MORPHOGENETIC POTENTIAL OF CALLUSES DERIVED FROM GAMMA IRRADIATED SAFFLOWER SEEDS

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Abstract. In order to induce morphological diversities in safflower experimental mutagenesis was applied in combination with *in vitro* culture. According to the tasks of inducing somaclonal variability seeds irradiated with gamma rays in doses of 50, 100, 150 Gy were used. Two types of explants (fragments of leaves cotyledons and hypocotyls) were applied for inducing *in vitro* morphogenesis. As callus-inducing mineral base medium was used by MURASHIGE & SKOOG (1962) with additions of growth regulators (6-Benzylaminopurine and 2,4-Dichlorophenoxyacetic acid). The derived calli were cultivated for induction of morphogenesis, been identified the hormones balance added to the culture media, the interval between subcultivation. Based on proved researches, a greater diversity in the safflower morphogenetic potential was revealed, generated by *in vitro* culture in a complex with gamma radiation.

Keywords: *in vitro* morphogenesis, callus, gamma radiation, safflower.

Rezumat. Potențialul morfogenetic al calusului obținut din semințe iradiate de șofrănel. În scopul inducerii diversităţi morfologice la şofrănel a fost propusă aplicarea mutagenezei experimentală în combinaţie cu cultura *in vitro*. Conform sarcinilor de sporire a variabilităţii somaclonale au fost folosite semințele iradiate cu raze gama în dozele 50, 100, 150 Gy. Pentru inducerea morfogenezei *in vitro* au fost utilizate două tipuri de explante (fragmente de frunze cotiledonate şi hipocotil). Baza minerală a mediului de inducere a calusogenezei a servit MURASHIGE & SKOOG (1962) cu adaosuri a regulatorilor de creştere (6-benzilaminopurină şi acid 2,4-diclorfenoxiacetic). Calusurile derivate au fost sub-cultivate în vederea inducerii morfogenezei, fiind identificată balanţa hormonală, intervalul dintre pasaje. Pe baza cercetărilor a fost evidenţiată majorarea diversităţii potenţialului morfogenetic la şofrănel prin aplicarea culturii *in vitro* în complex cu radiaţia gama**.**

Cuvinte cheie: morfogeneză *in vitro,* calus, gama radiaţie, şofrănel.

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is an oilseed crop with multiple uses (CHAPMEN et al., 2010). 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, are extracted from safflower petals. Cultivated varieties are used as a source of quality oil (rich in linoleic acid).

However, there is a lack of information about safflower cultivars and their characteristics, so the objective of variety improvement is very important and actual.

In vitro technology has been known to be an 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. According to literature data, safflower regeneration through tissue culture has been limited by low frequency and lack of an efficient protocol that is suitable for most safflower cultivars. It has been limited by the many factors, namely genotype, age of seedling and callus (FAN & GUO, 2013); type of explants (CHAWLA, 2000); medium components, plant growth regulators and other additives (RAO et al., 2008; FAN & GUO, 2013; XUE et al., 2015).

The aim of the present work involved the study of impact of experimental mutagenesis associated with *in vitro* culture on morphogenetic potential of safflower calluses.

MATERIAL AND METHODS

The study involved the seeds irradiated with gamma rays in doses 50, 100, 150 Gy (as source of gamma rays was used the RXM-V-20 system, the radiation element - ^{60}Co). Untreated seeds were used for control. For the sterilization of materials, conditions were established which involved the rinsing in water with drops of Tween-80 (0.1%) and running under 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).

The sterile seeds were inoculated in Magenta jars with MURASHIGE & SKOOG (MS, 1962) medium without hormones for inducing direct embriogenesis. 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). The fragments of cotyledon and hypocotyl were excised after 7-8 days of in vitro culture and have been inoculated on callus-inducing medium with mineral base after MURASHIGE & SKOOG (1962) and additions of growth regulators (6-Benzylaminopurine (BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D)). 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 and calluses with morphogenetic potential. The software package Statgraphics Plus 2.1 was used for statistical analysis. ANOVA test it was applied for variance analysis of callusogenesis intensity, morphogenetic potential and Student test in assessment of statistically significant differences between treatments.

RESULTS AND DISCUSSION

For all variants the seeds germination, callus intensity were evaluated based on assessment system. According to the observations, the seeds germination had been identified during the first 24 hours after inoculation (Fig. 1).

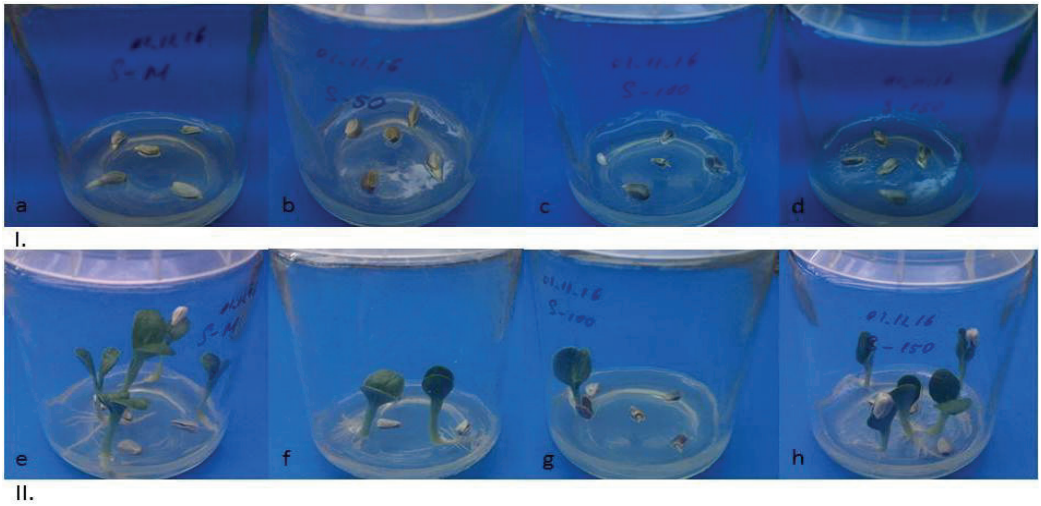

Figure 1. Safflower seeds germination (I) and direct regeneration (II): a) and e) control; b) and f) 50Gy; c) and g) 100Gy; d) and h) 150Gy.

At 2-3 days the impact of radiation on seed germination compared to the control (untreated) (Table 1) was appreciated. Thus, seeds irradiation doses 50 and 100Gy conducted to significant reduction of germination ($P \leq 0.01$) by 1.99 and 3.49 times respectively compared to the control. The impact of 150Gy dose was found insignificant.

Table 1. Safflower seeds germination (%) in dependence of irradiation doses.

Statistical parameters	Control	50Gy	100Gy	150Gy
Average value ± ES	93.33±6.67	46.67±6.67**	26.67±6.67**	81.10±1.10
min ÷ max	80÷100	40÷60	20÷40	80÷83.3
CV, %	12.37	24.74	43.30	2.34

**- significant at $P \leq 0.01$

Under dark and controlled conditions of temperature, intense proliferative processes were triggered in all experimental groups. Towards the end of the first passage (21 days after inoculation) the rate of explants with positive response varies between 90-94%, regardless of the type of explant or irradiation dose. It is necessary to be noted that the intensity of callus, estimated in degree, varies depending on the experimental variant studied. Based on bifactorial dispersion analysis, we find a significant 95% influence of gamma rays, while the explant type and their interaction have no significant impact on the intensity of callus (Table 2).

Table 2. Analysis of variance for callusogenesis intensity (ANOVA test).

Source of variance	Sum of Squares	Degrees of freedom	Mean Square	F-ratio
Radiation (R)	4.866	3	1.622	3.77*
Explant (E)	0.314	1	0.314	0.73
R-E interaction	0.260	3	0.086	0.20
Total	24.847	52		

* significant at $P \leq 0.05$.

The increased intensity was observed for hypocotyl / 50Gy - 2.86, and the lowest in the variant hypocotyl / 100Gy - 1.67 (Table 3).

Table 3. Dependence of callusogenesis values on the irradiation dose and type of explant.

Treatments	Explant	Callusogenesis intensity, note
Control	hypocotyl	2.71±0.22
	cotyledon leaves	2.51±0.19
50Gy	hypocotyl	2.84±0.46
	cotyledon leaves	2.33±0.38
100Gy	hypocotyl	1.67±0.37
	cotyledon leaves	1.72±0.29
150Gy	hypocotyl	2.52±0.19
	cotyledon leaves	2.43±0.23

Evaluating the data distribution, it was found that the mean values in control and experimental variants 100 or 150Gy range between 1-3 notes, but for 50 Gy represent 2-3 notes.

The morpho-structurally study of callus shows a slight difference regarding nuance and structure in dependence of type of explant and applied dose (Fig. 2a, b) and number of passages (Fig. 3).

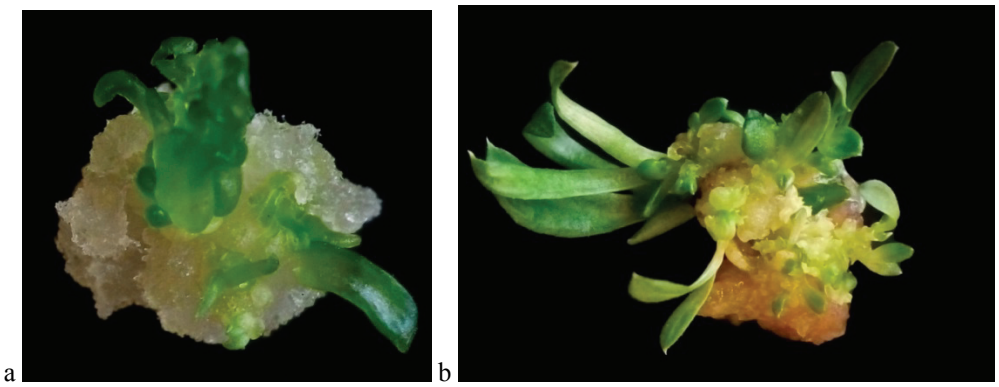

Figure 2. Types of morphogenic calluses. a – friable callus with organogenic potential obtained from safflower leaves, untreated; b – compact callus with embryogenic potential derived from safflower hypocotyl, 50Gy (original).

Thus, the initial callus of hypocotyl 50Gy had yellowish-white and friable structure (Fig. 3b), while leaf / 150Gy present compact structure of green color (Fig. 3c). After the second passage both histogene types had generated compact, green callus (Fig. 3h, i), which subsequently induce morphogenetic centers. It was mentioned that gamma irradiation accelerates the morphogenetic processes and induction of more shoots per explant (Fig. 3g) compared to the control. At the same period of subcultivation, only morphogenetic areas are initiated in untreated explants (Fig. 3f).

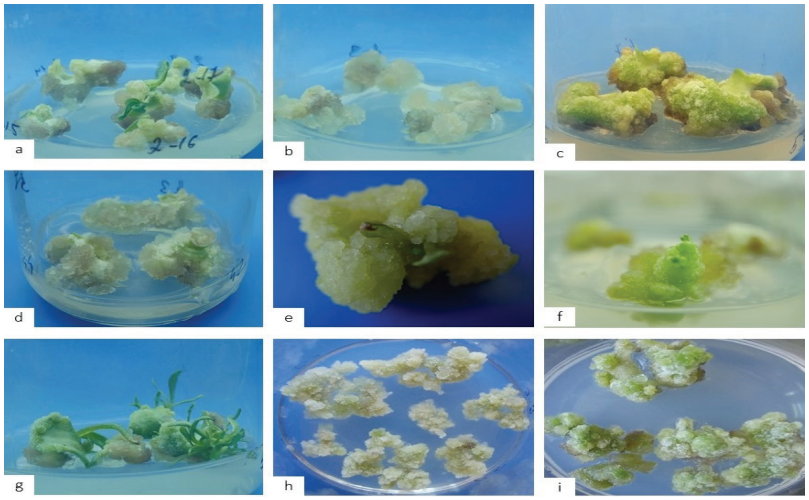

Figure 3. Callus initiation and regeneration for different types of safflower explants in dependence of irradiation doses and subcultivation duration: a) callus from cotyledon leaves; b) hypocotyl callus / 50Gy; c) callus from cotyledon leaves / 150Gy; d) callus from cotyledon leaves / control; e) callus from hypocotyl / 100Gy; f) induction of morphogenetic zones from cotyledon leaves / control; g) shots induction from hypocotyl callus / 50Gy; continually callus cultivation; h) hypocotyl / 50Gy; i) cotyledon leaves / 150Gy. Morphological aspect of callus at first passage (21 days of cultivation): a – e and second passage (after 30 days of cultivation): f – I (original).

The following assessment of morphogenetic intensity show that from an explant are obtained from 1 to 2.5 shoots. During the subcultivation, calluses with aerial rhizogenic capacity have been identified. In this case the subsequently cultivation conducted to the inhibition of the regenerative potential (Fig. 4a). Usually, the morphogenic areas, initiated from cotyledon leaves or hypocotyl, generated primordial shoots (Fig. 4b), which after elongation stage derived plantlets (Fig. 4c). According to the literature, explants of hypocotyl and cotyledons leaves have increased regenerative potential.

a b c

Figure 4. Induction of aerial roots (a); shoots (b) and shoot elongation (c) from morphogenic calluses of hypocotyl in control variant.

All these processes are primordially determined by the genotype (NIKAM & SHITOLE, 1999; BASALMA et al., 2008; RADHIKA et al., 2006; MOTAMEDI et al., 2011).

In our study, it was shown that the number of shoots per explant / callus varies depending on the experimental studied variant. Based on bifactorial dispersion analysis, a significant impact for interaction of gamma radiation and explant was found at 95% and alone gamma radiation at 90%, whereas the type of explant does not significantly influence the regenerative potential (Table 4).

Table 4. Analysis of variance of regenerative potential (ANOVA test).

Source of variance	Degrees of freedom	Sum of Squares	Mean Square	F-Ratio	Source contribution (%)
Radiation (R)	3	79.691	26.564	3.00*	21.61
Explant (E)	1	0.038	0.038	0.00	ns
R-E interaction	3	90.024	30.008	3.39**	24.41
Total	30	368.774			

*; ** significant difference from the control at $P \leq 0.05; 0.01$

After analyzing the interaction factors of gamma radiation / type of explant based on average values, it was fond that the highest regenerative potential (9.33) is attested in the variant 50Gy / cotyledon leaves and exceeds the control by about 9 times. The stimulatory or inhibitory impact of gamma rays has a tissue specificity of the explant.

The study carried out reveals a greater diversity in safflower morphogenetic processes generated by *in vitro* culture during subcultivation. Thus, if after passage IIIth was attested regeneration of plantlets from callus in all experimental treatments, while the fourth *in vitro* passage generates in the control group antogenic induction (direct formation of inflorescence) and antogenic induction associated with *albino* mutation in regenerants derived from irradiated seeds, 50Gy. At the same time, in the experimental treat (50Gy) sporadically were established callus with morphogenic activity which development has an atypical pathway of *in vitro* indirect regeneration: antogen induction and lack of elongation of the shoots.

According to the literature information, the most effective method of improvement, especially of qualitative characters, it is the complex use of *in vitro* culture with experimental mutagenesis, applying physical factors. Gamma rays are successfully combined with *in vitro* culture to ameliorate different species: apple, potato, pineapple, palm (AHLOOWALIA & MALUSZYNSKI, 2001), and for some species with vegetative multiplication this technique can be an important way for plant improvement (MALUSZYNSKI, 1995). In safflower, gamma irradiation fulfills variability induced *in vivo* at the level of quantitative and qualitative characters (RAMACHANDRAN & GOUD, 1983), but also chromosomal instability (KUMAR & SRIVASTAVA, 2010; VERMA & SHRIVASTAVA, 2014).

In our investigations, in the lots derived from irradiated explants were obtained regenerants with orange and yellow flowers, while the initial form was red. At the same time, there are differences in leaves shape and presence of spines: most of the obtained plants have spineless leaves, while some plantlets presented spines such original form.

CONCLUSIONS

1. The calluses initiated from fragments of leaves cotyledons and hypocotyls of safflower proved to *in vitro* cultivation the morphogenetic potential influenced by the type of explant and dose of gamma radiation.

2. The morphogenetic capacity express tissue specificity of the interaction explant / radiation dose, was dependent of calluses aspects (color, consistence - compact or fluid), duration of subcultivation.

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