Antioxidant defences of Norway spruce bark against bark beetles and its associated blue-stain fungus

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SUMMARY

Bark beetles and their fungal associates are integral parts of forest ecosystems, the European spruce bark beetle (*Ips typographus* Linnaeus, 1758) and the associated pathogenic blue stain fungus *Ceratocystis polonica* (SIEM.) C. MOREAU, are the most devastating pests regarding Norway spruce [*Picea abies* (L.) H. KARST.]. Bark beetles commonly inhabit weakened and felled trees as well as vital trees. They cause physiological disorders in trees by destroying a phloem and cambium or interrupt the transpiration flow in the xylem. Conifers have a wide range of effective defence mechanisms that are based on the inner bark anatomy and physiological state of the tree. The basic function of bark defences is to protect the nutrient- and energy-rich phloem, the vital meristematic region of the vascular cambium, and the transpiration flow in the sapwood. The main area of defence mechanisms is secondary phloem, which is physically and chemically protected by polyphenolic parenchyma (PP) cells, sclerenchyma, calcium oxalate crystals and resin ducts. Conifer trunk pest resistance includes constitutive, inducible defences and acquired resistance. Both constitutive and inducible defences may deter beetle invasion, impede fungal growth and close entrance wounds. During a successful attack, systemic acquired resistance (SAR) becomes effective and represents a third defence strategy. It gradually develops throughout the plant and provides a systemic change within the whole tree's metabolism, which is maintained over a longer period of time. The broad range of defence mechanisms that contribute to the activation and utilisation of SAR, includes antioxidants and antioxidant enzymes, which are generally linked to the actions of reactive oxygen species (ROS). The presented review discusses the current knowledge on the antioxidant defence strategies of spruce inner bark against the bark beetle (*Ips typographus*) and associated blue stain fungus (*Ceratocystis polonica*).

Key words: antioxidants, ascorbate-glutathione system, blue-stain fungus *Ceratocystis polonica* (SIEM.) C. MOREAU, Norway spruce (*Picea abies* (L.) H. KARST.), phenolics, systemic acquired resistance (SAR)

THE BIOLOGY BEHIND THE ASSOCIATION OF SPRUCE BARK BEETLE WITH BLUE-STAIN FUNGI

Over their long lifetimes, conifers are targets of numerous attacks by different pests such as insects, other herbivores, fungi, and bacteria. They pave their way towards nutrient-rich phloem through the bark. Phloem and cambium represent a relatively small part of a tree's trunk, which could be even

faster and more easily damaged and destroyed than other tissues (Franceschi et al. 2005).

The majority of bark beetle species are considered as rather harmless species in their native ranges, colonising mainly weakened or dead trees and thereby represent an important ecological factor in forest rejuvenation (Müller and Job 2009, Smith et al. 2011, Novak et al. 2014). However, these species pose potential risks in the case of significant increase in abundance of populations and within changing or new environments. They should not be ignored when evaluating

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risks and threats to forest ecosystems or when determining quarantine measures for pests and pathogens. Forest pest insects and their associated micro-organisms are capable of movement through national boundaries and have a potential impact on forest-dependent industries (Christiansen and Bakke 1988, Linnakoski et al. 2012, Sallé et al. 2005, Raffa et al. 2008, Faccoli 2009, Smith et al. 2011).

One of the most destructive forest insects in Europe is the spruce bark beetle *Ips typographus* (Linnaeus 1758.) (Curculionidae, Scolytinae), which affects Norway spruce and is with its fungal associates, the pathogenic blue-stain fungus *Ceratocystis polonica* (SIEM.) C. MOREAU the integral parts of the forest ecosystems (Wermelinger 2004, Klepzig et al. 2009, Linnakoski et al. 2012, Novak et al. 2014). Their symbiotic relationship has been described as mutualistic (Whitney 1982). Although a number of studies have been devoted to resolving the nature of bark beetle-fungus interactions, these interactions remain poorly understood (Jurc 2011, Six and Wingfield 2011).

The fluctuations in bark beetles' populations are interactions between at least three main biological components: the host tree, bark beetles, and pathogen fungi (Novak 2014).

Normally has *Ips typographus* two (spring and summer) generations per year (Fig. 1). If global warming extends the growing season, a higher proportion of the second generation may reach the cold at the hardy adult stage and survive the winter (Holger et al. 2006). Bark beetles construct galleries under the bark within the phloem layers of woody plants, mostly in weakened or recently killed trees, where they lay eggs and their brood feed and develop during the winter (Six and Wingfield 2011).

Adult beetles form wounds through the bark into the phloem, as a source of food and the place of reproduction, to the cambium. Parental adults construct an initial egg gallery, which is continued by larvae into the larval galleries (Raffa et al. 2008). During the constructions of galleries, bark beetles serve as vectors for several types of micro-organisms that enter into the host tree. They benefit the fungus because it is transferred into a nutrient-rich environment, where competition is limited (Whitney 1982, Beaver 1989, Paine et al. 1997). Specific roles of fungal associates in the *Ips typographus* symbiotic system and some of its underlying molecular mechanisms have been the topics of considerable previous work (Krokene and Solheim 1998, Hammerbacher et al. 2013, Urbanek Krajnc et al. 2014).

Now it is known that within a symbiotic system bark beetles serve as vectors for fungi, which would otherwise be unable to reach a new host (Six and Wingfield 2011), alternatively fungi benefit the beetle as sources of nutrients, or they may weaken tree defences (Linnakoski et al. 2012). The concept that tree-killing bark beetles require fungal pathogens to overcome tree defences and to incur tree mortality has received the most attention over the years. This hypothesis, which is referred to as the classic paradigm, has formed the basis for the majority of research conducted on these interactions (Six and Wingfield 2011). Blue-stain fungi are thought to be primarily responsible (Johnson and Croteau 1987) or required (Whitney 1982) for mortality regarding conifers attacked by bark beetles.

Such conclusions are based on observations that:

- beetles are capable of vectoring or dispersing the fungi,
- beetles are rarely found in the absence of staining fungi,
- sapwood of beetle-killed trees is stained (Paine et al. 1997).

Although the mechanisms are not fully understood, a tree is killed as a result of the simultaneous activities and interactions of both organisms, rather than the successive actions of beetles and pathogen (Nebeker and Hodges 1993, Paine et al. 1997). On the other hand, Six and Wingfield (2011) suggest that fungal phytopathogenicity has a more important role for the fungi, rather than supporting the bark beetles in tree killing. It may be a factor helping the fungi to survive within a living tree.

DEFENCE STRATEGIES OF NORWAY SPRUCE BARK

Due to the variety of pests the conifers evolve complex anatomical and chemical defences against pathogen attack, which may deter beetle invasion, impede fungal growth, and seal entrance wounds (Rohde et al. 1996, Bonello et al. 2001, Baier et al. 2002, Wermelinger 2004, Franceschi et al. 2005). Bark anatomy and the physiological condition of a potential host tree are crucial for the success of an attack/infection. Until now, three types of tree's defence strategies have been described: constitutive, induced defences and systemic acquired resistance (Franceschi et al. 2005). Defence mechanisms are based on the bark anatomy and

Fig.1: Generalised life-cycle of the spruce bark beetle (*I. typographus*), which normally has two generations per year - spring and summer generations. Bark beetles construct galleries, lay eggs and at the same time inoculate fungi into the bark phloem of woody plants (Six and Wingfield 2011).

its physiological condition. The main area of defence mechanisms is secondary phloem, which represents both physical and chemical protections (Hudgins and Franceschi 2004).

Constitutive and induced defence

An effective defence is provided by a multi-stage system, which runs as a series of different resistance mechanisms. Conifer trunk includes constitutive and inducible defences, which have attracted much attention over recent years. Most of the new knowledge has been obtained on resin-producing and resin-storing structures. Furthermore, quantitative and qualitative changes in the local metabolism have been studied around the entrance hole. These investigations have focused on phenolics, terpenoids and protein-based chemical defences (Brignolas et al. 1998, Evensen et al. 2000, Nagy et al. 2004, Wermelinger 2004, Franceschi et al. 2005, Erbilgin et al. 2006, Zeneli et al. 2006). Without prior attack the bark implements mechanical and chemical constitutive passive defence mechanisms. It involves initiation of a wound periderm, and based on bark anatomy also formation of cells with phenols, resin ducts, concentric layers of polyphenolic parenchyma (PP) cells, sclerenchyma and calcium oxalate crystals (Hudgins and Franceschi 2004, Franceschi et al. 2005, Hudgins et al. 2004, Krekling et al. 2004).

An invasion activates inducible defence systems, which include secondary resin production, synthesis of new phenolics, and protein-based chemical defences. The induced defence detects attacks and closes the initial wounds by the formation of resin ducts, which contribute to increased synthesis of terpenes and resin flow. The induced defence is more effective and represents a more sustainable method of defence, as is provided by qualitative and quantitative changes in the chemical compositions of the trees' metabolisms. Furthermore, induced defence increases the formations of new PP cells and phenolic compounds, which are toxic for invasive organisms (Hudgins and Franceschi 2004, Franceschi et al. 2005, Schmidt et al. 2005, Witzel and Martin 2008). Pathogens use mechanical force or release cell wall degrading enzymes to break down this barrier. At the cell wall, they also trigger pathogen-associated molecular patterns (PAMPs) either inadvertently or as a consequence of plant degradative enzymes (e.g. the release of chitin oligomers by plant chitinases). Plants, in turn, appear to sense these PAMPs and damage to their cell walls and activate a variety of defences, including the production of reactive oxygen species (ROS), the production and export of anti-microbial compounds and fortification of their cell walls. PAMPs also activate a localised and systemic acquired resistance (LAR and SAR), initiating the synthesis of pathogenesis-related proteins (PR proteins), installation of lignin and accumulation of phenols and toxins (Hématy and et al. 2009). In addition, sensing PAMPs may activate intracellular defences such as the salicylic acid pathway, perhaps priming the plant for the next stage of warfare.

Collectively, constitutive and inducible compounds may deter beetle invasion, impede fungal growth, and seal entrance wounds (Bonello et al. 2001, Nagy et al. 2004, Wermelinger

2004, Franceschi et al. 2005, Zeneli et al. 2006, Rodrigues et al. 2009).

SAR - systemic acquired resistance

The last and very important defence strategy is composed of a systemic acquired resistance (SAR), which becomes effective during successful attack and ensures faster and easier responses to attacks in the future (Evensen et al. 2000, Percival 2001, Nagy et al. 2004, Franceschi et al. 2005, Bonello et al. 2006, Witzel and Martin 2008). This mechanism gradually develops throughout the plant during pest colonisation, and provides a systemic change in the whole tree metabolism. This leads to the production of fewer carbohydrates but more proteins, which are needed for defence (Viiri et al. 2001, Wermelinger 2004). The broad range of defence mechanisms, which contribute to the appearance of SAR, include antioxidants (ascorbic acid, cysteine, glutathione, total phenols) and antioxidant enzymes (Foyer and Rennenberg 2000, Riedle-Bauer 2000, Urbanek Krajnc 2004, 2009), which are generally linked to the actions of ROS (Hayat et al. 2009). Although anti-oxidative defence systems are often used as stress indicators for the diagnosis of disturbances in forest trees (Foyer and Rennenberg 2000, Riedle-Bauer 2000, Tausz et al. 2003, 2004, Grill et al. 2001, Tegischer et al. 2002), to date only a few investigations have dealt with anti-oxidative system of attacked Norway spruce (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2014).

While several studies have demonstrated that the induction of phenolics occurs on a local scale (Evensen et al. 2000, Franceschi et al. 2000, 2005), several recent studies have also found evidence of the systemic induction of soluble low molecular weight phenolics (Bonello et al. 2001, 2006, Wallis et al. 2008). A SAR hypothesis postulated by Bonello et al. (2006) illustrated the interplay between SAR and induced susceptibility in trees against microbes and herbivores. The authors postulated that the time course of pathogen infection may have a bell-shaped effect on the strength of SAR that is similar to the spatial variations of constitutive secondary metabolites generated by resource availability. In the earliest stages of pathogen infection, SAR responses are predicted to rapidly and systemically increase concentrations of compounds involved in defence against pathogens and insects. However, if the pathogen is able to grow despite the deployment of localised defence responses, the infection will progress, and the tree will become increasingly stressed by the resulting resource limitations (e.g. reduction of sap flow, limited nutrient and water absorption, decreased carbon assimilation and growth). Consequently, a degradation of molecules involved in SAR would follow.

SAR has been actively studied in herbaceous plant species, and, over recent years, in woody plant species (Bonello et al. 2006, Eyles et al. 2009), and is fast emerging as an intriguing, eco-friendly concept for enhancing tree resistance. However, before applying SAR becomes possible, there is a need to increase our knowledge of the mechanisms of defence in forest trees. Over recent years, research has focused on studying the impact of exogenous methyl jasmonate (MJ) and SA treatment on the inductions of certain metabolites

which are involved in SAR. Many authors (Franceschi et al. 2002, Hudings and Franceschi 2004, Hudings et al. 2004, Schmidt et al. 2005, Erbilgin et al. 2006) have examined the effect of MJ treatment in combination with the induced resistance of Norway spruce, where the positive impacts have been observed of MJ treatment and bark beetle attacks or *Ceratocystis polonica* infection. They reported that MJ treatment stimulated the synthesis and accumulation of terpenes (Kozłowski et al. 1999, Franceschi et al. 2002, Martin et al. 2002, 2003, Fäldt et al. 2003, Hudgins and Franceschi 2004, Hudgins et al. 2004, Miller et al. 2005, Erbilgin et al. 2006, Zeneli et al. 2006).

In our previous field experiment (Urbanek Krajnc et al. 2011), 100 mM SA was applied to the bark sections of Norway spruce prior to being attacked by bark beetles, in order to study interactions with antioxidants and their significance for mediating stress-tolerance under natural conditions. SA-treatments significantly elevated the total SA levels over the whole sampling period. Total glutathione (tGSH) and total cysteine (tCys) increased, respectively, two weeks after treatment, in comparison with controls. One month after pheromone dispensers were placed on trees, an intensification of the ascorbate-glutathione system occurred within moderately-affected bark but to a greater extent after SA-treatment. Total SA levels within SA-treated moderately-affected trees remained at the control level until June. In contrast, strong attack was characterised by a successive increase in total SA and degradation of tGSH as well as total phenolics (tPH), a moderate increase in total ascorbate (tASC) and an oxidation of the ascorbate-glutathione pool within non-treated bark.

Furthermore, at the end of the three month experiment, the SA-treated bark had less entrance holes, and exhibited fewer and shorter maternal galleries than the control-bark. From this perspective, exogenous SA was successfully implicated as an activator of SAR in Norway spruce, providing tolerance against the complex interactive effects of bark beetle attack and environmental factors (Urbanek Krajnc et al. 2011).

OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE OF CONIFER METABOLISMS

Antioxidants play an important role in chemical defence as they prevent the oxidations of other substances such as inactivate oxygen compounds and inhibit the formations of free radicals (Brodnjak Vončina 2006). Antioxidants are classified into three groups: real antioxidants that bind free radicals; reducing agents, while the third group consists of antioxidant synergists (Kugler 2011).

Both biotic and abiotic stress factors trigger plant metabolism changes and mostly react with the reactive oxygen species (ROS; H_2O_2). Oxidative stress reflects an imbalance between the systemic manifestations of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox states of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell.

Antioxidants can slow down or even totally stop the oxidations of these molecules by hunting the free radicals and by removing oxidative damaged biomolecules (Kugler 2011, Veberič 2010, Harris and Hansen 2012). The antioxidants do not accumulate in the cytoplasm but in the vacuoles and other organelles such as plastids and mitochondria, where they are urgently needed as an effective "redox shuttle" (Kreft et al. 2000).

Phenolics

Among antioxidants phenolics represent a more important component of the inducible defence strategy regarding conifer bark. They range from simple, low molecular weight, single aromatic-ringed compounds to large and complex tannins and derived at polyphenols that provide different chemical and structural defence strategies.

In regard to the synthesis and accumulation of phenolic compounds, the barks of all conifer families have polyphenolic parenchyma cells (Krekling et al. 2000, Franceschi et al. 2000, 2005, Schmidt et al. 2005). Within their vacuoles, polyphenolic parenchyma cells contain variable amounts of phenolic bodies that are thought to serve as antifeedant and antifungal agents (Beckman 2000). Polyphenolic parenchyma cells are also major sites for storing starch and lipids (Krekling et al. 2000). In this capacity, they can be seen as targets for beetles and fungi, and constitutive phenolics can be hypothesised to protect the cells themselves, as well as prevent fungal penetration towards the cambial zone. In any case, the multiple layers of polyphenolic parenchyma cells providing physical and chemical resistance to penetration of the bark (Franceschi et al. 2000, 2005, Schmidt et al. 2005).

In our previous study on Norway spruce, the increase in total phenolics' concentrations two weeks after a bark beetle attack was recognized as an immediate inducible response to the attack (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011). Additionally, phenolics accumulated, to a higher extent, in the control tissue, which was explained as a wound reaction induced by mechanical injury (Christiansen et al. 1999, Franceschi et al. 2005, Ralph et al. 2006, Urbanek Krajnc 2009). The increase in total phenolics during the initial bark beetle colonisation was accompanied by the degradations of total ascorbic acid and tGSH, as well as oxidation of the ascorbate-glutathione pool. These initial events pointed out that the antioxidant defence is a multi-tier system with a spatial and temporal component. The spatial component is determined by the positions of PP cells' concentric rings from the periderm surface to the cambial zone (Franceschi et al. 2005) and the temporal component consisted of seasonal, continuous and enhanced production of phenolics in the attacked tissue. However, at later sampling dates, the trees with moderate attack were characterised by increased and steady-states of the tPH concentration, whereas the strong attacks were shown by drastically decreased concentrations of tPH (Urbanek Krajnc 2009). As the PP cells are the primary sites of phenolic biosynthesis within the secondary phloem (Franceschi et al. 2000, 2002, 2005), an assumption can be made that the synthesis of phenolics is lacking when the phloem is damaged, by the establishment of a complete

breeding system.

The more important phenolics in the Norway spruce phloem are stilbenes, flavonoids, as well as tannins (e.g. Lieutier et al. 2003, Schmidt et al. 2005, Witzell and Martin 2008, Hammerbacher et al. 2011). In general, phenolics fulfil different defensive functions. Most of the phenolics contribute to resistance indirectly. For instance, some low molecular weight phenolic compounds may function as precursors for other defensive compounds (e.g. lignin; Bonello and Blodgett 2003), or they may confer resistance as a group, rather than as individual compounds (Wallis et al. 2008). Catechin is a building block of condensed tannins for which antimicrobial activity via protein precipitation and iron depletion has been suggested (Witzell and Martin 2008).

Significant quantities of stilbene in pine bark were found years ago (Sjöström 1993), while now it is known that pine bark extract contains several types of stilbene (the more important being astringin and isorapontin) and flavonoids. Changes in the concentrations of stilbenes and flavonoids in Norway spruce in response to injury or fungal infection are considered to be an active defence response (Brignolas et al. 1995, 1998, Viiri et al. 2001, Havsteen 2002, Schmidt et al. 2005, Witzell and Martin 2008, Li et al. 2012, Hammerbacher et al. 2011, 2013). Stilbenes are known to inhibit fungal growth by interfering with microtubule assembly (Woods et al. 1995, Adrian et al. 1997), disrupting plasma membranes and uncoupling electron transport within fungal spores and germ tubes (Pont and Pezet 2008, Adrian and Jeandet 2012). Stilbenes have also been shown to protect plants against oxidative stress (He et al. 2008), to deter herbivores (Torres et al. 2003), and to inhibit the growth of competing plants (Fiorentino et al. 2008).

Specific phenolic compounds in spruce inner bark against *Ceratocystis polonica* infection were previously interpreted by Urbanek Krajnc et al. (2014). Increases in tPH, catechin, astringin, isorhapontin and taxifolin were monitored. By analysing each single phenolic compound, on later sampling dates, it could be observed that within certain time shifts the phenolics followed the eco-physiological concept and basically fit with the temporal sequence of changes in tPH concentrations after the moderate bark beetle attack, previously reported on the spruce/bark beetle pathosystem (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011). The initial decline in catechin was followed by an increase in catechin concentration until September, when taxifolin accumulated within the infected bark at significantly higher levels until June, and later the concentrations dropped toward the control levels. On the other hand, a slight accumulation of astringin observed in response to fungi infection, when the concentration at later sampling dates dropped. The same was for isorhapontin concentration, where a dramatic initial increase was observed, and later it simply diminished (Urbanek Krajnc et al. 2014).

Ascorbate-glutathione cycle

Besides phenolics, ascorbate–glutathione system plays an important role in plant metabolism and defence. The ascorbate–glutathione cycle is considered to be the main pathway for ROS removal, and both ascorbate and glutathione

are recognised as the heart of the redox hub within the cell (Foyer and Noctor 2012).

Amongst antioxidants, glutathione is a low molecular sulphur metabolite, which plays multiple roles within tree–environment interactions and defences (Grill et al. 2001, Tausz et al. 2004, Zhao et al. 2008, Noctor 2006, 2012). It functions as a reductant in the enzymatic detoxification of ROS within the glutathione–ascorbate cycle and as a thiol buffer in the protection of proteins via direct reaction with ROS or by the formation of mixed disulphides (Zhao et al. 2008). In this role it has been suggested as a general redox sensor and signalling agent in plant cells (Meyer and Hell 2005, Cameron and Pakrasi 2010, Noctor et al. 1998, 2012).

Owing to its redox-active thiol group, GSH has often been considered as playing an important role in plant defence against oxidative stress (Grant et al. 1996). Trees under stress seem to generally require and synthesise higher concentrations of glutathione (Gullner and Kömives 2001, Tausz et al. 2003, 2004). Glutathione synthesis depends on

Fig. 2: A model for the mechanism of SA action within the generation of active oxygen species, which induces the expression of the defence-related genes via an increase in $[Ca^{2+}]_i$. SA and a trace of H_2O_2 are used for the SA^- -generating peroxidase reaction. Then the resultant SA^- reacts with O_2 to produce $O_2^{\cdot -}$ that triggers an increase in $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ may induce further physiological responses including the induction of PR genes. Ascorbate reconverts SA^- to SA yielding monodehydroascorbate. An excess of ascorbate may inhibit the SA^- -dependent generation of $O_2^{\cdot -}$. While the generation of $O_2^{\cdot -}$ is going on, SA inhibits the decomposition of H_2O_2 by catalase and a Fenton-type reaction. SA also lowers the $HO^{\cdot}$ level by blocking the Fenton reaction and by directly trapping $HO^{\cdot}$. Thus SA protects the cells from highly reactive $HO^{\cdot}$, while producing the less reactive $O_2^{\cdot -}$ and H_2O_2 through a peroxidase-catalysed reaction, as the intermediate signals.

the distribution and cycling of sulphur in trees. Glutathione biosynthesis is restricted to the cytosol and the plastids, but mitochondria, which lack the capacity for glutathione biosynthesis, have been described as major sites of glutathione accumulations (Zechmann et al. 2007).

In all cells where GSH is found, besides reduced tripeptide form, the oxidised form of GSH (glutathione disulphide: GSSG) is also present in small quantities (< 20 %). While glutathione reductase (GR) uses NADPH to reduce GSSG to GSH, various free radicals and oxidants are able to oxidise GSH to GSSG (Fig. 2).

Both GSH and GSSG are considered as markers of oxidative stress in the plant, by increasing the antioxidant pool and as actors in cell signalling. Based on the current understanding of redox status and the roles of ROS, thiols, oxidisers and cellular antioxidants, scientists have redesigned a definition of oxidative stress (Harris and Hansen 2012), now it is based on changes in translation of thiol proteins, important for the control of the redox potential. Thiol redox couples such as GSH / GSSG, cysteine / cystine (Cys / cySS) form independent centres within the cells, which are linked to changes in the redox potential.

Glutathione is central to the regeneration of ascorbate within the ascorbate-glutathione cycle (Tausz et al. 2003, 2004). Glutathione, as an antioxidant, together with ascorbate, removes ROS, including hydrogen peroxide, superoxide and hydroxide radicals, which generate in cells as a result of oxidative stress (Xiang et al. 2001, Alscher 2006). In addition to being the most abundant water-soluble antioxidant in plant cells (Smirnoff and Wheeler 2000), ascorbic acid (AA) is also required for the re-conversion of SA, as ascorbate is highly reactive against phenoxyl radicals generated by peroxidases during oxidative stress (Fig. 2) (Kawano and Muto 2000, Smirnoff and Wheeler 2000). Together with glutathione, it is present in oxidation-reduction processes within the ascorbate-glutathione cycle. Dehydroascorbate (DHA) formed when ascorbate is oxidised, must be reduced again in order to prevent decreases in the total ascorbate pool (Likar and Regvar 2003, Šuštaršič 2012). At alkaline pH values, GSH rapidly reduces DHA to ascorbate in a non-enzymic reaction. In plant tissues this reaction is catalysed by dehydroascorbate reductase (DHAR) (Noctor et al. 1998). The ascorbate glutathione cycle is essential for the removal of ROS and connects the conversion of ascorbic acid and glutathione.

Ascorbic acid is due to the two hydroxyl groups being one of the stronger reductants in the cell and allows the detoxification of reactive oxygen species. In the cell metabolism AA operates in oxidation-reduction processes, in the ascorbate-glutathione chain, where it is reversibly oxidised to DHA. At this stage, the oxidised form of ascorbic acid is restored via glutathione, when the GSH is oxidised to GSSG, and at the same time increases the GSSG / GSH ratio and the activity of GR (Foyer and Noctor 2012).

Although the antioxidant response is relatively well-documented on the level of spruce needles (Tegischer et al. 2002, Tausz et al. 2004), there is little known about changes in the ascorbate-glutathione chain at the inner bark level during bark beetle and fungus infection. In two of our previous experiments, the roles of thiols and ascorbate in the spruce

inner bark were studied during the five month sampling period after the spruce inner bark was exposed to beetle attack and *Ceratocystis polonica* infection (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2014).

The initial reaction of Norway spruce to *Ceratocystis polonica* infection was characterised by a significantly more oxidised glutathione pool which was accompanied by higher GR activity. At the same time, the tCys dropped slightly below the levels of the non-infected samples and the cysteine redox state shifted towards a more oxidised value. Similarly, in one of the previous experiments, 2 weeks after the exposures of spruce trees to bark beetles slight but insignificant decreases were measured for tCys as well as tGSH, which were accompanied by a slightly more oxidised glutathione redox state (Urbanek Krajnc 2009). Until July, the infected inner bark was characterised by a gradual increase in tGSH, which was accompanied by a significantly increasing GR activity. Furthermore, within the infected inner bark the tCys remained elevated over the whole sampling period. Based on a previous experiment Urbanek Krajnc et al. (2009, 2014), concluded that the antioxidant shift within the *Ceratocystis polonica* infected bark indicated a successful defence reaction, which was characterised by a higher accumulation of thiols and a more reduced redox state. In September, the glutathione system reached a steady-state, and the GR activity and tCys concentrations remained increased. Consequently, thiols accumulated in May were accompanied by a significant degradation of tASC within the *Ceratocystis polonica* infected inner bark until July, followed by a significant accumulation in September. However, when comparing the tASC contents in the *Ceratocystis polonica* infected samples, the concentrations increased continuously from the spring onwards to September, which reflected increased pressure on the ascorbate–glutathione cycle (Urbanek Krajnc 2009, 2014).

CONCLUSIONS

Our previous field experiments (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011, 2014) demonstrated that the sequence of changes in the endogenous levels of antioxidant molecules within the affected Norway spruce inner bark strengthened the general eco-physiological stress-response concept as suggested by Larcher (2003) and Tausz et al. (2004).

The time-course analysis of the individual molecules enabled us to establish a relationship between salicylic acid, phenolics and the ascorbate–glutathione system in response to the complex interactive effects of bark beetle attack, infection by blue-stain fungus, and environmental factors (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011, 2014). Based on the ascorbate–glutathione concept of Foyer and Noctor (2011), phenolics within the concentric layers of PP cells can be viewed as a wheel of the bark defence mechanism, driven by the ascorbate–glutathione system, as a central part or redox hub of the wheel that integrates metabolic information and environmental stimuli to tune defence responses against pathogen and pests. The presented article reviews the current knowledge on physiological and biochemical defense

responses of Norway spruce defence against bark beetles and associated fungi and also allows predictions of antioxidative defence responses in other conifer pathosystems.

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Antioksidativni obrambni odziv lubja navadne smreke ob napadu podlubnikov in z njimi povezanimi glivami modrivkami

IZVLEČEK

Čeprav so podlubniki in z njimi povezane glive sestavni del gozdnih ekosistemov, sta osmerozobi smrekov lubadar (*Ips typographus* LINNAEUS) in z njim povezana patogena gliva modrivka [*Ceratocystis polonica* (SIEM.) C. MOREAU], ena najbolj uničujočih škodljivcev navadne smreke [*Picea abies* (L.) H. KARSTEN]. Lubadarji navadno naseljujejo oslabela in podrtá drevesa, v specifičnih pogojih pa lahko napadejo tudi vitalna. S poškodbami floema in kambija ter motnjo transpiracijskega toka v ksilemu, drevesu povzročijo številne fiziološke motnje. Iglavci imajo široko paleto obrambnih mehanizmov, ki temeljijo na anatomiji lubja in fiziološkem stanju drevesa. Njihova osnovna funkcija je zaščita hranilno in energetsko bogatega floemskega tkiva, meristematske aktivnosti vaskularnega kambija in transpiracijskega toka v sekundarnem ksilemu debla. Glavno področje obrambnih mehanizmov je namreč sekundarni floem, ki predstavlja fizično in kemično zaščito lubja pred škodljivci, saj vsebuje polifenolne parenhimatske celice, sklerenhim, kristale kalcijevega oksalata in shizogene smolne kanale. Obrambni sistem lubja iglavcev zajema tri vrste obrambnih strategij – konstitutivno in inducirano obrambo ter sistemsko pridobljeno odpornost. Konstitutivna in inducirana obramba zavirata kolonizacijo podlubnikov, rast gliv in zapirata ranitvena mesta. Med uspešnim napadom pa je za drevo ključnega pomena še vzpostavitev sistemsko pridobljene odpornosti (SAR). Le ta se postopoma širi po rastlini ter izzove sistemske spremembe v metabolizmu drevesa, ki se ohranjajo skozi daljše časovno obdobje. Med številnimi obrambnimi odzivi, povezanimi z aktivacijo SAR, sta pomembni sinteza antioksidantov in aktivacija antioksidativnih encimov, ki varujejo rastlino pred reaktivnimi kisikovimi spojinami (ROS). Prispevek zajema pregled dosedanjega znanja o strategijah antioksidativnega odziva lubja smreke pri napadu smrekovega lubadarja (*Ips typographus*) in z njim povezane patogene glive modrivke (*Ceratocystis polonica*).