Tree Physiology 29, 1059–1068
doi:10.1093/treephys/tpp041

A temporal analysis of antioxidative defense responses in the phloem of *Picea abies* after attack by *Ips typographus*

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Received January 28, 2009; accepted May 28, 2009; published online June 17, 2009

Summary The temporal gradation of antioxidants was investigated on the phloem tissue of Norway spruce [*Picea abies* (L.) Karst.] in response to weather conditions and colonization levels of *Ips typographus* L. (Col., Scolytidae). Two weeks after pheromone dispensers were placed on trees, the initial reaction of Norway spruce to bark beetle attack resulted in moderately lowered levels of total glutathione (tGSH) and total cysteine. Likewise, the total ascorbic acid dropped slightly below the control levels, whereas the concentration of dehydroascorbic acid increased in comparison to the first sampling date. This transient degradation and oxidation of glutathione and ascorbate system was accompanied by moderately increased concentrations of total phenolics. One month later, the shift in antioxidant balance after moderate attack differed quantitatively from the reaction after massive attack. An intensification of antioxidant defense occurred within moderately affected bark. Total cysteine and tGSH contents were markedly raised, whereas the concentrations of total ascorbic acid and total phenolics were slightly increased by moderate attack. On the other hand, massive bark beetle colonization caused a strong decrease in tGSH and total phenolics, whereas total cysteine and total ascorbic acid values remained at control level. Dependent upon the intensity and the success of the attack, a progressive degradation of antioxidants was determined at later sampling dates, which was accompanied by an obvious oxidation of the ascorbate and glutathione pools. With an unsuccessful defense upon massive attack, the thiols and total phenolics did not reach a new steady state, but deteriorated until the end of the brood beetles' development. In contrast, the dynamic antioxidative response within the moderately affected trees indicated an acclimation stage in the middle of July. It was characterized by a higher accumulation of tGSH, total ascorbic acid and total phenolics as well as a more reduced redox state of glutathione. A sequence of changes in the endogenous levels of antioxidant defense molecules in the bark beetle-affected Norway spruce showed consistency with the general ecophysiological stress–response concept, and provided important avenues for evaluating the role and effectiveness of antioxidants in systemic acquired resistance against the complex interactive effects of bark beetle attack and environmental factors.

Keywords: antioxidants, ascorbic acid, cysteine, glutathione, Norway spruce, phenolics.

Introduction

Among the different bark beetle species, *Ips typographus* (L.) is the most dominant and important pest of Norway spruce [*Picea abies* (L.) Karst.] in Europe. Its economic significance is reflected in its period fluctuations and is displayed by the damage caused to forest timber. Under certain environmental conditions, such as windstorms, it can, over a short time, breed in great numbers and represents one of the most dangerous invaders among herbivore insects attacking living trees (Økland and Bjørnstad 2006). It causes physiological disturbances in hosts, destroys phloem and cambium and can also disrupt the xylem flow of water. A major breach in the bark can lead to tree death (Wermelinger 2004, Franceschi et al. 2005). The risk of attack is related to the exposition, age, nutrient and water supply of the trees, as well as the density of the beetles and weather conditions. Furthermore, the efficiency of human control measures, such as salvage, trapping, chemical insecticides and biological agents, affects the survival of bark beetles, host availability and susceptibility (Baier et al. 2002, Wermelinger 2004, Jurc 2006).

Conifers evolve complex anatomical and chemical defenses against bark beetle colonization, which have attracted much attention over recent years (Rohde et al. 1996, Bonello et al. 2001, Mattanovich et al. 2001, Baier et al. 2002, Wermelinger 2004, Franceschi et al. 2005, Schmidt et al. 2005, Erbilgin et al. 2006, Zeneli et al. 2006). Most of this new knowledge has been obtained regarding morphological and biochemical changes in the local metabolism around the entrance holes. Recently, defense molecules such as phenolics, terpenoids and

protein-based chemical defenses were investigated under field and laboratory conditions (Brignolas et al. 1998, Evensen et al. 2000, Fäldt et al. 2003, Martin et al. 2003, Nagy et al. 2004, Wermelinger 2004, Franceschi et al. 2005, Schmidt et al. 2005, Erbilgin et al. 2006, Zeneli et al. 2006).

The first and basic function of bark defenses is to protect the nutrient- and energy-rich phloem, the vital meristematic region of the vascular cambium and the transpiration stream in the sapwood. The first and basic defense strategy is constitutive defense, which involves initiation of a wound periderm, traumatic resin duct formation as well as concentric layers of polyphenolic parenchyma (PP) cells and sclerenchyma. An invasion also activates inducible defense systems, which include secondary resin production, synthesis of new phenolics and protein-based chemical defenses. Collectively, these constitutive and inducible compounds may deter beetle invasion, impede fungal growth and seal entrance wounds. During successful bark beetle attack, systemic acquired resistance (SAR) becomes effective and represents a third defense strategy of an attacked tree (Christiansen et al. 1999, Evensen et al. 2000, Bonello et al. 2001, Percival 2001, Nagy et al. 2004, Franceschi et al. 2005). Systemic acquired resistance gradually develops throughout the plant and provides a systemic change to the whole tree metabolism. This leads to the production of fewer carbohydrates but more proteins that are needed for defense. They are accompanied by a moderate increase in the procyanidine values during successful defense (Rohde et al. 1996, Viiri et al. 2001, Wermelinger 2004, Franceschi et al. 2005).

A broad range of defense mechanisms, which contribute to the appearance of the SAR, also include the synthesis of antioxidants and the activation of antioxidant enzymes (Lamb and Dixon 1997, McDowell and Dangl 2000, Barna et al. 2003, Durrant and Dong 2004, Vallad and Goodman 2004, Noctor 2006). An antioxidant defense system is generally linked to the action of reactive oxygen species and is determined by the pool size of the antioxidants. Changes in these parameters reflect the impact of environmental stresses on plant metabolism (Foyer and Rennenberg 2000, Rennenberg 2001, Herbinger et al. 2002, 2005, Tausz et al. 1996, 1998*a*, 1998*b*, 1999, 2001, 2004, Šircelj et al. 2005, Zechmann et al. 2005, 2006, 2007*a*, 2007*b*, Noctor 2006). Until now only a few investigations have dealt with an antioxidative system in bark beetle-affected Norway spruce, although antioxidative defense systems have often been used as stress indicators for the diagnosis of disturbances in forest trees (Kronfuß et al. 1998, Wonisch et al. 1998, 2003, Batič et al. 1999, Polle et al. 1999*a*, 1999*b*, Ribarič Lasnik et al. 1999, Tausz and Grill 2000, Grill et al. 2001, Mattanovich et al. 2001, Tausz et al. 1996, 1998*a*, 1998*b*, 1999, 2001, 2002, 2003, 2004, Tegischer et al. 2002, Pučko et al. 2005). Pučko et al. (2005) reported an increase in ascorbic acid and α-tocopherol in spruce needles after massive bark beetle infestation. Mattanovich et al. (2001) demonstrated that high breeding success was accompanied by a severe depletion of glutathione in the stem bark 8 weeks after the trees were felled. These investigations deal with antioxidants in the needles and bark of non-vital Norway spruce trees, but a time-course analysis of antioxidants in bark beetle-affected trees under natural weather conditions has not been part of any study yet. To our knowledge, time-course analysis of antioxidants and pigment molecules has only been carried out on the leaves of apple trees, which had been subjected to progressive drought. In apple trees, the initial response was a slight oxidation of the glutathione pool, followed by increased glutathione and ascorbate concentrations. When the stress increased, glutathione and ascorbate concentrations dropped and the redox state became more oxidized, which marked the degradation of the system (Tausz et al. 2004, Šircelj et al. 2005). This temporal analysis of antioxidants during progressive drought confirmed that ascorbate and glutathione follow a general ecophysiological stress–response concept according to the principles given in Larcher (2003) and Tausz et al. (2004).

In this study, a temporal analysis of antioxidants (ascorbic acid, cysteine, glutathione and total phenolics) in the phloem tissue of Norway spruce was performed when the bark was exposed to increasing levels of bark beetle attack ranging from successful defense up to tree death. It was of interest to evaluate whether the sequence of events in the antioxidant response during bark beetle attack followed the general ecophysiological stress–response concept (Larcher, 2003, Tausz et al. 2004) and whether, under certain conditions, the response is dynamic or at a steady state.

Materials and methods

Technical preparation and selection of trees

An unmanaged spruce monoculture was selected in Meranovo, Spodnji vrhov dol, Pohorje, Slovenia (latitude 46° 32′17.25″–46° 32′23.36″, longitude 15° 33′12.53″–15° 33′17.30″ and altitude: 474–493 m; exposition: N–NW; inclination: 5°). The stand structure was characterized as second growth Norway spruce even-aged stand with dense patches of small diameter trees (35-year-old clonal Norway spruce trees, tree height: 20 ± 2 m; crown length: 8 ± 0.7 m; DBH: 25 ± 2 cm).

The experimental field (600 m^2) was divided into two parcels (bark beetle colonized and control) with a 50-m distance between parcels. The 5-m border around the infested parcel was treated with insecticide (Fastac®, 0.3%, BASF, Pinus, Rače, Slovenia) twice during the maximum beetle-flight activity, to protect single susceptible trees and prevent the migration of bark beetles. Test trees (n = 8 for control and n = 8 for colonized trees) were randomly selected within both parcels. The distances between test trees ranged from 13 to 15 m. Sampling dates were selected according to the weather conditions (Figure 1) and the activity period of the bark beetles (Figure 2). Air temperature and relative humidity during the period of sampling were measured

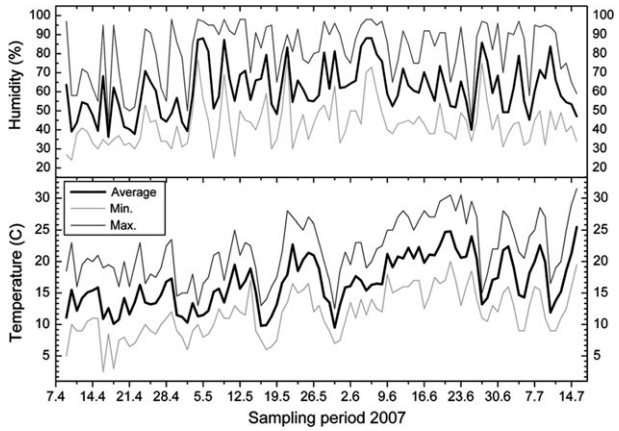

Figure 1. The average, minimum and maximum day temperatures and relative humidity during the 3-month sampling period in 2007.

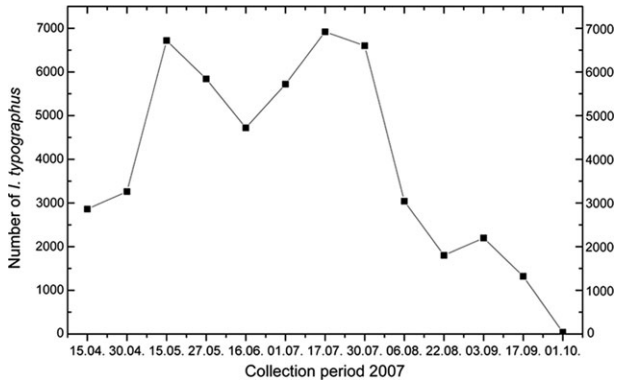

Figure 2. Flight period of *I. typographus* at the experimental plot Meranovo in 2007, recorded according to the weekly catches in pheromone traps.

using thermo/hygrographs. The activity period of the bark beetles was recorded from April until October, according to weekly catches in the slit traps baited with pheromones (Pheroprax®, Cyanamid Agrar, Germany) 500 m from the experimental plot Meranovo.

Pheromone-induced attack by bark beetles

On 13 April 2007, a pheromone dispenser (Pheroprax®, Cyanamid Agrar, Germany) was placed on the north side of each tree within the bark beetle-affected parcel, 2 m above the ground, to induce bark beetle attack. At this time, the average night temperature was about 10 °C over 3 days. The pheromone dispensers remained on the stems for 3 weeks. At the beginning of the experiment, the beetle population in this area was moderate.

Sample preparation for biochemical analysis

To obtain a temporal sequence of defensive chemicals, test trees were sampled five times from 13 April to 15 July 2007. The first samples were taken on 13 April 2007. The second sampling date was 2 weeks later (26 April 2007), and the next sampling dates followed over 1-month intervals from the middle of May until the middle of July 2007.

On each test tree, four samples containing bark and secondary phloem (6 × 6 cm) were collected for biochemical investigation at 1.3 and 3.3 m above the ground, two on the east and two on the west side. The bark was removed and the total phloem was immediately frozen in liquid N_2 and was transferred to the freezer (−80 °C). Since the antioxidants follow a pronounced diurnal rhythm with the maximum amount during daylight (Martin et al. 2003, Tausz et al. 2003), the samples were taken on a clear day between 11:00 and 14:00 solar time. Further processing (freeze-drying, pulverization) was done according to Tausz et al. (2003). For biochemical analysis, four bark samples of each tree were mixed together in equal volume proportions and were used as one sample.

Determination of antioxidants

Total ascorbic acid and dehydroascorbic acid were analyzed by an isocratic reversed-phase chromatography method according to Tausz et al. (2003) and Herbinger et al. (2005). Thiols [total cysteine, total glutathione (tGSH) and oxidized glutathione] were determined by a gradient high pressure liquid chromatography, after the labeling of thiol groups with monobromobimane, as described by Kranner and Grill (1993). Total phenolic compounds were determined spectrophotometrically according to Ainsworth and Gillespie (2007).

Assessment of the beetle attack

The degree of attack on the experimental trees was recorded over 2-week intervals on a three-partite scale, according to Rohde et al. (1996). The number of entrance holes was assessed on the east- and west-facing stem sections between 1.5 and 2.5 m above ground. At the end of experiment, the trees were felled, and the outer cork bark was carefully shaved away on both sides of the trees (east/west sides) between 1.5 and 2.5 m above ground. The number of entrance holes and galleries (tunnel length > 10 mm), the mean and total lengths of all maternal galleries and the number of larval galleries were recorded according to Erbilgin et al. (2006).

At the end of June, the first young beetles emerged from the barks of severely affected trees. Two trees that had been mass attacked and killed were excluded from the experiment at the end of June, as their bark was crowded with well-developed beetle galleries. The experiment was finished in the middle of July, when the first generation of bark beetles completed their life cycles in most of the affected trees. To prevent the emergence and migration of a beetles' second generation, the trees were felled, the logs were debarked and the bark was burned.

Statistics

Based on previous data evaluation (Tausz et al. 2003), methodical variations are known to be substantially lower (variance coefficients of repeated extractions, typically 4–9%) than biological variations of field samples (typical variation coefficient 8–36%), hence special emphasis was placed on considering sampling standardization in the field with respect to sampling time (seasonal and diurnal) and representative sampling of individuals and tissues.

The results of biochemical analyses represented mean values and standard deviations (SDs) of eight replicate samples. They were statistically evaluated with the help of the Kruskal–Wallis test, followed by post hoc comparisons according to Conover (Bortz et al. 2000). Significant differences were indicated by different letters (a–e). Decision rule: $P < 0.05$ was regarded as significant. Calculations were performed on the Statistica 6.0 software package (Stat Soft Inc. 2001).

Results

Flight period and I. typographus colonization

The population level of *I. typographus* in the area was moderate at the beginning of the experiment. The first entrance holes were monitored on all the test trees 3 days after pheromone dispensers were placed on the trees (Table 1). Some rainy days at the end of April and at the beginning of May (Figure 1) were characterized by a reduced number of captures (Figure 2) and new entrance holes (Table 1). Continuously increasing day temperatures and a 2-week period of dry weather (14–28 May 2007, Figure 1) coincided with the first maximum beetle-flight activity (Figure 2) and increased the number of new entrance holes on test trees (Table 1). In the middle of June, the flight activity was moderate and increased gradually due to the emergence and migration of the beetles' first generation from test trees until the end of June. The breeding success and the development of young beetles were favored by the hot, rain-free period between 8 and 27 June 2007 (Figure 1). Consequently, new attacks were recorded on test trees at the end of June and at the beginning of July (Table 1). Beetle-flight activity reached its second maximum in the middle of July, which coincided with hot weather and the attraction of the second generation of bark beetles. The flight activity decreased in August and ended in the first week of October (Figures 1 and 2). On two trees, the attack was averted in time, and no galleries were formed. One strongly attacked tree died at the end of June.

Antioxidative response to progressive bark beetle attack

In the present investigation, the sequence of antioxidative responses in the phloem tissue of trees attacked by bark beetles was evaluated in comparison with the tissue of unaffected (control) trees. Continuously increasing concentrations of tGSH and total cysteine were determined in the phloem tissue of control trees during the sampling period (Figures 3 and 4). The total ascorbic acid increased significantly, by almost 40%, on the second sampling date, and the ascorbate pool became more oxidized. The concentrations remained almost constant during the next sampling dates, whereas the redox state became more reduced (Figure 5). In control trees, a similar trend was determined for total phenolics, which increased by 30% on the second sampling date and remained almost constant until the end of the sampling period (Figure 6).

In affected trees, a sequence of events can be observed from the initial attack to optimal colonization of bark beetles' first generation. On 26 April 2007, concentrations of tGSH and total cysteine dropped slightly below the levels of the unaffected samples and the glutathione redox state shifted toward a more oxidized value due to the initial bark beetle colonization (Figures 3 and 4). Similarly, the total ascorbic acid concentration dropped by 22% below the control levels and the concentration of dehydroascorbic

Table 1. Number of entries and degree of attack by *I. typographus* during the sampling period 2007, monitored on east- and west-facing halves of the trees between 1.5 and 2.5 m above ground.

Tree no.	Number of entries by *I. typographus*								Degree of attack[1]
	16.04.	18.04.	03.05.	10.05.	28.05.	11.06.	05.07.	11.07.	
1	8	8	8	8	9	9	10	10	+
2	5	8	10	27	52	75	83	152	++
3	5	8	8	13	14	15	19	39	++
4	1	1	3	3	3	6	8	8	+
5	2	2	2	2	3	4	5	5	+
6	8	9	9	9	12	14	15	18	++
7	4	6	6	11	17	19	19	19	++
8	2	2	2	2	2	4	5	7	+

[1]Degree of attack: +, moderate attack (isolated entry holes, no or only very short galleries of the adult beetle); ++, strong attack (many entry holes, complete galleries of the adult beetle and larval galleries).

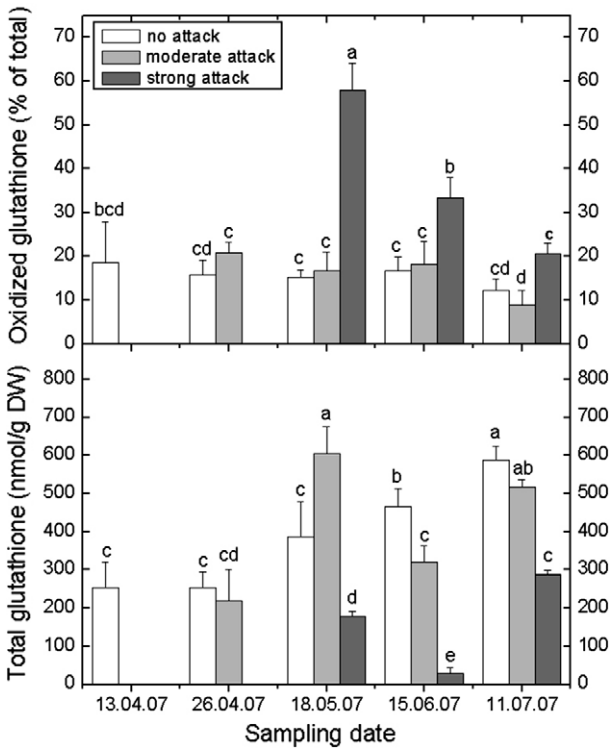

Figure 3. Concentrations of tGSH and oxidized glutathione (% of total) in the phloem tissue of unattacked trees and trees which had been attacked to varying degrees by *I. typographus* during the period of sampling. Each value is the mean of eight independent tree samples ± SD. Different letters (a–e) indicate statistically significant differences ($P < 0.05$) between control and affected trees. For the degree of attack, compare Table 1.

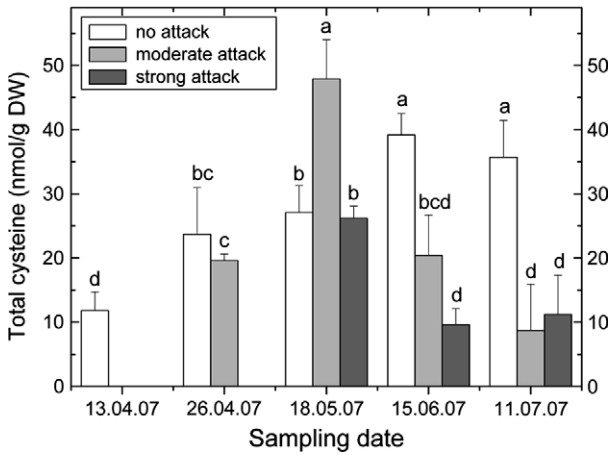

Figure 4. Total cysteine concentrations in the phloem tissue of unattacked trees and trees which had been attacked to varying degrees by *I. typographus* during the period of sampling. Each value is the mean of eight independent tree samples ± SD. Different letters (a–d) indicate statistically significant differences ($P < 0.05$) between control and affected trees. For the degree of attack, compare Table 1.

acid increased (Figure 5). This slight degradation and oxidation of glutathione and ascorbate pools was accompanied by a higher concentration of total phenolics (Figure 6).

On 18 May 2007, the antioxidative shifts after moderate attack differed quantitatively from the reaction after massive colonization. Total glutathione increased by 56% and total cysteine by 77% in moderately affected bark (Figures 3 and 4). The accumulation of thiols was accompanied by a slight increase in total ascorbic acid (Figure 5) and total phenolic contents (Figure 6). Furthermore, moderately affected bark exhibited a more reduced ascorbate pool (Figure 5), whereas the glutathione redox state remained unaffected (Figure 3). In contrast, massive bark beetle colonization had caused a 53% decrease in tGSH content, while the percentage of oxidized glutathione had risen nearly to 60% (Figure 3). Total phenolic contents were also diminished (Figure 6), whereas total cysteine and total ascorbic acid remained at control levels (Figures 4 and 5).

On 15 June 2007, the first young beetles emerged from the barks of severely affected trees, which were then removed after sampling. Some trees had already been affected by the second bark beetle colonization, as fresh entrance holes were observed (Table 1). Dependent upon the intensity and the success of the attack, a progressive degradation of antioxidants was determined, which was accompanied by the oxidation of the glutathione pool. In the moderately affected bark, the concentration of tGSH decreased by 30% (Figure 3), while total cysteine was lowered by 50% (Figure 4). Total ascorbate contents were moderately raised, whereas the redox states of ascorbate and glutathione remained equal to those of controls (Figures 4 and 5). The moderately increased concentrations of total phenolics in May eased until June (Figure 6). The severely affected tissue was characterized by very strong decreases in tGSH (−94%) and total cysteine (−75%) contents, which were accompanied by a strong increase in the percentage of oxidized glutathione (Figures 3 and 4). Total ascorbic acid was also lowered, while the percentage of dehydroascorbic acid remained unchanged (Figure 5). Furthermore, a 76% degradation of total phenolics was characterized as at an advanced disease stage (Figure 6).

On 11 July 2007, the concentration levels of tGSH in moderately affected bark remained still under control levels, although the concentrations were higher than in June. This moderate increase was accompanied by a more reduced redox state of glutathione (Figure 3). Total cysteine concentrations remained strongly diminished (Figure 4), whereas total ascorbic acid and total phenolics had accumulated by more than 70% and 20% (Figures 5 and 6). In severely affected trees, tGSH and total cysteine had decreased by 51% and 68% (Figures 3 and 4), but a more or less restored glutathione redox balance was detectable. Likewise, total phenolic levels dropped significantly after massive bark beetle colonization (Figure 6), whereas total ascorbic acid had accumulated by 35% (Figure 5).

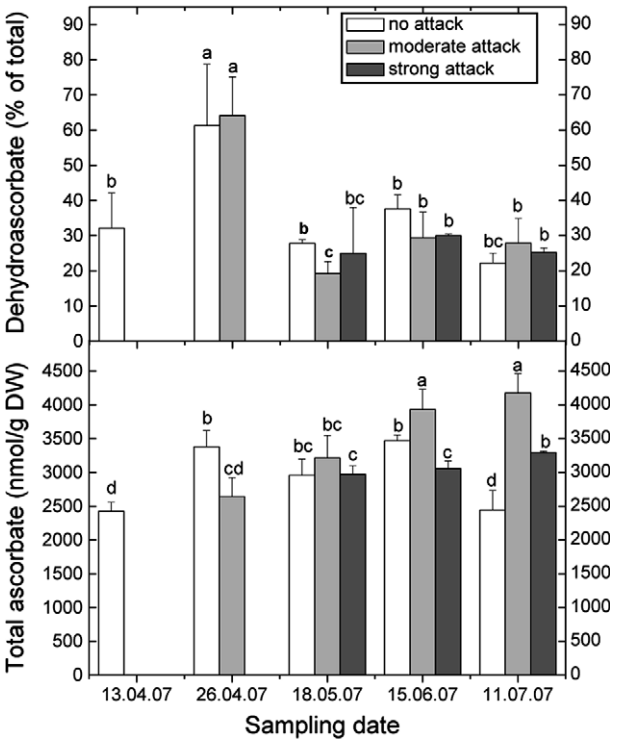

Figure 5. Concentrations of total ascorbate and dehydroascorbate (% of total) in the phloem tissue of unattacked trees and trees which had been attacked to varying degrees by *I. typographus* during the sampling period. Each value is the mean of eight independent tree samples ± SD. Different letters (a–d) indicate statistically significant differences ($P < 0.05$) between control and affected trees. For the degree of attack, compare Table 1.

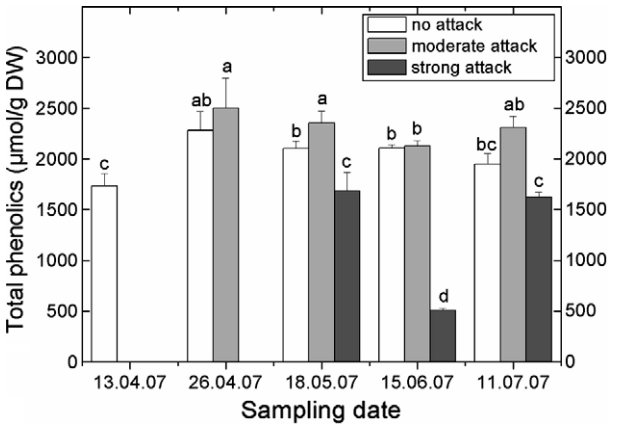

Figure 6. Total phenolic contents in phloem tissue of unattacked trees and trees which had been attacked to varying degrees by *I. typographus* during the period of sampling. Each value is the mean of eight independent tree samples ± SD. Different letters (a–d) indicate statistically significant differences ($P < 0.05$) between control and affected trees. For the degree of attack, compare Table 1.

Discussion

In this study, a temporal analysis of antioxidant contents was performed from initial attack to optimal colonization of Norway spruce with regard to weather conditions, activity period and colonization by bark beetles. The initial reaction of Norway spruce to bark beetle attack 2 weeks after exposure to bark beetles resulted in a slight but insignificant decrease in total cysteine and tGSH. The glutathione redox state shifted toward a slightly more oxidized value. High temperatures in the middle of May contributed to increased flight activity. Consequently, fresh attacks on test trees and light drought stimulated the antioxidant defense system of the Norway spruce trees. Thus, in the middle of May, obvious accumulations of both total cysteine and tGSH were detected in the bark after a moderate bark beetle attack. Most likely, this increase was initially triggered by the glutathione redox state itself or by increased production of reactive oxygen species or both (Noctor et al. 2002, Tausz et al. 2004, Šircelj et al. 2005, Noctor 2006). On the other hand, tGSH decreased drastically in strongly affected trees. The glutathione redox state shifted toward a more oxidized value, whereas total cysteine remained at control levels.

These results clearly showed that the antioxidant shifts, after a successful defense characterized by moderate attack, differed quantitatively from the reaction after a strong bark beetle attack. Similarly, quantitative differences within moderately and strongly affected bark were also reported for the main protein amino acids and carbohydrates (Rohde et al. 1996). Furthermore, the relationship of breeding success to tGSH was previously discussed by Mattanovich et al. (2001). Trees with high breeding success had about 50% less tGSH than trees with low breeding success, although sulfur contents did not show significant changes. Similarly, in our study tGSH had decreased by 30% in moderately affected bark and by 94% in severely affected tissue. It is assumed that SO_4^{2-} reduction in the roots, and its allocation in the form of cysteine to the trunk, is necessary to meet the demand of reduced sulfur in the phloem tissues of the trunk (Blaschke et al. 1996, Mattanovich et al. 2001). Interestingly, no transport of glutathione, cysteine and γ-glutamylcysteine was found to occur from needles into the basipetal section of the stem, although the reduction in SO_4^{2-} is a light-dependent process that takes place predominantly in mature leaves (Brunold 1990).

In the middle of July, the antioxidant shift within moderately affected samples indicated a successful defense reaction. It was characterized by acclimation of an antioxidant system, which was noticed by a higher accumulation of tGSH and a more reduced redox state of glutathione. Upon massive bark beetle attack, tGSH values were higher in the middle of July than in June and glutathione redox balance was more or less restored, but the antioxidant responses were insufficient and the glutathione system had not reached a new steady state. These events correspond

to the general stress–response concept suggested by Larcher (2003) and Tausz et al. (2004).

The total ascorbic acid underwent similar temporal changes as to tGSH. The ascorbate–glutathione cycle, which uses reduced glutathione as an electron donor to regenerate ascorbate from its oxidized form, dehydroascorbate, is considered the main pathway for reactive oxygen species' removal from the chloroplasts (Noctor and Foyer 1998, Noctor et al. 2002, Noctor 2006). Thus, the increased rates of reactive oxygen species production during bark beetle attack lead to an increased load on the ascorbate–glutathione cycle. Two weeks after the initial bark beetle attack, total ascorbic acid dropped below the levels of unaffected samples and the concentration of dehydroascorbic acid increased. Between May and July in lightly attacked phloem tissues, total ascorbic acid increased continuously to about 70% in July. In mass-attacked trees, the total ascorbic acid concentrations dropped below control levels in June and reached a new steady state, with a 35% increase in July. To our knowledge, there is no information about the ascorbate levels in affected bark as yet, but similar slightly higher average ascorbate levels were found within previous and current years' needles of mass-attacked trees on the Pohorje location, by Ribarič Lasnik et al. (1999).

Among antioxidants, phenolics represent a more important component of the inducible defense strategy with regard to conifer bark. For the synthesis and accumulation of phenolic compounds, the barks of all conifer families have axial phloem parenchyma cells, referred to as PP cells (Krekling et al. 2000, Franceschi et al. 2005). Within their vacuoles, PP cells contain variable amounts of phenolic bodies that are thought to serve as antifeedants and as antifungal agents (Beckman 2000). Polyphenolic parenchyma cells are also a major site for the storage of starch and lipids (Krekling et al. 2000). In this capacity, they can be seen as a target for beetles and fungi, and constitutive phenolics can be hypothesized to protect the cells themselves, as well as prevent fungal penetration toward the cambial zone. In any case, the multiple layers of PP cells between which are the crushed remnants of the sieve cells can be viewed as providing physical and chemical resistance to penetration of the bark (Franceschi et al. 2000*a*, 2000*b*, 2005, Schmidt et al. 2005). It is unfortunate that the temporal gradation of phenolics during the successful bark beetle attack has not been extensively studied, as yet. The increase in phenolic concentration 2 weeks after bark beetle attack can be recognized as an immediate inducible response to a bark beetle attack. Additionally, phenolics as well as ascorbic acid accumulated, to a higher extent, in the control tissue, which can be explained as a wound reaction induced by mechanical injury at the first sampling date (Christiansen et al. 1999, Franceschi et al. 2005, Ralph et al. 2006). The increase in total phenolics during the initial bark beetle colonization was accompanied by the degradation of total ascorbic acid and tGSH, as well as oxidation of the ascorbate–glutathione pool. These initial events pointed out that antioxidant defense is a multitiered system with a spatial and temporal component. We can presume that, in both cases, the phenolics are favored against ascorbate and thiols. The spatial component is determined by the positions of PP cells' concentric rings from the periderm surface to the cambial zone analogous to concentric castle defenses (Franceschi et al. 2005). The temporal component of the system consisted of seasonal, continuous and enhanced production of phenolics in the attacked tissue. However, at later sampling dates, the trees with strong attack were characterized by drastically decreased concentrations of total phenolics. Since the PP cells are the primary sites of phenolic biosynthesis in the secondary phloem (Franceschi et al. 2000*a*, 2000*b*, 2002, 2005), an assumption can be made that the synthesis of phenolics is lacking, when the phloem is damaged with the establishment of a complete brood system.

Despite the clear-cut results of this study, the antioxidant response needs to be evaluated regarding not only the degree of bark beetle attack, but also with respect to the weather conditions. We can assume that the strong deterioration of the antioxidant system in June can be linked to the establishment of a complete brood system, and also a hot, rain-free period between 8 and 27 June 2007. During drought stress, plants employ stomatal closure to minimize water loss. This limits the availability of CO_2 and promotes photo-oxidative stress in the chloroplasts (Tausz et al. 2004). It has been recently reported that drought influenced the secondary chemistry of the bark (Turtola 2005) and antioxidant defense processes in the needles of Norway spruce (Tausz and Grill 2000, Tausz et al. 2004). However, only a short-term antioxidative response to mild drought has been studied for coniferous trees, as yet. It was characterized by an increase in the ratio of oxidized to reduced glutathione as the first symptom in the antioxidative system of spruce needles (Tausz and Grill 2000, Tausz et al. 2001, 2004). In the present results, a deterioration of antioxidants during the rain-free period in June was followed by an acclimation reaction within moderately affected bark in the middle of July. This acclimation phase was characterized by increased levels of total phenolics and total ascorbic acid in July. A slight increase in tGSH was also measured when compared to concentrations determined in June, although the levels remained below the control. This adaptive response, which can be seen during moderate stress, may include, not only damage repair mechanisms (antioxidative scavenging of toxic oxygen species), but also mechanisms to avoid water loss (osmolyte accumulation) and the protection of those cellular components induced by qualitative and quantitative changes of the pigments (Tausz et al. 2004, Šircelj et al. 2005). If acclimatory responses are too slow or too weak, these multiple stress factors deplete the antioxidative system, and the balance between oxidative load and scavenging tips toward degradation. Advanced bark beetle attack is, therefore, characterized by the progressive oxidation and degradation of the glutathione and

ascorbate pools, as well as deterioration of phenolic compounds, followed by tree death. The same sequence of events in the antioxidative system, as described above, was also reported for apple trees subjected to progressive drought. First, the glutathione redox state decreased significantly after 10 days, followed by an increase in tGSH and total ascorbic acid concentrations after 15 days. Later on, when the stress became more intense, degradation and further oxidation of glutathione and ascorbate was observed (Šircelj et al. 2005).

In the present experiment, the changes in the antioxidative system during bark beetle attack have been pointed out as a dynamic process with a temporal and spatial component. It was triggered depending on both the intensity of attack and the weather conditions. In general, the initial response led to acclimation during a successful defense marked by the intensification of the antioxidant contents. If the pressure of the beetles became very high and was favored by a dry weather period, then the deterioration processes soon led to tree death. This strengthens the generally well-accepted hypothesis that antioxidants follow the general stress–response concept, as suggested by Larcher (2003) and Tausz et al. (2004).

Acknowledgments

This research was funded by Slovenian Research Agency (ARRS, Z1-9602). The author thanks Prof. Dr. Božidar Krajnčič, Chair of the Department of Biology and Plant Physiology, for valuable suggestions during the research. Mag. Ignac Vučko and Peter Kramer from the Maribor University Agriculture Centre, as well as Alojz Pucko from the Slovenian Forest Service, are acknowledged for permission to use the experimental field and support during the experimental work. The author also thanks Katja Urbanek, Robi Gjergjek, Jožica Korez, Boris Sapač and Metka Visočnik for field assistance. Prof. Dr. Maria Müller, Prof. Dr. Günther Zellnig, Dr. Bernd Zechmann, Dr. Edith Stabentheiner and Dr. Astrid Wonisch from the Institute of Plant Sciences, University of Graz, are also acknowledged for effective collaboration.

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