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In vitro selection of alfalfa plants resistant to *Fusarium oxysporum* f. sp. *medicaginis**

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Summary. From two lines of *Medicago sativa* characterized by a high regeneration capability, calli resistant to culture filtrate of *Fusarium oxysporum* f. sp. *medicaginis* have been selected. In these calli regeneration capability was greatly reduced and only one plant per callus was recovered. Regenerated plants have been evaluated for resistance to culture filtrate and for in vivo resistance to the pathogen. Three plants out of eight were resistant to the fungus and a high correlation between resistance to culture filtrate and in vivo resistance was observed.

Key words: *Medicago sativa* – *Fusarium oxysporum* – Culture filtrate – Disease resistance

Introduction

The genetic improvement of alfalfa for forage yield is not easily achieved by working on polygenic characters. It could be more easily accomplished by considering traits with a narrow genetic base, such as disease resistance, particularly when they seriously affect forage production (Elgin 1981). This seems to be the case in *Fusarium* wilt of alfalfa, which is caused by *Fusarium oxysporum* f. sp. *medicaginis*, and which has been observed in many areas throughout the world (Graham et al. 1979), Italy included (Raynal 1986; Gondran 1986).

In vitro culture is a promising tool for genetic improvement. It may simplify two steps of the plant breeding process: the induction of novel variability and the screening of large populations. Tissue culture revealed an effective method in increasing genetic variability and its utilization plays an even more important role for polyploid and vegetatively propagated species (Foroughi-Wehr et al. 1986).

As the mechanism of somaclonal variation is not yet well understood, the process of inducing novel variability via in vitro culture has not been completely rationalised. The best results are expected for single or few loci controlled traits, where single mutations are sufficient for the expression of the desired phenotypes. Therefore this technique could be particularly useful for introducing disease resistance when it is controlled by a few genetic loci.

A selection criterion widely used up to now is the growth of the callus in the presence of culture filtrates (Sacristan 1982). Although this method does not assure that plants regenerated from resistant calli will be also resistant to the pathogen, it has been effective for several plant-parasite systems (Gengenbach et al. 1978; Hartmann et al. 1984; Ling et al. 1985).

The purpose of this research is to obtain plants resistant to *Fusarium oxysporum*, via in vitro selection to culture filtrate, starting from a variety of alfalfa widely cultivated in central Italy.

Materials and methods

Two genotypes (P1 and P2) selected from the variety "Adriana" for their high regeneration ability through somatic embryogenesis (Arcioni et al., unpublished) were utilized. Adriana is widely cultivated in central Italy and is susceptible to *Fusarium oxysporum*. The cultivar F208/05, resistant to the fungus (supplied by Dr. I. Bocsa, Agricultural Research Institute, Kompolt, Hungary), was also included in our experiments.

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The pathogen (*Fusarium oxysporum* f. sp. *medicaginis*) was supplied from the Commonwealth Mycological Institute, Kew Surrey, England. To ensure the persistence of virulence, susceptible alfalfa plants were inoculated according to the procedure of Frosheiser and Barnes (1978) and the virulent fungus was again isolated at the Institute of Plant Pathology, Perugia, Italy. The pathogen was kept at 24 °C in Czapek Dox agar solidified medium and subcultured every month. For toxin production, the fungus was surface cultured in 200 ml Czapek Dox Broth in 500 ml flasks at 24 °C for 21 days. The medium was filtered first through a double layer of gauze, then through a filter paper and finally filter sterilized (Ø = 22 μm).

Toxic media were prepared by replacing part of the distilled water of the normal medium with an equivalent volume of culture filtrate. To avoid thermal degradation of toxic compounds in the culture filtrate, it was added after autoclave sterilization of the medium.

Callus was induced from leaves of 2-month-old plantlets grown in the growth chamber (23 °C, 12,000 lux, 12 h photoperiod). Scratched leaves, previously sterilized (10 min in 5% v/v Domestos bleach) and rinsed (tap-water, 6 changes), were incubated (22 °C, 1,500 lux) in B5 solidified medium (Gamborg et al. 1968) supplemented with 2,4-D (2 mg/l) and kinetin (0.1 mg/l). Cultures were routinely transferred every 3 weeks.

Selection of calli resistant to culture filtrate was performed with two different approaches: direct selection and selection with habituation. In the first method, 6-week-old calli were transferred to toxic B5 medium containing 10% culture filtrate. Growing sectors of calli were maintained on the toxic medium for 3 months. In the second approach, the selection procedure was performed as previously described but was preceded by 9 weeks of culture on B5 medium containing 1%, 5% or 7.5% culture filtrate. The selection took place only on the 10% culture filtrate medium because at the lower concentrations the callus growth was still appreciable.

The efficiency of in vitro selection was tested by comparing the growth (percent increase of weight after 3 weeks of culture) of selected and unselected calli (0.2–0.4 g calli) on toxic (10%) and non toxic media.

Plant regeneration from resistant calli was achieved on hormone-free MS (¼ strength) solidified medium (Murashige and Skoog 1962). Embryo formation occurred after 2 months and subculture was performed after 1 month. Embryos were then removed, cultured again on the same medium, and plantlets transferred to the soil and kept for 3 weeks under transparent plastic to prevent dessication (23 °C, 8,000 lux, 12 h photoperiod).

The chromosome number of the plants regenerated from the resistant calli was assessed in root-tips, pretreated with hydroxiquinoline 0.002 M for 4 h, then fixed in ethanol-acetic acid 3:1 v/v and stained with Feulgen. Ten metaphases per plant were counted.

In order to evaluate in vitro the induced resistance to culture filtrate, 1-month-old calli (0.2–0.4 g) induced from leaves of some regenerated plants were grown on B5 toxic (10%) and non toxic media.

Resistance to culture filtrate of regenerated plants was also detected by their tissue permeability. For this, 70 leaf discs (Ø = 3 mm) per plant from randomly collected leaves were suspended in a 25% (v/v) aqueous solution of culture filtrate (30 ml). The suspension was kept under moderate agitation and leaf electrolyte loss was measured by changes in electrical conductivity of the solution after 40, 90, 120, 160 and 200 min. The values of electrical conductivity were expressed as the difference between treatment (25% culture filtrate solution plus leaf discs) and the control (25% culture filtrate solution only). The experiment was repeated 3 times and the results evaluated by the analysis of variance.

Evaluation of the in vivo response to *Fusarium oxysporum* was carried out according to the method of Frosheiser and Barnes (1978), inoculating 25 vegetative propagules of each plant regenerated and 25 plants of P1, P2 and F208/05. The inoculated plants and uninoculated controls where grown in the growth chamber for 6 weeks (22 °C, 12,000 lux, 16 h photoperiod). Response to the infection was assessed with the visual rating system (Frosheiser and Barnes 1978), scoring plants for their root discoloration (0 = no discoloration, resistant; 5 = fully discolored, susceptible). Average severity index (ASI) of each genotype was computed and compared with control plants by the *t*-test. The correlation coefficient between data obtained in vitro (tissue permeability) and resistance in vivo was calculated on the basis of the mean values of each genotype.

In vivo resistance to *Fusarium oxysporum* was also evaluated for plants regenerated from calli induced from P1 (RP1) and P2 (RP2) genotypes. In all, 25 plants per population of regenerants were cloned (5 scions per plant), inoculated and scored for disease resistance as previously described.

Results and discussion

Table 1 shows the increase in fresh weight (in percentage of initial weight) of calli of susceptible and resistant genotypes grown on media at different concentrations of culture filtrate. Calli of P1 and P2 were characterized by a slow growth rate which is a typical feature of *M. sativa* genotypes with good regeneration capability (Lupotto 1983). Increasing the concentration of culture filtrate up to 7.5% affected callus growth only slightly. At concentrations higher than 7.5% P1 and P2 calli died, while F208/05, although inhibited, was still growing on the 10% culture filtrate. The callus of this last

Table 1. Callus fresh weight increase as percentage of initial weight after 4 weeks of culture on media containing different *Fusarium oxysporum* f. sp. *medicaginis* culture filtrate concentrations. Values shown are means ± SE

Genotypes	Concentration of culture filtrates						
	Control	1%	5%	7.5%	10%	15%	25%
P1	189±33	160±27	175±30	120±19	−22± 4	−20±9	−29±9
P2	91±15	100±18	110±21	70± 9	−15± 3	−31±5	−35±4
F208/05	353±20	310±25	302±31	280±21	123±24	10±3	−12±8

Table 2. Fresh weight increase (as percentage of initial weight) of selected and unselected calli after 4 weeks of culture on B5 and on the same medium containing 10% *Fusarium* culture filtrate (B5 10%). Values shown are means ± SE

Culture media	P1		P2		F208/05	
	Selected callus	Unselected callus	Selected callus	Unselected callus	Selected callus	Unselected callus
B5	48±20	165±31	68±18	81±26	253±19	324±31
B5 10%	65±22	−28±10	44±22	−13± 8	210±26	123±24

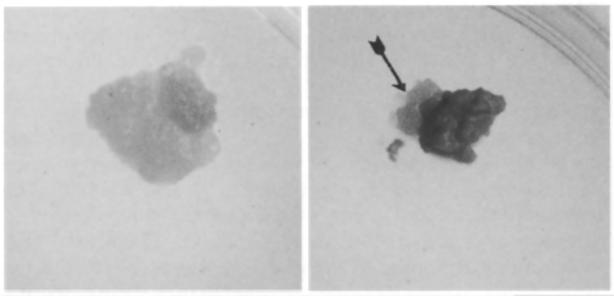

Fig. 1. *Medicago sativa* calli after three weeks of culture on B5 (*left*) and on B5 with 10% culture filtrate (*right*) media. *Arrow* indicates surviving cells

cultivar survived after 1 month on 15% culture filtrate but died on medium containing 25% toxic filtrate.

Apparently growth inhibition was not due to the presence of the pathogen's growth medium alone because no significant differences between normal B5 and B5 with 10% Czapek-Dox Broth media were found. The remarkable differences between our results and the data of Hartman et al. (1984) were probably due to the different fungal strains and growth procedures adopted. In the presence of 10% culture filtrate, calli of P1 and P2 were necrotic but occasionally they showed small clumps of surviving cells (Fig. 1). On the media containing 15% culture filtrate, a complete growth inhibition was observed. On the basis of these observations in preliminary experiments, the selection of resistant cell lines was initially attempted on medium with 10% culture filtrate for 7 months, but after such a long time the calli completely lost plant regeneration capability. Thus the two different procedures of selection mentioned in "Materials and method" (direct selection and selection with habituation) were later adopted.

Resistant cell lines of P1, P2 and F208/05 were obtained from both selection procedures. The resistance of the selected calli was evaluated by measuring their fresh weight increase on toxic and non toxic B5 media and comparing these data with analogous measures of unselected calli. No significant differences were observed among calli selected from the same genotype. Average values are reported in Table 2. Selected calli of P1, P2 and F208/05 grew on both normal B5 and B5 with 10% culture filtrate media, while the unselected P1 and P2 died when cultured on the medium with *Fusarium* culture filtrate. Culture filtrate selection caused a reduction of callus growth rate and this does not seem an inhibitory effect of culture filtrate but a stable alteration maintained also in the absence of toxic compounds. This phenomenon is particularly evident in P1-derived calli.

Plant regeneration

Resistant calli of P1 and P2 showed a regeneration ability much lower than that of unselected material. They produced a reduced number of embryos, most of which were abnormal and failed to grow into complete plantlets. Thus plant recovery was very low (10% of the control).

Six plants were regenerated from selection with habituation and were designated by the name of the original genotype followed by the culture filtrate concentration used for habituation (P1-1, P1-7.5, P2-5 a, P2-5 b, P2-7.5 a, P2-7.5 b) and two plants were obtained from direct selection (P2 a and P2 b). Conclusions on the efficiency of the two selection methods could not be made due to the low number of regenerated plants.

In cultivar F208/05, no plants were obtained from selected or unselected calli. This can be explained by plant regeneration in allogamous forage legumes being genotype specific (Pezzotti et al. 1984; Arcioni et al. 1985).

Evaluation of plants regenerated from selected cell lines

It is widely reported that in vitro regeneration frequently produces alterations of chromosome number. In our experiments, all the regenerated plants had the chromosome number characteristic of the species (2n = 32) and did not show particular alterations in morphological traits which are usually associated with chromosome number variation; this could be due to the relatively short callus phase.

Latunde-Dada and Lucas (1983) reported an association between the increased ploidy level and tolerance to *Verticillium albo-atrum* and hypothesized that the resistance was due to gene dosage effect. Hartman et al. (1984) also observed an increased chromosome

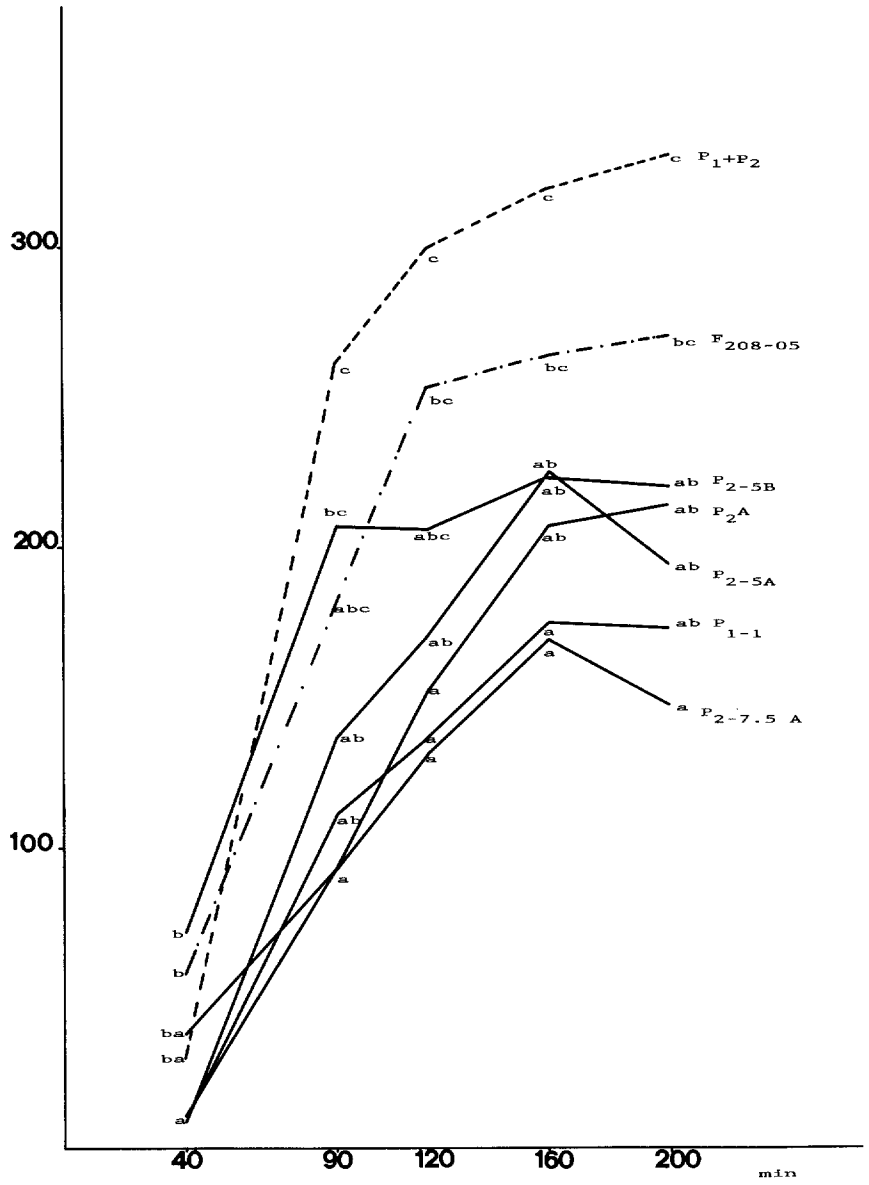

Fig. 2. Electrical conductivity values of leaf discs suspended in 0.25% (v/v) aqueous solution of culture filtrate. Values at each incubation time followed by the same letters do not differ at $P \leqq 0.05$

number in the plants resistant to *F. oxysporum* but in a second experiment with a shorter duration of callus phase, they obtained resistant plants with an unalterated chromosome number. Our results confirm this last finding and indicate that chromosome alteration is not mandatory for the achievement of disease resistant lines.

Leaf-derived calli of two regenerated plants (P1-1 and P2-7), were evaluated for their growth on toxic and non toxic B5 media and no significant differences were found. The remaining regenerants were not tested. The capacity of the callus originating from a plant regenerated after selection to grow in the presence of culture filtrate is an indication that the induced resistance was probably of a genetic nature.

Alteration of cell permeability is one of the characteristic features of the reaction of a plant to disease infection (Naef-Roth 1972). In several species, for instance, loss of electrolytes occurs in tissues infected with the fungus or treated with its toxin (Wheeler and Black 1963; Samaddar and Scheffer 1968) or with its culture filtrates (Collins and Scheffer 1958).

The results in Fig. 2 show that the regenerants lost fewer electrolytes than the susceptible parents. These differences became significant after 90 min from the treatment with the toxic medium. After 120 min P2a, P2-5a, P2-5b did not significantly differ from F208/05, the resistant variety, while P2-7.5a and P1-1 showed values significantly lower than that of F208/05. Furthermore the statistical analysis of pooled data at

different times indicated that the plant P2a also significantly differed from the resistant variety.

The conductivity test (Fig. 2) and the callus growth in toxic medium (Table 1) of the resistant variety F208/05 indicate that this genotype, known to be resistant in vivo, shows in vitro a higher tolerance to culture filtrates than susceptible genotypes. On this basis we propose the use of these in vitro techniques for early screening in traditional plant breeding and for selecting resistant genotypes (see also Buiatti et al. 1985).

The in vivo response to the pathogen is reported in Table 3. Plants P1-1, P2-7.5a and P2a had average severity index (ASI) values of 0.7, 1.0 and 0.9 respectively, indicating that they were significantly less affected by the infection than parental plants P1 = 2.5 and P2 = 2.4. However they did not differ significantly from F208/05 (ASI = 1.0). Nevertheless they can be considered resistant according to Frosheiser and Barnes (1978). On the other hand, regenerants P1-7.5, P2-5a, P2-7.5b and P2b, with the same ASI values as the susceptible parents can be defined as susceptible. Plant P2-5b resulted in intermediate resistance because it differed neither from the resistant nor from the susceptible control. It is interesting to note that resistant plants also exhibited the lowest values of tissue permeability as measured by the test reported in Fig. 2. This finding was statistically confirmed by the positive and significant correlations between ASI and electrical conductivity values (Table 4).

In spite of the encouraging results of in vivo response to the pathogen, the stability in subsequent generations and the genetic nature of the observed resistance has to be demonstrated by progeny analysis. The genetic origin of the induced resistance is, however, supported by its preservation for long time (16 months from transfer of callus to regeneration medium until in vivo disease evaluation) and by its transmission through scions. It has been reported (Hijano et al. 1983) that the known inheritance of *Fusarium* resistance in alfalfa is controlled by two genes: a dominant gene, FW1, and one with an additive type of action, FW2. Appropriate crosses will reveal which gene is responsible in our material for the induced resistance.

Tissue culture by itself can provide large genetic variability (Larkin and Scowcroft 1981) that can be successfully used for practical applications. Disease resistant plants were obtained in sugarcane (Heinz et al. 1977), in potato (Matern et al. 1978; Shepard et al. 1980), in rape (Sacristan 1982) and in alfalfa (Latunde-Dada and Lucas 1983) without any specific selection procedure but simply by testing the regenerants for disease resistance. In order to verify whether this approach is also successful with our material, several plants were regenerated from leaf derived calli of P1 (RP1) and P2 (RP2) and evaluated for in vivo response to the fungus (Table 5). Significant variability for disease resistance was recorded only among RP1 regenerants, but no resistant plants were found. The differences between our results and those previously reported (Latunde-Dada and Lucas 1983) could be due to the different starting genotypes and in vitro techniques, to

Table 3. In vivo responses to *Fusarium oxysporum* of plants regenerated from culture filtrate resistant calli and susceptible (P1, P2) and resistant (F208/05) controls. *n.s.*: not significant

Plant designation	ASI[a]	Significance of *t*-test (Selected vs parents P1, P2)	Significance of *t*-test (Selected vs F208/05)
P1-1	0.7	***	n.s.
P1-7.5	2.5	n.s.	**
P2-7.5a	1.0	**	n.s.
P2a	0.9	**	n.s.
P2-5a	2.2	n.s.	*
P2-5b	1.6	n.s.	n.s.
P2-7.5b	2.2	n.s.	*
P2b	2.0	n.s.	*
P1	2.5		**
P2	2.4		**
F208/05	1.0		

[a] Average severtiy index (ASI)
* Significant ($P \leq 0.05$); ** Significant ($P \leq 0.01$); *** Significant ($P \leq 0.001$)

Table 4. Correlation coefficients between average severity indexes (ASI) and electrical conductivity values measured at different times after incubation. Plants regenerated from culture filtrate resistant calli altogether with their susceptible progenitors P1 and P2 and the resistant variety F208/05 were considered. *n.s.*: not significant

	Incubation time (min)				
	40	90	120	160	200
Correlation coefficients	0.24 n.s.	0.85*	0.80*	0.82*	0.71 n.s.

* Significant ($P \leq 0.05$)

Table 5. In vivo responses to *Fusarium oxysporum* of RP1 and RP2 plants regenerated respectively from the susceptible P1 and P2 genotype. *n.s.*: not significant

Regenerants	Average severity index		
	Mean	Range	Significance of variance
RP1	3.1	1.8–5	**
RP2	3.2	2.2–5	n.s.

** Significant ($P \leq 0.01$)

the shorter duration of callus phase and to the type of regeneration. It cannot, however, be ruled out that resistant plants could arise in a larger population. In any case, the low frequency of resistant plants and the amount of work necessary for their detection show the convenience of culture selection on toxic media (Sacristan 1982; Thanutong et al. 1983; Scala et al. 1984).

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