BIOLOGIA PLANTARUM 61 (1): 178-186, 2017 DOI: 10.1007/s10535-016-0654-y

Reactive oxygen species and sugars may be the messengers in kinetin-induced death of field bean root cortex cells

M. DONIAK[1], A. KAŹMIERCZAK[1]*, A. BYCZKOWSKA[1], and S. GLIŃSKA[2]

Department of Cytophysiology, Faculty of Biology and Environmental Protection, University of Łódź, PL-90236 Łódź, Poland[1]
Faculty of Biology and Environmental Protection, University of Łódź, PL-90237 Łódź, Poland[2]

Abstract

Kinetin-induced programmed cell death of field bean (*Vicia faba* spp. *minor*) root cortex cells led to aerenchyma formation. The process was accompanied by appearance of a greater amount of reactive oxygen species (ROS), greater superoxide dismutase (SOD) and catalase (CAT) activities, as well as by thickening cell walls and changes in sugar amounts, particularly in cell wall-bound sugars. The obtained results justify the supposition that ROS scavengers together with an increased amount of sugars (soluble, storage, and cell wall-bound) and thick cell walls protected the cells against death. Thus, kinetin played a dual role because it induced programmed death of chosen cells and simultaneously stimulated protective mechanisms against death in other cells. These results confirm an earlier suggestion that cell death induced by kinetin is a specific process during which its progression is hallmarked by metabolic and morphological features.

Additional key words: aerenchyma, catalase, fluorescence microscopy, superoxide dismutase, transmission electron microscopy.

Introduction

Programmed cell death (PCD), defined as a sequence of specific events (Kacprzyk *et al*. 2011), has a fundamental role in development of uni- and multi-cellular organisms (Gadjev *et al*. 2008). Compared to animals, in plants PCD is poorly understood. There have been several attempts to classify cell death in plants so far (Galluzzi *et al*. 2007, 2012, Kacprzyk *et al*. 2011, Van Doorn 2011, Van Doorn *et al*. 2011). Kunikowska *et al*. (2013) described kinetin-induced death of cortex cells in field bean seedling roots and provided a convenient, interesting, scientific *in planta* model, which allows researchers to study cell death at morphological, metabolic, and cell signalling levels (Doniak *et al*. 2014, 2015). It was found that death of cortex cells is accompanied by a higher plasma membrane permeability, which results in leakage of cell electrolytes leading to a greater culture medium conductivity, greater activities of cellular secreted dehydrogenases, decrease in the number of mitochondria, and release of reactive oxygen species (ROS) to cytoplasm (Kunikowska *et al*. 2013), which suggests that permeability of mitochondrial, nuclear, and plastid membranes increases (Kim *et al*. 2012).

Plasma membrane as well as nuclear envelope permeability changes (one of the essential PCD hallmarks; Domínguez and Cejudo 2012) are the basis of a specific method allowing PCD detection (Byczkowska *et al*. 2013). The method is based on the fact that only one of two fluorescence probes used, *i.e.*, ethidium bromide, can penetrate the nuclear envelope when it is changed, while acridine orange can penetrate every cellular membrane even if it is not changed. So, the amount of ethidium bromide in nuclei depends on the degree of nuclear membrane permeability or its damages. Thus, the method is the only one which, simultaneously showing PCD-dependent nuclear envelope and chromatin (condensation and its marginalisation) changes and

Submitted 3 October 2015, *last revision* 10 February 2016, *accepted* 14 March 2016.
Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; CAT - catalase; CMA_3 - chromomycin A_3; Ctrl - control; DAPI - 4',6-diamidino-2-phenylindole; NBT - nitroblue tetrazolium; PCD - programmed cell death; PHB - sodium phosphate buffer; ROS - reactive oxygen species; SOD - superoxide dismutase; TEM - transmission electron microscopy.
Acknowledgements: This work was supported by the University of Łódź, grants Nos. 545/487, B1551000000988.02, and 545/502. We thank Ms. M. Fronczak for her help in preparing this manuscript in English.
* Corresponding author; fax: (+48) 42 6354514, e-mail: kazand@biol.uni.lodz.pl

values of fluorescence intensity, allows to show alive cells and those undergoing PCD. Additionally, staining with 4',6-diamidino-2-phenylindole (DAPI) easily confirms nuclear hallmarks (Domínguez and Cejudo 2012) of PCD induced by kinetin and shows invaginations and a slight increase in volume of nuclei as well as micronuclei formation (Kunikowska *et al*. 2013, Doniak *et al*. 2014), which might be "plant apoptotic cell bodies" (Domínguez and Cejudo 2012). Moreover, studies showed that in PCD induced by kinetin, decrease in the number of G1- and G2-phases and of the fraction of hypoploid cells appeared as the effect of DNA degradation but without DNA ladder formation (Kunikowska *et al*. 2013, Doniak *et al*. 2014). A PCD induced by kinetin is also manifested by a nuclear DNA content decrease, a greater number of DAPI-stained and a lower number of chromomycin A_3 (CMA_3)-stained chromocentres, a greater fluorescence intensity of CMA_3-stained chromocentres, condensation of DAPI-stained and loosening CMA_3-stained chromatin, fluctuation of DNA methylation, fluctuation of activities of exo-/endo-nucleolytic Zn^{2+} and Ca^{2+}/Mg^{2+}-dependent nucleases, alterations in histone H1 and core histone kinase activities, and decrease in cellular ATP amount (Doniak *et al*. 2014). The hallmarks of kinetin-induced PCD also include greater amount of cytosolic Ca^{2+} (Kunikowska *et al*. 2013, Doniak *et al*. 2015) and emerging small and then lytic vacuoles, which become acidic. There are also greater diameter and mass of apical parts of roots and lower cortex cell length, leading to shortening of the whole roots (Kunikowska *et al*. 2013).

The cell death hallmarks, defining a specific kinetin-induced PCD, indicate that they may be classified as vacuolar (Kunikowska *et al*. 2013) or autolytic (Doniak *et al*. 2014) ones, the most common types of cell death during plant development (Van Doorn 2011, Van Doorn *et al*. 2011). Induction of cell death concerns *ca*. 50 % of cortex cells and leads to aerenchyma formation (Kunikowska *et al*. 2013, Doniak *et al*. 2015). Neither whole seedlings of *V. faba* ssp. *minor* nor whole roots die, therefore kinetin might play a dual role in PCD. It can induce death of certain cells but protecting the others. Thus, the studies on ROS amounts, activities of antioxidative enzymes, as well as sugar content were conducted to explain this phenomenon.

Materials and methods

Plants and treatments: Twenty seeds of *Vicia faba* L. ssp. *minor* Peterm. cv. Nadwiślański were germinated in Petri dishes (15 cm in diameter and 3 cm high) on two blotting papers moistened with distilled water in the dark for 3 d. Then six of the seedlings with a nearly equal length (2.0 ± 0.2 cm) of roots were transferred into a glass container (8 cm in diameter and 4 cm in height) with two blotting papers moistened with 10 cm^3 of water (Ctrl; control) or with 10 cm^3 of 46 μM kinetin and cultivated for 48, 72, and 96 h, or only for 72 h, in a humidified incubator in darkness (Fig. 1 Suppl.). Temperature and relative humidity were controlled at 23 ± 1 °C and 92 ± 2 %, respectively. Then, 2-cm apical parts of roots were cut off and used for analytic measurements or microscopic observations.

Reactive oxygen species, superoxide dismutase, and catalase activities: ROS detection was carried out in cortex cells of apical fragments of the root seedlings. The plant material was washed with a 0.1 M sodium phosphate buffer (PHB, pH 7.4) and stained with 0.05 % (m/v) nitroblue tetrazolium (NBT; *Sigma*, St. Louis, USA) in PHB (Kunikowska *et al*. 2013) in darkness for 1 h. Then, the material was fixed with 2.5 % (v/v) glutardialdehyde in PHB for 15 min, washed two times with PHB, thin longitudinal sections of roots were prepared, photographed using an *Optiphot-2* microscope (*Nikon*, Tokyo, Japan) equipped with a *DDX* camera and with the *Act-1* image software (*Precoptic*, Warsaw, Poland), and analysed.

The apical fragments of roots, which were used for measurement of superoxide anion ($O_2^{\bullet-}$) amount according to Hideg (2009), were incubated in a mixture of a 10 mM potassium-phosphate buffer, pH 7.4 (1.2 cm^3) with 10 mM NaN_3 (150 mm^3) and 0.05 % (m/v) NBT (75 mm^3) in darkness for 5 min. Then, 1 cm^3 of the mixture was incubated at 85 °C for 15 min, cooled, and absorbance was measured at 580 nm using an *Amersham Biosciences*, (Uppsala, Sweden) UV-VIS spectrophotometer, which was used for all spectrophotometric measurements. The amount of $O_2^{\bullet-}$ was calculated using an NBT absorbance coefficient (ε) of 30 mM^{-1} cm^{-1}.

Content of H_2O_2 was assayed according to Sunil and Narayana (2008). The apical fragments of roots were frozen on solid carbon dioxide, homogenised with a 50 mM Tris-HCl buffer (pH 7.45), and centrifuged at 5 000 *g* and 4 °C for 10 min. A 50 mm^3 of the supernatant was supplemented with cold (-20 °C) 96 % (v/v) ethanol to form its 80 % final concentration and centrifuged as above. The 0.6 cm^3 of a sample with a known amount of H_2O_2 was supplemented with 2 % (m/v) potassium iodide (0.1 cm^3) and 2 M hydrochloric acid (0.1 cm^3), and the mixture was shaken vigorously. When yellow colour appeared, the mixture was supplemented with 0.01 % (m/v) toluidine blue (0.2 cm^3) and a 2 M sodium acetate solution (0.2 cm^3) and absorbance was measured at 628 nm. The amount of H_2O_2 was calculated using a calibration curve prepared with 0.2 - 14 μM H_2O_2.

To measure superoxide dismutase (SOD) and catalase

(CAT) activities, 100 mg of apical parts of seedling roots were homogenised at 4 - 6 °C with 0.3 cm^3 of a 50 mM Tris-HCl buffer, pH 7.45, supplemented with 10 μM phenylmethylsulfonyl fluoride and centrifuged at 5 000 *g* and 4 °C for 10 min. The residues were re-extracted under the same conditions and the supernatants were combined and used to determine the amount of proteins with a Coomassie G-250 reagent according to Bradford (1976).

Activity of SOD was assayed using the method of Janknegt *et al*. (2007). A 0.82 cm^3 of a reaction mixture was with or without the enzyme extract and with 0.65 cm^3 of a 0.1 M K-phosphate buffer (pH 7.8), 20 mm^3 of riboflavin (1 mg cm^{-3}), 50 mm^3 of methionine (3 mg cm^{-3}), and 50 mm^3 of NBT (5 mg cm^{-3}). The samples were incubated in darkness at 4 - 6 °C for 30 min and then under irradiance (four 18 W *Flora* lamps) for 15 min. Absorbance was measured at 560 nm. Activity of SOD was expressed in U g^{-1}(f.m.) min^{-1} or U mg^{-1}(protein) min^{-1}, pointing that U was a 50 % inhibition of NBT reduction.

Activity of CAT was determined according to Durner and Klessig (1996). A 1.5 cm^3 of a reaction mixture was composed of a 100 mM K phosphate buffer, pH 7.0, 6 mm^3 of concentrated (30 %) H_2O_2, and 50 mm^3 of the enzyme extract. A decrease in absorbance at 240 nm was measured at 25 °C after 15 and 60 s of incubation. Activity was calculated as an amount of decomposed H_2O_2 using a standard curve prepared with known amounts of H_2O_2 in a range of 0.2 to 14 μM.

Sugar amount assays: To analyse sugars amount, the apical parts of roots (100 mg) were homogenised in 80 % (v/v) methanol (200 mm^3) at 23 °C. The homogenate was centrifuged at 5 000 *g* for 10 min and re-extracted. The supernatants were combined and used for determination of soluble sugar using the anthrone spectrophotometric test or to determine total sugar amount using the reflectometric test.

The pellets were evaporated at 30 °C for 30 min to remove methanol and extracted with 500 mm^3 g^{-1}(f.m.) of 35 % (m/m) perchloric acid ($HClO_4$; *POCH*, Gliwice, Poland) at 4 - 8 °C for 24 h, centrifuged at 5 000 *g* for 20 min and re-extracted. The combined supernatants were used to determine storage sugars (starch and hemicelluloses) using the anthrone test. The pellets were supplemented with 500 mm^3 g^{-1}(f.m.) of 67 % (m/m) sulphuric acid (H_2SO_4; *POCH*, Gliwice, Poland) and shaken at 4 - 8 °C for 24 h, centrifuged at 10 000 *g* for 20 min, and the supernatants were used to determine cell wall sugars using the anthrone test.

Alternatively, the $HClO_4$-pellets were re-suspended in a mixture of glacial acetic acid and 65 % nitric acid (6:1) and digested in a boiling water bath for 20 min. The samples were cooled, centrifuged at 1 000 *g* for 20 min, and the pellets were washed with ethanol, dried, and supplemented with 500 mm^3 g^{-1}(f.m.) of 67 % (m/m) H_2SO_4 at 4 - 8 °C for 24 h and centrifuged at 5 000 *g* for 20 min. The supernatants were used to determine cellulose amount using the anthrone test.

The anthrone test was prepared according to Kaźmierczak (2007). A reaction mixture consisting of 0.9 cm^3 of an anthrone reagent (200 mg anthrone in 100 cm^3 of 72 %, m/m, sulphuric acid) and 50 mm^3 of an extract of a sample was heated at 110 °C for 11 min and cooled. Absorbance was measured at 630 nm. Blank solutions were prepared using proper volumes of methanol, $HClO_4$, or H_2SO_4 instead of an extract. Amount of sugars was estimated using a calibration curve prepared with glucose as a standard in a range of of 1 to 50 μg (total volume was 0.95 cm^3).

Reflectometric determination of sugar amount was carried out according to the procedure of *Merck* (Darmstadt, Germany) using the *RQflex 10 plus* system and *TS-1* reagent with special test strips. Methanol extracts of 2-cm long apical parts of field bean roots were used. In the test, free D-glucose and D-fructose are converted to 6-phosphate-glucose, which is converted by a dehydrogenase cofactor (NAD) into 6-phosphate-gluconate and NADH. Next, diaphorase with NADH reduces tetrazolium salts to blue phormazane.

Cellulose and callose in walls of cortex cells of apical root parts were identified and estimated using an *Optiphot-2* microscope and fluorescence microscope (*Nikon*, Tokyo, Japan) according to Kaźmierczak (2007, 2008).

To detect cellulose, roots were fixed with 4 % (m/v) paraformaldehyde in a buffer for transmission electrom microscopy TEM (50 mM Tris, 5 mM $MgCl_2$, and 5 %, v/v, dimethyl sulfoxide, pH 7.0) at room temperature for 60 min, and thin handmade cross sections were prepared. Then, pectic polysaccharides from cell walls were removed by treatment with 100 mM Na_2EDTA, pH 7.0 at 85 °C for 4 h. Next, the sections were washed three times in the buffer for TEM and stained for 15 min with 0.001 % (m/v) calcofluor white (*Fluostain*; Sigma) and washed with the buffer for TEM. Blue fluorescence of cellulose under UV radiation (an *UV-2A* filter) was photographed and analysed.

For identification of callose, roots were fixed at once after preparation with 2.5 % (v/v) glutaraldehyde in a 0.1 M K-phosphate buffer, pH 7.0, at 23 °C, and thin handmade longitudinal sections were prepared. Then, the sections were treated with a boiling 10 mM Tris, 1 mM Na_2EDTA, pH 8 buffer for 30 min, stained with 0.05 % (m/v) aniline blue (*Water blue*; *Fluka*, Germany), a specific dye for callose identification (Scherp *et al*. 2001), in 4 mM K_2HPO_4 (pH 9.0) for 15 min, and washed three times with the same buffer. Green/yellow fluorescence of callose under blue radiation (a *B2A* filter) was photographed and analysed.

Relative amount of cellulose and callose, expressed in fluorescence a.u., was assayed by measurement of total fluorescence intensity with the *Scn Image* software using

colour photos (Kaźmierczak 2007, 2008).

Flavonoids and phenols amounts: Flavonoids amount was measured according to Marinova *et al*. (2005). Extracts were prepared in the same way as those for the soluble sugar measurement. A reaction mixture was composed of the supernatant (0.1 cm^3), H_2O (50 mm^3), and 5 % (m/v) $NaNO_2$ (30 mm^3) and incubated at 23 °C for 6 min. Then, 10 % (m/v) $AlCl_3 \cdot 6\ H_2O$ (60 mm^3) was added. The reaction was stopped with 1 M NaOH (0.2 cm^3). Absorbance was measured at 510 nm. The amounts of flavonoids were expressed in mg g^{-1}(f.m.) of catechin, which was used to prepare a calibration curve in a range of 0.345 to 175 mM. Catechin was assessed in a sample prepared in the same way as the experimental ones.

To determine phenols amount, samples were prepared in the same way to those used for the measurement of SOD and CAT activities. A reaction mixture containing 250 mm^3 of the Folin-Ciocalteu reagent, 0.5 cm^3 of 7 % (m/v) Na_2CO_3, and H_2O was incubated for 25 min and centrifuged at 5 000 *g* and 4 °C for 10 min. Absorbance was measured at 725 nm. The amounts of phenols were estimated using ferulic acid, which was used to prepare a calibration curve in a range of 0.515 to 7.725 mM. Ferulic acid was assessed in a sample prepared in the same way as the experimental ones.

Transmission electron microscopy: For ultrastructural observations of root cortex cells made according to Glińska and Gapińska (2013), 2-cm long apical parts of roots of the control and kinetin treated seedlings were fixed in 2 % (v/v) glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.5, at 4 °C for 2 h. Then, they were rinsed with the same buffer and post-fixed in 1 % (m/v) osmium tetroxide at 4 °C for 2 h. The material was dehydrated in a graded ethanol series (30, 50, 70, 90, 95 and 100 %) and embedded in Epon-Spur's resin mixture. Ultrathin sections were analysed after staining with a saturated solution of uranyl acetate and with lead citrate. Observations by transmission electron microscopy (TEM) were carried out using *JEM 1010* (*JEOL*, Peabody, USA) at 80 kV.

Statistical analyses: The results of the cytomorphological measurements were statistically verified using Student's *t*-test, whereas the results of the metabolic research were verified by Mann-Whitney's *U*-test, and the *P* value was applied to show significant differences between the results.

Results

In the cortex cells of 2-cm long apical parts of roots of the *V. faba* ssp. *minor* seedlings, a ROS burst was prominently visible after staining with NBT in the material treated with kinetin for 72 h (Fig. 1*C*), and it was less observed in the plants treated for 48 h (Fig. 1*B*) and 96 h (Fig. 1*D*); it was not noticed in the control (Ctrl; Fig. 1*A*). However, the spectrophotometric measurements of the extracts from whole 2-cm root fragments reveal that in the seedlings after treatment with kinetin for 48, 72, and 96 h, the amounts of ROS were about 30, 20 and 70 % lower ($P < 0.05$) than in the Ctrl (Fig. 2*A*); when the amounts of ROS were expressed per mg of protein (Fig. 2*B*), they were lower in the 48- and 96-h-kinetin variants but in the 72-h-kinetin variant, they were 2-fold higher ($P < 0.05$) than in the Ctrl. The results from this variant are consistent with those observed microscopically (Fig. 1*C*).

Activities of SOD in the root extracts of the plants treated with kinetin for 48, 72, and 96 h were similar to those in the samples of the respective Ctrl ($P > 0.05$; Fig. 2*C*) when expressed in U mg^{-1}(f.m.), whereas SOD activities expressed in U mg^{-1}(protein) were about 2-, 2-, and 3-fold greater ($P < 0.05$) at 48, 72, and 96 h than in the respective Ctrl (Fig. 2*D*).

Changes in ROS metabolism in the kinetin variant were accompanied by specific morphological changes in mitochondria. Two types of mitochondria were observed, more spherical and containing more developed mitochondrial crests (Fig. 3*A*,*B*) and irregularly shaped ones (Fig. 3*C,D*).

The amounts of H_2O_2 expressed in μmol g^{-1}(f.m.) were higher ($P < 0.05$) by about 20 % in the kinetin treated plants for 48 and 72 h in comparison to the respective Ctrl (Fig. 4*A*), however, after 96, it was about 2-fold lower as compared to the Ctrl ($P < 0.05$; Fig. 4*A*). The amounts of H_2O_2 expressed in μmol mg^{-1}(protein) in the kinetin treated plants for 48 and 72 h were greater ($P < 0.05$) by about 20 and 200 % as compared to the respective Ctrl, but after 96 h, it was similar to the Ctrl (Fig. 4*B*).

Activities of CAT expressed in mmol(H_2O_2) g^{-1}(f.m.) measured in the extracts from 2-cm long apical parts of the kinetin treated seedling roots for 48 and 72 h were greater ($P < 0.05$) by about 150 and 20 % as compared to the respective Ctrl (Fig. 4*C*) but after 96 h, they were similar (Fig. 4*C*). Specific activities of CAT in the kinetin treated roots for 48, 72, and 96 h were greater ($P < 0.05$) by about 50, 300, and 40 % as compared to the Ctrl, respectively (Fig. 4*D*).

The analyses of flavonoids and phenols show that their amounts in the extracts from 2-cm long apical parts of the kinetin treated plants for 72 h and in the Ctrl were similar (Table 1).

The spectrophotometrically measured amounts of soluble (Fig. 5*A*) and storage (Fig. 5*B*) sugars of 2-cm long apical parts of the field bean seedlings were about

Table 1. The amounts of flavonoids [mg(catechin) g^{-1}(f.m.)] and phenols [mg(ferulic acids) g^{-1}(f.m.)] in 2-cm long apical parts of untreated (Ctrl) and kinetin treated for 72 h bean seedlings. Means ± SEs (n = 6) of two replicates of three independent experiments.

Variants	Flavonoids	Phenols
Ctrl	5.5 ± 1.1	12.1 ± 1.9
Kin	7.1 ± 1.4	13.2 ± 3.1

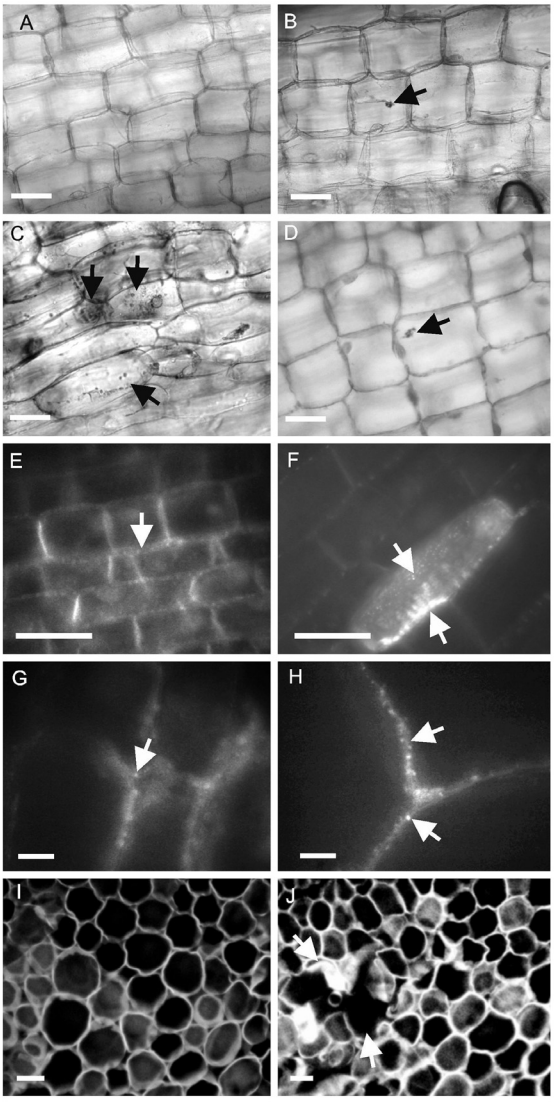

Fig. 1. Micrographs visualizing ROS production in cortex cells on longitudinal sections (*A-D*), callose in cell walls on longitudinal (*E-H*), and cellulose in cell walls on cross (*I-J*) sections of cortex from six roots of control (*A*,*E*,*G*,*I*) and seedlings treated with kinetin for 48 h (*B*), 72 h (*C*,*F*,*H*,*J*), and 96 h (*D*) in two independent experiments. The *black arrows* in *A-D* indicate ROS accumulation, the *white arrows* in *E-H* and *J* indicate callose grains in plasmodesma and aerenchyma spaces, respectively. The *scale bars* in *A-E*, *G* = 50 μm, in *F*,*H* = 10 μm, and in *I*,*J* = 100 μm.

4- and 2-fold greater ($P < 0.05$), respectively, but only in the kinetin treated plants for 96 h as compared to the Ctrl (Fig. 5*A*,*B*).

The total amount of sugars reflectometrically measured as the amount of a fructose and glucose mixture in 2-cm long apical parts was about 5.60 ± 1.5 mg g^{-1}(f.m.) in the Ctrl, whereas 8.55 ± 1.2 mg g^{-1}(f.m.) in the kinetin treated plants for 96 h.

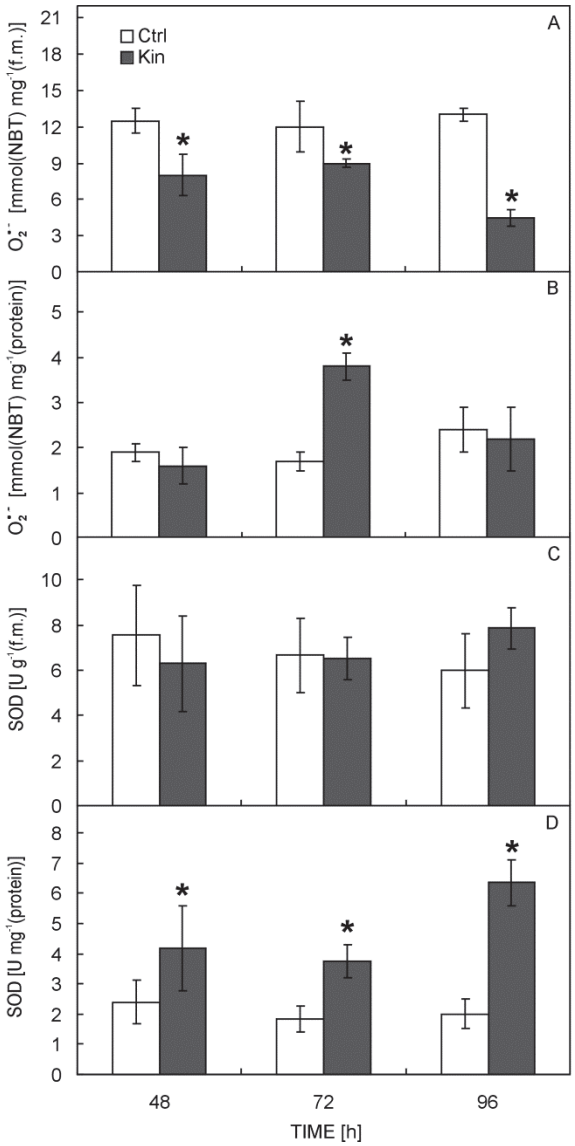

Fig. 2. The amount of $O_2^{\bullet-}$ (*A*,*B*) and activities of superoxide dismutase (SOD, *C*,*D*) in 2-cm long apical parts of control seedlings and seedlings treated with kinetin for 48 - 96 h. Means ± SEs (n = 6) of two replicates of three independent experiments; * - indicates significant differences at $P < 0.05$ between the results from the treated and untreated plants.

The amounts of cell wall-bound sugars measured by the anthrone test were higher in the kinetin treated plants for 72 and 96 h than in the respective Ctrl (Fig. 5*C*; $P < 0.05$). In the walls of cortex cells stained with calcofluor

Table 2. The amount of cellulose in cell walls cytophotomerically measured on cortex cross sections, expressed as total fluorescence intensity (TFI) [a.u.], and the amount of cellulose spectrophotometrically measured [mg(glucose) g^{-1}(f.m.)] in 2-cm long apical parts of roots from untreated and kinetin-treated seedlings for 72 h. Means ± SEs (n = 6) of three replicates of three independent experiments; * - indicates significant differences at $P < 0.05$ between the results from the treated and untreated plants.

Variants	TFI	Cellulose
Ctrl	1.39 ± 0.22	1.95 ± 0.42
Kin	1.83 ± 0.09*	2.90 ± 0.83*

white (Fig. 1*I*,*J*), the cytophotometric measurements showed higher cellulose content in the cell walls of the kinetin-treated roots than in the Ctrl (Table 2). The spectro-photometric measurements using the anthrone test revealed higher ($P < 0.05$) cellulose content in the cell walls of a kinetin-treated roots than in the Ctrl (Table 2). In the cross sections of kinetin-treated (Fig. 1*J*) roots, in contrast to the Ctrl (Fig. 1*I*), aerenchyma spaces were observed. The callose amount cytophotometrically determined in the walls of aniline blue stained cortex cells (Fig. 1*E-H*) was greater ($P < 0.05$) by about 30 % in the kinetin treated plants compared to the Ctrl. The ultramicrographs confirmed the presence of plasmodesmata in cell walls only in the Ctrl variant (Fig. 3*E*).

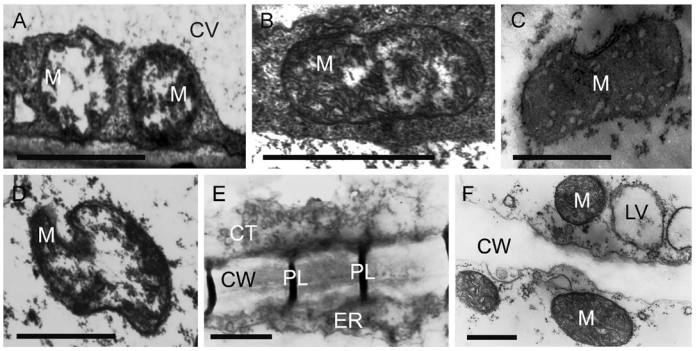

Fig. 3. Ultramicrographs of mitochondria (*A*,*B*,*C*,*D*,*F*) and cell walls (*E*,*F*) of root cortex cells of control (*A*,*B*,*E*) and 72-h kinetin-treated (*C*,*D*,*F*) seedlings. CT - cytoplasm, CV - central vacuole, CW - cell wall, ER - endoplasmic reticulum, M - mitochondrion, LV - lytic vacuole, PL - plasmodesmata. The *scale bars* = 1 μm.

Discussion

Apical parts of field bean roots and their cortex cells become a very good, *in situ* (*in planta*) scientific model to study the PCD (Byczkowska *et al*. 2013, Kunikowska *et al*. 2013, Doniak *et al*. 2014, 2015). Experiments with 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor (Byczkowska *et al*. 2013), or kinetin (Kunikowska *et al*. 2013), a cytokinin hormone which plays a crucial regulatory role in cell differentiation (Barciszewki *et al*. 2007), show that morphological and some of metabolic aspects of death processes induced by these chemicals in cortex cells of the field bean seedling roots were similar. Formation of a gas-filled space, *i.e*., aerenchyma, is the most morphological important effect of both ACC treatment and kinetin treatment (Byczkowska *et al*. 2013, Kunikowska *et al*. 2013, Doniak *et al*. 2015). The main event of aerenchyma formation consists in cells removing from the root cortex by elimination of protoplasts and then walls of these cells; thus, the space which facilitates gas diffusion between roots and the aerial environment is formed (Drew *et al*. 2000, Videmšek *et al*. 2006). Aerenchyma formation was confirmed in the present study.

There are many results indicating that ROS and calcium ions may be second messengers (Kaludercic *et al*. 2014). In cellular metabolism and plant differentiation, ROS play a dual, both signalling and damaging, role (Gadjev *et al*. 2008, Bolouri-Moghaddam *et al*. 2010, Kaludercic *et al*. 2014). In plants ROS are generated in chloroplasts, mitochondria, peroxisomes, cytosol, endomembrane system, and nucleus, as well as they appear in apoplast as a result of energy transfer reactions and electron leakage from saturated electron transport chains as well as of various activities of oxidative enzymes (Gadjev *et al*. 2008).

The present results show a greater amount of $O_2^{\bullet-}$ after 72 h, when it was expressed per protein unit, and a greater amount of H_2O_2 after 48 and 72 h of the kinetin treatment, when its amount was expressed per fresh mass unit. Greater specific activities of SOD and CAT in the kinetin treated plants compared to the roots of the untreated seedlings indicate that the kinetin-induced PCD was mediated by ROS. The results show that a relative amount of H_2O_2 and CAT activities were greater than the $O_2^{\bullet-}$ amount and SOD activities. It might have resulted from the fact that SOD eliminates $O_2^{\bullet-}$ and produces H_2O_2 which activates CAT. These results are consistent with

data present in literature (Gadjev *et al*. 2008), suggesting activation of protective mechanisms reducing a ROS-harmful influence, hence *ca*. 50 % of cortex cells of apical parts of field bean roots after 72-h-kinetin treatment remain alive (Kunikowska *et al*. 2013). Moreover, the mechanism of ROS metabolism sufficiently counteracts their damaging impact on root cortex because phenols and flavonoids amounts, factors playing important roles in ROS defense mechanisms (Soylu *et al*. 2003), were similar in the untreated and kinetin-treated material.

Overproduction of ROS changes morphology of mitochondria. They are elongated and irregularly shaped (Wakabayashi and Spodnik 2000). In the present study, during the kinetin-induced PCD, the influence of ROS on morphology of mitochondria in cortex cells was confirmed by TEM. The fact that kinetin-induced PCD is transduced *via* mitochondria was further proved by their lower number and a decrease in total cellular dehydro-genase activities, which was suggested earlier (Kunikowska *et al*. 2013). These facts suggest that ROS play a pivotal role in control of kinetin-induced death of cortex cells.

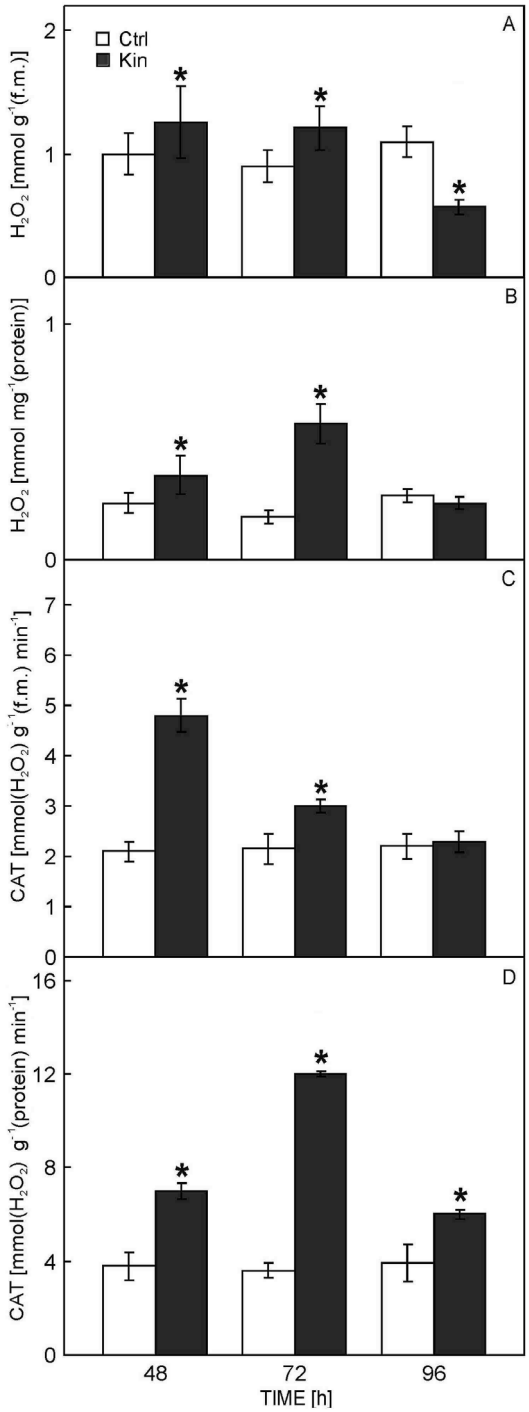

Fig. 4. Content of H_2O_2 (*A*,*B*) and catalase (CAT) activities (*C*,*D*) in 2-cm long apical parts of control seedlings and seedlings treated with kinetin for 48 - 96 h. Means ± SEs ($n = 6$) of two replicates of three independent experiments; * - indicates significant differences at $P < 0.05$ between the treated and untreated plants.

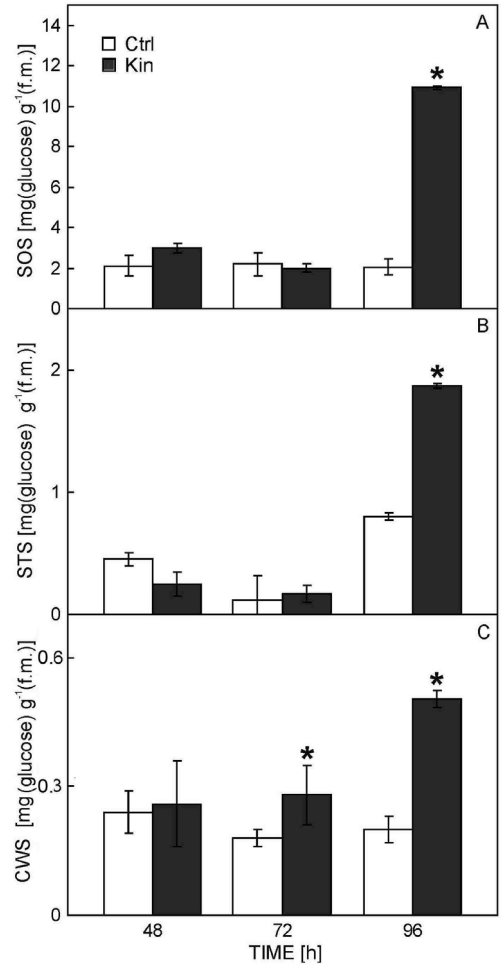

Fig. 5. The amounts of soluble (*A*; SOS), storage (*B*; STS) and cell wall bound (*C*; CWS) sugars in 2-cm long apical parts of roots of control seedlings and seedlings treated with kinetin for 48 - 96 h. Means ± SEs ($n = 6$) of two replicates of three independent experiments. * - indicates significant differences at $P < 0.05$ between the treated and untreated plants.

Up till now, the effects of sugars on induction of PCD have been rarely studied (Granot 2003). The results presented in this paper show that PCD in plants might be manifested by modifications of sugar content. It was found that in the kinetin-treated 2-cm long apical parts of the field bean seedling roots, the amounts of spectrophotometrically measured free and storage sugars were greater but only after 96 h, whereas the total amount of sugars (fructose and glucose) was greater after 72 h of the kinetin treatment. Fructose might have played a signalling role in the kinetin-induced PCD, likewise such a role of fructose was indicated by Ruan (2012). Moreover, the spectrophotometrically measured amount of cell wall sugars was greater after the 72- and 96-h treatments with kinetin. Their greater amount after the 72-h treatment might have been connected with greater amounts of cellulose and callose confirmed by spectrophotometric and cytofluorospectrophotometric measurements. Thus, these results indicate that kinetin-induced cell death, which led to aerenchyma formation, was mainly connected with a greater amount of cell wall-bound sugars. This phenomenon is reflected in a two-fold thicker cell wall (Doniak *et al*. 2015) and a greater width and lower length of root cortex cells resulting in a greater mass of 2-cm long apical parts of roots (Kunikowska *et al*. 2013, Doniak *et al*. 2015). This fact suggests that the wall sugar amounts might increase, which is important for protection of the root structure *via* mechanic resistance of walls of non-dying cells.

In the present research, an increase in the number of green fluorescent grains in cell walls of root cortex cells of the kinetin-treated seedlings after aniline blue staining was observed. This fact indicates that in the walls of cortex cells, plasmodesmata are clogged with callose (Kaźmierczak 2008). It was confirmed by TEM micrographs showing that in the walls of cortex cells of the material treated with kinetin, no plasmodesmata were observed. These results suggest that callose might isolate living cells from dying ones, which might be one of the additional protective mechanisms against death of all cortex cells. Therefore, callose is important for a wide range of processes in plant development and/or in response to multiple biotic (Kuźniak *et al*. 2013) and abiotic (Chen *et al*. 2009) stresses. Callose is necessary during development of *Anemia phyllitidis* gametophytes because application of deoxyglucose, an inhibitor of its synthesis, disturbs their morphogenesis (Kaźmierczak 2008). It is reflected in a lower number and size of gametophyte cells and fewer antheridia as a result of death of cells (Kaźmierczak 2008).

Saccharides play important roles in plants both as nutrients and as regulatory molecules forming a network with plant hormones and ROS production, and signalling and scavenging pathways. It has been suggested that a mitochondria-associated hexokinase plays a central role in the synergistic interaction of sugars (or sugar-like substances) as well as phenolic compounds, which are parts of an integrated redox system, quenching ROS, and contributing to reinforcement stress tolerance (Bolouri-Moghaddam *et al*. 2010).

Analyses of ROS and sugars amounts as well as activities of ROS scavenging enzymes allow to suppose they play a dual role in PCD induced by kinetin; either triggering death or protecting cortex cells. Kinetin stimulated ROS synthesis and their accumulation led to dysfunction of mitochondria. On the contrary, kinetin-induced ROS scavenging enzymes limited spreading ROS in plant tissues, thus protected them against damage. The greater amount of cell wall sugars and fructose might be stimulated by accumulated ROS (Ruan 2012).

Assuming that the process of cell death is the sequence of events which are manifested, *e.g*., by an increase in amount of cell metabolism elements including signalling particles (ROS, fructose, and the enzymes of ROS metabolism) may trigger changes in content of other cellular elements (soluble and storage sugars, and cell wall bound sugars - cellulose and callose). Subsequently, a high content of sugars may start processes leading to death of cortex cells, but they can also be used to build a cell wall. Cell wall sugars were used for reinforcing the structure of root cortex cell walls, strengthening cortex tissue and whole roots, and regulating plasmodesma-dependent signalling pathways. Plasmodesmata clogged with callose form symplasmic domains (Chen *et al*. 2009), which prevents specific cell-to-cell migration of signals, among them of gibberellins (Lucas and Lee 2004).

Our earlier results suggest that kinetin-induced cell death can be classified as vacuolar (Kunikowska *et al*. 2013) or autolytic (Doniak *et al*. 2014). The most important conclusion of this paper is that the kinetin-induced PCD in root cortex is a specific type of death since a greater amount of ROS can be the hallmark of both necrosis and hypersensitive response related PCD (Van Doorn *et al*. 2011) as well as of a non-autolytic (Van Dorn 2011) type of death. Thus, we suggest that this cell death should not be classified by its specific hallmarks but by factors which induce this death process, so we propose a term “kinetin-specific root cortex cell death”.

References

Barciszewski, J., Massino, F., Clark, B.F.C.: Kinetin – a multi-active molecule. - Int. J. Biol. Macromol. **40**: 182-192, 2007.

Bolouri-Moghaddam, M.R., Roy, K.L., Xiang, L., Rolland, F., Van den Ende, W.: Sugar signalling and antioxidant network connections in plant cells. - FEBS J. **277**: 2022-2037, 2010.

Bradford M.M.: A dye binding assay for protein. - Anal. Biochem. **72**: 248-254, 1976.

Byczkowska, A., Kunikowska, A., Kaźmierczak, A.:

Determination of ACC-induced cell programmed death in roots of *Vicia faba* ssp. *minor* seedlings by acridine orange and ethidium bromide staining. - Protoplasma **250**: 121-128, 2013.

Chen, X.Y., Kim, J.Y.: Callose synthesis in higher plants. - Plant Signal Behav. **4**: 489-492, 2009.

Doniak, M., Barciszewska, M.Z., Kaźmierczak, J., Kaźmierczak, A.: The crucial elements of the 'last step' of programmed cell death induced by kinetin in root cortex of *V. faba* ssp. *minor* seedlings. - Plant Cell Rep. **33**: 2063-2067, 2014.

Doniak, M., Byczkowska, A., Kaźmierczak, A.: Kinetin-induced programmed death of cortex cells is mediated by : ethylene and calcium ions in roots of *Vicia faba* ssp. *minor*. - Plant Growth Regul. **78**: 335-343, 2016

Domínguez, F., Cejudo, F.J.: A comparison between nuclear dismantling during plant and animal programmed cell death. - Plant Sci. **197**: 114-121, 2012.

Drew, M.C., He, C.J., Morgan, P.W.: Programmed cell death and aerenchyma formation in roots. - Trends Plant Sci. **5**: 123-127, 2000.

Durner, J., Klessig, D.F.: Salicylic acid is a modulator of tobacco and mammalian catalases. - J. biol. Chem. **271**: 28492-28501, 1996.

Gadjev, I., Stone, J.M., Gechev, T.S.: Programmed cell death in plants: new insights into redox regulation and the role of hydrogen peroxide. - Int. Rev. cell. mol. Biol. **270**: 87-144, 2008.

Galluzzi, L., Maiuri, M.C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., Kroemer, G.: Cell death modalities: classification and pathophysiological implications. - Cell Death Differ. **14**: 1237-1266, 2007.

Galluzzi, L., Vitale, I., Abrams, J.M., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., Dawson, T.M., Dawson, V.L., El-Deiry, W.S., Fulda, S., Gottlieb, E., Green, D.R., Hengartner, M.O., Kepp, O., Knight, R.A., Kumar, S., Lipton, S.A., Lu, X., Madeo, F., Malorni, W., Mehlen, P., Nuñez, G., Peter, M.E., Piacentini, M., Rubinsztein, D.C., Shi, Y., Simon, H-U., Vandenabeele, P., White, E., Yuan, J., Zhivotovsky, B., Melino, G., Kroemer, G.: Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. - Cell Death Differ. **19**: 107-120, 2012.

Hideg, E., Vitányi, B., Kósa, A., Solymosi, K., Bóka, K., Won, S., Inoue, Y., Ridge, R.W., Böddi, B.: Reactive oxygen species from type-I photosensitized reactions contribute to the light-induced wilting of dark grown pea (*Pisum sativum*) epicotyls. - Physiol. Plant. **138**: 485-492, 2009.

Glińska, S., Gapińska, M.: The effect of pre-incubation of *Allium cepa* L. roots in the ATH-rich extract on Pb uptake and localization. - Protoplasma **250**: 601-611, 2013.

Granot, D., Levine, A., Dor-Hefetz, E.: Sugar-induced apoptosis in yeast cells. - FEMS Yeast Res. **4**: 7-13, 2003.

Janknegt, P.J., Rijstenbil, J.W., Van de Poll, W.H., Gechev, T.S., Buma, A.G.J.: Comparison of quantitative and qualitative superoxide dismutase assays for application to low temperature microalgae. - J. Photochem. Photobiol. B **87**: 218-226, 2007.

Kacprzyk, J., Daly, C.T., McCabe, P.F.: The botanical dance of death: programmed cell death in plants. - Adv. bot. Res. **60**: 169-271, 2011.

Kaludercic, N., Deshwal, S., Di Lisa, F.: Reactive oxygen species and redox compartmentalization. - Front. Physiol. **5**: 1-15, 2014.

Kaźmierczak, A,: Ethylene is a modulator of gibberellic acid-induced antheridiogenesis in *Anemia phyllitidis* gametophytes. - Biol. Plant. **51**: 683-689, 2007.

Kaźmierczak A.: Cell number, cell growth, antheridiogenesis, and callose amount is reduced and atrophy induced by deoxyglucose in *Anemia phyllitidis* gametophytes. - Plant Cell. Rep. **27**: 813-821, 2008.

Kim, Ch., Meskauskiene, R., Shengrui Zhang, S., Lee, K.P., Ashok, M.L., Blajecka, K., Herrfurth, C., Feussner, I., Apel, K.: Chloroplasts of *Arabidopsis* are the source and a primary target of a plant-specific programmed cell death signaling pathway. - Plant Cell **24**: 3026-3039, 2012.

Kunikowska, A., Byczkowska, A., Kaźmierczak, A.: Kinetin induces cell death in root cortex cells of *Vicia faba* ssp. *minor* seedlings. - Protoplasma **250**: 851-861, 2013.

Kuźniak, E., Kaźmierczak, A., Wielanek, M., Głowacki, R., Kornas, A.: Involvement of salicylic acid, glutathione and protein S-thiolation in plant cell death-mediated defence response of *Mesembryanthemum crystallinum* against *Botrytis cinerea*. - Plant Physiol. Biochem. **63**: 30-38, 2013.

Lucas, W.J., Lee, J-Y.: Plasmodesmata as a supracellular control network in plants. - Nat. Rev. mol. cell. Biol. **5**: 712-726, 2004.

Marinova, D., Ribarova, F., Atanassova, M.: Total phenolics and total flavonoids in bulgarian fruits and vegetables. - J. Chem. Technol. Metall. **40**: 255-260, 2005.

Ruan, Y.L.: Signaling role of sucrose metabolism in development. - Mol. Plants **5**: 763-765, 2012.

Scherp, P., Grotha, R., Kutschera, U.: Occurrence and phylogenetic significance of cytokinesis-related callose in green algae, bryophytes, ferns and seed plants. - Plant Cell Rep. **20**: 143-149, 2001.

Soylu, S., Baysal, Ö., Soylu, E.M.: Induction of disease resistance by the plant activator, acibenzolar-S-methyl (ASM), against bacterial cancer (*Clavibacter michiganensis* subsp. *michiganensis*) in tomato seedlings. - Plant Sci. **165**: 1069-1075, 2003.

Sunil, K., Narayana, B.: Spectrophotometric determination of hydrogen peroxide in water and cream samples. - Bull. Environ. Contam. Toxicol. **81**: 422-426, 2008.

Van Doorn, W.G.: Plant programmed cell death and the point of no return. - Trends Plant Sci. **10**: 478-483, 2005.

Van Doorn, W.G., Beers, E.P., Dangl, J.L., Franklin-Tong, V.E., Gallois, P., Hara-Nishimura, I., Jones, A.M., Kawai-Yamada, M., Lam, E., Mundy, J., Mur, L.A.J., Petersen, M., Smertenko, A., Taliansky, M., Van Breusegem, F., Wolpert, T, Woltering, E., Zhivotovsky, B., Bozhkov, P.V.: Morphological classification of plant cell deaths. - Cell Death Differ. **18**: 1241-1246, 2011.

Van Doorn, W.G.: Classes of programmed cell death in plants, compared to those in animals. - J. exp. Bot. **14**: 4749-4761, 2011.

Videmšek, U., Idemšek, U., Turk, B., Vodnik, D.: Root aerenchyma - formation and function. - Acta agr. slov. **87**: 445-453, 2006.

Wakabayashi, T., Spodnik, J.: Structural changes of mitochondria during free radical-induced apoptosis. - Folia morphol. **59**: 61-75, 2000.

Yan, C., Huang, D., Zhang, Y.: The involvement of ROS overproduction and mitochondrial dysfunction in. PBDE-47-induced apoptosis on Jurkat cells. - Exp. Toxicol. Pathol. **63**: 413-417, 2010.