Neth. J. Pl. Path. 98 (1992) 107-115

Fusaric acid: phytotoxicity and in vitro production by Fusarium oxysporum f.sp. lilii, the causal agent of basal rot in lilies

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Accepted 6 January 1992

Abstract

The production of phytotoxins by *Fusarium oxysporum* f.sp. *lilii*, a pathogen causing bulb and scale rot in lilies, was investigated. To determine the toxic activity of culture filtrate, a bioassay with in vitro grown scale bulblets or with callus was set up. The fungus produces toxic components in different culture media. The highest toxicity was observed in Czapek Dox medium. HPLC and GC/MS analyses revealed the presence of fusaric acid at toxic concentrations in this medium. The production of fusaric acid in time coincides with the increase of toxic activity in the culture filtrate. It is concluded that at least part of this toxicity is due to fusaric acid.

Additional keywords: Lilium, resistance, in vitro selection, phytotoxins.

Introduction

The soil-borne fungal pathogen *Fusarium oxysporum* f.sp. *lilii* is the causal agent of bulb and scale rot in lilies (Imle, 1942). This disease seriously threatens the cultivation of lily bulbs and flowers. Chemical protection is difficult since the fungus readily develops resistance against some of the pesticides. Besides, the number of available pesticides decreases due to environmental considerations. Therefore, resistant cultivars of lily would be very valuable. One strategy to get resistance could be in vitro selection (Löffler and Mouris, 1990). This method implies the selection of resistant cells induced by somaclonal variation. In vitro selection has been successfully applied by Gengenbach and Green (1975) for the selection of Helminthosporium resistant corn plants. Since then, this technique was used frequently, also aimed at the selection of Fusarium resistance, in various crops (Behnke, 1980; Hartman et al., 1984; Arcioni et al., 1987; Chawla and Wenzel, 1987). In spite of many efforts, incidentally successes have been achieved. For example, Wenzel and Foroughi-Wehr (1990) tested progenies of selfed barley plants which were selected in vitro for resistance against fusaric acid. They did not find any segregation for resistance against fusaric acid. Moreover, the level of resistance of the progeny equalled that of the non-selected control. They concluded that too little genetic change due to somaclonal variation occurred. In contrast, Heath-Pagliuso and Rappaport (1990) confirmed with genetic studies of regenerated plants, that resistance against Fusarium was induced in callus tissue of

celery by somaclonal variation. It seems necessary to consider the value of somaclonal variation and in vitro selection further.

One of the prerequisites for in vitro selection is a suitable selective agent, preferably a toxin produced by the pathogen. Fusarium species are known to produce several toxins, among which fusaric acid (Drysdale, 1982). This toxin was studied extensively in the past (Kern, 1972) and, partly because of its potential as selective agent for in vitro selection, again more recently (Matsui and Watanabe, 1988; Mégnégneau and Branchard, 1988; Jullien, 1988). However, the role of fusaric acid during pathogenesis is still not clear. Ouchi et al. (1989) demonstrated that tomato plants, infected with fusaric acid degrading Pseudomonas bacteria, were protected partially against Fusarium wilt caused by *Fusarium oxysporum*. This indicates that fusaric acid might play a role in pathogenesis and that insensitivity of the plant for this toxin may enhance the resistance against the pathogen.

Up to now, no reports have been published about the production of fusaric acid by bulb rot producing Fusarium species. This paper describes the production of fusaric acid by *Fusarium oxysporum* f.sp. *lilii*. Moreover, sensitivity for this compound of both differentiated and non-differentiated tissue of lily is demonstrated.

Materials and methods

Fungi. A highly aggressive isolate of *Fusarium oxysporum* f.sp. *lilii* (CPRO/Fol-11) was kindly supplied by E. Roebroeck from the Laboratory of Flower Bulb Research (LBO) at Lisse. The isolate was isolated from the Asiatic lily hybrid 'Esther' and stored for long term preservation on 'Protect Bacterial Preservers' beads (Technical Service Consultants) at −80 °C. For inoculum production, the fungus was grown on Czapek Dox medium.

Plant material. Scales of the Asiatic lily hybrid 'Golden Melody' and *Lilium longiflorum* 'Gelria' were superficially disinfected in 1% sodium hypochlorite for 30 minutes, washed in sterile water and sliced in 2-3 mm thick segments. The sliced scales of 'Golden Melody' were incubated on LS-medium (Linsmaier and Skoog, 1965) with 3% sucrose and 0.8% agar (further abbreviated as LS1) containing 0.5 μM naphtylacetic acid (NAA) for the induction of adventitious bulbs.

Sliced scales of 'Gelria' were incubated on LS1 medium containing 0.5 μM benzylaminopurine (BAP) and 0.5 μM NAA for the induction of callus. Callus was subcultured on the same medium every 3 months.

Development of a bioassay. Wells of a multiwell plate (Greiner, 12 wells) were filled with 3.5 ml LS1 medium each, containing 0, 0.001, 0.01, 0.1 or 1 mM fusaric acid (Sigma, 5-butylpicolinic acid). Small adventitious bulblets of 'Golden Melody' were incubated on this medium in duplicate in the dark at 23 °C. After 5 weeks the growth of the bulblets was visually assessed.

Alternatively, a callus bioassay was used. Wells of a multiwell plate (Greiner, 24 wells) were filled with 1 ml LS1 medium each, containing 0.5 μM NAA, 0.5 μM BAP and 0, 0.05, 0.1, 0.5, 1 or 5 mM fusaric acid. Callus pieces of 'Gelria' (3-4 mm diameter) were incubated on this medium in four replicates in the dark at 23 °C. After 18 days, the calli were evaluated for growth and colour. Discolouration was scored

using an index ranging from 0 (healthy, light yellow callus) to 4 (strong discolouration).

Toxin production in four culture media. The following four culture media were tested for their ability to induce the production of toxic compounds by the fungus: 1) Czapek Dox (Oxoid) supplemented with 25 mM sodium acetate, 2) Richard's medium modified according to Dobson et al. (1967) and supplemented with 25 mM sodium acetate, 3) a low- nutrient medium (SNA) consisting of KH_2PO_4 (1 g), KNO_3 (1 g), $MgSO_4.7H_2O$ (0.5 g), KCl (0.5 g), glucose (0.2 g), sucrose (0.2 g) and sodium acetate (2.05 g) per liter of distilled water, and 4) a medium to detect possibly inducible toxins containing 400 g ground scales of the Asiatic lily hybrid 'Esther', sucrose (5 g), KH_2PO_4 (0.1 mol) and sodium acetate (25 mmol) per liter (extraction medium). Acetate was added to these media since it is a precursor of fusaric acid and thus may enhance its production (Dobson et al., 1967). All media were autoclaved for 20 minutes at 120 °C before use.

Two agar plugs containing mycelium of the fungus were inoculated in 500-ml Erlenmeyer flasks with 100 ml culture medium in duplicate. The flasks were incubated at 23 °C in an orbital incubator (100 rpm). After 3 weeks cell free media were collected by differential filtration up to 0.2 μm. Culture filtrates were stored at −20 °C until further use. The toxicity of the culture filtrates was assessed using the callus bioassay as described before. In this assay, half-strength culture filtrate was tested for toxicity in four replicates. After 9 days, the discolouration of the callus was determined.

Analysis of fusaric acid in culture filtrates. Culture filtrate from five-week-old cultures of the fungus grown in Czapek Dox was tested for the presence of fusaric acid using Reversed Phase HPLC (High Performance Liquid Chromatography) on a Brownlee RP-C18 column (5 μm, 220 × 2.1 mm). A 20 μl aliquot of culture filtrate, Czapek Dox culture medium or pure fusaric acid (1 mM) was injected and eluted with methanol-2% H_3PO_4 (7:3 v/v) for 10 minutes at a flow rate of 0.5 ml/min and a pressure of circa 4500 psi. The absorption of the eluent was recorded with a UV-detector at 270 nm. For GC/MS (Gas Chromatography/ Mass Spectrometry) analysis the culture filtrate was eluted with methanol-0.1% acetic acid (7:3) and fusaric acid peaks were collected after HPLC purification and dried under nitrogen. The samples were redissolved in hexane-ethylacetate (9:1) and methylated with diazomethane. A 2 μl aliquot was injected split-less in a 50-meter CP Sil8 column (injector Temp. = 260 °C), eluted for 40 minutes using a temperature gradient of 70 °C to 200 °C. The compounds were detected with an HP5970 mass spectrometer.

Time course of toxin production. The presence of toxic activity and fusaric acid in Czapek Dox medium inoculated with the fungus was followed in time (three replicates). After 0, 1, 2, 3, 4 and 5 weeks, samples of the culture were collected and prepared for the callus bioassay and HPLC analyses as described before. In the callus bioassay, half-strength culture filtrate was tested in duplicate and evaluated after 9 days. All samples were assayed for fusaric acid with HPLC. The concentration of fusaric acid in the culture filtrate was calculated on the basis of a calibration curve obtained with pure fusaric acid.

Results

Development of a bioassay. After 5 weeks, the growth of the bulblets of 'Golden Melody' was inhibited at fusaric acid concentrations of 10 μM and higher (Fig. 1). Especially root development was reduced. At a concentration of 1 mM fusaric acid the bulblets died.

Also the growth of callus of 'Gelria' on LS-medium with fusaric acid is inhibited (Fig. 2). After 18 days, calli grown at 0.1 mM fusaric acid showed some discolouration. The calli grown at 0.5 mM fusaric acid or higher were strongly discoloured and died. Especially at the higher fusaric acid concentrations, calli became bleached and greyish rather than brown. This bleaching was found throughout the experiments and seems to be a characteristic of fusaric acid.

Toxin production in four culture media. With the callus bioassay, culture filtrates of three out of the four media tested showed toxic activity (Fig. 3). Only in the low-nutrient SNA medium no toxic activity was found. Calli grown on non-inoculated control media showed no or sometimes slight browning. The activity of the extraction medium did not exceed the activity of the artificial media. The strongest toxic activity was found in culture filtrate of Czapek Dox medium. Especially this culture filtrate induced a greyish discolouration typical for fusaric acid. Mycelial growth of the

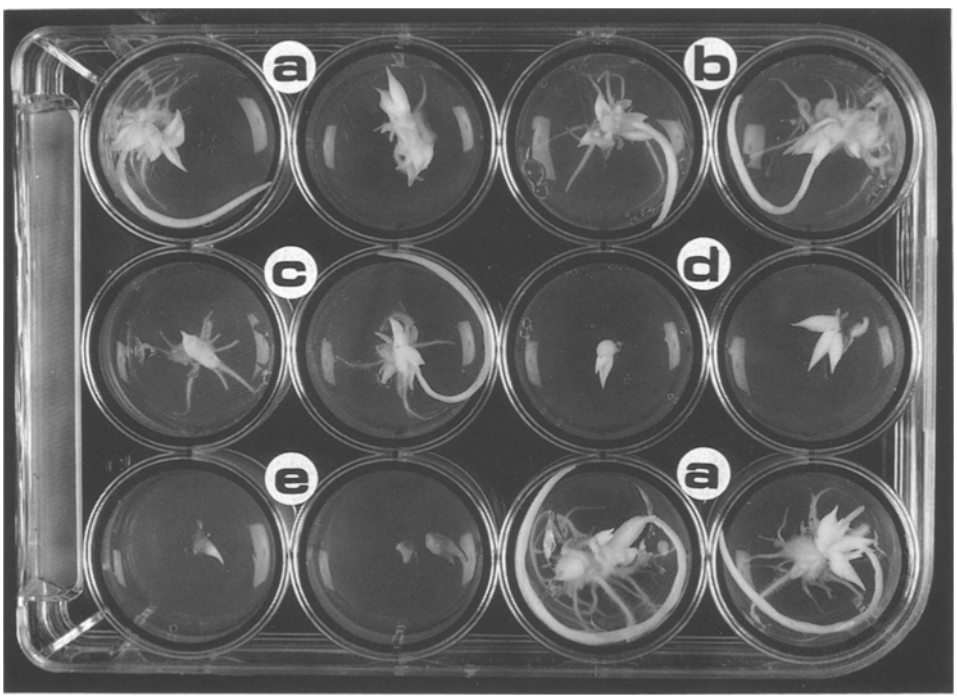

Fig. 1. Decrease of bulblet growth of the lily cultivar 'Golden Melody' caused by fusaric acid after 5 weeks. A: control; B: 1 μM fusaric acid; C: 10 μM fusaric acid; D: 100 μM fusaric acid; E: 1 mM fusaric acid.

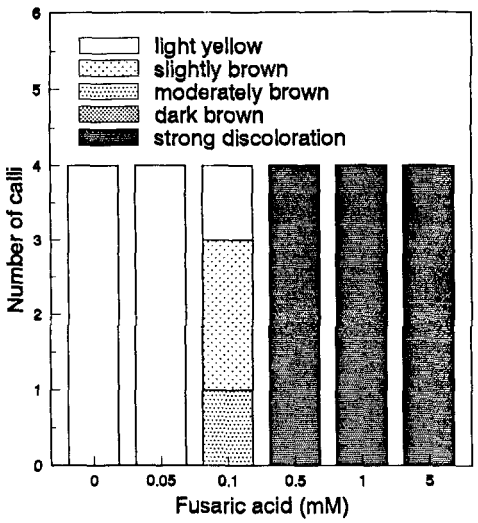

Fig. 2. Discolouration of calli of the lily cultivar 'Gelria' at various concentrations of fusaric acid.

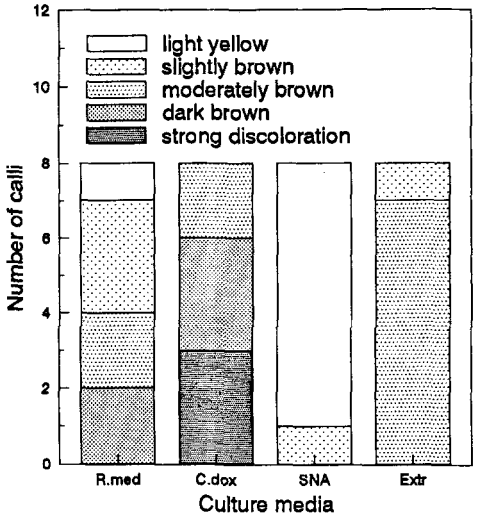

Fig. 3. Toxicity for lily callus of culture filtrate of *F. oxysporum* f.sp. *lilii* grown in four different culture media observed as dicolouration of calli.

fungus depended on the medium used. The highest dry-weight was found in Extraction medium and Richard's medium (1.2-1.5 g 100 ml $^{-1}$), followed by Czapek Dox (0.4 g 100 ml $^{-1}$) and SNA (0.2 g ml $^{-1}$).

Analysis of fusaric acid in culture filtrate. HPLC analysis of culture filtrates showed two peaks, one of which corresponds to fusaric acid (Fig. 4B,C). This material, which is absent in non-inoculated medium (Fig. 4A), was collected and further analyzed with GC/MS. The GC-spectra of pure fusaric acid and of the putative fusaric acid fraction isolated with HPLC from culture filtrate both showed a peak at a retention time of 22 min (Fig. 4D,E). Those peaks were further analyzed by mass spectrometry, which showed an equal mass/charge pattern for both peaks (Fig. 4F,G). Therefore the compound detected by HPLC in culture filtrate is fusaric acid. These results show that the fungus produces fusaric acid in vitro.

Time-course of toxin production. Accumulation of fusaric acid in the medium coincides with increase of toxicity as found in the bioassay. After 2 weeks, fusaric acid was found in the culture filtrate. The concentration increases almost linearly in time (Fig. 5). According to the bioassay, toxic activity started after one week. At week 3 all calli were completely greyish, which is a characteristic symptom for the presence of fusaric acid.

Discussion

It is well known, that fusaric acid is toxic for many prokaryotes and eukaryotes (Marasas et al., 1984), among which many plants. Recently, growth of soybean plants

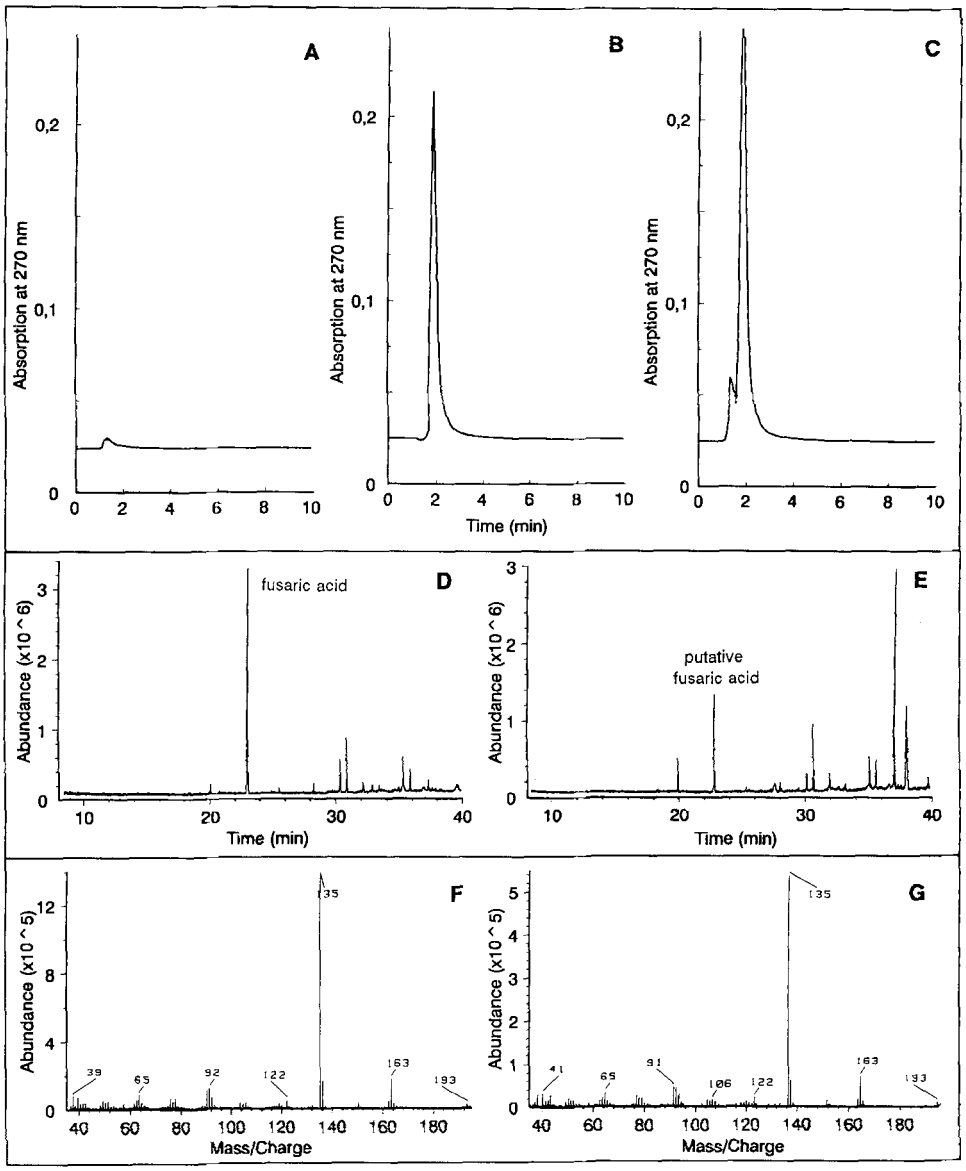

Fig. 4. HPLC elution profiles of Czapek Dox culture medium (A), pure fusaric acid (B) and culture filtrate of *F. oxysporum* f.sp. *lilii* grown for 5 weeks in Czapek Dox (C); GC spectra of pure fusaric acid (D) and the putative fusaric acid isolated with HPLC from culture filtrate (E) and MS spectra of the fusaric acid peaks from GC-runs of pure fusaric acid (F) and the putative fusaric acid isolated from culture filtrate (G).

was found to be affected by 1 ppm (ca 6 μM) fusaric acid (Matsui et al., 1988). In this paper we confirm the toxicity of this compound for lily. Formation of roots and bulblets was decreased in the presence of fusaric acid at concentrations of 10 μM or

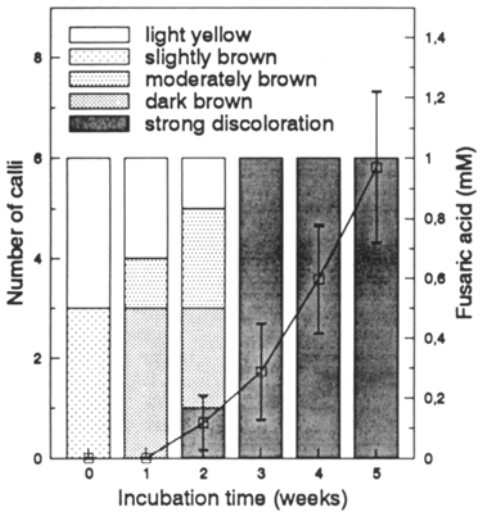

Fig. 5. Development of toxicity for lily callus observed as dicolouration of calli and concentration of fusaric acid in culture filtrate of *F. oxysporum* f.sp. *lilii* grown in Czapek Dox medium during 5 weeks.

higher. Also non-differentiated callus tissue of lily was sensitive for fusaric acid. A concentration of 0.5 mM fusaric acid in LS-medium killed the callus pieces within 18 days. These findings correspond with those of Mégnégneau and Branchard (1988), who demonstrated inhibition of callus growth of muskmelon at 0.1 mM fusaric acid, and those of Jullien (1988), who found an LD_{50} for callus of asparagus at 0.35 mM fusaric acid.

Both the bulblet and callus assays can be used to demonstrate the toxic activity of fusaric acid. The latter one is preferred since it is an easy and rapid assay. From the callus assay it becomes evident, that sensitivity is expressed in non-differentiated tissue. This is important for in vitro selection, e.g. with fusaric acid as a selective agent.

A typical effect of fusaric acid on callus is a greyish discolouration rather than browning of the callus. This lack of browning may be related to the inhibition of polyphenol oxidases and peroxidases (Drysdale, 1982).

With the callus assay four different culture media were screened for their potential to induce production of toxic activity. Three of those media were artificial, whereas the fourth medium contains autoclaved lily bulb material. The latter medium was included to test for possibly inducible toxins, although to our knowledge such toxins have not been described for Fusarium. In this medium, toxic activity was found, but less than in Czapek Dox medium, suggesting that inducible toxins are not involved.

The low-nutrient medium SNA was included to find out whether toxins would be formed under nutrient stress. Since hardly any toxic activity was found in SNA-medium although the fungus grows rather well in that medium, there are no indications that toxins are stress metabolites.

Since the toxicity in culture filtrate of Czapek Dox was the highest, we analyzed this medium for toxic compounds. HPLC and GC/MS analyses showed the presence of fusaric acid in the culture filtrate of Czapek Dox. The concentration amounted to approximately 1 mM after 30 days. This concentration is comparable to that found

by Matsui and Watanabe (1988; circa 2 mM after 35 days), but much lower than that mentioned by Dobson (1967; circa 7 mM after 7 days).

More than one component could be responsible for the observed toxic activity of the culture filtrate. However, the production of fusaric acid coincides with the increase of toxic activity in the culture filtrate. Moreover, the symptoms observed after exposure of the callus to culture filtrate were typical for the presence of fusaric acid. Therefore these symptoms seem to be at least partially due to fusaric acid.

In conclusion, fusaric acid can be formed by *Fusarium oxysporum* f.sp. *lilii*. This compound is toxic for differentiated as well as non-differentiated tissue of lily. Up to now, it is not known whether the toxin plays an important role in pathogenesis. This question is subject of present research.

Acknowledgements

We thank E. Knecht en E. Vermeer from the Department of Plant Physiology of the Agricultural University (Wageningen) for carrying out the GC/MS analyses. We thank R.W. van den Bulk and J.P.F.G. Helsper for critical comments on the manuscript and A.A.C. de Gier for correcting the English.

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