Impact of beauvericin on membrane properties of young initial leaves of maize with different susceptibility to *Fusarium*

J. Pavlovkin[1], I. Mistríková[2], K. Jašková[1], L. Tamás[1]

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

ABSTRACT

In the present study the impact of beauvericin (BEA) on the cell membrane properties and respiration of young initial leaves of maize were studied using two maize cultivars differing in their susceptibility to *Fusarium* sp. BEA significantly depolarized E_M of leaf parenchymal cells and this depolarization showed time and dose dependency regardless on the sensitivity of maize cultivars to *Fusarium*. However, the extent of BEA-induced depolarization was 2–5 times higher in sensitive cv. Pavla than in tolerant cv. Lucia. Membrane permeability and K^+ leakage from leaves cells treated with BEA was higher in sensitive cv. Pavla but the differences were not so considerable than the depolarization of E_M. Treatment of maize young initial leaves with 40 μmol BEA significantly inhibited respiration. In accord with electrophysiological measurements inhibition of respiration was higher in sensitive cv. Pavla showing 70% inhibition already after 90 min of BEA treatment while in tolerant cv. Lucia inhibition represented only 27%. The biological activity of BEA seems to be mediated by the ability of BEA to affect membrane permeability and ion transport. This is probably the initial effect of BEA on plant cell leading to subsequent effect on other cell organelles (mitochondria) and cell metabolism.

Keywords: beauvericin; membrane potential; membrane permeability; respiration; phytotoxicity; young maize leaves; *Zea mays* L.

Members of the genus *Fusarium* produce a range of chemically different phytotoxic compounds such as beauvericin, enniatin, moniliformin, fusaproliferin, and trichothecenes. These possess a large variety of biological activities and metabolic effects including wilting, chlorosis, necrosis, germination and growth inhibition, and effects on calli (Neuhold et al. 1997).

A relatively recently identified toxin beauvericin was isolated and purified by Moretti et al. (1995). 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* and various *Fusarium* fungal species infecting maize, wheat and rice, worldwide (Moretti et al. 1995). The increased interest in beauvericin (BEA) is due to its potential toxicity and synergistic effects with other fusariotoxins (Logrieco et al. 1996). BEA is a specific cholesterol acyltransferase inhibitor (Tomoda et al. 1992), induces apoptosis in mammalian cells (Macchia et al. 2002), human leukemia cells (Chen et al. 2006) and shows cytotoxicity of the invertebrate cell line SF-9 (Ojcius et al. 1991, Logrieco et al. 2002). However, until now, phytotoxic activity of BEA has only been sporadically investigated (Paciolla et al. 2004, Pavlovkin et al. 2006). Paciolla et al. (2004) reported that BEA-induced tomato protoplast death is caused by imbalance of the ascorbate system and subsequent oxidative stress. Šrobárová et al. (2009) suggested that the nuclear fragmentation in BEA-treated cells is an important feature of apoptosis and implies that the

Supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and Slovak Academy of Sciences, Project No. VEGA 0002 for Ján Pavlovkin and No. 0050 for Ladislav Tamás.

intracellular phytotoxin BEA plays an important role, possibly as a mediator in cell-death signalling.

The aim of the study was to investigate the impact of BEA on membrane properties of young initial leaves of maize. Our hypothesis comes out from the knowledge that BEA phytotoxicity in maize leaves is based on the disturbance of cell membrane functions (e.g. changes in membrane potential, membrane permeability etc.) which subsequently affects the respiration and metabolism of treated cells. All measurements were performed on young initial leaves isolated from coleoptiles of maize cultivars differing in the sensitivity to *Fusarium* sp. at seedling stage.

MATERIAL AND METHODS

Plant material. Two maize cultivars provided by Zeainvent, Trnava, Slovakia, tolerant (cv. Lucia) and sensitive (cv. Pavla) to *Fusarium* infection were used. Seeds were surface-sterilized with sodium hypochlorite for 2 min and rinsed in sterile distilled water. The seeds germinated on moistened filter paper in Petri dishes in the dark at 25°C. Four-day-old etiolated coleoptiles with a length of 20–30 mm were harvested and young initial leaves were isolated after peeling off the coleoptiles with tweezers. The length of etiolated young initial leaves was ca. 20 mm and all experiments were performed under green light or in dark.

Chemicals. Fusicoccin (Sigma, 30 µmol in 0.1% ethanol) an H^+-ATPase stimulator was used to monitor the functionality of the membrane H^+-ATPase (Marrè 1979). Beauvericin (BEA, ca. 99% purity) was purchased from Sigma Chemical Co. (St. Louis, USA). Stock solution 0.5 mg/mL of BEA was prepared by dissolution of the crystalline material in 10 mL of MeOH and stored at –20°C. To obtain the final (40 µmol) concentration of BEA 1 mL of stock solution was diluted with perfusion solution. This concentration was based on our previous publication Pavlovkin et al. (2006) and showed significant reversible effect on all measured parameters.

Electrophysiological measurements. The electrophysiological experiments were performed on intact, 20 mm-long young initial leaf segments. The membrane potential (E_M) was measured by recording the voltage between a 3 mol KCl-filled glass micropipette (tip diameter was 0.5 µm, tip potential –5 to –15 mV) inserted into the parenchymal cells to 2 mm behind leaf tip and a reference electrode in the perfusion solution containing 0.2 mmol KCl, 1 mmol NaH_2PO_4, 1 mmol Ca $(NO_3)_2$ and 0.5 mmol $MgSO_4$, adjusted to pH 5.8 using 0.1 mol HCl. Before the electrophysiological experiments, the young initial leaf segments were preincubated for 2 h at the 22°C in an intensively aerated bathing medium. Subsequently, one leaf segment was transferred into a perfusion 4 mL volume plexiglass chamber and constantly perfused by perfusion solution (5 mL/min). The microelectrodes were inserted into the parenchymal cells under microscope by means of a micromanipulator (Leitz, Germany). During measurements 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.

Potassium (K^+) determination. For K^+ determination the 20 mm long young initial leaf segments of maize were incubated for 12 h in aerated 0.2 mmol $CaSO_4$ without BEA (control) or supplemented with 40 µmol BEA (BEA-treated) in darkness. Each 4 h ten segments of leaf segments were removed form the incubation medium, washed, and frozen at –20°C. Crude extracts were prepared by addition of deionised water to the frozen tissue. The K^+ content in an extract was determined with an ion-selective electrode and was related to the fresh weight of leaves.

Respiration analyses. 20 mm long young initial leaf segments were used for measurement of total respiration rate (VT; nmol O_2/g/DW/s). Respiration was measured polarographically using oxygen, Clark-type electrode (YSI 5300, Yellow Springs, Ohio, USA) at 25°C. The young initial leaf segments were sealed in a water-jacketed vessel containing fully aerated 10 mmol Na-phosphate buffer (pH 6.8).

RESULTS

Membrane potential. The main objective in short-term electrophysiological experiments was to characterise the dynamics of BEA-induced changes of E_M during exposure of young initial maize leaves to 40 µmol BEA. After insertion of the microelectrode into the parenchymal cell and stabilization of E_M, the values in control leaves were –129 ± 7.6 mV (mean ± SD, n = 27) in a tolerant cv. Lucia, and –127 ± 11.3 mV (mean ± SD, n = 29) in sensitive cv. Pavla (Figure 1). The maximal depolarization was recorded in intervals 12–60 min and the extent of E_M depolarization was more considerable in sensitive cv. Pavla than in tolerant cv. Lucia (Figure 2). The E_M value of

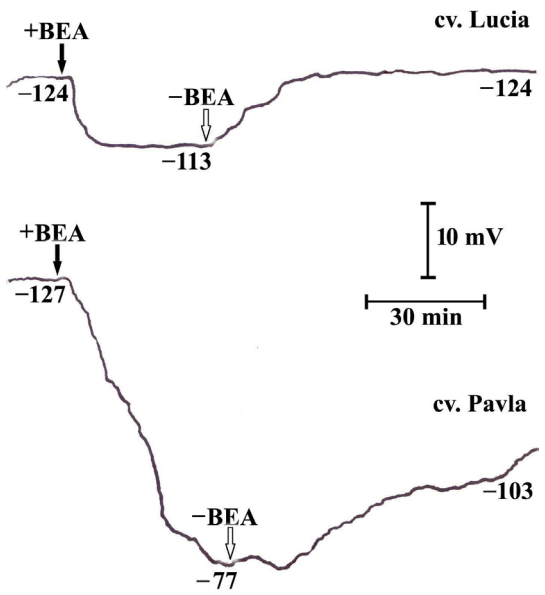

Figure 1. Tracing of chart recordings showing the effects of 40 µmol beauvericin (BEA) on E_M of young initial leaf parenchymal cells of maize cvs. Lucia and Pavla

leaf parenchymal cells of sensitive cv. Pavla declined during the first 30 min of BEA treatment to ca. 60% of the initial value, while in tolerant cv. Lucia the decline represented only ca. 10%. Similar tendency was recorded in cells exposed to prolonged BEA treatment (up to 12 h) (Figure 3) but the rate of depolarization was much slower than in the first two hours.

Effect of fusicoccin (FC). In long-term experiments application of FC (plasma membrane H^+-ATPase activator) to the perfusion solution caused an immediate hyperpolarization of E_M in cells treated with 40 µmol BEA for 12 h. Due to FC the value of E_M became more negative than the observed original potential in tolerant cv. Lucia (29 ± 4 mV, mean ± SD, n = 3), however in sensitive cv. Pavla the hyperpolarization was very low (9 ± 3 mV, mean ± SD, n = 3) or completely absent (n = 2).

Membrane permeability. BEA treatment negatively influenced the permeability properties of leaf parenchymal cells. Decrease in K^+ content was more pronounced in sensitive cv. Pavla and after 12 h of BEA treatment K^+ content in treated leaf segments represented only 51.61% that of control. In tolerant cv. Lucia BEA-induced reduction of K^+ content represented only 31.04%.

Respiration measurements. Significant decrease in respiration of young initial leaf segments was recorded even after short BEA treatment, especially in sensitive cv. Pavla. Inhibition of respiration increased with the time of BEA treatment. In sensitive cv. Pavla it increased from 34.06% (30 min treatment) up to 70.43% (90 min treatment), while in tolerant cv. Lucia inhibition represented only 12.54% (30 min) or 25.88% (90 min) that of control.

DISCUSSION

BEA is a cyclic hexadepsipeptide produced by various phytopathogenic *Fusarium* species (Moretti et al. 1995) with apoptotic properties (Ojcius et al. 1991, Paciolla et al. 2004) influencing membrane properties of root cells (Lemmens et al. 1997, Pavlovkin et al. 2006, Santini et al. 2008, Šrobárová et al. 2009).

According to our results the most sensitive parameter for monitoring the plasmalema membrane properties is the measurement of cell membrane potential (E_M). Depolarization of E_M started almost immediately after application of BEA to the perfu-

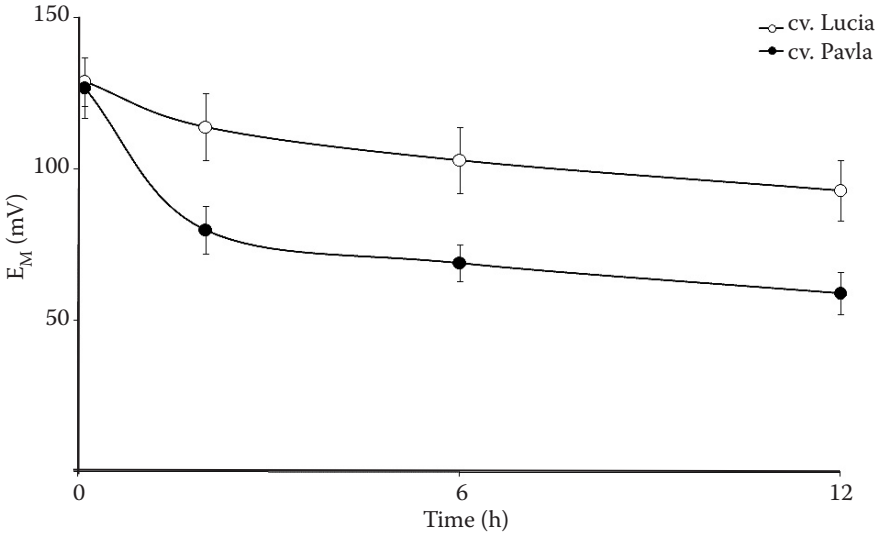

Figure 2. Changes of E_M in parenchymal cells of maize leaves at different concentrations of beauvericin (BEA); values are mean ± SD (n = 5–8) after 60 min treatment by BEA. Standard errors are shown as vertical bars

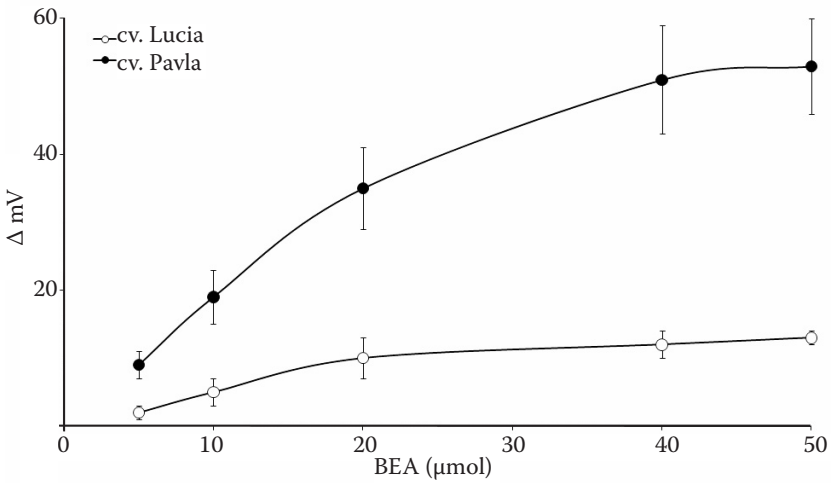

Figure 3. Time effects of 40 μmol beauvericin (BEA) on E_M of maize leaf parenchymal cells; values are mean ± SD (n = 5–8). Standard errors are shown as vertical bars

sion solution but the extent and rate of depolarization differed with the sensitivity of maize cultivars to BEA. The more expressive depolarization was registered in sensitive cv. Pavla (Δ mV 50 ± 8 mV). In tolerant cv. Lucia E_M depolarization was only mild but very fast (Δ mV 11 ± 4 mV), representing ca. 22% of the depolarization value of cv. Pavla.

BEA especially in short-term experiment (up to 4 h) specifically influenced only the active component of E_M. The results with fusicoccin show that the function of H^+-ATPase is not directly influenced by BEA even after 12 h of BEA treatment. The response may indicate independent sites or mode of action for alteration of H^+-ATPase activity by BEA and fusicoccine. Inconsiderable or absent hyperpolarization in sensitive cv. Pavla 60 min after FC application is mainly a reflection of insufficient supplying of ATP to leaf cells PM-H^+-ATPase due to damage of mitochondria function rather than a direct damage of PM-H^+-ATPase.

The decrease in E_M was accompanied by the loss of electrolytes, particularly K^+ ions. Reduction of K^+ content in leaf segments started immediately after BEA application and was higher in sensitive cv. Pavla than in tolerant cv. Lucia. However, magnitudes of K^+ decrease in leaf segments have not entirely corresponded with the changes in E_M value of cells.

The BEA-induced K^+ leakage from leaf parenchymal cells is in agreement with other authors describing efflux of electrolytes from tissues treated with non-host-specific fungal toxins (Ojcius et al. 1991, Pavlovkin et al. 2004, 2006, 2011). According to Macchia et al. (2002) perturbation of the normal gradient of physiologically important cations at the cellular membranes may disturb cell metabolism and finally lead to cell apoptosis. Our results confirmed that mainly in sensitive cv. Pavla the lost of 50% K^+ from leaf segments during 12 h of BEA-treatment should be responsible for the lack of cell E_M repolarisation during recovery experiments and considerable inhibition of respiration.

Especially, in sensitive cv. Pavla significant inhibition of leaf respiration was recorded already after 30 min of BEA treatment and inhibition increased

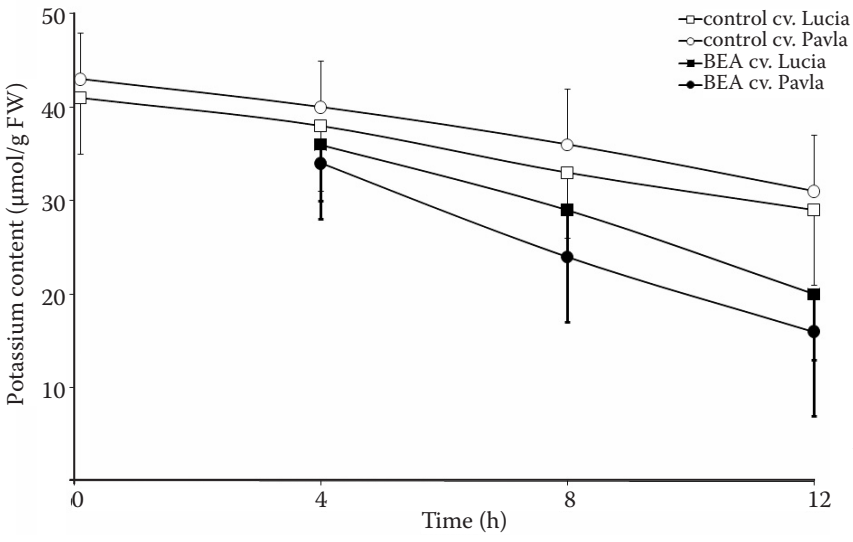

Figure 4. Potassium content in young initial leaves of maize cvs. Lucia and Pavla after 40 μmol beauvericin (BEA) treatment (0–12 h); mean values ± SD (n = 5). Standard errors are shown as vertical bars

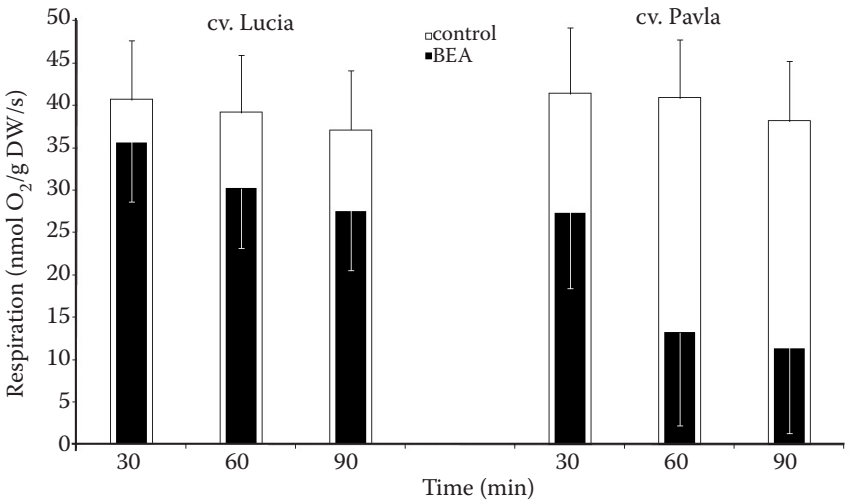

Figure 5. Time course of leaf respiration of maize cvs. Lucia and Pavla. Young initial leaf segments were exposed to 40 µmol beauvericin (BEA) (0–12 h); mean values ± SD (n = 10). Standard errors are shown as vertical bars

with time (70.43% after 90 min). In tolerant cv. Lucia inhibition did not exceed 25.88% of control. This inhibition could be caused by BEA impact on electron transport through the cytochrome pathway or oxidative phosphorylation. Experiments with isolated pea steam and maize root mitochondria showed that mycotoxin zearalenone, produced by a number of species of the genus *Fusarium* causes an inhibition of the oxidative phoshorylation, uncoupling and inhibits mitochondrial ATPase activity (Vianello and Macri 1978). Similar results were described on soybean cell suspension using mycotoxin patulin (Polacco and Sands 1997) and possible involvement of mitochondria, especially mitochondrial-derived ROS generation, as an important regulator in controlling apoptotic cell death in oats was induced by toxin victorin (Yao et al. 2002). On the other hand, respiration fall may also be caused by the decrease of growth rates which lowers the overall requirements for respiratory ATP, and thus the rate of total respiration (Atkin et al. 2000). Another possibility is that BEA could damage the mitochondria or cause mitochondrial oxidative burst which can lead to reduction of ATP synthesis in mitochondria. Little or full absence of E_M hyperpolarization in sensitive cv. Pavla 60 min after FC application supports our opinion about insufficient supply of ATP rather than direct damage of PM-H^+-ATPase.

Based on our results we can conclude that BEA may act as a virulence factor in the disease caused by *Fusarium* pathogens. BEA decreased E_M of leaf parenchymal cells but did not influence PM-H^+-ATPase. The extent of depolarization was closely related to the sensitivity of maize cultivars to BEA. Tolerant cv. Lucia showed considerably lower membrane depolarization than the sensitive cv. Pavla. The biological activity of BEA seems to be mediated by the ability of ionophoric compounds to affect transport across the cell membrane, leading to perturbation of the ionic balance on plasma membrane. Such impact of BEA could increase its cytotoxicity to other organelles in parenchymal cells and might induce a wide range of toxic effects.

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Received on July 27, 2011

Corresponding author:

Dr. Jan Pavlovkin, Ph.D., Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 9, 845 23 Bratislava, Slovakia
e-mail: jan.pavlovkin@savba.sk