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FULL RESEARCH PAPER

Fusaproliferin effects on the photosystem in the cells of maize seedling leaves

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Abstract The possible role of the fusariotoxin, fusaproliferin in plant pathology was investigated with respect to cell membrane potential. Electron microscopy was used to study both the early effect of fusaproliferin on the host's plasma membrane and ultrastructure responses in the cells of maize leaves. The seedlings of resistant (Lucia) and susceptible (Pavla) to the fusaproliferin maize cultivars were grown in the presence of fusaproliferin at different concentrations, namely 5 and 35 μg ml^{-1}, respectively, and electrophysiological measurements were compared with those obtained using two different toxic compounds, namely fusicoccin and *3-3(3,4 dichlorophenyl)-1,1-dimethylurea* (DCMU). It was observed that only the higher concentration of fusaproliferin induced the onset of visible symptoms on the leaves. Comparing the effect of fusaproliferin to that of fusicoccin and DCMU at the higher toxin concentration, it was observed that functional differences in membrane potential induced severe damage to the mesophyll and outer chloroplast membrane; the extent of changes in electrophysiology and ultrastructure disturbances depended on the toxin concentration and was greater in the susceptible cv. Pavla. Results indicated that fusaproliferin could be involved in *Fusarium* pathogenesis either as a virulence factor or by enhancing the activity of other toxins that might be concomitantly present in infected plants.

Keywords Fusaproliferin · Electrophysiological measurement · Leaves · Mesophyll · Bundle sheath · Chloroplast

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Introduction

Members of the genus *Fusarium* produce a range of chemically different phytotoxic compounds, such as fusaric acid, fumonisins, beauvericin, enniatin, moniliformin and trichothecenes. These possess a large variety of biological activities and metabolic effects including wilting, chlorosis, necrosis, growth inhibition, inhibition of seed germination, and effects on calli (Neuhold et al. 1997; Desjardins and Hohn 1997). The possible use of some fungal toxins as bioherbicides has been extensively reviewed (Duke and Lydon 1993), and some *Fusarium* metabolites, such as enniatins and fumonisins, were evaluated for their herbicidal properties (Abbas et al. 1991).

In planta experiments demonstrated that deoxynivalenol is phytotoxic to wheat tissues (Wang and

Miller 1988). The role of mycotoxins in plant disease were studied by Adams and Hart (1989); they determined that deoxynivalenol serves no role as a pathogenicity or virulence factor, but Atanassov et al. (1994), found that isolates of *F. graminearum* that produced more deoxynivalenol were more aggressive on wheat by reducing grain weight more than isolates that produced less of the toxin *in planta*. A large variation in aggressiveness and in the type and amount of mycotoxin produced has been found among *Fusarium* isolates collected from various geographic locations (Gang et al. 1998; Miedaner et al. 2001) and variations in mycotoxin production were correlated to aggressiveness of the isolates in their experiments. The phytotoxicity of the *Fusarium* trichothecene and fumonisin mycotoxins has led to speculation that these toxins are involved in plant pathogenesis. Recently this subject has been addressed by examining virulence of trichothecene and fumonisin non-producing mutants of *Fusarium* in field tests (Proctor et al. 2002). Trichothecene non-producing mutants of *F. graminearum* caused less disease than the wild-type strain and the fumonisins-producing species *F. verticillioides*, which causes maize ear rot. Although the analyses of virulence of fumonisins non-producing mutants of *F. verticillioides* are not complete, to date, the mutants have been as virulent on maize ears as their wild-type progenitor strains. In our previous work (Šrobárová et al. 2004) with fusariotoxins (moniliformin, fumonisins B_1, zearalenone, zearalenol, deoxynivalenol and fusaproliferin), large amounts of osmiophilic globules were present within the periplasmic space and in the cytoplasm of maize cells. Some of the toxins are phytotoxic (e.g. moniliformin) and fumonisin damages cell membranes and reduces chlorophyll synthesis (Lamprecht et al. 1994).

Mycotoxins, secondary metabolites produced by a variety of fungi under appropriate circumstances (e.g., temperature and moisture), can have carcinogenic, mutagenic, or teratogenic properties, and it has been observed that the presence of mycotoxins in foods and feeds can represent a severe health risk for both humans and animals. A relatively recently identified toxin fusaproliferin was isolated and purified by Ritieni et al. (1997) and it has been shown that it can induce teratogenic effects, e.g. cephalic dichotomy, macrocephaly and limb asymmetry, in chicken embryos bioassays (Ritieni et al. 1997). Later its toxicity to *Artemia salina*, SF-9 insect cells, and IARC/LCL 171 human B-lymphocytes was identified (Logrieco et al. 1996). Fusaproliferin, a bicyclic sesterterpene derived from five isoprenic units contains several chiral centres in the molecule (Santini et al. 1996). Fusaproliferin is produced by several isolates of *Fusarium proliferatum* and *F. subglutinans* and it has been found as a natural contaminant in different commodities in Europe and in the United States (Placinta et al. 1999). In plants, only the effects of fusaproliferin on chloroplasts were investigated (Nadubinská et al. 2003) and the co-production of fusaproliferin with fumonisin and moniliformin, known for their phytotoxicity, was observed in maize kernels (Pascale et al. 2002). This literature gap and the clear relationship between toxins and their phytopathological effects prompted us to investigate the effect of fusaproliferin on maize seedlings, and in order to reach this goal two different cultivars of maize, one resistant and one susceptible to *Fusarium* were selected. Many mycotoxins have an important role in plant disease development and they may be considered a possible phytotoxic agent produced by microscopic fungi during their colonization. To date fusaproliferin has been well investigated for its role on mycotoxicosis towards animal and cell lines; the main goal of this work is to recognize the phytotoxic activities of fusaproliferin in plant diseases.

Materials and methods

Chemicals

All solvents (methanol, acetonitrile, and hexane) were of HPLC grade and purchased from J. T. Baker (Deventer, Holland). Deionized water was purified with a Millipore Milli-Q Plus system (Millipore, Espoo, Finland).

Toxin extraction

Fusaproliferin was produced according to the method proposed by Randazzo et al. (1993). Autoclaved maize grains were inoculated with a known fusaproliferin producer, *F. proliferatum* ITEM 1494. After incubation, fusaproliferin was extracted from the matrix, purified with liquid–liquid extractions, and purified by silica column and preparative thin layer

chromatography (TLC) following the method of Ritieni et al. (1995). For the fusaproliferin extraction, 100 g of sample were homogenized in a Waring blender for 5 min with 150 ml of methanol (Baker, Deventer, Holland, 99.5%). Samples were filtered using Whatman No. 4 filter paper, and residual methanol was removed under reduced pressure. This extraction procedure yielded 1.5 g of raw organic extract that was used to evaluate the fusaproliferin concentration. A standard stock solution (10 μg ml^{-1}) was prepared in methanol and stored in the dark at 4°C.

Chemical analysis

The amount of fusaproliferin was determined by high-perfomance liquid chromatography (HPLC). Sample extracts were filtered through Whatman no. 4 filter paper and concentrated under reduced pressure at 408°C (Heidolph Instruments, Schwabach, Germany) to 3 ml. Pre-purification was performed on a C18 column (Varian, Palo Alto, CA, United States) pre-conditioned with 3 ml of methanol, and samples were eluted with 2 ml of the same solvent. The eluate was concentrated to 1 ml and filtered through an Acrodisk filter (0.22 mm); 20 μl were loaded onto the column for HPLC analysis. Analyses of fusaproliferin was performed according to the method of Monti et al. (2000) with minor modifications using a Shiseido Capcell Pak C_{18} (250 m, 4.6 mm, 5 μm) column, LC-10AD pumps, and a diode array detector (Shimadzu, Tokyo, Japan). The solvent system was CH_3CNH_2O (65:35, vol/vol) with a constant flow at 1.5 ml min^{-1}. The starting solvent ratio was kept constant for 5 min and then linearly modified to 70% CH_3CN over 10 min. Mycotoxin identification was performed by comparing retention times and UV spectra of purified samples to those of pure standards and by co-injecting the sample and the pure standards. All analyses were run in duplicate. Calculated standard deviation was always <5%. From the calibration curve, a detection limit of 20 ng for fusaproliferin with ratio noise to signal of three was detected; this limit corresponded to 1 mg kg^{-1} of dried sample. Mycotoxin quantification was carried out by comparing peak areas of samples with the calibration curve of reference standards. For LC-MS HPLC, conditions described above for the analytical separations were applied, but a different HPLC system was used. A Perkin-Elmer LC series 200 connected to a 785A UV/VIS detector was coupled with an API-100 single quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Canada). A flow rate of 20 μl min^{-1} was split from the LC eluent into the ion spray source. A probe voltage of 5,300 V and a declustering potential of 50 V were used. Full-scan spectra were acquired from 400 to 800 amu using a step size of 0.5 amu and a dwell time of 4.2 ms. The instrument mass to charge ratio scale was calibrated with the ions of the ammonium adducts of polypropylene glycol.

Plant material

For our investigations two maize cultivars provided by Zeainvent, Trnava, Slovakia were used, one resistant (cv. Lucia) and one susceptible (cv. Pavla) to *Fusarium* infection (Šrobárová et al. 2004). Seeds (20 of each cultivar) were surface-sterilized with sodium hypochlorite (1% available chlorine) for 2 min and rinsed three times in sterile distilled water for 2 min. The seeds were germinated on moistened filter paper in Petri dishes in the dark at 21°C for 3 days. The seedlings were selected for uniformity and the filter paper was cut into small pieces (1 cm^2), each containing one germinated seed.

The pieces of filter paper with the seedlings were placed on a larger moistened filter paper in a jar, on the surface of potato dextrose agar (PDA; Difco Laboratories, Detroit); 40 g was suspended into the 1 l distilled H_2O, containing three different concentrations of fusaproliferin: 0 (control), 5 and 35 μg ml^{-1}. Seedlings (five in each experiment) were grown at 21/15°C (day/night) and a photoperiod of 16/8 h. After 8 days of cultivation the leaves were removed for sampling. For the membrane potential (E_M) measurements, segments (10×15 mm) were removed from the central part of the third leaf of 8 day-old plants.

The higher toxin concentration in the agar substrate (35 μg ml^{-1}) caused chlorophyll and structural responses in maize seedlings in our previous experiments (Nadubinská et al. 2003), while the lower level (5 μg ml^{-1}) was the lowest concentration effective in an embryotoxicity bioassay (Ritieni et al. 1997). Fusaproliferin, isolated and purified as described by Ritieni et al. (1997), was dissolved in methanol (Mikrochem, Bratislava, >99%) and the stock solution was diluted in PDA to a 0.5 mg ml^{-1} concentration.

Electrophysiological measurements

The leaf segments were mounted in a 4 ml volume plexiglass chamber and were constantly perfused (10 ml min^{-1}) with bathing solution containing 1 mM KCl and 1 mM $CaSO_4$, adjusted to pH 5.7 using 0.1 M HCl. The membrane potential (E_M) of both control and fusaproliferin (35 μM) treated segments were then measured using micropipettes filled with 3 M KCl. The tip diameter was 0.5 μm, tip potential −5 to −15 mV, and a micromanipulator was used to insert micropipettes into single palisade parenchyma cells of corn leaves. The E_M was recorded as previously described (Pavlovkin et al. 2004). For the E_M measurements in the light the irradiance at the tissue surface was 115 μW cm^{-2}. For those E_M measurements in the dark, a green filter was placed in front of the light source during the micropipette impalement of the cells. E_M measurement was carried out at 22°C. Fusicoccin (Sigma, 30 μM in 0.1% ethanol), a H^+-ATPase stimulator, was used to monitor the functionality of the membrane H^+-ATPase (Marre 1980). To induce anoxic conditions, the perfusion solution was saturated with N_2 gas by flushing. The flow of the perfusion solution through the measuring chamber at 10 ml min^{-1} was sufficient to maintain anoxia.

Electron microscopy

For electron microscopy, segments from the central part of the 3rd leaf were fixed with 3% glutaraldehyde and 1% OsO_4, buffered with sodium cacodylate to pH 7.2, dehydrated in ethanol and embedded in Spurr's medium according to Glauert (1975). Ultra-thin sections from five embedded specimens of each treatment were stained with uranyl acetate and lead citrate, and investigated with the EM Tesla BS 500.

Results

Membrane potential

The membrane potential (E_M) in palisade parenchyma cells of 8 day-old control leaf segments treated was −168±6 mV (n=12) in Lucia and −168±4 mV (n=7) in Pavla in the light and conditions (Fig. 1a, b). Leaf segments treated 48 h by 35 μm fusaproliferin lost their capacity to maintain a high E_M in the light and dark conditions. After 48 h treatment with fusaproliferin the E_M values in the light for Lucia was −150± 9 mV (n=16) and −143±13 mV (n=9) for Pavla, and in the dark for Lucia −166±12 mV (n=17) and −102± 13 in Pavla (n=17). The cells of control plants not exposed to fusaproliferin were able to rescue their E_M values, repolarising within minutes after the light was turned on (Fig. 1a). In the presence of fusaproliferin, membranes in the light did not repolarise although E_M transiently recovered in the tolerant variety, Lucia (Fig. 1b). In the susceptible variety, Pavla, the values of E_M was only slightly higher than those recorded before the light was turned on (Fig. 1b). The most

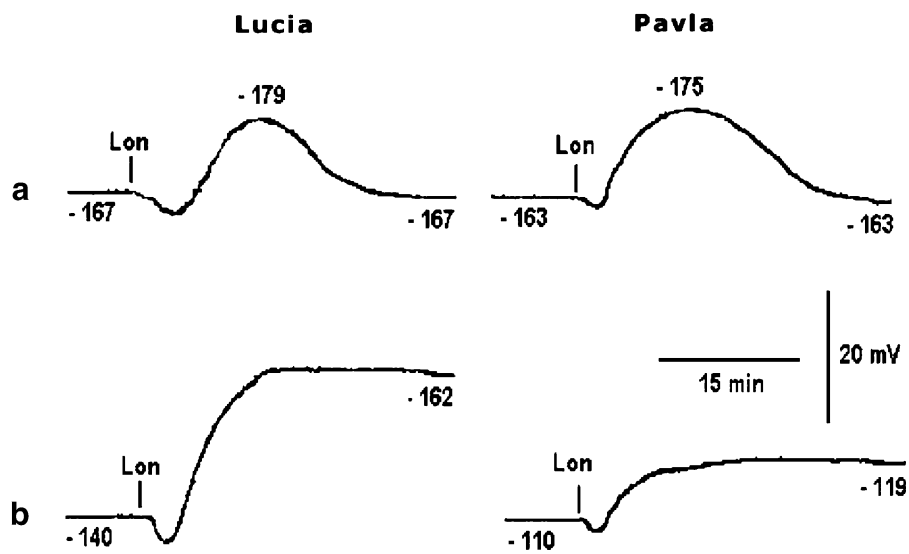

Fig. 1 Response of E_M of palisade parenchyma leaf cells of maize cvs Lucia (resistant) and Pavla (sensitive) to light (Lon) in control (**a**) and with 35 μM fusaproliferin-treated segments (**b**)

commonly observed change in chloroplast structures was the loosening of the tight arrangement of grana thylakoids and a disordered orientation of the stroma thylakoids (Fig. 2). In Fig. 3a the chloroplast grana dilation of thylakoids is illustrated. In mesophyll cells of the susceptible cultivar, vacuolation of cytoplasm due to plasmolysis was observed. The observed structural responses were more pronounced and evident in the susceptible cultivar. Following the exposure to 35 μg ml^{-1} fusaproliferin concentration, several changes in the chloroplast structure were observed in both cultivars, macroscopic symptoms were chlorosis, and a shrivelled appearance or water-soaking, but the outer membrane of the chloroplasts was only sporadically disrupted (Fig. 3a). In the bundle sheath (Fig. 3b) as well as in the mesophyll (Fig. 3c) chloroplasts, a remarkable disturbance of the thylakoid arrangement occurred and plastoglobuli were more frequent. The mesophyll chloroplasts of the susceptible cv. Pavla were filled with large osmiophilic globules (Fig. 3d).

Effect of anoxia

Two components of E_M were separated by anoxic conditions, i.e. by perfusion of the roots with N_2 saturated solution. Anoxic conditions in control roots (treated with N_2 but not with fusaproliferin) in the dark resulted in depolarization of E_M of palisade parenchyma cells to −110±3 mV (n=3) for Lucia and to −113±5 mV (n=3) for Pavla (Fig. 2a). These values are considered to be those of the diffusion potential (E_D). The palisade parenchyma cells of maize leaf segments treated for 48 h by 35 μM fusaproliferin after anoxia in the dark showed E_D values −91±6 mV (n=3) for Lucia and −79±8 mV (n=5) for Pavla. The magnitude of depolarization by anoxia was only small in comparison with that observed for the control cells used as a

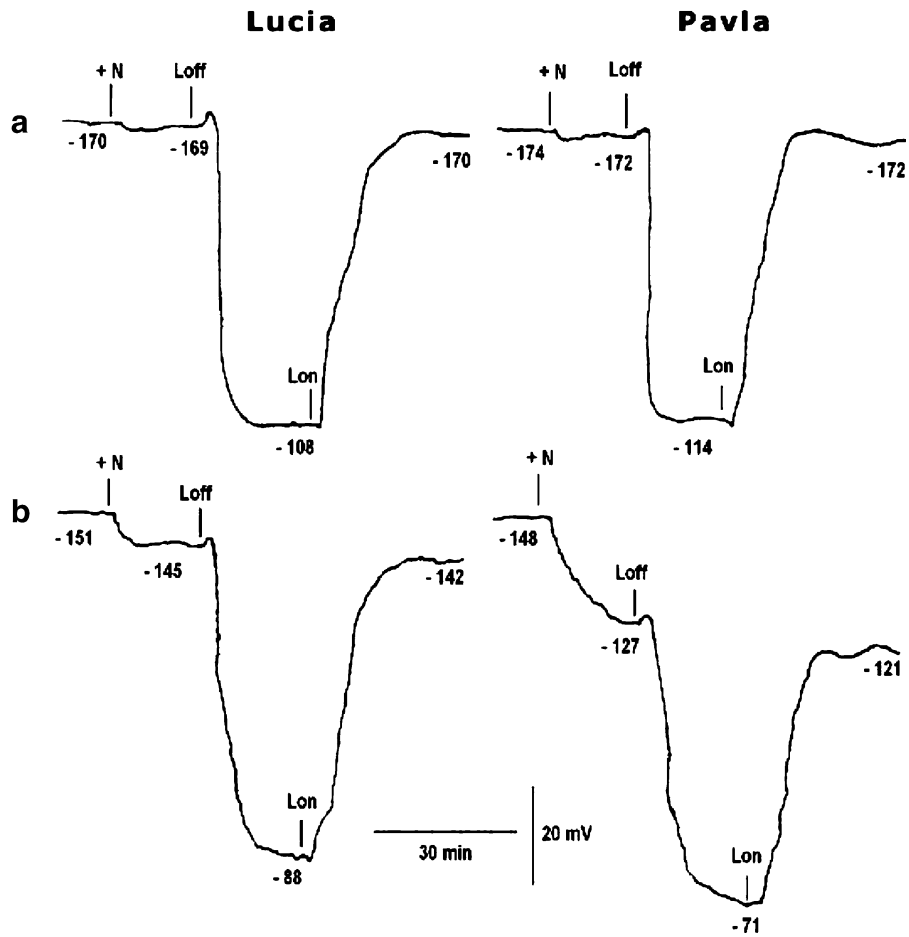

Fig. 2 Effect of anoxia (N_2) on E_M of palisade parenchyma leaf cells of maize cvs Lucia (resistant) and Pavla (sensitive); **a** control, **b** treatment with 35 μM fusaproliferin (48 h). *Loff* light off, *Lon* light on, *+N* addition of N_2-anoxia

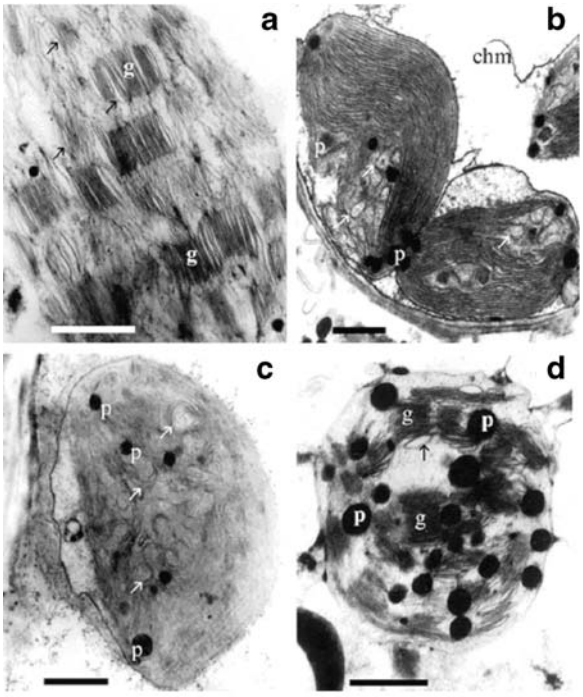

Fig. 3 Chloroplasts in leaves of Pavla (susceptible) (**a**, **b**, **d**), and Lucia (resistant) (**c**) maize cultivars treated with 5 μg l^{-1} fusaproliferin, leaves without visible symptoms (**a**) or 35 μg l^{-1} fusaproliferin, leaves with visible symptoms (**b**–**d**). **a** Loosening of grana (g) and thylakoid dilation (*arrows*). **b** Disorder in thylakoid arrangement (*arrows*) and damaged continuity of chloroplast outer membrane (chm). **c** Disintegrated grana and disordered thylakoids (*arrows*). **d** Grana (**g**) with dilated thylakoids (*arrows*) and an increased number of plastoglobuli (p). *Bars* represent 1 μm

reference and these results suggest that fusaproliferin did not produce a significant enhancement of the passive transport in cells in Pavla or Lucia.

Immediately after the application of anoxia in the light the E_M of control plants was transiently depolarized in both cultivars. After 48 h treatment with fusaproliferin the E_M lowered by 4 to 8 mV in Lucia and by 17 to 24 mV in Pavla but the E_M did not recover in fusaproliferin-treated leaves under anoxic conditions, as was observed in control cells. In dark conditions a second depolarization was observed (Fig. 2b), and although treated tissue was kept under illumination, the E_M did not recover completely under anoxic conditions as observed in the control cells (Fig. 2a).

Comparison with effect of fusicoccin

An amount of 30 μM fusicoccin, a known powerful hyperpolarizing phytotoxin, rapidly and permanently hyperpolarized the membranes of the palisade parenchyma cells of maize leaves in both control and fusaproliferin-treated tissues in the light. The shift in E_M values after 30 min was approximately 30–38 mV, with none of the recorded hyperpolarisations being statistically different between Lucia (34±4 mV, n=4) and Pavla (35±4, n=4). However, in fusaproliferin-treated tissue, the hyperpolarisation was slightly reduced and in Lucia reached 32±4 mV, (n=4) and in Pavla 26±5 mV (n=6). These results indicate that fusaproliferin did not permanently affect the P-ATPase during the first 48 h of treatment. The response to the fusicoccin treatment revealed that the electrogenic pump operated in the light approximately to the same extent in healthy as in toxin-treated tissues.

Comparison with effect of 3-3(3, 4 dichlorophenyl)-1, 1-dimethylurea (DCMU)

DCMU is considered a very specific and sensitive inhibitor of photosynthesis. It blocks the plastoquinone binding site of photosystem II, blocking the electron flow from the generation site in photosystem II, to plastoquinone. This interrupts the photosynthetic electron transport chain and thus blocks the capability of the plant to transform light energy into chemical energy (ATP). DCMU only blocks electron flow from photosystem II; it has no effect on photosystem I or other reactions in photosynthesis, such as light absorption or carbon fixation in the Calvin cycle. In our experiments DCMU was used to determine whether photosynthesis is an energy source for E_M maintenance in cells treated by fusaproliferin. The response of E_M to 5 μM DCMU in palisade parenchyma cells was characterized by transient E_M changes similar to the changes registrated after switching the light off in both untreated (Fig. 4a–d) and fusaproliferin-treated tissues (Fig. 4e–h). Our results show that the functional properties of the plasmalemma of maize palisade parenchyma cells basically were not changed by fusaproliferin treatment during the 48 h period in the light. The diffusion potential (E_D) was changed in diseased tissue only slightly under anoxic conditions. However, the energy-dependent portion of E_M was changed by fusaproliferin treatment. A higher E_M was observed only with light, but not in the dark (Fig. 1); the membrane depolarization caused by fusaproliferin could be by uncoupling of the H^+ pump from the energy supply in

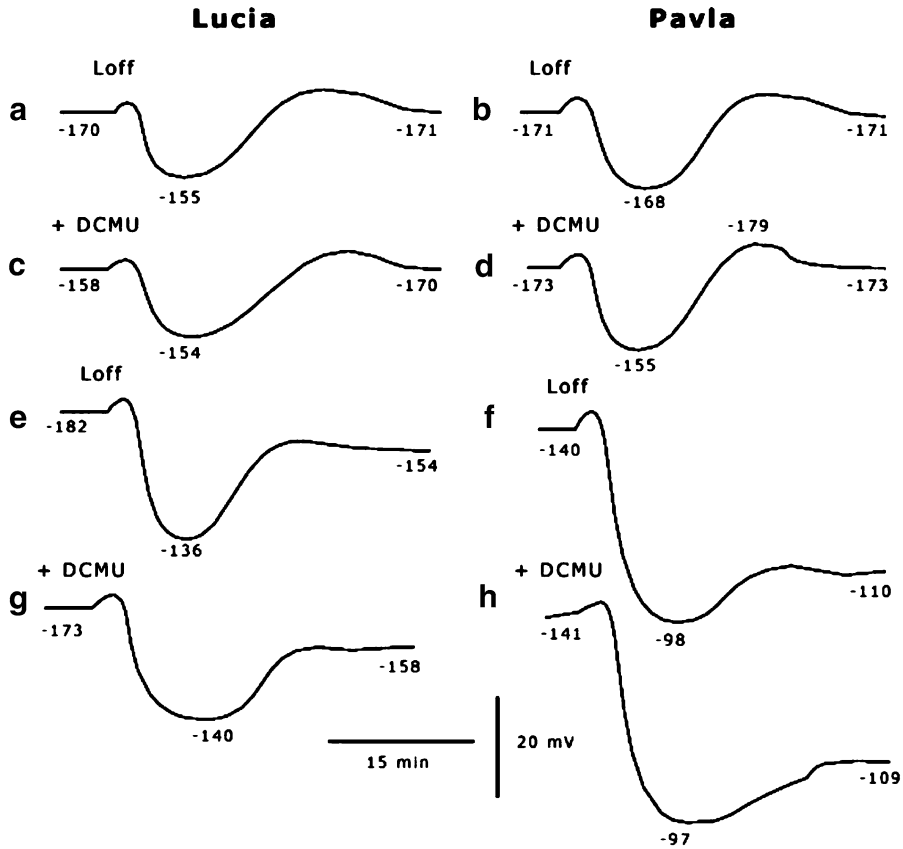

Fig. 4 Effect of 5 μM 3-3 (3, 4 dichlorophenyl)-1, 1-dimethylurea on E_M of control (**a**–**d**) and 35 μM fusaproliferin-treated palisade parenchyma leaf cells of maize cvs Lucia (resistant) and Pavla (sensitive) (**e**–**h**). The experiments **c**, **d**, **g**, **h** were performed with light; experiments **a**, **b**, **e**, **f** were performed without light (Loff)

the dark via fusaproliferin-induced changes in the energy sink. In the latter, the active system component of E_M was completely lost under dark conditions, even though the energy supply was enhanced.

Discussion

Analyses of membrane potential (E_M) in palisade parenchyma cells of 8 day-old maize plants suggest that these biological effects may reflect fusaproliferin-induced changes on the cells of maize. Membranes of leaf segments in the light did not repolarise, and lost their capacity to maintain a high E_M under light and dark conditions. The alteration of plasma membrane permeability and electrolyte leakage is a well known effect of some fusariotoxins, such as zearalenone and fusaric acid (Vianello and Macri 1978; Pavlovkin et al. 2004), but only indirect experiments have been performed to study fusaproliferin activity in plants. According to the results of Zonno and Vurro (1999), among 14 assayed toxins most active in depressing germination capability of *Striga hermonthica*, seven belonged to *Fusarium* toxins, and fusaproliferin belonged to the third group of toxins with a low level of activity. The changes in membrane potential could be associated with a decrease in the electron transport components leading to decreased content of chlorophyll after fusaproliferin treatment on maize seedlings both in *vivo* and *in vitro* (Nadubinská et al. 2003). The content of chlorophyll *a* was slightly decreased after *in vitro* treatment with fusaproliferin, while chlorophyll *b* increased. The authors reported that *in vivo* chlorophylls *a* and *b* in the susceptible cv. Pavla were reduced less by fusaproliferin (9.4%) than in the resistant cultivar (18.2%). Similarly, a reduction of photosynthetic capacity in maize and banana plants was induced by *F. verticillioides* living as an endophyte (Pinto et al. 2000). These effects may reflect a reduction of chlorophyll content and an impairment of electron transport in the thylakoid membranes. This would suggest a reduction in the maximum yield of photosynthesis due to the toxins produced by the endophytic fungi. Nevertheless, no

symptoms were observed in the leaves similar to those described in this work.

In addition to fusaproliferin, *F. verticillioides* can also produce moniliformin and fumonisin (Pascale et al. 2002), which are known for their phytotoxicity and ability to induce macroscopic effects on infected plants. In our experiment visual symptoms occurred both in the apical and marginal part of the leaves of the plants cultivated in the presence of the higher fusaproliferin concentration (35 μg ml^{-1}), such as chlorosis, a shrivelled appearance or water-soaking. The affected parts of the leaves contain, developmentally, the oldest cells, and their death was probably induced by fusaproliferin. This non-specific symptom can be induced by numerous pathogens, e.g. *Phytophthora* spp., *Ascochyta rabiei* (Farr et al. 1989; Ilarsan and Dolar 2002); however, more specific responses of infected plant tissues can be expected as specific non-covalent interactions, as have been recently detected between both single- and double-stranded model oligonucleotides and fusaproliferin with a 1:1 stoichiometry (Pocsfalvi et al. 2000). The mesophyll chloroplasts of both cultivars were filled with large osmiophilic globules. These may contain lipids resulting from thylakoid membrane disintegration, as occurs during senescence (Kaup et al. 2002) or induced by numerous biotic stress, as in cotton plants infected by *Fusarium oxysporum* f.sp. *vasinfectum*, (Shi et al. 1991) and abiotic stresses. Disruption and swelling of thylakoids is the most universal structural response of the inner membrane system of cells to stress, and can occur in bean plants under conditions of water deficit and temperature stress (Dekov et al. 2000) or in barley plants when high concentrations of salicylic acid are applied (Stoyanova and Uzunova 2001). Up to now the possible role of toxins in *Fusarium* plant diseases has been supported only for deoxynivalenol and fumonisins (Munkvold 2003).

The differences observed between experiments performed with and without light were possibly due to differential intracellular CO_2-dependent pH regulation during photosynthesis and respiration (Daly 1981). The changes were similar to those observed during disease development of cotton cotyledons inoculated with *Xanthomonas campestri* pv. *malvacearum* (Novacky and Ullrich-Eberius 1982).

Determining the activity of purified toxins has generally provided the basis for establishing their role in the host-pathogen relationship. The data presented here indirectly indicates that fusaproliferin may act as a virulence factor in the disease caused by *Fusarium* pathogens. Fusaproliferin could have an important role in disease development, in the expression of symptoms through interactions between fusaproliferin and nucleic acids during the first steps of cellular splitting in the meristem or during leaf development. Fusaproliferin could also have an important role in the endophytic mode of life of its producers. Moreover the data support the conclusion that photosystem II is disturbed by fusaproliferin, although this could result from action of the toxin on other organs.

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