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BIOTECH TECHNIQUES FOR ACCELERATED BREEDING OF SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) RESISTANT TO *FUSARIUM OXYSPORUM* SCHLECHT

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Abstract

The paper presents the results of multi-year research into cell selection of the Krasa Stupinskiy safflower varieties to increase their resistance to *Fusarium oxysporum* Schlecht. The selection was carried out on the well-proliferating callus tissue obtained from the cotyledon leaves and hypocotyl segments isolated from sterile safflower seedlings using the culture filtrate (CF) of the pathogen in 5, 10, 20 and 50-percent concentration as a selective factor. CF toxicity was determined based on seed germination and callus tissue growth. It has been found that the CF in different concentrations has a toxic effect on the biometric parameters of the seedlings as well as on the callus tissue growth. Higher concentrations increase the phytotoxic effect, leading to complete inhibition of seed germination when reaching 100%. Optimum CF concentration for cell selection has been 15%. The callus cells could adapt to the pathogenic effect due to cell wall lignification. The obtained regenerants are currently being investigated. The objective of the presented study was to investigate the morphogenetic potential of isolated safflower tissues *in vitro,* to select optimum culture conditions for cellular selection and to produce fusariosis-resistant cultivars.
Keywords: Safflower, *Carthamus tinctorius* L., Culture filtrate, *Fusarium oxysporum*, phytopathogen, callus culture.

Introduction

Nowadays in Russia, there is a huge interest in the technologies to grow new cultivars, introduced plants and cultures that are new to Russian agricultural scientists and practitioners. N.I. Vavilov highly praised adventitious plants and called for wider use of wild flora as a potential source of herbal and biologically active substances. The issue has recently become even more topical in the light of import substitution because Russia is currently importing most of the plant oils and biological substances the country needs.

Increasing the quantity and quality of harvested agricultural plants through their better adaptation to the environment, including both biotic and abiotic factors is one of the trends in plant selection. In this respect, cultivating plants in the higher latitudes of our country is an important problem because of the high cost of the cultivation process (Temirbekova, 2018).

One such perspective agricultural plant to be brought further to the north is safflower (*Carthamus tinctorius* L.) that belongs to the Asteraceae family and originates from Egypt and India. After many years of research, a team of Russian selectionists headed by S.K. Temirbekova have developed the Krasa Stupinskiy safflower variety included in the Russian State Register of Selection Inventions in 2013 and recommended to be grown in all the regions of the Russian Federation.

It is noteworthy that the safflower in general, and the Krasa Stupinskiy in particular, are susceptible to different phytopathogens that may result in inhibition and even death of the plant.

One such disease is fusariosis caused by *Fusarium oxysporum* Schlecht. ex Fr.

Using biotech techniques is one of the new perspective ways to increase selection efficiency. These techniques that allow one to develop new varieties of plants become a basis for further selection and include somatic hybridization; obtaining regenerates from continuously cultivated callus tissues; developing haploid plants, and other techniques that significantly reduce selection duration. An important role here is played by cellular and tissue-specific selection, the methods selecting the cell populations that remain stable in presence of a selective factor and regenerate a whole plant from such a population. These techniques are essential for developing plants that are not susceptible to biotic environmental factors such as phytopathogens (Kalashnikova, 2012).

Cellular selection has been successfully applied both in Russia and abroad to develop the phytophthora - resistant potato, alternariosis - resistant tomato piricullariosis -resistant rise, fusariosis – resistant flax and lucerne, sclerotinia – resistant clover and other cultivars (Butenko, 1999). As a rule, the protein and nonprotein toxins extracted from fungal pathogens are used as a selective factor. However, the disease resistance of the regenerants selected this way is not always a match to the resistance of the plants grown *in vivo*. In this respect, one has to develop nontraditional approaches to increase plant resistance to phytopathogens (Kalashnikova, 2003).

The same holds true for the safflower, which, before the presented study, had never been considered as an object for cellular and tissue-specific selection to obtain the cultivars resistant to different diseases, fusariosis in particular.

Materials and Methods

In this study, the seeds of the Krasa Stupinskiy safflower variety bred at the All-Russian Selection and

Technological Institute of Horticulture and Nursery were used a starting material. Their hypocotyl segments, apical buds and cotyledon leaves isolated from their sterile seedlings served as primary explants.

The seeds were sterilized in 0.1 % solution of mercuric chloride or 7% solution of sodium hypochlorite during 4, 8 and 16 minutes to be rinsed in sterile distilled water and cultivated on the hormone-free Murashige and Skoog medium containing 3% of sucrose and 0.7 % of agar.

To induce callus formation and morphogenesis, the growth medium also included cytokine-inducing (Epin, Dropp, 2 isopentenyl adenine (2iP)) and auxine-inducing (indole acetic acid) agents in different concentrations.

The isolated explants were cultivated in the Petri dishes in a light room (16 -hour photoperiod; 25ºC; white luminescent lamps of 3000 lx; 70% of relative humidity).

The pure-culture *Fusarium oxysporum* pathogen was extracted from the Krasa Stupinskiy safflower seeds by O.O. Beloshapkina, D. Sc., Professor of Chair of Plant Protection of Moscow Timiryazev Agricultural Academy.

The culture filtrate (CF) of the pathogen was obtained by growing the fungal isolates in 300 ml of Czapek's medium in a shaker rotating at 100 rpm. Each tube contained 10^8 fungal conidia. The fungal suspension was filtered through filter paper and autoclaved. The obtained CF was then added in the growth medium in a concentration of 5, 10, 20 and 50% of the total medium volume.

CF toxicity was determined based on seedling length and callusogensis intensity following the O.A. Berestetsky technique. The measurements were performed on the 7th (seedlings) and 30th (callus tissue) days.

The callus cells were studied in squash temporary preparation using a KR-12 microscope and a digital Sony photo camera, their viability estimated after being treated with Evans blue dye.

The works were carried out in aseptic conditions; the tools, growing media and plant materials were sterilized in accord with the guidelines devised at Chair of Genetics, Biotechnology, Selection and Seed Breeding of Moscow Timiryazev Agricultural Academy (Kalashnikova *et al*., 2014)

Mathematical processing of the experimental data was performed based on the methods of mathematical statistics (Dospekhov, 1985; Smiryaev, 2007). Disperse and regressive data analysis was carried out in MS Excel.

Results and Discussion

The objective of the first stage of the study was to develop a technique for proper plant material sterilization that provided growing of a culture that is free from both internal and external infections. At this stage, 2 sterilizing agents (0.1 % mercuric chloride and 7% sodium hypochlorite) were assessed. The time exposition for the agents varied from 4 to 16 minutes.

The obtained results demonstrated that seed germination capacity depended on a type of sterilizing agent and its time exposition. In case of the mercuric chloride, the percentage of well-grown seedlings varied from 12.5 to 83.3% of sterile seedlings for different time expositions. The maximum effect was obtained for an 8-minute exposition when we observed formation of multiple thick seedlings (10.3 cm in shoot and 3-4 cm - in root length) of proper morphology. When the agent was applied for 16 minutes, it resulted in a sterile culture with single seedlings, which can be explained by excessive sterilization of the seeds that manifested itself in changing seed color from bright to dark to be a sign of the necrotization that inhibited germination.

Using the sodium hypochlorite led to the number of well-grown seedlings reduced 2.5-3.5 times if compared to the mercuric chloride and their shoot length did not exceed 4.7 cm.

Thus, the performed investigation into sterilization techniques allowed us to select the best conditions for seed sterilization and obtain the well-grown seedlings that were later used in our works of cellular selection *in vitro*.

As a rule, such works begin with selecting the optimum growing media that provide high proliferation activity of callus cells and their proper morphogenesis. Changing the mineral and hormonal composition of a medium, one can control the morphogenesis of cultivated cells and plant organs *in vitro* that make it possible to solve different problems of cellular biotechnology.

Our investigation demonstrated that the isolated hypocotyl segments, apical buds and cotyledon leaves of safflower seedling had different capacities for callus tissue generation. The experiments showed the callus tissue obtained from hypocotyl segments had the highest proliferation capacity, and the tissue from cotyledon leaves – the lowest.

The obtained callus tissue was later used for the cellular selection *in vitro* designed to cultivate dedifferentiated cells in the growth media containing a selective factor. To obtain the plant forms resistant tocertain phytotoxins, one commonly uses the cultural filtrate of that very phytopathogen. This approach takes 8-10 months of continuous cell cultivation under stress conditions. In our study, the cultural fluid of the pathogen was used as a selective factor and the growth cycle comprised 5 passages.

At the first stages of the selection, it was necessary to estimate CF phytotoxic activity and concentration applicable for cellular selection, which was done using safflower seeds.

Table 1 : Safflower seedling biometrics for different concentrations of *Fusarium oxysporum* CF.

Growth time	CF concentration, %	Shoot length, mm	Root length, mm
15 days	25	65,2 ± 6,3	54,9 ± 6,0
	50	58,7 ± 6,1	42,4 ± 6,4
	75	31,7 ± 6,0	23,5 ± 6,1
	100	0	0

Table 2 : *Fusarium oxysporum* CF phytotoxicity.

Growth time	CF concentration, %	Shoot phytotoxicity	Root phytotoxicity
15 days	25	31,2	44,8
	50	51,3	57,6
	75	88,3	76,5
	100	0	0

Note: Table data are percentages in relation to a control.

The results can be seen in Tables 1and 2 and demonstrate that CF in different concentrations had a toxic effect on safflower seedling biometrics. Increasing the concentration to 100% boosted the phytotoxic effect to the degree of complete germination inhibition. It is also noteworthy that CF concentrations of 25, 50 and 75% had a higher inhibitory effect on the seedlings' roots than on their shoots.

Based on the obtained results a decision was made that CF concentration in the callus culture medium should not exceed 35 %.

In a following series of experiments, CF effect on callusogensis intensity was investigated. These experiments were necessary to determine the stress factor concentrations to be used while cellular selection. The obtained results are displayed in (Fig. 1).

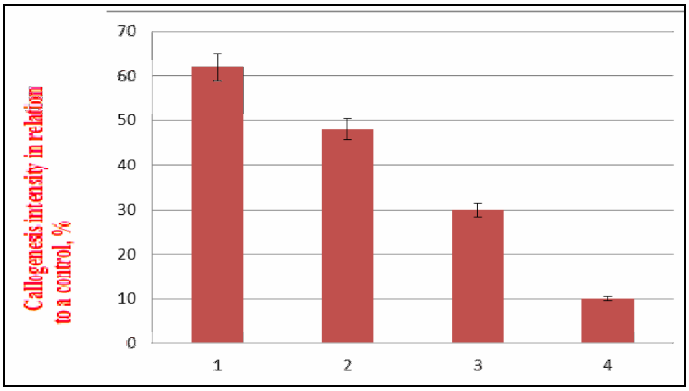


Fig. 1 : Safflower callusogensis intensity in media with different concentrations of *Fusarium oxysporum* CF: 1 - 5%, 2 - 15%, 3 - 25%, 4 – 35%.

The experiments demonstrated that increased CF concentrations in the medium led to a significant reduction in callusogensis intensity, which was mainly due to necrocytosis. From all the concentrations studied, the most favorable was that one of 15% that allowed 48% of the cells to survive, which was in consistency with the generally known fact that selective-factor concentration for cellular selection should be chosen in a way to provide a 50:50% ratio of live and dead cells.

Thus, the performed series of experiments enabled us to select an optimum CF concentration (15%) to perform cellular selection (Fig. 2).

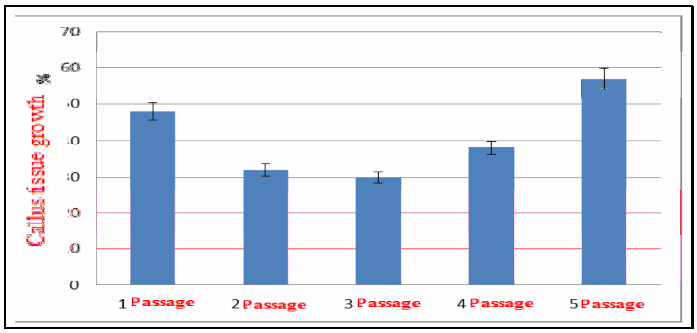
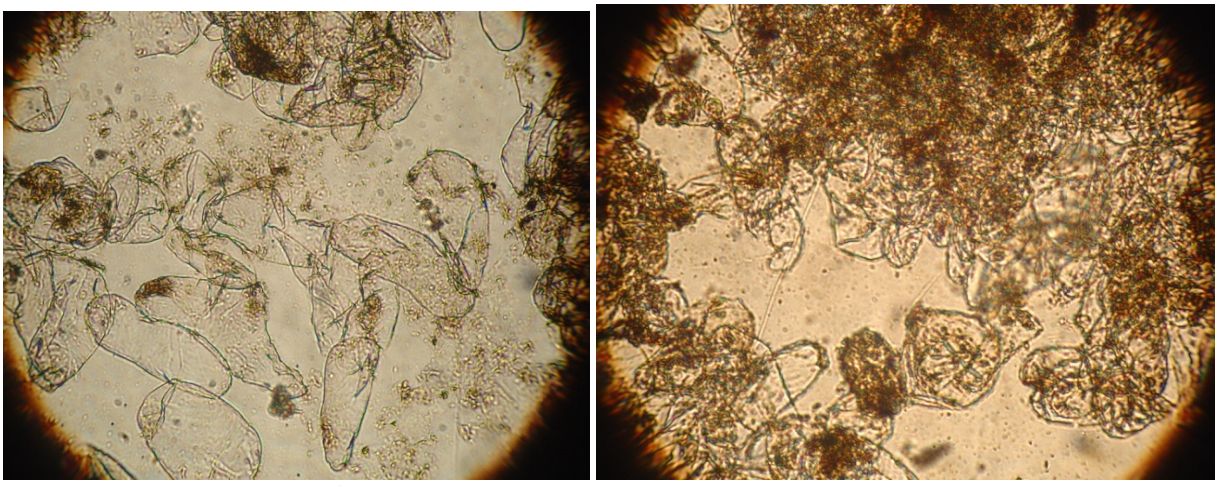


Fig. 2 : Safflower callogenesis intensity for Fusarium oxysporum CF concentration of 15%.

The diagram demonstrates that callusogenesis intensity was significantly suppressed in the early passages. However, starting from passage 4, the intensity started to increase to reach 58% in passage 5 being the evidence that the cells had adapted to the stress factor.

As for the reasons for this adaptation, lignin synthesis in cell wall was considered because it makes a cell inaccessible for a stressor (Zaprometov, 1979). To prove that point, a study into the morphophysiological characteristics of the callus cells cultivated both in experimental and control groups was carried out (Fig. 3).



a- passage 1 **b- passage 5**

Fig. 3. Safflower callus cells from different passages: a – passage 1; b – passage 5.

The investigation demonstrated that the callus tissues were composed of heterogenic (both in shape and size) cells and that was typical for both early and late passages. Their cultivation had certain patterns. In particular, in case of continuous cultivation under stress conditions, we observed changes in the morphology. For instance, in the early passages, the tissues were composed of large oval-shaped cells with big vacuole, while in passages 4 and 5, the cells remained oval-shaped but significantly reduced in size, which was probably due to the changes in cell-wall structure caused by its lignination. Analogous results have been obtained for the carrot, potato, sunflower and other cultures, which confirms cell cultures increase their resistance to different biotic factors thanks to lignin synthesis in cell wall and the content of total soluble phenolic compounds (Kalashnikova, 2003)

The stable callus cultures were later used to grow the regenerant plants (Fig. 4) that are currently being investigated.

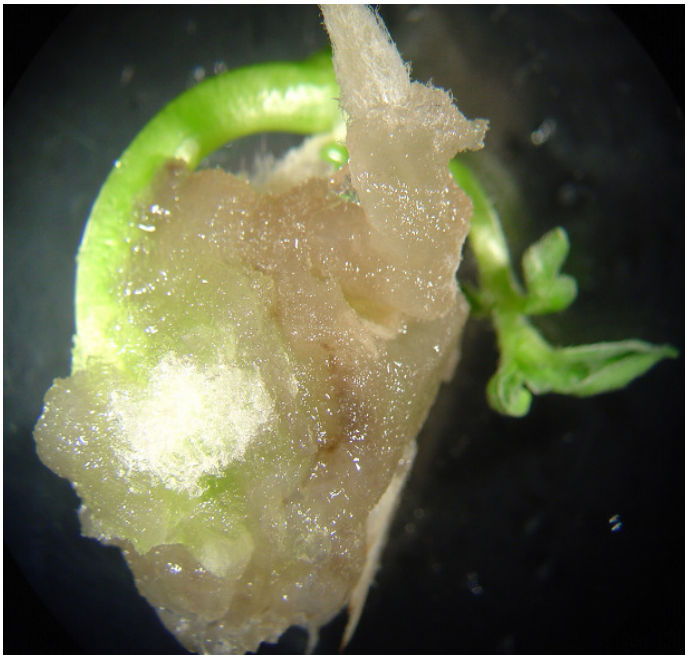


Fig. 4 : Safflower regenerants grown from selected callus tissue.

The performed studies allow us to conclude that in order to carry out successful cellular selection of the safflower resistant to *Fusarium oxysporum* and other phytopathogens one is required to use a cultural filtrate (in a concentration of no less than 15%) as a stress factor and cultivate the callus tissues under these conditions during 5 passages. This selection scheme allows obtaining stable cell and tissue cultures as well as regenerant plants.

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