Xylanase-induced cell death events in detached tobacco leaves

Z.P. Yordanova¹, V.M. Kapchina – Toteva¹, E.J. Woltering², R.B. Batchvarova³, E.T. Yakimova²,⁴
University of Sofia, Faculty of Biology, Department of Plant Physiology, Sofia, Bulgaria¹
Wageningen University & Research Centre, Agrotechnology and Food Science Group (AFSG), Wageningen, The Netherlands²
AgroBioInstitute, Sofia, Bulgaria³
Institute of Ornamental Plants, Nevganov, Sofia, Bulgaria⁴
Correspondence to: Elena Yakimova
E-mail: elena_iakimova@abv.bg

ABSTRACT
Plant-pathogen interactions are associated with plant defense mechanism known as hypersensitive response (HR), which is a form of programmed cell death (PCD). In the present work we have tested the potency of chemicals, proven as PCD inhibitors in other systems, to prevent the spread of cell death in detached tobacco leaves challenged with β-xylanase from Aspergillus awamori K1. Xylanase induced cell death that was accompanied by electrolyte leakage and increased levels of the stress metabolites hydrogen peroxide and malondialdehyde. Lesion development, ion leakage and the production of stress metabolites were suppressed if the infiltration site was pre-infiltrated with broad range caspase inhibitor benzyoxycarbonyl-Asp-2,6-dichlorobenzoylomethylketone (Z-Asp-CH2-DCB), cysteine protease inhibitor iodoacetamide (IA), serine protease inhibitor 4-(2-aminoethyl) benzenesulfonfyl fluoride hydrochloride (AEBSF), the antioxidant l-galactone-γ-lactone (L-gal) and the ethylene blocker (S)-trans-2-Amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride (AVG). Massive ROS accumulation, as determined by staining with 3,3'-diaminobenzidine and 2',7'-dichlorofluorescein diacetate, occurred in xylanase-infiltrated lesions and was substantially reduced by the inhibitors. To the best of our knowledge, this is the first report showing that β-xylanase produced by Aspergillus awamori K1 induces cell death response in tobacco and proteolysis, ROS and ethylene are involved in the mediation of the signaling.

Keywords: Nicotiana tabacum, oxidative stress, programmed cell death, proteolysis, xylanase

Introduction
Programmed cell death (PCD) is a genetically and environmentally controlled process that functions during development of multicellular organisms and is involved in their responses to biotic and abiotic stresses. The living systems employ PCD to eliminate redundant, damaged or infected cells. Elaborated plant defence mechanism, known as hypersensitive response (HR) is described in association with plant-pathogen interactions. HR is a form of PCD that occurs as a rapid, localized death of tissues at the site of infection, limiting further pathogen multiplication and spread (9). Bacteria, fungi, viruses, nematodes, insects or their purified toxins can elicit HR accompanied by cell death signalling involving oxidative stress, calcium, proteolytic activities and other biochemical events (9, 17). In mammalian systems, PCD occurs in a form of apoptosis, which is associated with activation of executioner machinery of a specific class of cysteinyl aspartic proteases named caspases and shows typical morphological features, such as cytoplasm shrinkage, nuclear condensation, DNA fragmentation and formation of apoptotic bodies that contain cellular debris and are digested by phagocytosis. Although not all morphological markers of apoptosis are found in plants and no structural homologues of animal caspases have been identified, there is evidence that other plant proteases – metacaspases, vacuolar processing enzymes (VPEs) and subtilisin-like serine proteases (saspases) can function in a caspase-like manner (21). Specific peptide inhibitors of animal caspases have been shown as efficient abolifiers of plant PCD (4). The irreversible inhibitor of animal caspase-1 Acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and the reversible caspase-3 inhibitor Aetyl-Asp-Glu-Val-l-aspartic acid aldehyde (Ac-DeVD-cho) are demonstrated to attenuate bacteria- and tobacco mosaic virus (TMV)-induced HR in tobacco leaves (5), and broad range caspase inhibitors zVAD-fmk and BocD-fmk are shown to block PCD in tobacco cell suspension cultures treated with the ethylene-inducing fungal elicitor xylanase (7). Ethylene is a gaseous phytohormone that participates in many aspects of plant developmental processes (3) and is illustrated to accompany cell death in response to environmental cues, chemicals and at plant-pathogen interactions (4). Ethylene-Inducing Xylanase (EIX), a 22-kD proteinautious elicitor, induces hypersensitive response, stimulates ethylene biosynthesis, and provokes other defense events in plants such as tomato (Lycopersicon esculentum) and tobacco (Nicotiana tabacum) (1, 7, 15). Xylanase-elicited increase of ethylene biosynthesis has been accompanied by accumulation of its precursor l-aminocyclopropane-1-carboxylic acid (ACC) and by augmentation of ACC synthase (ACS) and ACC oxidase activities (1).
In this work, the potency of chemicals, proven as PCD inhibitors in other systems, to prevent the spread of cell death in detached tobacco leaves challenged with xylanase from *Aspergillus awamori* was examined. Lesion development was suppressed if the elicitor was applied to leaves pre-infiltrated with the inhibiting compounds: a broad range caspase-specific peptide inhibitor benzoxycarbonyl-Asp-2,6-dichlorobenzoyloyxymethylketone (Z-Asp-CH2-DCB), cysteine protease inhibitor iodoacetamide (IA), serine protease inhibitor 4-(2-Aminoethyl) benzensulfonil fluoride hydrochloride (AEBSF), the antioxidant l-galactone-γ-lactone (L-gal) – a natural precursor of ascorbic acid and the ethylene blocker (S)-trans-2-Amino-4-(2-aminoethoxy)-3-butenoic acid (AVG). Augmentation of ion leakage, massive reactive oxygen species (ROS) accumulation and increased amounts of the stress-marker metabolites hydrogen peroxide (H2O2) and malondialdehyde (MDA) were detected in xylanase-infiltrated lesions. The stress symptoms were substantially attenuated in the presence of tested inhibitors.

**Materials and Methods**

**Plant material**

Tobacco (*Nicotiana tabacum* L.) plants, cv. Nevrokop 261, were cultivated in pots under controlled environmental conditions (16h light/8h dark, light intensity 150 µE/s/m², temperature 26°C and 60–70% relative air humidity, RH). For the experiments, at least three pairs of fully expanded leaves (3rd position from the top) from 2-months-old plants (40 cm height) were harvested and exposed to xylanase and chemical treatments.

**Infiltration procedure and chemical treatments**

The detached leaves were infiltrated (by means of injecting) with β-xylanase preparation (further referred to as xylanase) from micromycete strain *Aspergillus awamori* K1. The enzyme preparation was provided by the Department of Biotechnology, Faculty of Biology, University of Sofia, Bulgaria, where it has been isolated and tested for its hydrolytic potential essentially as described by Ilieva et al. (11). Drops of 10 µl xylanase (30 mg/ml, dissolved in 0.02 M acetate buffer) were injected into the leaf mesophyll by hypodermic syringe through the epidermal layer of the upper side of detached tobacco leaves. Cell death inhibitors in an equal volume to the enzyme preparation were injected as pre-treatments, one hour before the application of xylanase. The enzyme preparation was introduced at the zone of the chemical treatments. In general, the used substances were tested in a range of concentrations (from nM to mM) with and without the addition of xylanase. Lowest concentrations giving significant restriction of lesion formation are presented. In the stock solutions, Z-Asp-CH2-DCB, AEBSF and AVG were dissolved in dimethyl sulfoxide (DMSO) and diluted 1000 times to the final applied concentration (final solvent concentration 0.1% v/v). IA and L-gal were dissolved in water. DMSO and the acetate buffer were tested alone and at the indicated concentrations no leaf toxicity was detected. Following infiltration, the leaves were kept in climatic cabinet (KBWF 240) at 26°C and >90% RH under photoperiod of 16h light/8h dark. Cell death development was scored 72 hours post inoculation (hpi), by assessing the formation of brown necrotic lesions.

**Cell death determination**

For visualization of the dead cells, Evans Blue staining was used according to the method of Keogh et al. (13) with slight modifications. Leaf discs (1.0 cm in diameter) from the inoculated areas were collected 72 hpi and boiled for 1 min in a mixture of phenol, lactic acid, glycerol and distilled water containing 20 mg/L Evans Blue (1:1:1:1), prepared immediately before use. Tissues were then clarified in 96% ethanol for 30–60 min.

**ROS imaging**

H2O2 accumulation in the leaves was visualized by 3′,3′-diaminobenzidine (DAB) staining as described by Thordal-Christensen et al. (19) with slight modifications. Leaf segments from the inoculated areas (1.0 cm in diameter) were excised 72 hpi, placed in 50 ml tubes, covered with 0.1% w/v DAB and incubated in an orbital shaker for 18 h. The dye was discarded and chlorophyll removed by boiling in 15% (v/v) ethanol for 15 min. DAB is rapidly absorbed by the plant tissues and is polymerized locally in the presence of H2O2, giving a visible brown stain with intensity corresponding to the amount of H2O2.

In addition to DAB staining, intracellular ROS were detected using 2′,7′-dichlorofluorescein diacetate (DCF-DA), following the protocol of Sakamoto et al. (16) with modifications. To determine intracellular ROS, 72 h after exposure to experimental treatments, leaf segments were washed with distilled water and incubated for 60 min in the presence of 10 µM DCF-DA.

**Microscopic observations**

Evans Blue stained dead cells and DAB stained ROS were examined under binocular microscope (Carl Zeiss, Jena, Germany) at 160x magnification and the fluorescence of DCF-DA stained samples was determined by Nikon Eclipse microscope, TS 100, filter B-2A, exciter 450–490, DM 505, BA 520, magnification 200x. Images were taken with Nikon Dxm 1200 digital camera (Nikon Inc., Melville, NY, USA).

**Biochemical assays**

For the biochemical assays, circular leaf discs (1.0 cm in diameter) including the infiltration area were collected 72 hpi as an average sample from at least 10 leaves. Ion leakage and the contents of hydrogen peroxide and malondialdehyde were measured.

**Ion leakage**

Ion leakage was determined according to Song et al. (18) with slight modifications. The leaf discs (0.1 g) were placed for 2h in Petri dishes with 10 ml de-ionized water at 25°C. After the incubation the conductivity of the bathing solution was measured.
Hydrogen peroxide and MDA analysis
A 0.3 g leaf tissue was homogenized in 3 ml 0.1% trichloroacetic acid (TCA) on ice, centrifuged at 14,000 rpm for 20 min and the supernatant was used for the assay. MDA was determined according to Dhindsa et al. (6) including TCA/TBA (thiobarbituric acid) addition and a heat/cool cycle. Absorption was read at 532 nm and 600 nm and MDA concentration was calculated using its molar absorptivity 155 μmol/cm for MDA (10).

The endogenous level of H$_2$O$_2$ was determined spectrophotometrically at 390 nm after incubation of the leaf extracts with 1 M KJ (12). Hydrogen peroxide content was calculated using a standard curve and expressed in mM. g$^{-1}$FW.

Results and Discussion
Detached tobacco leaves were infiltrated with xylanase from Aspergillus awamori. In order to allow the cell death inhibitors to penetrate the cells, the chemicals were administrated 1h before xylanase. Broad necrotic lesions developed around the point of xylanase infiltration (Fig. 1B, Fig. 1D). To determine whether caspase-like proteases are involved in the response to xylanase, the leaves were pre-infiltrated with 10 μM of the broad range caspase inhibitor Z-Asp-CH2-DCB. Significant suppression of lesion formation was detected 72h after pre-infiltration of the leaves with 100 μM of the antioxidant L-gal, immediate precursor of ascorbic acid (Fig. 1E). Strong inhibition of lesion development was also detected when the leaves were pre-treated with 10 μM of the cysteine protease inhibitor IA and 100 μM of the serine protease inhibitor AEBSF (Fig. 1F, Fig. 1G). The restriction of lesions by IA and AEBSF indicates the participation of proteolysis in xylanase-induced cell death. These results are in line with our previous findings that caspase-like and serine proteases play a role in Alternaria alternata AT toxin-induced PCD in detached tobacco leaves (22). Although similar indication about a role of caspase-like proteases in xylanase-induced PCD has been provided with tobacco suspension cells (7), caspase inhibitors and antioxidants have not been found efficient in preventing cell death induced by xylanase from Trichoderma viridae whereas serine protease inhibitors did inhibit the cell death (23). This suggests that there is a signalling pathway in which a serine protease might be responsible for the signal transduction, which is independent of the oxidative burst that leads to hypersensitive cell death of tobacco cells (23). The activation of certain cell death signalling cascade might depend on the origin of the elicitor and on the model system under study. Moreover, the activation of different signal transduction pathways can be a mechanism of avoiding the redundancy of metabolic events that are simultaneously provoked at PCD in response to stress stimuli.

ROS production is among the first plant responses to pathogen attack. Suppression of cell death was observed 72h after pre-infiltration of the leaves with 100 μM of the antioxidant L-gal, immediate precursor of ascorbic acid (Fig. 1H). This shows an involvement of ROS in xylanase-induced cell death and corresponds to the findings of other authors for xylanase-potentiated ROS production in tomato suspension cells (14). Significant inhibition of lesion development was also detected when the leaves were pre-treated with 100 μM of the ethylene synthesis inhibitor AVG, which indicates the involvement of ethylene in the cell death in xylanase-treated tobacco leaves (Fig. 1I) and suggests that xylanase elicits ethylene dependent signaling (1, 7, 15).

To detect the occurrence of dead cells, leaf discs containing xylanase-infiltrated tissue were stained with Evans Blue and microscopically examined. Evans Blue is permeable only in the dead cells that show blue colour after the dye uptake. Evans Blue positive cells were observed in the central area of the lesions after infiltration with xylanase and a halo of brown stained cells appeared in the surrounding tissue (Fig. 1J). Similar pattern was observed in tobacco leaves infiltrated with AT toxin from Alternaria alternata tobacco pathotype (22). In the central zone of the samples pre-treated with 10 μM Z-Asp-CH2-DCB, 10 μM IA, 100 μM AEBSF, 100 μM L-gal and 100 μM AVG a ring of Evans Blue positive cells appeared as well,
but much narrower circle of brown cells was observed (Fig. 1L-P). The reason for the occurrence of brown stained cells is not clear. It might be due to deposition of polyphenols in the dead cells that could have hindered the uptake of the dye by the neighbouring cells or the brown ring might consist of dead cells that have died due to necrosis. An indication of necrotic cell death in these cells might be due to the fact that Evans Blue binds to proteins of cytoplasm and is not expected to stain the necrotic cells where the cell constituents are entirely destroyed. In difference to PCD, necrosis is unprogrammed (accidental) type of cell death that occurs in presence of toxic compounds and at acute states and does not show the morphological PCD features (cytoplasm shrinkage, nuclear condensation, etc.). The cells undergoing necrosis are characterized by compromised plasma membrane, cell swelling, lysis and release of the cellular components in intracellular spaces (20). Formation of a ring of necrotic cells around the area of cells actively undergoing PCD at the primary side of infection is involved in the mechanism of the HR and helps the plant to prevent the spread of disease by limiting the nutritional sources in a case of biotrophic pathogens (9).

**Fig. 2.** Effect of cell death inhibitors on electrolyte leakage in detached tobacco leaves cv. Nevrokop 261, infiltrated with xylanase from Aspergillus awamori. The leaves were pre-infiltrated with 10 µM of broad range caspase inhibitor Z-Asp-CH2-DCB, 10 µM of the cysteine protease inhibitor IA, 100 µM of the serine protease inhibitor AEBSF, 100 µM of the antioxidant L-gal and 100 µM of the ethylene blocker AVG one hour before infiltration with xylanase. Ion leakage was measured 72 hpi in average samples of circular discs of leaf tissues (1.0 cm in diameter, including the infiltration area). Error bars indicate ± SEM (n=1).

At late stages of cell death, electrolytes from dying cells leak into the intercellular space. This allows ion leakage to be used as a marker of cell death (5). We have assayed the cell death by means of conductivity 72h after infiltration with xylanase alone and in presence of specific inhibitors. In xylanase treated leaf areas the cell death symptoms (Fig. 1D) were associated with nearly threefold increase of ion leakage in comparison to non-treated leaf discs (Fig. 2). Administration of 10 µM Z-Asp-CH2-DCB, 10 µM IA, 100 µM AEBSF, 100 µM L-gal and 100 µM AVG substantially reduced the electrolyte leakage (Fig. 2) in average with reduced size of the formed lesions (Fig. 1E-I).

ROS generation is one of the earliest responses triggered after pathogen recognition and has been shown to occur upon xylanase elicitation (2, 8, 14). H₂O₂ was detected in the dead cells lesions using DAB as a substrate. DAB staining occurred throughout the lesions and in the surrounding area of the xylanase infiltrated tissue (Fig. 3B). In the leaf areas pre-infiltrated with 10 µM Z-Asp-CH2-DCB, 10 µM IA, 100 µM AEBSF, 100 µM L-gal and 100 µM AVG lower intensity of DAB staining was detected indicating reduced generation of ROS in the surrounding tissue (Fig. 3C-G). This shows that the tested inhibitors are potent suppressors of xylanase-induced oxidative burst. The intracellular ROS levels were also detected by using the fluorescent probe DCF-DA. Strong fluorescence was detected in xylanase treated tissue and the accumulation of ROS corresponded to the one found in DAB stained leaf discs (Fig. 3I). Weaker fluorescence was observed after pre-treatment with 10 µM Z-Asp-CH2-DCB, 10 µM IA, 100 µM AEBSF, 100 µM L-gal and 100 µM AVG (Fig. 3J-N). Chlorophyll fluorescence in the living cells appeared in red (Fig. 3H). These results confirm that xylanase induces cell death which is associated with oxidative stress.

To further elucidate the involvement of oxidative stress in xylanase-induced cell death signalling, the stress markers hydrogen peroxide and malondialdehyde were studied. Following xylanase treatment the cell death symptoms were accompanied by remarkable increase of H₂O₂ amount in comparison to non-treated leaves (Fig. 4A). Application of cell death inhibitors diminished the level of H₂O₂, leading to 41, 40 and 32% inhibition in presence of 10 µM Z-Asp-CH2-DCB, 10 µM IA and 100 µM AEBSF respectively. Pre-infiltration with 100 µM L-gal caused 50% inhibition of H₂O₂ production. ACS inhibitor AVG reduced the H₂O₂ amount with 29%. This is an additional evidence that xylanase induces cell death processes that involve a stimulation of ROS.

MDA is end product of peroxidation of polyunsaturated fatty acids and is widely used as a marker of membrane damage (10). Although no lesions of dead cells were detected, a certain level of MDA was measured in non-treated control leaves. In the lesions with dead cells that developed after infiltration with xylanase the amount of MDA increased 2.5 times by comparison with the control. Pre-infiltration of the leaf discs with 10 µM Z-Asp-CH2-DCB, 10 µM IA, 100 µM AEBSF, 100 µM L-gal and 100 µM AVG reduced the level of MDA (Fig. 4B) and this corresponded to suppressed lesion formation (Fig. 1E-I).
Effect of cell death inhibitors on the content of hydrogen peroxide (A) and malondialdehyde MDA (B) in detached tobacco leaves cv. Nevrokop 261, infiltrated with xylanase from *Aspergillus awamori*. The leaves were pre-infiltrated with 10 µM of broad range caspase inhibitor Z-Asp-CH2-DCB, 10 µM of the cysteine protease inhibitor IA, 100 µM of the serine protease inhibitor AEBSF, 100 µM of the antioxidant L-gal and 100 µM of the ethylene blocker AVG one hour before infiltration with xylanase. The amounts of H2O2 and MDA were measured 72 hpi in average samples of circular discs of leaf tissues (1.0 cm in diameter, including the infiltration area). Error bars indicate ± SEM (n=3).

Fig. 4. Effect of cell death inhibitors on the content of hydrogen peroxide (A) and malondialdehyde MDA (B) in detached tobacco leaves cv. Nevrokop 261, infiltrated with xylanase from *Aspergillus awamori*. The leaves were pre-infiltrated with 10 µM of broad range caspase inhibitor Z-Asp-CH2-DCB, 10 µM of the cysteine protease inhibitor IA, 100 µM of the serine protease inhibitor AEBSF, 100 µM of the antioxidant L-gal and 100 µM of the ethylene blocker AVG one hour before infiltration with xylanase. The amounts of H2O2 and MDA were measured 72 hpi in average samples of circular discs of leaf tissues (1.0 cm in diameter, including the infiltration area). Error bars indicate ± SEM (n=3).

Conclusions
Our work illustrates that β-xylanase from *Aspergillus awamori* K1 induces cell death in detached tobacco leaves. By inhibitory studies we have shown that xylanase-stimulated cell death signaling involves caspase-like, cysteine and serine proteases and that oxidative stress and ethylene play an important role in mediation of the signalling cascade. The death of the cells in the lesions was accompanied with active electrolyte leakage, enhanced H2O2 accumulation and MDA production that were substantially prevented by application of caspase, cysteine and serine protease inhibitors, ethylene synthesis inhibitor and by an antioxidant. The involvement of caspase-like proteases and ROS in the cell death process suggests that β-xylanase-induced cell death is a form of PCD that resembles apoptotic-like signalling. To the best of our knowledge this is the first report indicating that β-xylanase produced by *Aspergillus awamori* K1 induces cell death in tobacco and the signalling involves biochemical events in similarity to those found in response to other fungal elicitors. The obtained data provide additional information and might be a clue to better understanding of the mechanisms of plant hypersensitive response.

Acknowledgements
This work was supported by project VU-B-9/05 National Science Fund, Ministry of Education and Science, Bulgaria.

REFERENCES