IN VITRO INDUCTION AND MULTIPLICATION OF CALLUS CULTURES PRODUCING SECONDARY METABOLITES IN *Pimpinella anisum* SPECIES

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Abstract. The aim of our research consisted in optimising the secondary metabolite production in *Pimpinella anisum* callus lines cultures by determining the type and concentration of the efficient elicitors. In order to obtain a consistent amount of callus *in vitro*, we started to obtain plantlets by *in vitro* germination of seeds, free of contaminants, as source of vegetal material, after which the inocula were subcultivated on different variants of the basal medium Murashige - Skoog (MS). We used yeast extract, benzylaminopurine (BAP), 2.4-dichlorophenoxyacetic acid (2.4-D), naphthalenacetic acid (NAA) and indolylacetic acid (IAA) in different combinations and concentrations, as growth factors. The culture medium variant supplemented with 100 mg/L yeast extract associated with 5 mg/L BAP and 1 mg/L NAA triggered the optimal value regarding growth of callus biomass. The calli grown on a variant of nutritive culture medium supplemented with NAA and BAP in equal concentrations (1 mg/L) had the highest concentration of flavonoids (30±2.49 mg ERU/g fresh weight) and polyphenols (11,5 ±0.49 mg EAG/g fresh weight). Triterpenoid accumulation in callus lines was most stimulated by subcultivation on a medium with 2.4-D and IAA in concentration of 1 mg/L, associated with yeast extract (100 mg/L).

Keywords: anise callus cultures, secondary metabolites, *in vitro* plant growth factors.

Rezumat. Inducerea și multiplicarea *in vitro* a culturilor de calus producătoare de metaboliți secundari la specia *Pimpinella anisum*. Scopul cercetărilor noastre a constat în optimizarea producerii de metaboliți secundari la liniile calusale de *Pimpinella anisum* prin determinarea tipului și a concentrației optime de elicitori. Pentru a obține o cantitate considerabilă de calus *in vitro* la specia menționată, au fost germinate semințe de anason în condiții sterile, apoi, fragmente din plantulele rezultate au fost inoculate pe diferite variante ale mediului bazal Murashige–Skoog (MS) suplimentat cu diferiti factori de creștere. Cele nouă variante de mediu utilizate au continut extract de drojdie, benzilaminopurină (BAP), acid diclorofenoxiacetic (2.4–D), acid naftalenacetic (ANA) și acid indolilacetic (IAA) în diferite concentrații și combinații. Valori optime în ceea ce privește creșterea masei de calus s-au obținut pe varianta de mediu de cultură suplimentată cu 100 mg/L extract de drojdie în asociere cu 5 mg/L BAP și 1 mg/L NAA. Cel mai mare conținut de flavonoizi (30±2.49 mg ERU/g greutate proaspătă) și polifenoli (11,5±0.49 mg EAG/g greutate proaspătă) s-a obținut pe varianta de mediu de cultură ce contine NAA și BAP înconcentrații egale (1 mg/L). Acumularea de triterpenoizi a fost stimulată de subcultivarea pe mediu suplimentat cu 2.4-D și IAA în concentrație de 1 mg/L, în asociere cu extractul de drojdie (100 mg/L).

Cuvinte cheie: culturi de calus de anason, metaboliți secundari, factori de creștere ai plantelor *in vitro*.

INTRODUCTION

Studies concerning *in vitro* culture of plants with conservative and economical importance provide many opportunities regarding aspects such as enhancing seed germination, obtaining plant material by micropropagation of shoots, roots and callus cultures, and production of bioactive compounds with medicinal properties or economical values.

Pimpinella anisum L. (Apiaceae family) is an annual aromatic herb native to the eastern Mediterranean region and Southwest Asia and a traditionally medicinal plant which contained many bioactive compounds with a wide range of pharmacological activities (CHIRISTAKI et al., 2012; SHOJAII & ABDOLLAHI, 2012). The carminative, antibacterial, antiviral, antifungal, anti-inflammatory and antioxidant activities were mentioned among these (MOHAMED et al., 2015). The fruit contains volatile oils consisting mainly of trans-anethole (AMER & OMAR, 2019). Because of their natural products content, including flavonoids, terpenes, and essential oils with higher antioxidant activity, *Pimpinella anisum* seeds were extensively studied (SALIM & AALI, 2021). To meet the commercial demand of anise cultivation, different techniques for *in vitro* propagation were developed, such as callus production. Among the first studies, CHAND et al., 1997 obtained *in vitro* callus cultures to anise from shoot apex, stem explants and seeds. Other authors, SAXENA et al., (2012) studying the effect of cytokinins BAP and Kinetin on organogenesis in anise, obtained primary callus as the first developmental stage in the indirect organogenesis process.

The aim of our paper was to obtain different types of callus cultures in *Pimpinella anisum*, with a higher rate of secondary metabolite production, by using different growth factors and elicitors.

MATERIAL AND METHODS

In order to obtain plant material, mature seeds of *Pimpinella anisum*, commercially available (Agrosem, Romania) were used. Aseptic seeds were obtained by washing with running tap water for 30 minutes, then using a series of disinfection baths: in ethanol 70 % for 1 minute, 0.1 % $HgCl_2$ solution for 3 minutes and rinsed with sterile bidistilled water.

Seeds germination was stimulated by adding 0.5 mg/L benzylaminopurine (BAP) and 0.1 mg/L naphthalenacetic acid (NAA) to the basal MS medium (MURASHIGE & SKOOG, 1962). After a week, different fragments from obtained plantlets were sampled and inoculated on the MS medium with 30g/l sucrose and supplemented with 1 mg/L 2.4- dichlorophenoxiacetic

acid (2,4-D) in order to initiate the callusogenesis process. Primary callus was obtained after two weeks of cultivation in the growth room at a temperature of 22-24±2ºC. The callus fragments of same mass of 0.2 g were subcultivated on nine culture media variants containing different combination of growth factors as BAP, NAA, 2,4-D or indole acetic acid (IAA) in association with yeast extract (YE) (Table 1).

Table 1. Variants used for *in vitro* callus cultures.

Culture media variants	Growth factors added to MS basal medium (mg/L)				
	BAP	NAA	2,4-D	IAA	YE
V1	0.5	0.1			
V2	0.1		1	10	
V3	5	1			100
V4			2		200
V5			1	1	100
V6	1	2			
V7	1	1			
V8	3	0.5			100
V9			0.25	0.5	100

The culture dishes were kept in the same conditions at a temperature of 22-24±2ºC with a photoperiod of 16 hours light/8 hours darkness.

The biomass production was evaluated as the growth rate, which was calculated as a ratio between the callus weight after 30 days of cultivation on nutritive media and the initial weight of the inoculum (0.2 g).

Biochemical analyses. About 1g callus was ground with mortar and pestle and extracted with 100% methanol at a ratio of 1:5 (m/v), at room temperature, with stirring at 180 rpm, for 72 hours. The supernatant obtained by centrifugation at 10,000 G for 20 minutes was used for subsequent analysis.

Total polyphenolic content determination. For polyphenol concentration, the classic method described by MIHAILOVIC et al. (2013) was used. The reaction mixture (0.5 ml of extract, 2.5 ml of Folin-Ciocalteu reagent, and 2 ml of 7.5% Na_2CO_3) was let for 30 minutes at room temperature, and absorbance was read at 765 nm. The total phenolic content, expressed as gallic acid equivalents/ fresh weight (GAE mg/g sample) represented the average of three repetitions.

The flavonoid content determination. For estimation of the total content of flavonoids, the method adapted by CAI et al. (2010) was applied. A quantity of 250 µl diluted extract was mixed with 1 ml of distilled water and 75 µl of 5% $NaNO_2$ solution. The samples were incubated for 5 min at room temperature, and then 75 µl of 10% $AlCl_3$ solution was added. After 6 minutes, the reaction was stopped with 2 ml of 4% (w/v) NaOH and diluted up to 5 ml with distilled water. Absorbance was determined at 510 nm against blank with methanol. Results were expressed as rutin equivalents/fresh weight (RE mg/g sample) and represented the average of three repetitions.

The triterpenoid content determination. The triterpenoid concentration was measured following the method proposed by KE et al. (2014). Samples (50 µl dry extract) were kept on ice and mixed with 400 µl of 5% vanillin-glacial acetic acid solution and 800 µl perchloric acid. After a 15 min incubation at 60 °C, 5 ml of glacial acetic acid was added and samples were thoroughly mixed. The absorbance of each sample was read at 545 nm and compared to a blank with methanol. Results were expressed as mg of ursolic acid equivalents/fresh weight (UAE mg/g sample) and represented the average of three repetitions.

RESULTS AND DISCUSSIONS

The source of plant material free of contaminants was represented by plantlets germinated from seeds on an MS medium added with BAP (0.5 mg/L) and NAA (0.1 mg/L), the germination being very delayed on the basal MS medium without hormones (Fig. 1). Primary callus was obtained from different fragments of one-week plantlets on a medium supplemented with 1 mg/L 2.4-D.

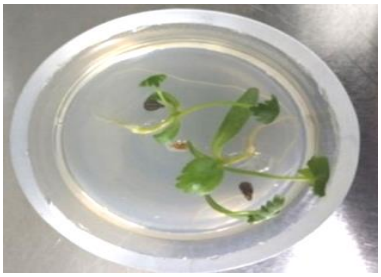

Figure 1. *Pimpinella anisum* plantlets obtained on MS supplemented with BAP (0.5 mg/L) and NAA (0.1 mg/L) (orig.).

The different types of calli were initiated from the same inocul on culture media, supplemented with various concentrations and combinations of growth factors (Fig. 2). In our case, the medium variants V2 and V4 could not maintain the viability of callus cells presenting advanced necrosis processes. Probably higher hormone concentrations or imbalanced concentrations from these medium variants had negative effects on the proliferation process and cell viability. In generally, yeast extract added to the *in vitro* basal MS culture medium in concentration of 100 mg/L can sustain the growth of callus mass as nutritive factor, but in a higher concentration than 200mg/l it had an unwanted effect (V4).

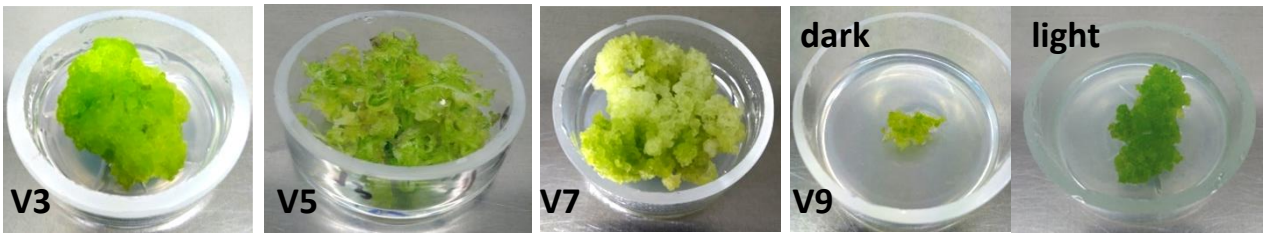

Figure 2. The different type of callus obtained on basal MS media supplemented with 5 mg/L BAP, 1 mg/L NAA and 100 mg/L YE **(V3)**, 1 mg/L 2,4-D, 1 mg/L IAA and 100 mg/L YE **(V5)**, 1 mg/L BAP and 1 mg/L NAA **(V7)** and 0.25 mg/L 2,4-D, 0.5 mg/L IAA and 100 mg/L YE **(V9)** (orig.).

Our results showed that the best culture medium for obtaining a consistent biomass of *Pimpinella anisum* callus was the V3 variant, which included 100 mg/L yeast extract associated with 5 mg/L BAP and 1 mg/L NAA in the MS nutritive medium (Fig. 3). Also, high growth rates above 10 were registered on V6, V7 and V8, but the larger SD values suggested the need to optimize biomass production in these callus lines. The results of ARAFA and USAMA (2022) present shoot tip explants producing efficiently callus at *Pimpinella anisum* on MS medium supplemented with 1 mg/L NAA, 1 mg/L 2.4–D and 2 mg/L kinetin after four weeks of *in vitro* cultivation. Regarding the fresh weight of callus obtained by *in vitro* culture on different variants of basal MS medium, MOUBARAK et al (2021) mention that the largest quantity was achieved on medium supplemented with 0.5 mg/L BAP and 1 mg/L 2,4 D, while the addition of IBA in association with BAP proved to be the weakest option for callus proliferation. Other authors, TANIDA and SHIOTA (2019) mention the specific action of anise callus regarding the 2,4-D degradation system.

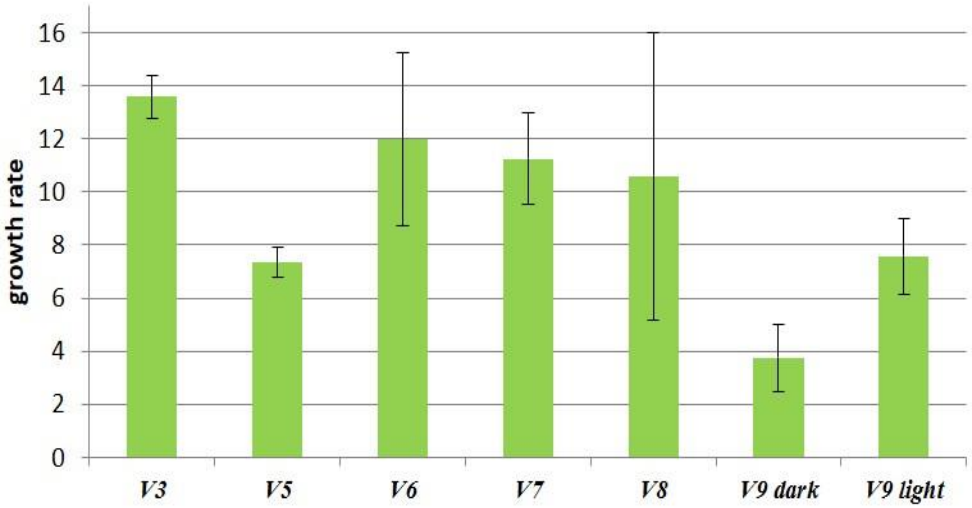

Figure 3. The growth rates of *Pimpinella anisum* calli on different culture media.

The variant of culture medium supplemented with NAA and BAP in equal concentrations (1 mg/L) determined the highest content of polyphenols of 11.55±0.49 mg GAE/g fresh callus weight and flavonoids of 30±2.49 mg RE/g fresh weight (Fig. 4A and B). Callus grown on V9 medium had the lowest content in polyphenols. Triterpenoid concentration of callus lines was most stimulated by V5 culture medium which contained 2.4-D and IAA in 1 mg/L concentration, associated with yeast extract (100 mg/L) (Fig. 4C). *In vitro* culture studies of TANIDASI & SHIOTA (2019) and ARAFA & ALY (2022) also mention yeast extract as a growth promoting factor with stimulatory effect on the bioactive compounds of callus cultures to *Pimpinella anisum*. The cultures exposed to light were more effective regarding the studied parameters than the samples incubated in the dark in the V9 variant. The results showed that *in vitro Pimpinella anisum* calli can synthesize important secondary metabolites like phenolics, flavonoids and triterpenoids and these can be modulated through the concentration and/or combination of growth factors.

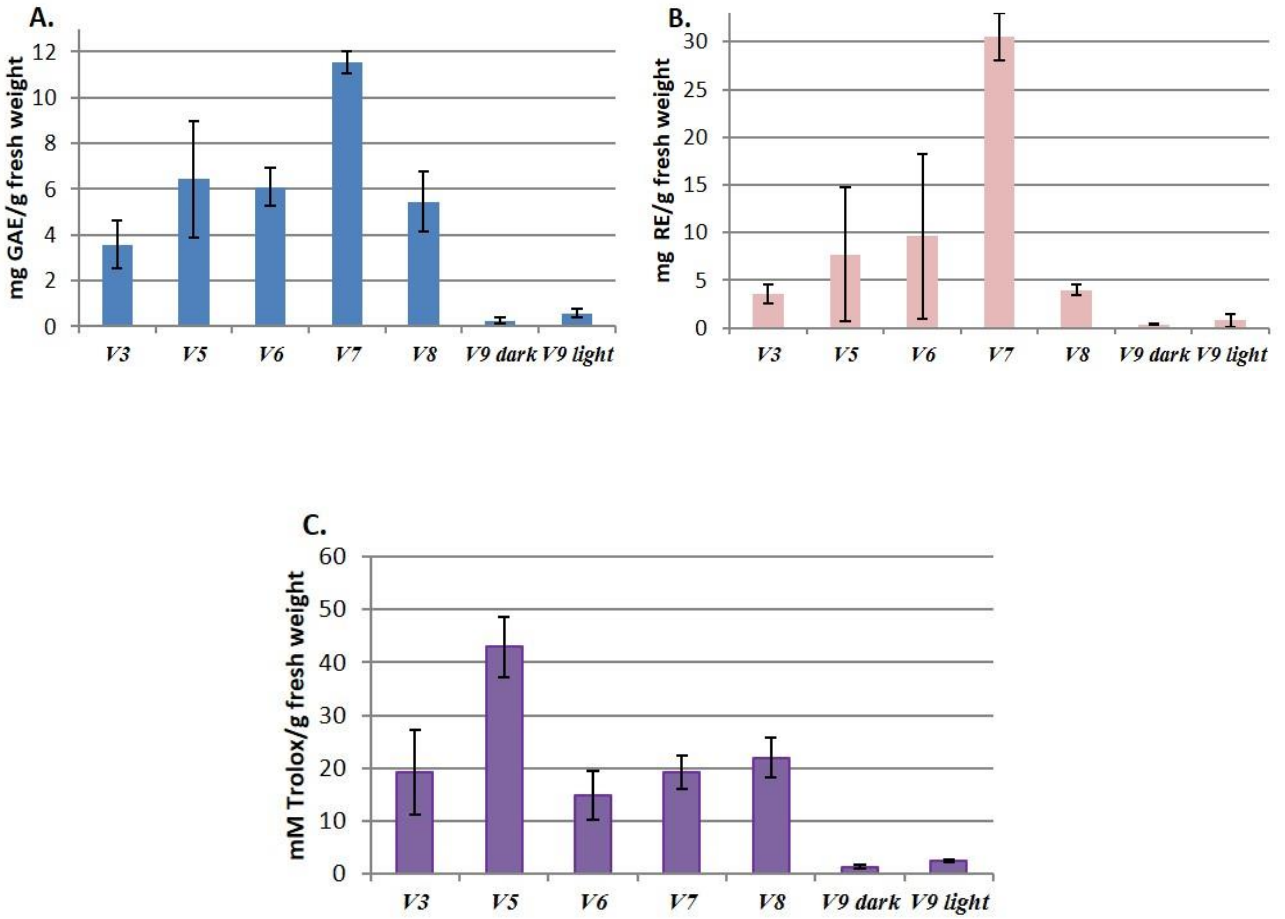

Figure 4. The total polyphenol (A), flavonoid (B) and triterpenoids (C) content in *Pimpinella anisum* calli on different culture media.

CONCLUSION

The type, concentration and combination of growth factors can stimulate the production of secondary metabolites and biomass production of *Pimpinella anisum* callus lines. Our preliminary results regarding the modulation of secondary metabolites production showed a high production of polyphenols, flavonoids and triterpenoids in *in vitro* callus cultures, but further studies remain to develop optimized biotechnologies for obtaining a considerable amount of callus which synthetise useful compounds to *Pimpinella anisum* aromatic species.

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