European Journal of Plant Pathology **102:** 127–132, 1996.

Effects of fusaric acid on cells from tomato cultivars resistant or susceptible to *Fusarium oxysporum* f. sp. *lycopersici*

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Accepted 1 June 1995

Key words: cell suspension culture, ion leakage, *Lycopersicon esculentum*, tomato, toxicity, fusaric acid

Abstract

Cell suspension cultures were set up from two tomato cultivars, one resistant, ('Rio grande') and one susceptible ('63.5') to *Fusarium oxysporum* f. sp. *lycopersici*. Growth rates of the two cell cultures were comparable. Toxicity of fusaric acid, expressed as the fresh weight loss, was analyzed: It was significant in both cases after 10 h, but toxicity was twice as high for '63.5' suspension cells. In the same way, electrolyte leakage caused by fusaric acid was three times more important for '63.5' suspension cells. Moreover, fusaric acid treatment resulted in an acidification of the extracellular medium for '63.5' suspension cells (0.4 pH unit), whereas an alkalization was observed for 'Rio grande' suspension cells (0.2 pH unit). Preliminary experiments suggest that fusaric acid was partially metabolized by 'Rio grande' suspension cells, however, no detoxified forms of fusaric acid were detected either in cells or in culture filtrates. For these two tomato cultivars, the differences in sensitivity to fusaric acid of cultivated cells correspond to the differences in plant susceptibility to *Fusarium oxysporum* f. sp. *lycopersici*.

Abbreviations: BAP = 6-benzylaminopurine; γ = conductivity; 2,4-D = 2,4-dichlorophenoxyacetic acid; EtOAc = ethyl acetate; FA = fusaric acid; ρ = resistivity.

Introduction

Fusaric acid (FA: 5-*n*-butyl-pyridine-2-carboxylic acid), a well known non-specific toxin, is produced by many *Fusarium* species which cause wilt disease on crops such as cotton, watermelon, pea, banana and tomato. However, the involvement of FA in pathogenicity has not been clearly established. Some data suggest it has a role in pathogenesis [Gäumann, 1958; Barna *et al.*, 1983; Mégnégneau and Branchard, 1988; Chakrabarti and Basu Chaudhary, 1980; Toyoda *et al.*, 1988] while others assume that it does not have a direct role and may act associated with other factors [Davis, 1969; Kuo and Scheffer, 1964; Shahin and Spivey, 1986].

Concerning the mode of action of FA, this toxin is known to impair the water permeability of protoplasts [Gäumann, 1958], and to cause electrolyte loss in tomato leaves [Linskens, 1955] and in sorghum seedlings [Dunkle and Wolpert, 1981]. FA can also affect the electrical membrane potential [Marrè *et al.* 1993], the membrane conductance and the cellular ATP level [Köhler and Bentrup, 1983; D'Alton and Etherton, 1984].

The aim of this study was to compare the effect of FA on tomato suspension cells obtained from plants which are resistant to *Fusarium oxysporum* f. sp. *lycopersici* (race 0 and 1) 'Rio grande' or susceptible '63.5'. FA toxicity, its effect on electrolyte leakage and its possible metabolization by tomato suspension cells were investigated.

Materials and methods

Culture conditions

Cell suspension cultures were set up from seedling explants of two tomato cultivars. 'Rio grande'

(Petoseed obtention) is resistant to *Fusarium oxysporum* f. sp. *lycopersici* (resistant to the 2 races), it has the 2 genes I and I-2 and it is also resistant to *Verticillium dahliae* by the gene Ve. The hybrid F_1-Montfavet 63.5 ('63.5') (obtained at the Station d'Amélioration des Plantes Maraichères Montfavet, France) does not have these resistance genes. Seeds were sterilized by dipping in 80% EtOH (2 min), followed by a treatment with 1.3% NaOCl solution and finally rinsed in sterile distilled water [Tisserat, 1985]. Germination on solid agar medium was performed in sterile culture tubes which were kept in a growth chamber at 24 °C under continuous fluorescent light (1500 Lux). 15-day old seedlings were transferred on agar medium supplemented with 22.5 mg l^{-1} BAP. Induction and growth of calli were performed with stem explants from 3-week old seedlings on Chandler's medium [Chandler *et al.*, 1972], supplemented with 0.1 mg l^{-1} 2,4–D and 0.3 mg l^{-1} BAP. Calli were subcultured every 3 weeks for 4 to 5 months, and subsequently used in order to initiate suspension cells. Cell suspension cultures of *Lycopersicon esculentum* f. sp. *lycopersici* cv 'Rio grande' and '63.5' were grown in Chandler's medium on a rotary shaker (150 rpm) in a growth chamber as described above. Suspension cells were subcultured every 6 days by transferring 13 g of cells (fresh weight) into 220 ml of fresh medium.

Determination of cell growth

Suspension cells were transferred under sterile conditions in 16 cm long tubes (5 ml/tube) and put in a growth chamber on a rotary shaker (72 rpm). Three replicates were daily analysed. Cells were removed from the medium by filtration and fresh weights were determined immediately.

Extracellular pH and conductivity measurement

Suspension cells (100 ml) were collected at the exponential growth phase, washed with 3 × 20 ml buffer (175 mM mannitol, 0.5 mM $CaCl_2$ $2H_2O$, 0.5 mM K_2SO_4, 2mM Mes, pH 5.75) and resuspended in buffer (1 g fresh weight for 10 ml buffer). 10 ml aliquots were transferred into 25 ml erlenmeyer flasks. After a 3 h stabilization period on a rotary shaker at 25 °C, suspension cells were treated with an aqueous sterile FA solution [100 μg/ml, (0.56 mM) final concentration]. FA was purchased from Sigma, used as an aqueous solution sterilized by filtration through a Millipore membrane (GV 0.22 μm). Ion leakage measurements (extracellular pH and resistivity) were measured for 8.5 h with a PHM 62 standard pH meter and a CD 60 electronic Tacussel resistivimeter, respectively. Conductivity (γ, $\mu S\ cm^{-1}$) was calculated using the following equation: $\gamma = 1/(\rho \times K)$, (K = 0.84 cm). Results are expressed as ΔpH where $\Delta pH = pHt - pHc$, and $\Delta\gamma = \gamma t - \gamma c$, t for the treated cells, c for the control cells.

Toxicity of fusaric acid and FA distribution

5 ml of 4-day old suspension cells of '63.5' and of 'Rio grande' were transferred into sterile tubes and treated with different amounts of FA [0 to 100 μg ml^{-1} (0.56 mM) final concentration] for 5, 10 and 20 h. Tubes were set on a rotary shaker in the growth chamber in the conditions described previously. Cellular viability was observed under light microscope using vital staining (neutral red). Cells were removed from the medium by filtration. Toxicity was expressed as $\Delta P/Pc \times 100$ where $\Delta P = Pt - Pc$, [Pt: fresh weight of treated cells and Pc: fresh weight of control cells]. Then, FA was extracted from the cells and from the culture filtrates separately in order to obtain information on FA distribution and on its potential metabolization.

Extraction of fusaric acid

The method of extraction of FA from culture filtrates and cells was adapted from a previously described method [Barna *et al.*, 1983]. The pH of the filtrate was adjusted to 3.5 with 5N HCl and FA was extracted 3 times with EtOAc. Organic extracts were pooled and concentrated by rotary evaporation under vacuum at 30 °C. Samples were dissolved in 200 μl EtOH and stored at −20 °C until further use. Treated tomato cells were kept frozen until extraction. After thawing, they were sonicated in 10 ml H_2O for 2 min. Then, cell debris was removed from the aqueous fraction by filtration. This step was repeated twice, the aqueous fractions were pooled, the pH was adjusted to 3.5 and FA was extracted with EtOAc as described for culture filtrates.

HPLC quantitation of fusaric acid

Aliquots of 25 μl were taken from the 200 μl ethanolic extracts and the solvent was evaporated to dryness under N_2. Samples were methylated with diazomethane at room temperature for 15 min in EtOAc. Then, diazomethane and EtOAc were removed under N_2 and samples were stored at −20 °C. Samples were dissolved in 75 μl EtOH and 20 μl were analysed by HPLC using a Merck Lichrocart Cartridge RP-C18 (5

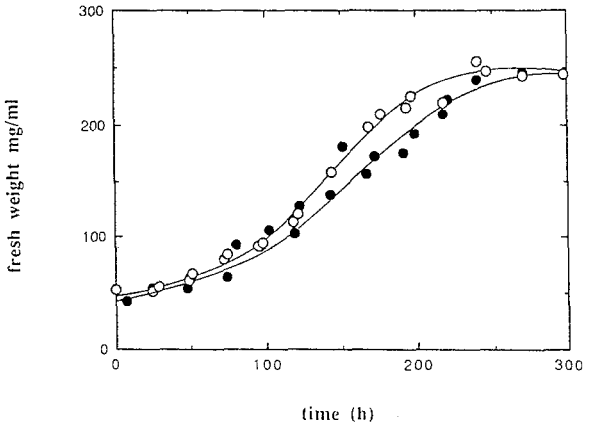

Fig. 1. Time course of cell growth of *Lycopersicon esculentum* f. sp. *lycopersici* cv. '63.5' (●) and 'Rio grande' (○) grown in Chandler medium. Suspensions were subcultured every 6 days with 13 g cells (fresh weight) in 220 ml fresh medium.

μm, 250 mm × 4 mm) and the mobile-phase was composed of a mixture of acetonitrile: H_2O containing 1% acetic acid, with the following linear gradient steps: initial conditions (25:75); 0–8 min (40:60); 8–12 min (100:0); 12–17 min (100:0); 17–22 min (25:75), with a flow rate of 1 ml min^{-1}. Quantitation was obtained using a UV LC-spectrophotometer Waters model 481 set at 254 nm. Pure FA was methylated and used for calibration.

Results

Growth rate of tomato cell suspension cultures

Growth rate, generation time and biomass level at the stationary phase were determined with suspension cells from '63.5' and 'Rio grande' tomato cultivars (Fig. 1). After a 24 h lag period, the growth of suspension cells 'Rio grande' increased exponentially, reached the stationary phase after 225 h and contained 243 mg ml^{-1} fresh weight. A 76 h generation time was calculated during the linear phase. A similar profile was obtained for '63.5' suspension cells with a 20 h lag period and 237 mg ml^{-1} fresh weight at the beginning of the stationary phase which was reached after 225 h. Its generation time was 77 h. Both suspension cell cultures showed comparable growth rates. For the following experiments, cells were treated with FA 4 days after subculturing. This corresponded to the beginning of the linear growth phase.

Fusaric acid toxicity

Toxicity of FA to tomato cells was observed under light microscope using neutral red as vital staining of

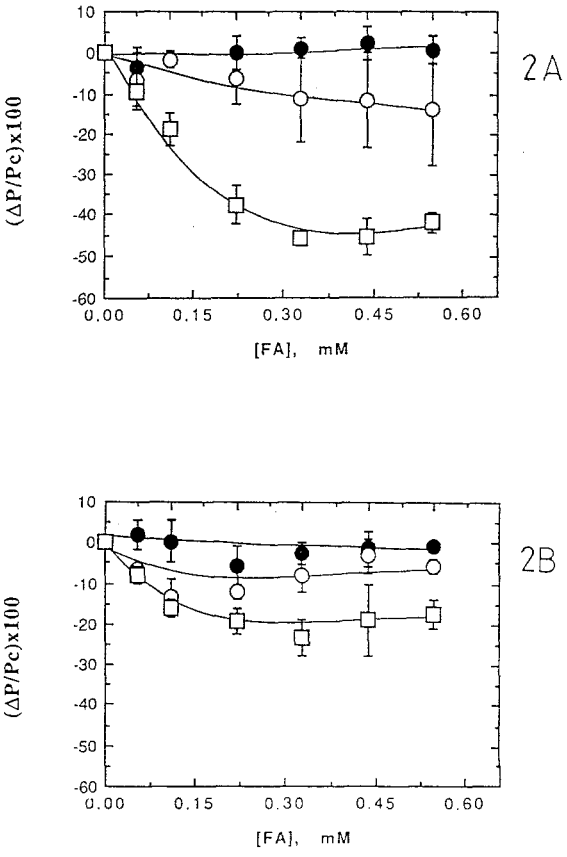

Fig. 2. Fusaric acid toxicity. Culture were kept under constant light, at 24 °C on a rotary shaker (72 rpm) for 5 (●), 10 (○), 20 h (□). 4-day old suspension cells from *Lycopersicon esculentum* f. sp. *lycopersici* were treated with FA (0 to 0.56 mM). Toxicity was expressed as (ΔP/Pc) × 100. (A) '63.5' suspension cells; (B) 'Rio grande' suspension cells.

the cells and was quantified by the cell fresh weight loss (FA concentration: 0–0.56 mM).

With '63.5' cells after 5 h, the vacuoles of treated and untreated cells were highly stained with neutral red and no plasmolysis was observed. Between 10 and 20 h, the vacuole staining of treated cells was less intense and some cells were plasmolysed which was not the case for control cells. The fresh weight of the cells was not affected by a 5 h-treatment with FA (up to 0.56 mM) (Fig. 2A). After a 10 h-treatment, a significant weight loss was observed for FA concentrations between 0.22 to 0.56 mM. After a 20 h-treatment, 0.11 mM FA already induced a ΔP/Pc of −20% which reached a plateau at −45% with increasing concentrations.

With 'Rio grande' no plasmolysis was observed either on FA-treated or on control cells. There was no significative difference in fresh weight loss after 5 h

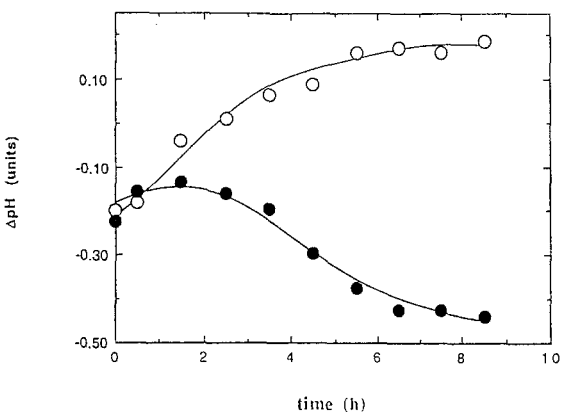

Fig. 3. Time course of the effect of fusaric acid on pH of '63.5' (●) and 'Rio grande' (○) suspension cells expressed as ΔpH between treated and control cells. Cells were collected at the exponential phase, resuspended in buffer (175 mM mannitol, 0.5 mM $CaCl_2$ $2H_2O$, 0.5 mM K_2SO_4, 2mM Mes, pH 5.75) and treated with FA (0.56 mM) after a 3 h stabilisation period on a rotary shaker at 24 °C.

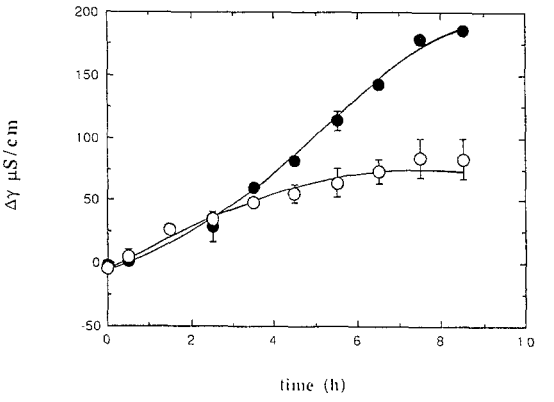

Fig. 4. Time course of the effect of fusaric acid on conductivity of '63.5' (●) and 'Rio grande' (○) suspension cells expressed as Δγ between treated and control cells. The conditions are those described for Fig. 3.

or 10 h of treatment with FA (up to 0.56 mM) (Fig. 2B). After 20 h, the loss of fresh weight increased with FA concentrations up to 0.22 mM, then remained constant (ΔP/Pc around −20%) for a higher FA concentration.

Therefore, significant fresh weight losses of treated cells as compared to control cells only occured after a 10 h-treatment. After 12 h, (data not shown) ΔP/Pc was more important for '63.5' than for 'Rio grande' suspension cells. After a 20 h-treatment the difference between the two suspension cells was more pronounced. Relative loss of fresh weight of '63.5' suspension cells reached a maximum of about 45%, whereas it did not exceed 20% with 'Rio grande' cells. With these cultivars, a higher susceptibility to *Fusarium oxysporum* f. sp. *lycopersici* corresponded to a higher toxicity of FA.

Similar toxic effects of FA treatment on callus and cell growth have been previously reported in the literature. FA concentrations above 0.02 mM decreased the growth of muskmelon calli but the extent of this decrease depended on the genotype studied, whereas at a 0.11 mM concentration, callus growth was stopped for both genotypes. These observations were done after one month of culture [Mégnégneau and Branchard, 1988]. For *Asparagus officinalis* cells, toxicity was expressed over a 10-day period as a delay of cell division (0.04–0.06 mM) and lethality (0.08–0.20 mM) [Jullien, 1988]. In the present study, the effects of two parameters, treatment duration and toxin concentration were investigated and evidence was obtained for the involvement of both factors in toxicity. It has to be noted that the FA concentration range used in this work is about 5 times higher than those used for the studies done with muskmelon or asparagus. In our case, the loss of fresh weight cannot be ascribed only to plasmolysis but could be due to an alteration of cell division. For example, it was shown [Zaichenko and Bogomolova, 1984] that FA inhibits enzymes of glycolysis (alcoholdehydrogenase and glucose-6-phosphate dehydrogenase) in *Dendrodochium toxicum*, this might alter cell division.

Extracellular pH and conductivity measurement

Effect of FA on plasma membrane permeability was reflected by changes in pH and in conductivity (γ) of the extracellular medium. For both cell suspension cultures, the extracellular pH decreased immediately after the addition of FA (0.56 mM) (Fig. 3). This is due to a direct acidification of the medium by FA, since the pH of the medium without cells also dropped by 0.2 pH unit after the addition of FA. During the first 30 min after FA addition, a slight alcalization occured for both suspension cells. Afterwards, the ΔpH of '63.5' suspension cells remained constant for 2.5 h, decreased to −0.4 pH unit between 2.5 and 6.5 h and remained constant thereafter. The ΔpH of 'Rio grande' suspension cells increased gradually to +0.2 pH unit during the 6h-treatment.

Δγ values of '63.5' and of 'Rio grande' suspension cells were recorded for 9 h (Fig. 4). Δγ of the '63.5' suspension cells increased gradually up to 190 $\mu S\ cm^{-1}$, whereas Δγ of 'Rio grande' suspension cells

Table 1. Fusaric acid distribution after a 5 min and a 20 h treatment. FA was extracted from cells and culture filtrates of '63.5' and 'Rio grande' cell suspension cultures. Results are expressed as the percentage of the amount initially added

Cultivars	Duration of the treatment	% FA extracted from cells	% FA extracted from culture medium	% total FA recovered
63.5	5 min	8 ± 1	92 ± 2	100 ± 3
Rio grande	5 min	15 ± 2	85 ± 3	100± 5
63.5	20 h	23 ± 2	77 ± 2	100 ± 4
Rio grande	20 h	28 ± 2	32 ± 5	60 ± 7

increased more slowly and reached a plateau at 90 μS cm^{-1} after 6 h. Cells obtained from cultivars which are susceptible to *Fusarium oxysporum* f. sp *lycopersici* might present a higher membrane permeability to ions than cells from resistant ones as it was previously mentioned for other tomato cultivars [Storti *et al.*, 1992].

To summarize, FA-treatment induced a decrease of the extracellular pH of '63.5' suspension cells, an increase of the extracellular pH of 'Rio grande' cell cultures and a more pronounced electrolyte leakage for '63.5' suspension cells than for 'Rio grande'. Therefore, with these cultivars, suspension cells obtained from plants susceptible to *Fusarium oxysporum* f. sp *lycopersici* are slightly more sensitive to FA than suspension cells obtained from resistant plants, sensitivity being characterized by both fresh weight losses and electrolyte leakage.

Fusaric acid distribution

The amounts of FA extracted from the cells and from the culture medium after a 20 h treatment were compared with the amount extracted after a few minutes treatment and expressed as the percentage of the total FA added (500 μg/assay) (Table 1). Just after homogenization (5 min after FA addition), FA was extracted from the two suspension cells (cells and culture media) and 100% of the FA added was recovered. However, FA distribution was different: in cells from '63.5' culture, the amount of FA was lower (8 ± 1 %) than in cells from 'Rio grande' culture (15 ± 2%). After 20 h of treatment with FA, 100% of the FA added was recovered from '63.5' suspension cells whereas only 60% from 'Rio grande' suspension cells was. The amounts of FA in the two cell extracts were similar (23 ± 2 and 28 ± 2% for '63.5' cells and 'Rio grande' cells respectively), but the amount of FA in '63.5' culture filtrate (77 ± 2%) was about 2.5 fold the amount recovered from 'Rio grande' culture filtrate (32 ± 5%).

A possible explanation is that FA could be metabolized in 'resistant' cells. Another study [Kluepfel, 1957] pointed out that tomato plant could detoxify FA. However, in our experimental conditions, no such detoxified forms of FA were found. This might indicate that FA is partly degraded by 'Rio grande' cells. It could also be bound to the cells or to secondary metabolites which made it unextractable.

Discussion

From these experiments, where we analysed extracellular pH modifications, electrolyte efflux and FA distribution, differential responses were observed between '63.5' and 'Rio grande' suspension cells. Just after FA addition to the suspension cells, extracellular pH values were similar for both. FA could penetrate the cells in the neutral form and accumulation could occur in the cytosol in the charged form or in the vacuole. For '63.5', the external FA concentration did not change significantly and the acidification of the extracellular medium occured concomitantly with the increase in conductivity. For 'Rio grande' cells, FA external concentration decreased. This could contribute to alcalinize the medium but cannot be responsible alone for this effect. Part of the FA recovered from the cells could be bound to membranes or cell walls or accumulated in the cytosol as shown previously [Marrè *et al.*, 1993]. The differential response in conductivity may be correlated to membrane permeability changes which are more important for '63.5' than for 'Rio grande'.

In conclusion, two suspension cells from tomato plants which are susceptible or resistant to *Fusarium oxysporum* f. sp. *lycopersici* were compared for their

sensitivity to FA. This study pointed out differential sensitivity of these cultivars to FA. These differences might be related to their susceptibility to *Fusarium oxysporum* f. sp *lycopersici*. Further experiments are under progress in order to compare a larger number of tomato genotypes.

Acknowledgements

This work was supported by the Conseil Regional de Bourgogne. We thank the Caisse Régionale du Crédit Agricole for its contribution.

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