Effects of beauvericin on root cell transmembrane electric potential, electrolyte leakage and respiration of maize roots with different susceptibility to *Fusarium*

J. Pavlovkin[1], I. Mistríková[2], M. Luxová[1], I. Mistrík[1]

[1]*Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovak Republic*
[2]*Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic*

ABSTRACT

Effect of beauvericin on root cell transmembrane electric potential (E_M), electrolyte leakage and respiration of roots were studied in two maize cultivars (*Zea mays* L.) with different susceptibility to this toxigenic metabolite produced by *Fusarium*. Beauvericin treatment induced rapid and significant depolarisation of membrane potentials of the outer cortical cells of maize roots of tolerant cv. Lucia. The range of depolarisation was dose dependent with maximum depolarisation of 55 mV (55 ± 7 mV, n = 7) at 200μM beauvericin. In contrast, membrane potentials of beauvericin susceptible cv. Pavla was only slightly depolarised by identical concentrations of beauvericin and the value of depolarisation represented only half of the value of tolerant cv. Lucia (27 ± 6 mV, n = 8). The values of membrane potentials of root cells of tolerant cv. Lucia were higher (137 ± 9 mV, n = 26) and more electrogenic (60 ± 2 mV, n = 3) than in susceptible cv. Pavla (125 ± 7 mV, n = 28), (47 ± 2 mV, n = 3), respectively. Our results confirmed that 2 h treatment with 50μM beauvericin does not cause irreversible changes in plasma membrane H^+-ATPase, because fusicoccin, an H^+-ATPase activator diminished the depolarizing effect of beauvericin on the E_M. Further experiments revealed beauvericin-induced increase of membrane conductivity in root cells of Pavla but not in root cells of Lucia. Time-coarse experiments showed that 25μM beauvericin induced slight, but significant inhibition of root respiration in both cultivars during the first two hours of treatment, and the inhibition was higher in cv. Lucia than in cv. Pavla. The depolarisation of E_M in the outer cortical cells of maize roots may be the result of a cumulative effect of beauvericin on ATP supply, activity of H^+-ATPase and mainly on the permeability of plasmalemma. Increased beauvericin tolerance in maize might be associated with the increased ability of tolerant plant to maintain normal ion fluxes and membrane potentials across the plasmalemma of root cells in the presence of beauvericin.

Keywords: beauvericin phytotoxicity; membrane potential (E_M); diffusion potential (E_D); conductivity (μS); respiration; fusicoccin (FC); maize root (*Zea mays* L.)

Several *Fusarium* species occurring worldwide on cereals as causal agents of blight (scab) of small-grain cereals and ear rot of maize are capable to accumulate in infected kernels several mycotoxins some of which have a relevant impact on human and animal health (Macchia et al. 2002, Šrobárová et al. 2004). Plant pathogenic fungi frequently produce metabolites that most likely play an important role in the disease development.

Beauvericin (BEA), a cyclic hexadepsipeptide of alternating L-N-methylphenylalanyl and D-α-hydroxyisovaleryl residues (Logrieco et al. 2002), initially regarded as an entomopathogenic mycotoxin, toxic to the brine shrimp *Artemia salina* (Hamill et al. 1969), was originally described as a secondary metabolite of the entomopathogenic fungus *Beauveria bassiana*. More recently, beauvericin has been recognized as an important toxic compound synthesized by several *Fusarium* strains, infecting maize, wheat and rice, worldwide (Moretti et al. 1995). Because of the occurrence of beauvericin producing fungi in such commodities,

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this mycotoxin might enter the food chain, causing so far unknown consequences to the health of domestic animals and humans (Logrieco et al. 2002). Beauvericin effects on mammalian cells was studied (Ojcius et al. 1991) but the effect of beauvericin on membrane function of host plant cells is mostly unknown.

Early investigations showed that one of the first toxin isolated from *Fusarium* sp. fusaric acid causing wilt disease of a great variety of plants (Gäumann 1957) alters the permeability of plant membranes, increases electrolyte leakage (Linskens 1955, Marrè et al. 1993), causes modification of membrane potential (D'Alton and Etherton 1984, Marrè et al. 1993), inhibits respiratory activity (Marrè et al. 1993) and decreases ATP levels (D'Alton and Etherton 1984) in several plant species. Other toxin produced by fungi family *Fusarium,* trivially named zearalenone inhibits oxidative phosphorylation, causes depolarization of membrane potential in cells of corn roots and stimulates leakage of electrolytes and organic compounds (Vianello and Macri 1978). Zearalenone, and deoxynivalenol are able to induce ion channels in the lipid bilayer (Ziegler et al. 1994). The authors presume that, at least certain mycotoxins might express their toxic activity on host cell membranes that are the primary places of interaction with pathogens by depolarizing transmembrane potential or affecting activity of membrane ion pumps and transporters.

The purpose of our work was to elaborate whether or not beauvericin, a toxic compound synthesized by several *Fusarium* strains, can affect the permeability, membrane potential and respiration rate of root cells of maize cultivars differing in susceptibility to *Fusarium* and what mechanism such changes might be based on.

MATERIAL AND METHODS

Plant material. Maize (*Zea mays* L. cv. Lucia – tolerant, and Pavla – susceptible) seeds were surface sterilised with 12% H_2O_2, placed in filter paper rolls soaked with distilled water and germinated in dark at 25°C and 98% RH. Three-day-old maize seedlings with 5 cm long primary seminal roots were used for the experiments.

Electrophysiological measurements. Measurements of the membrane potential (E_M) were carried out at 22°C on outer cortical cells of intact seedlings by standard microelectrode techniques described earlier (Pavlovkin et al. 1993). The seedlings were attached to a Plexiglas holder with a soft rubber ring and mounted in a vertical 5 ml plexiglas cuvette perfused at a flow rate 4 ml/min with a standard solution containing 0.1mM KCl and 0.5mM $CaSO_4$, adjusted to pH 5.7, in which various concentrations of beauvericin (Sigma) were added (beauvericin stock solution: 5 mg of BEA was dissolved in 10 ml DMSO). The microelectrode was inserted into the outer cortex cells 5 mm behind the root tip. Insertion of the microelectrode was observed under microscope and the electrode was pushed not further than 30–40 μm into the tissue. Fusicoccin (Sigma), a H^+-ATPase stimulator was used (in 0.1% ethanol, final concentration 30μM) for monitoring the functionality of membrane H^+-ATPase (Marrè 1979).

Anoxic conditions. To induce anoxic conditions the perfusion solution was saturated with N_2 gas by flushing. The flow of perfusion solution through the measuring chamber at 5 ml/min was sufficient to maintain anoxia in the measurement chamber (Pavlovkin et al. 1986). Membrane potential of cells kept under anoxia represents value of diffusion potential (E_D), which is the passive component of electrical membrane potential (E_M).

Membrane permeability. 2.5 cm long apical segments of primary seminal maize roots were aged in 0.5mM $CaCl_2$ solution for 2 h. After this time, incubation solution was changed for a fresh one supplemented with various concentrations of beauvericin (25 or 50μM) and initial conductivity was determined by conductivity meter OK-109-1 (Radelkis, Hungary). In short term experiments conductivity changes were monitored every hour and expressed as a difference between the value of particular conductivity measured, and the value of the initial conductivity.

Respiration measurements. Two cm long apical segments cut from the primary roots of maize seedlings were transferred into glass cuvette with 3 ml fully aerated 10mM phosphate buffer (pH 6.8). The rate of oxygen consumption was measured polarographically using a Clark-type oxygen electrode (YSI 5300, Yellow Springs Instrument, USA). The respiration rate was expressed as a percentage of respiration of control roots.

RESULTS

Electrophysiological measurements

Membrane potential (E_M). Replacement of aging solution (0.5mM $CaSO_4$) by standard nutrient solution in the perfusion chamber has slowly

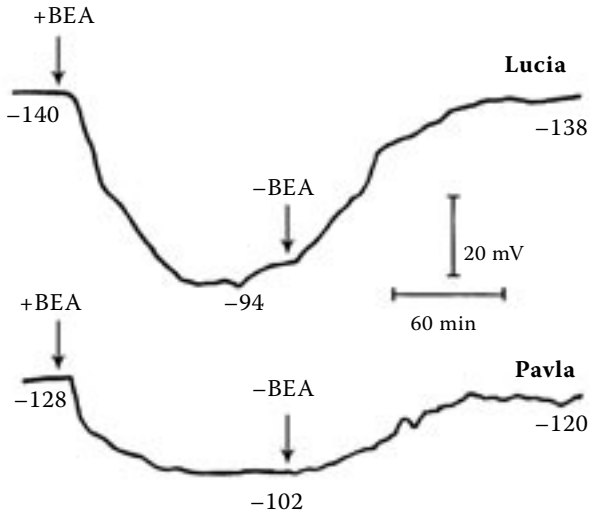

Figure 1. Tracing of chart recordings showing the effects of 50μM beauvericin on E_M of root outer cortical cells of maize cultivars Lucia and Pavla

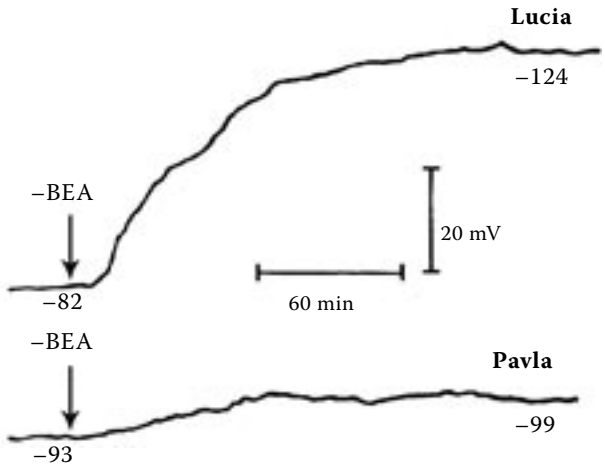

Figure 2. Partial repolarisation of E_M of outer cortical cells of intact maize roots after 6 h treatment with 50μM beauvericin

hyperpolarised the value of membrane potential (E_M) of the outer cortical root cells during the first two hours. Thereafter, membrane potential (E_M) was constant, at least, for one day and represented −137 ± 9 mV (n = 26) for cv. Lucia. The values of membrane potential (E_M) of the outer cortex cells of intact maize roots were −125 ± 7 mV on average (n = 28) for cv. Pavla. Upon addition of BEA (50μM) to the perfusion solution, an immediate decrease of the E_M was observed. The maximum depolarisation was maintained to 1–2 h, in some cases slow repolarisation of E_M (3–6 mV) were observed in tolerant cv. Lucia but not in susceptible cv. Pavla (Figure 1). In all measurements extent of E_M depolarisation of outer cortical cells of maize roots was greater in tolerant cv. Lucia (Δ mV 47 ± 4 mV, n = 8) than in susceptible cv. Pavla (Δ mV 18 ± 7 mV, n = 13). During long term experiments the root cortical cells of cv. Pavla treated for 6 h with 50μM BEA showed slightly more negative E_M values (−92 ± 5 mV, n = 7) than root cortical cells of cv. Lucia (−80 ± 7 mV, n = 11). After removing BEA from perfusion solution E_M partially recovered to −120 ± 8 mV (n = 7) in root cortical cells of cv. Lucia, but recovery in cv. Pavla was negligible and reached −99 ± 6 mV (n = 11) (Figure 2). The effect of BEA on E_M of root cells was dose dependent with a threshold of 5μM of BEA (2 h treatment). BEA at 10μM concentration induced depolarisation 8 ± 4 mV (n = 3) in tolerant cv. Lucia approximately after 90 min but surprisingly not in root cortical cells of susceptible cv. Pavla. At all higher concentrations of BEA (25, 50, 100, 200μM), the beauvericin-induced depolarisation was significantly greater in root cortical cells of tolerant cv. Lucia than in susceptible cv. Pavla (Figure 3).

N_2 conditions. Two components of E_M were separated by anoxic conditions, i.e. by perfusion the roots with N_2 saturated solution. Anoxic conditions in control roots (treated with N_2 but not with BEA) resulted in depolarisation of E_M of root cortical cells to −79 ± 2 mV (n = 3) in cv. Lucia and to −80 ± 3 mV (n = 3) in cv. Pavla (Figure 4A, B). These values are considered to be the values of the diffusion potential (E_D). The energy dependent component (E_P) of E_M represents the difference between E_M and E_D and was higher in root cortical cells of cv. Lucia (Δ mV = 60 ± 4, n = 3) than in cv. Pavla (Δ mV = 44 ± 3, n = 3). The outer corti-

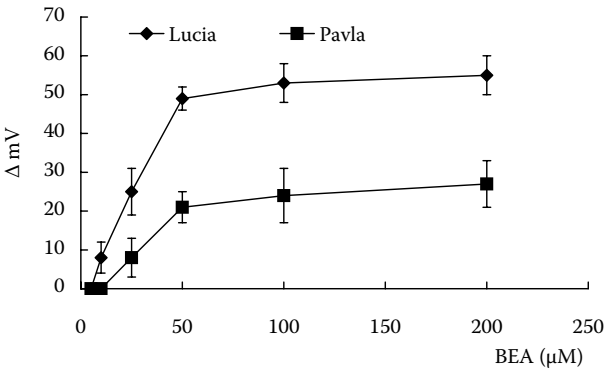

Figure 3. Changes of E_M of outer cortical cells of intact maize roots at different concentrations of beauvericin; values are mean ± SD (n = 3–5)

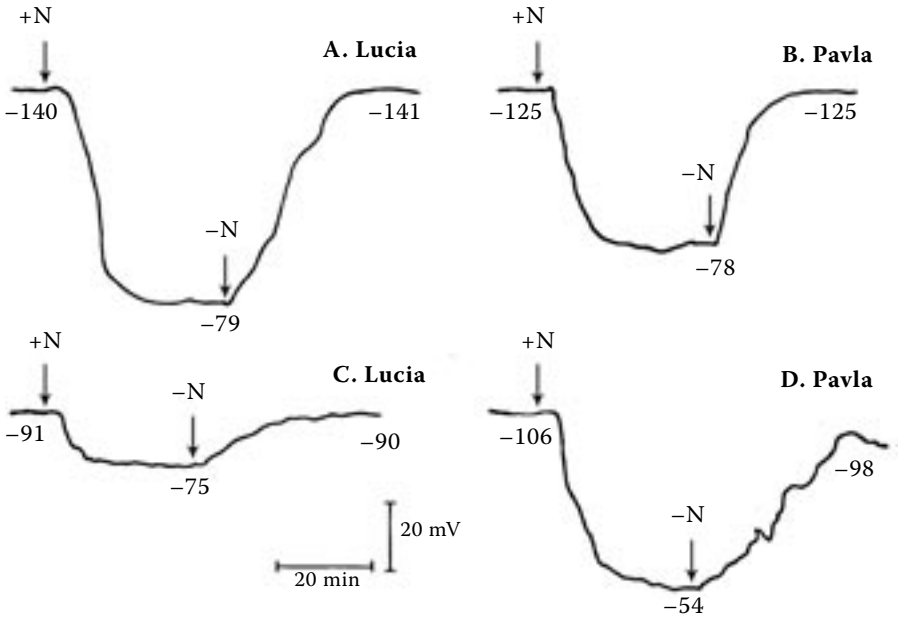

Figure 4. Effect of anoxia (N_2) on E_M of outer cortical cells of intact maize roots; A, B – control, C, D – treatment with 50μM beauvericin for 4 h

cal cells of intact maize roots treated for 4 h by 50μM BEA before anoxia showed E_M values from −87 to −96 mV (−92 ± 5 mV, n = 16) in roots of cv. Lucia and from −99 to −114 mV (−105 ± 7 mV, n = 23) in roots of cv. Pavla; this means that the magnitude of depolarisation by anoxia was only 18 ± 2 mV (n = 9) in roots of cv. Lucia, but almost 50 mV (Δ mV = 48 ± 6, n = 13) in roots of cv. Pavla (Figure 4C, D). The effect of anoxia on the E_M and E_D values of root cortical cells during long term BEA treatment are presented on Figure 5. A rapid decrease of E_D values after anoxia in root cortical cells of susceptible cv. Pavla continues with prolonged exposition to BEA, while E_D values of root cortical cells of tolerant cv. Lucia remain stabile during 6 h treatment with 50μM BEA. Further experiments suggested that BEA significantly decreased passive transport in outer cortical cells in susceptible cultivar Pavla but not in those of tolerant Lucia.

Effect of fusicoccin (FC). 30μM FC, a powerful hyperpolarizing phytotoxin rapidly and permanently hyperpolarised the membranes of the outer cortex cells in both cultivars (data not shown). The Δ E_M values after 30 min reached approximate 30–40 mV in control cells, with none of the

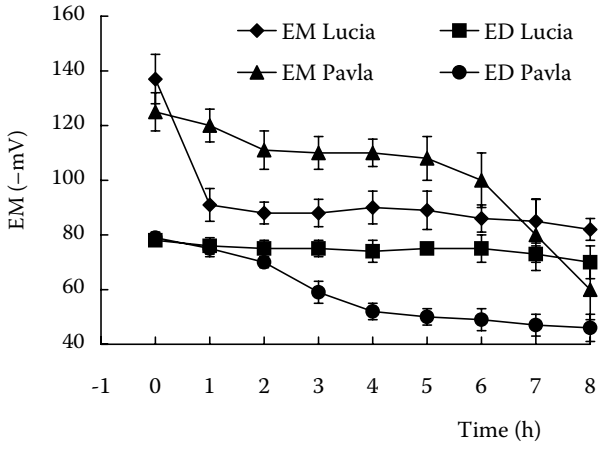

Figure 5. Effect of 50μM beauvericin on E_M and anoxia on E_D of outer cortical cells of intact maize roots treated for 8 h with BEA

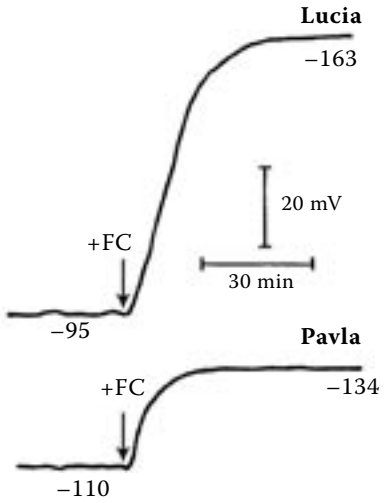

Figure 6. Representative potential measurements of outer cortical cells of intact maize roots; effect of 30μM fusicoccin (FC) on membrane hyperpolarisation after exposure of roots to 50μM beauvericin for 2 h

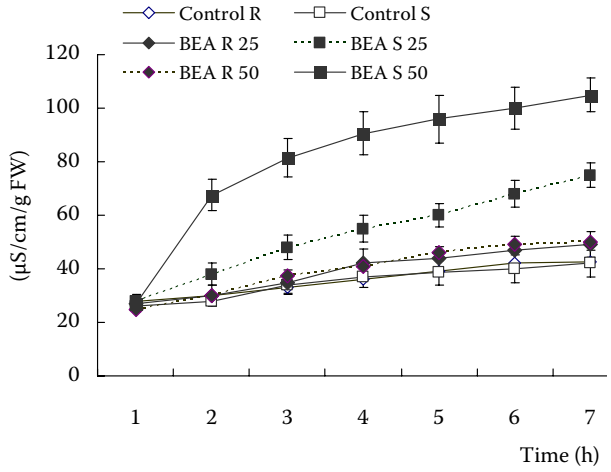

Figure 7. Time course of electrolyte leakage from maize root segments after beauvericin (25 or 50µM) treatment for 6 h; mean values ± SD (n = 3)

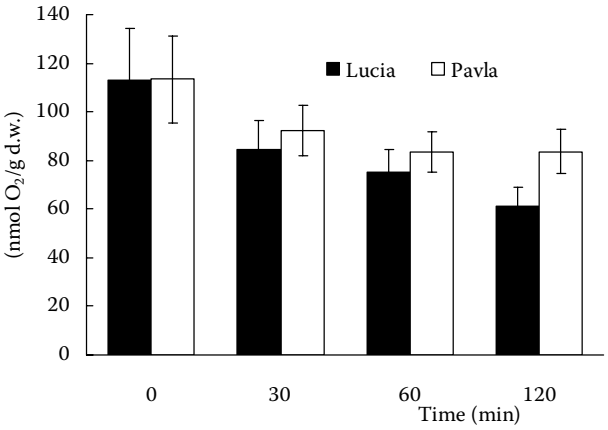

Figure 8. Time course of respiration of maize root segments treated with 50µM beauvericin; mean values ± SD (n = 3)

recorded hyperpolarisations being statistically different between Lucia and Pavla. After 2 h treatment with 50µM beauvericin, FC hyperpolarised the membrane to –161 ± 7 mV (n = 3) on average in root cortical cells of cv. Lucia and to –134 ± 5 (n = 5) on average in cv. Pavla (Figure 6). These results indicate that beauvericin did not permanently affect the P-ATPase during the first two hours of treatment.

Membrane permeability. The effect of BEA (25 or 50µM) on membrane permeability of maize root cells resulted in significant changes in electrolyte leakage from the roots of susceptible cv. Pavla but not from those of tolerant cv. Lucia (Figure 7).

Respiration measurements. Treatment of maize roots by 25µM of BEA induced a significant decrease in root respiration especially in tolerant cv. Lucia (Figure 8). Already 30 min of BEA treatment almost 40% reduction in root respiration of tolerant cv. Lucia was observed while only a slight reduction of root respiration occurred in susceptible cv. Pavla during this time. After 2 h of BEA treatment root respiration was decreased in tolerant cv. Lucia by 60% and by 33% in susceptible cv. Pavla.

DISCUSSION

The plasma membrane of the host cell is the first "living" structure that is target for toxic metabolites produced by pathogens. Such toxicity could result from various mechanisms including oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as H^+-ATPase, or changes in the composition and fluidity of membrane lipids and activity of membrane channels and transporters. A strong indication of alterations induced by the non-host-specific toxins at the plasma membrane comes from the electrophysiological measurements of E_M. All non-host-specific toxins so far studied, with the exception of *Fusarium* sp., depolarised the membranes of their hosts shortly after the application of the toxin preparation (Vianello and Macri 1978, D'Alton and Etherton 1984, Marrè et al. 1993). The electrophysiological technique of E_M measurements allows monitoring of membrane transport properties *in situ* with minimal disturbance of the vital cellular functions. Because beuvericin is an important toxic compound synthesized by several *Fusarium* strains we performed a number of electrophysiological measurements for a better characterisation of its impact on transport and permeability properties of maize root cells differing in their sensitivity to this toxin. Regardless to the sensitivity of maize cultivars to *Fusarium* sp. BEA treatment resulted in rapid and reversible depolarisation of E_M. The extent of E_M depolarisation of root cells of tolerant cv. Lucia was almost twice as high as in root cells of susceptible cv. Pavla (Figure 1). The rapidity and reversibility of the BEA-induced depolarisations indicates that BEA acts at the membrane surface. According to Olivetti et al. (1995) we presume, that tolerant cv. Lucia responds to BEA by significantly depolarizing the E_M, and this large depolarisation (or the ion fluxes related to it) may act as a signal that is transduced into metabolic responses enabling this maize cultivar to eventually acquire tolerance to BEA. Much lower depolarisation of

E_M in root cortical cells of susceptible cv. Pavla should not be sufficient to act as a signal, or the metabolic pathways conferring BEA tolerance may be absent in this cultivar. Except differences in the magnitude of E_M depolarisation between these two maize cultivars, our results confirmed that both cultivars differ in functional properties of their cell plasma membrane during BEA treatment. The functional properties of plasma membrane of root cells of tolerant cv. Lucia were basically not changed during the six-hour exposure to BEA. The E_D was the same as in healthy roots, where E_D is determined by the diffusive K^+ equilibrium potential (E_{K^+}) (Pavlovkin et al. 1986). E_D of both healthy and diseased tissue was about –75 to –80 mV, as estimated by application anoxia (N_2). On the other hand E_D continuously decreased in root cells of susceptible cv. Pavla with the time of treatment and after 4 h the E_D values dropped from initial –80 mV to –50 to –60 mV. This value was lower than E_D of the control root cells or E_D of root cells of tolerant cv. Lucia and the magnitude of membrane depolarisation under anoxia increased with the time of BEA treatment.

It is evident that both components E_D, and E_p were simultaneously affected during the first 2 h of BEA treatment. Our data indicated that FC hyperpolarised the membranes in both cultivars, suggesting that the BEA had not dramatically altered the function of the plasma membrane H^+ extrusion pump. The differences were found between two maize cultivars when FC more significantly hyperpolarised membrane of root cells of tolerant cv. Lucia than root cells of susceptible cv. Pavla even though E_M depolarisation after 50μM BEA was larger in tolerant cv. Lucia than in susceptible cv. Pavla.

The decrease in E_D was accompanied by the loss of electrolytes from root cells. In agreement with other authors (Linskens 1955, Vianello and Macri 1978, Marrè et al. 1993) describing a loss of electrolytes from tissue treated by some non-host-specific toxins, we found an extraordinary high electrolyte leakage form root cells treated with BEA of susceptible cv. Pavla. An increase in electrolyte leakage was immediate and continued up to 6 h especially when a higher dose (50μM) of BEA was used. On the other hand there were no differences in electrolyte leakage between two BEA concentrations in roots of tolerant cv. Lucia. Our results show that BEA does basically not affect the functional properties of the plasma membrane of root cells of tolerant cv. Lucia during the first six hours of BEA treatment even at the concentration of BEA that almost doubles the amount of electrolyte leakage from susceptible cv. Pavla.

These cultivar differences are very interesting because it was described earlier by Ojcius et al. (1991) that BEA acts as an efficient ionophore for monovalent and divalent cations, and according to Macchia et al. (2002) perturbation of the normal gradient of important physiological cations, at the cellular membranes, may lead to apoptosis. An increase in electrolyte leakage and a rapid decrease of the intracellular K^+ content was also described during bacteria-induced hypersensitive reaction (HR) by Pavlovkin et al. (1986). Correspondingly, the calculated Nernst potential of K^+ (E_{K^+}) became less negative, simultaneously with $E_{D,}$ which is similar to our results (Figure 5). Electrolyte leakage consisted in HR experiments of about 80% K^+ efflux. We assume that a significant increase of the membrane conductivity also in BEA experiments reflects the inhibition of passive potassium efflux in tolerant cv. Lucia and its stimulation in susceptible cv. Pavla.

From the above-mentioned results it is clear that the reaction of root cells to BEA differs in relation to their susceptibility to *Fusarium*. Differences observed between two cultivars, especially differences in membrane depolarisation and permeability properties of root cell plasma membrane indicate that some constitutive and inducible characteristics are responsible for tolerance of maize plants to *Fusarium* fungi. Dual mechanism of BEA linked to passive and active membrane transport phenomenon and the magnitude of E_M depolarisation may play an important role in signal transduction and induction of defence mechanisms while differences in plasma membrane may act as structural barriers of host cells to pathogens.

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Corresponding author:

RNDr. Ján Pavlovkin, Ph.D., Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 14, 845 23 Bratislava, Slovak Republic
phone: + 421 259 426 115, fax: + 421 254 771 948, e-mail: jan.pavlovkin@savba.sk