GENETIC VARIABILITY OF SOME ROMANIAN SUNFLOWER GENOTYPES UNDER *IN VITRO* STRESS INDUCED BY *PHOMOPSIS HELIANTHI* FILTRATE

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ABSTRACT

During 2002-2003 at A.R.D.I. Fundulea, a lot of experiments for *in vitro* testing and selection of some Romanian sunflower genotypes with tolerance to *Phomopsis helianthi* have been performed. Fourteen out of the 30 tested genotypes were selected for their good response to *in vitro* culture. As follows of the treatment applied on MS culture medium supplemented with 150 ml/l filtrate and on the basis of the results obtained regarding the leaf index, chlorophyll content, TKW, seed oil percentage and its composition, seven genotypes with increased resistance to this pathogen were selected. The determinations were performed by the Minolta Chlorophyll Meter (SPAD units) for chlorophyll content, RMN method for oil content and gaschromatography method (Shimadzu-GC-14B) for fatty acid content from oil. ANOVA for the leaf index revealed a very different behaviour of the tested lines, with significant positive or negative differences between genotypes, depending on both tolerance degree to disease and response to *in vitro* culture. Eight genotypes in which the leaf area was not diminished by the treatment as compared with control were identified. As regards the chlorophyll content, the average/variant diminished with 5.2 SPAD units compared to the control. In variant treated with filtrate,TKW drastically decreased, at six genotypes and the oil content strongly diminished at seven out of the 14 genotypes. The oleic acid content was reduced more in comparison with the control in all lines excepting the LC 4010 line.

Key words: chlorophyll content, leaf index, *Phomopsis helianthi* filtrate.

INTRODUCTION

Phomopsis helianthi, considered one of the most harmful pathogens of sunflower, can compromise the crop in favourable years of its development.

Genetic studies indicate that resistance to *Phomopsis helianthi* is polygenic (Tourvieille et al.,1988) or oligogenic (Skoric, 1985) and that additive genes action prevails (Vear et al., 1997).

The best protection against this pathogen is the tolerance and resistance of sunflower hybrids, but for this it is necessary to identify genes of resistance which can be inherited. Vrânceanu et al. (1983) reported that resistance to *Phomopsis helianthi* is controlled by a small number of genes with partial dominance and for obtaining resistant hybrids, both parental lines should have a certain level of resistance. Other authors affirm that resistance to *Phomopsis* is controlled by recessive genes, but depends on the number of genes which enter in interaction (Tourvieille and Vear, 1990).

Phenotypical resistance to *Phomopsis* was associated with „stay-green" of sunflower stems and there is a positive correlation with *Macrophomina phaseoli* and *Phoma macdonaldii*.

The wild species of sunflower are an important source of resistance for practical sunflower breeding, but this goal is very difficult due to the incompatibility between species.

In the last years several studies have been carried out in order to obtain plants with an increased level of disease resistance, using metabolites produced by the pathogen as selection agent applied on plant level (Masirevic et al., 1988).

For early selection a laboratory method is highly desirable because testing a large number of genotypes can easily be performed in limited space and time (Dozet and Vasic, 1995).

In 1994 a faster method for *in vitro* sunflower screening using *Phomopsis helianthi* filtrate and embryo culture was reported by Raducanu et al.

MATERIALS AND METHODS

For *in vitro* testing for resistance to *Phomopsis helianthi* pathogen, a number of 14 Romanian inbreed lines were used. As explants, immature embryos, 10 days after pollination were inoculate on MS medium, supplemented with 150 ml *Phomopsis helianthi* filtrate and incubated for 21 days at 27°C, 12/12 light/dark. After this period, phenotypical normal plants were transplanted in pots with a mixture of heavy soil and sand in 1:1 proportion and were grown under

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controlled conditions until technical maturity. On these plants in different stages of vegetation the following data were registered: leaf index, chlorophyll content,TKW, seed oil content and its composition .

The determinations have been performed by the Minolta Chlorophyll Meter (SPAD units) for chlorophyll content, RMN method for oil content and gascromatography method (Shimadzu-GC-14B) for fatty acids content from oil.

The fatty acids were analysed according to the conventional method (Schulte and Weber, 1989). The transesterification of triglycerides to fatty acid methyl esters was performed with trimethylsulfoniumhydroxid (TMSH). The capillary column (25 MX 0,32 MM ID) by 25 m lengths on a Shimadzu gaschromatography with flame ionization detector (FID) was used. Injector and temperature detector were kept at 270ºC and 280ºC, respectively. The carrier gas was the nitrogen, with a flow rate of 20 ml/min.

To calculate the total area of the peaks an electronic integrator was used. The area of each fatty acid peak was expressed as a percentage of the total area.

The leaf index was calculated by the following formula:

$$L \times l \times 0.66$$

(L = length; l = width; 0.66 = correction coefficient).

RESULTS AND DISCUSSION

ANOVA for leaf area revealed a very different behaviour of the studied lines at both control and treatment variants. A significant effect of genotypes and treatments as well as a significant interaction between genotypes and treatments was established (Table 1).

Taking into account that during the first vegetation stage (21 days), the plants were grown under *in vitro* conditions, their subsequent development under *in vivo* conditions, was very much affected. Depending on the genotype response to *in vitro* culture, ANOVA reveals significant positive or negative differences between genotypes. Thus, statistically ensured negative values vs. control average were registered to LC 4001, LC 4002, LC 4016, LC 4022 and LC 4023 lines. The LC 4010, LC 4019, LC 4020 and LC 4024 lines registered a very good response *in vitro* and with a normal development after their transfer into soil, with the leaf index values significant higher than average values of the control. As regards the influence of *Phomopsis* filtrate on this trait, eight genotypes were identified at which the leaf surface was not reduced as compared with the control. We notice the LC 4020 and LC 4024 lines at which the leaf area had significant positive values (62.566 cm^2, 49.866 cm^2 respectively).

Table 1. ANOVA regarding the leaf area of some Romanian sunflower genotypes after *Phomopsis helianthi* treatment

Source of variation	SS	DF	MS	F value
Genotype (A)	34198.51	13	2630.65	60.049***
A error	1139.02	26	43.808	-
Treatment (B)	2584.12	1	2584.12	67.034***
A x B	4020.47	13	309.267	8.022***
B error	1079.37	28	38.549	

For all tested genotypes the leaf area was reduced, on an average, with 3.821 cm^2 after the *Phomopsis* filtrate treatment (Table 2).

As regards the chlorophyll content parameter, at all tested genotypes of treated variant, the average/variant was reduced with 5.2 SPAD units comparing with the control. Also, the genotypes reaction to *in vitro* culture was emphasized for this trait. Thus, at control, the LC 4002 and LC 4023 lines have a chlorophyll content significantly reduced comparing with the treatment variant average (22.433 SPAD units at LC 4002 line and 22.866 SPAD units at LC 4023 line). F factor values demonstrates that the genotypes had a different reaction in the presence of *Phomopsis* filtrate depending on both genetic characteristics and tolerance degree to this pathogen (Table 3).

The LC 4023 line was very significantly affected at treatment variant, with a chlorophyll content of only 18.9 SPAD units. The LC 4011 and LC 4019 lines had a very good resistance to

Table 2. The effects of *Phomopsis helianthi* filtrate on leaf area of some Romanian sunflower genotypes

No.	Genotypes	Average leaf area (cm^2/genotype)			
		Control		Treatment	
		Average	Difference from average	Average	Difference from control
1	LC 4001	17.366	-16.125^{oo}	22.800	-10.691
2	LC 4002	12.933	-20.558^{ooo}	162.000	-17.291**
3	LC 4005	27.566	-5.925	25.466	-8.025
4	LC 4006	41.800	8.308	26.600	-6.891
5	LC 4007	23.866	9.625	14.100	19.391***
6	LC 4010	62.166	28.675^{ooo}	44.000	10.508
7	LC 4011	36.166	2.675	34.833	1.341
8	LC 4016	14.600	-18.891^{oo}	15.166	-18.325**
9	LC 4018	37.433	3.914	16.100	-17.391**
10	LC 4019	55.633	22.141^{ooo}	31.833	-1.658
11	LC 4020	110.666	77.175^{ooo}	62.566	29.075***
12	LC 4022	16.266	-17.225^{oo}	11.533	-21.958***
13	LC 4024	68.300	34.802^{ooo}	49.866	16.375***
14	LC 4025	21.766	-11.725^{o}	20.166	-13.325*
Average		39.037		35.216	

$^{o,oo,ooo)}$ Significantly different from average for P<0.05; P<0.01; P<0.001

*,**,***) Significantly different from control for P<0.05; P<0.01; P<0.001

Table 3. ANOVA regarding the chlorophyll content of some Romanian sunflower genotypes after *Phomopsis helianthi* filtrate treatment

Source of variation	SS	DF	MS	F value
Genotype (A)	3418.58	13	262.968	19.575***
A error	349.271	26	13.433	-
Treatment (B)	460.615	1	460.615	43.124***
A x B	593.004	13	45.615	4.271***
B error	299.075	28	10.681	

Table 4. The effect of *Phomopsis helianthi* filtrate on chlorophyll content of some Romanian sunflower genotypes

No.	Genotypes	Average chlorophyll content (SPAD/units)			
		Control		Treatment	
		Average	Difference from average	Average	Difference from control
1	LC 4001	24.500	-5.508	28.500	-1.508
2	LC 4002	22.433	-7.575	17.600	-12.408**
3	LC 4005	26.633	-3.375^{oo}	20.766	-9.241**
4	LC 4006	30.233	0.225	23.866	-6.141**
5	LC 4007	30.400	0.391^{ooo}	25.400	-4.608
6	LC 4010	35.800	5.791	36.000	-6.141**
7	LC 4011	35.800	5.791	39.866	9.858**
8	LC 4016	38.800	8.917^{oo}	31.933	4.925
9	LC 4018	42.733	12.725	31.566	1.558
10	LC 4019	42.866	12.725	36.800	6.792**
11	LC 4020	42.733	12.725	31.266	-1.258
12	LC 4022	26.166	-3.841^{ooo}	24.266	-5.741
13	LC 4024	38.500	8.492^{ooo}	20.800	-9.408**
14	LC 4025	22.866	-7.142^{o}	18.900	-11.108***
Average		32.890		27.680	

$^{o,oo,ooo)}$ Significantly different from average for P<0.05; P<0.01; P<0.001

*,**,***) Significantly different from control for P<0.05; P<0.01; P<0.001

Phomopsis with a more increased chlorophyll content, statistically ensured vs. control average (Table 4).

After the plants harvesting from the vegetation house, determinations of TKW and oil content from seed, were performed. For the TKW

parameter, F factor values statistically ensured for both genotype and treatment and interaction genotype x treatment, were obtained. ANOVA for TKW parameter reveales the genotypes LC 4022, LC 4005, LC 4006 and LC 4020 at which this trait registered very significantly positive values reported to the average obtained at control. At the treatment variant, TKW was significantly diminished at six genotypes: LC 4007, LC 4010, LC 4019, LC 4022, LC 4024 and LC 4023. At LC 4005 line, TKW was of 18.9 g, exceeding with 4.726 g the average value of treatment variant (12.833) (Tables 5 and 6).

Table 5. ANOVA regarding the TKW of some Romanian sunflower genotypes after *Phomopsis helianthi* filtrate treatment

Source of variation	SS	DF	MS	F value
Genotype (A)	819.046	13	63.003	21.632***
A error	75.726	26	2.912	-
Treatment (B)	150.934	1	150.934	117.678***
A x B	77.645	13	5.972	4.657***
B error	35.912	28	12.826	

In comparison with TKW, the oil content was drastically reduced at seven out of the 14 studied genotypes, at treatment variant. Very significant negative values were registered at the following genotypes: LC 4001, LC 4007, LC 4011 and LC 4016. For this trait, all genotypes responded at treatment by decreases of oil content excepting the LC 4019 and LC 4002 lines, at which the oil percentage had significant positive values comparing with control (Tables 7 and 8).

The analysis of standard solutions of derived fatty acids was done in order to determine the retention time useful for each peak identification, retention time which was equal with seven minutes (Table 9).

The results obtained on the basis of analyses by gas chromatograph are presented in table 10.

Based on these analyses, LC 4022 line with an increased content of oleic acid in control variant (78.96%), was emphasized. At treated variant, although the content was reduced, it is however placed over the oleic acid content of all genotypes from the control variant.

Table 6. The effect of *Phomopsis helianthi* filtrates on TKW of some Romanian sunflower genotypes

No.	Genotypes	Average TKW (g)			
		Control		Treatment	
		Average	Difference from average	Average	Difference from control
1	LC 4001	13.766	-0.408	13.533	-0.640
2	LC 4002	18.733	4.559^{000}	18.366	4.192**
3	LC 4005	20.966	6.792^{000}	18.900	4.726***
4	LC 4006	21.223	7.059^{000}	15.600	1.426
5	LC 4007	12.300	-1.874	10.366	-3.807**
6	LC 4010	16.800	2.626^{0}	10.600	-3.574**
7	LC 4011	17.566	3.392^{00}	15.466	1.292
8	LC 4016	14.666	0.492	13.033	-1.106
9	LC 4018	14.000	-0.174	10.933	-3.240**
10	LC 4019	14.033	-0.140	10.466	-3.707
11	LC 4020	18.933	4.759^{000}	12.933	-1.240
12	LC 4022	12.700	-1.474	11.100	-3.074**
13	LC 4024	11.700	-2.474^{0}	9.400	-4.774***
14	LC 4025	9.700	-4.474^{000}	8.966	-5.207***
Average					

^{0},^{00},^{000}) Significantly different from average for P<0.05; P<0.01; P<0.001

*,**,***) Significantly different from control for P<0.05; P<0.01; P<0.001

Table 7. ANOVA regarding the oil content of some Romanian sunflower genotypes after *Phomopsis helianthi* filtrate treatment

Source of variation	SS	DF	MS	F value
Genotypes (A)	1302.19	13	100.169	48.209***
A error	54.022	26	20.778	-
Treatment (B)	516.031	1	516.031	160.344***
A x B	195.958	13	15.073	4.684***
B error	90.111	28	3.218	

Table 8. The effect of *Phomopsis helianthi* filtrate on the oil content of some Romanian sunflower genotypes

No.	Genotypes	Average oil content (%)			
		Control		Treatment	
		Average	Difference from average	Average	Difference from control
1	LC 4001	29.333	-4.972°°	27.200	-7.104***
2	LC 4002	37.700	3.395°	27.600	6.671***
3	LC 4005	39.266	4.961°°	33.633	0.671
4	LC 4006	39.200	4.895°°	36.333	2.028
5	LC 4007	27.200	-7.104°°	29.033	-5.271***
6	LC 4010	41.900	7.595°°	36.000	1.695
7	LC 4011	29.933	-4.371°°	27.366	-6.938***
8	LC 4016	36.233	1.928	28.433	-5.871***
9	LC 4018	38.033	3.728°°	33.333	1.005
10	LC 4019	41.100	7.795°°	38.500	4.195*
11	LC 4020	43.866	9.561°°	35.400	1.095
12	LC 4022	38.866	4.561°°	30.000	-4.305*
13	LC 4024	33.700	-0.538	30.133	-4.171*
14	LC 4025	37.566	3.261°	34.033	-2.272
Average		36.706		31.928	

°; °°; °°°) Significantly different from average for P<0.05; P<0.01; P<0.001

*,**,***) Significantly different from control for P<0.05; P<0.01; P<0.001

Table 9 . Retention time for fatty acids from the standard solution

No. peak	Retention time (min.)	Fatty acid (formula)	The fatty acid
1	12.55	C 16 : 0	Palmitic
2	17.48	C 18 : 0	Stearic
3	17.48	C 18 : 1 trans	Oleic
4	20.36	C 18 : 2	Linoleic
5	22.65	C 18 : 3	Linolenic

Table 10. Fatty acids content of 14 sunflower genotypes

Genotype	Palmitic acid C16 : 0	Stearic acid C18 : 0	Oleic acid C18 : 1	Linoleic acid C18 : 2	Linolenic acid C18 : 3
LC 4001 C*)	5.42	5.43	42.37	45.60	n.d.
LC 4001 Ph*)	8.61	2.79	35.75	51.02	n.d.
LC 4002 C	7.63	2.34	42.13	42.18	
LC 4002 Ph	7.10	3.15	39.37	45.42	
LC 4005 C	6.25	4.27	30.87	53.30	1.08
LC 4005 Ph	6.99	5.53	31.60	50.10	0.69
LC 4006 C	6.18	n.d	36.79	52.50	n.d.
LC 4006 Ph	6.20	4.23	34.69	50.63	0.78
LC 4007 C	9.10	4.02	51.50	35.37	
LC 4007 Ph	9.20	n.d	50.02	34.98	
LC 4010 C	7.50	3.20	57.38	31.92	
LC 4010 Ph	7.35	3.50	58.14	30.12	
LC 4011 C	7.33	3.20	54.70	32.85	
LC 4011 Ph	7.43	4.44	50.92	34.51	
LC 4016 C	6.64	3.23	29.22	56.11	0.63
LC 4016 Ph	8.86	5.19	28.64	54.38	
LC 4018 C	5.44	3.98	50.58	34.06	
LC 4018 Ph	6.79	2.87	46.87	41.95	
LC 4019 C	7.33	3.20	54.70	32.85	
LC 4019 Ph	7.62	3.22	47.56	38.56	
LC 4020 C	6.11	3.92	46.83	41.17	
LC 4020 Ph	5.67	4.84	36.93	45.61	
LC 4022 C	6.85	4.43	78.96	6.98	
LC 4022 Ph	6.12	3.56	69.75	1.63	
LC 4024 C	5.71	4.67	66.51	2.10	
LC 4024 Ph	5.65	6.27	44.59	3.77	
LC 4023 C	6.18	3.14	39.66	5.02	
LC 4023 Ph	6.85	4.43	36.79	5.50	

*) C = control; Ph = treated variant

At all the other genotypes, excepting the LC 4010 line, the oleic acid content decreases after the treatment in favour of increasing of linoleic acid content. Excepting three lines (LC 4005, LC 4006 and LC 4016), the presence of linolenic acid was not identified.

CONCLUSIONS

ANOVA calculated for leaf area, chlorophyll content, TKW and oil percentage parameters, shows the presence of a significant effect of genotypes and treatment as well as a significant interaction between genotype and treatment. This demonstrates that the genotypes distinctly respond in the presence of *Phomopsis* filtrate depending on the genetic characteristics and tolerance degree to this pathogen.

The *Phomopsis* filtrate introduced into culture medium induced a strong enough stress for the genotypes classification from the viewpoint of tolerance to *Phomopsis helianthi* pathogen. A number of seven lines at which at least three parameters did not registered statistically ensured diminutions after treatment were selected in comparison with the control variant (LC 4005, LC 4006, LC 4011, LC 4019, LC 4020, LC 4022 and LC 4024).

The results obtained at gas chromatograph underline the fact that from the five fatty acids from sunflower oil, the oleic acid decreases after treatment in all genotypes, excepting the LC 4010 line. At the same time, the linoleic acid percentage increases after treatment in nine out of the tested lines. We positively notice the fact that the linolenic acid which reduces the oil stability, was detected only in three genotypes but in very small quantities.

ACKNOWLEDGEMENTS

The authors thank Prof. Dr. h c. Alexandru Viorel Vrânceanu and Dr. Maria Pacureanu-Joita for providing sunflower lines and scientific support.

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Table 1. Reproduction ability of the *E. integriceps* recent generations, as compared with multiannual average (1970-2000) and with the specific years: favourable (1986) and unfavourable (1989).

Natural generation of *E. integriceps*	Prolificacy (egg/female)		
	under field conditions	under controlled conditions	
		average	maximum/female
1970-2000	40.2	57.9	311
1986	56.3	71.3	298
1989	18.8	27.1	87
1996	47.1	69.9	302
1997	46.6	68.6	197
1998	37.5	53.8	209
1999	38.8	54.5	219
2000	39.3	55.7	208

Table 2. Prolificacy level of some *E. integriceps* populations (fertile females), from generations with different fat body levels, collected from the field, at the beginning of migration and studied under controlled conditions.

Fat body	Generation	Prolificacy (egg/female)	
		average	maximum

23.4	1989-1990	32.1	97
22.5	1972-1973	33.4	127
26.5	1971-1972	46.4	148
27.9	1977-1978	67.5	186
28.0	1984-1985	83.6	210
29.7	1985-1986	95.3	234
29.8	1994-1995	104.7	246

Table 3. Level and stages of fat body diminution at *E. integriceps* (multigeneration average).

Stages	Fat body level		Diminution	
	limits	average	limits	average
Diapause beginning	33.03-37.58	35.69	0	0
End of diapause	21.97-27.64	25.43	24.57-36.33	27.39
End of oviposition	8.12-10.39	8.78	66.50-78.69	74.43

Table 4. Mortality registered at the *Eurygaster integriceps* populations, during diapause in different generations, from Romanian area

E. integriceps natural population	Mortality (%)	
	Limits in counties	Total area (mean)
2000-2001	4.6-35.7	8.7
1995-1996	3.7-36.4	10.2
2001-2002	5.1-32.3	12.7
1985-1988	3.8-41.2	14.8
1999-2000	4.8-97.6	24.5
1973-1974	11.6-85.0	39.5
1988-1989	17.5-68.4	48.2

Table 5. Fat body value at *Eurygaster integriceps* populations, established on female groups, distributed in weight classes, at the beginning of diapause (multigeneration average).

Weight (mg)	% from the total of population		Fat body (%)	
	limits	average	limits	average
below 0.110	3.7-7.7	5.6	26.2-26.6	26.4
0.111-0.118	7.6-23.1	13.3	26.5-28.8	28.7
0.119-0.126	15.9-24.7	19.7	32.8-33.5	33.6
0.127-0.134	32.5-34.8	33.7	34.9-36.4	35.4
over 0.145	22.4-30.8	28.6	35.7-39.8	38.7

Table 6. Fat body value at *Eurygaster integriceps* populations, established on male groups, distributed in weight classes, at the beginning of diapause (multigeneration average).

Weight (mg)	% from the total of population		Fat body (%)	
	limits	aver-age	limits	aver-age
below 0.105	7.0-19.7	12.3	25.3-26.7	26.2
0.106-0.113	16.8-19.9	17.3	27.2-28.5	27.7
0.114-0.121	20.3-29.5	23.7	29.4-33.8	31.5
0.122-0.129	19.2-32.7	28.5	31.2-35.5	32.6
over 0.130	15.5-23.9	19.4	31.4-36.6	33.8

Table 7. Mortality (%) registered at *Eurygaster integriceps* female populations, depending on the fat body (multigeneration average).

Fat body (%)	Mortality (%)			
	During August-October		During November-March	
	limits	average	limits	average
26.4	17-22	20.4	59-64	61.3
28.7	13-15	12.9	43-54	47.6
33.6	9-17	12.5	41-52	46.2
35.4	4-11	6.6	29-34	33.6
38.7	4-7	5.8	26-35	30.9

Table 8. Mortality (%) registered at *Eurygaster integriceps* male populations, depending on the fat body (multigeneration average).

Fat body (%)	Mortality (%)			
	During August-October		During November-March	
	limits	average	limits	average
26.2	22-31	22.6	62-71	67.1
27.7	11-24	20.4	53-62	57.4
31.5	12-19	14.3	39-47	44.0
32.6	9-18	12.7	30-44	37.6
33.8	5-14	9.1	24-45	32.3

Table 9. Sterility and prolificacy registered at the *Eurygaster integriceps* populations, depending on the fat body (multigeneration average).

Fat body (%)	Females sterility (%)		Mean prolificacy (egg/female)		
	limits	aver-age	limits	aver-age	maxi-mum
26.4	100	100	0	0	0
28.7	60-72	63.5	4.1-6.6	5.4	42
33.6	54-63	57.3	16.2-22.8	19.5	78
35.4	35-44	39.1	26.4-33.1	30.3	135
38.7	25-32	29.8	38.9-51.7	45.8	194

Table 10. Multiplication index at the *Eurygaster integriceps*

populations, depending on the fat body (multigeneration average).

Fat body (%)	Multiplication (egg/female)	index
	limits	average
26.4	0	0
28.7	0.37–2.47	1.54
33.6	4.54–9.62	6.95
35.4	28.57–40.18	35.22
38.7	49.38–64.83	56.47