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Efficiency of selection–biotechnological system of selection for creation of breeding source material of sunflower resistant to herbicides and broomrape

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Abstract: The sunflower is a strategically important oil crop. Every year the area under this crop grows, and the rapid returning of sunflowers back to the fields provokes the formation of new more aggressive races of broomrape (*Orobanche cumana* Wallr.). Broomrape is a parasite that interferes with the normal development of sunflower and can lead to significant crop losses. For creating a sunflower hybrid (F_1) it is needed to cross the parental components, which have a complex of important traits, among which there is a resistance to the herbicides and a broomrape. Considering that the creation of each of the components of the hybrid involves many years of painstaking work in the breeding process, modern approaches and methods are used to accelerate the creation of a new source material. Thus, using the technology of cultivating immature embryos *in vitro* culture, it is possible to reduce the time to create lines resistant to herbicides, for example. And during selection for resistance to pathogenic organisms, testing is most often used against an artificial infectious background, both in the field and in laboratory conditions, in order to differentiate the material on this basis. The **aim** of this work was to establish the effectiveness system when creating an initial breeding material resistant to herbicides and broomrape. As a

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result of testing the lines on an artificial infectious background, was identified plants which have high resistance to the G-race broomrape and were isolated from hybrid combinations resistant to tribenuron-methyl and imidazolinones. Thus, among the analyzed plants which are resistant to tribenuron-methyl, four lines were isolated, which are highly resistant to the G-race broomrape from a hybrid combinations BH0118/SURES–2 (101/1, 101/4, 101/6, 101/7), and BH0318/SURES–2 (101/21, 101/24, 101/28, 101/30), and five lines (101/11, 101/12, 101/16, 101/17, 101/18) from a hybrid combination BH0218/SURES–2. Among imidazolinone-resistant sunflower lines – line 3 was isolated as highly resistant to the G-race broomrape.

Keywords: broomrape; *Helianthus annuus* L.; *in vitro* culture; *Orobanche cumana* Wallr.; resistance; sunflower.

Introduction

Sunflower (*Helianthus annuus* L.) is the second most important crop in the world, after corn, the cultivation of which is based on the use of the phenomenon of cytoplasmic male sterility (CMS) (Seiler et al. 2017). This system includes the creation of three main components: sterile line (S *rfrf*), maintainer (N *rfrf*), and restorer line (N *RfRf*) (Letian and Yao-Guang 2014). Today, the needs of production are increasing, and it is necessary to have genotypes with a set of traits, such as: resistance to herbicides (imidazolinone or sulfonylurea group), resistance to drought and the most harmful pathogens, resistance to broomrape (*Orobanche cumana* Wallr.). The current need for sunflower production are hybrids resistant to herbicides and broomrape, especially in the southern part of Ukraine.

Taking into account the duration of the creation of a highly productive sunflower hybrid (about 12 years), various techniques and methods are increasingly involved in breeding programs that make it possible to accelerate the creation of the initial breeding material. Such techniques include: methods of molecular biology (molecular marker, marker-assisted breeding), biotechnological methods (culture of immature embryos, culture of cells and tissues *in vitro*), assessment of resistance to pathogens (artificial infectious background).

The culture of immature embryos *in vitro* is successfully used in the study of somatic embryogenesis, organogenesis, regeneration and genetic transformation of sunflower (Finer 1987; Lucas et al. 2000; Malone-Schoneberg et al. 1994; Montathong et al. 2019; Soroka and Lyakh 2009). Also, the use of a culture of immature sunflower embryos *in vitro* is an effective method of accelerating the breeding process, due to obtaining two or three generations of sunflower per year.

Considering that with the use of a culture of immature embryos becomes possible the accelerated reproduction, homozygous by obtaining several

generations per year (Dagustu et al. 2010, 2012; Nenova et al. 2014), the use of this technique is relevant.

The culture of immature embryos began to be used in the 80s in order to solve such issues as the creation of interspecific hybrids, accelerating of the selection process, studying somatic embryogenesis, organogenesis, regeneration and genetic transformation in sunflower (Chandler and 1983; Finer 1987; Friedt 1992; Malone-Schoneberg et al. 1994; Marin 2000; Montathong et al. 2019). Considering that the limiting factor in selection is time, the use of immature embryo culture is one of the effective tools for quickly isolating lines with desirable traits. Therefore, after our study with lines and hybrid combinations of sunflower (Babych et al. 2020a,b) with a culture of immature embryos *in vitro*, the next stage was to assess this material against an infectious background in laboratory conditions.

Also, at present, an urgent need for production is the cultivation of hybrids that are resistant to the flower parasite – broomrape. The broomrape is common in all countries that grow sunflower as the major oilseed crop among the production crops in large areas, especially in central and eastern Europe, Spain, Turkey, Kazakhstan and China (Iuoras et al. 2004).

The work to study and use QTL, RFLP, RAPD, and SSR molecular markers is actively carried out to identify broomrape resistance genes (Dimitrijevic and Horn 2018; Imerovski et al. 2012, 2015; Louarn et al. 2016; Molinero-Ruiz 2014).

The breeding for resistance to pathogenic organisms has its own specific features. The creation of lines resistant to the broomrape includes several stages:

1) selection and crossing of a genotype with a complex of valuable traits with a resistance donor, which includes the *Or*-gene (or genes) to create a new source material;
2) assessing the resistance of the lines to broomrape on an artificial infectious background;
3) assessment of the resistance of hybrid combinations to broomrape against an infectious background, in order to select the best, with the corresponding characteristics (yield, plasticity, stability, resistance to herbicides, disease resistance, resistance to broomrape, high oil content in seeds and oil yield per hectare);
4) analysis of the inheritance of the trait in the F_1 hybrids, in order to isolate lines-donors of broomrape resistance (Dimitrijevic and Horn 2018).

Since the publication of the results of the laboratory vegetation method for selection of broomrape-resistant lines in a greenhouse by Panchenko A. in 1975 (Panchenko 1975), this method has been repeatedly modified (Grezes-Besset 1994; Labrousse 2004), but now it is successfully used and considered as one of the most effective methods.

In addition, it significantly speeds up the breeding process. Antonova et al. (2017) showed how throughout 1.5 years it is possible to create and isolate genotypes with full resistance to *O. cumana* using an artificial infectious background in a greenhouse, as well as using Biotron–5 cameras (Antonova et al. 2017). The determined features of genetic control of broomrape resistance allow to direct the breeding process, which saves time for creating the initial material in sunflower breeding. So, as a result of Velasco's et al. (2011) hybrid combinations analysis of Bc_1F_1, Bc_1F_2, Bc_1F_3, Bc_2F_1, Bc_2F_2 and Bc_2F_3 by crossing of sunflower lines susceptible to the most virulent race of broomrape (*G*) with a resistant wild form of *Helianthus debilis* on an artificial infectious background for five years (2005–2010), it was found that the trait has monogenic control and dominant inheritance (Velasco 2011).

These studies confirm that the use of molecular genetics and laboratory vegetation methods is currently an effective tool for isolating genotypes for breeding of sunflower hybrids resistant to herbicides and broomrape.

Therefore, we took the stage of testing of the selected lines for valuable traits (for example, resistance to herbicides, regenerative ability of lines, etc.) and for resistance to the broomrape on an artificial infectious background, as one of the steps to develop an effective system for selecting the source material of sunflower. The **aim** of this work is to evaluate the fertility restorers of sunflower, which was in previously used in the study of regenerative capacity (lines resistant to imidazolinones) and fertility restorers, which were isolated using a biotechnological method (culture of immature embryos) for resistance to tribenuron-methyl.

Materials and methods

During the development of an integrated system for the selection of the source material of sunflower resistant to herbicides and broomrape, was evaluated a selection material, which was used at the stage of sunflower lines regenerative ability research (Babych et al. 2020a), and on the basis of these researches the effective system, which was further used for accelerated receipt of selection material with the improved and valuable economic signs, was developed. Also, the material was used in the development of an effective system for selecting the source of sunflower material resistant to tribenuron-methyl using the method of culture of immature embryos *in vitro* (Babych et al. 2020b).

Plant material

As a source material was used the breeding material – sunflower restorer lines (I_8) of the Ukrainian Scientific Institute of Plant Breeding (VNIS). These lines were chosen because they have a number of valuable traits:

a) line 2 – mid–early (vegetation period is 110 days), with the resistant to diseases such as phomopsis, phomosis, sclerotinia, coal rot, downy mildew and alternaria, it is resistant to the herbicides of the imidazoline group;
b) line 3 – mid–early (vegetation period is 110 days), with the resistant to herbicides of the imidazoline group;
c) line 19 – early (vegetation period is 100 days), with the resistant to herbicides of the imidazoline group;
d) line 35 – late maturity (vegetation period is 120 days), with the resistant to herbicides of the imidazoline group, resistance to the downy mildew, and alternaria.
e) BN0118 – early (vegetation period is 100 days), undersized (156 cm), with resistance to downy mildew, and to the F-race broomrape, linoleic type, with a high combinational capacity by yield, intended for classical cultivation technology;
f) BN0218 – mid–early (vegetation period is 110 days), with resistance to the F-race broomrape, linoleic type, intended for classical cultivation technology;
g) BN0318 – really late (vegetation period is 120 days), tall (187 cm), linoleic type, with a high combinational capacity by yield, intended for classical cultivation technology, with resistance to downy mildew and to the F-race broomrape;
h) SURES–2 line (PI633750) – obtained from the USA genetic bank GRIN (the Germplasm Resource Information Network) (Accessed 2001).

Regenerative capacity of sunflower lines

In a study of lines resistant to imidazolinones for regeneration capacity, it was found that line 35 has a high regenerative capacity. In total, four sunflower lines were involved in the study of regenerative capacity by direct organogenesis *in vitro*: 2, 3, 19 and 35. Seeds of immature embryos selected on day 21 after pollination were used for *in vitro* culture. The cotyledons were used as explants.

This study included the induction of adventitious shoots, elongation of adventitious shoots, rooting of regenerated plants and adaptation of regenerated plants under the greenhouse conditions.

Induction of adventitious shoots: During the induction of adventitious shoots was used macro- and micronutrients Murashige-Skuga medium (MS) (Murashige and Skoog 1962), supplemented with vitamins B_5 (Gamborg et al. 1968), 3% sucrose, and also 5 mg/L $AgNO_3$. The basic environment was supplemented by different concentration of phytohormones. Induction of morphogenesis was performed in two ways: explants were cultured without and with the light for 21 days at 25 °C.

Elongation of adventitious shoots: Elongation of adventitious shoots was performed on: MS medium, with vitamins B_5, 3% sucrose, 5 mg/L $AgNO_3$, and also with different concentration of cytokinins and gibberellin (10–12 days).

Rooting and plants adaptation: For root induction, was used macro- and microelements of MS medium, vitamins B5, 2% sucrose, with the addition of auxin 1 mg/L, indole-3-buteric acid (IBA). Regenerating plants were adapted in a greenhouse with a photoperiod of 16/8 and a temperature of 25 °C.

As a result of the study, optimal cultivation conditions were selected to obtain the maximum frequency of sunflower regenerates and an effective rooting system of adventive shoots was developed, which makes it possible to adapt regenerated plants to septic conditions (Babych et al. 2020a).

Identification of the sunflower lines resistant to tribenuron-methyl with using *in vitro* of immature embryos culture

The study began in summer 2017 with the crossing of fertility restorer lines BH0118, BH0218, and BH0318 with the tribenuron-methyl resistance donor SURES–2 (TBM gene – resistance AHASL1–2). After 21 days, immature embryos were removed from each basket of combinations (SURES–2/BH0118, SURES–2/BH0218, SURES–2/BH0318) – 30 seeds per each, which were introduced into *in vitro* culture (Babych et al. 2020b). As a result of cultivation in aseptic conditions *in vitro*, 25–27 plants passed to the stage of plant adaptation in a greenhouse. In total, about 100 days passed from the moment of selection of 21-day-old immature embryos to technical maturity, which was observed in the greenhouse after adaptation of plants obtained in *in vitro* culture. We were observed that the flowering was not simultaneous. All plants were isolated for self-pollination and I_1 seeds were obtained.

In spring 2018, I_1 seeds, obtained from these hybrid combinations, were sown in a field and treated with a selective agent (tribenuron-methyl). As a result of herbicide treatment, plants that were resistant to tribenuron-methyl were isolated for self-pollination. Later, in July 2018, 21-day-old immature embryos were selected from self-pollination plants resistant to tribenuron-methyl and reintroduced into *in vitro* culture for another cycle.

In 2019, after repeated treatment with a selective agent, 10 lines, homozygous by the resistance to tribenuron-methyl were isolated for each hybrid combinations (Babych et al. 2020b). They became the object for further improvement and selection of lines resistant to tribenuron-methyl and broomrape.

Testing of the broomrape resistance

Testing of these lines was carried out in the department of Disease and pest plants' immunity of VNIS.

To assess the breeding material of sunflower, the seeds of broomrape were collected in the phase of physiological ripeness of the host plant in Zaporizhia, Kharkiv, Kirovograd, Odessa, Donetsk, Lugansk and Kherson regions. The collected broomrape seeds were sieved in order to separate dry plant residues. Seeds of sunflower samples were sown in pots with infected peat composition (5 L of peat (=1 kg 300 g), 2 kg of sand, 2 g of broomrape seeds).

After 30–35 days, the sunflower plants were carefully removed from the peat composition, and kept records of availability of broomrape tubers. Accounting was performed visually – to determine the presence or absence of broomrape tubers on each of the tested plants.

Limagrain company hybrids were used as the standards (St), namely: LG 50505 (resistant to the G-race broomrape) – resistance standard (St R "resistance"), and LG 5665 (resistant to E-race broomrape) – susceptibility standard (St S "Susceptible").

Results and discussions

In the early stages, the work on the development of a comprehensive system for the selection of material of sunflower resistant to herbicides and broomrape included the study of the regenerative capacity of sunflower lines (Babych et al. 2020a) and accelerated creating of sunflower materials resistant to tribenuron-methyl with using the method of culture of immature embryos *in vitro* (Babych et al. 2020b), and the next stage was the evaluation of the selected source material for the resistance to broomrape on an artificial infectious background (Figure 1).

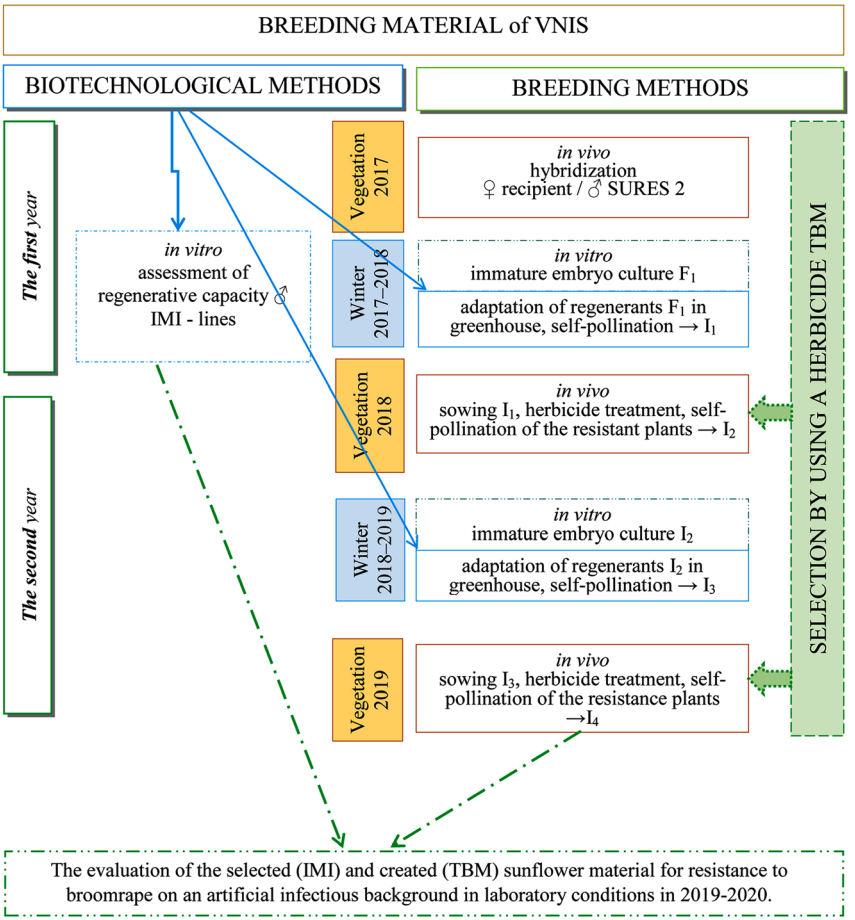

Figure 1: An integrated selection–biotechnological system for the creation of herbicide and broomrape resistant source material of sunflower.

During the study of lines resistant to imidazolinones for their regenerative capacity, which included the induction of adventitious shoots, elongation of adventitious shoots, rooting of regenerated plants and adaptation of regenerated plants under greenhouse conditions, line 35 was isolated by its high regenerative capacity. As a result of the study, optimal cultivation conditions were selected to obtain the maximum frequency of sunflower regenerate and an effective rooting system of adventive shoots was developed, which makes it possible to adapt regenerated plants to septic conditions (Babych et al. 2020a).

The next step in the development of a system for the selection of the breeding material of sunflower resistant to herbicides and broomrape was a combine of the biotechnological method (immature embryos culture) with classical methods of breeding (crossing, homozygosis) and selection of resistant plants under the action of a selective agent (herbicide of the sulfonylurea group).

The study began in the summer of 2017 by crossing the lines of fertility restorers BH0118, BH0218, and BH0318 with the tribenuron-methyl resistance donor methyl SURES–2 (TBM gene – resistance AHASL1–2). After 21 days, immature embryos were removed from each basket of combinations (SURES–2/BH0118, SURES–2/BH0218, SURES–2/BH0318), which were introduced into *in vitro* culture (Babych et al. 2020b).

In the spring of 2018, I_1 seeds obtained from these hybrid combinations were sown in a field and treated with a selective agent (tribenuron-methyl). As a result of herbicide treatment, plants that were resistant to tribenuron-methyl were isolated for self-pollination. Later, in July 2018, 21-day-old immature embryos were selected from self-pollination plants resistant to tribenuron-methyl and reintroduced into *in vitro* culture for another cycle.

In 2019, after repeated treatment with a selective agent, 10 lines homozygous for resistance to tribenuron-methyl were isolated for each hybrid combinations (Babych et al. 2020b). They became the object for further improvement and selection of lines resistant to tribenuron-methyl and broomrape.

Therefore, the breeding material – lines 2, 3, 19, 35, are resistant to imidazolinones, differentiated by their regenerative capacity (Babych et al. 2020a), and the lines created during 2017–2019 using the culture of immature embryos *in vitro*, with hybrid combinations resistant to tribenuron-methyl (Babych et al. 2020b), were assessed for resistance to broomrape on an artificial infectious background in laboratory conditions in the winter period of 2019–2020. The results of the study are presented in Table 1.

As a result of evaluation of the lines obtained from hybrid combinations received by crossing with donor resistance to tribenuron-methyl, by using the laboratory-vegetation method, the high-resistant to the G-race of broomrape lines were identified (because the formation of broomrape tubers was not observed on these samples). Thus, from the hybrid combination BH0118/SURES–2 were

Table 1: Resistance to the broomrape of the source breeding material created by biotechnological methods in a complex system of selection of herbicide-resistant genotypes, laboratory-vegetation method of A. Panchenko.

Pedigree	Code number	Replications	Quantity of plants	Quantity of susceptible plants		Quantity of resistant plants	
				Plants	%	Plants	%
Hybrid combinations resistant to tribenuron-methyl							
BH0118 × SURES-2	101/1	1	10	0	0.0	10	100.0
		2	10	0	0.0	10	100.0
	101/2	1	6	6	100.0	0	0.0
		2	9	9	100.0	0	0.0
	101/3	1	5	5	100.0	0	0.0
		2	8	8	100.0	0	0.0
	101/4	1	10	0	0.0	10	100.0
		2	10	0	0.0	10	100.0
	101/5	1	10	1	10.0	9	90.0
		2	6	1	16.7	5	83.3
	101/6	1	10	0	0.0	10	100.0
		2	10	0	0.0	10	100.0
	101/7	1	9	0	0.0	9	100.0
		2	9	0	0.0	9	100.0
	101/8	1	10	10	100.0	0	0.0
		2	9	9	100.0	0	0.0
	101/9	1	8	8	100.0	0	0.0
		2	6	6	100.0	0	0.0
	101/10	1	10	1	10.0	9	90.0
		2	10	2	20.0	8	80.0
	Total amount		175	66	37.7	109	62.3
BH0218 × SURES-2	101/11	1	7	0	0.0	7	100.0
		2	5	0	0.0	5	100.0
	101/12	1	7	0	0.0	7	100.0
		2	5	0	0.0	5	100.0
	101/13	1	10	10	100.0	0	0.0
		2	9	9	100.0	0	0.0
	101/14	1	9	9	100.0	0	0.0
		2	10	10	100.0	0	0.0
	101/15	1	6	6	100.0	0	0.0
		2	9	9	100.0	0	0.0
	101/16	1	3	0	0.0	3	100.0
		2	5	0	0.0	5	100.0
	101/17	1	10	0	0.0	10	100.0
		2	10	0	0.0	10	100.0

Table 1: (continued)

Pedigree	Code number	Replications	Quantity of plants	Quantity of susceptible plants		Quantity of resistant plants	
				Plants	%	Plants	%
	101/18	1	10	0	0.0	10	100.0
		2	10	0	0.0	10	100.0
	101/19	1	11	10	90.9	1	9.1
		2	10	9	90.0	1	10.0
	101/20	1	8	8	100.0	0	0.0
		2	7	7	100.0	0	0.0
	Total amount		161	87	54.0	74	46.0
BH0318 × SURES-2	101/21	1	10	0	0.0	10	100.0
		2	10	0	0.0	10	100.0
	101/22	1	7	1	14.3	6	85.7
		2	6	1	16.7	5	83.3
	101/23	1	8	1	12.5	7	87.5
		2	10	4	40.0	6	60.0
	101/24	1	8	0	0.0	8	100.0
		2	7	0	0.0	7	100.0
	101/25	1	6	6	100.0	0	0.0
		2	8	7	87.5	1	12.5
	101/26	1	7	4	57.1	3	42.9
		2	6	6	100.0	0	0.0
	101/27	1	10	1	10.0	9	90.0
		2	9	1	11.1	8	88.9
	101/28	1	6	0	0.0	6	100.0
		2	7	0	0.0	7	100.0
	101/29	1	9	2	22.2	7	77.8
		2	10	3	30.0	7	70.0
	101/30	1	10	0	0.0	10	100.0
		2	8	0	0.0	8	100.0
	Total amount		162	37	22.8	125	77.2
Lines resistant to herbicides of imidazoline group							
2	L1/1	1	10	0	0	10	100
		2	10	0	0	10	100
	L1/2	1	10	1	10	9	90
		2	10	2	20	8	80
	Total amount		40	3	7.5	37	93
35	L1/3	1	10	10	100	0	0
		2	10	9	90	1	10
	L1/4	1	10	4	40	6	60

Table 1: (continued)

Pedigree	Code number	Replications	Quantity of plants	Quantity of susceptible plants		Quantity of resistant plants	
				Plants	%	Plants	%
		2	7	4	57	3	43
	Total amount		37	27	73	10	27
3	L1/5	1	10	0	0	10	100
		2	9	0	0	9	100
	L1/6	1	10	0	0	10	100
		2	10	0	0	10	100
	Total amount		39	0	0	39	100
19	L1/7	2	10	3	30	7	70
		1	9	2	22	7	78
	L1/8	1	10	3	30	7	70
		2	10	1	10	9	90
	Total amount		39	9	23	30	77
Standart							
LG 50505 (St R)	ST1	1	10	0	0	10	100
	ST2	2	10	0	0	10	100
	Total amount		20	0	0	20	100
LG 5665 (St S)	ST3	1	10	10	100	0	0
	ST4	2	10	10	100	0	0
	Total amount		20	20	100	0	0

isolated four lines (101/1, 101/4, 101/6, 101/7); from the hybrid combination BH0218/SURES–2 – five lines (101/11, 101/12, 101/16, 101/17, 101/18); and four lines (101/21, 101/24, 101/28, 101/30) were isolated from the hybrid combination BH0318/SURES–2.

Among the sunflower lines resistant to the imidazolinone group of herbicides (2, 3, 19, 35), the line 3 was identified as highly resistant (no signs of parasite infestation were found in 100.0% of plants) to the G-race broomrape. The isolated by us lines resistant to the G-race of broomrape in the future can be used as donors of resistance to this parasite.

In the work of Antonov et al. (2017) it is shown an effective assessment of resistance to broomrape on an artificial infectious background to obtain lines constant in resistance to the G-race of broomrape for a short period of time (1.5 years).

Considering that the laboratory-vegetation method of selection of sunflower lines resistant to the broomrape in the greenhouse was developed in 1975 by A.

Panchenko, it is still successfully used at present, which confirms our study. And the combination of testing lines on an artificial infectious background in a comprehensive selection system for the created source material is a good tool for sunflower breeding.

However, at present, more and more researches are aimed at studying the mechanism and interaction of sunflower with the flowering parasite (Martín-Sanz et al. 2020). Thus, in the work of Martín-Sanz et al. (2020) is shown the mechanism of stability of the broomrape in the inbred line (PHSC1102), a mapping of the population obtained by crossing a stable line with an unstable one was performed. Thus, this line can also act as a donor of resistance to the broomrape, and according to the laboratory-vegetation method of selection of broomrape-resistant sunflower lines in the greenhouse – it could be possible to significantly speed up the selection process.

However, the most effective method of isolating plants with broomrape resistant will be to assess resistance to broomrape on an artificial infectious background.

Conclusion

In the course of research by combination of biotechnological methods (assessment of regenerative capacity, immature embryos culture) with classical breeding methods (crossing, homozygous, selection of herbicide-resistant plants, namely, herbicide sulfonylurea group) and phytopathogenic (broomrape) breeding – the integrated selection-biotechnological system was developed.

The effectiveness of the integrated selection–biotechnological system for the created breeding material is the halving of time required to isolate sunflower lines with herbicide and broomrape resistance, by combining biotechnological methods with classical breeding methods.

Among the analyzed plants from each hybrid combination, which are resistant to tribenuron-methyl, four lines were isolated, which are highly resistant to the G-race broomrape BH0118/SURES–2 (101/1, 101/4, 101/6, 101/7), and BH0318/SURES–2 (101/21, 101/24, 101/28, 101/30), and five lines (101/11, 101/12, 101/16, 101/17, 101/18) with a hybrid combination BH0218/SURES–2.

Among imidazolinone-resistant sunflower lines, differentiated by regenerative capacity, according to the results of an assessment on an artificial infectious background in laboratory conditions, line 3 was isolated as highly resistant (no signs of parasite infestation were found in 100.0% of plants) to the G-race broomrape.

Thus, when using the integrated selection–biotechnological system, the lines of sunflower resistant to herbicides and broomrape were isolated out of source breeding material.

The lines are recommended for further use in breeding programs to create high-yielding herbicides and broomrape resistant hybrids.

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