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In vitro selection of barley and wheat for resistance against *Helminthosporium sativum*

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Summary. Calli derived from immature embryos of barley and wheat genotypes were screened for their resistance to purified culture filtrate produced by the fungus *Helminthosporium sativum* P.K. and B. Two selection methods were used: a continuous method in which four cycles of selection were performed one after another on toxic medium and a discontinuous method in which a pause on non-toxic medium was given after the second or third cycle of selection. The latter was superior as it allowed the calli to regain their regeneration ability. About 3,000 calli from two barley genotypes and 2,000 from two wheat genotypes were used for selection. The selection with the pathotoxins resulted in 6% to 17% surviving calli. Toxin tolerant callus lines of barley were characterised by protein isozymes. Zymograms showed one more isozyme than with the unselected sensitive callus. Barley and wheat plants have been regenerated from callus lines surviving the toxin treatment and in vivo testing against pathogen revealed that the majority of these plants were less sensitive.

Key words: In vitro selection – *Helminthosporium sativum* – Disease resistance – Protein isozymes – Barley – Wheat

Introduction

Breeding for resistance is a high priority. At present, in addition to classical approaches progress is anticipated through the use of cell and tissue culture. The recovery of disease-resistant plants by selection of cell and callus cultures resistant to fungal culture filtrate or synthetic toxins of the pathogen is a direct application of the cell culture approach to crop improvement (Wenzel 1985). Selection in plant cell cultures using pathotoxins as a screen for resistance have been reported for dicots in *Brassica napus* to *Phoma lingam* (Sacristan 1985), in potato to culture filtrate of *Phytophthora infestans* (Behnke 1979) and *Fusarium oxysporum* (Behnke 1980; Schuchmann 1985), in tobacco to toxins from *Pseudomonas syringae* and *Alternaria alternata* (Thanutong et al. 1983) and in alfalfa to culture filtrate of *Fusarium oxysporum* (Hartman et al. 1984). In cereals, where not only selection but also regeneration from in vitro cultures is still a limiting factor, successful reports on selection are published for oats insensitive to the pathotoxin victorin (Rines and Lukes 1985) and in maize to culture filtrate of *Helminthosporium maydis* Nisikado and Miyake (Gengenbach and Green 1975). In this experimental series toxins from *Helminthosporium sativum* P.K. and B. were used for selection. This fungus produces helminthosporal (Ludwig 1957) and victoxinine toxins (Pringle 1976), which produce seedling blight, root rot, head blight and leaf spot in cereals and grasses including barley and wheat. The selection system of embryogenic callus induced from immature embryos was used, as reported for barley and wheat (Bayliss and Dunn 1979; Sears and Deckard 1982; Eapen and Rao 1985; Hanzel et al. 1985; Goldstein and Kronstad 1986; Chawla and Wenzel 1987).

Materials and methods

Plant material

Callus cultures were established from immature embryos of the wheat varieties 'Atys' and 'Pitic 62' and the spring barley variety 'Dissa' and one F_2 family 'W 193', as described by Chawla and Wenzel (1987).

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Isolation of toxin

Helminthosporium sativum P.K. and B. collection strain no. 62606 (supplied by Dr. H. Nirenberg, Berlin) were used in the study. For the preparation of toxic culture filtrate, the mycelial suspensions for seeding production flasks were prepared by transferring fungal material to 250 ml Erlenmeyer flasks containing broken glass rods and 25 ml modified Fries medium as described by Pringel and Scheffer (1963). They were incubated for 4 days at 25 °C, then the flasks were shaken for 30 min and 1 ml mycelial suspension was added in 20 ml of modified Fries medium as described by Luke and Wheeler (1955). After additional incubation at 25 °C for 28 days, the culture filtrate containing the pathotoxic compounds was filtered through several layers of cheesecloth and then through Sartorius filter (0.2 μ) to discard the mycelium and spores. The culture filtrate was concentrated in vacuo at 40 °C to 10% of the original volume. An equal volume of cold methanol was added and the solution was stored overnight at 5 °C. Precipitates were removed by filtration through Whatman no. 1 filter paper and washed with cold methanol. The methanol was removed in a vacuum freeze drier, and the oily substance formed was dissolved in a small quantity of methanol. Further dilutions were with water. Toxicity of purified culture filtrate was tested by the root bioassay method (Pringle and Braun 1957).

Testing of lethality of toxins on callus. Callus was divided into small pieces and five inocula of calli were placed in petri dishes containing different concentrations of toxin. Final fresh weight increase of each inoculum was determined after 3 weeks. Five levels of toxin concentration were selected and their growth rates were compared (Table 1). At the highest toxin concentration (200 units), almost all the calli died and an overall negative growth rate was observed. At the next lowest concentration (100 units), 10%–30% of the calli survived the toxicity of culture filtrate and a growth rate of 30% was observed. At lower levels of 20 and 5 units, no great effect on growth of calli was observed (Fig. 1 a). Thus 100 units of toxin were used for selection of calli. A control experiment was also conducted with uninoculated *H. sativum* growth medium processed in the same way as the toxin preparation. Growth of calli was tested at different levels of concentration; almost no effect of these callus growing media was observed (Fig. 1 b).

Callus selection and regeneration procedures. Selection began 3–4 months after callus initiation. The callus was divided into small pieces (approx. 30 mg fresh weight) which were put in a petri dish containing 10 ml toxic medium. Two selection methods were employed: continuous and discontinuous. For the continuous method, four cycles of selection were performed by transferring healthy calli onto the toxic medium after a 3 week interval. In the discontinuous method, the calli were grown once on non-toxic medium after the 2nd or 3rd cycle, but 4 cycles were always performed on the toxic medium. After these selections, resistant calli were grown on maintenance medium and parts of calli were regenerated. Regenerated plants wer transferred to soil and grown under semicontrolled greenhouse conditions.

Biochemical characterisation of callus. The protein isozymic patterns of calli resistant to the toxin and calli not exposed to it (referred to as sensitive callus) were analysed by polyacrylamide gel electrophoresis. Coomassie blue R 250 in combination with methanol and acetic acid was used for staining the gels.

Table 1. Comparison of growth rates of immature embryo derived calli of barley on different levels of toxin concentration in the callus growing medium. Means were obtained from weights of five inocula/treatment after 3 weeks

Sample no.	Percentage increase in fresh weight at toxin units				
	0	5	20	100	200
1	240	168	61	8	−40
2	170	127	63	31	−15
3	267	161	73	40	19
4	176	137	112	37	16
$\bar{x}$	214	149	78	30	−20

In vivo testing of regenerated plants against pathogen. Leaves of pants regenerated from callus after selection and non toxin-treated control plants were sprayed with 0.2% tween solution. After ½ h, during which the leaves dried up, they were inoculated with 5 μl spore suspension (10^6/ml), and kept in humid chambers. In susceptible plants, dark brown spots appeared on the leaves within 3–4 days of inoculation. The plants regenerated from in vitro cultures were categorised as non-sensitive (R), intermediate (I) and susceptible (S).

Results

Calli were placed on toxic medium and screened using the two selection methods. These methods were employed because the very high level of toxin concentration used at the beginning could have destroyed the capacity of resistant callus lines to regenerate. Hence in one method a pause on non-toxic medium was given to allow maintenance or regaining of regeneration ability. After the first cycle of selection on the toxic medium, wheat genotypes showed 24% survival of calli and barley 'Dissa' and 'W 193' showed 29% and 23% survival respectively (Table 2). In the second cycle, about 8% of the calli still died but thereafter insensitivity to toxic medium was exhibited and in further selections fewer calli died (Fig. 1 c, d). The number of surviving calli after 4 cycles of selections on toxic medium were 11% to 12% for wheat genotypes and 12% and 9% for 'Dissa' and 'W 193', respectively. The two methods of selection did not show many differences in the number of survival of calli when the selection was completed for a particular genotype. 'Dissa' gave a large number of calli resistant to the toxic compounds of *Helminthosporium* compared to the 'W 193' genotype but a large number of 'W 193' calli regenerated roots during selection cycles.

Testing surviving callus lines. Calli from the selected cell lines of barley were placed on toxic and non-toxic me-

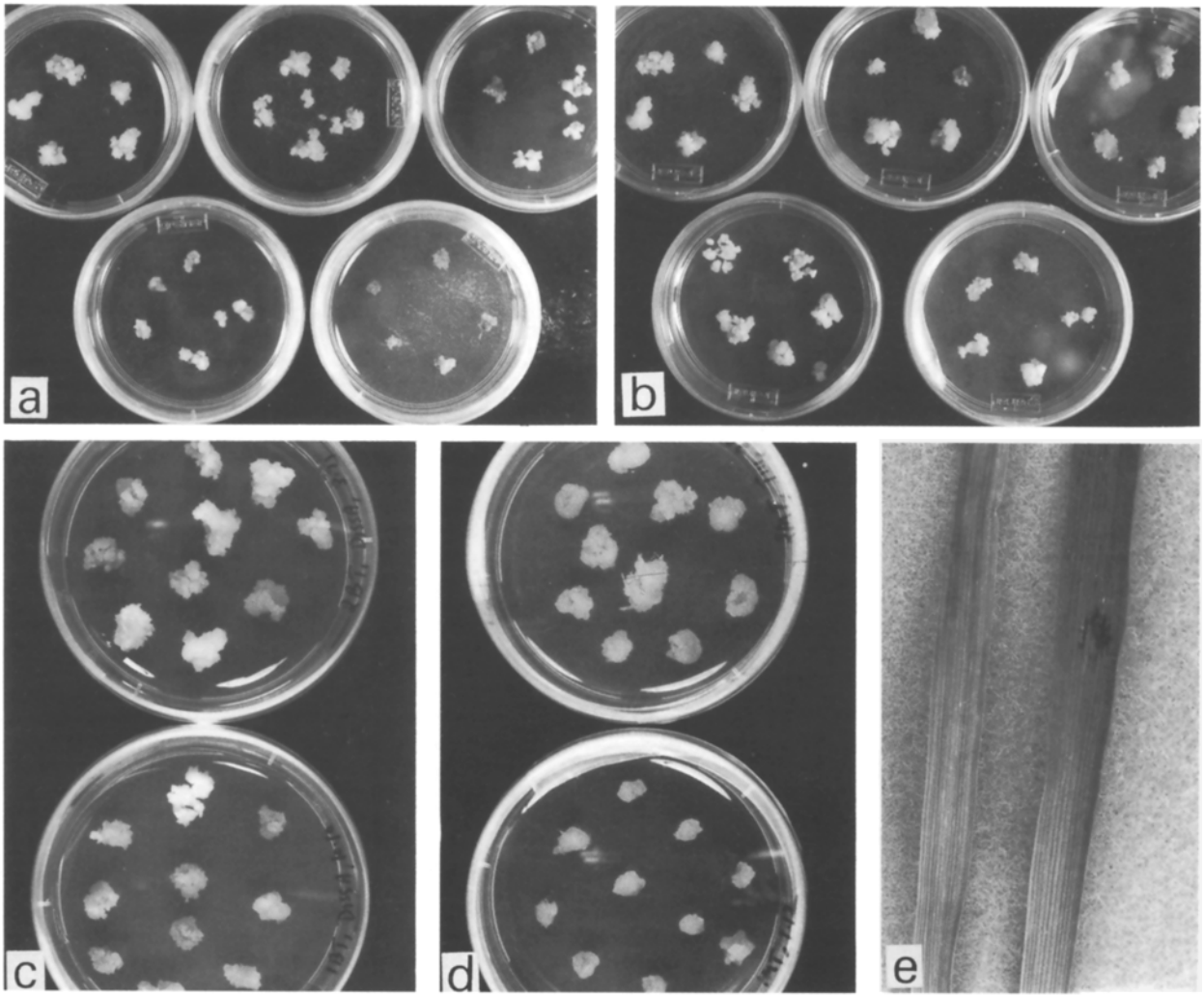

Fig. 1. **a** Effect of pathotoxin at concentrations of 0, 5, 20 (*upper row*), 100 and 200 units on callus growth of barley; **b** the same experiment with extract units from uninoculated fungal culture medium added. **c** Barley calli and **d** wheat calli after the first and second cycles of selection on toxic media. White callus is alive and dark structures are dead. **e** In vivo reaction of barley leaves of an in vitro selected (*left*) and an unselected control plant to 5 µl spore suspension with 10^6 *Helminthosporium sativum* spores per ml

dia and increase in fresh weight was measured after 3 weeks. A large amount of variation in fresh weight increase in both media was observed. There was less increase in fresh weight on toxic medium than on non-toxic medium. As such the insensitive callus lines showed stability of this trait to the pathotoxic compounds after they had been grown on non-toxic medium. As a large amount of variation was observed within a particular medium, a statistical comparison of the growth rate of the selected lines in two media will not give authentic results.

Characterisation of callus. Toxin insensitive lines of 'Dissa' and randomly taken sensitive lines of callus were studied for isozymes of proteins. It was observed that toxin selected lines had one band of protein (isozyme) more than the sensitive lines (Fig. 2). Otherwise there were no differences in the isozymic pattern.

In vivo reaction of regenerated plants against pathogen. After selection on toxic medium, surviving calli were grown on maintenance medium without hormones and parts of active calli were placed on regeneration medium. A total of 20 barley and 6 wheat lines have been regenerated after selection (Table 3). Plant regeneration frequency in barley after the two selection procedures varied considerably. The regeneration potentiality of the calli from the continuous method was less than in discontinuous method for both genotypes. From 'Dissa', 12 and 24 plants have been regenerated by continuous and discontinuous methods, respectively. So, far in vivo testing of 33 regenerated plants of 'Dissa' to the patho-

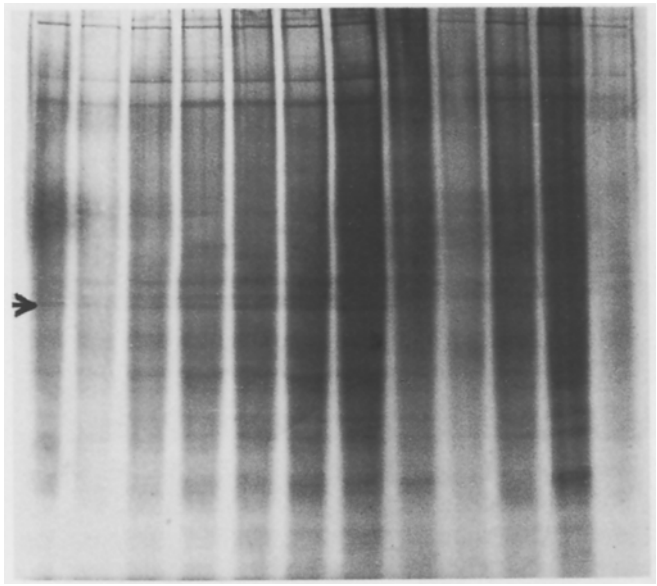

Fig. 2. Zymogram showing protein isozymic pattern in callus lines insensitive to *Helminthosporium sativum* (*first 7 tracks*) and sensitive lines of barley (*last 5 tracks*)

Table 2. Response of immature embryo derived calli of barley and wheat to the toxin preparation of *Helminthosporium sativum*

Genotype	No. of calli	No. of surviving calli after selection cycles			
		1	2	3	4
Continuous method					
Barley					
Dissa	1,145	320 (28%)	230 (20%)	190 (17%)	176 (15%)
W 193	450	93 (21%)	56 (12%)	35 (8%)	26 (6%)
Wheat					
Atys	1,360	331 (24%)	243 (18%)	189 (14%)	158 (12%)
Pitic 62	500	118 (24%)	85 (17%)	66 (13%)	54 (11%)
Discontinuous method					
Barley					
Dissa	1,310	376 (29%)	–	–	218 (17%)
W 193	410	94 (23%)	–	–	39 (10%)

gen has revealed 17 insensitive plants (9 lines), 13 with very few brown spots (5 lines) – called intermediate – and 3 plants (2 lines) which were susceptible (Fig. 1e). From 'W 193', 11 plants could be regenerated but until now all are albino. In wheat from the 6 lines (9 plants) tested against the pathogen, 7 plants were resistant and 2 showed an intermediate reaction. Neither insensitive nor resistant reactions were observed in 150 randomly taken, unselected plants. Thus, selection for toxin insensitivity in callus cultures resulted in plants that were less sensitive to the causal organism.

Discussion

In the 1970's numerous attempts were made to obtain novel disease-resistant plants through induced mutations. With the development of cell culture technique, it was hoped that mutants with disease resistance would show up in vitro more frequently due to somaclonal variation than they did using older approaches. However, initial hopes were diminished by two main factors: (a) difficulties in regenerating plants after long periods of in vitro selection against toxins and (b) the traits selected in culture systems were sometimes not expressed in the regenerated plants. The high genetic variability in in vitro cultures, as well as the correlation sometimes observed between in vitro and in vivo responses of a host to the pathogen supports the use of in vitro approaches in resistance breeding (Wenzel 1985). The present experimental evidence demonstrates that cereals which are rather recalcitrant to in vitro culture techniques can become amenable to in vitro selection.

Gengenbach and Green (1975) in their selection experiment on maize increased the concentration of toxin in the media slowly, but an intense selection was applied from the very beginning to kill about 80% of the calli. The selection in the first cycle killed around 70%–80% of the calli in both genotypes and these insensitive calli were further transferred to the subsequent cycles of selections to maintain this. Continuous and discontinuous modes of selection were employed in barley because after long culture on toxic medium they could lose the regeneration ability. The numbers of resistant callus lines obtained after selection by the two methods were the same, but the discontinuous method showed the apparent advantage of high regeneration ability over the continuous method.

The selected callus lines were characterised biochemically. They were clearly genetically different from the unselected callus which has an extra isozyme. It cannot be said that protein found in the selected lines was synthesised in vivo due to genetic change because of toxin pressure or that it was a translation of mRNA already present in the cell. Genetic experiments to confirm nuclear or cytoplasmic inheritance remain to be done. These will be of particular interest, as the *Helminthosporium maydis* resistance was located on the mtDNA (Brettell et al. 1980). In contrast to Gengenbach and Green's (1975) experiment, not all re-

Table 3. Regeneration frequency of wheat and barley calli insensitive to pathotoxins and in vivo reaction of regenerated plants to *Helminthosporium sativum*. R: insensitive; I: intermediate; S: susceptible

Genotype	Calli	Reg.	Shoots	Green	Albino	Disease reaction			
						Plants (Lines)	R	I	S
Barley									
Dissa	599	65	44	36	8	33 (20)	17	13	3
W 193	91	15	11	–	11	–			
Wheat									
Atys	92	45	4	4	–	4 (3)	3	1	–
Pitic 62	44	11	7	7	–	5 (3)	4	1	–

generated plants were completely insensitive or sensitive but a few showed incomplete tolerance, i.e. many fewer symptoms and at a later stage, and very few plants were susceptible, when in vivo inoculated with the pathogen. The variation in reaction to the pathogen obtained in the experiments indicates that the nuclear genome probably influences resistance to *Helminthosporium sativum* in wheat and barley rather than the plasmon, which should result in a qualitative reaction.

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