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In vitro techniques for selecting wheat (*Triticum aestivum* L.) for Fusarium-resistance. I. Double-layer culture technique

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Summary

Calluses of spring and winter wheats (*Triticum aestivum* L.) were selected for *Fusarium* resistance *in vitro*, using the double-layer culture technique. Potato-dextrose agar medium in vials was inoculated with mycelia of *Fusarium graminearum* and *F. culmorum*. After one week, fungal cells were killed by autoclaving and the agar medium containing the thermostable toxic metabolites was overlayered with MS callus-growing medium. Later, wheat calluses were placed on the upper medium for 4–5 weeks, and from the surviving calluses plants were regenerated. R_2 seedling populations from self-fertilized R_1 plants of 4 varieties were tested for *Fusarium* resistance by artificial infections in the greenhouse, and 3% of the regenerated R_2 plants have been found to be more resistant than the original cultivars.

Introduction

Fusarium spp. are serious pathogens causing seedling blight, root rot and head blight in wheat and other cereal crops worldwide. Wheat can be infected by *Fusarium* spp. in all developmental stages (Atanasoff, 1920; Snijders, 1987). These fungi produce a number of toxic compounds that can affect both human health and animal productivity (Austwick, 1984; Wang & Miller, 1988). Breeding for *Fusarium* resistance is difficult, although efficient minor resistance genes are known (Yu, 1982; Gocho, 1985; Mesterházy, 1989). Mielke (1988) found no absolute resistance or tolerance in wheat to *F. culmorum*. Immunity was also not found, but this does not mean that highly significant differences in resistance and tolerance do not exist (Mesterházy, 1989; Snijders, 1990). Therefore, *in vitro* selection of somaclonal variants insensitive to toxic metabolites produced by these pathogens seems to be a viable approach of obtaining resistant or tolerant plants as experienced already by various authors on different plant species (Daub, 1984; Hartman et al., 1984; Wenzel, 1985; Arcioni et al., 1987; Chawla & Wenzel, 1987; Pauly et al., 1987; Latunde-Dada & Lucas, 1988; Binarová et al., 1990). In most cases the toxin resistance expressed in the regenerated plants correlated with the level of their disease resistance. Furthermore, the resistance has been transmitted to the progeny of selected plants in all investigated cases (Daub, 1986).

This paper describes the *in vitro* selection of wheat calluses for insensitivity to toxic metabolites of *F. graminearum* and *F. culmorum* via a double-layer culture technique, and the results of testing the R_2 seedlings for *Fusarium* resistance.

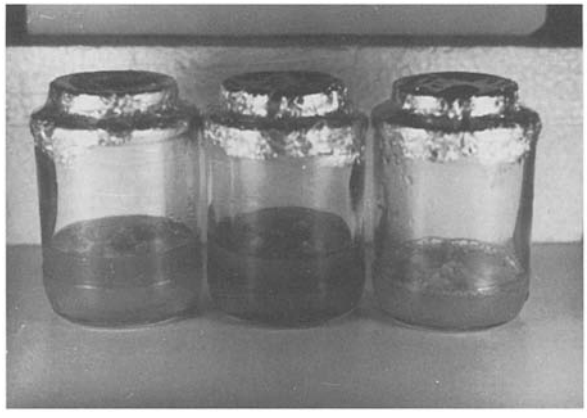

Fig. 1. The double-layer culture. From left to right: double-layer callus culture without *Fusarium*, the same with *Fusarium* and single-layer callus culture on MS medium

Materials and methods

a. Plant materials and callus cultures

Callus cultures of 9 spring and winter wheat (*Triticum aestivum* L.) cultivars (Lerma Rojo 64, Sakha 8, Sakha 69, Siete Cerros, Tobari 66 and GK Bence, GK Kincsö, GK Mini Manó, GK Ságvári, respectively) were established from immature inflorescences, immature and mature embryos on solidified MS medium (Murashige & Skoog, 1962) supplemented with 1 or 2 mg/L 2,4-D, or mesocotyls in 2,4-D solution (5–8 mg/L, Bartók & Sági, 1990). Cultures were grown at 26° C in darkness.

b. Double-layer culture technique

The double layer technique classically utilized in microbiology (Lepoivre et al., 1986) was modified as follows: vials containing 33 ml of potato-dextrose agar medium (consisting of extract of 200 g potato tubers, 20 g glucose and 7 g agar per liter, thickness: 12 mm), were inoculated with mycelium of *F. graminearum* (isolate No. 12216) or *F. culmorum* (isolates No. 12375 or 12551). These cultures were grown at 26° C for 4 days in a 16/8 h light-dark cycle, then at 5° C for 3 days in continuous light. Subsequently, the vials were autoclaved at 120° C for 15 min to kill the fungal cells (Joffe, 1974). After 2–3 h the cooled agar medium containing the thermostable toxic compounds (Patey & Gilbert, 1989) was overlayered with 33 ml of MS callus-growing medium supplemented with 1 mg/L 2,4-D (thickness: 12 mm). After diffusion of the toxic materials from the fungal culture into the upper nutrient layer (7 days), 5-weeks-old wheat calluses were placed on the upper medium (5 calluses/vial) for 4–5 weeks at 26° C in 16 h photoperiods (Fig. 1), and toxin-sensitivity was assessed on the basis of callus weight or necrosis rating. The surviving calluses were transferred to MS regeneration medium (0 or 0.1 mg/L 2,4-D added). The regenerated plants (R_0) were transplanted in pots and grown to maturity in the greenhouse.

c. Evaluation of R_2 plants for resistance

The 2nd selfed generation (R_2 progeny) of Sakha 69, Sakha 8, Lerma Rojo 64 and Tobari 66 was evaluated for reaction to *F. graminearum* and *F. culmorum* separately by a greenhouse seedling test based upon seed germination, number of killed plants, plant height and dry matter production calculated for 60 seeds, each in 6 replication according to Mesterházy (1978). Two resistant genotypes (74-2, 84-42) and the original cultivars were used as controls.

Results and discussion

An initial test indicated that callus growth on the upper medium layer was strongly inhibited (Fig. 2). Effect of *Fusarium* toxins on callus weight of the susceptible variety GK Ságvári was assessed after 4 weeks and the level of inhibition is given in Table 1. Majority of the calluses ceased growth, turned brown and died within 6 weeks after being transferred on the upper layer, similarly to the calluses of other varieties. Consequently, the double-layer technique seems to be suitable for selection of wheat calluses resistant to toxic *Fusarium* metabolites. Table 2 demonstrates the growth of calluses on toxic medium as affected by the genotype. Most calluses of the resistant GK Bence, the susceptible

GK Ságvári and of the very susceptible GK Mini Manó exhibited good early growth on the upper layer, independently on *Fusarium* spp., while calluses of the intermediate GK Kincsö and Sakha 8 tolerated *F. graminearum* and those of the resistant Sakha 69 *F. culmorum* isolate No. 12551 only. Double-layer callus cultures without the fungus (controls) exhibited maximum growth in all cases. The different *Fusarium*-sensitivity of the varieties can probably influence their *in vitro* selection for resistance. Dependence of *in vitro* selection results on the genotype has been mentioned by Chawla & Wenzel (1987) and Mégnégneau & Branchard (1988) also. However, somaclonal variation may have an effect, too (Daub, 1986; Latunde-Dada & Lucas, 1988; Kaleikau et al., 1989).

Regenerating ability of the resistant calluses from all genotypes was lower than that of the unselected calluses as found by Arcioni et al. (1987) as well. From the 9 genotypes tested, plants were regenerated from *in vitro* selected calluses of 5 only, i.e. Lerma Rojo 64, Sakha 8, Sakha 69, Tobari 66 and Siete Cerros (Table 2), mostly from immature inflorescences and young embryo-derived calluses. In the cultivars GK Kincsö, GK Bence, GK Mini Manó and GK Ságvári no plants were obtained from selected calluses. Toxin treatment reduced the regenerative capacity of calluses in the experiments of Latunde-Dada & Lucas (1988) also.

Table 1. Changes of fresh weight of mesocotyl-derived calluses of GK Ságvári wheat in the double-layer technique after 4 weeks with and without *F. graminearum*

Callus weight, g	*MS medium* (control I)	*Double-layer*	
		without F. graminearum (control II)	*with*
Initial[a]	1.18	1.10	1.31
After 4 weeks[a]	1.73	1.48	1.11
Difference[b]	0.55	0.38	− 0.20
Difference in per cent	46.61	34.55	− 15.27

[a] Total fresh weight of 30 calluses each. [b] Differences between double-layer calluses with *F. graminearum* and both control calluses are significant, but between control I and control II calluses are not significant (T-test, P 0.1)

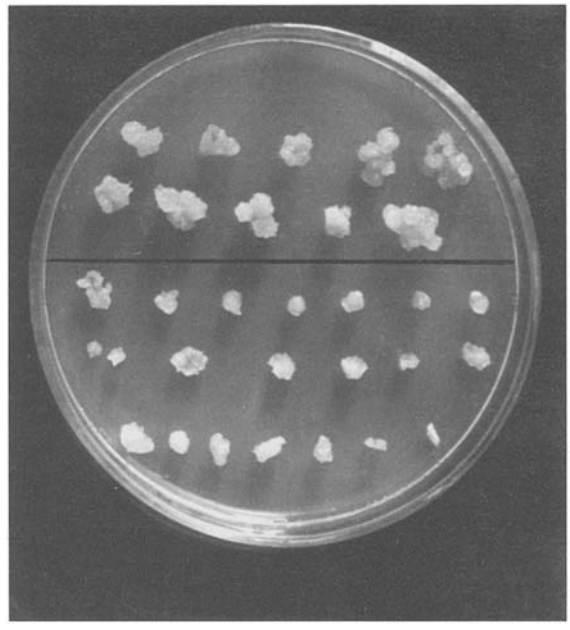

Fig. 2. Wheat calluses kept for 4 weeks on MS medium (upper two rows) and calluses grown in double-layer culture with *Fusarium* (lower three rows)

Plant regeneration in cereals is known to be genotype and explant specific (Rengel, 1987; Kaleikau et al., 1989), and its seems that callus growth and plant regenerating ability is not equally sensitive to the toxic Fusarium-metabolites. Reaction of R_2 progeny to *F. graminearum* and *F. culmorum* in the seedling test revealed that large variation exists within and between the lines from selected and unselected callus cultures in most resistance parameters, for instance 0 to 71.6% (Sakha 8, shoot length) or 8.3 to 78.9% (Sakha 69, germination, Table 3). Resistance of the spring wheat cultivar Sakha 69 is equal to or better than that of the reference cultivars. On this basis, Sakha 8 can be regarded as less resistant, and Lerma Rojo 64 and Tobari 66 as susceptible. Resistance differences between lines from selected and unselected callus cultures, on the one hand, and between lines from selected calluses and the original varieties on the other hand, are significant in many cases (Table 3).

Mean comparisons demonstrated that one unselected R_2 line of Tobari 66 was significantly more resistant than the original cultivar. This superior

somaclone (F2/18-B) had a resistance similar to that of the genotypes 74-2 and 84-42 (Tables 3, 4, Fig. 3). However, many R_2 lines were significantly more susceptible than the cultivars from which they were derived. In an R_2 wheat population not selected *in vitro* previously, Maddock & Semple (1986) also found a line with improved *Septoria* resistance. Complete resistance in R_2 tomato plants was obtained from non-selected callus tissue to bacterial wilt, too (Toyoda et al., 1989).

Resistance differences of R_2 lines derived from selected and unselected calluses seemingly does

Table 2. Callus growth and plant regeneration of various wheat genotypes in the double-layer culture technique

Fusarium spp. and isolate	Genotype	Explant	Total no. of calluses	Number of calluses				Number of regenerants
				N	S	M	G	
F. graminearum	7C	I	15	5	5	3	2	
No. 12216	LR	YE	16	5	2	0	9	8
	T66	YE	12	6	1	5	0	
	Kö	ME	61	32	9	4	16	
	S8	I	16	0	0	2	14	8
	S69	I	18	8	7	3	0	1
	B	ME	36	0	10	8	18	
	MM	ME	12	4	2	1	5	
	SV	ME	16	0	0	2	14	
F. culmorum	LR	I	21	16	2	3	0	3
No. 12375	T66	I, YE	12	7	2	1	2	1
	Kö	ME	48	35	11	0	2	
	S8	I	17	14	3	0	0	
	S69	I	18	15	0	3	0	3
	B	ME	24	0	3	0	21	
	MM	ME	30	3	0	4	23	
	SV	ME	16	0	0	2	14	
F. culmorum	7C	I	13	12	0	0	1	1
No. 12551	LR	YE	13	0	9	4	0	
	T66	I, YE	14	8	3	1	2	1
	Kö	ME	60	50	4	6	0	
	S8	I	12	6	6	0	0	
	S69	I	24	7	5	6	6	4
	B	ME	36	1	2	13	20	
	MM	ME	24	5	18	1	0	
	SV	ME	12	0	0	6	6	
Control	LR	I	10	0	0	0	10	2
	T66	YE	15	0	0	0	15	5
	Kö	ME	30	0	12	4	14	
	S8	I	12	0	0	0	12	6
	S69	I	18	2	1	0	15	9
	B	ME	18	0	0	1	17	
	MM	ME	22	2	3	6	11	
	SV	ME	16	0	0	3	13	
Total:			737	243	120	92	282	52

Abbreviations. Genotypes: 7C = Siete Cerros, LR = Lerma Rojo 64, T66 = Tobari 66. Kö = GK Kincső, S8 = Sakha 8, S69 = Sakha 69, B = GK Bence, MM = GK Mini Manó, SV = GK Ságvári. Explants: I = immature inflorescence, YE = immature embryo, ME = mature embryo. Callus growth: N = no growth, S = slight growth, M = moderate growth, G = good growth

not depend on the resistance of the genotype. However, since the *in vitro* processes can also be involved, induced and non-induced somaclonal variation can or cannot be of similar value for resistance against *Fusarium* spp. Because of partial or complete sterility, R_2 lines of Siete Cerros, those of Lerma Rojo 64 from unselected calluses and of Tobari 66 from selected calluses could not be included into this comparison.

As summarized in Table 4, from the 30 R_2 lines tested, only one (Tobari 66, F2/18-B, unselected, 3%) showed an improved resistance over the original cultivar, 9 (30%) possessed a similar character and 20 (66,7%) proved to be more susceptible.

Table 3. Reaction of R_2 plants selected by the double-layer culture technique and from unselected calluses as compared to that of the original genotypes to *F. graminearum* and *F. culmorum* in the seedling test. Data are averages presented as % of the controls

Cultivar	Original cv. or R_2 line	Isolate	Germination %	Mortality %	Shoot length %	Dry matter %
Sakha 69	original cv.		97.5	17.5	94.2	92.5
	sel. F7/1-A	F.g.12216	78.9	44.7	76.4	58.3
	F8/38-B	F.c.12551	69.5	38.9	73.1	71.3
	F8/2-B	F.g.12216	52.6	57.9	46.8	46.4
	F4/9-B	F.c.12375	44.5	72.2	64.5	39.3
	F8/2-C	F.g.12216	35.3	70.6	32.4	44.3
	F8/2-A	F.g.12216	20.0	85.0	15.0	15.3
	F4/9-A	F.c.12375	8.3	91.7	16.6	11.7
	F8/38-A	F.c.12551	12.0	93.8	15.7	10.0
	unsel. F4/23-H		80.0	35.0	97.5	86.0
	F4/23-F		23.4	80.0	18.5	13.7
	F4/23-I		20.6	82.4	17.8	31.7
	F4/23-C		20.6	88.2	16.5	9.3
Sakha 8	original cv.		82.5	52.5	94.4	72.7
	sel. F8/4-A	F.g.12216	47.1	52.9	71.6	61.5
	F8/4-B	F.g.12216	55.6	55.6	53.3	42.1
	F8/10-A	F.g.12216	27.8	80.6	36.7	31.5
	F8/10-B	F.g.12216	21.1	84.2	18.5	16.4
	F8/20-A	F.c.12375	6.3	93.8	6.0	6.5
	F4/3-A	F.g.12216	0.0	100.0	0.0	0.0
	F8/10-C	F.g.12216	0.0	100.0	0.0	0.0
	unsel. F8/48-B		9.4	96.9	6.0	1.7
	F8/48-A		6.3	96.9	0.9	1.7
	F4/22-D		0.0	100.0	0.0	0.0
	F4/22-C		0.0	100.0	0.0	0.0
Lerma Rojo 64	original cv.		13.3	93.4	7.3	4.2
	sel. F2/8-B	F.c.12375	33.3	72.2	28.0	30.6
	F2/8-D	F.c.12375	25.0	75.0	29.8	30.8
	F2/3-A	F.g.12216	23.6	85.3	22.0	35.7
	F2/3-C	F.g.12216	7.9	92.1	3.5	8.6
	F2/8-C	F.c.12375	0.0	100.0	0.0	0.0
Tobari 66	original cv.		32.2	75.0	22.7	23.0
	unsel. F2/18-B		73.7	32.9	94.1	103.3
	F2/17-A		31.6	71.1	30.9	35.6
74-2	reference cv.		92.5	12.5	77.2	91.7
84-42	"		92.5	15.0	120.2	90.2
	LSD 5%		23.3	24.1	33.2	31.5

Table 4. Disease reaction of regenerated R_2 lines selected by the double-layer culture technique for resistance to *F. graminearum* and *F. culmorum* as compared to that of the original cultivars (based upon the significant differences in Table 3)

Cultivar	Type[a]	R_2 line	No. of lines	Disease reaction		
				similar	susceptible	resistant
Sakha 69	R	selected	8	1	7	0
		unselected	4	1	3	0
		total	12	2	10	0
Sakha 8	R/I	selected	7	1	6	0
		unselected	4	0	4	0
		total	11	1	10	0
Lerma Rojo 64	S	selected	5	5	0	0
		unselected[b]	–	–	–	–
Tobari 66	S	selected[b]	–	–	–	–
		unselected	2	1	0	1
		gross total	30	9	20	1
		%	100	30.0	66.7	3.3

[a] Disease reaction, compared to that of the reference cultivars. R = resistant, R/I = resistant/intermedier, S = susceptible. [b] Sterile or semisterile regenerants, not tested

Thus, the double-layer culture technique seems to be an useful method of selecting *Fusarium*-resistant calluses of wheat and is definitely cheaper than the *in vitro* selection methods using commercially available *Fusarium* toxins. Nevertheless, its effectivity does not reflect in the number of resistant regenerants. It is possible that the *Fusarium* toxins do not play a significant role in the blight pathogenesis, the resistance at seedling stage will be lost during regeneration or has not been transmitted to the progeny. By means of *in vitro* selection for fusaric acid-resistance, Wenzel & Foroughi-Wehr (1990) obtained barley plants resistant to fusaric acid, but their *Fusarium*-resistance was not checked.

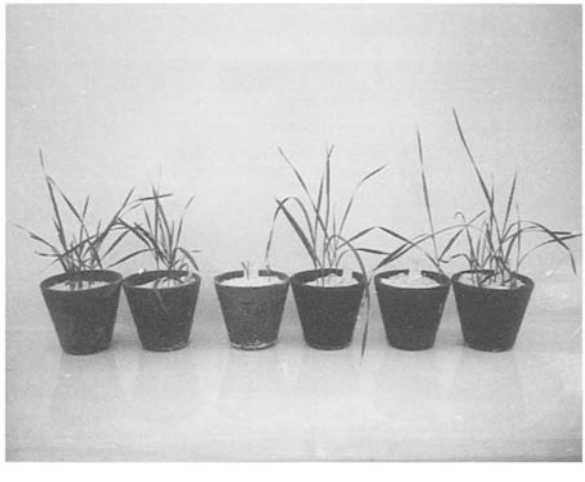

Fig. 3. Fusarium resistance of Tobari 66 control and R_2 wheat seedlings derived from unselected calluses. From left to the right: Tobari 66, F2/18-B (controls), Tobari 66 + *F. graminearum*, F2/18-B + *F. graminearum*, Tobari 66 + *F. culmorum*, F2/18-B + *F. culmorum*

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