DOI: 10.1007/s10535-014-0433-6 BIOLOGIA PLANTARUM 58 (3): 551-560, 2014

From birth to death – *Populus trichocarpa* fibrous roots functional anatomy

A. BAGNIEWSKA-ZADWORNA[1]*, A. STELMASIK[1], and J. MINICKA[1,2]

Department of General Botany, Institute of Experimental Biology, Adam Mickiewicz University, Umultowska 89, PL-61614 Poznań, Poland[1]
Department of Virology and Bacteriology, Institute of Plant Protection-National Research Institute, Węgorka 20, PL-60318 Poznań, Poland[2]

Abstract

The main task of annually grown fibrous roots is to supply aboveground organs with water and nutrients. The key for this function, the development and mortality of primary tissues during a root lifespan is still poorly understood, especially in plants grown under field conditions. The goal of this study was to investigate the differentiation and maturation of fibrous roots from the initial appearance until the senescence and death. We monitored the histogenesis and anatomy of first order fibrous roots of black cottonwood (*Populus trichocarpa* Torr. & Gray) sampled at a known age. The daily examination of the first order fibrous roots revealed that only primary tissues were differentiated during the first seven days after their initiation and were maintained during the root lifespan. We observed all stages of exodermis and endodermis formation regulation a fibrous root water and nutrients uptake capacity. A cytological analysis, examined if any cellular symptoms of autophagy could be detected in senescent roots, indicated that vacuolar cell death was involved in root mortality. Our results are the first report strongly suggesting that programmed cell death (PCD) could be engaged in the senescence of ephemeral fibrous roots. The anatomical results advance our understanding of how roots absorptive ability is related to precise timing of tissue development during primary growth and of fibrous root senescence after fulfilment of its tasks.

Additional key words: black cottonwood, programmed cell death, root senescence, transmission electron microscopy .

Introduction

The evolution of a root system is undoubtedly one of the most important adaptations that enable plants to survive in terrestrial environments (Harper *et al.* 1991). This evolutionary success is possible due to the ability of higher plants to develop various types of primary roots, known as heterorhizis (Hishi 2007). Primary roots may be divided into: *1*) short and thin roots (usually up to 1 mm in diameter) known as fibrous, fine or feeder roots; and *2*) long, thick (> 1 - 2 mm of diameter), fast-growing pioneer roots which form the framework of the root system, also known as coarse or framework or skeleton roots (Kolesnikov 1971, Lyford 1980, Sutton and Tinus 1983, Bagniewska-Zadworna *et al.* 2012). Most root systems of trees involve tip-ended, first order fibrous roots (Jagodziński and Kałucka 2011). The main function of fibrous roots is the absorption of water and mineral nutrients from the rhizosphere. The function of a fine root is closely related to its anatomical structure and lifespan (Valenzuela-Estrada *et al*. 2008). During the first days of growth, roots undergo anatomical changes at various distances from the apical meristem that affect their permeability to water and nutrients. Xylem is an important structural element that partly determines uptake and transport of water and ions to the aboveground parts of a plant. Previously, we traced the process of xylogenesis in both fibrous and pioneer roots with

Submitted 23 October 2013, *last revision* 5 March 2014, *accepted* 10 March 2014.
Abbreviations: PCD - programmed cell death; TEM - transmission electron microscopy.
Acknowledgements: This work was partially supported by grant No. NN309007437 from the Polish Ministry of Science and Higher Education and was a preliminary study for the project 2012/07/E/NZ9/00194 from The National Science Centre. We thank Dr. Marc Goebel, the Department of Natural Resources at Cornell University and two anonymous reviewers for discussions and valuable comments on the manuscript, and the Institute of Dendrology, Polish Academy of Sciences, for access to their poplar experimental field site.
* Corresponding author; fax: (+48) 618295611 , e-mail: agabag@amu.edu.pl

regard to their different functions and found that in fibrous roots, functional vessels develop on the third to fourth day following root emergence (Bagniewska-Zadworna *et al.* 2012). Apart from xylem vessels, both the hydrophobic exodermis and the endodermis determine water radial flow after its uptake by finest roots (Steudle and Peterson 1998, Ma and Peterson 2001, Endstone *et al.* 2003, Martinka *et al.* 2012). The exodermis forms from the outside layers of cortex parenchyma and prevents water flow from a root back into soil, protecting it also against microorganisms as well as offering mechanical support (Ma and Peterson 2003). Tissue differentiation in the primary roots is of major importance in terms of functions performed by an individual root. The lack of secondary structure, like secondary xylem or periderm, in fibrous first order roots affects the effectiveness of the whole root water uptake (Eissenstat and Achor 1999, Huang *et al.* 2010, Zadworny and Eissenstat 2011, Bagniewska-Zadworna *et al.* 2012). Majdi *et al*. (2005) suggested that given the complexity and heterogeneity within the root system it would be crucial to define fine roots not only by morphological traits but also by their function. Apart from the root anatomy, root lifespan can also affect the absorption efficiency. General theories on plant tissue lifespan are mainly based on the observations of leaf lifespan, however, there is an uncertainty if these theories can also explain the patterns of fibrous root life cycles. Most fibrous roots, in contrast to the pioneer roots, are as short-lived as leaves (Eissenstat and Yanai 2002), but Pregitzer (2002) rightly commented that one of the most remarkable gaps in our knowledge is that we still do not know what controls the mortality of individual roots. Some data suggest that lifespan of fibrous roots could be shorter in deciduous trees than in evergreen species (Vogt and Bloomfield 1996). In woody plants, most of the fibrous laterals are formed *de novo* every year, growing no more than a few centimeters, with the growth taking place during a few days (Wells and Eissenstat 2001, Eissenstat and Volder 2005, Xia *et al.* 2010). Root lifespan has been a subject of broad interest among scientists for many years. Previous studies indicate that the lifespan of individual roots might depend, besides their diameter, on many environmental factors and also on the root branching order. The later describes the location of a root in the root system, its depth, the growth potential of a plant, and water and nutrients availability (Joslin *et al.* 2000, Wells and Eissenstat 2001, Baddeley and Watson 2005). In poplar, fibrous roots are relatively short-lived (Coleman *et al.* 2000), ranging from 30 to 300 d (Block *et al.* 2006, McCormack *et al*. 2012). In aging fine roots, morphological changes are observed (browning, blackening, and shrinking) and they are linked to fine root death and disappearance (Hendrick and Pregitzer 1993, Comas *et al.* 2000), leaving only "scars" in higher order, "mother" roots (Pregitzer *et al.* 2000). Thus, enhanced root disappearance observed in late summer and autumn (Burke and Raynal 1994, Hendrick and Pregitzer 1996) suggests that the finest roots of first order exhibit annual lifespan (Majdi *et al.* 2001, Wells *et al.* 2002, McCormack *et al*. 2012). In many cases, higher rates of fine root mortality are observed in autumn, sometimes linked with decreasing temperatures (Pregitzer *et al.* 2000, Wells and Eissenstat 2001).

There is still a large gap in our knowledge on what causes fibrous root cell death. It is suggested that fine root death might be passive (Eissenstat and Yanai 1997), with plants reducing their investment in sugar supply and defense compounds leaving roots more vulnerable to pathogens (Eissenstat and Volder 2005). However, there are data suggesting that the active process of programmed cell death (PCD) leads to cell self-destruction, but at the same time, it can also condition the organism growth and survival (Greenberg 1996, Van Doorn and Woltering 2004). Programmed cell death is known especially during the xylogenesis that leads to the degeneration of organelles and finally to complete elimination of the protoplast (O'Brien and Thimann 1967, Srivastava and Singh 1972, Fukuda 2000). PCD is also involved in the development of primary roots of *Cactaceae* during the formation of the primary root system important for rapid seedling establishment in a desert environment (Shishkova and Dubrovsky 2005), and possibly during senescence of the cortex of a fine root (Lascaris and Deacon 1991, Liljeroth and Bryngelsson 2001). A specific example of genetically programmed cell death in plants is leaf senescence (Leopold 1961, Barlow 1982, Pontier *et al*. 1999, Delorme *et al*. 2000, Simeonowa and Mostowska 2002, Gepstein 2004, Kołodziejek *et al.* 2007). During senescence, accumulated carbon and nitrogen compounds are translocated from senesced organs (*e.g*., leaves in autumn) to other parts of the plant (Lam 2004, Rogers 2006, Della Mea *et al.* 2007). Similar processes take place during fibrous roots senescence. However, Nambiar (1987) suggested that in senescent roots of *Pinus radiata*, there is little translocation of nutrients.

We are not aware of a correlation of PCD with seasonal senescence of fibrous roots. Thus, we address the issue of the possible analogy between fibrous root- and leaf-lifespans and hypothesize that PCD is engaged in the senescence of ephemeral fibrous roots. The PCD process in plant cells involves autophagy including cell degradation and recovery (Van Doorn and Woltering 2005, 2010). Autophagy is commonly divided into subcategories: micro-autophagy and macro-autophagy, characterized by an engulfment of smaller or larger portions of cytoplasm, subsequently entering the vacuole and degrading by hydrolases inside vacuole lumen. The third autophagy process, the mega-autophagy or mega-autolysis, is an accumulation of hydrolytic enzymes in enlarged vacuoles leading to the increased permeability of tonoplast, its final disruption, and digestion of the remaining part of the cell (Van Doorn and Woltering

2005). However, according to the latest classification of all autophagy-like processes in plants, it should be called "vacuolar cell death" (Van Doorn *et al.* 2011) or "autolytic PCD" (Van Doorn 2011).

The main purpose of this study was to investigate the anatomical structure of first order fibrous roots during their appearance (initial growth), their differentiation and maturation, as well as their structural symptoms of natural senescence and death in black cottonwood. Moreover, we carried out the detailed cytological analysis of senescent and dying first order fibrous roots in order to check whether autophagy is a form of cell death that plays an important role resulting in root death.

Materials and methods

Plants: All experiments were performed on mature trees of *Populus trichocarpa* (Torr. & Gray) at the experimental field of the Institute of Dendrology, Polish Academy of Sciences in Kórnik (52° 14' 40" N and 17° 06' 27" E) (Bagniewska-Zadworna *et al.* 2012). The daily rate of root growth was examined using root boxes (rhizotrons) with transparent windows enabling observations of individual roots from their early growth without disturbance and allowed continuous sampling fibrous roots of known age. New fibrous root growth visible on the acetate film was traced daily with different colour extra-fine markers. Individual fine root segments (at least 20 from each age category) were cut directly through the acetate film at soil depths ranging from 5 to 30 cm. Each harvested root was immediately cut into segments that corresponded to increments of daily growth and were analysed individually. Fibrous roots were identified in the field based on their morphology (a smaller diameter than pioneer roots). At the end of the growing season, between September and November, fibrous roots of a maximum length of 6 - 10 mm started to turn brown and they were collected for a further analysis. For anatomical and cytological tests, senescent roots were identified and categorized according to the morphological symptoms as described Comas *et al.* (2000) and Block *et al.* (2006). At least 20 individual roots from each pigmentation category: white (functional), browning (senescent), and dark brown (dying) were analysed.

Root anatomical studies: For histological analysis, harvested samples of fibrous roots were fixed immediately in 2 % (v/v) glutaraldehyde and 2 % (v/v) formaldehyde (overnight; pH 6.8; *Polysciences*, Warrington, USA). The samples were rinsed three times with a cacodylate buffer (0.05 M; pH 6.8; *Polysciences*) and dehydrated in a graded ethanol series (10 - 100 %, v/v). The samples were treated with butanol, infiltrated and embedded in *Paraplast Plus* (*Sigma*, St. Louis, USA) and *Technovit 7100* resin (*Heraeus Kulzer*, Wehrheim, Germany). Cross-sections were cut with a *HM 340E* rotary microtome (*Microm*, Walldorf, Germany) at a thickness of 12 µm. Afterwards, the cross-sections were double-stained with 1 % (m/v) Safranin O and 0.1 % (m/v) Fast Green. We evaluated the anatomy of the first order fibrous roots for cell wall lignification/suberization and examined *Paraplast* sections under a light and fluorescence microscope equipped with *Colibri*.2, a LED radiation source giving excitations at 365, 470, 555, and 615 nm (*Axioscope A1*, *Carl Zeiss*, Jena, Germany). For histological observations, senescent and dying roots were embedded in *Technovit* and stained with 0.1 % (m/v) Toluidine blue (pH 4.4).

Root vitality test - FDA staining technique: We tested the viability on the first order fibrous roots with fluorescein diacetate (FDA, *Sigma*). After the harvest, fibrous roots were washed with distilled water and cut into 150 µm thick cross-sections with a *Leica VT1200S* vibratome (*Leica Biosystems*, Nussloch, Germany). Four cross-sections were made from five individual roots. We stained the cross-sections in a glass Petri dish with 0.002 mg cm^{-3} FDA in a 0.1 M phosphate buffer, pH 7.4, at 20 °C for 15 min. Afterwards, the samples were rinsed twice in a 0.1 M phosphate buffer. Only alive cells show a fluorescent response to FDA vital staining (Rotman and Papermaster 1966), therefore, this technique provides detailed information about the physiological status of fibrous root cross section, living cells actively convert the non-fluorescent FDA into a fluorescent compound called fluorescein. Fluorescein appears as light green fluorescence when excited at 470 nm (*Axioscope A1*).

Root cytological studies: Fibrous roots for cytological studies were fixed in 2 % glutaraldehyde and 2 % formaldehyde (pH 6.8) at room temperature for 2 h and at 4 °C overnight. Afterwards, samples were rinsed three times with a cacodylate buffer (0.05 M; pH 6.8, *Polysciences*) and postfixed with 1 % (v/v) osmium tetraoxide at room temperature for 2 h. The fixed material was counterstained and embedded in low viscosity resin using the method described by Zenkteler and Bagniewska-Zadworna (2005). Ultrathin sections (0.1 µm) were cut with a diamond knife on an ultra-microtome *EM UC6* (*Leica-Reichert*, Bensheim, Germany) and collected on *Formvar*-coated copper grids. The sections were stained with uranyl acetate and lead citrate, and examined with a *JEM 1200 EX II* transmission electron microscope (*Jeol*, Tokyo, Japan) operating at an accelerating voltage of 80 kV. For cytological studies, we harvested at least five root segments from each age category and each morphological

type, classified into living (functional), senescent, and dying root. An average of three copper grids per sample was examined under the electron microscope.

Results

Within the first 10 d of rhizotron observations, we found first order fibrous roots with primary growth only. Therefore, only primary development was examined, and anatomical analyses were performed from first to seventh day of root growth to recognize all steps of tissue differentiation.

At the end of the first day of fibrous root growth, all tissues started to differentiate as observed in cross sections. During the first day of fibrous root initiation at the root length of > 1.5 mm, the root cap also started to disappear. On the second day, the rhizodermis consisted of a single layer of small circular cells (Fig. 1*A*). Most of the cross-section area of the first order fibrous roots contained parenchyma cortex cells (Fig. 1*A*) with an average number of cell layers of 8.08 ± 0.58. Cortex parenchyma cells observed in the cross sections were oval or circular, thin-walled, with vacuoles filling almost their entire interior. The endodermis, the innermost layer of the cortex, marked the boundary of the fibrous roots stele and consisted of one to two layers of regularly arranged and tightly adherent cells (Fig 1*B,D*). The endodermis in first order roots started to differentiate as layer encircled stele visible at the distance of 400 μm from the root tip on the first day of root growth. However, initially, cell walls in this layer were thin and did not show autofluorescence (Fig. 1*B,D*).

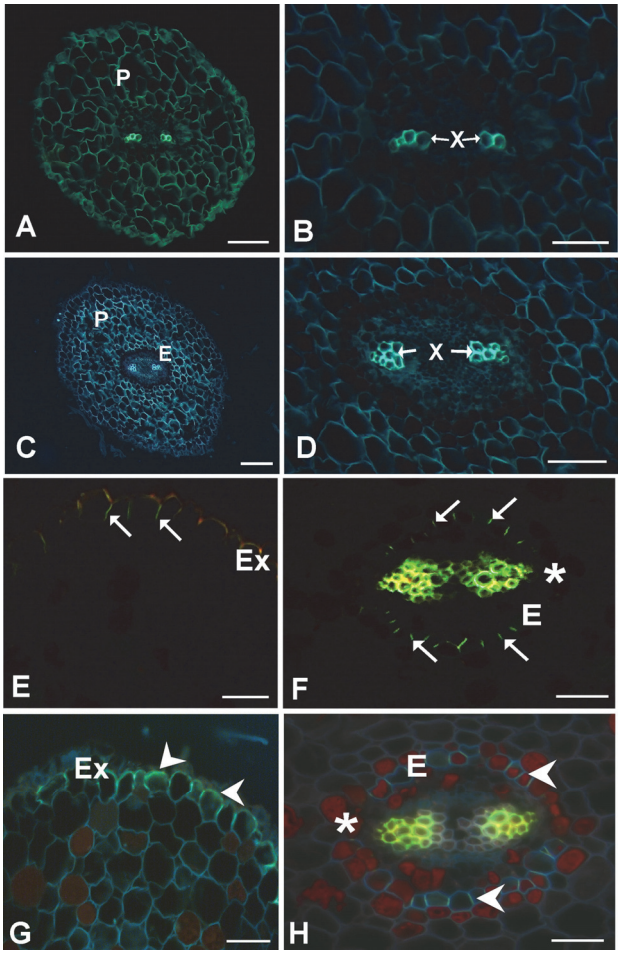

Fig. 1. Comparative anatomy of primary tissues differentiation of *Populus trichocarpa* fibrous roots viewed byfluorescence (UV) microscopy. *A,B* - the second day 5 mm from the tip; *C,D* - the third and fourth days 7 - 8 mm from the tip; *E,F* and *G,H* - the fifth and seventh days 9 - 10 mm from the tip. Fluorescence of exodermis and endodermis, Casparian bands (*E,F, arrows*), and suberin lamellae (*G,H*, *arrowheads*) are seen. Passage cells are indicated by *stars*; P - cortex parenchyma cells, E - endodermis, Ex - exodermis, X - xylem. *Scale bars* in *A,C* = 100 μm, in *B,D-H* = 50 μm.

On the second day of growth, the most recognizable was the lignification of first tracheary elements formed inside the stele at a height above 5 mm from the root tip (Fig. 1*A,B*). The fibrous roots were diarch showing only two primary xylem poles. At this stage, each xylem pole consisted of two or three tracheary elements with lignified walls easily recognizable under the fluorescent microscope due to lignin autofluorescence (Fig. 1*B*). In addition to the two xylem poles, two groups of phloem cells were detected.

From third to seventh days of fibrous root growth, we observed the expansion of xylem poles (Fig. 1*C-H*). The rhizodermis was gradually replaced by cells from the outermost layer of the cortex with thicker cell walls incrusted with lignin and adcrusted with suberin that formed the exodermis (Fig. 1*E,G*), detected with different filter combinations. Both the exodermis and endodermis of stage I with Casparian bands, visible under the fluorescent microscope on their anticlinal walls, were observed at the fifth day of root growth at a height > 9 mm from the root tip (Fig. 1*E,F*). Initially developed Casparian strips in both exodermis and endodermis were strongly lignified showing the same fluorescent pattern as the xylem vessels (Fig. 1*E,F*). At this stage, we observed numedrous root hairs that increased the absorbing surface of fibrous roots. Subsequently, in seven-day-old first order fibrous roots, the exodermis and endodermis of stage II with suberin lamellae were detected. Ten millimeters behind the root tip we observed a strong suberin deposition in the exodermis and endodermis (Fig. 1*G,H*) with three to five passage cells close to the protoxylem (Fig. 1*H*). The endodermic cells were heavily filled with tannins (visible after safranin staining even under UV), located on the outskirts of the vacuole (Fig. 1*H*). We usually observed one to two layers of thin-

walled pericycle cells next to endodermal cells.

We identified senescent fibrous roots to study the anatomical symptoms of death based on morphological symptoms as root shrinkage and with brown or black pigmentation. The roots morphologically classified as functional showed proper anatomic structure (Fig. 2*A*). Functional roots with a perfectly cylindrical shape were significantly differed from those displaying visible symptoms of senescence, *i.e.*, shrinking. The analysis of the roots classified as senescent revealed obvious changes in their structure (Fig. 2*B-F*). Most distinct signs of senescence were in the cortex parenchyma cells, whose cell walls were folded (Fig. 2*B,C*). Along with the changes in the cortex, pericycle cells in several roots were also shrunk and interrupted (Fig. 2*C*). Discovered alterations involved the whole cortex or were only partially visible on one side of cross-sections of fibrous roots. In some cases, the cell walls of fibrous roots disappeared forming large intercellular spaces (Fig. 2*D*). At a later phase in roots morphologically classified as dying, individual cortex parenchyma cells were unrecognizable being fully contracted (Fig. 2*E,F*). At this stage, cells inside the stele also underwent several alterations in the vascular tissue structure, such as cell shrinkage and collapse (Fig. 2*C,F*). In some cases, the cortex was completely separated from the stele (Fig. 2*E*).

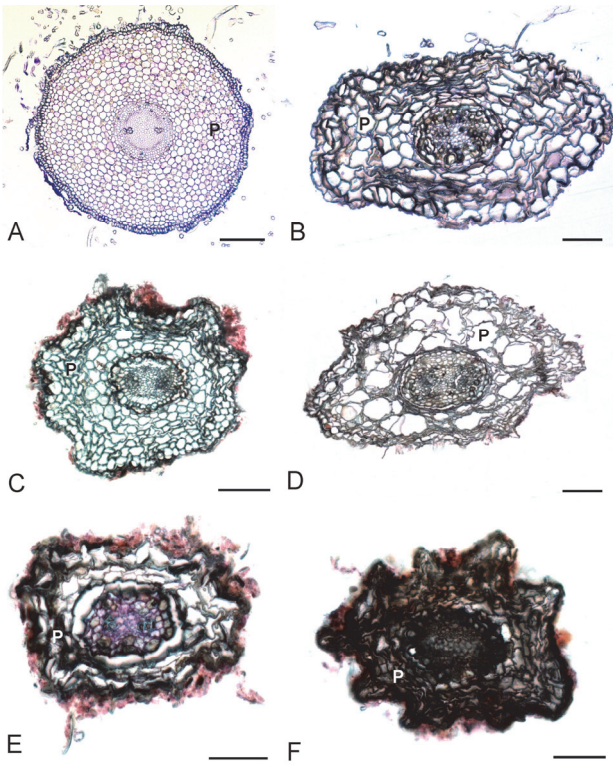

Fig. 2. The structure of functional (*A*), senescent (*B-D*) and dying (*E,F*) fibrous roots of *Populus trichocarpa*. Note changes within cortex parenchyma cells causing cell wall folding, disappearance, and cell collapsing. P - parenchyma cells. *Scale bars* = 100 μm.

Vascular cambium, secondary vascular tissues, or periderm were undistinguishable (Fig. 2*B-F*).

Based on the morphological parameters, roots were classified as functional (white or yellow), senescent (light brown), and dying (dark brown to black), and tested separately for cytological changes. We confirmed the different stages of viability mentioned above with FDA and observed significantly reduced FDA fluorescence in senescent light brown roots (Fig. 3*C,D*), than in functional roots (Fig. 3*A,B*). Dead parenchyma cells did not exhibit any fluorescence and roots that exhibited dark brown or black tissue pigmentation showed no fluorescence at all.

Based on our anatomical results, we focused on the root cytological observation of cortex parenchyma cells, as they were subjected to the most structural changes during root senescence. In addition, we also observed xylem cells to verify if final symptoms of senescence and death were also present in the stele.

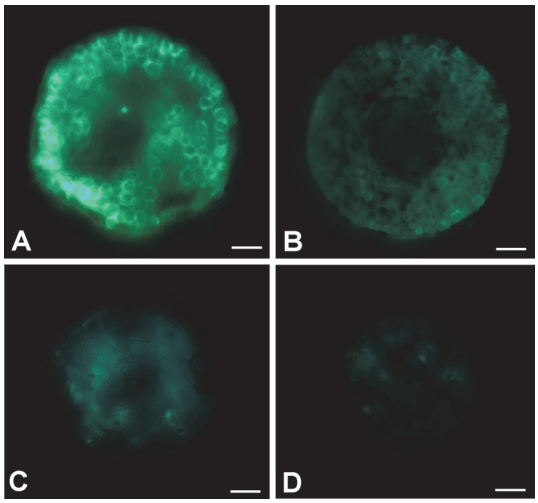

Fig. 3. The results of the FDA viability test performed on functional (*A-B*) and senescent (*C-D*) fibrous roots of *P. trichocarpa*. Only functional root cells were able to cleave FDA to form fluorescein which displayed light green fluorescence when excited at a wavelength of 470 nm. The fluorescence gradually disappeared in senescent roots and was undetectable in dying roots. *Scale bars* = 50 μm.

Parenchyma cells of functional roots were circular and thin-walled (Fig. 4*A*). The major visible compartment of these cells was a single empty-looking vacuole filling almost the entire cell, or vacuole filled with tannins, usually located next to the tonoplast (Fig. 4*A*). The cytoplasm and other organelles were also located along cell walls forming a relatively narrow strip. Large intercellular spaces were observed between cells (Fig. 4*A*).

The ultrastructure of senescent and dying roots differed significantly from that of the functional roots. Senescent light brown roots showed only small changes in ultrastructure. Vacuoles were usually electron lucent

(Fig. 4*B-E*), nuclei and other organelles did not exhibit any structural changes. Cisternae of rough endoplasmic reticulum were well distributed in parenchyma cells (Fig. 4*B*). In the cytoplasm, several vacuole-like vesicles were observed (Fig. 4*B*). In many cells, portions of cytoplasm, including small organelles, were sequestered forming autophagosomes, with double membrane derived from endoplasmic reticulum (Fig. 4 *B*). In the cortex parenchyma cells of light brown roots, tonoplast invagination, with a cytoplasmic content, into a vacuole, and vacuole-like vesicles fusion with central vacuole were observed (Fig. 4*C-E*), however, no changes in nuclei, mitochondria, and other organelle structure were identified (Fig. 4*E*). At the next stage when cortex parenchyma cells derived from dark brown roots, organelles became unrecognizable, whereas double-membrane autophagic structures were noticeable in the close vicinity of vacuoles. Moreover, we frequently detected vacuoles that contained several autophagic-like bodies with cytoplasm derived structures (Fig. 4*F,G*). When autophagic bodies merged with a vacuole, they disappeared and their content was unrecognizable (Fig. 4*G*).

The analyses of dark brown to black roots confirmed the internal anatomical observation of folded and creased cell walls (Figs. 4*H-K*). At this final stage of root life, tonoplasts collapsed and complete disintegration of other organelles were observable (Fig. 4*H*). From that time, cell death was rapid and restricted to cytoplasm acidification. Indications of numerous microorganisms penetrating the dead roots (Fig. 4*I*), *e.g.*, fungi and bacteria, which was noticeable in dead cells (Fig. 4*J,K*). Moreover, micro-organisms appeared also in the area of the root stele, and were present inside xylem vessels too (Fig. 4*L,M*).

Discussion

The main function of fibrous roots is the absorption of water and nutrients from soil as reflected in their anatomical structure. During the initial growth of fibrous roots, the development of specific tissues in the first days of root life is still poorly understood, especially when grown under field conditions. Recently, Bagniewska-Zadworna *et al.* (2012) examined daily growth of fibrous and pioneer roots observing the formation of their tracheary elements. Here, we focused on the structure of *Populus trichocarpa* fibrous root tissue that determines its adaptability to maximum water and nutrients uptake, as well as preventing the loss of water back into soil. Intensive water uptake by fibrous roots occurs at the root hair zone with root hairs responsible for an additional root-soil contact and water and nutrients acquisitions (Gregory 2006). Thus, below the root hair zone closer to the root tip, fibrous roots can be ineffective in water uptake. In black cottonwood, fibrous roots mostly differentiate root hairs 7 - 8 mm from the root tip which corresponds to the third and fourth day after root emergence and the differentiation of functional tracheary elements (Bagniewska-Zadworna *et al.* 2012). It seems that 4-d-old roots are prepared for absorption, however, on the fifth day of root growth, hydrophobic tissues like the exodermis and endodermis with Casparian bands develop. These two types of cell layers are formed to regulate radial water movement in roots (Endstone *et al.* 2003, Ma and Peterson 2003) providing parallel symplastic, apoplastic, and transcellular water passages across different tissues (Steudle and Peterson 1998, Martinka *et al*. 2012).

The number of xylem vessels in the conductive tissue influences the transport of water and nutrients. We observed only a few functional tracheary elements per xylem pole developing on the third to fourth day after root initiation. This observation indicates that an additional regulation of water movement was not as necessary as later on, when the hydraulic flow in functional xylem cannot be interrupted by water and ions outflow. The function of endodermis is also important during drought events and other environmental stresses. In such situations, the endodermis and exodermis develop closer to root tips (Endstone *et al.* 2003). Furthermore, the exodermis with thicker cell walls than the rhizodermis provides an additional mechanical support and isolation of living parenchyma cortex cells from soil particles and pathogens. However, the exodermis and endodermis should be considered mainly as key regulators controlling water and nutrients radial movement in roots.

The anatomical analysis of primary fibrous roots also revealed that all tissues are differentiated during the first seven days after their initiation. Given that fibrous roots grow in a relative short time and usually stop growing within a few days, they are never longer than a few centimeters (Eissenstat and Volder 2005) and this length usually remains for the rest of their lifespan.

Fibrous roots are also relatively short lived and retain their original function of water and nutrients uptake throughout their lifespan (Eissenstat and Yanai 2002, Eissenstat and Volder 2005). Still, our knowledge on relationships among root age, anatomical structure, and mortality is limited. In our study, we investigated whether the mortality of first order fibrous roots under the field conditions was a passive or active process, and whether mortality was genetically programmed through the activation of autophagy. The analysis of fibrous roots classified as senescent revealed many alterations in the structure of cortex parenchyma cells. We observed that cell walls were folded and sometimes hollow, and especially in dying roots, parenchyma cell walls were usually completely creased and crushed. Others observed that in some lateral roots, the cortex dies sooner than the

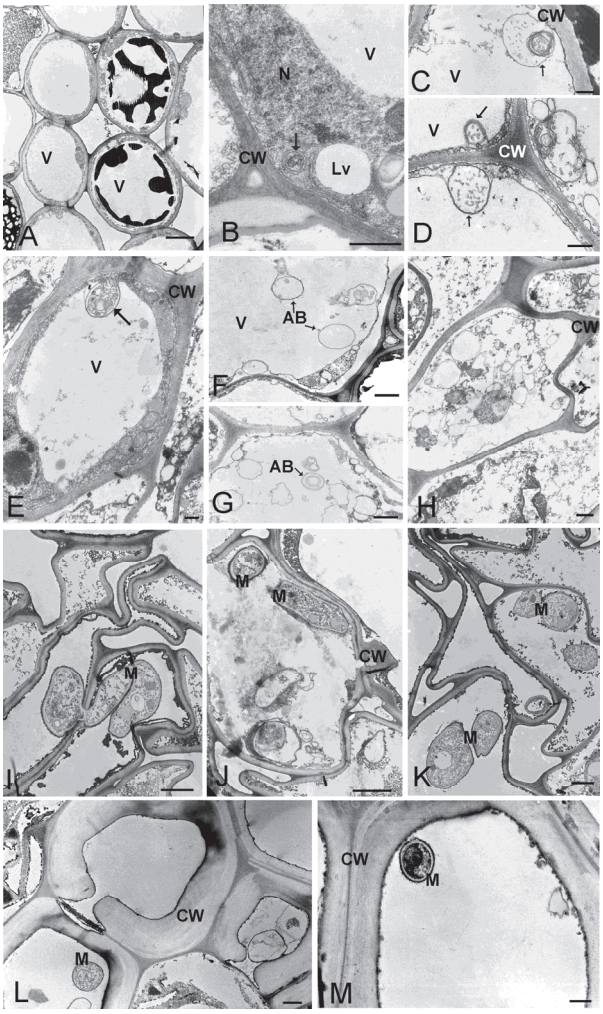

Fig. 4. Different stages of programmed cell death during senescence of fibrous roots of *P. trichocarpa*. Control cortex parenchyma cells (*A*) and all stages of fibrous root senescence in cortex parenchyma (*B-K*) and xylem vessels (*L,M*) of dying roots. Note preautophagic structures – large portions of cytoplasm with a double membrane derived from endoplasmic reticulum and vacuole-like vesicles (*B*), tonoplast invagination into a vacuole and formation of autophagic bodies with a cytoplasmic content (*C-E*, *arrows*), autophagic-like bodies with cytoplasmic derived structures inside a vacuole (*F,G)* in senescent roots and parenchymatic cell walls folded and creased *(H-K*), complete disintegration of remaining organelles (*I-K*) and microorganisms appearance inside parenchyma cells of dying roots (*I-K*) as well as inside xylem vessels (*L,M*). CW - cell wall, V - vacuole, Lv - vacuole-like vesicles, AB - autophagic-like bodies, N - nucleus, M - microorganisms (fungi and/or bacteria) inside cells. *Scale bars* in *A, I-K* = 2 μm, in *B-H, N,M* = 500 nm.

entire root (Eissenstat and Volder 2005), whereas a root of an annual plant growing in a pot might retain a functional pericycle to produce new laterals (Spaeth and Cortes 1995). However, Brundrett and Kendrick (1988) and Eissenstat and Achor (1999) observed that the cortex of citrus or sugar maple roots remains functional during root lifespan. A root water uptake capacity is higher in primary roots, thus the lack of secondary development does not reduce efficient water and nutrients movement through roots (Peterson *et al*. 1999).

Apparently, when the cortex tissue is sloughed by the expansion of the stele, the vascular cylinder undergoes secondary development (Soukup *et al*. 2004, Gregory 2006). However, we only observed primary structures in the first order fibrous roots even with pigmentation similarly as we observed them previously for functional roots in this species (Bagniewska-Zadworna *et al.* 2012), with no development of vascular cambium or phellogen as secondary meristems. Thus, first signs of death in cortex cells of fibrous roots of *P. trichocarpa* can be identified as first symptoms of the death of the entire first order root.

The detailed cytological analysis was performed to find out if any cellular symptoms of autophagy could be detected in cells during roots senescence. A common assumption is that various types of autophagy may simultaneously occur in the cell (Van Doorn and Woltering 2005, Bagniewska-Zadworna *et al.* 2010, 2012). Here, we identified all symptoms of vacuolar cell death including mega-autophagy with collapsed lytic vacuoles along with the release of hydrolases during the final stage of root senescence. In senescent fibrous roots, the first visible symptom was the formation of vacuole-like vesicles and autophagy-like structures through sequestration of large portions of cytoplasm including small organelles within the cytoplasm. These structures are involved in the initial cell degradation (Van Doorn 2011). Moreover, in cortex parenchyma cells, as observed in browning roots, tonoplast invagination, with a cytoplasmic content, into the large central vacuole was frequently noticed, and autophagic-like bodies were present inside vacuoles. These observations suggest that a lytic vacuole (Wink 1993) and PCD are involved in root senescence, similarly as PCD is responsible for seasonal leaf senescence (Gepstein 2004). Thus, mortality occurred in darkened roots and could be linked with leaf senescence as in other ephemeral organs (Hendrick and Pregitzer 1993).

The phenomenon of autophagy as gradual process is described in details during wood fiber development (Curtois-Moreau *et al.* 2009) and hybrid embryo abortion (Bagniewska-Zadworna *et al.* 2010). PCD processes are also found in roots: during xylogenesis in *P. trichocarpa* roots (Bagniewska-Zadworna *et al.* 2012) and during the formation of aerenchyma in root cortex cells (He *et al.* 1996, Lenochová *et al*. 2009). Considering the previous and current findings, we document that when fibrous roots lose the ability to absorb water and nutrients at the end of the vegetation season, root mortality is inevitable. This is closely related to the beginning of active PCD, but perhaps once saccharides and defense compounds are extremely low in roots, the result is mega-autophagy with vacuole rupture and hydrolytic enzymes. Additionally,

limiting soil resources (both water and nutrients) as well as other external signals from soil can influence the rate of fine root turnover (Forde 2002, Green *et al.* 2005). Eissenstat and Volder (2005) suggested that once a root is useless, it should be left as prey for herbivores and pathogens. This fact was actually confirmed by our observations in cortex parenchyma cells of dying roots. At the last stage of senescence in dark brown roots, root cells were infected with numerous pathogenic microorganisms and soon after the colonization by microorganisms, which we also noticed inside xylem vessels, root tissue started to break down.

In summary, our findings show, how first order fibrous roots changed in their anatomy and cytology during their primary development, although maintaining their water and nutrients uptake. Variations in the root morphology were not only the result of root growth, development, and foraging strategies, but also of root senescence and mortality. In addition, our results indicate that PCD was a crucial process for both plant growth and development as well as for death of selected, usually ephemeral organs. On the one hand, this strategy enables plants to maintain cell homeostasis and controlled development, reproduction, and senescence. On the other hand, it allows plants to adjust to environmental conditions, as limiting soil resources (both water and nutrients) and other external signals from soil can influence the rate of fine root turnover (Forde 2002, Green *et al.* 2005). We hope that this new understanding of root development and growth patterns in black cottonwood is of considerable interest to foresters as well as scientists estimating the rates of fine root production and belowground carbon turnover in forests dominated by the temperate tree species.

References

Baddeley, J.A., Watson, C.A.: Influences of root diameter, tree age, soil depth and season on fine root survivorship in *Prunus avium*. - Plant Soil **76**: 15-22, 2005.

Bagniewska-Zadworna, A., Byczyk, J., Eissenstat, D.M., Oleksyn, J., Zadworny, M.: Avoiding transport bottlenecks in an expanding root system: xylem vessel development in fibrous and pioneer roots under field conditions. - Amer. J. Bot. **99**: 1-10, 2012.

Bagniewska-Zadworna, A., Wojciechowicz, M.K., Zenkteler, M., Jeżowski, A., Zenkteler, E.: Cytological analysis of hybrid embryos of intergeneric crosses between *Salix viminalis* and *Populus* species. - Austr. J. Bot. **58**: 42–48, 2010.

Barlow, P.W.: Cell death - an integral plant of plant development. - In: Jackson, MB, Grout, B, Mackenzie, IA. (ed.): Growth Regulators in Plant Senescence. Pp. 27-45. British Plant Growth Regulator Group, Wantage 1982.

Block, R.M.A., Rees, K.C.J., Knight, J.D.: A review of fine root dynamics in *Populus* plantations. - Agroforestr. Syst. **67**: 73-84, 2006.

Brundrett, M.C., Kendrick, B.: The myccorhizal status, root anatomy, and phenology of plants in a sugar maple forest. - Can. J. Bot. **66**: 1153-1173, 1988.

Burke M.K., Raynal D.J.: Fine-root growth phenology, production, and turnover in a northern hardwood forest ecosystem. - Plant Soil **162**: 135-146, 1994.

Coleman, M.D., Dickson, R.E., Isebrands, J.G.: Contrasting fine-root production, survival and soil CO_2 efflux in pine and poplar plantations. - Plant Soil **225**: 129-139, 2000.

Comas, L.H., Eissenstat, D.M., Lakso, A.N.: Assessing root death and root system dynamics in a study of grape canopy pruning. - New Phytol. **147**: 171-178, 2000.

Courtois-Moreau, C., Pesquet, E., Sjödin, A., Muñiz, L., Bollhöner, B., Kaneda, M., Samuels, L., Jansson, S., Tuominen, H.: A unique programme for cell death in xylem fibers of *Populus* stem. - Plant J. **58**: 260-274, 2009.

Delorme, V.G.R., McCabe, P.F., Kim, D.J., Leaver, C.J.: A matrix metalloproteinase gene is expressed at the boundary of senescence and programmed cell death in cucumber. - Plant Physiol. **123**: 917-928, 2000.

Della Mea, M., De Filippis, F., Genovesi, V., Fracassini, D.S., Del Duca, S.: The acropetal wave of developmental cell death of tobacco corolla is preceded by activation of transglutaminase in different cell compartments. - Plant Physiol. **144**: 1211-1222, 2007.

Eissenstat, D.M, Achor, D.S.: Anatomical characteristics of roots of citrus rootstocks that vary in specific root length. - New Phytol. **141**: 309-321, 1999.

Eissenstat, D.M, Volder, A.: The efficiency of nutrient acquisition over the life of a root. - In: Bassiri Rad, H. (ed.): Nutrient Acquisition by Plants: an Ecological Perspective. Pp. 185-220. Springer-Verlag, Berlin - New York 2005.

Eissenstat, D.M, Yanai, R.D.: The ecology of root life span.- Adv. Ecol. Res. **27**: 1-62, 1997.

Eissenstat, D.M, Yanai, R.D.: Root life span, efficiency and turnover. - In: Waisel, Y., Eshel, A., Kafkafi, U. (ed.): Plant Roots: the Hidden Half. Pp. 221-238. Marcel Dekker, New York 2002.

Endstone, D.E., Peterson, C.A., Ma, F.: Root endodermis and exodermis: structure, function and responses to the environment. - J. Plant Growth Regul. **21**: 335-351, 2003.

Forde, B.G.: Local and long-range signaling pathways regulating plant responses to nitrate. - Annu. Rev. Plant Biol. **53**: 203-224, 2002.

Fukuda, H.: Programmed cell death of tracheary elements as a paradigm in plants. - Plant mol. Biol. **44:** 245-253, 2000.

Gepstein, S.: Leaf senescence - not just a 'wear and tear' phenomenon. - Genome Biol. **5**: 212, 2004.

Green, J.J., Dawson, L.A., Proctor, J., Duff, E.I., Elston, D.A.: Fine root dynamics in a tropical rain forest is influenced by rainfall. - Plant Soil **276**: 23-32, 2005.

Greenberg, J.T.: Programmed cell death: a way of life for plants. - Proc. nat. Acad. Sci. USA **93**: 12094-12097, 1996.

Gregory, P.: Roots and the architecture of root systems. - In: Gregory, P. (ed.): Plant Roots. Growth, Activity and Interaction with Soils. Pp. 18-44. Blackwell, London 2006.

Harper, J.L., Jones, M., Sackville Hamilton, N.R.: The evolution of roots and the problems of analyzing their behaviour. In: Atkinson, D. (ed.): Plant Root Growth, an Ecological Perspective. Pp. 3-22. Blackwell, London 1991.

He, C.J., Morgan, P.W., Drew, M.C.: Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. - Plant Physiol. **112**: 463-472, 1996.

Hendrick, R.L., Pregitzer, K.S.: The dynamics of fine root length, biomass, and nitrogen content in two northern hardwood ecosystems. - Can. J. Forest. Res. **23**: 2507-2520, 1993.

Hendrick, R.L., Pregitzer, K.S.: Temporal and depth-related patterns of fine root dynamics in northern hardwood forests. - J. Ecol. **84**: 167-176, 1996.

Hishi, T.: Heterogeneity of individual roots within the fine root architecture: causal links between physiological and ecosystem functions. - J. Forest. Res. **12**: 126-133, 2007.

Huang, G., Zhao, X., Zhao, H., Huang, Y., Zuo, X.: Linking root morphology, longevity and function to root branch order: a case study in three shrubs. - Plant Soil **336**: 197-208, 2010.

Jagodziński A.M., Kałucka I. Fine root biomass and morphology in an age-sequence of post-agricultural *Pinus sylvestris* L. stands. - Dendrobiology **66**: 71-84, 2011.

Joslin, J.D., Wolfe, M.H., Hanson, P.J. Effects of altered water regimes on forest root systems. - New Phytol. **147**: 117-129, 2000.

Kolesnikov, V.A.: The Root System of Fruit Plants. - MIR, Moskva 1971.

Kołodziejek, I., Kozioł-Lipińska, J., Waleza, M., Korczyński, J., Mostowska, A.: Aspects of programmed cell death during early senescence of barley leaves: possible role of nitric oxide. - Protoplasma **232**: 97-108, 2007.

Lam, E.: Controlled cell death, plant survival and development. - Nat. Rev. mol. cell. Biol. **5**: 305-315, 2004.

Lascaris, D., Deacon, J.W.: Comparison of methods to assess senescence of the cortex of wheat and tomato roots. - Soil Biol. Biochem. **23**: 979-986, 1991.

Lenochová, Z., Soukup, A., Votrubová, O.: Aerenchyma formation in maize roots. - Biol. Plant. **53**: 263-270, 2009.

Leopold, A.C.: Senescence in plant development – death of plants or plant parts may be of positive ecological or physiological value. - Science **134**: 1727-1732, 1961.

Liljeroth E., Bryngelsson, T.: DNA fragmentation in cereal roots indicative of programmed root cortical cell death. - Physiol. Plant. **111**: 365-372, 2001.

Lyford, W.H.: Development of the root system of northern red oak *(Quercus rubra* L.*)*. - Harvard Forest Paper **21**: 31, 1980.

Ma, F., Peterson, C.A.: Development of cell wall modifications in the endodermis and exodermis of *Allium cepa* roots. - Can. J. Bot. **79**: 621-634, 2001.

Ma, F., Peterson, C.A.: Current insight into the development, structure and chemistry of the endodermis and exodermis of roots. - Can. J. Bot. **81**: 405-421, 2003.

Majdi, H., Damm, E., Nylund, J.E.: Longevity of myccorhizal roots depends on branching order and nutrient availability. - New Phytol. **150**: 195-202, 2001.

Majdi, H., Pregitzer, K., Moren, A.S., Nylund, J.E., Agren, G.I.: Measuring fine root turnover in forest ecosystems. - Plant Soil **276**: 1-8, 2005.

Martinka, M., Dolan, L., Pernas, M., Abe, J., Lux A.: Endodermal cell-cell contact is required for the spatial control of Casparian band development in *Arabidopsis thaliana*. - Ann. Bot. **110**: 361-371, 2012.

McCormack, M.L., Adams, T.S., Smithwick, E.A.H., Eissenstat, D.M.: Predicting fine root lifespan from plant functional traits in temperate trees. - New Phytol. **195**: 823-831, 2012.

Nambiar, E.K.S.: Do nutrients retranslocate from fine roots? - Can. J. Forest. Res. **17**: 913-918, 1987.

O'Brien, T.P., Thimann, K.V.: Observation on the fine structure of the oat coleoptile III. Correlated light and electron microscopy of the vascular tissues. - Protoplasma **63**: 443-478, 1967.

Peterson, C.A., Enstone D.E., Taylor J.H.: Pine root structure and its potential significance for root function. - Plant Soil **217**: 205-213, 1999.

Pontier, D., Gan, S., Amasino, R.M., Roby, D., Lam, E.: Markers for hypersensitive response and senescence show distinct patterns of expression. - Plant mol. Biol. **39**: 1243-1255, 1999.

Pregitzer, K.S.: Fine roots of trees – a new perspective. - New Phytol. **154**: 267-270, 2002.

Pregitzer, K.S., King, J.S., Burton, A.J., Brown, S.E.: Responses of tree fine roots to temperature. - New Phytol. **147**: 105-115, 2000.

Rogers, H.J.: Programmed cell death in floral organs: how and why do flowers die? - Ann. Bot. **97**: 309-315, 2006.

Rotman, B., Papermaster, B.W.: Membrane properties of living mammalian cells as studied by enzymic hydrolysis of fluorogenic esters. - Proc. nat. Acad. Sci. USA **55**: 134-141, 1966.

Shishkova, S., Dubrovsky, J.G.: Developmental programmed cell death in primary roots of Sonoran desert *Cactaceae*. - Amer. J. Bot. **92**: 1590-1594, 2005.

Simeonowa E., Mostowska A.: [Biochemical and molecular aspects of leaf senescence] - Post. Biol. Kom. **28**: 17-32, 2002. [In Polish]

Soukup, A., Malá, J., Hrubcová, M., Kálal, J., Votrubová, O., Cvikrová M.: Differences in anatomical structure and lignin content of roots of pedunculate oak and wild cherry-tree plantlets during acclimation. - Biol. Plant. **48**: 481-489, 2004.

Spaeth, S.C., Cortes, P.H.: Root cortex death and subsequent initiation and growth of lateral roots from bare steles of chickpeas. - Can. J. Bot. **73**: 253-261, 1995.

Srivastava, L.M., Singh, A.P.: Certain aspects of xylem differentiation in corn. - Can. J. Bot. **50**: 1795-1804, 1972.

Steudle, E., Peterson, C.A.: How does water get through roots? - J. exp. Bot. **49**: 775-788, 1998.

Sutton, R.F., Tinus, R.W.: Root and root system terminology. - Forest Sci**. 29**: 1-138, 1983.

Valenzuela-Estrada, L.R., Vera-Caraballo, V., Ruth, L.E, Eissenstat D.M.: Root anatomy, morphology, and longevity among root orders in *Vaccinium corymbosum* (Ericaceae). - Amer. J. Bot. **95**: 1506-1514, 2008.

Van Doorn, W.G.: Classes of programmed cell death in plants, compared to those in animals. - J. exp. Bot. **62**: 4749-4761, 2011.

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 Differen. **18**: 1241-1246, 2011.

Van Doorn, W.G., Woltering, E.J.: Senescence and programmed cell death: substance or semantics? - J. exp.

Bot. **55**: 2147-2153, 2004.
Van Doorn, W.G., Woltering, E.J.: Many ways to exit? Cell death categories in plants. - Trends Plant Sci. **10**: 117-122, 2005.
Van Doorn, W.G., Woltering, E.J.: What about the role of autophagy in PCD? - Trends Plant Sci. **15**: 361-362, 2010.
Vogt, K.A., Bloomfield, J.: Tree root turnover and senescence. - In: Waisel, Y., Eshel, A., Kafkafi, U. (ed.): Plant Roots: the Hidden Half. 2nd Ed. Pp. 287-306. Marcel Decker, New York 1996.
Wells, C.E., Glenn, D.M., Eissenstat, D.M.: Changes in the risk of fine root mortality with age: a case study in peach, *Prunus persica* (Rosaceae). - Amer. J. Bot. **89**: 79-87, 2002.
Wells, C.E., Eissenstat, D.M.: Marked differences in survivorship among apple roots of different diameters. - Ecology **82**: 882-892, 2001.
Wink, M.: The plant vacuole - a multifunctional compartment. - J. exp. Bot. **44**: 231-246, 1993.
Xia, M., Pregitzer, K.S., Guo, D.: Ephemeral root modules in *Fraxinus mandshurica*. - New Phytol. **188**: 1065-1074, 2010.
Zadworny, M., Eissenstat, D.M.: Contrasting the morphology, anatomy and fungal colonization of new pioneer and fibrous roots. - New Phytol. **190**: 213-221, 2011.
Zenkteler, E., Bagniewska-Zadworna, A.: Ultrastructural changes in rhizome parenchyma of *Polypodium vulgare* during dehydration with or without abscisic acid pretreatment. - Biol. Plant. **49**: 209-214, 2005.