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A Comparative Study on Callus Induction and Indirect Morphogenesis in Two Papaveraceae Species

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Abstract. The aim of the study was callus induction of two species of Papaveraceae family (*Chelidonium majus* L. and *Glaucium flavum* Crantz) using various types of explants on *in vitro* media supplemented with different combinations of plant growth regulators (PGRs). Their indirect morphogenetic capacity has been subsequently investigated, too. Entire *in vitro* seedlings or organs excised from them were used as explants for callus induction. High percentage of seedlings and root explants from *G. flavum* formed calli in almost all tested combinations of PGRs. In *C. majus*, the roots were the most appropriate among all tested explant types, followed by seedlings and hypocotyls, regardless of the PGRs used for stimulation of callogenesis. For both studied species, only in the combination with N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU) no callogenesis was observed. The combination of α-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) stimulated morphogenetic processes in the seedlings – shoot organogenesis in *C. majus* and somatic embryogenesis in *G. flavum*.

Key words: Greater celandine, *Chelidonium majus* L., Yellow horned poppy, *Glaucium flavum* Crantz, *in vitro* cultivation.

Introduction

Chelidonium majus L. and *Glaucium flavum* Crantz are both medicinal species belonging to Papaveraceae family and biosynthesize large amounts of isoquinoline alkaloids with promising biological activities. *G. flavum* is a vulnerable species with a decreasing area of occurrence, whose gathering is forbidden from its native populations in Bulgaria according to the Medicinal Plants Act (2000). The main alkaloids in this species are glaucine and protopine. *G. flavum* is mainly famous for its antitussive activity (Aleshinskaya, 1976; Stoykov, 1964). Additionally, its newly found antiproliferative and anticancer activities have recently been investigated (Bournine et al., 2013). Its medicinal properties and narrow distribution demand its *ex situ* conservation. *C. majus*, on the other hand, is a valuable medicinal species with common occurrence. It contains many alkaloids, among them chelidonine, chelerythrine, sanguinarine, protopine, and berberine. It has a wide variety of biological activities: antiulcer, anticancer, hepatoprotective, anti-arthritic, anti-inflammatory and analgesic, antibacterial,

antifungal, antiviral, etc. (Maji & Banerji, 2015). The species is an ingredient in some remedies and becoming more and more popular object of numerous studies aimed at revealing its cytotoxic and antitumor activity. It is cultivated as a crop in some countries in Europe (Zielinska et al., 2018). The rising interest in the species makes it a topical object of different ways of *in vitro* cultivation that might be useful in future breeding of *C. majus'* genotypes which could possess desired characteristics. Overall, it is important that different *in vitro* methods be selected which could facilitate the multiplication of both species.

Indirect morphogenesis is among the most successful *in vitro* methods applied to the species of Papaveraceae family to which *G. flavum* and *C. majus* belong. In the indirect morphogenesis the cultures pass through a stage of callus formation, which facilitates the use of methods such as indirect somatic embryogenesis and morphogenesis, suspension and protoplast cultivation. The successful application of suitable *in vitro* methods for rapid propagation would be beneficial for the multiplication of cultivars with higher alkaloid content than that of plants grown in their natural habitats. Auxins and cytokinins are the most commonly used plant growth regulators (PGRs) and usually combined in the media for *in vitro* tissue cultures. It is well known that auxins play crucial role in callus formation and cytokinins support their effect in the callus induction (Zakaria et al., 2011). The number of PGR combinations applied for callogenesis in Papaveraceae species is small. The combinations α-Naphthaleneacetic acid (NAA) + Kinetin (Kin); NAA + 6-Benzylaminopurine (BAP); 2,4-Dichlorophenoxyacetic acid (2,4-D) + BAP; and 2,4-D + Kin are the most commonly used in studies. *G. flavum* is a recalcitrant for *in vitro* cultivation species, which explains the limited number of research papers on this aspect (Doycheva et al., 2017; Mohamed et al., 2014). However, *C. majus* is more popular as an object of study through different *in vitro* methods – callogenesis, somatic embryogenesis, direct and indirect organogenesis (Otgonpurev et al., 2013; Vântu, 2011; Vinterhalter & Vinterhalter, 2002; Zielinska et al., 2018). The object of the study was callus induction of two species of Papaveraceae family (*C. majus* and *G. flavum*) and comparison of their propagation response using various types of explants on *in vitro* media supplemented with different combinations of plant growth regulators.

Materials and Methods

Seeds were gathered from native plants of *C. majus* grown in the village of Mramor, near Sofia (N 42.78855, E 23.27943), and from native plants of *G. flavum* grown on the Black sea coast located near the city of Pomorie, Bulgaria (N 42.58634, E 27.63191). Sterilized seeds were germinated on a B5 agar solidified medium (Gamborg et al., 1968). Entire *in vitro* seedlings or organs excised from them (hypocotyls and cotyledons from *C. majus* and roots from both species) were used as explants for callus induction. Explants were cultivated in Petri dishes with MS-based medium (Murashige & Skoog, 1962) supplemented with different combinations and concentrations of the following PGRs: NAA, 2,4-D, Kin, BAP, Thidiazuron (TDZ), N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU), p-Chlorophenoxy-acetic acid (4-CPA), and 4-Amino-3,5,6-trichloropicolinic acid (Picloram). Seven media differing in their PGRs combinations and/or concentrations were tested: 1.0 mg/l 2,4 D + 0.5 mg/l TDZ + 0.5 mg/l BAP (medium MS1); 1.0 mg/l 2,4 D + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS2); 1.0 mg/l 4-CPPU + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS3); 1.0 mg/l 4-CPA + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS4); 1.2 mg/l NAA + 0.7 mg/l BAP (MS5); 1.0 mg/l NAA + 0.5 mg/l BAP (MS6); 1.0 mg/l Picloram + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS7). The media were solidified with 7 g/l Plant agar and autoclaved at 121°C for 20 minutes.

Cultivation was performed at 23,2°C under dark conditions. Each treatment had 3 replications with 20 explants in each. The frequency of callus formation is presented as a percentage of the number of primary explants, and its amount is determined approximately. The statistical significance was evaluated using Student's t-test. ($p \leq 0.05$).

Results and Discussion

High percentage of seedlings and root explants from *G. flavum* formed calli in all tested combinations of PGRs (Table 1). Only in the combination with 4-CPPU no callogenesis was observed. During the sub-cultivation of the formed calli on the same media on which they were induced, somatic embryogenesis (SE) was observed when the combination NAA + BAP was applied to *G. flavum* on media MS5 and MS6, supplemented with different concentrations of these PGRs. SE was observed in 51.59%±22.81 of the seedlings cultivated on MS6 (1.0 mg/l NAA+0.5 BAP) while 31.02%±17.58 of those cultivated on MS5 (1.2 NAA + 0.7 BAP) formed somatic embryos. Although SE occurred in higher frequency on the medium supplemented with lower concentrations of NAA and BAP, the average number of somatic embryos formed per explant did not differ considerably on media MS6 and MS5: 23±6.18 (1.0 NAA + 0.5 BAP) and 22±0.51 (1.2 NAA + 0.7 BAP). SE was not observed on root explants on any of the applied PGR combinations. Successful callogenesis on MS6 was reported in previous studies on *G. flavum* (Doycheva et al., 2017; Mohamed et al., 2014). However, until now this combination of PGRs has not induced SE in this species. The little increase in NAA and BAP concentrations did not increase the amount of calli induced or the number of somatic embryos formed in *G. flavum*.

Table 1. Callus formation and quantity of seedlings and root explants of *G. flavum*. *Legend:* Mean ± standard deviation; mean values followed by different letter within the same column are statistically different. N/A – not available. (-) no callus; (+) poor callus quantity; (++) moderate callus quantity; (+++) large callus quantity.

Medium and PGRs (mg/l)	Seedlings		Roots	
	Callus formation (%)	Callus quantity	Callus formation (%)	Callus quantity
MS + 1.0 2,4 D + 0.5 TDZ + 0.5 BAP	100.0a±0.0	(++)	100.0a±0.0	(++)
MS + 1.0 2,4 D + 0.5 TDZ + 0.2 BAP	100.0a±0.0	(+)	90.0a±0.0	(++)
MS + 1.0 4-CPPU + 0.5 TDZ + 0.2 BAP	0.0b±0.0	(-)	0.0b±0.0	(-)
MS + 1.0 4-CPA + 0.5 TDZ + 0.2 BAP	100.0a±0.0	(++)	100.0a±0.0	(+)
MS + 1.2 NAA + 0.7 BAP	100.0a±0.0	(++)	100.0a±0.0	(+)
MS + 1.0 NAA + 0.5 BAP	100.0a±0.0	(++)	100.0a±0.0	(+++)
MS + 1.0 Picloram + 0.5 TDZ + 0.2 BAP	100.0a±0.0	(+)	N/A	N/A

Of all the PGRs used for stimulation of callogenesis in *C. majus*, roots were the most appropriate for callogenesis among all tested explant types (Table 2). That was observed by other authors in *Papaver somniferum* L., too (Laurain-Mattar et al., 1999; Pathak et al., 2012). In the case with *C. majus* roots were followed by seedlings and hypocotyls. Cotyledons formed calli at the smallest degree. Callogenesis in this species was the most common in the explants cultivated on media MS5 and MS2. The PGR combination 4-CPPU + TDZ + BAP did not induce callogenesis in any of the used explant variants. The increase in the concentration of NAA and BAP (media MS6 vs. MS5) resulted in an increase in the callogenesis on hypocotyls and especially on cotyledons from *C. majus*.

The combination of NAA and BAP stimulated morphogenetic processes in the seedlings of *C. majus* and *G. flavum*, but shoot organogenesis and not somatic embryogenesis was stimulated in the former. But in contrast to *G. flavum*, only 10% of *C. majus* seedlings formed shoots. On average, 58 shoots per explant were induced. The shoots were transferred on B5 medium, where rhizogenesis was observed.

The combination of PGRs which included 4-CPPU inhibited callogenesis in both species. This could be explained with the presence of this cytokinin itself or with the disruption of the auxin:cytokinin ratio, in which normally auxins predominated over cytokinins. The supplementation of auxins ten times more than cytokinins is the most frequently applied ratio in PGRs combinations for callogenesis. Such proportion was used in all other combinations used except for MS1 where auxins and cytokinins were in equal concentrations.

Conclusions

The current research in *C. majus* and *G. flavum* revealed dependence of *in vitro* callogenesis on the type of the explant used. Thus, root explants were the most appropriate for callus formation in both species. Moreover, callogenesis could be affected by the type of PGRs applied, their combinations and concentrations. Therefore, the PGR combination containing 4-CPPU didn't induce callogenesis in both species.

The morphogenetic response of callus also depended on the plant species. The two studied species took different morphogenetic directions – somatic embryogenesis in *G. flavum* and shoot organogenesis in *C. majus*.

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