Study on an *in vitro* screening test for resistance to *Sclerotinia sclerotiorum* in sunflower

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ABSTRACT

An *in vitro* method that assayed callus induction on a medium amended with culture filtrate of *Sclerotinia sclerotiorum* was evaluated. Four double haploid R-lines obtained through the method of gamma-induced parthenogenesis at Dobroudja Agricultural Institute were involved (DH-R-128, DH-R-116, DH-R-7 and DH-R-2). The experiment was carried out at two levels, under field and laboratory conditions. After field infection, lines DH-R-128 and DH-R-116 demonstrated high to moderate resistance. Under laboratory conditions, *S. sclerotiorum* filtrate was added to the nutrition medium for callus induction from sunflower hypocotyl explants. Three variants of filtrate concentration in the nutrition medium were tested. The callus induction reaction of *Helianthus annuus* L. explants cultivated on a medium amended with *S. sclerotiorum* filtrate was evaluated. It was established that the higher filtrate concentrations suppressed the reaction of the explants to various degrees for the different lines. In lines DH-R-116 and DH-R-128 a better callus induction reaction was observed in comparison to the other two lines. The results showed that the test for resistance to *S. sclerotiorum* based on the callus induction allowed to identify materials with high to moderate resistance to the pathogen. The test cannot distinguish the differences between high and moderate levels in resistance and in susceptibility.

Key words: callus induction – *in vitro* test – resistance – *Sclerotinia sclerotiorum* – sunflower

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is a very important oil seed crop worldwide and is a main source of vegetable oil in Bulgaria. Multiple factors determine the productivity of sunflower hybrids and varieties. In this respect, a significant effect is brought about by the causal agents of different diseases such as sclerotinia (*Sclerotinia sclerotiorum),* downy mildew (*Plasmopara helianthi)*, phoma (*Phoma macdonaldii)*, and phomopsis (*Phomopsis helianthi*). Each of these diseases can significantly decrease sunflower productivity, but *S. sclertiorum* (Lib.) de Bary is considered to be one of the most devastating pathogens distributed in almost all production regions. The losses may be up to 100% under suitable conditions (20 ^{o}C and 70% air humidity) in fields where the fungus is spread (Maširević and Gulya, 1992; Rashid, 1993). In spite of the efforts of many researchers, no chemical control on the spreading of the pathogen has been found yet (Nelson and Lamey, 2000). At this stage, the only way to reduce damage from a sclerotinia attack is by the development of forms with genetic resistance; this is a priority in a number of research projects and investigations (Ronicke et al., 2004, 2005). Simultaneously, alternative approaches have been sought for fast selection and screening of the new forms developed which have demonstrated some degree of resistance to the pathogen (Grezes-Besset et al., 1994; Verzea et al., 2004).

The aim of this investigation was to study the effect of the cultural filtrate from *S. sclertiorum* on the callus induction reaction of cultivated sunflower (*Helianthus annuus* L.) and to find out if there is a correlation between *in vitro* and *in vivo* reaction of the plant material to the pathogen.

MATERIALS AND METHODS

The investigation was carried out at two levels – under laboratory and field conditions. We worked with four doubled haploid R-lines developed by the method of gamma-induced parthenogenesis at Dobroudja Agricultural Institute, Bulgaria. The lines were of different origin: lines DH-R-116 and DH-R-128 were produced from hybrid materials with parental forms obtained as a result of interspecific hybridization; lines DH-R-2 and DH-R-7 were obtained from *H. annuus* L. hybrids.

Preparation of inoculum and inoculation of lines under field conditions

Ten plants from each of the studied lines were inoculated by the Straw-method (Encheva and Kiryakov, 2002) at stage 5-6th pair of leaves. A petiole of the fourth pair of leaves from each plant was cut, so that 3 cm of it was left on the stem. A plastic straw (30 x 6 mm) with one end closed was inserted in the place of

incision. The straw contained an agar disc from the periphery of a 3 day old culture of isolate Ss-1 on nutrient medium PDA at 22±1°C. The reaction of the plant was rated three times every 7 days according to a 6-degree scale as follows: 0 – no symptoms at the place of inoculation; 1 - a whitish spot on the petiole (high resistance); 2 – a spot at the base of the petiole reaching to the stem (resistance); 3 – a spot spreading on a part of the stem (intermediate resistance), 4 – a spot spreading on the entire stem (susceptibility); 5 – breaking of the stem (high susceptibility). The last rating of the plant's reaction determined the final evaluation of their resistance to the pathogen.

Laboratory methods

S. sclerotiorum isolate SsPh1 was cultivated on a liquid medium (PDB) for seven days at room temperature (22-25°C), then it was filtered by the method of Miklas et al. (1992) applied to bean. Following cold sterilization in a laminar box, the obtained filtrate was stored at +4° C within 7 days.

Twenty seeds from each line were sterilized in 2.5% solution of potassium hypochlorite for 20 minutes; after the seed coat was removed, the seeds were then plated on medium MS (Murashige and Skoog, 1962) for formation of young plantlets. After 7-9 days cultivation, hypocotyl explants were removed and transferred onto medium for callus induction. The medium consisted of MS + 1.5 mg/l NAA + 0.5 mg/l BAP amended with *S. sclerotiorum* cultural filtrate. Four variants were tested depending on the amount of added filtrate: variant 1 (control, without added filtrate) and variants with added filtrate in the following concentrations: 5 ml/l (variant 2); 10 ml/l (variant 3); and 20 ml/l (variant 4). The number of calli induced was evaluated after one month of cultivation.

The experiment was designed in 5 replications (5 Petri dishes per variant for each line). Cultivation of explants was done under controlled conditions: 25±1ºC, light 6000 lux and photoperiod 16/8 hr.

RESULTS AND DISCUSSION

Field evaluation

After infection under field conditions best results were observed in line DH-R-128, where the reaction of infected plants was within the range 0-3 (Table 1).

Table 1. Field evaluation of the reaction of double haploid R lines to artificial infection with *S. sclerotiorum* inoculum

Genotype	**Plant reaction to *Sclerotinia sclerotiorum***									
plant №	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
DH-R-128	3	1	1	0	1	0	0	2	0	1
DH-R-116	1	1	2	3	3	3	1	1	2	2
DH-R-2	2	4	4	3	2	4	3	4	3	4
DH-R-7	5	5	5	5	5	5	5	5	5	5

In contrast to line DH-R-128, all plants of line DH-R-7 were completely damaged by the pathogen. A moderate reaction was observed in the other two lines, inclining towards the moderate resistance margin in line DH-R-116 and towards the susceptibility margin in line DH-R-2. Taking the origin of the lines as a starting point, no definite conclusion about their reaction to the disease can be reached; however, the expression of this reaction can be analyzed generally. Emphasis is mainly placed on the wild species of genus *Helianthus* as a main source of resistance genes to both *S. sclerotiorum* and all diseases of sunflower of economic importance (Škorić, 1992; Cerboncini et al., 2002). This, to a great extent, confirmed the result we obtained in line DH-R-128, and partially, in line DH-R-116. Parallel to this, forms having a lower susceptibility to sclerotinia attack have been found in cultivated sunflower as well (Castano et al., 1993; Degener et al., 1999 a,b). In the present study, the line DH-R-2 can be referred to as being in this category.

Callus induction reaction

All DH-R-lines had normal processes of callus induction in the control (variant 1); line DH-R-128 showed a slightly weaker reaction than the rest of the lines (Table 2).

The higher concentrations of the filtrate prevented the callus induction reaction of the plants, the degree of suppression being different for the different lines. Regardless of the differences, the general trend of the reaction of the lines is expressed as a progressive decrease in callus initiation with the increase in the *S. sclerotiorum* filtrate concentration (Fig. 1).

Table 2. Callus induction in a culture of hypocotyls explants of *Helianthus annuus* L. on a medium amended with *Sclerotinia sclerotiorum* filtrate

Genotype		Number of plated explants							Number of calli obtained						
DH-R-116	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	28	32	31	30	31	**152**	**30.4**	28	32	31	30	31	**152**	**30.4**
	variant 2	30	29	20	40	34	**153**	**30.6**	30	29	20	37	30	**146**	**29.2**
	variant 3	32	31	29	29	33	**154**	**30.8**	32	31	26	18	30	**137**	**27.4**
	variant 4	32	16	43	32	41	**164**	**32.8**	22	7	9	15	11	**64**	**12.8**
DH-R-128	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	25	33	29	30	29	**146**	**29.2**	25	33	29	25	21	**133**	**26.6**
	variant 2	25	37	32	29	32	**155**	**31.0**	19	33	30	19	32	**133**	**26.6**
	variant 3	33	28	23	25	25	**133**	**26.6**	12	10	17	7	12	**58**	**11.6**
	variant 4	31	28	19	27	28	**133**	**26.6**	11	0	4	17	7	**39**	**7.8**
DH-R-2	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	24	28	16	20	25	**113**	**22.6**	24	26	14	20	23	**107**	**21.4**
	variant 2	20	22	25	21	24	**112**	**22.4**	2	9	11	6	7	**35**	**7.0**
	variant 3	29	21	25	30	26	**131**	**26.2**	0	0	0	0	15	**15**	**3.0**
	variant 4	30	25	22	20	33	**130**	**26.0**	0	0	0	0	0	**0**	**0**
DH-R-7	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	20	25	28	22	23	**118**	**23.6**	15	25	28	22	23	**113**	**22.6**
	variant 2	23	17	17	20	22	**99**	**19.8**	11	6	3	7	5	**32**	**6.4**
	variant 3	17	21	17	23	20	**98**	**19.6**	0	3	3	4	0	**10**	**2.0**
	variant 4	24	26	22	20	22	**114**	**22.8**	1	0	0	1	0	**2**	**0.4**

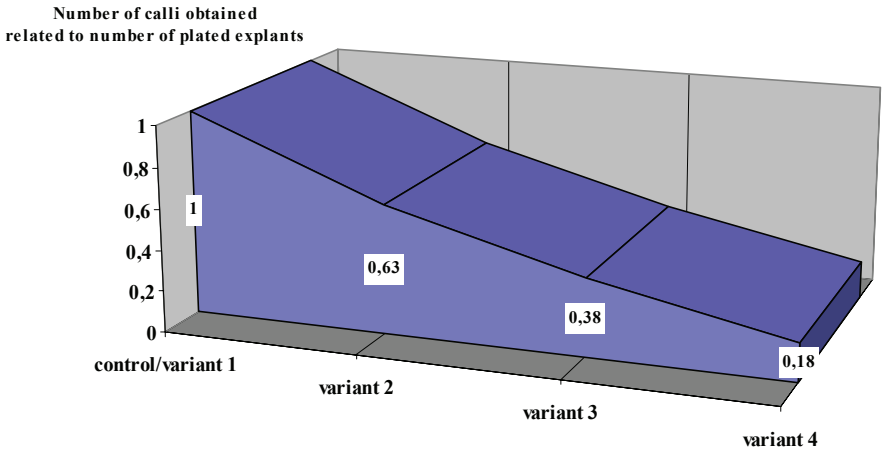

Fig. 1. General evaluation of the callus induction reaction on the investigated lines according to *S. sclerotiorum* filtrate concentration.

This trend was expressed to a higher degree in lines DH-R-2 and DH-R-7; in variants 3 and 4 their callus induction intensity was zero (Fig. 2).

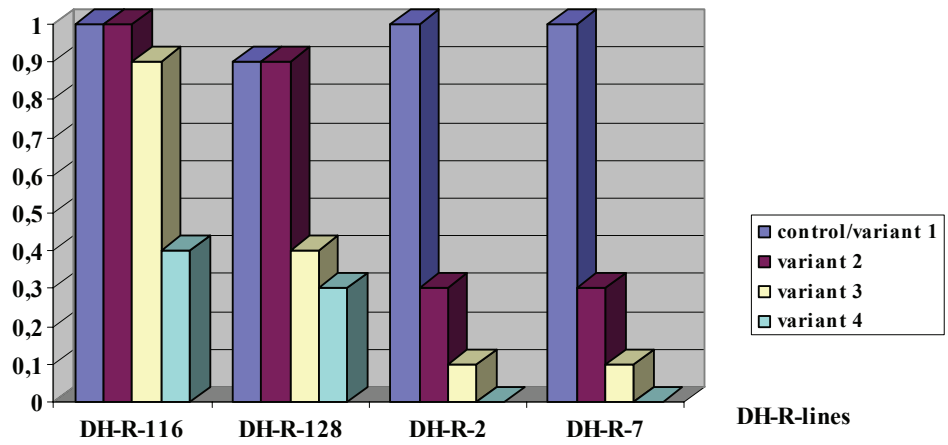

Fig. 2. Callus induction reaction of sunflower DH-R-lines according to the genotype and *S. sclerotiorum* filtrate concentration in the nutrient medium.

Differences were not observed in lines DH-R-116 and DH-R-128 between the control and variant 2. The callus induction in these lines was impeded by the higher concentrations of the filtrate in variants 3 and 4, but, in contrast to the other two investigated lines, callus initiation was not completely blocked.

The results from the *in vitro* investigation showed that line DH-R-116 had the best callus induction reaction in all four variants. Some similarity in the reactions was established for lines DH-R-116 and DH-R-128, with the exception of variant 3, where a sharp decrease in the callus genesis of line DH-R-128 was observed. This decrease was observed in variant 4 of line DH-R-116. In variant 4, the differences between the two lines were insignificant.

The comparison between the other two lines (DH-R-2 and DH-R-7) did not show any significant differences in the callus initiation of all four variants.

The comparison of the results from the field evaluation of the resistance to the *in vitro* reaction of the investigated lines revealed a certain correspondence expressed in the generalization that lines DH-R-128 and DH-R-116 showed resistance under field conditions and the best callus induction reaction, and lines DH-R-2 and DH-R-7 showed a susceptibility to *S. sclerotiorum* under field conditions and weaker in the *in vitro* reaction. By considering the results in detail, the fact comes out that although line DH-R-128 had the best evaluation in the field trial, it ranked second after line DH-R-116 under laboratory conditions. The lack of differences in the *in vitro* reaction of line DH-R-2, which had a relatively lower level of susceptibility to *S. sclerotiorum* under field conditions than line DH-R-7, also indicated that the laboratory test should most probably be more precise involving additional steps/variants between variant 3 and variant 4, so that the differences between the lines could be expressed.

CONCLUSIONS

The *in vitro* test for resistance to *S. sclerotiorum* based on the callus induction reaction is good enough to differentiate high to moderate resistant materials from materials with high to moderate susceptibility to the pathogen. The test cannot detect the differences between high and moderate levels in resistance and in susceptibility.

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