CALLUSOGENETIC AND MORPHOGENETIC CAPACITY OF SAFFLOWER EXPLANTS

CAPACITATEA DE CALUSOGENEZĂ ŞI MORFOGENEZĂ A EXPLANTELOR DE ŞOFRĂNEL

SMEREA Svetlana[1], ANDRONIC Larisa, SCHIN Victoria
e-mail: andronic.larisa@yahoo.com

Abstract. *For in vitro culture were optimized the conditions of sterilization (concentration of sterilization solution and the duration of treatment), the composition of nutritive media for direct embryogenesis of seeds and callusogenesis. Two types of explants, fragments of leaves cotyledons and hypocotyls, were used for inducing in vitro callusogenesis. As callus-inducing medium served Murashige & Skoog mineral base (1962) with 4 variants of additions of growth regulators (6-Benzylaminopurine and 2,4-Dichlorophenoxyacetic acid). The higher frequency of callusogenesis was found for both types of explants on medium with 2,4-D 0,25mg/L + BAP 0,5 mg/L and 2,4-D 0,25mg/L + casein hydrolysates 500 mg/L. Based on the ANOVA test it was established that the positive response is determined significantly only by the culture medium (hormonal balance) at 99.9%.*
Key words: safflower, callus, callusogenesis, morphogenesis, explant

Rezumat. *Pentru inducerea culturii in vitro au fost optimizate condiţiile de sterilizare: stabilită concentraţia soluţiei de sterilizare şi durata tratamentului, compoziţia mediilor nutritive pentru embriogeneza directă a seminţelor şi calusogenezei. În calitate de explant au fost utilizate fragmente de frunze cotiledonate şi hipocotil de şofrănel. Ca mediu de inducere a calusurilor a fost utilizată baza minerală conform Murashige & Skoog (1962) cu 4 variante de adiţii a regulatorilor de creştere (6-benzilaminopurină şi acid 2,4-diclorfenoxiacetic). Frecventa mai majoră a calusogenezei a fost stabilită pentru ambele tipuri de explante pe mediile suplimentate cu 2,4-D 0,25mg/L + BA 0,5 mg/L şi 2,4-D 0,25mg/L + hidrolizat de cazeină 500 mg/l. În baza testului ANOVA a fost stabilit că, mediul nutritiv (balanţa hormonală) influenţează semnificativ la nivel de 99,9 % răspunsul pozitiv al explantelor de şofrănel.*
Cuvinte cheie: şofrănel, calus, calusogeneză, morfogeneză, explant

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is a very ancient crop that has origin of domestication from approximately 4000 years ago in the Fertile Crescent region, ranged from southern Israel to Western Iraq (Chapmen *et al.,* 2010). Safflower has multiple use. Until this century, before cheaper aniline dyes became available, safflower was mainly grown for dye. Cultivated varieties are applied as source of

[1]Institute of Genetics, Plant Physiology and Protection, Academy of Sciences of Moldova, Chisinau, Republic of Moldova

quality oil (rich in linoleic acid). From safflower petals are extracted carthamidin (yellow dye, water-sol) and carthamin (red dye, water-insoluble) which are very important as a source of medicinal preparations, natural food colour and dyes for colouring fabrics.

Safflower is cultivated in 800 000 ha in the world with a yield of 650 000 tones (Emongor, 2010). The main producers are India, USA, Mexico, Ethiopia, Argentina, China, Kenia, Canada, Spain, Italy, Turkey, Iran and Russia.

Safflower plants are tolerant to severe drought and salinity, grow in dry hot climates, and can be cultivated, under poor environmental conditions (Kizil *et al*., 2008).

Domestication of safflower has resulted in traits such as reduced shattering, smooth seeds, reduced duration of early vegetative growth stage, restriction of branching to the upper part of the stem, and reduced seed dormancy (Berville *et al*., 2005). Breeding programs have resulted in the release of cultivars with high oil content and/or increased disease resistance. But breeding works were increasingly restricted by lack of germplasm (McGuire *et al.,* 2012). On average, the collections created 99.3% were spiny and 98.9% were orange flowered. There is strong selection pressure against spineless types by birds and livestock, bushy types would bene favored. It is considered that orange flowers are a hold-over from a period over 150 years ago when orange and red flowers were a source of carthamin, an important dye of commerce. So, red-flowered types should have been more frequent.

In vitro technology has been known to be effective procedure for diversification of crop plants. The genetic variability may be achieved by means of somaclonal variation or combination of in vitro culture with experimental mutagenesis. Safflower regeneration through tissue culture has been limited by low frequency and lack of an efficient protocol that suitable for most safflower cultivars (Fan and Guo, 2013). The somaclonal regeneration processes had the many factors, namely genotype, age of seedling and callus (Fan and Guo, 2013); type and orientation of explants (Chawla, 2000); medium components, plant growth regulators and other additives (Rao *et al*., 2008; Fan and Guo, 2013; Xue *et al*., 2015).

The aim of present work involved the study of the reaction of safflower explant to *in vitro* culture in order to induced somaclonal morphogenesis.

MATERIAL AND METHOD

For inducing *in vitro* callusogenesis and morphogenesis were used the two types of explants (fragments of cotyledon leaves and hypocotyls) obtained from aseptic plantlets. Firstly, the seeds were rinsed in water with drops of Tween-80 (0.1%) and under running tap water for 15 min. Following, the seeds were surface sterilized for 1 min in 70% ethanol and then were disinfected with sodium hypochlorite solution (5.2%, as a commercial bleach, in dilution 1:1) for 17 min. After that, the seeds were rinses for three times 3 min each, in sterilized water to remove all traces of Clorox. The sterilization procedure and the incubation had been conducted in culture cabinet (laminar airflow hood).

According the purpose objectives, the sterile seeds were inoculated in Magenta jars with Murashige&Skoog (MS) medium without hormones for inducing direct embryogenesis (fig. 1). Culture medium was solidified with 0.8% agar and adjusted to pH 5.7 and incubated at 25±2^{0}C under illuminated conditions (16h photoperiod).

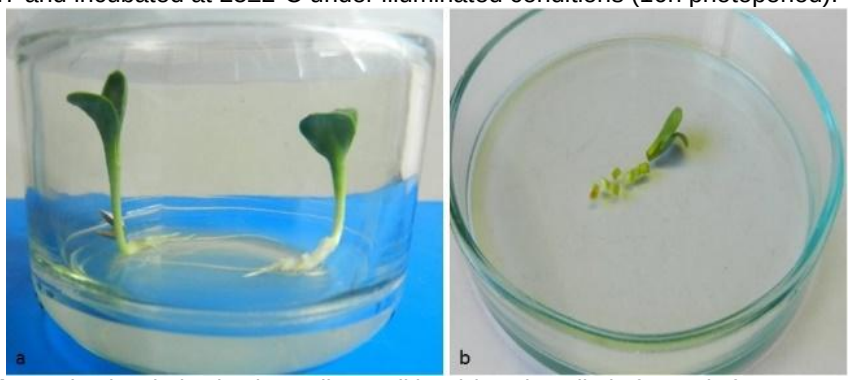

Fig. 1 Plantlets induction in sterile condition (a) and applied of aseptic fragments as explant for *in vitro* culture (b).

Leaves cotyledons and hypocotyls of 21 day old plantlets were sliced into 4-6 mm sections and used as explants in *in vitro* inoculation (fig. 1b). For callus induction was used MS medium (pH 5.8) supplemented with 6-benzylaminopurine (BAP), thidiazuron (TDZ), alpha-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic (2,4-D) in different combination and concentration added before autoclaving. It was tested 4 variants: NAA 1,5mg/L + BAP 0,5 mg/L (No 1), 2,4-D 0,25mg/L + BAP 0,5 mg/L (No 2), 2,4-D 0,25mg/L + casein hydrolysates 500 mg/L (No 3) and NAA 0,5mg/L + TDZ 1 mg/L (No 4). pH of the medium was adjusted to 5.7 before sterilization by autoclaving for 20 minutes under the pressure of P=1 atm, T=120^{0}C. Every 25 pieces per type of explant were inoculated on nutrient media in three repetitions. The explants were incubated in dark and temperature-controlled conditions (25 ± 2^{0}C) for 2-3 weeks. After initiating callus vessels with explants were passed under 16-hour light and 8 hours dark for initiating morphogenesis. Serial passages were conducted every 2-3 weeks on initial and intermediate mediums.

As parameter it was assessed the frequency of explants (fragments of cotyledons leaves and hypocotyls) with positive response. The software package Statgraphics Plus 2.1 was used for statistical analysis. ANOVA test it was applied for variance analysis of callusogenesis frequency, and Student test in assessment of statistically significant differences between treatments.

RESULTS AND DISCUSSIONS

At second day after seeds inoculation has been established the first germination. According to the observations made during the first 3-4 days it was revealed the mass contamination of culture in treatment with sterilization of explants in sodium hypochlorite (5.2%), dilution 1:1 (sodium hypochlorite: sterile water). No contamination was identified in case of applied of standard concentration of commercial bleach. The best results were obtained for *in vitro* cultivation of the fragments collected during the first 7-8 days of cotyledons

leaves (rate of calusogenesis 67.34%) comparative to response explant collected after 21 days (35%) (fig. 2).

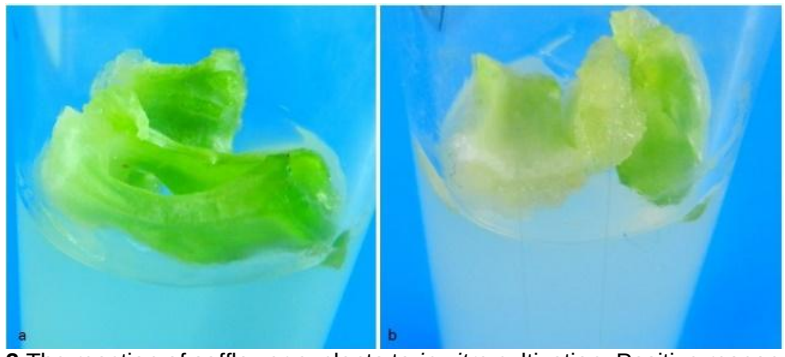

Fig. 2 The reaction of safflower explants to *in vitro* cultivation. Positive responze of fragments of cotyledon leaves (a) and hypocotyls (b).

The evaluation of response of explants (cotyledon leaves and hypocotyls) to *in vitro* culture was carried out after the first 4-5 days of incubation in the dark. So, it was confirmed the extension in volume of the majority of explants as a result of active cell proliferation. At 7-8 days of culture it was established the callus initiations at the fragment margins. Meanwhile, 3.8% of hypocotyl explants presented somatic regeneration on media No. 1 and No. 4 (with the addition of NAA) (tab. 1).

After of 18-19 days of culture in dark, were visualized colorless, yellow-green or brown calluses. The intensity of callusogenesis was assessed as high, medium or low in dependence of the nutritive medium. Sporadic were certified browning and necrotizing of tissue callus, especially at the margins of explants, which imposed the necessity for passage of explants on initial or intermediate media (fig. 2).

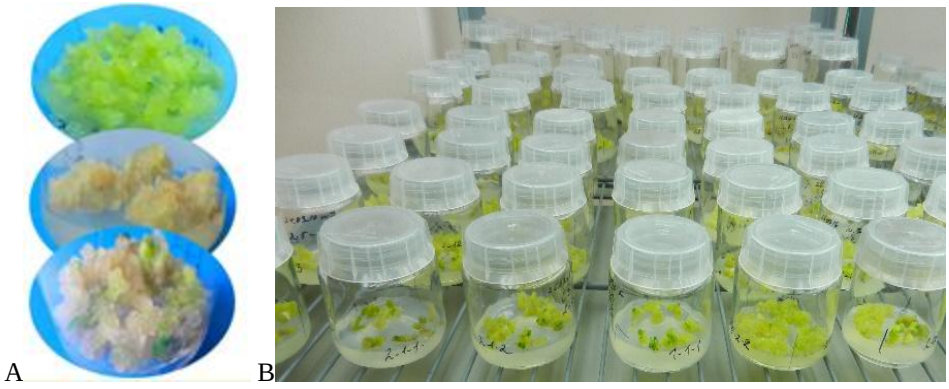

Fig. 3 *In vitro* cultivation of safflower explants. Morphological aspect of different types of calluses (A) and subcultivation of morphological calluses (B).

The obtained results highlight the increasing capacity of tissue proliferation, regardless of colour and structure of induced callus. The increased frequency was found for medium No. 2 (92.86%) and No.3 (87.22%), followed by the No.1 (72.62%). The lowest level of positive response of explants has been recorded for medium No. 4 (16.67%), caused by the lack of positive response for hypocotyls and low rate for cotyledon leaves (33.33%) (tab. 1).

Table 1

Average values of callusogenesis frequency in dependence of the nutrient medium and types of explant callus

Nutritive medium	Explant	Callusogenesis frequency, %	Average values
1	hypocotyl	66.67	72.62
	cotyledon leaves	78.57	
2	hypocotyl	85.71	92.86
	cotyledon leaves	100	
3	hypocotyl	80.00	87.22
	cotyledon leaves	94.44	
4	hypocotyl	0.00	16.67
	cotyledon leaves	33.33	
Average values			67.34

The interaction of sources of variation nutritive medium-explants demonstrate that the cultivation of cotyledon leaves on medium No. 2 induced maximal effect - 100%, on medium No.3 - 94.44%, and on the No.1 - 78.57%, while the positive response of hypocotyl fragments on same media have 85.71%, 80.0% and 66.67% respectively.

In order to evaluate the impact of culture media on callus frequency was established the rate of explant with positive response comparative to total number of inoculated explants. Based on the ANOVA test it was determined that the positive response is determined significantly by the culture medium (hormonal balance) at 99.9%, while the type explants do not show statistically significant differences (tab. 2).

Table 2

Analysis of variance for rate of callusogenesis (ANOVA test)

Source of variance	Sum of Squares	Degrees of freedom	Mean Square	F-ratio
Explant (E)	4494.03	1	4494.03	3.44
Nutritive medium (M)	47556.1	3	15852.0	12.13***
Interaction E-M	962.63	3	320.87	0.25
Total	112455.0	54		

*** - significant at P ≤ 0.001.

The subcultivation of calluses from both types of explants on initial media revealed that, yellow and brown calluses has fluid structure, while green are mixed calluses had compact areas.

In vitro morphogenesis involves obtaining of plantlets regeneration in results of organogenesis or somatic embryogenesis and is greatly influenced by exogenous and endogenous content of growth regulators, despite the initiation of morphogenetic zones in all types of histogenes. Further subcultivation of the calli was performed on the intermediate medium. This medium increases the *in vitro* activation of regenerative processes due to the grow of the content of cytokinins in the culture medium.

CONCLUSIONS

1. Evaluation of callusogenesis and morphogenesis frequency initiated from safflower explant, proved the positive influence of hormonal balance on reaction of fragments of leaves cotyledons and hypocotyls to *in vitro* cultivation.

2. The derived calluses established morphogenetic capacity not dependent of external aspects (colour - white or green; consistence - compact or fluid).

Acknowledgments: These investigations were supported by the project 6097 STCU/ASM.

REFERENCES

1. **Berville A., Breton C., Cinliffe K., Darmency H., Good A.G., Gressel J., Hall L.M., McPherson M.A., Medail F., Pinatel C., Vaughan D.A., Warwick S.I., 2005** - *Issues of fertility or potential for ferality in oats, olives, the Vigna group, ryegrass species, safflower and sugarcane*. In: Crop Ferality and Volunteerism. CRC Press, p.231255.
2. **Chapmen M.A., Hvala J., Strever J., Burke J.M., 2010** – *Population genetic analysis of safflower (Carthamus tinctorius; Asteraceae) releals a Near Eastern origin and five centers of diversity*. American Journal of Botany, 97: p.831-40.
3. **Chawla H.S., 2000** - *Introduction to plant biotechnology*. 2nd edn, Inc. Enfield, New Hampshire, USA, Science publisher, p. 39–56.
4. **Emongor V., 2010 -** *Safflower (Carthamus tinctorius L.) the underutilized and neglected crop: a review*. Asian Journal of Plant Siences, 9 (6), p. 299-306.
5. **Fan L., Guo M., 2013** - *Progress of safflower (Carthamus tinctorius L.) regeneration through tissue culture*. J. Med. Coll. PLA, 28(5), p. 289-301.
6. **Kizil S., Çakmak Ö., Kirici S., İnan M.A., 2008 -** *Comprehensive study on safflower (Carthamus tinctorius L.) in semi-arid conditions*. Biotechnol. & Biotechnol. Eq. 22(4), p.947-953.
7. **McGuire P.E., A.B. Damania, and C.O. Qualset (eds.), 2012** - *Safflower in California. The Paulden F. Knowles personal history of plant exploration and research on evolution, genetics, and breeding*. Agronomy Progress Report No. 313, Dept. of Plant Sciences. University of California. Davis CA USA.
8. **Rao N.N., Sujatha M., Narasu L., Kumar D.V., 2008** - *Establishment of regeneration and transformation protocols in safflower (Carthamus tinctorius L.)*. Proceeding of 7th International safflower conference, Wagga, Australia.
9. **Singh V., Nimbkar N., 2006** - *Safflower (Carthamus tinctorius L.)*. Chapter 6, p.168-194, In: Genetic Resources, Chromosome Engineering, and Crop Improvement: Oilseed Crops. Vol. 4, CRC Press.
10. **Xue Y., Li D., Gao Y., Guo M., 2015** - *Optimization of Carthamus tinctorius L. tissue culture system based on the combination of 1-naphthylacetic acid and 6-benzyl aminopurine*. Pharmaceutical Care and Research, 15(2), p. 91-94.