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Fusaric acid production by *Fusarium oxysporum* f.sp. *lilii* and its role in the lily basal rot disease

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Abstract

Fusarium oxysporum f.sp. *lilii* (Fol), the causal agent of lily basal rot, produced fusaric acid (FA) in aseptic culture. This toxin induced phytotoxicity symptoms on *in vitro*-grown lily bulblets of two different cultivars: the Fol-susceptible cultivar was more sensitive to the toxin than the Fol-resistant cultivar. When cultured in the presence of FA, the Fol-susceptible cultivar showed a greater tendency to accumulate FA within its tissues than the Fol-resistant cultivar. The polyphenol oxidase activity of the bulblets was inhibited by 1 mmol L^{-1} FA in both the cultivars, while at lower FA concentrations the enzyme activity increased only in the Fol-susceptible cultivar. Peroxidase showed a steady activity at the 1 mmol L^{-1} FA concentration in both the cultivars, while at lower FA dosages its activity increased. Within the Fol-infected *in vivo* tissues of both the lily cultivars, FA was detectable only in traces. The role of this toxin in the lily basal rot disease seems therefore to be of marginal importance.

Abbreviations: FA – fusaric acid; Fol – *Fusarium oxysporum* f.sp. *lilii*; GC – gas chromatography; MeOH – methanol; nm – nanometers; PO – peroxidase; PPO – polyphenol oxidase; R_f – retardation factor; R_t – retention time.

Introduction

Fusarium oxysporum f.sp. *lilii* (Fol) is the causal agent of the lily basal rot (Imle, 1942), a serious disease affecting lily cultivations (Linderman, 1981). Among other toxins produced by this fungus, fusaric acid (FA) has been recently investigated and its toxic effects towards lily tissues have been demonstrated (Löffler and Mouris, 1992). The importance of FA as a cause of phytotoxic effects proved to be fundamental in several *Fusarium*-induced diseases (Drysdale, 1982). For this reason, FA found applications as a selective agent for *Fusarium* resistance selection in some plant species (Jullien, 1988; Remotti et al., 1997a,b). Nevertheless, a correlation between the virulence of a *forma specialis* of *Fusarium oxysporum* and its ability to produce FA cannot always be demonstrated (Venkata Ram, 1958; Kuo and Scheffer, 1964), although in a few cases this relationship has been established (Davis, 1969; Kern, 1972). The real significance of FA in a *Fusarium oxysporum*-induced pathogenesis can therefore be variable, depending on both the fungus *forma specialis* and the host species. FA production by *Fusarium oxysporum* can vary as a function of the fungal growth conditions (Dobson et al., 1967; Löffler and Mouris, 1992); moreover, its phytotoxic effects can be more or less intense whether or not the toxin is produced and/or accumulated within the infected plant tissues (Davis, 1969), where FA is able to inhibit plant polyphenol oxidase (PPO) and peroxidase (PO) (Drysdale, 1982), enzymes involved in the defence browning reactions. With respect to the pathogenic interactions between Fol and lily, little is known about the FA production *in vitro* or *in vivo* by the fungus and about the toxic effects of this toxin towards lily tissues (Löffler and Mouris, 1992). To

better understand the role of FA in the lily basal rot disease, the present research has been therefore undertaken. With this aim, the FA *in vitro* production by Fol, in static or shaken culture, and the FA toxic effects towards *in vitro*-cultivated lily tissues were studied. FA accumulation within the *in vitro* plant tissues, the possible presence of this toxin within Fol-infected *in vivo* bulbs and its influence on PPO and PO activities in the FA-treated plant tissues were also studied.

Materials and methods

Fungal material

Fusarium oxysporum f.sp. *lilii*, isolate Fol-11, was used in all the experiments. The isolate was obtained from CPRO-DLO, Center for Plant Breeding and Reproduction Research, Wageningen, Holland.

Fungus in vitro *culture*

Mycelial discs, with a diameter of 0.5 cm, were obtained from 2-week-old cultures of Fol on potato dextrose agar (PDA) in Petri dishes and transferred into a glass flask containing 500 mL of Czapek–Dox nutrient medium. Forty flasks were set: 20 were stored as static cultures and 20 were kept in agitation in an orbitary shaker at 100 rpm. The culture duration was 4 weeks, at 24 °C, in darkness.

Conidia counting and evaluation of the biomass produced by Fol during the in vitro *culture*

At 7-day intervals, twenty 50 mL samples of Fol-inoculated medium were harvested from each flask. Each sample was centrifuged at 3000 g for 20 min, and the clear supernatant was used for the FA quantitation. The sediment from each sample was re-suspended in known volumes of distilled water to determine the conidial concentration per μL by microscopic examination through a Burker glass slide. The conidia counting was repeated 3 times for each flask. The suspensions were again centrifuged as above, the liquid removed and the sediment allowed to completely dry in an oven at 110 °C for 3 days, to determine the biomass dry weight L^{-1} in the different periods of culture. All the obtained values were statistically analysed through the Student–Newmann–Keuls test.

FA extraction from Fol culture media and quantitation

For FA quantitation, 50 mL medium were collected weekly from each flask, throughout the culture period. Each sample was acidified to pH 4.0 with HCl, and extracted 3 times with 10 mL ethyl acetate and then with 10 mL diethyl ether; the water fraction was discarded, while the 2 extracts were pooled and evaporated to dryness. The dry residues were dissolved in methanol (MeOH) and analysed for the presence of FA through paper chromatography on Whatman No. 1 paper sheets, eluted with butanol : acetic acid : water (4 : 1 : 1, v/v/v, descending phase). The R_f of sample putative FA was compared with pure, synthetic FA run under the same conditions. The quantitation of FA in each sample was performed through gas-chromatography (GC). A Hewlett-Packard HP 5890 – II instrument, equipped with a HP 3396 A integrator, was used. The capillary column was a HP 1 cross-linked methyl siloxane, 30 m × 0.53 mm × 2.65 μm phase thickness. The analytical parameters were splittless mode, attenuation 2^a: −0.5, purge: off. The oven temperature programme was: from 100 °C (initial temperature) to 180 °C with increases of 10 °C/min; then, from 180 to 200 °C with a rate of 20 °C/min. Helium was used as a carrier gas, with a flow of 35 mL min^{-1}, and the detector was a flame ionization detector (FID); the injector and the detector ports were set at 200 °C temperature; 1 μL of methanolic solution was injected for each run. Pure, commercial FA (Sigma, USA) was used as a reference, to determine its retention time (R_t) and to carry out the quantitation of the sample. Pure FA, dissolved in MeOH, was gas-chromatographed to obtain the corresponding integrated areas, and a standard reference curve was prepared for the FA quantitation.

Commercial samples of 3-butylpyridine were gas-chromatographed under the same conditions to exclude the possible decarboxylation of FA during the analysis.

Lily in vitro *culture*

Lily cultivars Connecticut King (Asiatic hybrid lily) and Star Gazer (Oriental hybrid lily), the first one known as resistant and the second one as susceptible to Fol, according to Löffler et al. (1995), were used for all the experiments.

The liquid culture medium (Murashige and Skoog, 1962) was supplemented with 30 g L^{-1} sucrose. The pH

of the medium was adjusted to 5.8 before autoclaving; a Whatman No. 1 paper bridge (6 × 1 cm) was inserted in each culture tube and then 20 mL of medium were dispensed in each tube (Kaputs, Bellco, USA; 20 × 2 cm). One hundred tubes per cultivar were set up.

The starting material was a leafy scale excised under aseptic conditions from *in vivo* bulbs, with a diameter of 6–8 cm, sterilized for 12 min with a solution of NaOCl, 1% free chlorine, and further rinsed with sterile water. Each scale was gently laid onto the paper bridge, kept in culture for 1 month and allowed to develop axillary bulblets.

Evaluation of FA toxic effects towards in vitro *lily bulblets*

Scale-generated lily bulblets from both cultivars Star Gazer and Connecticut King, with an average diameter of 1 cm and 3–5 leaves, were harvested from the *in vitro* material and aseptically transferred into test tubes, containing the same culture medium as above and a paper bridge as already mentioned. Three different concentrations of pure commercial FA were added to the liquid substrate: 0.01, 0.1 and 1.0 mmol L^{-1}. For each lily cultivar, 300 tubes per treatment were set up, and a further 100 without FA were prepared as a control. The experiments were repeated 3 times at different periods of the year. The culture conditions were those previously described. One month later, the visually appreciable phytotoxic effects of FA were recorded according to an arbitrary scale, where 4 classes were defined: healthy, light symptoms, heavy symptoms and decayed. The obtained data were statistically analysed using the Student–Newmann–Keuls test.

Lily in vivo *material inoculated with Fol*

Three hundred lily bulblets (4 cm diameter) for each cultivar were inoculated with Fol, while 100 uninoculated bulblets were used for each cultivar as a control. Bulbs were dipped into a Fol conidial suspension (10^5 conidia mL^{-1}) for half an hour, and then planted in a peat moss bench, under greenhouse growth conditions. Two months later, bulbs were harvested, carefully rinsed with distilled water and weighed; the visual symptoms of the Fol infection were classified according to the same disease scale used for the *in vitro* material, and the results were statistically analysed. Bulbs of both cultivars, showing evident symptoms of the Fol infection, were analysed for the content of FA according to the same protocol employed for the *in vitro* material.

Detection and quantitation of FA within both in vitro *and* in vivo *bulblet tissues*

Lily bulblets grown on FA-supplemented media from culture tubes, and *in vivo* bulblets inoculated with Fol, were weighed and rinsed for 3 h in distilled water containing a few drops of Tween 20. The bulblets were cut in two pieces, to obtain a basal part (roots included) and an apical part (leaves included); the bulb portions were analysed separately. Tissues were homogenized with MeOH in a Waring Blender and the homogenates were extracted for 5 h with boiling MeOH/ethanol (1 : 1, v/v) in a Soxhlet (1 g material/10 mL alcoholic solution). The alcoholic suspension was filtered through cheesecloth and centrifuged for 20 min at 3000 g. The sediment was discarded, and the clear supernatant was evaporated to dryness at low temperature under reduced pressure. The dry residue was resolubilized with double distilled water (1 mL/50 mg dry weight) containing 5% formic acid. This solution was loaded on top of a chromatography column (50 × 0.8 cm) filled with C_{18} silica gel (Vydac, Aldrich, USA) packed with MeOH which was replaced by a 5% formic acid solution. The elution was carried out using 200 mL absolute ethanol, and 90 fractions were collected (3 mL volume); the fractions absorbing at 268 nm were considered as containing FA (Heitefuss et al., 1960), and they were pooled, concentrated and analysed through GC.

PPO and PO activity within in vitro *bulblet tissues*

In vitro bulblets of cultivars Star Gazer and Connecticut King, grown on both FA-supplemented and FA-free medium, were harvested, weighed and homogenized at 4 °C temperature in a Waring blender with sodium phosphate buffer solution, 0.1 M, pH 7.0 (20 g fresh material/ 50 mL buffer, w/v). The homogenates were filtered through a cheesecloth, centrifuged and the sediment discarded. The clear surnatants were concentrated by means of a collodion bag (cut-off, 5000 Da) to give 5 mL samples. Polyphenol oxidase (PPO) activity was measured spectrophotometrically at 410 nm using catechol according to Flurkey (1985), while peroxidase (PO) activity was determined at 470 nm, using guaiacol and H_2O_2, following the protocol of Zieslin and Ben-Zaken (1991). The inhibition of enzyme activity

by FA was evaluated by comparing the PPO and PO activities of the untreated bulblets with those recorded for the bulblets treated with FA at 0.01, 0.1 and 1.0 mmol L^{-1} concentrations. Enzyme inhibition was expressed as percentages of activity in comparison to the control.

Determination of protein

The protein content in the tissue samples, analysed for the activity of PPO and PO, was determined following the method of Bradford (1976).

Results

Fol conidia, biomass and FA production rate by Fol in vitro *culture*

Fol *in vitro* cultures appeared to be strongly affected by the growth conditions (Tables 1 and 2). The shaken culture promoted very abundant conidial production and produced a reduced biomass development (as a mycelium), in comparison with the static culture. In the static medium, conidial production was relatively stationary during the 3 first weeks. After 21 days, there was not a statistically significant increase of the conidia number. Only at the end of the culture period (28th day) did the conidia production appear significantly greater. On the contrary, in the shaken medium statistically significant differences in the conidia production were observed among the 1st, 3rd and 4th week of culture. Throughout the culture period, in the shaken medium the number of produced conidia was always more than 10 times higher than that of the static culture. After 1 week, there were no statistically significant differences between static and shaken culture biomass dry weight. During the next 2 weeks, however, the static medium produced a greater increase of biomass in comparison with the shaken culture. At the end of the culture period, however, the large amount of conidia produced in the shaken culture germinated and gave rise to mycelial development. For this reason, only in the 4th week was the produced biomass statistically greater in the shaken medium than in the static culture.

The putative FA extracted from the exhausted culture media gave, after chromatography on paper sheets, a spot with a R_f of 0.87, revealed through its UV-light fluorescence. This value corresponded to the R_f obtained by co-chromatography of the commercial FA and is in accordance with literature (Kalyanasundaram and Venkata Ram, 1956; Nishimura, 1957). The UV spectrum of the putative FA obtained by elution of the chromatography spot with MeOH gave the same absorption peaks in Abs at 223 and 272 nm as obtained for the reference, synthetic FA. GC of the putative FA gave, under our analytical conditions, a R_t of 10.18 min (Figure 1). This value was coincident with that obtainable using the pure, commercial FA. The gas-chromatography of pure 3-butylpyridine gave a different R_t, and this proved that FA did not undergo a decarboxylation during the GC analysis.

The rate of FA biosynthesis within the two different culture systems, although higher in the static

Table 1. Conidia production in liquid Czapek–Dox medium inoculated with Fol kept in static or shaken culture

Days of culture	Conidia number μL^{-1*}	
	Static culture	Shaken culture
7	458^{a}	5500^{a}
14	460^{a}	5850^{a}
21	470^{a}	6000^{b}
28	833^{b}	7000^{c}

*Each value is the mean of 20 observations on as many different culture flasks, repeated 3 times (see Materials and methods). Values in each column followed by the same letter are not statistically different for $p = 0.05$ according to the Student–Newmann–Keuls test. Data were transformed in arcsin before the statistical analysis.

Table 2. Fusaric acid (FA) and biomass production rate by Fol during 4 weeks of *in vitro* culture

Days of culture	Dry weight (gL^{-1})		FA production (mmol L^{-1})	
	Static culture	Shaken culture	Static culture	Shaken culture
7	1.3^{aA}	0.9^{aA}	0.86^{aA}	0.47^{bA}
14	2.8^{aB}	1.9^{bB}	1.17^{aB}	0.92^{aB}
21	3.6^{aBC}	2.4^{bBC}	1.30^{aBC}	1.13^{aB}
28	4.1^{bC}	5.3^{aC}	1.52^{aC}	1.71^{aC}

Each value is the mean of 20 observations on as many different culture flasks, repeated 3 times (see Materials and methods). Values in each column followed by the same capital letter and in each row followed by the same lower case letter are not statistically different for $p = 0.05$ according to the Student–Newmann–Keuls test. Data were transformed in arcsin before the statistical analysis.

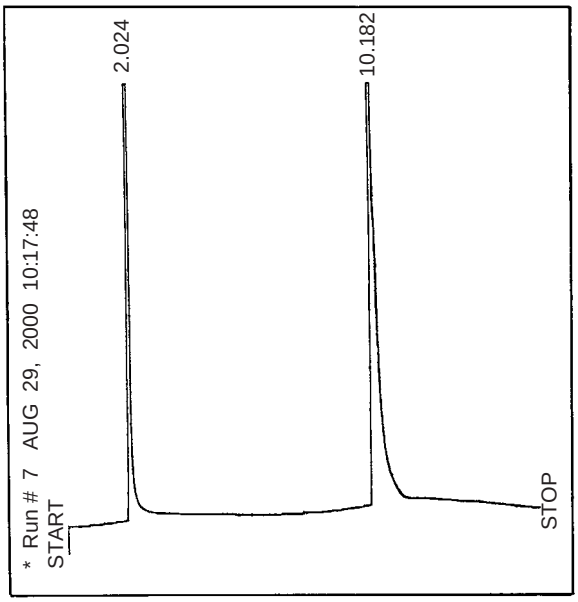

Figure 1. Detection by gas-chromotography (GC) of fusaric acid (FA) with its R_t of 10.18 min.

medium after the first week, followed the trend of the biomass production. No statistically significant differences between static and shaken culture occurred on the 14th, 21st and 28th day. The FA production was below 1 mmol L^{-1} in the first week and around 1 mmol L^{-1} from the 2nd to the 3rd week. In the last week of culture, the highest FA production was observed in the shaken medium (1.71 mmol L^{-1}), but in the static culture the production was very near to this value and not statistically different (1.52 mmol L^{-1}).

Toxic effects of FA towards in vitro *lily bulblets*

The two lily cultivars showed slightly different responses to the presence of FA in the growth medium (Figures 2 and 3). The Fol-resistant cultivar Connecticut King showed about 76% of healthy bulblets at the highest FA tested concentration, while the Fol-susceptible cultivar Star Gazer showed about 61% of healthy bulblets under the same growth conditions, with a statistically significant difference. At the 0.1 mmol L^{-1} FA concentration, the Fol-resistant cultivar showed about 87% and the Fol-susceptible cultivar about 73% of healthy bulblets, respectively, with a statistically appreciable difference. When the FA dosage was 0.01 mmol L^{-1}, the respective percentages were 92% and 82%, but these differences are not statistically significant. The effects of FA on the *in vitro* bulblets were mainly appreciable as growth slowering, leaves twisting and discoloration and browning of the basal part of bulblet. These effects were more remarkable at the highest FA concentration and more evident in the Fol-susceptible lily cultivar (Figure 4).

Response of the in vivo *bulblets to the Fol inoculation*

The results shown in Table 3 indicate that, following inoculation with Fol, the two lily cultivars give a statistically different resistance response to the disease. The Fol-resistant cultivar had a larger number of healthy bulbs in comparison to the Fol-susceptible cultivar. In addition, there were more bulbs with light symptoms within the Fol-susceptible than within the Fol-resistant cultivar, while the two cultivars did not statistically differ when the disease class 'heavy symptoms' was considered.

Detection and quantitation of FA within in vitro *lily tissues and* in vivo *Fol-inoculated bulbs*

FA was detectable within the FA-treated lily tissues only for the 1 mmol L^{-1} treatment. The amounts of FA ranged from 3 to 20 mg kg^{-1} fresh material, depending on the cultivar and on which portion of the bulblet was analysed. The highest FA concentration was found in the basal part of the Star Gazer bulblets (20 mg kg^{-1} fresh weight). In the same cultivar, however, the FA concentration was reduced to 3 mg kg^{-1} fresh weight when the apical portion of bulblet was analysed. In comparison with the Star Gazer cultivar, the Connecticut King cultivar contained a smaller FA concentration in the basal portion (8 mg kg^{-1} fresh weight), and no FA was detected within the tissues of the apical portion. The analyses carried out on the Fol-inoculated *in vivo* bulbs revealed the presence of small amounts of FA (about 3 mg kg^{-1} fresh material) within the infected tissues in both the lily cultivars but only in the basal portions of the bulbs.

PPO and PO activity within FA-treated and untreated in vitro *lily tissues*

The results are shown in Table 4. The PPO activity in the Star Gazer cultivar was significantly reduced at the 1 mmol L^{-1} FA concentration, while both at the low and at the average FA dosage there was an increase

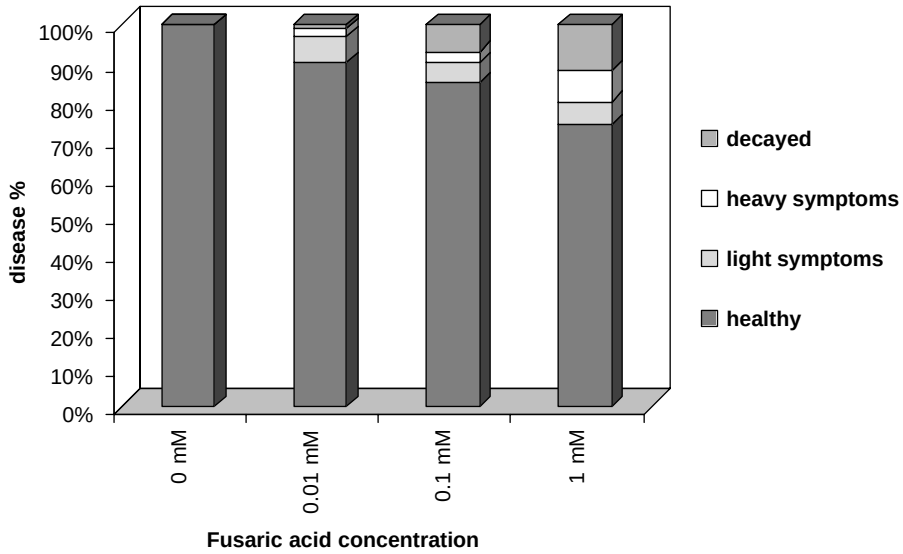

Figure 2. Toxic effects of 3 different dosages of fusaric acid (FA) towards *in vitro* lily bulblets after 30 days of culture: cv Connecticut King (resistant to Fol).

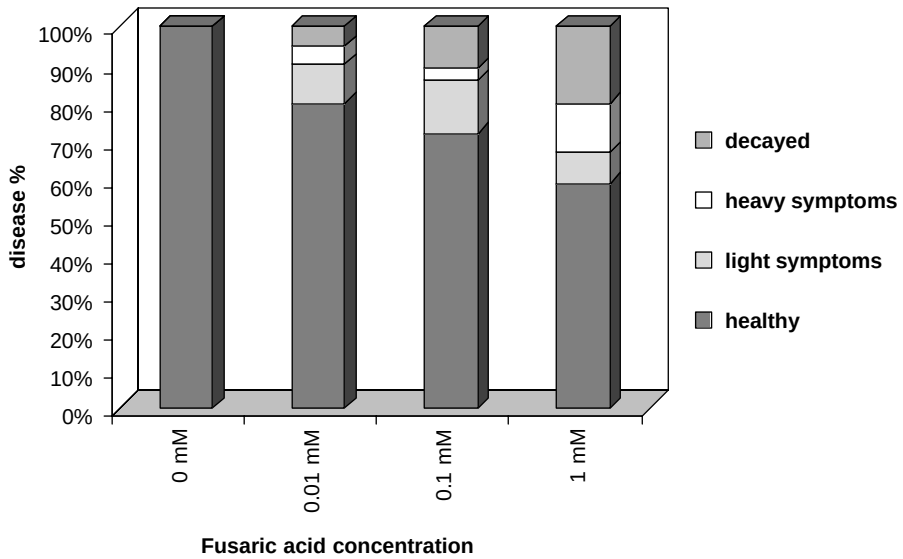

Figure 3. Toxic effects of 3 different dosages of fusaric acid (FA) towards *in vitro* lily bulblets after 30 days of culture: cv Star Gazer (susceptible to Fol).

in activity. An analogous trend could be observed for the Connecticut King cultivar, although the FA-induced increases of PPO activity were not statistically different from the starting value and the decrease observed at the highest FA concentration was less evident than in the Star Gazer cultivar.

With respect to the PO activity, both Star Gazer and Connecticut King showed a statistically appreciable increase at the low and at the average FA concentration. At the highest FA concentration, however, the PO activity was not significantly different from that of the untreated material.

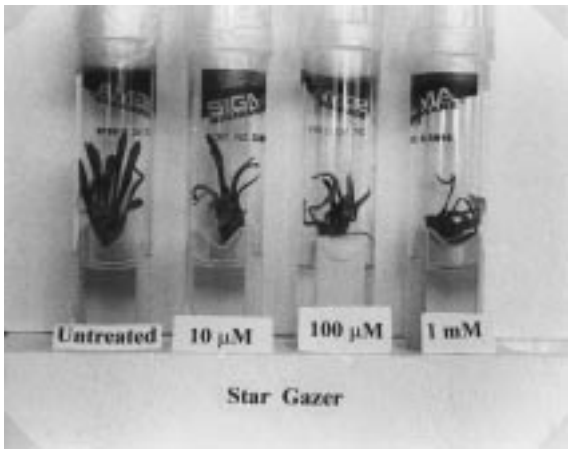

Figure 4. Effects of fusaric acid (FA) on Fol-susceptible Star Gazer cultivar *in vitro* lily bulblets, after 30 days of culture.

Table 3. Effects of Fol inoculation on *in vivo* bulbs of the Star Gazer (Fol-susceptible) and Connecticut King (Fol-resistant) lily cultivars, after 1 month of culture

	Star Gazer	Connecticut King
Healthy	61[a]	80[b]
Light symptoms	13[a]	6[b]
Heavy symptoms	8[a]	6[a]
Decayed	18[a]	8[b]

Each value, expressed as a percentage, is the average of 3 different observations on a 100-bulb sample. Values in each row followed by the same letter are not statistically different for $p = 0.05$ according to the Student–Newmann–Keuls test. Data were transformed in arcsin before the analysis.

Table 4. Percentages of polyphenol oxidase (PPO) and peroxidase (PO) activity within bulblet tissues treated with 3 different dosages of fusaric acid (FA)

FA mmol L^{-1}	PPO activity %		PO activity %	
	Star Gazer	Connecticut King	Star Gazer	Connecticut King
Untreated	100[a]	100[a]	100[a]	100[a]
0.01	110[ab]	100[a]	120[b]	115[b]
0.1	125[b]	103[a]	130[b]	120[b]
1.0	60[c]	90[b]	105[a]	100[a]

Data obtained by comparing, for each cv, the values of treated with those of the untreated samples, the latter assumed as 100% of activity. Each value is the average of 10 observations. Values in each column followed by the same letter are not statistically different for $p = 0.05$, according to the Student–Newmann–Keuls test. Values were transformed in arcsin before the analysis.

Discussion

For the same *in vitro* culture medium, the different conditions of static and shaken culture had a remarkable effect on Fol conidia formation, fungal growth and biomass production. FA analysis in the Fol culture medium confirmed reports of Löffler and Mouris (1992). However, the concentrations of this toxin found under our experimental conditions, at the end of the culture period, were greater (from 1.52 to 1.71 mmol L^{-1}) than those detected by the previous authors (about 1 mmol L^{-1}). Our results indicate that the FA production rate by Fol during the *in vitro* culture follows the biomass development, and that the biosynthesis of this toxin started at the beginning of the culture. FA was detected after 1 week of culture, in contrast with the results of Löffler and Mouris (1992) which did not find the toxin after an identical culture period. FA produced pathological effects, at the tested concentrations, in the lily tissues, as demonstrated through the experiments with the *in vitro* material. The Fol-susceptible cultivar was particularly sensitive to the FA effects, and the experiments with *in vitro* lily bulblets showed that the Fol-susceptible cultivar accumulates, within its tissues, a larger amount of FA than the Fol-resistant cultivar. This could possibly mean that, as reported for tomato plants (Klüpfel, 1957), FA would be subjected to a decomposition by plant tissues more rapidly in the Fol-resistant than in the Fol-susceptible cultivar. In *Pisum sativum*, *Solanum lycopersicum* and *Gossypium herbaceum* the different degrees of resistance to FA can be partly explained by different detoxification abilities (Rudolph, 1976). It is likely that in lily's cultivars the different tolerances to FA could find an analogous explanation. However, the low amount of FA detected within the tissues of both the Fol-infected lily cultivars indicates that the Fol mycelium, when it colonizes the bulbs, produces only small quantities of toxin, independent of the resistance level possessed by the tissues. From this point of view, differences in FA detoxification ability between a Fol-resistant and a Fol-susceptible lily cultivar become irrelevant: other factors (e.g., some other fungal toxin or enzyme) may have a more significant role. It is nevertheless important to stress that FA is able to evoke, within healthy lily bulbs, a defence response comparable to that elicited in other plants by the presence of a pathogen: FA at

the lower tested dosages induced in the Fol-susceptible cultivar a PPO activity increase, which is a typical defensive response elicited by a pathogenic fungus in several plant species (Diez and Gil, 1998; Heitefuss et al., 1960). The same holds true for the PO activity which, although unaffected by the high concentrations of FA tested, was slightly increased in both the lily cvs by the lower toxin concentrations: this is again a tissue reaction which mimics a defence response (Grisebach, 1981). FA in the Fol-lily pathogenic interaction seems therefore to act as an elicitor of the oxidative enzyme-mediated system of plant defence more than as an aggressin which, in the sense according to Yoder (1980), is an attack toxin able to cause severe damages to the plant tissues. This hypothesis is supported by finding only traces of FA within lily bulb tissues even when seriously infected by Fol. As for other previously studied pathogenic interactions, where only negligible levels of FA were detected within *Fusarium*-infected tissues (Davis, 1969), FA does not appear to be essential for pathogenicity in the lily basal rot disease.

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