Plant Cell, Tissue and Organ Culture **22**: 191–196, 1990.

Selection for resistance to filtrates of *Fusarium* spp. in embryogenic cell suspension culture of *Medicago sativa* L.

Pavla Binarová[1], J. Nedělník[2], M. Fellner[1] & Božena Nedbálková[2]
[1]*Institute of Experimental Botany, Czechoslovak Academy of Sciences, Sokolovská 6, 772 00 Olomouc, Czechoslovakia;* [2]*Research and Breeding Institute of Forage Crops, 664 41 Troubsko u Brna, Czechoslovakia*

Received 20 February 1989; accepted in revised form 28 February 1990

Key words: culture filtrate, disease resistance, embryogenic cell suspension culture, *Fusarium* spp., *Medicago sativa* L.

Abstract

A highly embryogenic cell suspension of alfalfa derived from a genotype sensitive to *Fusarium oxysporum* was successfully used for selection in vitro for resistance to culture filtrates of *F. oxysporum*, *F. solani* and *F. avenaceum*. Fifty two stable resistant cell lines were obtained and 500 plants regenerated from them. Among the 167 regenerants tested under glass there were 12–20% more plants with increased resistance to pathogens than in the group of plants regenerated from a control cell line. It was also found that the cell suspension cultures derived from genotypes of alfalfa with increased resistance to *Fusarium* spp. better tolerated filtrates of the pathogen. The results of a comparison of virulence of individual isolates of several species of *Fusarium* with toxicity of their filtrates to plants in vivo and in cell cultures were not unequivocal.

Introduction

Fungi of the genus *Fusarium* Link. ex. Fr. are one of the main causes of alfalfa necrosis under middle Europe conditions. Together with other pathogenic microorganisms, they contribute to vascular wilt and crown and root rot. The species most frequently isolated from necrotic tissues of the plants affected are *Fusarium oxysporum* Schlecht, *Fusarium solani* (Mart) Sacc and *Fusarium avenaceum* (Corda ex Fr.).

Fungi of the genus *Fusarium* are known to produce biologically active secondary metabolites displaying phytotoxicity in biotests on plants, but their role in pathogenesis is not yet quite clear (Hartman-Mitchell et al. 1983; Scheffer 1983). For selection in vitro a culture filtrate is usually used. Hartman et al. (1984) and Arcioni et al. (1987), selecting alfalfa callus culture for resistance to filtrate of *Fusarium oxysporum* f. sp. *medicaginis*, obtained resistant calli. Plants regenerated from them had increased resistance to infection in vivo with the fungus in question.

Cell suspension cultures have undoubted advantages over callus cultures for selection in vitro. We therefore decided to use a highly embryogenic cell suspension of *Medicago sativa* for selection for resistance to filtrates of *Fusarium* spp. The line is well characterised and has previously been used in selection for resistance to analogues of amino acids (Binarová et al. 1989).

Material and methods

The cell suspension culture

A highly embryogenic cell suspension culture of *Medicago sativa* L. (2n = 4x = 32) designated G13/K5 was initiated from recent breeding material. Details of the derivation, maintainance and growth characteristics of the suspension are to be found in Binarová & Novák (1985) and Binarová &

Doležel (1989). The cell suspensions designated ADV, NU and DE were similarly derived from genotypes of the varieties *Medicago sativa* Advantage, Nutiva and Derby, exhibiting increased resistance to *Fusarium oxysporum* (Nedělník 1988a).

Fungal microorganisms

All fungal isolates (*Fusarium oxysporum* Schlecht -isolates 162, 170, *Fusarium solani* (Mart) Sacc -isolates 143, 147, *Fusarium avenaceum* (Corda ex Fr.) Sacc—isolates 157, 158) were isolated from necrotic tissue of the roots of alfalfa plants. Determination of fungal cultures was performed according to the monograph of Booth (1971) and compared with stock material.

The virulence of various isolates was determined by a laboratory method. Seven days old plants were inoculated by individual isolates and test was evaluated after 4 weeks cultivation. Inoculated plants were divided into 4 groups in according morphological changes of leaves and hypocotyls (degree of infection). Virulence was expressed as average degree of infection (ADI) (Nedělník 1986, 1988b).

Preparation of toxic culture filtrates

The medium described by Bilay (Bilay 1977) containing 2% sucrose was inoculated with a spore suspension of the fungal isolate in question (20 ml suspension with a titre of 5.10^4 spores ml^{-1} per 100 ml medium). After four weeks stationary cultivation in a darkened incubator at a temperature of 24°C the mycelium and conidia were removed by centrifugation and the supernatant filtered through bacterial filters (0, 2 μm pore size). The supernatant was stored at −20°C.

Biological test of phytotoxicity of culture filtrates

Five weeks old alfalfa plants (cv. Palava) precultivated in a greenhouse were used for biotests of phytotoxicity. The plants were hydroponically cultivated for 10 days in culture medium and/or in the culture filtrates. After the end of the experiment changes in the weight of plants and their habit were evaluated according to a five point scale: (1) green leaves, (2) generally chlorotic leaves, (3) leaf necrosis with or without chlorosis and leaf curl in less than 50% of plants, (4) leaf necrosis and leaf curl in more than 50% of plants, (5) leaf wilt. Phytotoxicity of filtrates was expressed as average degree of phytotoxicity (scale 1–5).

Selection in vitro

A cell suspension in the exponential growth phase with a density around $2–10.10^4$ live cells ml^{-1} was plated out. The method employed was the pouring technique of Hughes (1984) using BL medium (Blaydes 1966) containing 5 μM 2,4-D and 1 μM KIN. Cell viability prior to plating was determined by means of fluorescein diacetate staining (Widholm 1972). Plated cells were cultivated in a darkened incubator at a temperature of 26 ± 1°C. The colony count on control and selection media was performed after 3 weeks incubation. Colony formation achieved on the control medium was expressed as plating efficiency (PE).

Individual culture filtrates obtained from 5 isolates of *Fusarium* as described above were used for selection. The inhibiting effect of filtrates on cell division and growth of cell colonies was expressed as the percentage fall in PE on media with toxic filtrates (the RPE—relative plating efficiency).

Development of resistant regenerated plants

The highly embryogenic cell suspension G13/K5 was used for selection for resistance to filtrates of several isolates of *Fusarium* spp. (147, 162, 170). The toxic concentration, used in further selection, was determined for each new filtrate as the concentration at which cell colony growth was reduced by 500–1000 times (i.e. RPE = 0.2–0.1%). This toxic concentration of filtrate varied in the range 5–20%. After three weeks culture on selection media the resistant cell lines were transferred to standard medium (BL 5/1) and cultivated for 2–3 months. After this the callus was again placed on medium containing toxin to assess the stability of the resistance. This was verified as the increment of fresh weight found after three weeks culture. Another part of the callus was transferred to BL

Table 1. Comparison of virulence of several isolates of *Fusarium* spp. and the toxicity of their culture filtrates to plants and cell suspension G13/K5.

Species of fungus	Isolate No	Virulence-average degree of infection (ADI) (Scale 0–3)	Average degree of phytotoxicity of filtrates in biotests on plants (Scale 1–5)	Fresh weight reduction of plants in biotest (%)	Toxicity of 10% filtrate in cell suspension G13/K5, relative plating efficiency (%)
F. solani	143	2.80	5.00	−45.1	1.80
F. solani	147	2.80	5.00	−35.3	5.18
F. avenaceum	157	2.16	3.83	−32.6	3.70
F. avenaceum	158	2.10	3.50	−33.3	9.60
F. oxysporum	162	1.53	3.67	−41.7	1.20

medium without growth regulators, where differentiation of somatic embryos occurred. Regenerated plants were gradually transferred to soil. In some of them the number of chromosomes in the root tips was determined by fuchsin staining (Carr & Walker 1961).

Test of sensitivity of regenerated plants to the isolates of Fusarium *spp.*

Prior to inoculation the plants were removed from the soil, and following washing of roots in distilled water the root tips were cut off and the plants immersed for 24 hours in inoculation spore suspension. Subsequently they were individually transferred to sterilised soil. The criterion for evaluation was the number of plants growing even after infection and the state of health of the root system 8 weeks after inoculation.

Results

Five isolates of three species of *Fusarium* spp. were tested for virulence. Culture filtrates obtained from these isolates as described above, were subjected to biotests on plants. Individual filtrates at a concentration of 10% were used for selection using cell suspension G13/K5.

The results of this experiment are summarised in Table 1. It emerges that the filtrate of fungal isolate 143, exhibiting the highest virulence (ADI = 2.8) is the most toxic in biotests on plants. In cell suspension the filtrate of this isolate most strongly inhibited cell division; on medium containing this filtrate about fifty times fewer cell colonies grew on control medium (RPE = 1.8). A similar positive relation was found between the degree of virulence of individual fungal isolates and toxicity to plants for the remaining isolates of *Fusarium* spp. One exception is isolate 162 *F. oxysporum*, whose virulence is relatively low, but whose filtrate exhibited high toxicity even in biotests on plants and even more so in selections in cell suspension.

A cell suspension G13/K5 derived from the genotype sensitive to *Fusarium oxysporum* and suspensions ADV, NU, DE derived from genotypes exhibiting increased resistance to *F. oxysporum* were plated on media containing 10% filtrate of *F. oxysporum*, isolate 170. The toxicity of the filtrate towards individual cell suspensions was again expressed as RPE. The results are summarised in Table 2. In the cell suspension derived from the sensitive genotype G13/K5 approximately 20–100 times fewer colonies grew on a medium with 10% filtrate than on a control medium. The cell suspension derived from the genotype with increased resistance to *F. oxysporum* tolerated toxic filtrate better. On a medium with 10% filtrate only 3–6 times fewer colonies grew compared with the control medium.

In all, 63 resistant cell lines were isolated in 9

Table 2. Toxicity of filtrate of *F. oxysporum* isolate 170, to cell suspensions derived from genotypes of alfalfa differing in sensitivity to *F. oxysporum*.

Cell suspension	ADI	Relative plating efficiency (%) Experiment No		
		1	2	3
G13/K5	2.67	5.9	0.79	2.26
NU	1.17	30.9	–	–
DE	1.13	20.5	–	5.58
ADV	2.12	15.6	3.50	–

Table 3. Fresh weight reduction of selected and unselected cell lines after 3 weeks of culture on BL5/1 media containing 5, 10, 15% *Fusarium* culture filtrate (as percentage of fresh weight after 3 weeks of culture on standard BL5/+ medium).

Toxin contentration (%)	Percentage fresh weight of control		
	Unselected cell line G13/K5	Selected cell line	
		No 17	No 19
0	100[a]	100[b]	100[c]
5	54	95	92
10	8	110	105
15	0	90	85

[a-c] Fresh weight after 3 weeks of culture on standard BL5/1 medium: a: 19 ± 8 g, b: 13 ± 6 g, c: 11 ± 4.5 g.

independent experiments. It was found that 52 of the 63 lines were stably toxin-resistant (Table 3).

Regeneration capacity was decreased to various extents in individual resistant lines compared to controls. About 500 plants were eventually regenerated. The number of plants with highly changed morphology was higher among the regenerants from resistant lines than that among regenerants from control cell lines (Fig. 1). On analysis of the chromosome number we did not find changes in chromosome number. In tests for sensitivity to *F. oxysporum* and *F. solani* there was no case in which these phenotypically different plants survived infection.

The results of a study of the reaction of regenerated plants to pathogen infection can be seen in Table 4. For the time being the number of plants obtained from toxin resistant cell lines tested is 167. In 30.95% and 37.35% of cases respectively a reduced sensitivity to infection with *F. oxysporum* and *F. solani* was demonstrated. In controls this reaction was found only in 18.18%. This means that toxin resistant cell lines gave 12–20% more plants with increased sensitivity to the pathogens in question. Increased ploidy was not apparent in any of these plants.

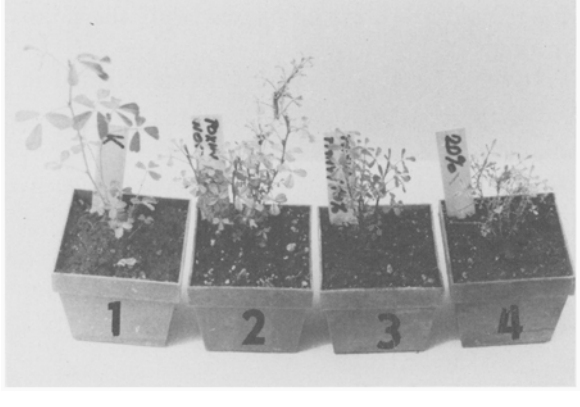

Fig. 1. Plants (no. 2, 3, 4) regenerated from selected cell lines are more often morphologically changes as compared to the control plants (1) regenerated from unselected cell line.

Table 4. Reaction of plants regenerated after selection with filtrates of *F. oxysporum* and *F. solani* to infection by the pathogens in question.

Resistant cell line	Initial number of regenerated plants	Number of plants surviving infection
G13/K5/170[+]	84	30.9
G13/K5/147[++]	83	37.3
G13/K5/K[+++]	22	18.2

[+] Cell lines obtained from selection for filtrate *F. oxysporum*, isolate 170.
[++] Cell line, obtained from selection for filtrate *F. solani*, isolate 147.
[+++] Control cell line.

Discussion

Selection for resistance to crude filtrates of pathogens in vitro may yield resistant plant, although it is not known exactly which toxins are present in the filtrates or what role they play in pathogenesis (Behnke 1980; Sacristan 1982). This is also true of filtrates of *Fusarium* spp. used successfully in the selection of alfalfa callus cultures (Arcioni et al. 1987; Hartman et al. 1984).

A positive relationship was found between the degree of virulence of a given isolate of *Fusarium* spp. and the degree of toxicity of its filtrate to both plants in vivo and cells cultured in vitro (Table 1). The results found for isolate *F. oxysporum* 162 are contradictory (exhibiting low virulence but high toxicity of the filtrate). Nedělník (1988b) and Manka & Chelkowski (1985) found a similar phenomenon in tests on plants, where lower virulence may not always be accompanied by a lower level of filtrate toxicity.

In *Fusarium* there are at present over 100 known toxic metabolites which structurally belong to the group of trichothecenes and zearalenone (Mirocha

& Christensen 1986). The level of knowledge of the role of these toxins in pathogenesis and the other physiological and biochemical bases of the interaction of *Fusarium* with plants is limited. Cell cultures derived from genotypes sensitive and resistant to *Fusarium* spp. differ by a large margin in growth and division capacities in the presence of toxic filtrates. Similarly, in the experiments of Arcioni et al. (1987) calli derived from resistant material of *M. sativa* were capable of growing on higher concentrations of toxin than calli from sensitive material.

In experiments with selection for resistance to filtrates of *F. oxysporum* f. sp. *medicaginis* in alfalfa only callus cultures have been used for the time being (Arcioni et al. 1987; Hartman et al. 1984). We have already seen in previous experiments the advantages of using an embryonic cell suspension for selection in vitro (Binarová et al. 1989). These consist chiefly in the use of mutagenesis, exposing a high number of units to selection, especially individual cells or proembryonic clumps, and obtaining a large number of regenerants. Cell suspension cultures also make it possible to test the toxicity of filtrates, which is very variable not only between isolates, but also between batches of filtrates of the same isolate, under standard conditions. Determination of a suitable growth-inhibiting concentration of filtrate then increases the probability of obtaining stable resistant cell lines.

Regeneration from a large number of resistant cell lines obtained in this way offered a chance to acquire 12–20% more plants with increased resistance to *Fusarium* spp. than in a set of plants regenerated without selection. Whether or not the resistance to *Fusarium* spp. in some of these plants is indeed genetically based, as documented by Hijano et al. (1983), will have to be demonstrated by genetic analysis of the seed progeny.

Not all cell lines stably resistant to *Fusarium* spp. filtrate regenerated plants with increased resistance to *Fusarium* in vivo. The same phenotype, i.e. resistance to toxic filtrates, may be due to different genetic or possibly epigenetic changes in different lines. Our results fully concur with the proposition of Daubt (1986) that in selection for crude pathogen filtrate one must isolate as many resistant cell lines as possible, since many of them may have been selected for resistance to components other than the putative toxin, but if enough are generated, some may carry the desired resistance.

A question frequently discussed is the effect of the toxic filtrates used for selection on changes in ploidy of the regenerated plants. Hartman et al. (1984), for instance, found polyploid plants with a greater in vivo resistance among regenerants of calli resistant to filtrate. The fact that the effect of gene dose may play a role in the increased resistance to pathogens is also mentioned by Latude Dada & Lucas (1983). In their experiments with alfalfa a low level of resistance to *Verticillium albo-atrum* in parents was simply amplified in polyploid somaclones.

In our experiments increased ploidy was not found in any of the regenerants which exhibited resistance to *Fusarium* spp. in vivo. The plants obtained by regeneration from resistant cell lines, which were phenotypically heavily altered, but sensitive to *Fusarium* infection were normal tetraploid. The bases of changes in habit which we find, though less often, even among regenerants from control cell lines, may be merely phenotypical. Arcioni et al. (1987) found among regenerants after selection for *F. oxysporum* resistance increased ploidy. Apart from the length of action of the toxic filtrates a role seems to be played here by the initial genotype used for selection. As is shown, by Alicchio et al. (1984), toxins of pathogens may affect chromosome number in cultivated cells by interacting with cell genotypes. In our experiments the cell suspension G13/K5 was used, with high stability of the 'normal' tetraploid content of nuclear DNA, both in the course of long-term culture and in the course of differentiation (Binarová & Doležel, 1989). This fact may in our case be the cause of the karyological stability shown even in selection for toxic filtrates.

We used large-scale selection, in which we managed to obtain some plants with increased resistance to *Fusarium* spp. Hartman et al. (1984) and Arcioni et al. (1987) selected callus cultures on a smaller scale, but achieved a higher number of resistant regenerated plants. We suppose this to be due to the different genetic background of the initial plant genotypes used for selection. Other works, too, indicate that the desired traits cannot be derived from just any source of parental material, and that in the case of disease resistance it would be better to start with the highest level of

resistance available in a desirable agronomic background (Daubt 1986). In such material the level of resistance can be increased much more effectively by selection in vitro than by simple screening of somaclonal variants of regenerated plants.

The results also support the concept of using cell culture technology to increase resistance to *Fusarium* spp. But they revealed the need to learn more about the biochemical and physiological bases of the interaction pathogen × plant in vivo and in cell culture in vitro. Some biochemical analyses along these lines are already under way.

Acknowledgements

The authors are grateful to Dr Jarmila Čihalíková for kindly performing to cytological work, to Miloslava Mazurová, Milena Čermáková and Jana Bácová for technical assistance.

References

Alicchio R, Antonioli C & Palenzona D (1984) Karyotypic variability in plants of *Solanum melongena* regenerated from callus grown, in presence of culture filtrate of *Verticillium dahliae*. Theor. Appl. Genet. 67: 267–271

Arcioni S, Pezzotti M & Damiani F (1987) In vitro selection of alfalfa plants resistant to *Fusarium oxysporum* f. sp. *medicaginis*. Theor. Appl. Genet. 74: 700–705

Behnke M (1980) General resistance to late blight of *Solanum tuberosum*, plants regenerated from callus resistant to culture filtrates of *Phytophtora infestans*. Theor. Appl. Genet. 56: 151–152

Bilay VI (1977) Fusarii. Kijev, p 442

Binarová P & Novák FJ (1985) A study on embryogenesis in two different types of cell culture of *Medicago sativa*. Acta Univ. Agric. Brno XXXIII(3): 293–297

Binarová P & Doležel J (1988) Alfalfa embryogenic cell suspension culture: growth and ploidy level stability. J. Plant Physiol. 133: 561–566

Binarová P, Novotný F & Nedbálková B (1990) Selection and characterization of alfalfa cell lines resistant to lysine plus threonine and/or ethionine. Biochem. Physiol. Pflanz. 185: 1–2 (in press)

Blaydes JF (1966) Interation of kinetin and various inhibitors of the growth of soybean tissue. Physiol. Plant. 19: 748–753

Booth (1971) The genus *Fusarium*. Kew Commonwealth Mycol Inst. p 237

Carr DH & Walker JE (1961) Carbol fuchsin as a stain for human chromosomes. Stain Technol. 36: 233–236

Daubt ME (1986) Tissue culture and the selection of resistance to pathogens. Annu. Rev. Phytopathol. 24: 159–186

Harman-Mitchell C, Knows TR & McCoy TJ (1983) Toxic components produced by *Fusarium oxysporum* f. sp. *medicaginis* and their use in alfalfa cell culture selection techniques. Phytopathology 73: 829

Hartman CL, McCoy TJ & Knows TR (1984) Selection of alfalfa (*Medicago sativa*) cell lines and regeneration of plants resistant to the toxins, produced by *Fusarium oxysporum* f. sp. *medicaginis*. Plant Sci. Lett. 34: 183–194

Hijano EH, Barnes DK & Frosheiser FI (1983) Inheritance of resistance to *Fusarium* wilt in alfalfa. Crop Sci. 23: 31–34

Hughes K (1984) Selection for herbicide resistance. In: Evans DJ, Sharp WR, Amirato PV & Yamada Y (Eds) Handbook of Plant Cell Culture, Vol 1 (pp 441–459). MacMillan Publ. Co., New York

Latunde Dada AO & Lucas JA (1983) Somaclonal variation and reaction to *Verticilium* wilt in *Medicago sativa* L. plants regenerated from protoplast. Plant Sci. Lett. 32: 205–211

Mirocha TJ & Christensen TM (1986) Mycotoxins and the fungi that produce them. Proc. Am. Phytopathol. Soc. 3: 110–125

Manka M & Chelkowski J (1985) Phytotoxicity and pathogenicity of *Fusarium nivale* towards cereal seedlings. Phytopathol. Z. 114: 1–5

Nedělník J (1986) The Sensitivity of some cultivars of red clovers to selected species of *Fusarium* spp. and the effect of fungicides on the growth of *Fusarium* spp. in vitro. Sb. UVTI. Ochr. Rostl. 22: 285–293

Nedělník J (1988a) The resistance of some varieties of *Medicago sativa* L. to *Fusarium oxysporum* Schlecht. Sb UVTIZ. Ochr. Rostl. 24: 191–197

Nedělník J (1988b) Pathogenicity and culture filtrate phytotoxicity in species of genus *Fusarium* Link. Ex Fr. Proc. XI Czechoslovak Plant Prot. Conf., Nitra 1988: 65–66

Sacristan MD (1982) Resistance responses to *Phoma lingam* of plants regenerated from selected cell and embryogenic cultures of haploid *Brassica napus*. Theor. Appl. Genet. 61: 193–200

Scheffer RP (1983) Toxins as chemical determinants of plant disease. In: Daly M & Deverall BJ (Eds) (pp 1–40). New York, Academic Press

Widholm (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47: 189–195