Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INITIATION AND SELECTION OF CALLUS CULTURES FROM FUMARIA ROSTELLATA KNAF. AS POTENTIAL PRODUCERS OF ISOQUINOLINE ALKALOIDS

Lidiya GEORGIEVA[1], Ivan IVANOV[1], Andrey MARCHEV[2], Ina ANEVA[3], Vasil GEORGIEV[4], Panteley DENEV[1], Atanas PAVLOV[2,5]

[1]Department of Organic Chemistry, University of Food Technologies, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria,Email: *lid_georgieva@abv.bg; ivanov_ivan.1979@yahoo.com; denev57@abv.bg*
[2]Department of Industrial Microbiology, Laboratory of Applied Biotechnologies, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Science, 139 Ruski Blvd., 4000 Plovdiv, Bulgaria, Email: *andrey.marchev@yahoo.com; at_pavlov@yahoo.com*
[3]Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Science, 1113 Sofia, Bulgaria, Email: *ina.aneva@abv.bg*
[4]Center for Viticulture and Small Fruit Research, College of Agriculture and Food Science, Florida A&M University, 6505 Mahan Drive, Tallahassee Fl 32317, USA,Email: *vasgeorgiev@yahoo.com*
[5]Department of Analytical Chemistry, University of Food Technologies, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria

Corresponding author email: *at_pavlov@yahoo.com*

Abstract

The aim of the study was development of protocols for obtaining callus cultures from Fumaria rostellata Knaf. For the purpose leaves, stems and flowers of the investigated plant were used as explants. Callus was initiated on a Murashige and Skoog nutrient medium, supplemented with 30 g/l sucrose, 5.5 g/l "Plant" agar and various concentrations (0.2; 0.5; 1.0; 2.0; 3.0 mg/l) of auxin (2,4-dichlorophenoxyacetic acid) and cytokine (6-benzylaminopurine). The best results for initiation of calli were observed at a low concentration of 2,4-D (0.2 mg/l or 0.5 mg/l) and a high concentration of BAP (2.0 mg/l or 3.0 mg/l). It is well known that Fumaria plants are rich sources of isoquinoline alkaloids and obtained in vitro cultures from F. rostellata could be used as potential technological matrixes for development of a commercial process for protopine production.

Key words*: Fumaria rostellata, callus, in vitro, 2,4-D, BAP.*

INTRODUCTION

Higher plants are sources of natural products used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides (Balandrin and Klocke, 1988). The search for new plant derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Phillipson, 1990). In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as an alternative to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Rao and Ravishankar, 2002, Steingroewer et al., 2013).

Plants of the genus *Fumaria* have been used in traditional medicine as anti-hypertensives, diuretics, hepatoprotectants and laxatives (to treat gastrointestinal disorders), as well as in the treatment of some skin diseases (rashes or conjunctivitis) (Martindale, 1996; Stubing and Peris, 1998; Suau et al., 2002).

The biological activity of *Fumaria* spp. is mostly associated with the presence of isoquinoline alkaloids, the most important of which is protopine. This alkaloid possess hepatoprotective activity (Rathi et al., 2008), inhibits histamine H1 receptors and platelet aggregation (Saeed et al., 1997), inhibits serotonin and noradrenaline transporters and has an antidepressant effect (Xu et al., 2006), as well as antimicrobial, antiviral (Orhana et al., 2007) and anti-inflammatory activities (Saeed et al., 1997).

Fumaria rostellata Knaf. belongs to the genus *Fumaria* (Fumariaceae), which consists of 60 species widely distributed all over the world

(Suau et al., 2002; Jaberian et al., 2013). The plant has branched stems, alternate and petiole leaves with small zygomorphic purplish pink flowers. Given the morphological similarities between species, the identification of different taxons is difficult and based on several specific morphological characters: presence of sepals, their length, the shape of the fruit, the length of the fruit pedicel and the length of the fruit pedicel bracteole. *F. rostellata* presents well developed calyx and corolla with sepal length between 3.5-4 mm and petal length between 8.5-9.5 mm. The plant spreads in the hills or lowland areas, through cultures, ditches and ruderal places (Paltinean et al., 2013). There are scanty data available about potential of *Fumaria rostellata* as a producer of protopine and therefore investigation of its alkaloid metabolism is of high importans.

During the last two decades *in vitro* systems of medicinal plants have been considered as a valuable alternative to intact plants as a source of bioactive substances (Pavlov A., 2014). However, only one report is currently available about *Fumaria in vitro* cultures. Tanahashi and Zeng (1985) were investigated presence of isoquinoline alkaloids in cell suspension cultures of *F. capreolata.* This way assessment of the potential of *in vitro* cultures of *Fumaria* spp. is of high interest.

The aim of the present study was development of protocols for callus obtaining from *F. rostellata* and evaluation of the possibilities for protopine production.

MATERIALS AND METHODS

Plant material

Plants of *F. rostellata* used in this study were collected in May 2013 from their natural habitats near Blagoevgrad, Bulgaria. Identification of the plant species was made through the references deposed in Herbarium of the Institute of Biodiversity and Ecosystem Research in Sofia and Herbarium of Sofia University.

Callus induction

Leaves, flowers and stems of the collected plants were washed with tap water and sterilized by using 70 % ethanol for 20 sec and 7 % (w/v) sodium hypochlorite for 20 min. The explants were triple-washed in sterile water, dried on filter paper, and transferred on callus induction media. Various combinations of induction media were used, all based on Murashige and Skoog (MS) medium, supplemented with 30.0 g/l sucrose, 5.5 g/l ‘‘Plant agar’’ (Duchefa, The Netherlands) and different concentrations (0.2; 0.5; 1.0; 2.0; 3.0 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4D, Sigma, USA) and 6-benzylamynopurine (BAP, Duchefa). Half of the explants were cultivated under illumination (16 h light/8 h dark) with light intensity of 110 $\mu mol/m^{-2}.s^{-1}$ (SYLVANIA Gro-Lux fluorescent lamps, F18W/GRO-LUX), and the other half was grown in darkness at 26ºC. The formed calli were separated from explants and transferred for self-growth in petri dishes under the same conditions with sub-culturing period of 21 days.

Extraction of alkaloids

Lyophilized biomass (0.15 g) was triple extracted with 5 ml ethanol in ultrasonic bath for 15 min. The combined extracts were concentrated under vacuum and dissolved in 3% sulfuric acid (2 × 2 ml). The neutral compounds were removed by extraction (three times) with diethyl ether. The alkaloids were fractionated after basification of the extracts with 1 ml of 25 % ammonia and extraction with chloroform (3×5 ml). The chloroform extracts were then dried over anhydrous sodium sulfate and evaporated to dryness.

TLC-method

For TLC qualitative determination of protopine chloroform – ethyl acetate – methanol – ammonium hydroxide (80:80:40:0.05, v/v/v/v) as a mobile phase was used. The protopine standard was dissolved in water in concentration 1 mg/ml and dried extracts of *F. rostellata* calli were disovled in 1 solution of 1N HCl in methanol (1.0 ml). The samples were spotted onto silica gel aluminium plates (ALUGRAM SIL G, 20x20), (Macherey-Nagel, Germany). The alkaloids were visualized by triplicate spraying with Dragendorff reagent.

RESULTS AND DISCUSSIONS

The species of genus *Fumaria*, including *F. rostellata* grow in grassy areas around crops

as a weed in degraded areas along roads and fences across up to 1000 m altitude. Sterilization procedure of explants from *F.rostellata* was consistent with the places from which plant material was picked. For the purpose explants were treated with 7% solution of sodium hypochlorite for 20 min., after presterilization with 70% EtOH for 15 sec. At these conditions explants remained alive and about 80 % of them were sterile.

Beside sterilization procedure the size of the explants was the second variable that influenced their viability. Smaller explants (up to 10 mm) did not survive sterilization procedure, probably due to tissue damage upon excision and treatment with EtOH and sodium hypochlorite during the process of sterilization of the explants (Goswami et al., 2013). It was proved that the size of 10 mm to 30mm were optimal for our purposes.

Sterilized leaves, stems and flowers of selected plant were transferred on Murashige and Skoog nutrient media, supplemented with different concentrations of growth regulators as it is described in the section Material and Methods. Explants were incubated at 26°C under illumination and in darkness. The first callus structures were observed 10 days after transferring explants on the selected media. Callogenesis of different explants (leaves, stems, flowers) of *F. rostellata* is shown in Figure 1.

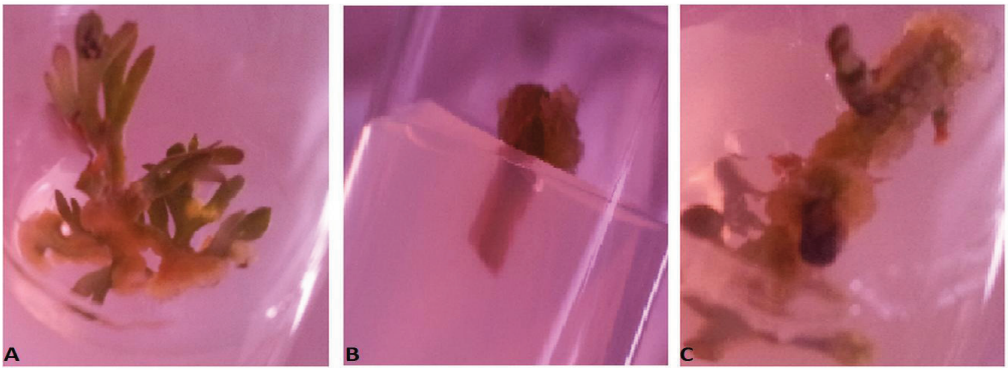

Figure 1 Callogenesis of different explants of *F. rostellata* after 10 days of cultivation: A) leaves; B) stems; C) flower stems

During incubation were contaminated with bacteria or fungi 36 % of all cultivated explants. It was observed, that the explants cultivated under illumination showed a high degree of survival (between 60 and 100 %). The lowest survival degree was observed at the cultivation on medium supplemented with 1.0 mg/l 2,4-D and 3.0 mg/l BAP) (Figure 2A). As a whole, the explants cultivated in darkness showed a lower survival degree. The best combinations of growth regulators in this case were 0.2 mg/l 2,4-D and 2.0 mg/l BAP and 1.0 mg/l 2,4-D and 1.0 mg/l BAP (Figure 2B). The new formed calli were transferred to fresh MS media and cultivated under illumination or in darkness at 26ºC. After 3–4 sub cultivation periods three morphology types of calli were observed. There was not any relationship between type of explants and calli types observed. Newly formed calli were of three morphological types- very compact and hard callus; soft and friable callus; a mixture of compact and friable callus. The type of formed callus strongly depended on used combination of plant growth regulators.

As it was expectable, the calli cultivated under illumination had green colour, while those cultivated on dark possessed yellow colouring

All survived explants cultivated under illumination induced callus (100% induction), while in darkness this value decreased to 87% (Figure 3).

.

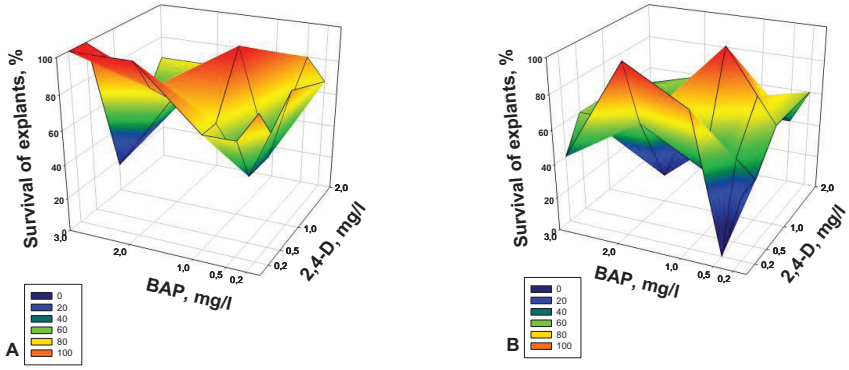

Figure 2 Survival of explants of *F rostellata* calculated as % of cultivated explants: A) under illumination; B) in darkness

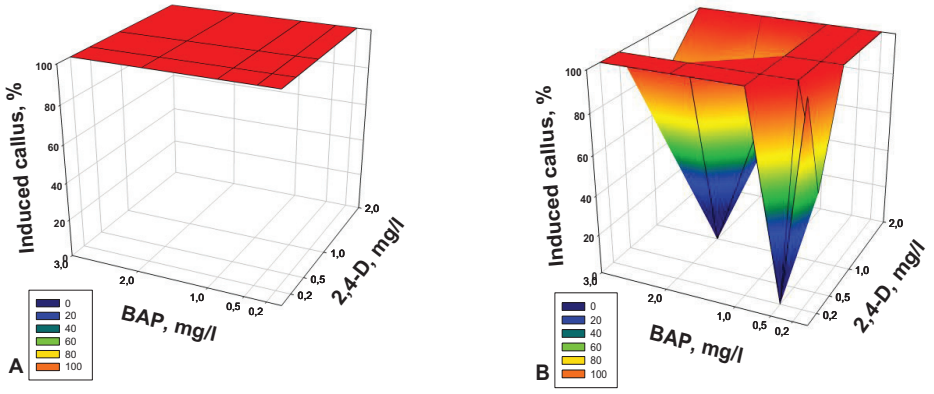

Figure 3 Induced callus of *F rostellata* calculated as % of survived explants: A) under illumination; B) in darkness

When explants were cultivated in darkness 0.2 mg/l 2,4-D and 0.5 mg/l BAP; 0.5 mg/l 2,4-D and 0.2 mg/l BAP; 1.0 mg/l 2,4-D and 2.0 mg/l BAP and 1.0 mg/l 2,4-D and 3.0 mg/l BAP were not appropriate combinations of growth regulators for development of calli with appropriate morphology.

As concequence of callus induction experiments, more than 100 lines were obtained Based on assessment of callus friability and growth 8 lines were selected for further experiments – 5FRL14 (0.2 mg/l 2,4-D and 2.0 mg/l BAP), 6FRL20 (0.2 mg/l 2,4-D and 3.0 mg/l BAP), 18FRL56 (1.0 mg/l 2,4-D and 1.0 mg/l BAP) and 23FRL60 (2.0 mg/l 2,4-D and 0.2 mg/l BAP) cultivated under illumination and lines 11FRL106 (0.5 mg/l 2,4-D and 1.0 mg/l BAP), 17FRL119 (1.0 mg/l 2,4-D and 0.5 mg/l BAP), 23FRL129 (2.0 mg/l 2,4-D and 0.2 mg/l BAP) and 23FRL130 (2.0 mg/l 2,4-D and 0.2mg/l BAP) cultivated in darkness.

TLC qualitative method for fast screening of protopine-synthesizing callus cutures was used (Figure 4). The alkaloid extracts of selected calli lines were spotted in volume of 50 µl (with an unknown concentration of protopine) and the protopine standard was spotted in a concentration of 20 µg/g. Obtained results showed that *Fumaria in vitro* cultures are prospective for further development of biosynthetic process. However for the more detaled assessment quantification by HPLC and/or GC should be performed.

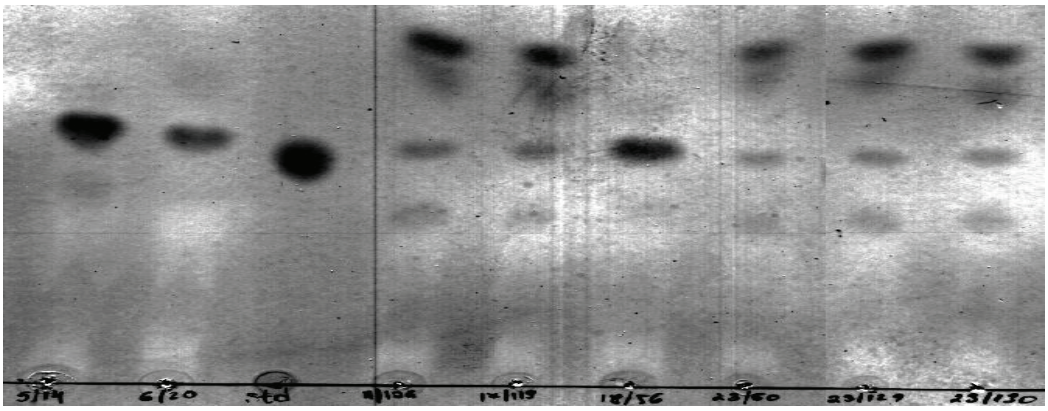

Figure 4 TLC of alkaloid extracts of selected callus lines of *F rostellata*

CONCLUSIONS

In this work, we have established for the fist time protocol callus induction of *Fumaria rostellata* Knaf. The best morphological characteristics and growth behavior were observed for callus lines cultivated on MS media supplemented with low concentration of 2,4-D (0.2 or 0.5 mg/l) and high concentration of BAP (1.0, 2.0 or 3.0 mg/l). Pimary analyses of protopine content showed that the *F. rostelata* in vitro cultures are prospective producers of this pharmacologically important alkaloid.

ACKNOWLEDGEMENTS

The authors thank for the financial support of this research by the Bulgarian Science Foundation, Bulgarian Ministry of Education and Science under contract number: DMU 03/77 – 2011.

REFERENCES

Balandrin J., Klocke J., 1988. Medicinal, aromatic and industrial materials from plants. In Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plant, Vol. 4. Springer-Verlag, Berlin, Heidelberg, 1–36.

Goswami N., Handique P., 2013. Explants size response to in vitro propagation of Musa (Aaa Group) "Amritsagar" Musa (Aab Group) "Malbhog" and Musa (Aab group) "Chenichampa" Banana. Indian Journal of Applied Research, 3(8): 40–43.

Jaberian H., Piri K., Nazari J., 2013. Phytochemical composition and in vitro antimicrobial and antioxidant activities of some medicinal plants. Food Chemistry, 136(1): 237–244.

Martindale W., 1996. Martindale: The Extra Pharmacopoeia, 31st edn. Pharmaceutical Press, London

Orhana I., Özçelik B., Karaoğlu T., Sener B., 2007. Antiviral and antimicrobial profiles of selected isoquinoline alkaloids from *Fumaria* and *Corydalis* species, Zeitschrift für Naturforschung C, 62(1-2): 19–26

Paltinean R., Wauters N. J., Tits M., Frederich M., Angelot L., Tamas M., Crisan G., 2013. Comparative morphological studies on some species of the genus *Fumaria*. Farmacia, 6(2): 371–377.

Pavlov A., 2014. Plant cells and algae in bioreactors II. Engineering in Life Sciences. in press, 14(6): 548–549.

Phillipson J.D., 1990. Plants as source of valuable products. In B.V. Charlwood, and M.J.C. Rhodes (eds.), Secondary Products from Plant Tissue Culture. Oxford: Clarendon Press, 1–21.

Ramachandra Rao S., Ravishankar G., 2002. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology Advances, 20: 101–153.

Rathi A., Srivastava K., Shirwaikar A., Rawat S., Mehrotra S., 2008. Hepatoprotective potential of *Fumaria indica* Pugsley whole plant extracts, fractions and an isolated alkaloid protopine. Phytomedicine, 15: 470–477.

Saeed A., Gilani H., Majoo U., Shah H., 1997. Anti-thrombotic and anti-inflammatory activities of protopine. Pharmacological research, 36(1): 1–7.

Steingroewer J., Bley Th., Georgiev V., Ivanov I., Lenk F., Marchev A., Pavlov A., 2013. Bioprocessing of differentiated plant in vitro systems. Engineering in Life Sciences, 13(1): 26–38.

Stubing G., Peris B., 1998. Plantas Madicinales de la Comunidad Valenciana. Generalitat Valenciana, Valencia.

Suau R., Cabezudo B., Rico R., Nájera F., López-Romero M., 2002a. Direct determination of alkaloid contents in *Fumaria* species by GC-MS. Phytochemical Analysis, 13: 363–367.

Suau R., Cabezudo B., Rico,R., López-Romero. M., Nájera F., 2002b. Alkaloids from *Fumaria sepium*

and *Fumaria agrarian*. Biochemical Systematics and Ecology, 30: 263–265.

Tanahashi T., Zenk N. M., 1985. Isoquinoline alkaloids from cell suspension cultures of *Fumaria capreolata*. Plant Cell Reports, 4: 96–99.

Xu F., Chu J., Qing Y., Li S., Wang S., Qing W., Fei J., Guo H., 2006. Protopine inhibits serotonin transporter and noradrenaline transporter and has the antidepressant-like effect in mice models. Neuropharmacology, 50: 934–940.