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ALTERATIONS IN WOOD AND BARK STRUCTURE OF APPLE TREE (*MALUS DOMESTICA*) CAUSED BY *NEONECTRIA DITISSIMA* FUNGUS

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ABSTRACT

The occurrence of fungi *Nectria* spp. affects the quality of forest stands and fruit trees significantly. The aim of the paper is to describe the changes in the anatomy of the bark and wood tissues of Malus domestica attacked by the fungus *Neonectria ditissima*. Significant structural alterations in the bark and wood tissues at the points of the perithecia occurrence were found. Significant and visible thickening (burl) on the branch at the point of injury was due to the excessive formation of lateral callus and wound after injury. The callus anatomy formed after the injury showed closed tumour features with a marked disorganization of the wood elements influenced by *Neonectria ditissima* infection spread in bark and cambium.

Key words: *Neonectria ditissima, Malus domestica,* Wood anatomy, Bark anatomy, Perithecia, Tumorous tissues, Light microscopy.

INTRODUCTION

The fungi of *Nectria* (Fr.) Fr. genus come from the family of Bionectriaceae and Nectriaceae and belong to order Hypocreales (ROSSMAN *et al.* 1999, MIHÁL and BLANÁR 2011). Several *Nectria* spp. are important vascular parasites of forest tree species, causing great damage, sometimes also epiphytoses. Epiphytosis was reported as an example of necrotic disease of beech bark in Slovakia (CICÁK and MIHÁL 2002). The first visible symptom of necrotic disease is the loss of tree crown density as a result of breakage the thin branches and their drying out. The most common feature of the disease is the formation of necrotic wounds on branches and trunks, from small and inconspicuous necroses, up to large, strongly deforming the trunks and branches, so-called 'breakage necroses'. Branches and trunk are often fractured at the point of necrosis, where strength of the wood is considerably weakened. The entry points of infection these parasites into vascular systems of the trees are mainly injury of bark by insect and wild animal, bacterial infections, bark injuries during felling and logging, damages caused by rockfall, and frost and sunburn cracks. However, infection can penetrate into the bark also through the undamaged areas, such as leaf scars or lenticels. The fungi of *Nectria* genus penetrate into wounded or undamaged bark tissues by means of the ascospores, conidia and hyphae of mycelia. Significant role in parasite propagation have abiotic (wind, water) and biotic factors (insects, birds, wild animal, humans), whereby sensitivity the of trees to infection is higher in autumn and winter (ZÚBRIK *et al.* 2008).

N. galligena is a signifficant fungi pathogen of fruit trees, especially of apple trees. This fungus induces a cancer, attacking only thin branches, damaged by mechanically, frost or insects (ZACHA *et al.* 1989). Local swelling of branches, so-called 'burl' are formed frequently. The trees attacked by mentioned disease consume a considerable amount of reserve nutrition substances during tumour formation, lacking later during normal growth. Subsequently, it also affects the quality of apples and reduce its production (ZACHA *et al.* 1989). The occurrence of *N. galligena* fungus has been found on the cancerous branches of some old apple trees (usually over 100 years old) in Revúca, where worms (*Cryptococcus* spp.) have also been detected. Therefore, it is possible to assume that the occurrence of worms is probably related to the infection of the branches by the spores of the mentioned fungus. Similar saproparasite *N. coccinea* is also a frequent cause of necrotic disease the tracheomycotic type. In addition to beech, this species of fungus was found in Slovakia only on *M. domestica* trees (MIHÁL 2002a, b).

So far, the species *N. ditissima* has been found in Slovakia in four orographic units and on four localities, where damages have occurred on old individuals only (ŠKUBLA 2003).

The aim of the paper was to describe the alterations of bark and wood anatomy of three-year old branch of *M. domestica* attacked by the fungus *N. ditissima*.

MATERIAL AND METHODS

Sample material

From crown base of the 15 m high *M. domestica* tree, age of 100 years growing in the locality of Revúcka vrchovina highlands (altitude of 330 m a. s. l.), one 3-year-old branch parts with maximal thickness of 2.5 cm and the length of 10 cm was taken. The burl on the branch was 5 cm long with the abundant perithecia occurrence (Fig. 1A). According to the morphological features of perithecia, the *N. ditissima* fungus has been identified on the burl. At the centre of the burl part, and simultaneously, at the same point of occurrence the perithecia was cut off a 1 cm thick the cross-section disc. A reference cross-section disc with the same thickness was also cut off from the uninfected part of the branch (Fig. 1A). Finally, disks were cut into 3 × 3 × 5 mm specimens, and subsequently used for preparation of microscopic sections.

Light microscopy

The preparation of slides was made according to adjusted methods (BARBOSA *et al.* 2010, RAPP and BEHRMANN 1998), using embedding media polyethylene glycol PEG 1500 (Sigma-Aldrich, St. Louis, United States). The difference from the original methodology was to shorten the penetration time of the PEG 1500 embedding to 12 hours at 60° C. and using a reinforcing layer of polish nail gel (RAČKO *et al.* 2018). After penetration of embedding media, specimens were oriented in right anatomical positions and stabilized into PEG blocks. Trimming of specimen blocks to a flat surface were made by sledge microtome (Reichert, Wien, Austria). Furthermore, reinforcing nail polish gel layer was applied on trimmed surface and dried approx 5–7 min. After sectioning, the microsection (15–20μm) was glued on the slide by Mayer albumin adhesive. Removal of the gel layer and PEG 1500 from the microsection surface and wood structure was performed with pure acetone during 1 min. Subsequently, microsections were stained with Toluidine blue O (Sigma-Aldrich, St. Louis, United States) during 5 min. Pinkish purple color will appear when the Tolutdine reacts with carboxylated polysaccharides such as pectic acid; green,

greenish blue or bright blue with polyphenolic substances such as lignin and tannins (O'BRIEN *et al.* 1964). The staining solution Astra blue + Safranine O (Sigma–Aldrich, St. Louis, United States) was prepared by separate dissolving both compounds, each in 100ml deionised water, and subsequently their mixure in a ratio of 1:1. Microsections were stained with this solution during 3 min. Subsequently, the dye was removed with a pure acetone, water and 96% ethyl alcohol solution (1:1:1 v/v/v). The microsection was dried by solution of 75% and 96% ethyl alcohol during 2 min. Finally, each concentration and microsection was mounted to Euparal (BioQuip Products Inc., Rancho Dominguez, United States).

Finally, the slides were examined with an Axio Lab.A1 microscope (Carl Zeiss Microscopy, Jena, Germany). A set of polarizing filters was also used for cellulose localization.

RESULTS AND DISSCUSION

The sample material was extensively attacked by *N. ditissima* fungus, where the numerous perithecia and apparent bark scars were visible on the burl (Fig. 1A, F, G). The formation of burl (closed canker) was caused by a mechanical wounding and subsequent pathogen penetration through the cracks and lenticels on the bark.

Xylem anatomy after wounding

Mechanical wounding was contained a considerable part of its circumference. Massive lateral callus formation was induced, which was able to close the wound during one year (Fig. 1B). Rapid double-sided wound closure inhibited the penetrating of infection from the lateral wound surfaces. However, lesions of infection in xylem spread from the longest exposed wound areas towards to pith. Furthermore, the infection also penetrated through the formed barrier zones into the newly formed wood (called as 'wound wood') (Fig. 1B, C). But 2 cm below (next to burl area), the infection in xylem and bark has already not found (Fig. 1D, E).

The xylem anatomy of the lateral callus and wound wood, formed after the injury had features of closed tumour with apparent hypertrophy, disorganization and distortion of the tissues (Fig. 2A,B). Tissues contained a lower proportion of narrow vessels, an higher proportion of fibres and an higher proportion of radial and axial parenchyma. Simultaneously, the tissues had often a chaotic arrangement. However, differentiation of narrow vessels in cambium is rather caused by mechanical wounding, than caused by tree canker (GHASEMKHANI 2012, LEV-YADUN and ALONI 1993). Also, it is known that most hypertrophic reactions of trees apparently are caused by various injuries, pathogenic organisms (especially bacteria), insects, or specific growth disorders (BEALS and DAVIS 1977). Disruption of the auxin flow in the cambium results in changes in the orientation of the cellular elements, as well as in their shapes and sizes (LARSON 1994). Irregular orientation of the xylem elements and large number of axial and ray parenchyma cells inducted by *Nectria* also found SAKAMOTO *et al.* (2004).

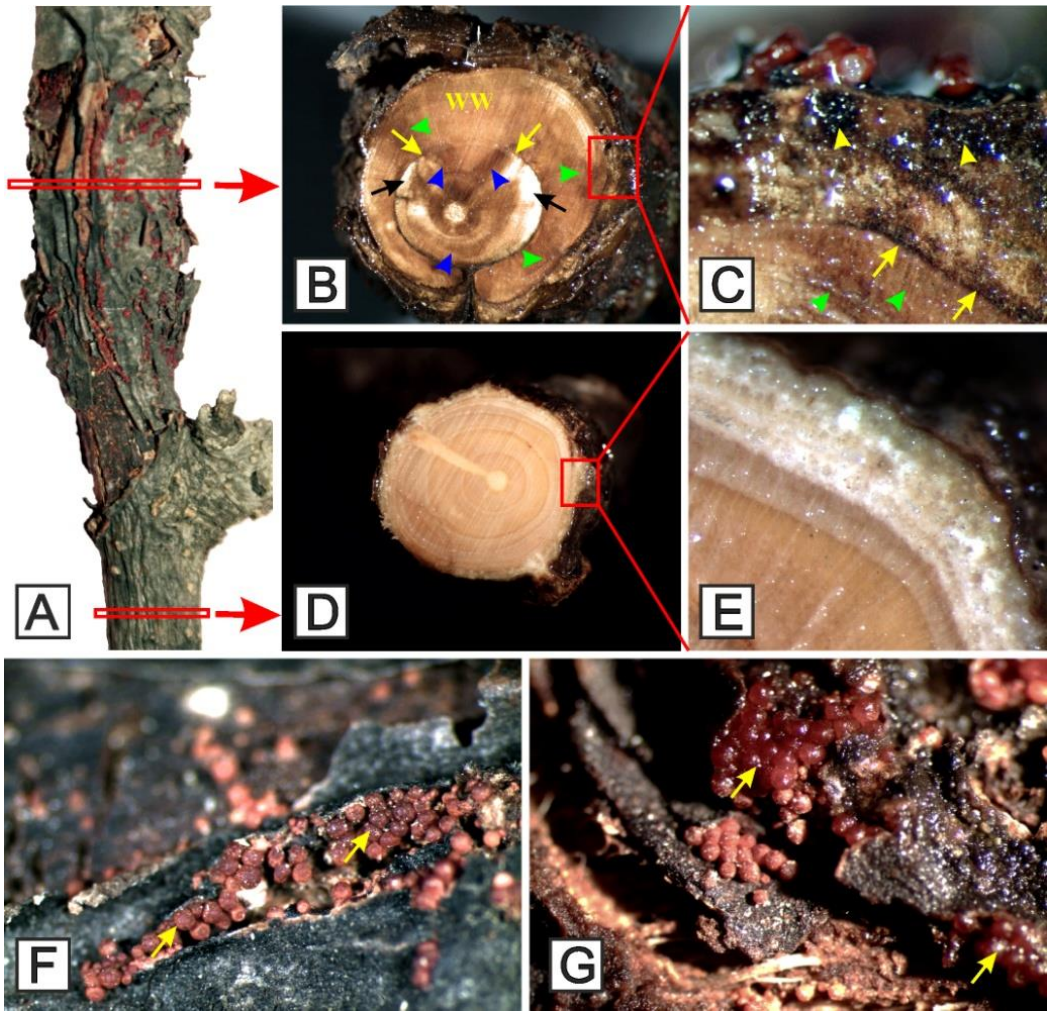

Fig. 1 Macroscopic features of *Malus domestica* branch infected by *N. ditissima* fungus (A) – The burl on three-years-old branch part. (B) – Cross section through the burl centre. Typical closed wound with abnormal wide growth ring and barrier zones (yellow arrows), formed after wounding (WW). Uninfected lateral parts of exposed tissues (black arrows). Lesions of infection spreading from exposed wound surface into the xylem (blue arrowheads). Lesions of infection spreading from bark side caused by *Nectria* (green arrowheads); (C) – Visible zones of infection in periderm (yellow arrowheads), cambium and part of phloem (yellow arrows). Lesions are also in xylem (green arrowheads) (D) and (E) – Cross sections of uninfected branch part. (F) and (G) – Massive occurrence of perithecia visible on bark surface.

However, such huge changes in the orientation of the lateral callus tissues did not only result in mechanical injury (Figures 2A, C, D). The chaotic course of the vessels, fibres and rays were apparently caused by the infection of the *N. ditissima* fungus penetrating through the bark. CROWDY (1949) stated that the pathogen can also penetrate into the xylem to an appreciable depth, invading the xylem parenchyma, vessels and fibres, while development of the canker depends on the balance between the development of the pathogen and the resistance of the host. The hyphae nectria is frequent in the lesion but become weaker and less frequent further away from the centre of infection (GHASEMKHANI 2012, SAKAMOTO *et al.* 2004). It indicates that the infection of *Nectria* spp. does not have a good ability to spread considerably in xylem and, therefore, they cannot be directly related to massive lesions spreading through wound wood as far as to cambium. Therefore, pathogens of other fungi that spread from the wounded area through the xylem overcame defensive barriers in xylem and caused weaker resistance of the host.

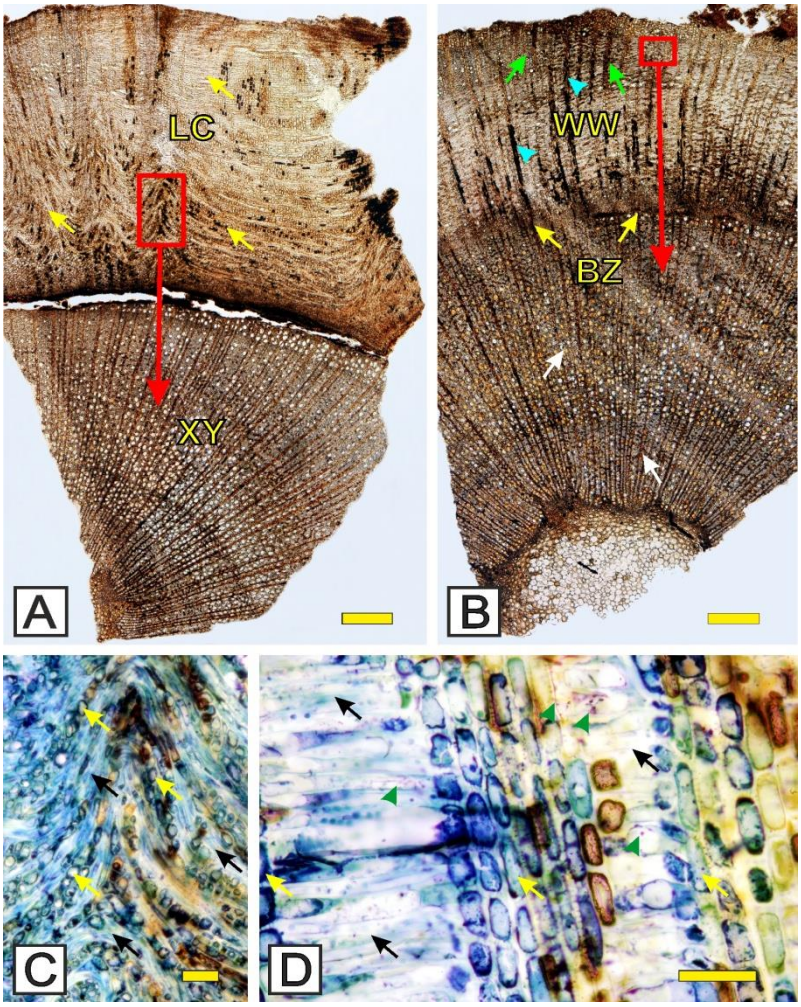

Fig. 2 The xylem anatomy of the *Malus domestica* infected by *N. ditissima* fungus. (A) – Xylem (XY) and lateral callus (LC) after one-year exposure of infection. Infection penetrated throught lateral callus and resulted to irregular and chaotic arrangement of LC tissues (yellow arrows); (B) – XY and wound wood (WW) before and after wounding. Huge amount of tyloses and phenolic compounds in vessels indicate spreading of infection from wound surface. The long and wide barrier zones (BZ) at the wound margins were formed (yellow arrows). Rays in WW were wider than in XY formed before wounding (blue arrowheads). Green arrows indicate the infection spreaded from the bark side; (C) – Whirled arrangement of uniseriate rays (yellow arrows) and fibres (black arrows). Vessels in these zones lacked. The presence of phenolic in these zones were frequent compounds (yellow-brownisch coloured zones); (D) – Change of the anatomical direction of vessels and fibres from longitudinal to tangential (black arrows) and locally colonized by hyphae (green arrowheads). Wider parenchyma cells in rays (yellow arrows) sometimes with yellow-brownisch coloured zones represent phenolic compounds. Cross sections in all figures; (A) and (B) unstained microsections scale bars – 200 µm; (C) and (D) Toluidine blue stained microsections scale bars – 50µm.

Bark anatomy infected by *N. ditissima*

Increased microbial activity in cambial area also caused the formation of thicker bark. The formation of large scars was induced by higher tangential dilation of the bark. As a result of this, the cracks were formed on its surface (in the periderm) and subsequently, gradually closing by. However, ascospores and conidia of *N. ditissima* fungus had already been presented on the bark surface at this time. Trees can become infected during propagation and the infection may remain latent for 3–5 years before appearing of symptoms (GHASEMKHANI *et al.* 2016, MCCRACKEN *et al.* 2003). The infection easier penetrated through these injuries

and lenticles further deeper into the bark structure, before closure the cracks by wound periderm. As can be seen in Fig. 1C, the periderm zone showed the characteristics of decay.

Observations of the microstructure of bark tissues in advance stage of decay confirmed also locally decomposed and destructed areas of the inner periderm and outer phloem in comparison with the healthy tissues (Fig. 3A, B). The spreading of the infection to the cambium induced increasing formation of dense sklereid clusters in phloem (Fig. 3C) that as also stated by (SAKAMOTO *et al.* 2004). Simultaneously, large amounts of crystals were detected there (Figure 3C). According to GHASEMKHANI *et al.* (2016) a hyphal aggregation that possibly represents an early stage of sporodochial formation was observed under phellem of infected *Malus* cultivars 110 days after inoculation by *N. ditissima*. Also phellem layer with U-shaped thickened cells were deformed and collapsed. In the next period, this layer was bulged and disrupted, where the perithecia got on the bark surface (Fig. 3B, C, D). However, one-year after wounding a massive formation of perithecia were evident and grew from bark scars (Fig.1F, G). Fig, 3 documents mature perithecium with formed ascospores.

The closed canker was formed in this stadium of activity the fungus where cambial zone was infected, but it not has been disrupted. However, progressive development of necrosis does not exclude the formation of target-like structures (open canker) due to the continuous cambium necrosis as stated SAKAMOTO *et al.* (2004).

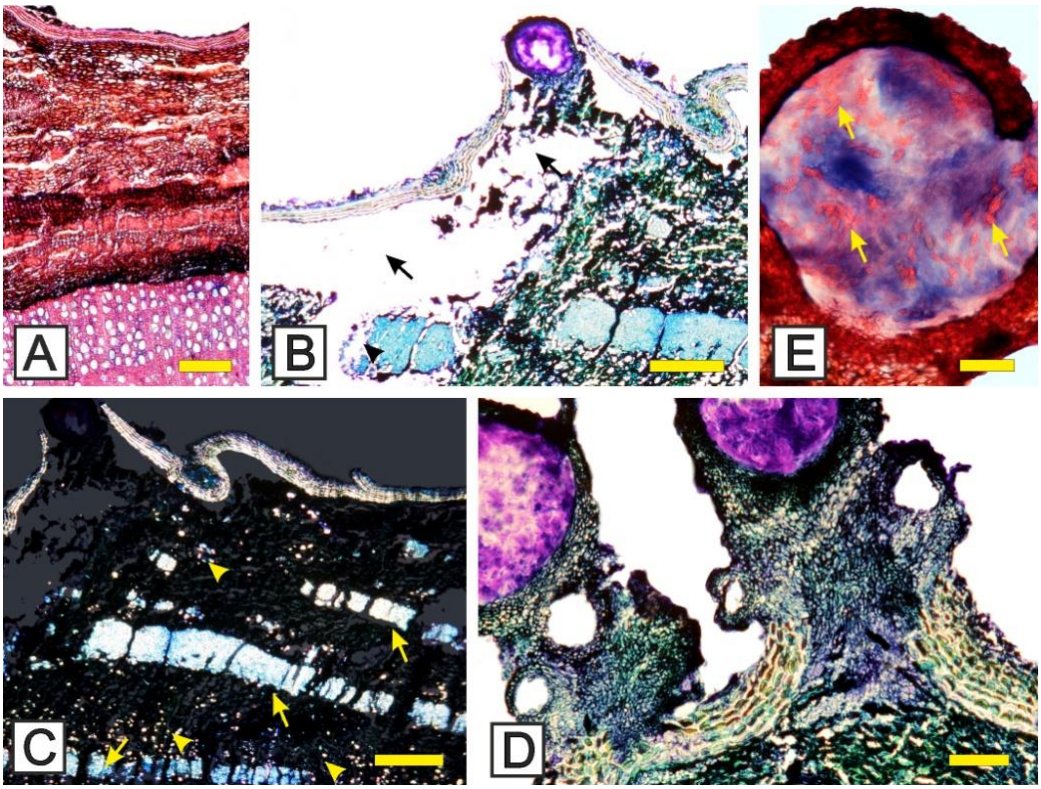

Fig. 3 The bark anatomy of the *Malus domestica* infected by fungus *Neonectria ditissima*. (A) – Uninfected bark tissues; (B) – Advanced stage of degradation of periderm tissues (black arrows) and partly outer phloem cells. Also sclereids were partly destroyed (black arrowheads); (C) – Excessive formation of sclereide clusters (yellow arrows) and prismatic crystals (yellow arrowheads) in phloem; (D) – Collapsed cortex and rhytidome by growing perithecia; (E) – Mature prithecium with visible ascospores (yellow arrows). Cross sections in all figures. (A) and (E) Astra blue and Safranine stained microsections; (B) and (D) Toluidine blue stained microsections; (C) Unstained microsection in polarised light; Scale bars: (A) and (B) – 100 µm; (C) – 200µm; (D) and (E) – 50µm.

CONCLUSION

We found out that the formation of burl (closed canker) of three-year old branch of *M. domestica* attacked by the fungus *N. ditissima* was inducted by a mechanical wounding and subsequent pathogen penetration through the bark. The one-year old xylem formed after the injury had features of closed tumour with apparent hypertrophy, disorganization and distortion of the tissues. The burl bark was locally degraded and scarred with high frequency of *N. ditissima* perithecia on its surface. Although the degradation progressed towards to cambium, the cambium had not been disturbed yet. However, the infection penetrated to the cambium and caused changes in the morphology of the tissues differentiated after wounding.

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