PRELIMINARY RESULTS REGARDING *IN VITRO* SCREENING FOR ROUNDUP RESISTANCE IN SUNFLOWER

Florentina Răducanu

ABSTRACT

Roundup is a non-selective herbicide controlling a wide spectrum of weeds. The active ingredient is glyphosate, an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthetase (EPSP synthetase). This enzyme (which is not present in animals) is present in both bacteria and superior plants: at plants is placed in chloroplasts. In 1999 at A.R.D.I. Fundulea, a study concerning *in vitro* screening of 30 sunflower genotypes (A, B, C inbred lines) to this herbicide was initiated. A preliminary experiment was performed in order to determine the dose of glyphosate required in the culture medium to inhibit callus growth relative to control by 50% (ID 50) m. The explants represented by immature embryos (10 days old) were cultivated on Har medium supplemented with ANA and BAP. After the incubation period calli were transferred on the same medium with Roundup (glyphosate), in the following concentrations: 2.0 ml/l; 3.0 ml/l; 4.0 ml/l; 6.0 ml/l. The obtained results showed some differences between genotypes regarding their response (callus viability), depending on the herbicide concentration. In only five genotypes the calli viability was higher than 50% (on 4.0 ml/l Roundup). For all studied genotypes, this parameter registered values lower than 30%, and a calli necrosis also appeared. Callus viability (number of calli on treatment variant / number of calli on control, %) diminished with increasing herbicide concentration. The experiment which used 3.0 ml/l Roundup showed that viability of calli was higher than 50% for all tested genotypes. So, this variant was the optimal concentration for the establishment of an *in vitro* selection system for Roundup resistance in sunflower. From all tested inbred lines, only T/99 - 6B, T/99 - 10B, and T/99 - 1088C lines presented some embryogenic calli, characterized by the presence of the cellular units. This type of callus usually constitutes the basis for regeneration or *in vitro* selection for tolerance/resistance to different stress factors.

Key words: callus viability, *in vitro* screening, Roundup, sunflower.

INTRODUCTION

Pre- and postemergent selective herbicides have been used to reduce weed populations that compete with agronomic crops for space and nutrients. Herbicide resistance has been mentioned as an area where this technology can be successfully used. The use of cell culture system can offer several advantages in the isolation of herbicide resistant plants. So, selection from culture systems provides new sources of genetic variability from which resistant phenotypes may be selected and *in vitro* selection of herbicide resistance from cell culture systems may produce cell lines with different forms of resistance.

There are many examples of herbicide resistance in cell cultures; however, only in a few cases, plants regenerated from resistant cell lines were noticed. In the cases of regenerated plants, they fall into three classes: resistant to normally lethal levels of the herbicide, tolerant to the herbicide and not resistant. Herbicide resistant plants have been regenerated from cell lines of *N. tabacum* resistant to amitrol and glyphosate, isopropyl N-carbonate (Aviv and Galun, 1977), picloram and paraquat (Hughes, 1978). Callus derived from both resistant and sensitive regenerated plants was herbicide resistant in most cases, indicating that the resistance trait was transmitted but not expressed in the whole plant.

Chaleff and Keil (1981) found that more than half of their picloram-resistant plant were also resistant to hydroxyurea.

Chaleff and Parsons (1978), Chaleff (1980) and Chaleff and Keil (1981) have only reported genetic analysis of herbicide-resistant mutants isolated *in vitro*.

Ellis (1978) established tomato cell suspension cultures from 4 cultivars showing different degrees of tolerance to metribuzin.

Occasionally, cell lines that are resistant to one herbicide are also found to be resistant to another apparently unrelated herbicide. Two out of five *N. tabacum* cell lines selected for resistance to amitrol are also resistant to glyphosate.

Conversely, two out of five lines selected for glyphosate resistance are also resistant to amitrol.

The glyphosate is an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthetase (EPSP synthetase). This enzyme (which is not present in animals) is present only in both bacteria and superior plants: in plants it is placed into chloroplasts. The glyphosate is not practically metabolized by plants because it is rapidly decomposed by soil microorganisms (Duke, 1999).

Plant resistance to these molecules was obtained through both enzyme overproduction and introducing of resisting forms. Theoretically, it is possible to transfer adequate bacterial genes into plants, but results have not yet been published.

MATERIAL AND METHODS

For this study, friable calli proliferated from immature embryos (1.2-1.8 mm lenght) from 30 Romanian sunflower genotypes (A, B, C inbred

lines) were used (Table 1). The immature embryos (400/genotype) were aseptically inoculated on modified Har medium, having the following composition:

- stock solution MS - 100 ml/l of medium
- inositol - 100 mg/l of medium
- adenine sulphate - 40 mg/l of medium
- casaminic acids - 50 mg/l of medium
- saccharose - 30 mg/l of medium
- agar - 7 g/l of medium
- NAA and BAP - 1,0 mg/l of medium

The parameters under study were: callus viability (%) expressed as viable number of calli/treatment variant; number of calli/control variant) during the first experimentation stage and mean callus weight established after both first and third passage in comparison with mean callus weight of control variant .

So for, there are not enough data regarding the Roundup herbicide utilization for sunflower *in vitro* selection. Therefore, the treatment initially chosen, were: V_0 = control; V_1 = 2.0 ml; V_2 = 4.0 ml; V_3 = 6.0 ml Roundup /l of medium.

Table 1. Calli of sunflower genoptypes subcultivated on media supplemented with Roundup herbicide

No.	Genotypes	Number of immature inoculated embryos	Number of calli	Capacity of callusing (%)
1.	T/99-1A	400	315	78.7
2.	T/99-1B	400	300	75.0
3.	T/99-2A	400	240	60.0
4.	T/99-2B	400	260	65.0
5.	T/99-3A	400	307	76.8
6.	T/99-3B	400	304	76.0
7.	T/99-4A	400	324	81.0
8.	T/99-4B	400	338	84.5
9.	T/99-5A	400	290	72.5
10.	T/99-5B	400	305	76.3
11.	T/99-6A	400	309	77.3
12.	T/99-6B	400	349	87.2
13.	T/99-7A	400	364	91.0
14.	T/99-7B	400	392	98.0
15.	T/99-8A	400	248	62.0
16.	T/99-8B	400	269	67.3
17.	T/99-9A	400	318	79.5
18.	T/99-9B	400	378	94.5
19.	T/99-10A	400	325	81.3
20.	T/99-10B	400	324	81.0
21.	T/99-1099C	400	400	100
22.	T/99-1103C	400	289	72.3
23.	T/99-1089C	400	314	78.5
24.	T/99-1064C	400	325	81.3
25.	T/99-1095C	400	355	88.8
26.	VL/99-1049C	400	400	100
27.	T/99-1085C	400	400	100
28.	T/99-1088C	400	370	92.5
29.	T/99-1054C	400	355	88.8
30.	T/99-1066C	400	315	79.3
	Average	400	326	81.5

To obtain calli, incubation conditions such as 28°C temperature and permanent darkness were ensured during four weeks.

The results obtained with 2.0 ml and 4.0 ml/l medium respectively, suggested that the optimum Roundup concentration, in order to ensure an efficient selection pressure would be 3.0 ml

Roundup/l of medium (second experimentation stage).

RESULTS AND DISCUSSION

The results obtained during the first experimentation stage, revealed genotype differences in the tolerance degree on the herbicide concentration from culture medium.

Thus, at 2.0 ml Roundup/l of medium, the callus viability was not affected in T/99-1B, T/99-3B and T/99-13C genotypes, (being 100%) and in 70% of genotypes callus viability values was up to 80%.

At 4.0 ml Roundup concentration/l of medium, the callus viability was higher than 50% in only five genotypes: T/99-1A (62.3%); T/99-1B (75%); T-99-5A (75%); T-99-9B (52.91%) and T-99-8A (53.8%). A very low tolerance to this herbicide was observed in pollen fertility restorer lines: T-99-16C (11.1%); T-99-11C (13.1%); T-99-14C (30%) as well as to male sterile lines: T-99-6A (24.4%) and T-99-9A (30%).

The 6.0 ml/l Roundup treatment, drastically reduced callus viability and after only two days from transfer, a strong necrosis was noticed. After 21 days in 20 genotypes, viability was between 6.6% and 30% (Figure 1).

No clear difference in tolerance to this herbicide was noticed between the line groups (A, B, C), but large differenced were observed between genotypes inside each group.

Making the average for each genotype, for all three treatment variants, the most affected by the applied treatments were T-99-11C (with 14.1% callus viability) and T-99-16C (with 19.8% callus viability) genotypes and the most tolerant one, proved to be T-99-4B (with 75% callus viability).

The results of the first experimentation stage, led to the conclusion that the utilized variants did not assure an optimum selection pressure (ID_{50}). Therefore, during the second experimentation stage, half of callus from the control variant (ten genotypes with high yielding level *in vitro* culture) was transferred on media with Roundup, using the following treatments: V_0 = control and V_1 = Har + 3,0 ml Roundup, V_2 = Har + 5.0 ml Roundup/l medium.

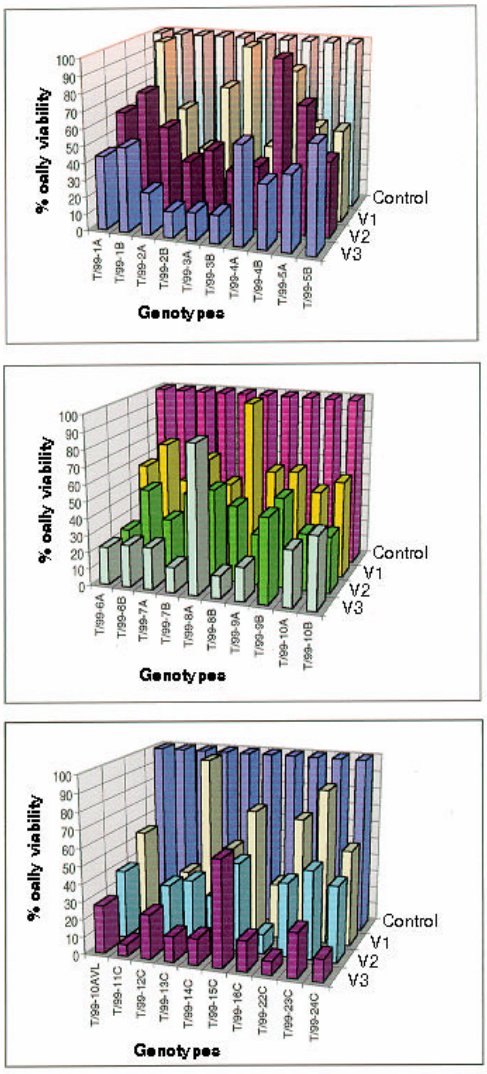

Figure 1.. The influence of Roundup herbicide on callus viability at some sunflower genotypes

Knowing the fact that the appearance of resisting somaclones to the different stress factors depends on *in vitro* passages number, three subcultures (at 28 days one of another) were performed.

The callus weight evolution after three passages measured by weighting after both first and last passage.

For the graphic presentation of genotypes behaviour to the induced stress factor, the means

genotype/first passage and genotype/ third passage in comparison with mean control/genotype, were calculated (Figure 2).

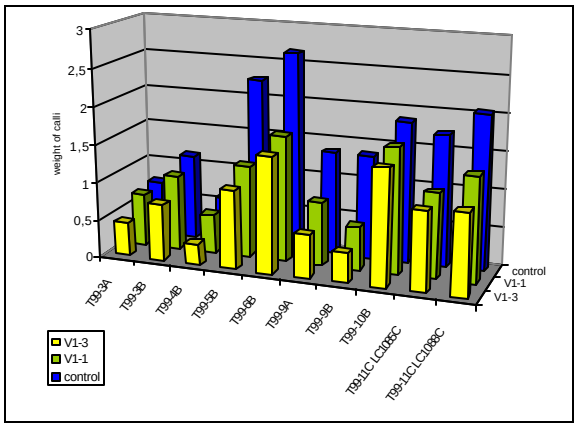

Figure 2. The influence of Roundup herbicide on weight of calli (g) after subculture *I-III*

In genotypes T/99-6B and T/99-10B, the weight of calli after the first passage was of 1.5 grams and 1.3 after the last passage, respectively: five out of ten genotypes lost very much of their weight.

CONCLUSIONS

During experimentation, three sunflower callus lines tolerant to the non- selective herbicide Roundup were selected.

The callus aspect was modified after each passage, generally having a non-embryogenic aspect. However, viable calli were obtained.in all genotypes without selection pressure.

It is necessary to carry on experiments with concentrations between 3.5 and 4.5 ml Roundup/l medium, so that the obtained viable and embryonic callus number can allow the initiation of an *in vitro* selection efficient program for sunflower resistance to Roundup herbicide

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Table 1

Average yield of experiments with winter wheat cultivars, under irrigation and dry-land in six localities from the South of Romania (2002)

Locality	Average yield under:		Yield percentage diminution
	irrigation (kg/ha)	dry-land (kg/ha)	
Caracal	8560	5601	34.6
Marculesti	4716	3075	34.8
Teleorman	5963	3594	39.8
V. Traian	6941	3794	45.3
Fundulea	4858	1918	60.5
Simnic	(8560)	380	95.6

Table 2

Percentage diminution of some plant features under water stress conditions as compared to irrigation

Locality	Plant number	Plant height	Grain filling period	Spike number	Grain/ear	TKW	Test weight
Caracal	0	14,9	15,0	7,9	10,2	14,1	0,9
Teleorman	0	10,0	19,2	12,0	12,0	11,9	1,0
V.Traian	34,9	21,0	16,9	42,5	12,2	2,9	8,1
Fundulea	4,9	28,8	24,9	6,9	28,9	29,5	3,9
Simnic	27,6	61,7	30,0	65,0	64,5	53,1	10,7
Media	13,5	27,3	21,2	26,9	25,6	22,3	4,9

Table 3

Minimum, maximum and average yields registered at Fundulea in 2002 in international trials WWEERYT with genotypes grouped depending on the originating country

Source	Average yield of the tested genotypes	Maximum yield of the tested genotypes	Minimum yield of the tested genotypes

	(kg/ha)	(kg/ha)	(kg/ha)
Romania	2368	2953	2073
Russia	2327	2453	1980
Ukraina-Odessa	2224	3013	1287
Hungary	2181	2780	1320
Ukraina-Mironovka	2108	2753	1500
Moldova	1927	2560	1293
Bulgaria	1898	2873	1313
Turkey	1893	2420	1487
Azerbaidjan	1460	1553	1367
Kazahstan	1422	1833	853
LSD 5%		243	275

Table 4

Correlations between yield under water stress conditions and different traits

Locality	Average yield diminution because of water stress (%)	Correlation coefficients between yield under water stress conditions and:						
		yield under irrigation	plant height under stress conditions	plant height under irrigation	heading time	spike/ m²	grain/ear	TKW
Caracal	34,6	0,48	0,29	-0,31	-0,12	0,20	0,11	-0,30
Teleorman	39,8	0,80	0,35	0,31	-0,85	0,58	-	-
Valu Traian	45,3	0,04	0,33	0,20	-0,40	0,42	0,40	0,22
Fundulea	60,5	0,00	0,46	-0,31	-0,46	0,52	0,30	-0,17
Simnic	95,6	-0,01	0,41	-0,62	-0,04	0,40	0,50	0,15

The bold characters are significant at the probability level of 0.05

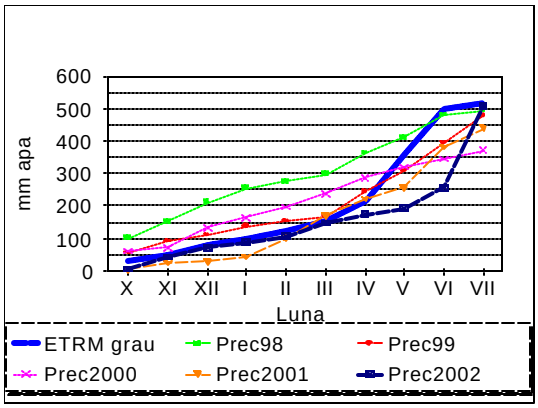

Figure 1. Average evapotranspiration and rainfall during 1999-2002 at Fundulea (mm water; month; wheat evapotranspiration; rainfall)

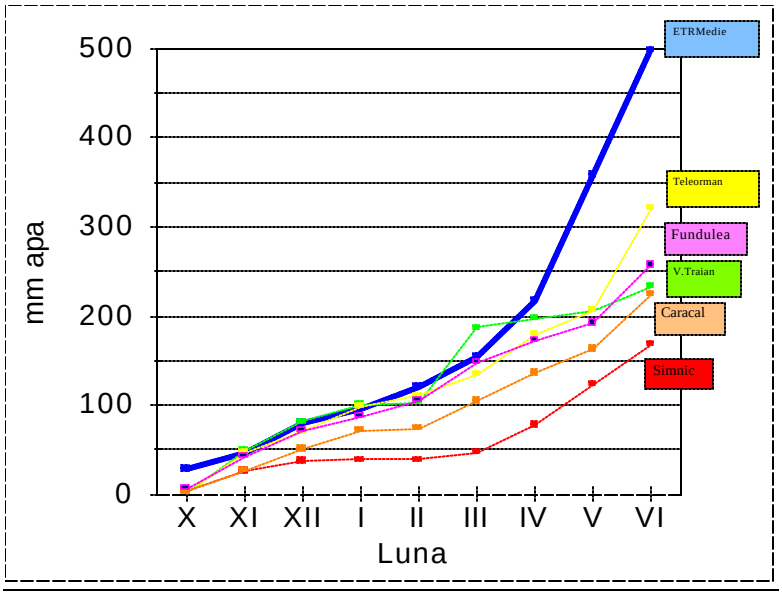

Figure 2. Average evapotranspiration and rainfall during the vegetation period in six locations of Southern of Romania in 2001-2002 year (mm water; month).

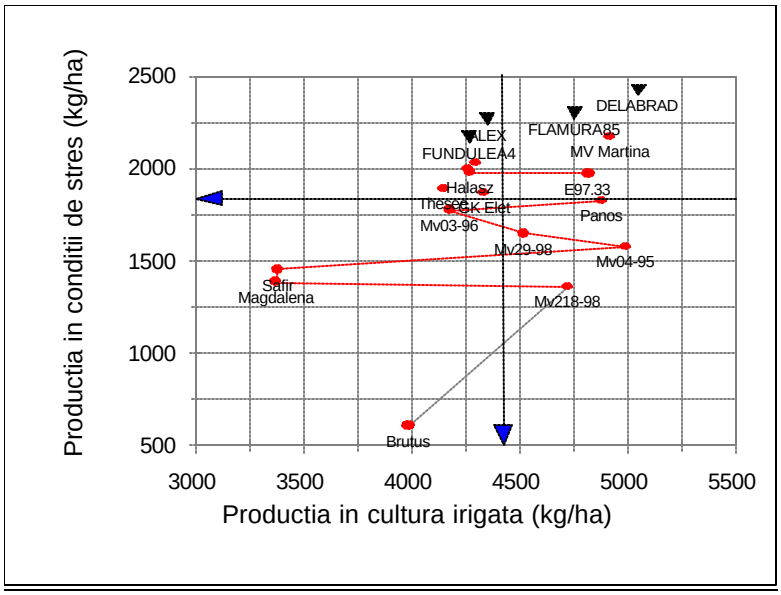

Figure 3. Yield obtained by some Romanian and foreign cultivars under irrigation and non-irrigation, in 2002 at Fundulea (arrows indicate the experiments average yield)(Yield under stress conditions; yield under irrigation).

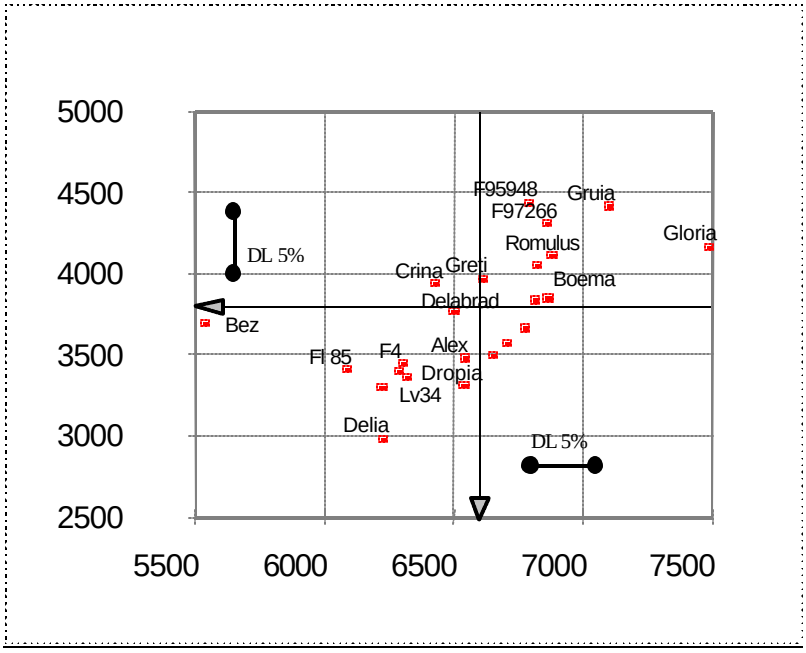

Figure 4. Average yields in four locations, obtained in 2002 by Romanian new lines and cultivars under irrigation and non-irrigation (arrows indicate experiments average yield)(Yield under non-irrigation; Yield under irrigation; LSD).