IN-VITRO SELECTION FOR FUSARIUM-RESISTANCE IN *Gladiolus*

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Abstract

The idea of using a toxin produced by a pathogen as a tool for resistance breeding dates from over 20 years ago. Several examples of germplasm screened for disease resistance using a toxin as discriminating agent are published. Such toxins may also successfully be applied in *in vitro* selection experiments. Before even attempting to exploit such compounds for breeding purposes, their role in plant pathogenesis has to be assessed. The current research is dealing with one of those toxins, fusaric acid. We searched for evidence that fusaric acid, a toxin produced by the pathogen *Fusarium oxysporum* f.sp. *gladioli*, the causal agent of gladiolus corm-rot, is involved in the rotting process of the corm. Although the fungus produces fusaric acid abundantly *in vitro*, a strong interaction between isolate and medium was found. Therefore the fusaric acid production was studied *in planta*. The toxin was detected in diseased corm tissue in amounts high enough to exert toxic effects on corm tissue. Subsequently, fusaric acid was applied in several bioassays. Fusarium-resistant cultivars were able to tolerate higher concentrations of the toxin than more susceptible ones. Fusaric acid induced part of the disease symptoms on shoots cultured *in vitro*. Callus tissue, however, was less suitable for such assays. Thirteen isolates of the fungus were tested for aggressiveness in a greenhouse and an *in vitro* assay. Results from both tests correlated strongly. Also the amount of ergosterol and fusaric acid were determined. Ergosterol reflects fungal growth and was strongly correlated with the visually observed symptoms. The amount of fusaric acid produced per unit fungus was not correlated with the visual symptoms. Therefore the hypothesis that the aggressiveness of isolates is due to their potency to produce fusaric acid was not affirmed. Since, however, the production and degradation of fusaric acid is a dynamic process, the determination of the amount of fusaric acid at a specific time location may be erratic.

The second part of the research concerned an attempt to select toxin-tolerant plants from an *in vitro* experiment. A cell suspension was challenged stepwise with increasing concentrations of fusaric acid (0.12 mM and 0.4 mM). Nine cell lines selected on 0.12 mM fusaric acid showed variable reactions when inoculated directly with conidia of the fungus. The growth of the fungus was reduced at least by 50% compared to that on non-selected callus. About 50% of the plantlets regenerated from selected calli showed increased tolerance for the toxin.

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Introduction

Corm-rot, caused by *Fusarium oxysporum* f. sp. *gladioli* is a common soil-born fungal disease in gladioli growing areas (Magie *et al.* 1972). The disease is characterized by leaf yellowing and corm rot which results in partial or complete flower and corm loss. Corm rot has been reported also for other *Iridaceae* as crocus, freesia and iris (McClellan, 1945). Partial resistance is present in some species (Straathof *et al.*, 1996) and breeding programmes are needed to transfer these resistance genes into cultivated varieties. The pathogenesis of corm rot is relatively unknown. Possibly fusaric acid, a toxin produced by many species of *Fusarium* (Drysdale, 1982) is involved. If so, it should be associated with the infected tissue in toxic amounts. If the toxin is specific, resistant plants should be insensitive to the toxin whereas susceptible genotypes should be sensitive. Furthermore, *in vitro* methods such as *in vitro* selection may be used by plant pathologists to prove the significance of a toxin in a particular plant pathogen interaction. In this paper we report in a short overview the evidence found for the possible importance of fusaric acid for corm-rot of gladiolus, along with the possibility to select fusaric acid insensitive gladioli using *in vitro* selection.

Discrimination Between Resistant and Susceptible Genotypes with Fusaric Acid

Ten *Gladiolus* genotypes, including three South African species, were tested for Fusarium resistance in a greenhouse assay and subsequently used in a number of bio-assays to measure their sensitivity for fusaric acid. The selected genotypes were: *Gladiolus callianthus* Marais, *G. garnierii* Klatt and *G. dalenii* Geel and seven *G. grandiflorus* Hort. cultivars ('Alfred Nobel', 'Amsterdam', 'Majolica', 'Peter Pears', 'Roselind' and 'White Prosperity'). *In vitro* cultured shoots of all genotypes were grown on medium supplemented with fusaric acid for two weeks. All genotypes showed with increasing concentrations of fusaric acid an increase of symptoms. At 0.35 mM fusaric acid, the Fusarium-resistant species and 'Roselind' grew well, while the susceptible 'Peter Pears' and 'Alfred Nobel' were highly affected by the toxin (Table 1). Similar responses were obtained when the electrolyte leakage from whole cormels caused by fusaric acid was measured. Cormels were immersed in a 5 mM solution of fusaric acid at pH 4.6 for 24 h. The cormels of the species and 'Roselind' were not affected by fusaric acid. The most affected cultivars were 'Peter Pears', 'Hawaii' and 'Majolica' (Table 1). However, when electrolyte leakage was measured from callus clumps immersed in a 0.35 mM fusaric acid solution, the results were less consistent. Also varying results were obtained when the growth rates of callus cultured on medium with fusaric acid were measured. Some genotypes, as 'Hawaii' and 'Majolica' grew well on this medium while being susceptible in the inoculation test (Table 1). An other type of discordant answer was observed with callus of 'Amsterdam'. This cultivar is resistant, but when callus is grown on medium with fusaric acid, the response appears to be sensitive.

The data obtained in the shoot assay and the ion release of the cormels correlated significantly with the data-set of the greenhouse (R= -0.74, R= 0.81) and with each other(R= -0.81). Non-significant correlations were found between the greenhouse test

and the bioassays involving callus tissue. The data-sets of the last two assays correlated to each other significantly (R= -0.86).

Table 1: Susceptibility of ten gladiolus genotypes for *F. oxysporum* (1) and sensitivity for fusaric acid according to different bio-assays (2-5).

	Assay				
	1	2	3	4	5
FA concentr.	-	0.35	5.0	0.35	0.35
Genotypes					
G. dalenii	1.0	84	4.7	99.6	-5.4
Roselind	1.4	92	5.8	104.7	0.1
G. callianthus	1.6	80	4.0	98.3	8.7
Amsterdam	2.1	84	9.8	72.3	19.6
White Prosperity	2.3	60	10.2	92.4	-
G. garnierii	2.6	84	5.9	103.3	4.3
Alfred Nobel	3.3	40	11.7	83.2	15.8
Peter Pears	4.1	24	12.1	47.7	21.8
Hawaii	4.6	56	10.4	98.9	3.8
Majolica	5.0	56	11.0	91.1	9.6
$LSD_{0.05}$	0.9	20.5	1.6	7.0	4.3

1 Disease score (1-6) in the greenhouse assay after 12 weeks of culture
2 Surviving shoots (%) after 15 days on medium supplemented with 0.35 mM fusaric acid
3 Electrolyte leakage of cormels immersed in a 5 M fusaric acid solution, measured after 72 h and expressed as mS
4 Relative growth of callus clumps on medium supplemented with 0.35 mM fusaric acid after 15 days;
5 Electrolyte leakage of callus immersed in a 0.35m M fusaric acid solution, measured after 24 h and expressed as mS

Production of Fusaric Acid *in vitro* and *in planta*

Different isolates of *Fusarium oxysporum* f. sp *gladioli* were tested for their aggressiveness on the susceptible cultivar Peter Pears in a greenhouse assay. Large differences in aggressiveness were found (Table 2). The same isolates were also used in a controlled *in vitro* infection, in which disinfected corms were artificially inoculated with the isolates. The disease score of this test coincided with the disease score assessed in the greenhouse. The *in vitro* infected corms were used for the extraction of fusaric acid and ergosterol, a steroid present only in fungal membranes (Seitz *et al.*, 1979). The ergosterol contents correlated well with the visual disease score data, indicating that the visual disease assessment reflects the actual presence of the fungus.

For all isolates, also the production of fusaric acid in both culture broth and in infected corms was assessed. Fusaric acid was readily produced in Czapek Dox liquid medium by all tested isolates as determined by HPLC. Large differences in the maximal amount of fusaric acid were detected after two weeks of culture. No correlation was found with the aggressiveness of the isolates (Table 2). The presence of fusaric acid in extracts of infected corm was measured using GC analyses. For 'Hunting Song', up to 2.5 µM fusaric acid g^{-1} (dry weight) corm tissue was detected. The highly infected outer part of the corm contained less fusaric acid than the lightly infected inner part, suggesting that

the toxin diffuses from the fungus to healthy tissue. When the fusaric acid production of the isolates was tested on 'Peter Pears' corms, a slight correlation between total fusaric acid production and aggressiveness of the isolates was found. When, however, the amount of fusaric acid was calculated per unit fungus, the correlation was lost. Therefore no indications were found for the hypothesis that the aggressiveness of isolates is based on their potency to produce fusaric acid. Yet the hypothesis is not falsified either, since production and degradation is a dynamic process. Therefore at specific times and at specific locations the fusaric acid concentrations may differ from those measured.

Table 2: Average disease scores from gladiolus corms of 'Peter Pears' grown in *Fusarium* infested soil in the greenhouse experiment (DS-G), the relative plant length, assessed in the green-house after 8 weeks (RL-G), the disease scores from the *in vitro* infection, evaluated after 8 weeks (DS-V), the amount of ergosterol per gram dry weight corm tissue (ER-V), the amount of fusaric acid per gram dry weight corm tissue (FA-V) and fusaric acid content detected in the culture filtrate 15 days after culture onset (FA).

Isolate	DS-G	RL-G [%]	DS-V	ER-V [mg g^{-1}]	FA-V [µmol g^{-1}]	FA [mM]
Control	0.0	100	0.5	0.002	0.000	0.000
Fog-22	0.0	107	1.3	0.006	n.d.	0.013
Fog-13	0.5	102	3.6	0.688	0.066	7.943
Fog-20	1.5	100	1.7	0.094	0.056	0.879
Fog-76	1.5	92	1.0	0.011	n.d.	4.775
Fog-15	3.0	79	4.0	1.034	0.374	0.706
Fog-11	3.3	81	4.7	2.970	0.270	0.015
Fog-2	3.7	78	4.7	1.302	0.198	0.607
Fog-57	3.7	71	4.7	2.270	0.056	0.262
Fog-70	4.2	77	4.0	0.839	0.286	0.966
Fog-34	4.3	28	5.0	3.093	0.450	1.712
Fog-7	4.3	41	5.0	4.094	0.682	2.093
Fog-63	4.8	49	5.0	5.108	0.400	0.942
Fog-21	4.8	33	5.0	2.598	0.264	2.091
$LSD_{0.05}$	0.53	12.0	0.64	0.812	0.320	2.058

n.d. - not detected

In Vitro Selection for Toxin Tolerant Plants

An embryogenic cell suspension culture (CSC) of 'Peter Pears', induced and maintained according to Remotti (1995), was challenged with 0.06-0.14 mM fusaric acid. At 0.06 mM fusaric acid, cell growth was considerably reduced. A concentration of 0.12 mM fusaric acid was considered optimal (LD_{95}) for a good selection pressure. Twelve developing colonies were isolated from medium with 0.12 mM fusaric acid and transferred to fusaric acid free medium. The cell-lines S4-4 and S5-5 were characterised further. S4-4 was subcultured on MS with 0.35 mM fusaric acid and on fusaric acid free medium whereas S5-5 was maintained only on fusaric acid free medium. Callus clumps of these lines and of the control callus were subcultured for 9 months, before testing their sensitivity

for fusaric acid on MS medium supplemented with increasing concentrations of fusaric acid. The size increase of each clump was measured with an image analyser. Cell line S5-5 was less inhibited by the toxin than line S4-4, but both lines grew significantly better than the control callus (Fig. 1). The insensitivity for fusaric acid was maintained during cultivation on fusaric acid free medium (Fig. 1). When the cell lines were inoculated with the fungus, the selected cell lines were able to reduce mycelial growth considerably.

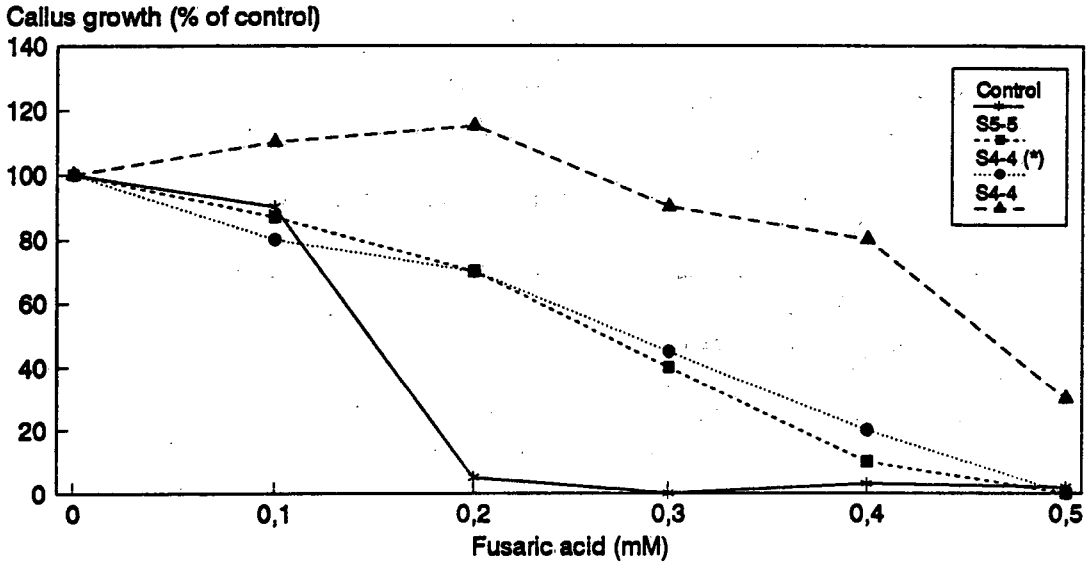

Figure 1: Growth of non-selected callus (control) and of two callus lines selected for fusaric acid-tolerance (S4-4, S5-5) on MS medium supplemented with various concentrations of fusaric acid. Line S4-4 and S5-5 are continuously maintained on fusaric acid-containing media, whereas S4-4(*) was subcultured for 9 months fusaric acid-free medium.

In a second selection experiment, 3 three-months old CSC were plated on fusaric acid-medium (0.12 mM fusaric acid). In total 395 single colonies developed. All were individually transferred to fresh medium supplemented with 0.40 mM fusaric acid and incubated during two weeks in the dark. In total 195 surviving calli were transferred to regeneration medium of which 194 plants (sometimes more than one per callus) were regenerated after six months. Plant regeneration was retarded significantly in respect to non-selected callus, which regenerated after three months. Part of the selected calli lost their regeneration ability. All plants developed well in culture, rooted easily and showed vigorous growth. Of the regenerated plants, 115 formed enough shoots *in vitro* to be tested for fusaric acid-sensitivity on medium with 0.35 mM fusaric acid. Of the regenerated plants 57 (50%) scored similarly to the control, 35 (30%) showed only few

symptoms and 23 (20%) were not affected at all by the toxin. The remaining plantlets were transferred to fresh medium for further root development, and prepared for greenhouse acclimatisation. When grown to maturity the resulting corms will be tested for *Fusarium* resistance.

Concluding Remarks

Fusaric acid affects the growth and development of gladiolus tissue. Shoots and cormels, but not always callus of fusarium-resistant genotypes tolerate the presence of the toxin better than fusarium-susceptible genotypes. Fusaric acid is found in infected tissue in toxic concentrations. These results indicate that fusaric acid is involved in the disease development. However, no indications were found that the aggressiveness of isolates is based on their ability to produce the toxin. Although fusaric acid is produced by most isolates both in liquid culture and *in planta*, no correlation with their aggressiveness was found. This may be due to the probably dynamic character of production and degradation of fusaric acid. Based on the results, we consider it likely that fusaric acid is involved in Fusarium-rot of gladiolus and can be used as agent for *in vitro* selection.

Cell-lines with decreased sensitivity to fusaric acid were selected *in vitro*. A multiple step selection, applying increasing concentrations of toxins proved successful for the final success of the selection. The cell-lines selected for fusaric acid insensitivity were able to inhibit the mycelial growth of an aggressive isolate. Toxin insensitive plantlets, regenerated from the selected cell-lines, are propagated and will be tested for Fusarium resistance in the future. This will ultimately judge the value of the use of fusaric acid for *in vitro* selection for fusarium-resistance in gladiolus.

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