cultures is now a very attractive way to use them as source for the production of various phytochemicals. It has added value, especially for endangered or rare plants. In this study, the propagation and cultivation of *A. thracicus* and *A. aitosensis* was investigated with regard to the establishment and development of their *in vitro* cultures. In a series of experiments, germination rate, influence of gelling agent, and carbon source were analyzed and compared. After determining the basic requirements for *in vitro* culturing, an elicitation experiment was carried out using methyl jasmonate (MJ), salicylic acid (SA), and yeast extract (YE).

Material and methods

Plant material

Seeds of *A. aitosensis* and *A. thracicus* were collected in June 2013. *A. aitosensis*: habitat located in the region of the town of Aytos (coordinates on Google Maps: 42.703065 N, 27.268543 E). *A. thracicus*: habitat located in the area of Bakadzhitsite, near the town of Yambol (coordinates on Google maps: 42.452016 N, 26.663550 E). The voucher specimens have been deposited in the herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, with ref. no. № SOM001362 and № SOM001363, respectively. The voucher specimens are shown in the Supplementary Materials section **Figure S1** and **Figure S2**.

Surface sterilization

Surface sterilization was performed by immersion in 70% EtOH for 60 s, followed by 30 min on Vortex® in 10% NaClO with the addition of 20 µl Tween 80, and the seeds were rinsed three times with sterile distilled water for 10 min each time. Seed coats were incised with a sterile scalpel. Unincised and incised seeds were aseptically placed in full strength Murashige and Skoog (MS) with added casein DoH media (see Supplementary Materials **Table S3**).

Seed germination

This experiment was carried out with Petri dishes filled with 20 ml of DoH medium (2% sugar; 6 g/l agar; measured pH 5.6) and 7 seeds in each, 10 dishes of each group, i.e. 70 scarified and 70 non-scarified seeds for each *Astragalus* species - *A. thracicus* and *A. aitosensis*. The experiment was performed once.

Media preparation

The culture media required for the experiments were prepared using commercial products from Duchefa™: Murashige and Skoog (MS) including vitamins and Gamborg B5, both dissolved in demineralized water with 3% sucrose added. The mineral and vitamin content of both media is given in the Supplementary Materials (**Table S3**). For the preparation of half strength media (½ MS and ½ B5), the same commercial products were used, but the measured amount was divided by 2. The pH of the media was adjusted to 5.80 using 1 N and 0.1 N solutions of HCl and NaOH under constant stirring. After measuring the pH, sucrose was added. The prepared medium was sterilized in Certoclav® at 121 °C for 20 minutes. ***Hormonal modifications:*** * stays for added BAP 1 mg/l and IAA 200 μg/l. ** means a modification with 1 mg/l Kin, 200 μg/l IAA and 100 μg/l 2,4-D. *** contains added 400 μg/l TDZ and 200 μg/l NAA Supplementary Materials (**Table S4**).

Growing conditions

All cultures were maintained at 24 ± 2 °C in a growth chamber with fluorescent light (20 klx light intensity) and a 16 h light/8 h dark photoperiod. The short-day conditions required for the vernalization stage of *ex vitro* plants were 8 h light/16 h dark photoperiod at 18±2 °C.

Callus induction

20-day old stem, leaf, and petiole explants were cut and placed under sterile conditions on different media compositions (MS, ½MS, B5 and ½B5) with different hormone regimens (*, **, ***; see Media preparation - data available in Supplementary Materials, **Table S4**). The flasks were kept in light growth conditions 16 / 8 h. After additional 20-25 days, a callus was induced and results were collected. The experiment was performed once.

Comparison of the gelling agent

The gelling agents used in this experiment were: plant agar at a concentration of 6 g/l and Gelrite™ at two concentrations with a difference of 20% (2.0 g/l and 2.4 g/l). The experiment was carried out in 10 petri dishes per group, each containing 7 callus explants. Results were collected on day 10.

Measurement of survival rate

Survival of callus was assessed per oculi. Fresh green callus was scored as 1; brown dead callus was scored as 0; green callus with dead parts was scored as 0.5 (**Fig. 1**).

Fig. 1: Parameters of the evaluation of callus vitality

The formula for the evaluation is:

$$\% = \frac{sum\ pieces\ of\ live\ callus}{total\ number\ of\ callus\ pieces\ in\ a\ Petri\ dish} \times 100$$

Comparison of survival rate according to carbon source

The survival rates of callus cultures of *A. thracicus* and *A. aitosensis* were compared according to the carbon source (sucrose and glucose), their concentrations (2-6%) on two different hormonal regimes MS* and MS**. Each group in the experiment consisted of 5 glass containers for biotechnological cultivation with 50 ml of nutrient medium and 7 pieces of callus, placed in each container.

Measurement of growth index and quantification of concentrations of flavonoid aglycons in callus cultures under induced stress conditions.

Callus lines of *A. thracicus* grown on MS* medium supplemented with 3% sucrose and the gelling agent Gelrite™ (2.0 g/l) were selected for the experiment. The experiments were performed in triplicates with 7 callus pieces placed on 25 ml of medium. The elicitors used were YE at concentrations of 5, 10, 20, 50, and 100 mg/l, MJ at concentrations of 10, 50, 100, 200, 500, and 1000 μmol/l, and SA at concentrations of 100, 200, 400, 500, 800, 1000, and 1600 μmol/l. The control medium contained no added elicitors. Evaluations of experiments were performed on 24, 48, and 72 hours.

Extraction of flavonoids from in vitro cultures and ex vitro plants from A. thracicus

100 mg of lyophilized plant material was placed into a centrifuge tube together with 2 ml of 80% methanol and immersed in an ultrasonic bath for 15 minutes. Tubes were centrifuged to separate the supernatant from the plant cell sediment. The solvent from the supernatant was evaporated in a vacuum desiccator at 30 °C. The dried extract was subjected to hydrolysis by dissolving in 1 ml of 1 N aqueous HCl and heating in a water bath (96 °C) for 1 hour. 1 ml of ethyl acetate was added to the hydrolysate and after separating the two phases, the upper ethyl acetate layer was carefully removed and evaporated to dryness at 30 °C. The dried residue obtained was dissolved in 1 ml of methanol and injected into the HPLC system.

Construction of a standard curve for the quantification of kaempferol, quercetin, and methylquercetin in in vitro cultures and ex vitro plants of A. thracicus

Quantification of kaempferol, quercetin, and methylquercetin was performed using the method of external standard with kaempferol. Solutions containing 6.25, 12.5, 25, 50, and 100 μg/ml of kaempferol were prepared by a series of dilutions from a 1 mg/ml solution of kaempferol in methanol. Measurements were made on LC20A HPLC Shimadzu system (Shimadzu, Duisburg, Germany), coupled to a UV detector (SPD-M20A). Column oven temperature: 40 °C. Detection wavelength used: λ = 330 nm. HPLC program: 5% to 35% of B, for 35 min, followed by increasing of B from 35% to 70% for a further 15 min (solvent A: deionized water + 0.2% HCOOH; solvent B: acetonitrile) and rinsing and equilibrating the column. Column: Ascentis® C18, 250 × 4.6 mm I.D., 5 μm (Supelco Analytical, USA). Flow rate: 1 ml/min. Injection volume: 20 μl. Each solution was analyzed in a triplicate by HPLC and a calibration curve (See Supplementary Materials **Figure S7**) was constructed using the average peak areas. The retention time for kaempferol was 36.9 minutes (Mr 286.23), for quercetin 37.3 minutes (Mr 302.24), and for methylquercetin 37.6 minutes (Mr 316.26), respectively. For mass measurements of each of these three peaks was used UPLC-ELSD-ESI-MS^nDionex Ultimate 3000RS UHPLC system (Thermo Scientific, Milford, USA) and an ESI-MS^n-detector (amaZon speed, Bruker, Bremen, Germany). equipped with an RP-18 Kinetex column (2.1 × 100 mm, 2.6 μm, Phenomenex, Torrence, CA, USA). The flow rate was set to 0.4 ml min^{-1} and the column oven temperature to 35 °C. Aliquots of 1 μl of sample were injected into the system using an autosampler. The statistical data of the curve were calculated by linear regression using GraphPad Prism 6.01. The equation of the curve is calculated as y = 22740x - 12916, r^2 = 0.9999, p <0.001 (Supplementary materials **Figure S7**).

The quantities of quercetin and methylquercetin were determined by reference to the standard curve constructed for kaempferol due to the similarities in the chromophores.

Micropropagation protocol for A. thracicus and flavonoid accumulation in ex vitro plants

1. Seeds were sterilized with 20 % sodium hypochlorite and 0.1% Tween 20, followed by scarification.
2. Ten days after germination, the plantlets were incised and placed on solid MS medium containing 1 mg/l BAP, 200 μg/l IAA, 3% sucrose, and 2.0 g/l Gelrite™ to induce callus formation.
3. Pieces of the resulting callus were transferred to MS medium with 2 mg/l BAP, 100 μg/l IAA, 6% glucose, and 2.0 g/l Gelrite™ to promote embryogenic callus and shoot development.
4. After three weeks, the shoots were moved to a root-inducing MS medium with 200 μg/l IAA until roots formed.
5. After another three weeks, plantlets were transferred to hormone-free MS medium to allow for further growth and strengthening.
6. The plantlets were removed from the *in vitro* containers, their roots carefully washed, and then planted in pots with soil substrate. They were grown in a greenhouse under long-day conditions (16 hours light at 23 ± 2 °C) for three months.
7. Once the plants were strong enough, they were transferred to a room with short-day conditions (8 hours light at 18 ± 2 °C) for three weeks for flower induction.
8. The plants were returned to long-day conditions in the greenhouse (16 hours light at 23 ± 2 °C).
9. Two weeks after the return to these conditions, plants started to flower.

RESULTS AND DISCUSSION

Seed germination

Seed coat of members of the Fabaceae family is relatively strong and tough, which significantly hinders the germination process. The scarification is a technique for mechanical or chemical damage of the seed coat in order to facilitate and accelerate the entry of water and mineral salts into the inner seed (Statwick 2016). It is important to note that mechanical scarification is carried out carefully, without damaging the cotyledons themselves. The control group consisted of non-scarified seeds that have undergone the same sterilization conditions and have been grown under the same temperature and light regiment. As the results of this experiment show, the creation of this small "scar" greatly facilitated the rupture of the seed coat and significantly increased the germination rate and shortened the germination period by 10-12 days (**Fig. 2**).

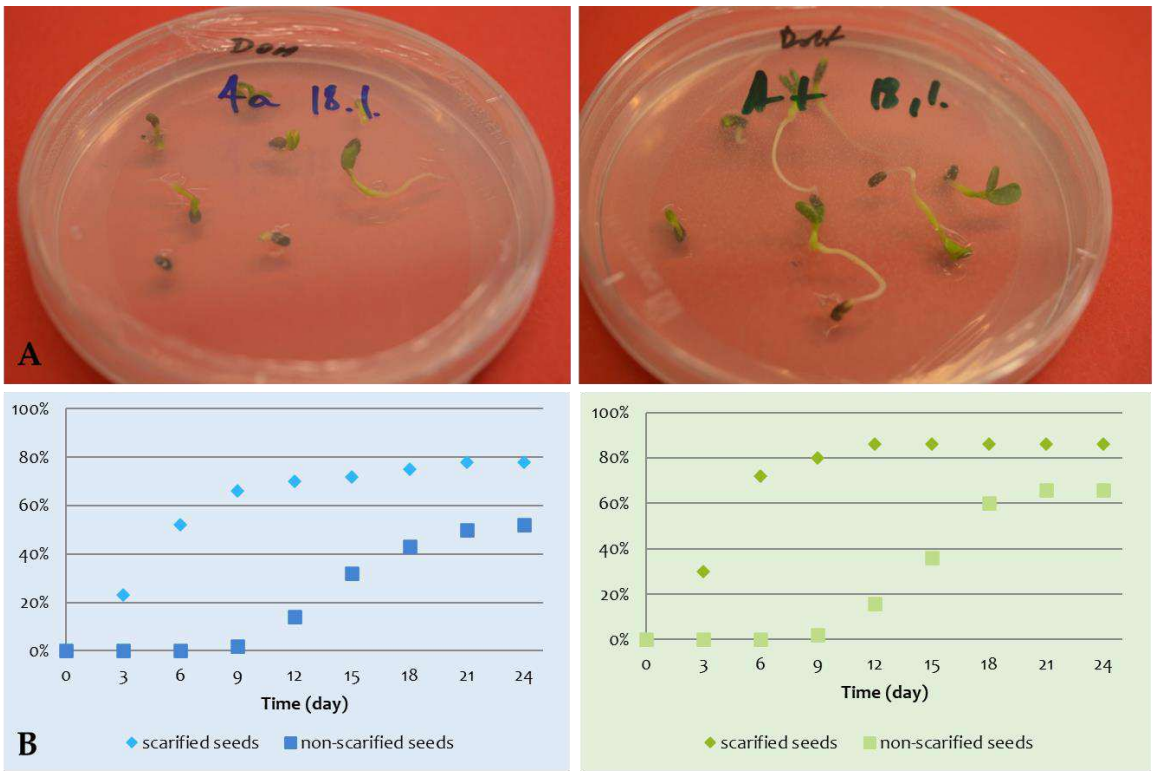

Fig. 2: Determination of seed germination. (A) Seed germination 20 days after sterilization. (B) Germination rate and time required in scarified and non-scarified seeds (78% scarified seeds vs. 52% non-scarified seeds for *A. aitosensis* and 86% scarified seeds vs. 66% non-scarified seeds for *A. thracicus*)

Of the two species studied, *A. thracicus* showed a higher germination potential, determined by the germination rate and the time required for germination. *A. thracicus* showed a maximum germination rate in scarified seeds (86%), reached on day 12, and 66% germination in non-scarified seeds on day 21. *A. aitosensis* showed a germination rate of 70% in scarified seeds on day 12, rising to 78% on day 21 and 52% in non-scarified seed on day 24.

Callus induction

After testing different media compositions (MS, ½MS, B5, and ½B5) and hormone concentrations (*, **, ***) added to the nutrient medium (data in Supplementary Materials **Table S3** and **Table S4**), it was found that MS* medium shows best potential for callus formation (**Fig. 3**). Therefore, 20-day old leaf, petiole and stem explants were cut and placed on MS* medium for callus induction. After a further 20-25 days, callus was induced.

Fig. 3: Callus formation rate from leaf or stem explants cultivated on media with different mineral and hormonal compositions. (**A**) *A. aitosensis* and (**B**) *A. thracicus*.

Comparison of the gelling agent effect

Gelling agents are traditionally considered to be an inert component of the culture media, primarily due to the large molecular size of the consisting polysaccharides (for example

253 agarose), which makes them virtually indigestible by the plant cell. What significantly
254 affects the plant cells is the level of admixtures (impurities) such as various phenolic
255 acids, salts, etc. in the gelling powder. Another very important characteristic of the gelling
256 agent is its retention strength, which measures the way in which water and mineral salts
257 are osmotically released from the solid nutrient medium.

258 Gelrite™ is a natural gelling agent that in recent years is increasingly replacing plant agar
259 usage as a medium solidifier in biotechnology. Gelrite™ is a highly purified natural
260 anionic polysaccharide produced by microbial fermentation, without the significant
261 variations in composition, typical of plant agar (Huang et al. 1995). In presence of soluble
262 salts, such as Mg^{2+} and Ca^{2+}, Gelrite™ forms hard, but brittle gels with similar osmotic
263 properties to these of plant agar, using only half of its amount. Another important feature
264 of Gelrite™ is that it does not contain the phenolic impurities, normally found in plant
265 agar, which can be toxic to some sensitive organisms. The strength of the gel formed
266 (according to the characteristics of the commercial product) is 1100 g/cm^2 for agar and
267 400-700 g/cm^2 for Gelrite™.

268 We compared the viability of *A. aitosensis* callus explants on media, solidified with three
269 different gelling conditions: plant agar at a concentration of 6 g/l and Gelrite™ at
270 concentrations 2.0 g/l and 2.4 g/l (representing a 20% increase in Gelrite™ concentration).
271 For each group of this analysis were used ten petri dishes, containing 7 callus explants in
272 each. The results were collected on day 10.

273 All media formulations showed relatively high viability rate. However, the highest value
274 was observed on the medium with 2.0 g/l Gelrite™, reaching 98%. When Gelrite™
275 concentration was increased to 2.4 g/l, this change led to a reduced viability rate
276 measured to 81%, representing a decrease of 17%, compared to the optimal value. In
277 contrast, media solidified with 6 g/l plant agar showed 77% of survival rate, which was
278 21% less than that, observed for 2.0 g/l Gelrite™. These results suggested that lower
279 concentration of Gelrite™ not only reduced medium cost, but may also create more
280 favorable osmotic conditions for callus viability, compared to higher Gelrite™
281 concentrations or standard plant agar.

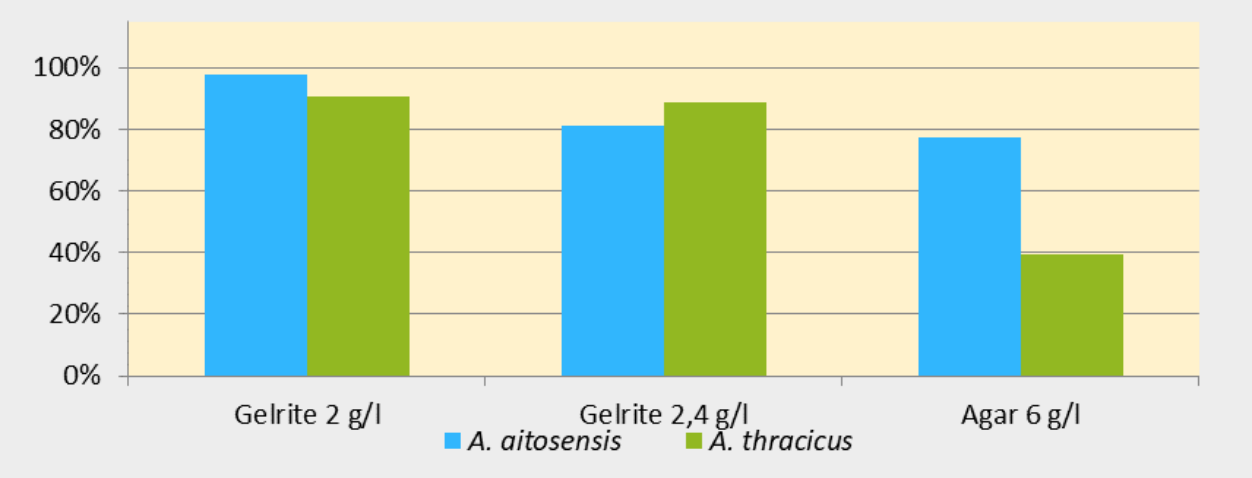

Fig. 4: Survival rate comparison of callus cultures from *A. aitosensis* and *A. thracicus* on media, solidified with different gelling agent used: Gelrite™ and Plant agar.

In contrast to *A. aitosensis*, the viability of *A. thracicus* callus explants was not significantly affected by the used concentrations of Gelrite™. Explant survival rates were measured to 91% on medium solidified with 2.0 g/l Gelrite™ and 89% with 2.4 g/l, which indicates low sensitivity to applied variations in concentration of Gelrite™. However, a markedly reduced viability was observed when plant agar was used as gelling agent . The survival rate on agar-solidified media declined to 39%, which is 52% decrease according to the optimum (**Fig. 4**). These results suggest that *A. thracucus* callus cultures show compliance to changes in Gelrite™ concentrations, but their viability is significantly compromised when grown on media, solidified with conventional plant agar.

Comparison of callus survival by carbon source

As described in Plant Propagation by Tissue Culture 3rd Edition, the type and concentration of carbon source has a strong influence on the osmoticum of nutrient media (Thorpe et al. 2008). It plays such an important role in plant physiology that it can even influence plant morphogenesis. Therefore, our third approach to determine the optimal growth conditions of *in vitro* cultures examined the effect of the carbon source and its concentration in two media with different hormonal regimes.

Callus cultures of *A. aitosensis* and *A. thracicus* were exposed to two carbon sources, sucrose and glucose, at five concentrations each (2%, 3%, 4%, 5%, and 6%; **Fig. 5**). In order to maintain the *in vitro* cultures studied in the state of callus, it was necessary to apply two different hormonal regimes, MS* and MS** (**Table S4**; Supplementary Materials).

Fig. 5: Survival rate (in %) measured for *in vitro* callus cultures of (**A**) *A. aitosensis* and (**B**) *A. thracicus* over three parameters: type of carbon source (sucrose and glucose), its concentration (2-6%) and hormonal composition of the MS** medium MS*.

A. aitosensis showed impaired development and comparatively low survival of its callus cultures compared to *A. thracicus*. Only 4 of the 10 tested media variations showed survival rates above 80%, those were 6% Glc-medium MS** (100%), 5% Glc-medium MS**

(90%), 4% Glc-medium MS** (83%) and 3% sucrose on MS**-medium (89%). In the nutrient media with the hormonal composition of MS* the highest survival rate (61%) was observed in 2%, 3% and 5% sucrose (**Fig. 5A**).

Callus cultures of *A. thracicus* showed significantly better performance with a higher survival rate (**Fig. 5B**). Unlike *A. aitosensis*, the growth of callus cultures of this species was not affected by the concentration of the carbon source provided and the observed survival rate was 100% in 17 out of 20 variations of the nutrient medium. The only deviations observed were in the MS** medium with 2% and 6% sucrose and 5% glucose. This is good evidence that the two species behave differently under the same conditions. Due to the poor cultivability of *A. aitosensis* callus lines, work with this species was subsequently discontinued.

Measurement of growth index and quantification of flavonoid aglycons in A. thracicus callus cultures under induced stress conditions

Another method to influence the development rate of callus cultures and their secondary metabolite production is the application of elicitors. According to literature, elicitation with MJ, SA, and YE has proven to be an effective strategy for enhancing flavonoid production in plant *in vitro* cultures (Halder et al. 2019). In plant metabolism, MJ and SA are intracellular signal molecules, which trigger plant defense mechanisms, respectively stress response. In that way, they significantly activate secondary metabolite biosynthetic pathways (see Supplementary Materials **Figure S5**), resulting in higher flavonoid yield (Jeyasri et al. 2023). On the other hand, YE is classified as an exogenous biotic elicitor. Exogenous elicitors are chemicals, normally originating from microbial pathogens such as polysaccharides, peptides, glycoproteins etc. They also show to be effective in enhancing secondary metabolite production (Zaman et al. 2022).

This experiment was carried out to determine the effect of biotic stressors on biomass accumulation in callus cultures and the measurement of flavonoid aglycones, quantified by HPLC.

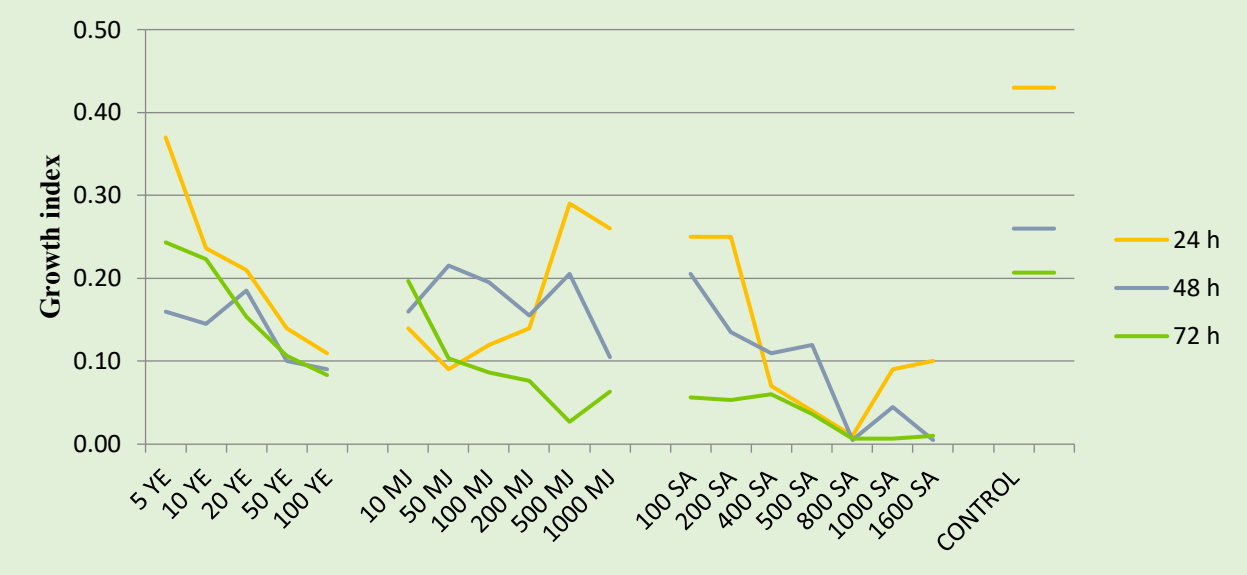

Fig. 6: Graphical representation of the measured growth index for *A. thracicus* callus cultures under different stress conditions by yeast extract (YE), methyl jasmonate (MJ), and salicylic acid (SA).

Fig. 6 illustrates the growth index for *A. thracicus* callus cultures subjected to elicitation with YE, MJ, and SA over different time intervals (24, 48, and 72 hours) in MS* medium. an inversely proportional correlation was observed between the callus biomass accumulation and the concentration of the used elicitor, indicating that higher levels of stressors were associated with reduced growth.

At the 72-hours exposure time point, all elicitors significantly reduced the growth rate of their cultures compared to the control (growth index: 0.21). Callus grown on a medium supplemented with 800 µmol/l SA exhibited minimal growth, with a reported growth index of 0.01. In contrast, the highest applied concentrations of YE (100 mg/ml) and MJ (1000 µmol/l) yielded growth to 0.08 and 0.06, respectively. While all three elicitors reduced callus proliferation, the degree of inhibition varied as salicylic acid showed the strongest suppressive effect on callus culture growth, followed by methyl jasmonate and yeast extract (**Fig. 6**).

Subsequently, HPLC analyses were performed to quantify the three main flavonol aglycones kaempferol, methylquercetin and quercetin after treatment with the three different elicitors: yeast extract (YE), methyl jasmonate (MJ) and salicylic acid (SA) (**Fig. 7**). Overall, the strongest effect on kaempferol biosynthesis in *A. thracicus* calli was observed for all three elicitors.

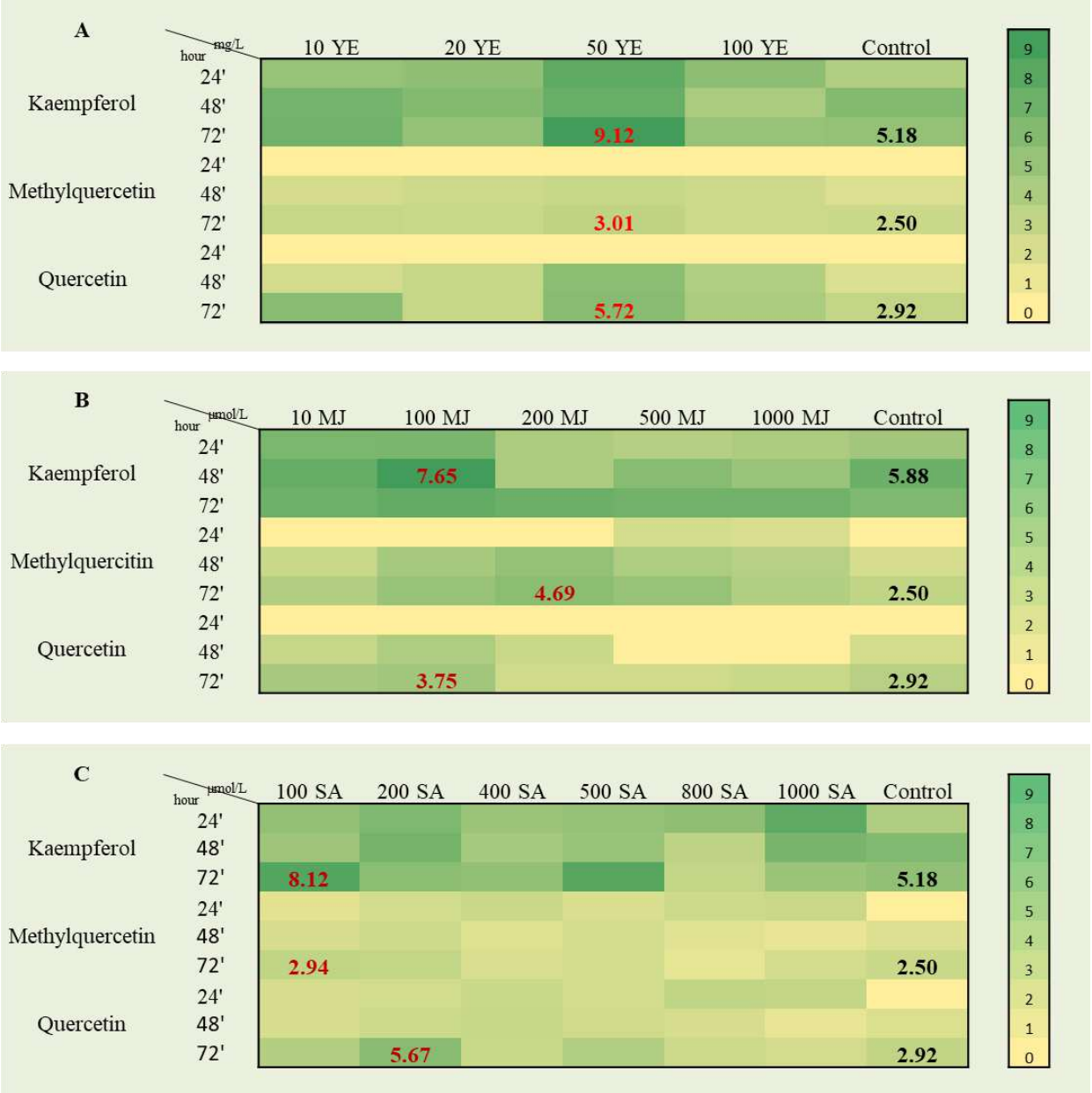

Fig. 7: Heat map diagrams for kaempferol, methylquercetin and quercetin (µg/g DW) yield after treatment with different concentrations of (7A) yeast extract (YE), (7B) methyl jasmonate (MJ) and (7C) salicylic acid (SA)

The highest concentrations of kaempferol (9.12 µg/g dry weight (DW)), methylquercetin (3.01 µg/g DW), and quercetin (5.72 µg/g DW) were obtained after 72 hours of elicitation with 50 mg/l YE (**Fig. 7A**). Compared to the corresponding control values – 5.18 µg/g DW for kaempferol, 2.50 µg/g DW for methylquercetin, and 2.92 µg/g DW for quercetin – a noticeable enhancement in kaempferol and quercetin accumulation is represented. This indicates that a concentration of 50 mg/l YE is optimal for the induction of kaempferol and quercetin biosynthesis. However, the increase of methylquercitin is marginal under

the same conditions, indicating that the influence of YE on its biosynthesis is not significant.

In the case of treatment with MJ, the maximum kaempferol accumulation (7.65 μg/g DW) was detected after 48 hours at 100 μmol/l MJ, compared to a control value of 5.88 μg/g DW) (**Fig. 7B**). Methylquercetin accumulation peaked to 4.69 μg/g DW at 200 μmol/l MJ (control: 2.50 μg/g DW), while quercetin reached 3.75 μg/g DW at concentration of 100 μmol/l MJ (control: 2.92 μg/g DW). These findings demonstrated that MJ also promotes flavonoid accumulation, particularly methylquercetin, but with differing optimal concentrations for each compound.

Elicitation with SA resulted in 8.12 μg/g DW of kaempferol after 72 hours at a concentration of 100 μmol/l SA (control: 5.18 μg/g DW) (**Fig. 7C**). A similar value (8.00 μg/g DW) was observed at 500 μmol/l of SA after the same period of exposure. Interestingly, when SA concentration was increased to 1000 μmol/l, peak of kaempferol biosynthesis (7.61 μg/g DW) was reached just after 24 hours, followed by a decline at 48 and 72 hours. Such a pattern of biosynthesis was not observed either for YE or MJ. The highest value for quercetin was 5.67 μg/g DW (control: 2.92 μg/g DW) at a concentration of 200 μmol/l SA. In contrast, no significant induction of methylquercetin biosynthesis levels could be detected following SA application, suggesting a compound-specific response to this elicitor.

Large number of papers discuss *in vitro* biosynthesis of flavonoids. Different research groups are using different protocols with significant fluctuations in parameters during the developing process, which would bring to a vast variation in the results gained. One of the most critical parameters influencing *in vitro* flavonoid production is the type of culture used for investigation. The choice of culture must be carefully chosen according to the metabolites of interest. As noted in a review by Krasteva et al. (Krasteva et al. 2016), for *in vitro* flavonoid production from *Astragalus* species most commonly used are callus or suspension cultures. Although some studies have utilization of organ cultures, like adventitious and hairy roots (Hussain et al. 2022), *in vitro* root cultures from *Astragalus* are more frequently reported to produce astragalosides or calycosin (Jiao et al. 2014; Park et al. 2015). Of a matter is also the age of the used culture, which could significantly influence its biosynthetic capacity, with reports ranging from newly established calli to cultures maintained for years (Grzegorczyk-Karolak et al. 2016).

Another major factor, which complicates the direct comparison of gained results with literature is the diversity in developing elicitation protocols. Commonly used elicitors such as YE, MJ, and SA are applied with wide variation of concentrations and times of exposure (Narayani and Srivastava 2017). For instance, YE has been reported in concentrations ranging from as low as only 1 mg (Zaman et al. 2022) to as high as 1000

mg per liter of medium (Akbary and Golkar 2023). MJ and SA are typically applied at concentrations between 50 to 200 µmol/l (Woch et al. 2023). The period of exposure of the callus cultures to the elicitor also considerably varies from short-term treatments of 24, 48, 72, or 168 hours (Tůmová et al. 2010; Anusha et al. 2016) to extended periods of 21 days (Mounika and Giri 2024), 28 days (Lescano et al. 2025), and even reaching up to 80 days (Szymczyk et al. 2021).

A third source of variability could be the process of extraction and analytical procedures used. In literature was found only one study (Mendoza et al. 2020), which describes similar method (including acidic hydrolysis, followed by a liquid/liquid extraction with ethyl acetate) for extraction and sample preparation, comparable to the one used in present report.

Taken together, all those factors in culture type, elicitation protocols and analytical methodologies make it challenging to directly compare the results of this study towards broader scientific literature and research background.

Micropropagation protocol for A. thracicus and flavonoid accumulation in ex vitro plants

After establishing a successful micropropagation protocol for *A. thracicus* (See Material and methods), the last step of this research was to determine the biosynthesis and accumulation of flavonoids in *ex vitro* plants grown in the greenhouse (**Fig. 8**). For that purpose, the content of three flavonol aglycones in four plants was analyzed using the same HPLC protocol as described above.

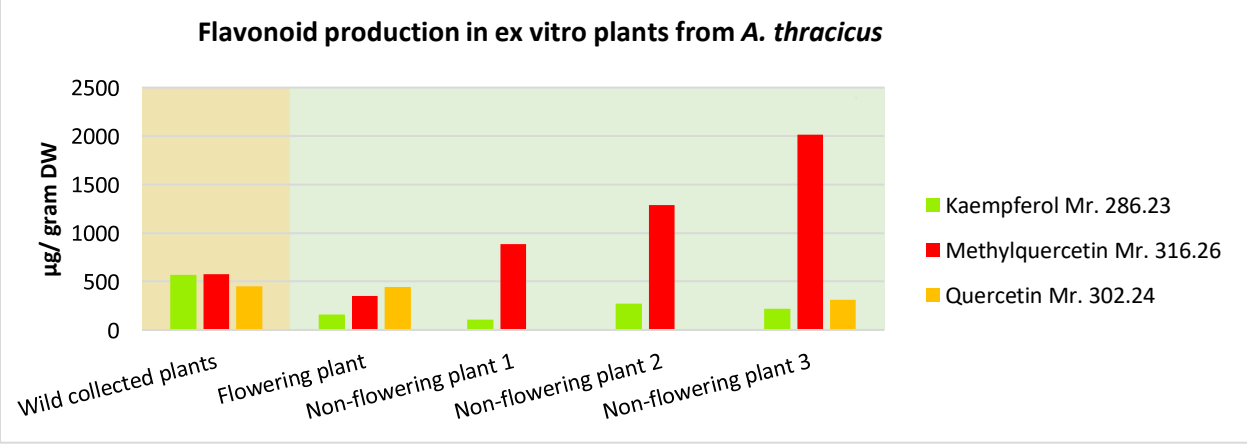

Fig. 8: Determination of flavonoid production in *A. thracicus* by HPLC. Plants used in the experiment are one flowering plant and three non-flowering plants obtained by micropropagation (1-3). Wild collected plants served as controls.

HPLC analysis of extracts from leaves of wild collected *A. thracicus* plant was used to compare the content with *ex vitro* plants gained with micropropagation – one flowering and three non-flowering plants (**Fig. 8**). The kaempferol aglycone content was highest in

wild collected material, measured as 569 μg/g DW, while *in vitro* – propagated plants (flowering and non-flowering) contained 269 μg/g DW, approximately half the amount measured in wild plant material.

However, the most significant variation was observed in methylquercetin content. In wild collected plant material, the content was measured to be 572 μg/g DW, while *in vitro* – propagated plants showed ranging accumulated methylquercetin: 351 μg/g DW in the flowering plant and 883 μg/g DW (non-flowering plant 1), 1290 μg/g DW (non-flowering plant 2), and 2012 μg/g DW (non-flowering plant 3) values, which significantly exceed the amount in wild-grown plant.

For the third analyzed aglycone – quercetin, a trend towards higher synthesis was observed in the flowering plant. In comparison to wild-collected plant (453 μg/g DW), the flowering plant contained an almost equal amount (444 μg/g DW). No traces of quercetin were detected in two of the non-flowering plants, while 312 μg/g DW was measured in the third, possibly indicating a transition from the vegetative to the generative phase.

In conclusion, this study demonstrates a successful method for conserving the endangered species *A. aitosensis* and *A. thracicus*. Furthermore, for *A. thracicus* was shown how flavonol aglycone content can be influenced by the application of various elicitators, suggesting its potential for future biotechnological exploitation as a source for the sustainable production of valuable secondary metabolites.

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Statements & Declarations,

Authorship contribution statement

Hristo Vasilev: Data curation, Funding acquisition, Investigation, Conceptualization, Writing - original draft. Karel Šmejkal: Data curation, Funding acquisition, Writing - original draft. Christian Schulze Gronover: Funding acquisition, Methodology, Writing - original draft. Dirk Prüfer: Writing and revision of original draft, Funding acquisition, Methodology.

Declaration of competing interest

The authors declare that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data can be provided on request.

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581 **Tables, legends to figures, figures.**

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583

584 **Fig. 1:** Parameters of the evaluation of callus vitality

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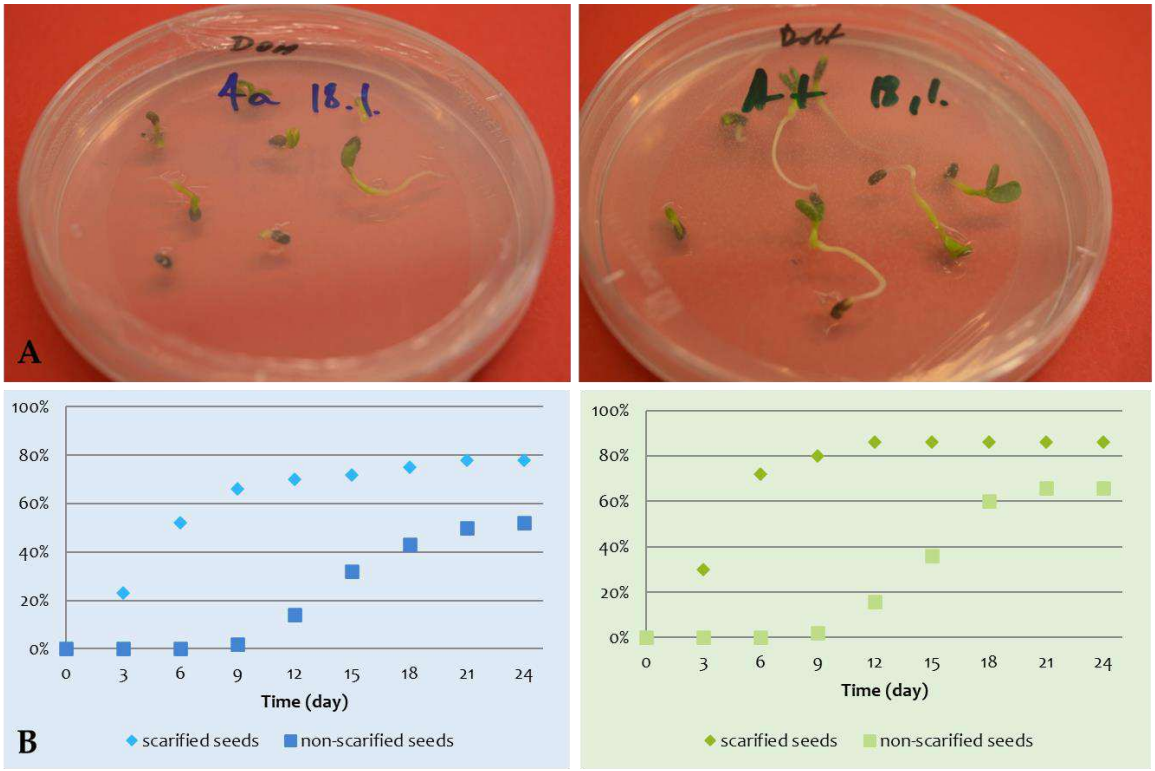
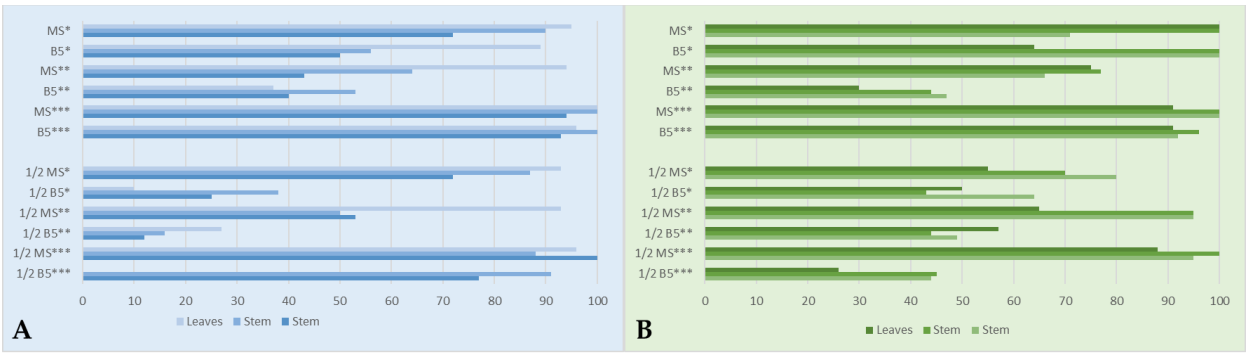


Fig. 2: Determination of seed germination. (**A**) Seed germination 20 days after sterilization. (**B**) Germination rate and time required in scarified and non-scarified seeds (78% scarified seeds vs. 52% non-scarified seeds for *A. aitosensis* and 86% scarified seeds vs. 66% non-scarified seeds for *A. thracicus*)



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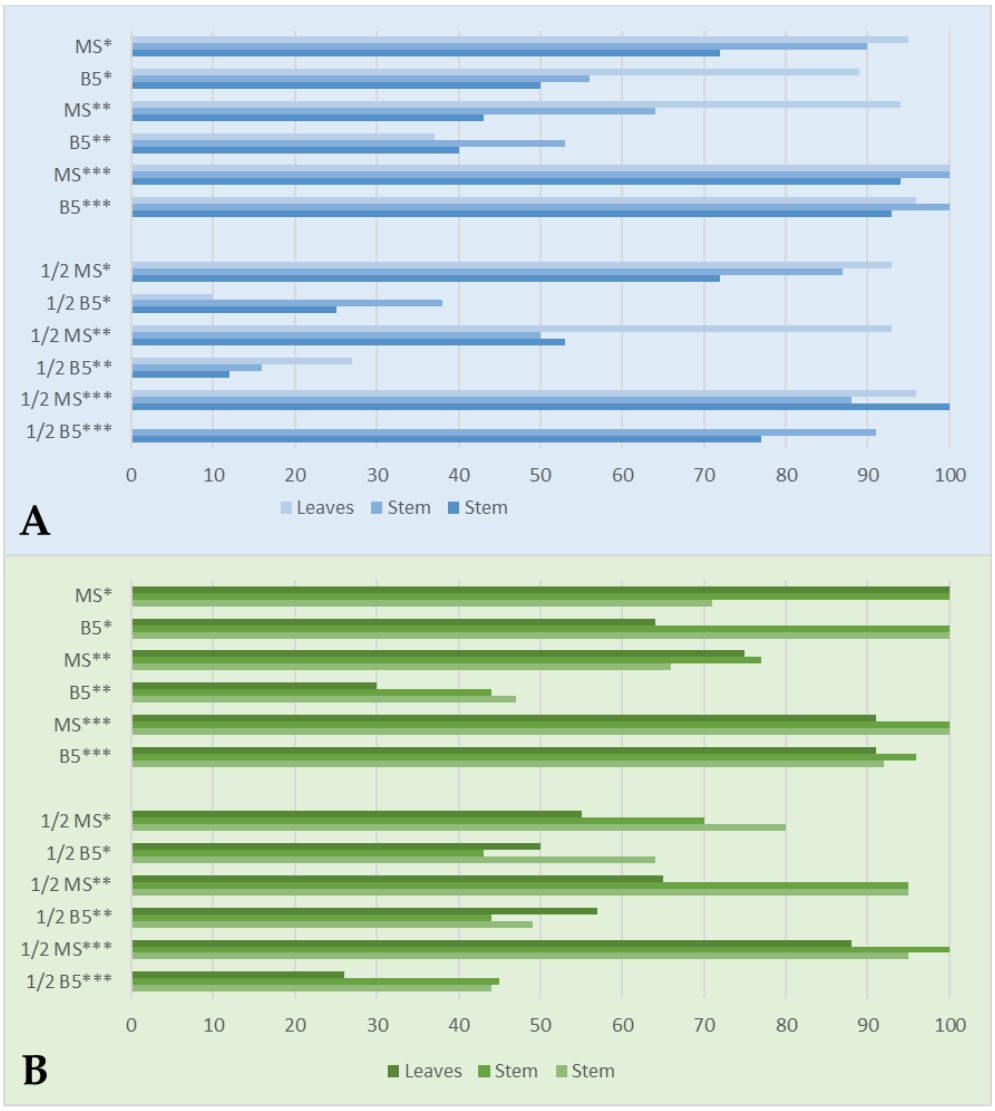

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595 **Fig. 3:** Callus formation rate from leaf or stem explants cultivated on media with different
596 mineral and hormonal compositions. (**A**) *A. aitosensis* and (**B**) *A. tharcius*).

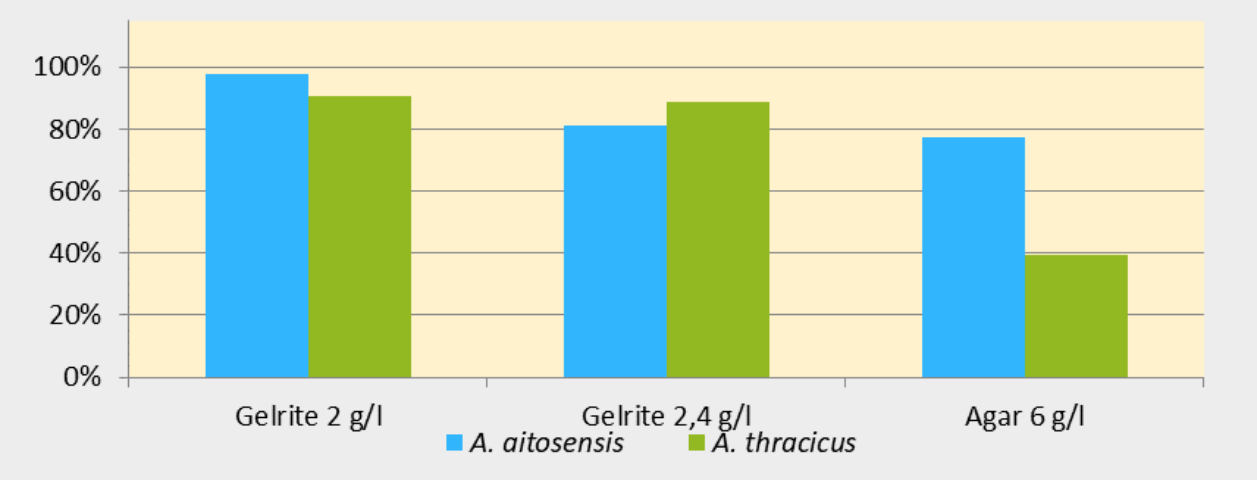
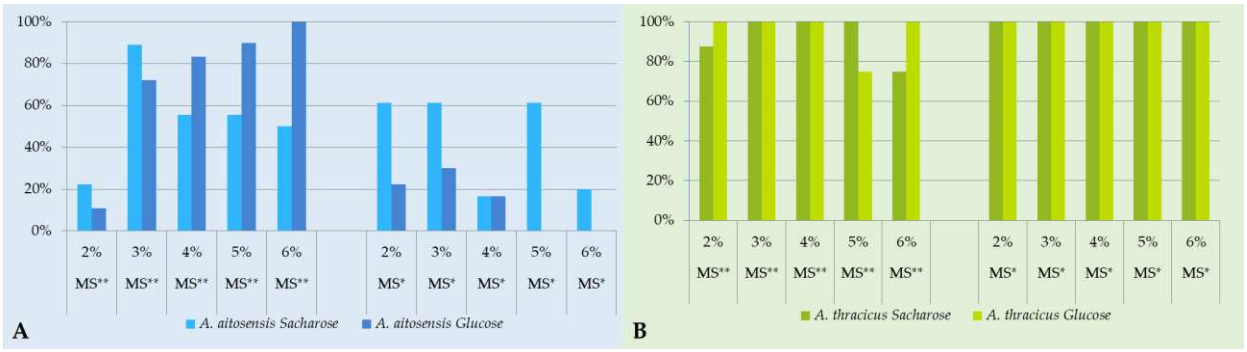


Fig. 4: Survival rate comparison of callus cultures from *A. aitosensis* and *A. thracicus* on media, solidified with different gelling agent used: Gelrite™ and Plant agar.



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602

603 **Fig. 5:** Survival rate (in %) measured for in vitro callus cultures of (**A**) *A. aitosensis* and (**B**) *A.*
604 *thracicus* over three parameters: type of carbon source (sucrose and glucose), its concentration (2-
605 6%) and hormonal composition of the MS** medium MS*.

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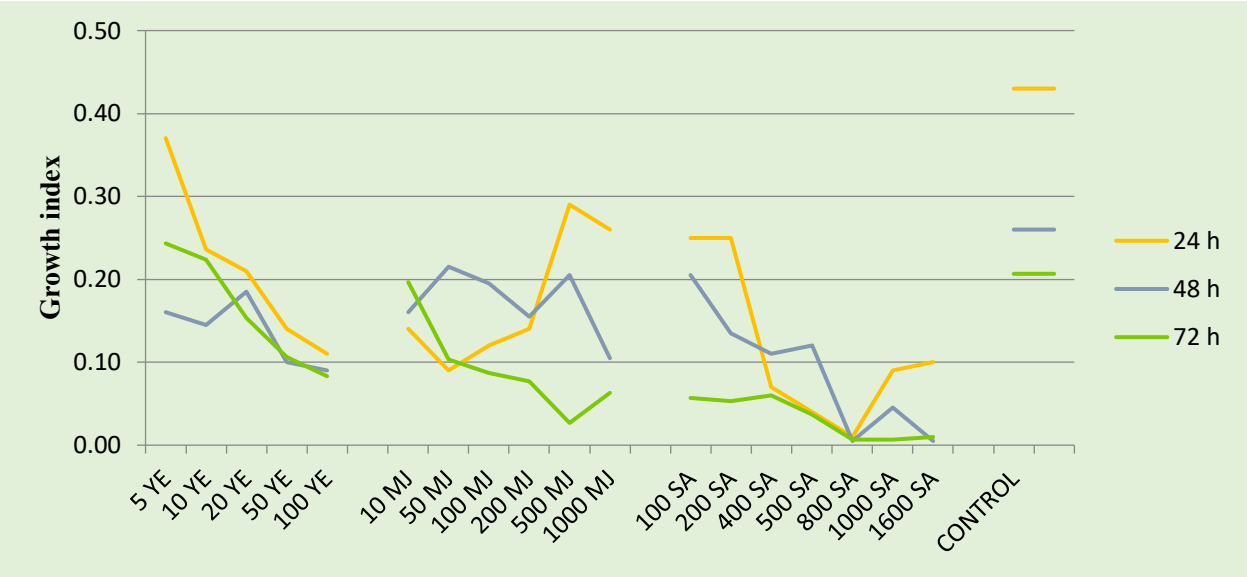

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608 **Fig. 6:** Graphical representation of the measured growth index for *A. thracicus* callus cultures
609 under different stress conditions by yeast extract (YE), methyl jasmonate (MJ) and salicylic acid
610 (SA).

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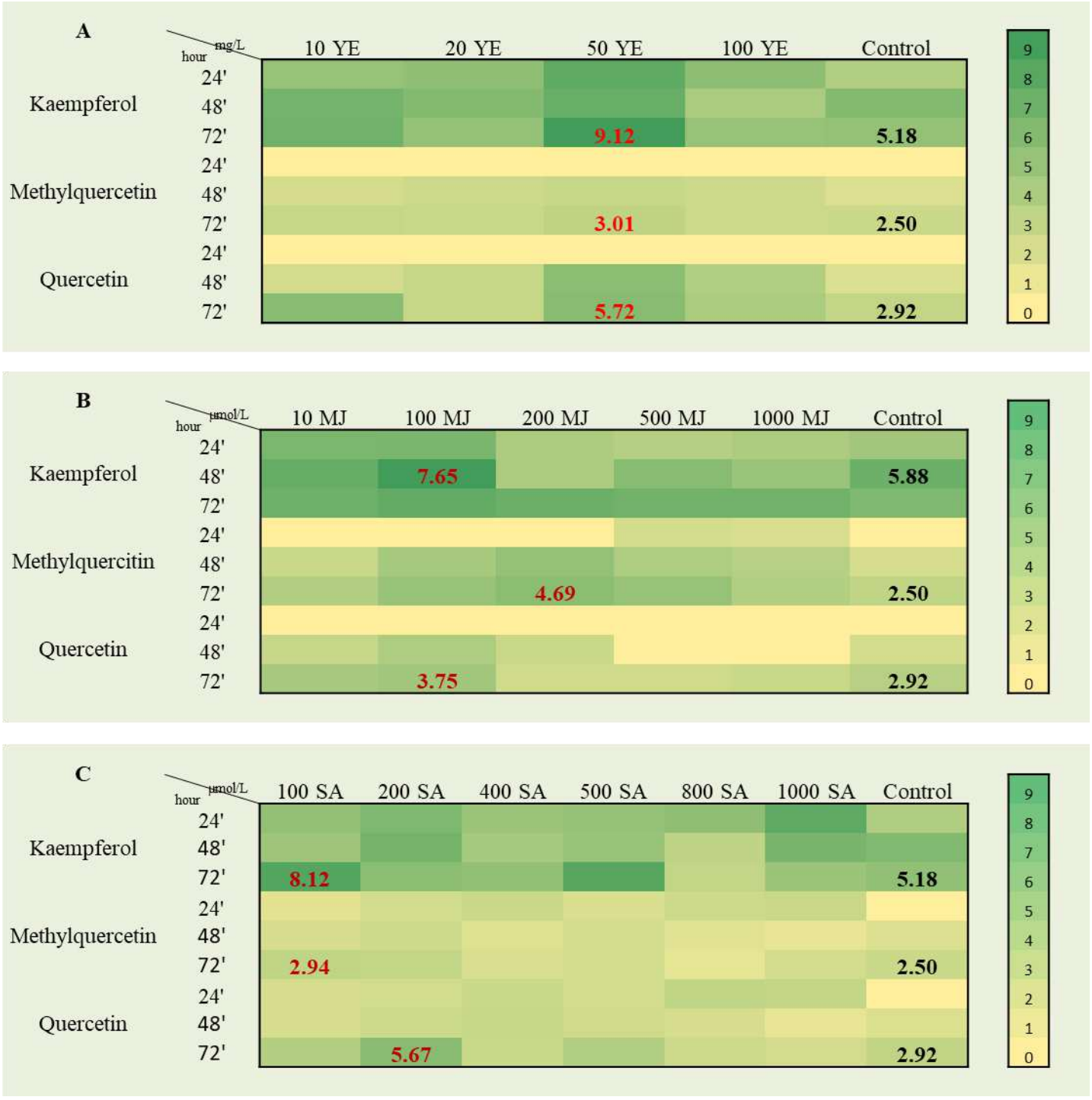

Fig. 7: Heat map diagrams for kaempferol, methylquercetin and quercetin (µg/g DW) yield after treatment with different concentrations of (**A**) yeast extract (YE), (**B**) methyl jasmonate (MJ) and (**C**) salicylic acid (SA)

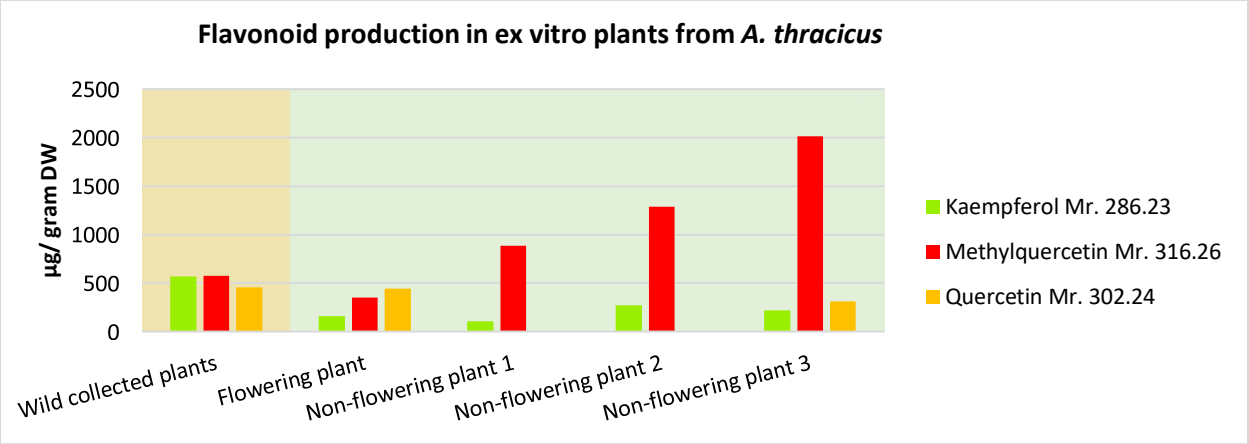

Fig. 8: Determination of flavonoid production in *A. thracicus* by HPLC-MS. Plants used in the experiment are one flowering plant and three non-flowering plants obtained by micropropagation (1-3). Wild collected plants served as controls.

Supplementary Files

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- SupplementaryMaterials20.07.2025.pdf