

# **IN VITRO SELECTION WITH FUSARIC ACID : A NOVEL APPROACH TO BREED FOR *FUSARIUM* RESISTANCE IN *GLADIOLUS***

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## Abstract

*In vitro* selection represents a feasible method to isolate variants with improved traits. Selection *in vitro* for disease resistance is facilitated when well characterized toxins are used as selective agents. However the number of metabolic compound with a known role in the development of the disease is limited. The prospective to use a specific fungal metabolite as selective agent were investigated. We report on the use of fusaric acid for the selection of *Fusarium*-resistant plants. Before performing the *in vitro* selection, the possible use of fusaric acid (FA) as selective agent was evaluated by applying two assays involving shoots and callus tissue of 10 genotypes. Callus tissue could reflect only partially the *Fusarium*, resistance level of the genotypes while shoots cultured in presence of the toxin could reflect the real resistance of the genotype.

Fusaric acid insensitive cell lines were selected form cell suspension challenged with 0.10-0.14 mM FA. The insensitivity to this toxin was retained even after the selective agent was removed. Callus originated from the cell lines grew well on a medium supplemented with 0.5 mM FA, when inoculated with a suspension of conidia the mycelial growth was significantly inhibited. Plantlets regenerated were tested with a shoot assay and half of them showed a reduced sensitivity to the toxin. Results suggest that selection of *Fusarium*-resistant variants through *in vitro* selection is a possible breeding approach. The final evaluation of the *Fusarium*-resistance level of the regenerated plantlets will be performed once the corms are grown to maturity.

## 1. Introduction

Corm rot caused by the soil borne pathogen *Fusarium* f. sp. *gladioli* is one of the most destructive diseases in gladiolus cultivation. Though sources of high resistance have been uncovered in wild South African species (Straathof, *et al.* 1996), in the assortment of the cultivated gladioli only limited sources of resistance are present. Breeding programs for gladioli are quite time consuming and the transfer of resistance genes from wild species to cultivated varieties involves frequently a number of backcrosses and selection steps, which therefore extend the time of release of the improved variety. Therefore new breeding methods are much wanted. One of these is *in vitro* selection, i.e. selection in tissue cultures of the target crop for the desired trait with a selective agent. For the final success of an *in vitro* selection experiment the initial choice of the selective agent is fundamental.

Out of the manifold toxins produced by fungal pathogens, only a restricted number acts as host specific toxins, and does therefore play a specific role in the development of diseases. Plants which normally are disease susceptible are also toxin sensitive, whereas disease

resistant plants are always toxin insensitive. Yet also non-host specific toxins may play an important role in the pathogenesis. FA, a toxin produced by *Fusarium oxysporum* is such a non-host specific toxin. It is known that FA is present in the diseased corms in toxic concentrations.

Hence, it may play a determinant role in the *Gladiolus-Fusarium* interaction and therefore may be used efficiently in selection programs. In this paper we describe our approach to use fusaric acid as selective agent in an *in vitro* selection experiment.

## 2. Materials and methods

### 2.1. Plant material

Cormels of three South African species (*Gladiolus callianthus*, *G. garnierii* and *G. dalenii*) and 7 large flowering cultivars of *G. grandiflorus* (Alfred Nobel, Amsterdam, Majolica, Peter Pears, Roselind, White Prosperity) were obtained from the germplasm collection of CPRO-DLO, The Netherlands. *In vitro* grown shoots, of all genotypes, were raised from the apical meristems of cormels. The cormels were disinfected, sliced and placed on modified MS medium as described by Remotti and Löffler (1995). The medium was amended with 0.5  $\mu\text{M}$  of BA. Shoot cultures were maintained at 24°C under a 16-h light photoperiod.

Central slices of the same cormels were used to initiate callus cultures on CI-3 medium (Remotti and Löffler 1995) containing 9  $\mu\text{M}$  2,4-D. The calli were maintained on CI-3 medium in the dark at 25°C and subcultured every four weeks. Compact callus was separated from friable callus and used for different experiments.

### 2.2. Development of assays

A greenhouse test was used to assess the resistance level of the 10 genotypes to *F. oxysporum* f. sp. *gladioli* according to Löffler, *et al.* (1996). For each genotype, three pots were filled with infected soil and one pot with non infected soil. In each pot three corms per genotype were planted. The pots were placed in a temperature controlled greenhouse at 18°C (16-h day/8-h night) in 4 randomized blocks. Twelve weeks after planting, the corms were evaluated for the presence of symptoms on a scale ranging from 1 to 6 (1: healthy; 2-5: respectively 5-10%, 15-25%, 30-50%, 60-90% necrotic areas or rot on the corm surface; 6: completely rotten).

To assess the FA sensitivity of the *Gladiolus* genotypes, a filter-sterile FA solution was added in different concentrations (0, 0.2, 0.35, 0.5, 0.7 mM) to the autoclaved mod. MS medium after cooling down to 40°C. Three pots were prepared for each combination of genotype and FA concentration. Into each vessel, three shoots were placed, selected for their uniformity and size. After 2 weeks, healthy shoots were scored 1, while the others scored 0.

For a callus growth assay only compact callus was used. For each genotype three six-well macro-plate were prepared and filled with 6 ml of CI-3 medium each containing 0, 0.2, 0.3, 0.35, 0.4 and 0.5 mM of filter-sterilized FA. Small samples of callus, with a weight of 45-50 mg were placed in the center of each well. The area covered by each clump was initially measured with an image analyzer as described by Remotti and Löffler (1995). After two weeks, the size of each clump was measured again. The size increase relative to the control well was calculated after 15 days.

### 2.3. *In vitro* selection

Friable callus of cv. Peter Pears was used to initiate a cell suspension according to Remotti (1995). After three months of subcultures, the suspension was plated in six-well macro plates. Each well was filled with 6 ml of solidified CI-3 medium amended with 0 - 0.14 mM FA. The plates were incubated in the dark at 24°C. Cell lines surviving on medium with 0.10, 0.12 and 0.14 mM were numbered and placed on CI-3 without toxin for further development. After 4 weeks 20 small clumps were sampled from the callus surface of each line and placed on CI-3 medium supplemented with 0.35 mM FA.

For further characterizations 2 lines out of the selected ones were chosen. The control callus and the two lines were maintained on modified MS medium. One line was split into two of which one part was retained on medium with 0.35 mM FA and the other on hormone-free medium. Once sufficient new callus was formed, equal amounts of callus were taken from the surface of the growing clumps and placed in the wells of a six-well macro plate, filled with 6 ml of CI-3 supplemented with 0, 0.2, 0.3, 0.35, 0.4 or 0.5 mM FA. The growth increase was measured by image analysis. A further characterization of the altered sensitivity to FA was done by inoculating the selected calli with conidia of a virulent isolate of *F. oxysporum* f. sp. *gladioli*. The extent of mycelial growth was estimated by measuring the amount of ergosterole on the callus mass (Seitz, *et al.* 1979).

A fresh cell suspension was prepared for an improved selection with FA, represented in Fig. 1. One ml of the cell suspension culture was plated on a Whatmann filter-paper No. 1 and placed on CI-3 supplemented with 0.12 mM FA. The surviving colonies were transferred after 10 days to fresh CI-3 with 0.4 mM FA and incubated for two more weeks. Thereafter the surviving calli were placed onto regeneration medium supplemented with 0.5 µM zeatin and 0.5 µM BA. Regenerated plantlets were transferred to medium with 0.5 µM BA for multiple shoot induction. Cloned plantlets were tested for FA reaction following the shoot assay previously described.

## 3. Results

### 3.1. *Fusarium* resistance and fusaric acid sensitivity

Between the tested cultivars, a large variation in resistance was detected. *G. dalenii* had a very high resistance to *F. oxysporum*. Most affected were 'Peter Pears', 'Hawaii' and 'Majolica' (Tab. 1). In most cases the corms of these genotypes were completely rotten.

The shoots of all genotypes showed increasing symptoms with increasing concentrations of the toxin. Table 1 shows the sensitivity of all genotypes for 0.35 mM FA. Shoots affected by FA had a bleached or necrotic shoot base or were completely necrotic. The South African species and 'Roselind' resulted to be the least affected by 0.35 mM FA.

The callus growth of all genotypes was reduced by increasing the concentration of FA, but not all genotypes to the same extent. The largest differences between genotypes could be observed at the concentration of 0.35 mM FA. Clearly highly affected by the toxin were 'Peter Pears', 'Amsterdam' and 'Alfred Nobel', which grew least on this concentration.

The shoot assay correlated well with the greenhouse test ( $R=-0.741$ ), while the callus growth assay did not ( $R=-0.367$ ).

### 3.2. *In vitro* selection

A total of 12 colonies developed on media with either 0.10, 0.12 or 0.14 mM FA. All clumps developed further on FA-free medium. Each line divided in 20 smaller units reacted differently on medium with 0.35 mM FA. The control callus died, 100 % growth and survival

was observed only for two cell-lines (S-4 and S-5) which were characterized further. Both lines were significantly less sensitive to FA than the control callus. Cell line S-5 was even less inhibited by the toxin than line S-4. Cell-line S-4, which was split into two parts and subcultured on FA containing or FA non-containing medium, showed the same response regardless of the medium on which it was subcultured (Fig. 2). Selected calli could reduce the infection from 85 to 50 % (Fig. 3)

For the improved selection three batches of cell suspension were used. These were plated on FA-medium (0.12 mM). After 10 days, 395 single colonies developed. All were individually transferred to fresh medium supplemented with 0.40 mM FA. About 50% of the calli developed further. Subsequently 195 calli were transferred to the regeneration medium. Regeneration of the selected calli took five months, compared to the three months needed for the control calli. Regeneration was proceeded by the appearance of green areas from which occasionally roots or shoot were formed. Not all calli could regenerate into plants. Shoots emerging from different sectors were considered as different individuals. From the calli 194 plants were regenerated.

Regenerated plants were propagated *in vitro* to obtain clones for further characterization. As a result of vitrification, malformations or contamination, 38 regenerants were lost. From the remaining 156 plantlets only 115 formed a sufficient number of shoots and could be tested for FA-sensitivity on medium. All control shoots (75 in total) showed signs of necrosis of bleached shoot basis after 15 days. Of the regenerated plants 57 (50%) scored similarly to the control, 34 (30%) showed only few symptoms and 22 (20%) were not affected at all by the toxin. A few plants from the last category even rooted and formed new shoots on the FA medium. The other remaining plantlets were transferred to fresh medium for further root development, and prepared for greenhouse acclimatization. The two plantlets regenerated from the cell-line S 4-4, were similarly tested and resulted to be only slightly or not at all affected by the toxin.

#### 4. Discussion

Fusaric acid affects the development of shoots and callus cultured *in vitro*. In both assays, the resistant genotypes reacted differently from the more susceptible genotypes. Significant differences between susceptible and resistant genotypes were found, however the use of callus in assays reflects the resistance level of the genotypes to a lesser extent than the use of shoots. 'Amsterdam', a cultivar which is known to be more tolerant to the disease, was found to be sensitive for FA in the callus growth assay, but was less sensitive to FA in the shoot assay.

Based on the significant correlation between the greenhouse test and the shoot assays, we conclude that FA may play a role in the corn-rot of gladiolus. However, the assays do indicate that selection with FA has also limitations since there is no strict correlation between FA-insensitive callus and *Fusarium*-resistance of the genotype tested. Yet in the current research cell-lines with decreased sensitivity to FA were isolated in *in vitro* selection experiments and the trait was also partially expressed at the plant level. Previously other successful selections using FA have produced resistant material (Shahin and Spivey 1986, Chawla and Wenzel 1987). Hartman, *et al.* (1984) have also described the possible use of culture filtrates for the *in vitro* selection of disease resistant genotypes. However, the use of a purified toxin represents a more appropriate selective agent, since culture filtrates are known to contain a variable number of phytotoxic compounds. The presence and the concentration of these metabolites produced by the pathogens depends mainly on the culture condition and on the pathogenicity of the isolates used. Toyoda, *et al.* (1984 a,b) showed that plants selected with culture filtrate may well be resistant to other factors than to the main pathogenic one.

Once the 12 selected cell-lines were grown to larger clumps and were split into 20 smaller units, they displayed different levels of FA-sensitivity. Similarly when inoculated with conidia of a virulent isolate the cell-lines displayed a sensible ability to inhibit the mycelial growth, this first test indicates that probably FA is needed by the isolate to condition the substrate for proliferation, and that tissue insensitive for the toxin may not be as suitable for the fungal growth. The heterogeneity in the response may be due to chimerism or to the differential expression of the trait. All cell-lines that did not pass the second selection step possibly possessed an insufficient level of tolerance to the toxin. Furthermore, about 50% of the cloned plantlets regenerated did not demonstrate an increased level of FA-insensitivity when tested at the shoot level. The fact that a number was still affected by the toxin can be explained by an incomplete resistance or by epigenetic or habituation factors. A definite evaluation of the altered expression of the regenerated material will be done once the regenerated plantlets which are cloned, transferred to greenhouse, and grown to maturity, with a standard screening test developed at CPRO-DLO, the Netherlands.

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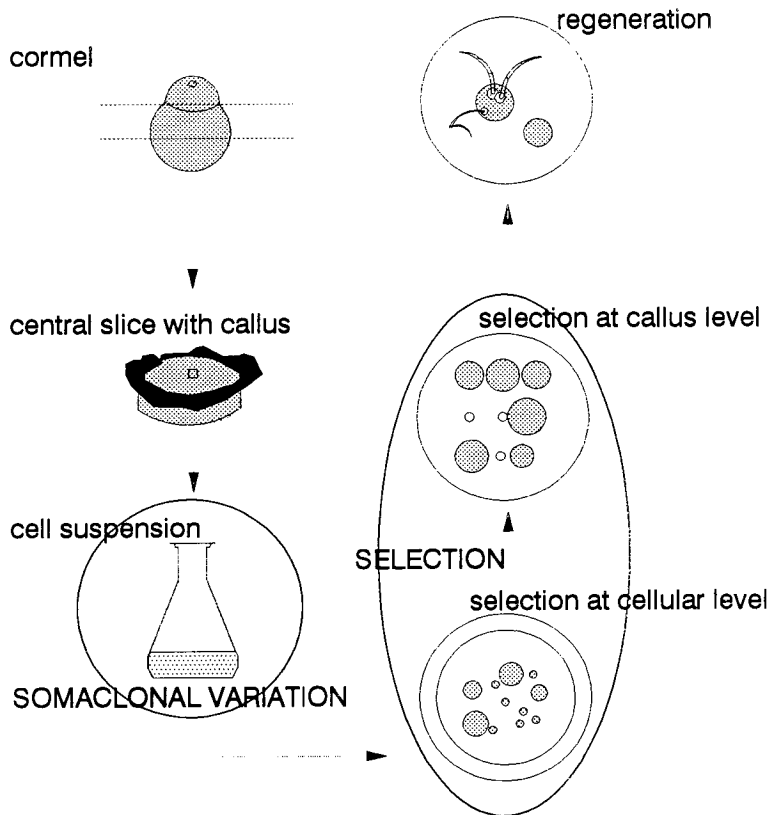


Figure 1 - Proposed scheme for the *in vitro* selection of gladiolus.

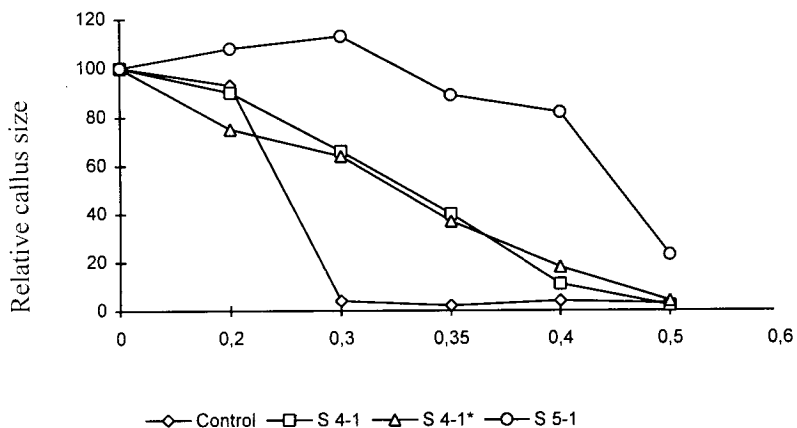


Figure 2 - Dependence of cell-lines S 4-1 and S 5-1 and control callus grown on medium supplemented with different FA concentrations, cell-line S 4-1\* was maintained on medium without the toxin.

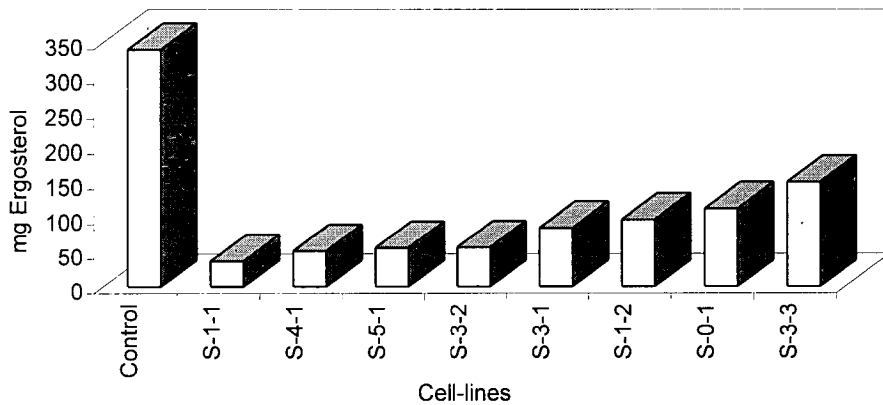


Figure 3 - Reduction of the mycelial growth of 8 cell-lines, the reduction is related to the amount of ergosterole in mg detected per gram callus.

Table 1 - Results relative to the greenhouse test, two assays and the ten genotypes tested.

Experiment number	Greenhouse test	Shoot growth	Callus growth
	[Disease score]	after 15 days [%]	after 15 days [%]
FA Conc. [mM]	1	2	4
Genotype	-	0.35	0.35
<i>G. dalenii</i>	1.0	84	100
Roselind	1.4	92	105
<i>G. callianthus</i>	1.6	80	98
Amsterdam	2.1	84	72
White Prosperity	2.3	60	92
<i>G. garnierii</i>	2.6	84	103
Alfred Nobel	3.3	40	83
Peter Pears	4.1	24	48
Hawaii	4.6	56	99
Majolica	5.0	56	91
LSD <sub>0.05</sub>	0.9	20.5	7