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SOMACLONAL VARIATION OF SUGAR BEET RESISTANT TO PATHOGENIC ROOT ROT *Fusarium oxysporum var. orthoceras*

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Sugar beet (*Beta vulgaris L.*) – one of the most important crop in the world. In Kazakhstan, it is a traditional and major source of domestic sugar. The industry of cultivation and production of sugar beet is one of the priority areas of agricultural development of the country. In this paper, we studied the regeneration ability of different genotypes of sugar beet explants on selective media with the culture filtrate of the pathogen fungus *F. oxysporum var. orthoceras*. From the roots and shoots of sugar beet the pathogen *Fusarium* root rot was isolated. Was obtained pure cultures of the isolated pathogen. As a result, of morphological and cultural descriptions, as well as microbiological analysis it was revealed that the isolated pathogen is *Fusarium Oxysporum*. The results showed the pathogenicity of the fungus. For regeneration *in vitro* of the sugar beet genotypes resistant to the pathogen the culture media was optimized to the culture filtrate of the fungus *F. oxysporum var. orthoceras*. The frequency of shoot regeneration, depending on the genotype, was 1,0–12,5 %. On these explants the multiple shoot formations were observed.

Key words: sugar beet, pathogen, fusarium, root rot, culture filtrate

INTRODUCTION

Sugar beet (*Beta vulgaris L.*) – one of the most important crop in the world. In Kazakhstan, it is a traditional and major source of domestic sugar. Such industries as sugar beet

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cultivation and sugar production are in priority of state agricultural politics. However, in a recent years, the yield of this crop has decresed, it can be explained by a number of economic and agronomic reasons, such as a low efficiency of fighting against pathogens, weeds and pests and also by the absence of varieties and hybrids of sugar beet resistant to herbicides, abiotic and biotic stresses.

The main pathogens of sugar beet are the bacteria *Erwinia carotovora*, *Pseudomonas aptata*, virus "BNVV" *Beet necrotic vein Virus*, deffusing by the soil-fungus *Polymyxa betae*, and fungi of the genera *Fusarium*, *Penicillium*, *Cladosporium*, *Rhizoctonia*, *Alternaria* (ABD-ELSALAM *et al.,* 2008; CAMPBELL *et al.,* 2006; HANSON *et al.,* 2007; LEPOIRVE and CARIES, 2005; MANDOLD *et al.,* 1998; WINDELS C.E. *et al.,* 2005).

The stress factor for sugar beet is also the *Fusarium* root rot, that produces toxic substances. Their mycotoxins are in the class of the most dangerous substances, and even in minor concentrations it has serious carcinogenic, terratogennic and immunogenic effects. The toxins of the widespread fungus - *F. oxysporum var. orthoceras* influence on the plants growth and development during the whole vegitation period, that is significantly reduce the productivity. The cultivation of sugar beet lines that are tolerant to this pathogenic fungus can increase the productivity of root crops approximetely up to 40%. Breeding *in vitro* significantly reduces the volume and duration of the traditional breeding (SHEVELUKHA, 1992) works.

The toxic metabolites of the fungus *F. oxysporum var. orthoceras* in the culture of callus tissue can be used as a selective agent in nutrient medium, which allows to affect directly on the cell and, therefore, to select the genotypes more resistant to the toxins. (LITVINOV, 1967).

The death of sugar beet infected by *Fusarium* according to Bilay (BILAI, 1977) can be explained by blockage of vascular system as well as the fungus toxins influence, in particular, the fusaric acid, leading to a breach of the osmotic pressure and cell turgor.

The centers of sugar beet cultivation in U.S. are Minnesota and North Dakota. They produce approximetly 48% of these product on an area of 300 000 hectares. In the fields of the United States *Fusarium* fungies have also been identified. Among the 35 sugar beet roots collected from the eight fields, 25 strain of *F. oxysporum* were isolated. The most virulent was *F. oxysporum f sp. Betae* (WINDELS *et al.,* 2005). HANSON *et al*., (2007) from Pennsylvania, has isolated an aggressive strain of *F. oxysporum-FC716*.

ZAPOLSKAYA (2000) identified and supplemented the species composition of sugar beet root pathogens, cultivated in the zone of central steppe of Ukraine.

In breeding, for the development of disease-resistant genotypes of agricultural crops and accelerating the breeding process biotechnology methods are helpful.

MATERIALS AND METHODS

Plant materials. In our study 10 varieties of sugar beet provided by the breeders of our institute (No. 2225, 1034, 1002, 2137, 2287, 2139, 2234, 2183, 2221, 2290) were used.

Selection of Fusarium rot pathogens. The roots were antisepticed and washed, then were and cut onto small pieces on the border of infected and healthy tissue. Then the roots were placed in bottles with sterile water. After one day the root pieces were dried on sterile filter paper and placed on a slightly dried agar plate in a Petri dish. From the colony developed in a day a subculture on wort agar was made.

The species of Fusarium rot pathogens was determined by Bilay method (BILAI, 1977). The fungus culture placed on agar netrient medium such as potato, sour potato medium, potato-

glucose and rice. An identification is carried out by Litvinov identification guide (LITVINOV, 1967).

To test the pathogenicity and aggressiveness of isolates of the fungus mycelium the *F. oxysporum* is applied to aseptic tissue pieces of sugar beet roots, placed in sterile petri dishes.

Obtaining culture filtrates of phytopathogenic fungies. Fungus pathogen is cultivated in Chapek liquid synthetic medium during 21 days. The cultural liquid is filtrated, autoclaved at 120°C for 30 min.

Seeds germination was determined according to State Standart, No. 12038-84 (GOST - 12038-84, 2010). Samples of sugar beet seeds were sown in Petri dishes in three replications and germinated in an incubator at a temperature 22-25°C during 10 days, followed by counting.

Defining the photo-pathogen properties of selected fungies was carried out on seedlings (SVIRSHCHEVSKAYA, 2005). After 10-15 day the germination, number of infected plants and the intensity of disease is recorded.

Defining the toxicity of selective factor according to sugar beet seedlings. The toxicity of culture filtrate was determined by the growth of the root and the whole plant (SHEVCHENKO, 1961).

To test the pathogenicity and aggressiveness of isolates of the mycelium, the *F. Oxysporum var. Orthoceras* is applied to aseptic tissue of healthy sugar beet roots, placed in the sterile petri dishes (SVIRSHCHEVSKAYA, 2005).

Determination of toxicity of selective factor to sugar beet seedlings. The toxicity of cultural filtrate is determined by the growth of roots and the whole plant (SHEVELUKHA, 1992).

Mediums. The plant material was cultivated on nutrient medium, containing MS macro and micro nutrients (MURASHIGE and SKOOG, 1962), carbohydrates, phitohormones and growth regulators (GAMBORG and EVELENIGH, 1968). The culture toxin *F. Oxysporum var. Orthoceras* (10%) and (30%) were used for selective mediums.

Regeneration and micropropagation of shoots in vitro. Explants were obtained from aseptic seedlings grown in culture *in vitro*. Frequency of regeneration of seedlings was defined as the ratio of regenreant-seedlings to the total number of explants, planted on the medium.

The treatment of the disease was conducted on a 5-point scale of Shevchenko (SHEVCHENKO, 1961).

0 - Highly resistant (HR), disease development 0-15%
1 - Stable (St), disease development 16-30%
2 - Average resistant (AR), disease development 31-50%
3 - Susceptible (S), disease development 51-70%
4 - Highly susceptible (HS), disease development 71-100%

Statistical analysis of experimental data. The obtained experimental results were processed using the standard methods of statistical analysis in "MS Excel" sofware.

RESULTS AND DISCUSSION

Extaction of root rot pathogen from sugar beet roots

Numerous fungies, such as *Mucor Mich*., *Penicillium Link*., *Fusarium* and other (Picture 1A, B) were extacted from sugar beet roots.

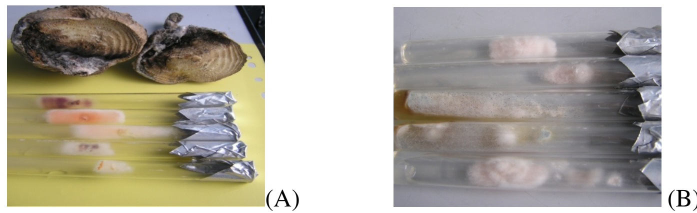

Fig 1. (A) transfer and (B) pure cultures of root rot pathogens

In order to obtain pure cultures of pathogens Fusarium root rot, fungies were repeatedly transplanted into the test tubes and petri dishes with Chapek medium. Purified cultures of extracted pathogens are shown in Picture 2 (A, B).

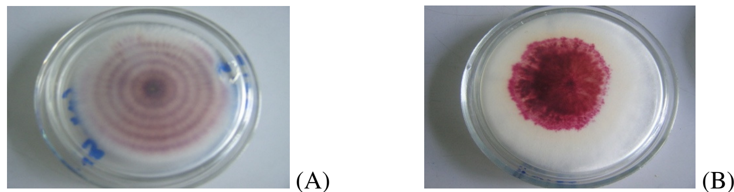

Fig 2. (A), (B) Pure cultures of sugar beet

The first fungus was purple with annular arrangement and smooth surface (Picture 2A), the second - purple, with air stroma (Picture 2B).

Identification of root rot pathogen of sugar beet

In order to identify the *Fusarium* rot pathogen (species of the *Fusarium* genus) the morphological and the cultural characteristics published by Litvinov (LITVINOV, 1967) were used. The standard mediums, such as a medium of rice, sour potato agar, potato agar with glucose were used.

The pigment descriptions, the conidias observation and measurements were made on the 15th, 30th and 45th day of growth on rice and potato mediums.

Two species of extracted fungies, shown in Picture 3 (A, B), were sown on the rice and potato mediums (simple agar, acid agar and glucose agar). The purple fungus, that forms rings (Picture 2 A) sawn on the rice medium gave pink stroma (Picture 3A, B) with an odor. Fungies cultivated on agar medium were off-white, spreaded and smooth (Picture 3C-F).

Cytological analysis of this fungus is shown Picture 4. The macro-and microconidia of this fungus were studied and measured (Picture 4 A-F).

As a result of morphological, cultural and microbiological studies, it was determined, that the fungus with violet rings is a *Fusarium oxysporum*.

The second fungus didn’t germinate on standard *Fusarium* culture medium. In our opinion, it was not the *Fusarium* fungi, therefore it was excluded from further study.

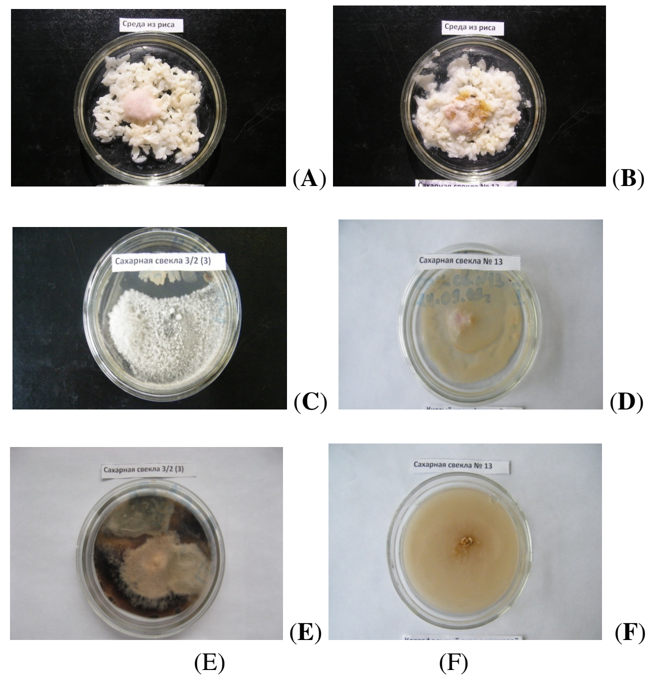

Fig 3. (A, B) The growth of *fusarium* fungies on rice, (C, D) sour potato and (E, F) potato glucose medium

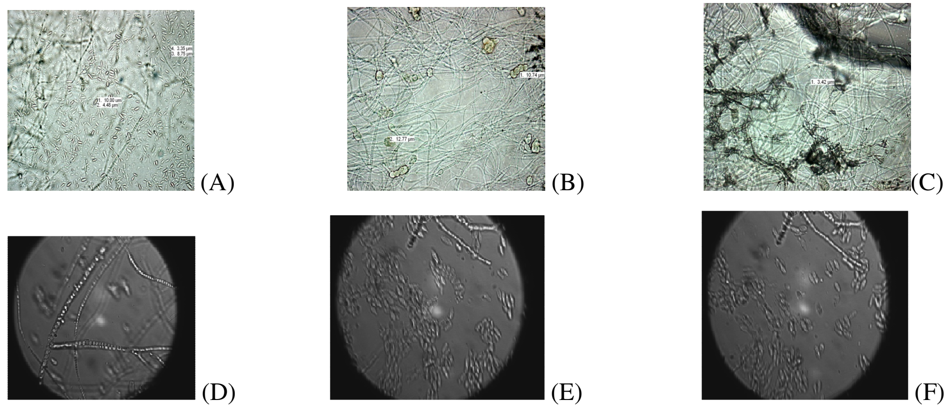

Fig 4. Macro (A, D) and microconidia (B, C, E, F) of the fungus *f.oxysporum*

F.oxysporum description: macroconidia formed in aerial mycelium, spindle - sickle, elliptically curved, most of the length have an equal diameter, have pedicellate or papilla, with 3 - 5 partitions: 3 dividers with overall dimension from 35 to 4,5 micrometer (um), and with 5 dividers 42 to 4,5 micrometer (um). The microconidias formed in the mycelium, often - false heads, always abundant. The chlamydospores are abundant, intermediate and apical. Aerial mycelium is painted in various shades of pink and purple, rare - in light – yellow or white colors.

Test on Fusarium root rot pathogens aggressiveness and obtaining the culture filtrate of F.oxysporum pathogen

To test the *F.oxysporum* fungus aggressiveness, the pathogen spores of *F.oxysporum* agent was applied on undamaged, clean tissue of sugar beet and within 5-10 days were monitored its development (Picture 5 A,B,C).

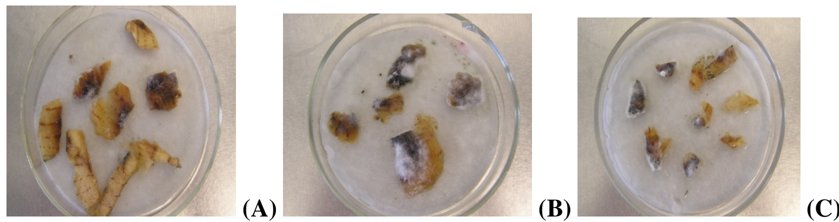

Fig 5. Studying pathogenicity of *F.oxysporum* fungies on sugar beet tissues

The pathogenicity of the fungus was established, because the tissue of sugar beet roots were completely decayed after determined time and was covered with white aerial raid. The culture filtrate (CF) of the fungus *F.oxysporum* was extracted by its cultivation in Chapek liquid synthetic medium. It was revealed, that on 12-14 day the accumulation of fungal biomass reaches its maximum (1,25-1,37 g per 100 ml of culture medium). Further cultivation did not results in biomass increase and after the 16th day the rate remained constant.

The optimal pH for the biomass accumulation is 5,0-6,0. The fungal growth occurs with slight swinging on a shaker.

Finding sterilization materials

The experiments showed that the main difficulty in introduction of sugar beet culture *in vitro* was a high infection of vegetating plants. Because of the infection a large amount of source material were inevitably dying during the mass introduction into culture. Even sterilization of solutions could not destroy it - infection was there during 2-5 weeks of cultivation (tab. 1).

Table 1. Sugar beet seedlings explants viability in vitro culture in conditions of using the various methods of sterilization

Solution	Concentration %	Exposure time h	Total explants	Be lost,%		Survived, %
				Necrosis	infection	
Hydrogen peroxide	3	3	20	53,0	38,0	9,0±0,04
		2	20	18,0	60,0	22,0±0,07
		1	20	24,0	53,0	23,0±0,06
		0,5	20	13,0	42,0	45,0±0,011
Bleach	15,0	3	20	3,0	90,0	7,0±0,03
		2	20	0	94,0	6,0±0,02
		1	20	2	90,0	8,0±0,04
		0,5	20	0	91,0	9,0±0,03
		0,3	20	0	88,0	12,0±0,01
Diotsid	0,1	3	20	6,0	10	84,0±0,03
		2	20	0	5,0	95,0 ± 0,09
		1	20	0	14	86,0±0,07
		0,5	20	0	17	83,0±0,05
		0,25	20	0	19	81,0±0,04

The concentrated diotsid 0.1% solution at the 2 h exposure provided the highest yield of healthy explants (95%). However, these plants developed weakly: light green color, growth point was absent, the leaf blades didn't extend and adventitious buds were absent. The optimal exposure appeared to be 1 hour diocidum: the adventitious buds and shoots of explants formed with sufficient efficiency.

Morphogenesis of sugar beet

The sugar beet samples Numbers 2225, 1034, 1002, 2137, 2282, 2139, 2234, 2183, №2221 and 2290 were planted on MS culture medium supplemented with 10% and 30% - concentrated toxin on 150 petri dishes each, and 150 Petri dishes of control without culture filtrate.

Table 2 shows that the percentage of callus formed in the second passage, ranged from 1.0 - 81.8%; the regeneration rate ranged from 1.0 - 11.0%. Sugar beet samples number 1034, 2225, 1002, 2183, 2282 showed resistance to the pathogen *F. oxysporum var. Orthoceras*; Average resistance showed the numbers: 2137 and 2221; susceptible to the deasease were: 2234, 2290, 2139. The control treatment was the highest in all the samples.

Table 2. Percentage of calli, rhizogenesis, regenerates of sugar beet resistant to the pathogen f. Oxysporum var. Orthoceras, after 2 passages

Media	I passage			II passage		
	% calli	% rhizogenesis	% regenerated plants	% calli	% rhizogenesis	% regenerated plants
1	2	3	4	5	6	7
			Resistant			
			№ 2225			
MC	36,3	-	-	81,8	37,7	11,0
MC+10% CF	11,2	-	-	39,3	16,0	10,0
MC+30% CF	8,1	-	-	9,0	7,2	3,4
			№1034			
MC	33,3	-	-	76,2	22,5	12,5
MC+10% CF	5,1	-	-	18,6	7,8	3,7
MC+30% CF	3,1	-	-	7,5	5,6	1,2
			№2282			
MC	22,9	-	-	55,5	14,4	7,2
MC+10% CF	13,9	-	-	18,1	7,5	3,5
MC+30% CF	12,7	-	-	13,0	5,1	2,1
			№1002			
MC	21,9	-	-	48,1	10,2	5,1
MC+10% CF	15,4	-	-	19,2	8,3	3,2
MC+30% CF	13,7	-	-	15,1	6,3	2,3
			№2183			
MC	32,4	-	-	60,1	27,2	9,1
MC+10% CF	12,1	-	-	30,1	16,4	5,2
MC+30% CF	7,2	-	-	10,3	5,2	2,1
			Average resistant			
			№2137			
MC	23,1	-	-	32,0	16,2	5,2
MC+10% CF	11,2	-	-	23,4	10,3	2,1
MC+30% CF	7,1	-	-	8,9	6,3	1,0
			№2221			
MC	24,5	-	-	34,2	14,7	3,6
MC+10% CF	9,7	-	-	12,3	9,7	2,2
MC+30% CF	6,2	-	-	9,5	5,4	1,0
			Susceptible (not regeneration)			
			№2234			
MC	21,5	-	-	18,1	-	-
MC+10% CF	8,4	-	-	4,2	-	-
MC+30% CF	7,3	-	-	2,3	-	-
			№2290			
MC	23,1	-	-	16,1	-	-
MC+10% CF	7,9	-	-	4,1	-	-
MC+30% CF	5,4	-	-	1,1	-	-
			№2139			
MC	20,8	-	-	12,5	-	-
MC+10% CF	8,2	-	-	4,0	-	-
MC+30% CF	6,2	-	-	1,0	-	-

Sugar beet regeneration

For the plant regeneration the calli were transplanted on the MS medium with gibberellic acid 1 mg/l - 450 tubes (Picture 6 A, B).

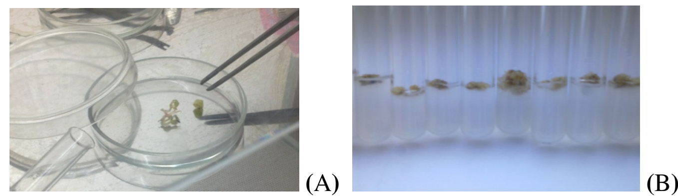
(A) (B)

Fig 6. Transfer of callus

It should be noted that, depending on the genotype, the number of explants with morphological abnormalities ranged between 1.0 - 12.5%. For the treatment MS +10% CF the best results (Picture 7) showed the samples No. 2225 and 2183.

In the treatment 30% of MS CF the shoots had the following morphological changes: a) yellowing of leaves b) changes in shape and curving of leaves, and c) highly elongated threadlike stalks with small leaves. Therefore, the selection of regenerants resistant to fusarium according their morphological characteristics is a necessary condition for receiving the regenerants genetically identical to the original shape, which determines the principle use of the method.

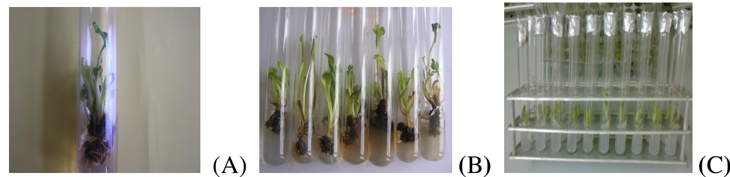
(A) (B) (C)

Fig 7. Production of plants regenerated

After preparation of mixture of peat + sand the regenerants of sugar beet were transplanted from tubes into the ground (Picture 8 A - D). The first week they were closed by film, and then were opened for the night, and closed for the day, i.e. the adaptation to the external conditions has been conducted.

Maintenance of basic physiological factors (temperature +26 ± 2ºC, 16-hour photoperiod, light 20,000 lux, humidity 60%) were under the control. In the *in vitro* experimental systems to create strictly defined, controlled conditions, the property of totipotency of plant cells is manifested to a greater extent and the morphogenetic development of regenerated plants of generative and vegetative organs of sugar beet through direct regeneration or callus through organogenesis.

Formed in the ground plants were characterized by evenness and morphological uniformity within each line. However, differences were observed between the lines of both the morphological features rosettes of leaves, and on the development of plants. At this point they are transferred to breeders in KRIACP.

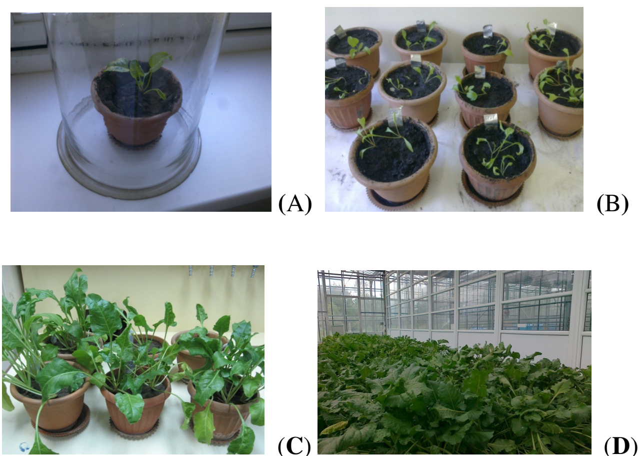

Fig 8. Transfer regenerated plants of sugar beet to soil

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REFERENCES

ABD-ELSALAM, K.A., A.H.BAHKALI, A.A.AL-KHEDHAIRY, J-A VERREET (2008): Development of a Conventional and Lightcycler PCR assay for Detection of *Fusarium solani.* Genes, Genomes and Genomics, *2(1)*:63-67.

BILAI, V.I. (1977): Fusarium. Kiev, Ukraine, Naukova Dumka, 442.

CAMPBELL, L., K.K. FUGATE, W.S. NIEHAUS (2011): Fusarium Yellows Affects Postharvest Respiration Rate, Sucrose Concentration, and Invert Sugar in Sugar beet. Journal of Sugar Beet Research, *48(1)*:17-40.

GAMBORG, O.L., D.E. EVELEIGH (1968): Culture methods and detection of glucanases in suspension cultures of wheat and barley. Can. J. Biochem, *1106*:95.

GOST - 12038-84 (2010): Methods for determination of germination of agricultural cultures.

HANSON, L.E., HILL A.L., L. PANELLA (2007): Interaction of varying *Fusarium oxysporum* isolates with different sugarbeet lines. Proc. the 34th Meeting of ASSBT (Agriculture), Slat Lake City, UT, USA, 28 Febrary – 3 March 2007, 157.

LEPOIRVE, P., N. CARIES (1986): Selection of sugar beet calli to obtain plants resistant to Cercospore beticola. Nucl. Techn. and in vitro cult. Plant improve. Proc. Int. symp., Vienna, 19-23 August 1986, 305 – 308.

LITVINOV, M.A. (1967): Determinant of soil microscopic fungi. Leningrad, Nauka, 304.

MANDOLD B., KRAUS J., MECHELKE W., G. BUTTNER (1998): Resistenz gegenular dem beet necrotic yellow vein virus (BNEVV) bei konventionelli geruchieten und gentechnisch ent vickelien linlen und sorten von Zuckrruben. Miolt. Bid. Bundesanst. Land – und Forstwist (Berlin), *357*:289 – 290.

MURASHIGE, T., F. SKOOG (1962): A revised medium for growth and bioassays with tobacco culture. Physiol.Plantarum, *15*:473-497.

SHEVCHENKO, V.N. (1961): Breeding methods of sugar beet for resistance against diseases. Plant immunity to diseases and pests, M.: Kolos, 118-126.

SHEVELUKHA V.M. (1992): Phytotoxic culture filtrate of *Fusarium oxysporum* to callus. Mycology and phytopathology, *25(4)*: 343-347.

SVIRSHCHEVSKAYA, A.M. (2005): Cultivation *in vitro* of cells and tissues of sugar beet to produce genetic breeding material. Proc. of the National Academy of Sciences, *49(6)*: 65-70.

WINDELS, C.E., J.R. BRANTNER, C.A. BRADLEY, M.F.R. KHAN (2005): First report of Fusarium oxysporum causing Yellows on sugar beet in the Red River Valley of Minnesota and North Dakota. Phatology, *89(3)*: 341.

SOMAKLONALNO VARIRANJE ŠEĆERNE REPE OTPORNE NA PATOGEN IZAZIVAČ TRULEŽI KORENA

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Izvod

U radu su prikazani rezultati istraživanja sposobnosti regeneracije različitih genotipova eksplanata šečerne repe na selektivvnim podlogama sa kulturom filtrata patogene gljive *F. Oxysporum var. Orthoceras*. Iz korena i izdanaka šećerne repe je izolovan patogen korena Fusarium i dobijene su čiste kulture izolovanih patogena. Morfolođki opis u kulturi patogena i mikrobiološke analize su pokazale da izolovani patogen *Fusarium Oxysporum* ima patogenu sposobnost. For regeneration *in vitro* of the sugar beet genotypes resistant to the pathogen the culture media was optimized to the culture filtrate of the fungus *F. oxysporum var. orthoceras*. Za regeneraciju in vitro genotipova rezistentnih na pathogen izvršena je optimizacija filtrate culture gljive *F. oxysporum var. orthoceras*. Učestalost regeneracije izdanaka, u zavisnosti od genotipa je 1,0-12,5%. Na ovim eksplantatima je uočeno višestruko formiranje izdanaka.

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