Genetics and Plant Physiology – 2016, Volume 6(3–4), pp. 167–175

Research Article

CHANGES OF SOME OXIDATIVE STRESS MARKERS IN BEECH LEAVES AFTER *ORCHESTES FAGI* INFESTATION

Lamlom S.[1], S. Georgieva[2], P. Dimitrova-Mateva[3], S. Anev[4], N. Tzvetkova[4], G. Chaneva[5]*

[1]*Faculty of Natural Resources and Environmental Sciences, University of Omar Al-Mukhtar, Al Bayda, Libya*

[2]*B.Sc. student, University of Forestry, Sofia, Bulgaria*

[3]*South-West State Enterprise - Blagoevgrad, Ministry of Agriculture and Food, Bulgaria*

[4]*Department of Dendrology, University of Forestry, Sofia, Bulgaria*

[5]*Department of Plant Physiology, Sofia University "St. Kl. Ohridski", Sofia, Bulgaria*

Received: *13 October 2016* Accepted: *04 November 2016*

Summary: The present work focused on the influence of leaf-mining weevil (*Orchestes fagi* L.) on the leaves of common beech (*Fagus sylvatica* L.). The study investigated the changes of the oxidative stress markers malone dialdehyde (MDA), total peroxides and free proline in beech trees located at two different altitudes (680 m asl and 1400 m asl) at the Petrohan Training and Experimental Forest Range, Western Balkan Mountains. Levels of infestation as well as the studied parameters were highly dependent on the location. The conditions at low altitude were favorable for the rapid development of *O. fagi* which resulted in significant changes of leaf growth and physiology. The peak of the invasion was reached in May – June, 2015. Malone dialdehyde was the most reliable oxidative marker among the examined parameters. The heavily damaged leaves at 680 m asl contained higher amount of MDA – 24% - 35% more than that in the leaves at the higher altitude. Statistically significant correlation between content of MDA and degree of infestation was established. However, the increased level of lipid peroxidation was not accompanied by the corresponding enhancement of total peroxides. The damage caused by *O. fagi* invasion was also manifested by the increase of free proline content, especially in the leaves at 680 m asl. In general, the herbivory resulted in serious biochemical changes occurring in the leaves, indicating a shift in the oxidative status of the beech trees.

Keywords: *Fagus sylvatica*; *Orchestes fagi*; MDA; total peroxides; proline.

Citation: Lamlom S., S. Georgieva, P. Dimitrova-Mateva, S. Anev, N. Tzvetkova, G. Chaneva, 2016. Changes of some oxidative stress markers in beech leaves after *Orchestes fagi* infestation. *Genetics and Plant Physiology*, 6(3–4): 167–175.

---

*Corresponding author: gchaneva@abv.bg

INTRODUCTION

Herbivory is the consumption of plant materials by animals or insects. It is a crucial ecosystem process whose extensively established consequences on primary production and vegetation structure and composition, derived from the sort and strength of feeding. Various kinds of herbivory influence plant tissues, which in turn influence the primary production, translocation and buildup of photosynthates to variable extents (Fahra-Rehman et al., 2009). It was established that the herbivory produced damages of leaf area (4.8% - 32.5%) and diminished tree rings growth (25% - 35%) initiating a physiological stress produced by partitioning of reserves to defensive chemicals (Trotter et al., 2002). Plants reacted to herbivory by introduction of numerous defensive processes to deal with the attack. Knowledge of plant defense against insect herbivory is not merely significant from an evolutionary opinion, however, it is also helpful in recognizing the level of its influence on agriculture (Fahra-Rehman, 2009).

In general, plant defensive approaches can be classified into two groups - constitutive and induced defenses. Constitutive defenses are perpetually found in plants, whereas induced defenses are produced at (or mobilized to) the spot of attack when a plant is hurt. Both groups are recognized to enhance the defensive capability and efficiency of attacked plants in opposition to a broad variety of insect herbivores. Knowledge of plant biochemistry, insect functioning and profusion may consequently be useful in developing realistic tools for insect management (Thaler et al., 2001). The interaction between herbivores and biotic environment is mainly derived from plant-mediated mechanisms, including constitutive characters like alterations in anatomy and physiology of plants, or herbivore persuaded alterations in host biochemistry (Ode, 2006; Ohgushi, 2005).

Orchestes fagi L. is the most significant pest on beech leaves in Bulgaria. Beech weevil infestation can produce a destructive effect on forest trees and may significantly decrease the yield of wood. It was established that beech weevil larvae triggered major modifications in the activity of some enzymatic and non-enzymatic defense systems, and in the secondary metabolites' matter in adult common beech trees (Miteva et al., 2007).

There are complex and uncertain interactions between insect herbivores and their host plants. Several differences in plant structure and metabolism have been recorded, indicating the vulnerability to insects and environmental persistence of pathogens (Cory and Hoover, 2006). Usually, plants react to herbivory by different morphological, biochemical and molecular mechanisms, which are extremely dynamic (War et al., 2012).

Lately the oxidative responses of plants to pathogens and other environmental stresses have received increasing awareness. Particularly interesting is the suggestion that oxidative response as well takes place following herbivores' attack. (Bi and Felton, 1995; Huang et al., 2007; War et al., 2012). An augmented production of reactive oxygen derivatives has been detected in a variety of pathogen-infected tissues (Baker and Orlandi, 1995). It was stated that adverse conditions such as drought, air pollution and herbivore infestation could produce oxidative stress in beech (Peltzer and Polle, 2001). Polle et al. (2001) also recognized that stress responsiveness of beech leaves was highly reliant on their developmental and seasonal. Additionally, infection of plants via a variety of pests often causes enhanced protection.

The aim of the present study was to examine the response of beech trees to *Orchestes fagi* attack and the oxidative stress eventually occurring as a result of the insect's activity.

MATERIALS AND METHODS

Site description

The study was performed in a young common beech (*Fagus sylvatica* L.) forest (12-18-years-old), situated in the Western Balkan Mountains. The sample plots are situated at two different altitudes - 680 m asl and 1400 m asl.

Plant material

At least 15 leaves were sampled on each sample plots three times (in May, June and August) during 2015. Sun exposed leaves of similar development stage, canopy position and exposition were randomly selected according to field sampling protocol (Tausz et al. 2003). Leaf samples were taken on clear days from 11.00 h to 14.00 h solar time from representative tree individuals. The collected leaves were scanned with a portable scanner (Canon Lide 110, Canon Inc., Japan) at 600 dpi resolution in PNG color mode format. The measurements of the total leaf area and the damaged leaf area were performed with open source software ImageJ (NIH, Bethesda, MD, USA) following standard protocols (Davidson, 2011; Pullen and Cunningham, 2010). The levels of infestation were calculated as the ratio between the damaged leaf area and the total leaf area in percentage.

Biochemical analyses

Malone dialdehyde (MDA) content was measured according to Dhindsa et al. (1981) after trichloroacetic acid/thiobarbituric acid addition. The absorbance was read at 532 nm subsequent to subtraction of non-specific absorption at 600 nm. The concentration of MDA was calculated by means of an extinction coefficient of 155 $mM^{-1}cm^{-1}$. Total peroxides content was determined spectrophotometrically at 390 nm after the reaction of the leaf extract with 1 M KI, according to Loreto & Velikova (2001). Determination of the free proline levels was done according to Bates et al. (1973) after extraction in 3 % (w/v) aqueous sulfosalicylic acid. Proline quantity was estimated after reading the toluol fraction at 520 nm.

Data processing and statistics

At least 3 values per leaf were used for data processing. Due to a random distribution of the data the Kruskal-Wallis One Way Analysis of Variance on Ranks for determination of significant differences between groups was used. To isolate the group or groups that differ from the others, we utilized the All Pairwise Multiple Comparison Procedures (Dunn's Method).

The coefficient of determination (R^2) of 2-nd order polynomial regression was used for assessment of degree of relation between any studied biochemical parameters and the degree of infestation. Significance of R^2 was tested with F-test (ANOVA for regressions, d.f. = 17) and P-value.

RESULTS AND DISCUSSION

The analysis of changes in the degree of infestation during the growing season showed that the beech leaves at low altitude were much more affected by the feeding of *O. fagi* – there was a significant difference (P-value <0.001) between the rate of *O. fagi* attack at both examined altitudes (Fig. 1).

At 680 m asl, the peak of the weevil attack was reached in May and remained almost unchanged during the season.

At the upper sample plot the highest degree of *O. fagi* invasion was registered in June, due to the delayed vegetation at this altitude. In August there was a slight decrease (statistically insignificant) in the rate of infestation.

The response of beech trees to *O. fagi* infestation was estimated by the changes in the level of lipid peroxidation, total peroxides and free proline content in the leaves. MDA formation is extensively utilized like a general indicator of the degree of oxidative damage as a result from oxidative stress. As was previously found by Bi and Felton (1995), herbivory produced a considerable rise in lipid peroxidation and ·OH formation. Hydroxyl radicals and singlet oxygen can react with lipids and form lipid peroxy radicals and hydroperoxide (Hollosy, 2002).

The attack of *O. fagi* on beech trees led to an augmented MDA accumulation in the leaves, which confirmed the development of free radical reactions and membrane damages in plants. Leaves of beech trees grown at the lower altitude contained enhanced MDA content compared with these at the higher altitude (P-value = 0.012) (Fig. 2). This trend was exceptionally distinct in the start of the vegetation period when MDA content in the leaves at the lower altitude was almost 4.5 times higher. The highest MDA values were recorded in June in trees in both locations.

The alterations of the amount of MDA in the early period of *O. fagi* attack could be translated as an escalation of lipid peroxidation rate since the antioxidative systems in the leaves have not yet been activated. Comparable outcomes were

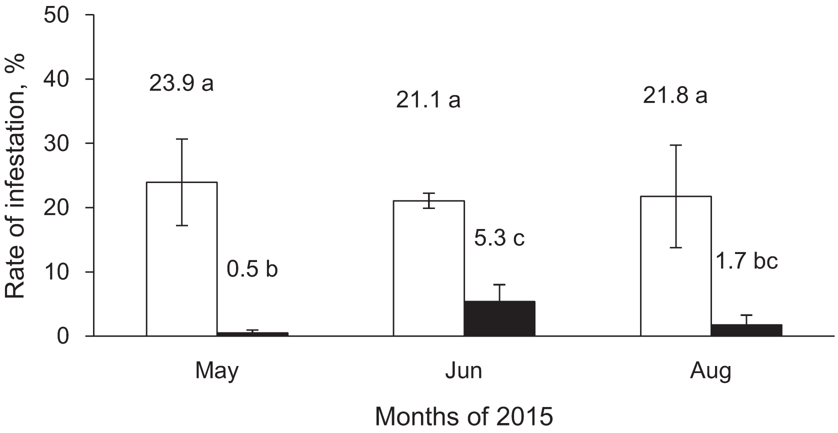

Figure 1. Rate of *Orchestes fagi* L. infestation (%) of beech leaves in the sample plots during 2015 (□ – 680 m als, ■ – 1400 m asl). The mean values ± standard error are shown.

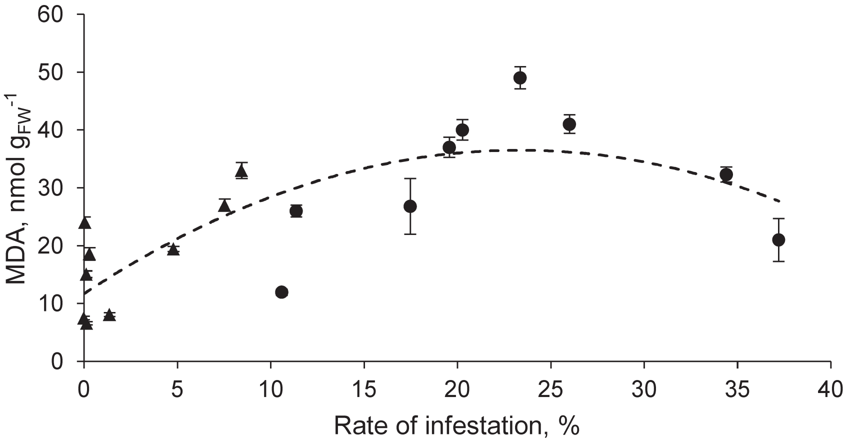

Figure 2. Changes in MDA content (nmol g $^{-1}$FW) in beech leaves in relation to the rate of *Orchestes fagi* L. infestation (%). (● – 680 m als, ▲ – 1400 m asl), $R^2 = 0.59$, P-Value < 0.001, d.f. = 17.

stated by Huang et al. (2007). In general, MDA itself was the parameter showing the highest correlation with the rate of infestation ($R^2 = 0.59$).

Variations in total peroxides' levels were investigated as a valuable indicator of oxidative damages in plant tissues. H_2O_2, in particular, is documented to be produced during normal processes of electron transfer chains in chloroplasts and mitochondria, however its overproduction persuaded by reactive oxygen species and additional stressors leads to particular disorders in plant metabolism (Foyer et al., 1994). Furthermore, H_2O_2 is regarded as one of the essential components of signal transduction in abiotic stresses in plants (Breusegem et al., 2001; Kumar et al., 2012).

A significant difference (P-value <0.001) between total peroxides' levels in the leaves from beech trees at 680 m asl and those at1400 m asl was found (Fig. 3).

The leaves of beech trees situated at the higher altitude showed enhanced levels of total peroxides (0.41 – 0.74 μmol g^{-1} FW), which was additional evidence strengthening the hypothesis that oxidative stress occurred after *O. fagi* infestation. However, such elevated levels of peroxides were not found in the low-altitude leaves, which would have corresponded to the registered MDA increase (Fig. 2). That parameter was permanently reduced (almost exponentially) with the increase of the rate of infestation. There was a weak correlation, which could be regarded as an indirect evidence of the declined superoxide dismutase (SOD) activity in low-altitude leaves, given that H_2O_2 is the product of the enzyme action. The same foundation could also clarify the reinforced catalase activity under the illustrated circumstances (Stefanova et al., 2016). These results supported our earlier conclusions that hydrogen peroxide, considered as an oxidative stress marker, reacted to a much lesser degree than MDA did (Chaneva and Petrova, 2014). Consequently, more research is needed on the enzyme antioxidative defense system in beech plants damaged by *O. fagi* feeding.

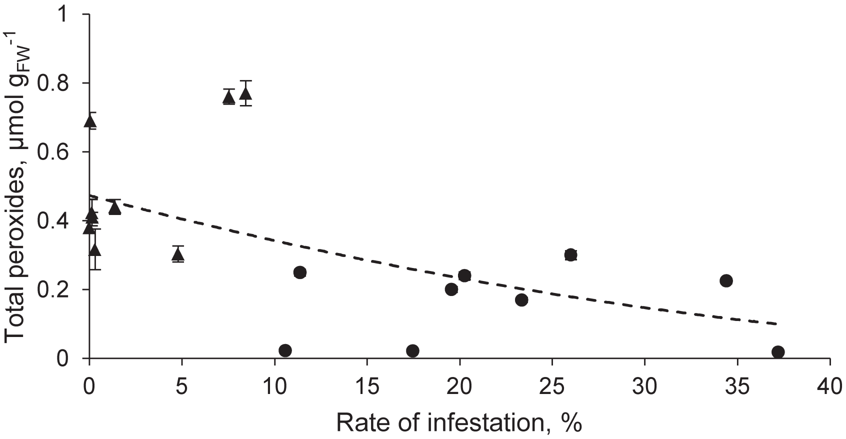

Figure 3. Changes in the content of total peroxides (µmol g $^{-1}$FW) in beech leaves in relation to the rate of *Orchestes fagi* L. infestation. (● – 680 m als; ▲ – 1400 m asl), $R^2 = 0.32$, P-Value = 0.048, d.f. = 17.

Free proline has been proved to perform a significant role in improving environmental stress in plants (Hong et al., 2000; Larher et al., 2003). In many plants, proline accumulates as a result of the imposition of a broad extent of biotic and abiotic stresses. Argument has surrounded the degree to which this change in nitrogen metabolism profits plants under unfavorable environmental situations. Most efforts to justify the phenomenon have concentrated on the capability of proline to mediate osmotic adjustment, stabilize subcellular structures and track free radicals (Hare and Cress, 1997). It recognized that plants' response to insect attacks and various abiotic stresses incorporate alterations in their cellular metabolism, in addition to the development of a variety of defense mechanisms (Mehdy, 1994) such as proline accumulation (Mahdavian et al., 2008). Proline influences the stability of some cytoplasmic enzymes, whose roles and kinetics could be disturbed due to the applied stress factors.

Based on our findings, the damage caused by *O. fagi* invasion was clearly visible in the increase of free proline content in the beech leaves. The increase of proline levels followed the vegetation season; it was higher in June and August, especially in the trees at the lower location (Fig. 4). The highest proline accumulation was measured at the end of the growing period – 0.91 µmol g^{-1} FW at 680 m asl and 0.4 µmol g^{-1} FW at 1400 m asl, which could be related to leaf aging. The enhancement of free proline content was higher in the heavily damaged leaves at the lower altitude. In general, there was a slight trend of free proline enhancement that followed the beech weevil's invasion.

Similar results (Farha-Rehman, 2008) demonstrated that in damaged maize leaves, the insect attack led to a proportionate loss of leaf proteins and an expansion in proline accumulation. The rise of nitrogen, stored as accumulated proline and valine residues, was detected to promote insects during drought stress (Haglund, 1980). It is central to record that the insect feeding changed the nitrogen

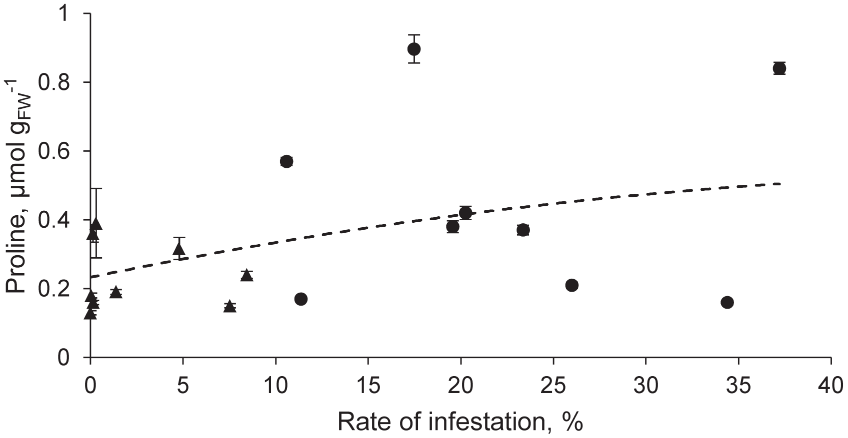

Figure 4. Changes in free proline content (µmol g $^{-1}$FW) in beech leaves in relation to the rate of *Orchestes fagi* L. infestation. (● – 680 m als; ▲ – 1400 m asl), $R^2 = 0.18$, P-Value = 0.228, d.f. = 17.

balance. Numerous proteins promoted in tomato foliage following herbivore damage were connected to decreases in herbivore functioning (Thaler et al., 2001).

Furthermore, oxidative changes have significant implications not only as a stress reaction of the tree plant, but as well as ways of resistance against the insect. It was stated that the ROS-mediated stress in the host plant corresponds to the augmented oxidative damage of the insects (Bi and Felton, 1995). This entails that the stress response can have a double significance - to reduce the herbivory effect and to raise the oxidative injury in the plant leaves.

The findings of our present work illustrated a major increase in MDA and free proline content in the highly damaged leaves from trees grown at the lower altitude. These changes could be considered as an indicator of oxidative damage resulting from the feeding of *O. fagi*. Based on the statistical analysis data, MDA could be pointed out as the most reliable oxidative marker examined in the present study. Further observations will be necessary to clarify the involvement of enzymatic mechanisms of protection against oxidative stress that occurring after *Orchestes fagi* invasion.

ACKNOWLEDGEMENTS

This work was supported by grant 93/2015 of the University of Forestry, Sofia, Bulgaria.

REFERENCES

Baker C, E Orlandi, 1995. Active oxygen in plant pathogenesis. Annual Review of Phytopathology, 33: 299–321.

Bates L, R Waldern, I Teare, 1973. Rapid determination of free proline for water stress studies. Plant. Soil., 39: 205–208.

Bi J, G Felton, 1995. Foliar oxidative stress and insect herbivory - primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. J. Chem. Ecol., 21(10): 1511–1530.

Breusegem FV, E Vranova, JF Dat, D Inze, 2001. The role of active oxygen species in plant signal transduction. Plant Sci., 161: 405–414.

Chaneva G, D Petrova, 2014. Effect of method of applying the herbicide paraquat on pea and maize. Oxid Commun., 37(4): 1090–1102.

Cory J, K Hoover, 2006. Plant-mediated effects in insect–pathogen interactions. Trends Ecol. Evol., 21(5): 278–286.

Davidson A, 2011. Measuring leaf perimeter and leaf area. In: PrometheusWiki. http://www.publish.csiro.au/prometheuswiki/tiki-pagehistory.php?page=Measuring leaf perimeter and leaf area&preview=27

Dhindsa R, P Plumb-Dhindsa, T Thorpe, 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot., 32(1): 93–101.

Fahra-Rehman, FA Khan, SB Anis, SM Badruddin, 2009. Plant defenses against insect herbivory. – In: Ciancio A., KG Mukerji (eds), Integrated Management of Arthropods and Insect Borne Diseases, Chapter 8: 185–204.

Foyer CH, P Descourvières, KJ Kunert, 1994. Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. Plant Cell Environ., 17: 507–523.

Hare P, W Cress, 1997. Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regul., 21(2): 79–102.

Hollosy, F, 2002. Effect of ultraviolet radiation on plant cells. Micron, 33:179–197.

Hong Z, K Lakkineni, Z Zhang, D Verma, 2000. Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol., 122: 1129–1136.

Huang W, J Zhikuan, H Qingfang, 2007. Effects of herbivore stress by *Aphis medicaginis* Koch on the malondialdehyde contents and activities of protective enzymes in different alfalfa varieties. Acta Ecol. Sin., 27: 2177–2183.

Kumar R, S Sharma, K Gadpaule, K Singh, R Sivaranjani, S Goswami, R Rai, 2012. Mechanism of action of hydrogen peroxide in wheat thermotolerance - interaction between antioxidant isoenzymes, proline and cell membrane. Afr. J. Biotechnol., 11(78): 14368–14379.

Larher F, A Gibon, P Trotel-Aziz, R Sulpice, A Bouchereau, 2003. An assessment of the physiological properties of the so-called compatible solutes using *in vitro* experiments with leaf discs. Plant Physiol. Biochem., 41: 657–666.

Loreto F, V Velikova, 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol., 127: 1781–1787.

Mahdavian K, M Ghorbanli, K Kalantari, 2008. The effect of ultraviolet radiation on the contents of chlorophyll, flavonoid, anthocyanin and proline in *Capsicum annuum* L. Turk. J. Bot., 32: 25–33.

Mehdy M, 1994. Active Oxygen Species in Plant Defense against Pathogens. Plant Physiol., 105: 467-472.

Miteva L, P Dimitrova, S Ivanov, E Ivanova, P Kershev, I Sergiev, D Ovcharov, V Alexieva, 2007. Beech weevil (*Orchestes fagi* L.) affects antioxidant defence of *Fagus sylvatica*. Poster session at international workshop “Plant Biology & Botany 2007”, 07-12 July 2007, Chicago, Illinois.

Ode PJ, 2006. Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. Annu. Rev. Entomol., 51: 163–85.

Ohgushi T, 2005. Indirect interaction webs: herbivore-induced effects through trait change in plants. Annu. Rev. Ecol. Syst., 36: 81–105.

Peltzer D, A Polle, 2001. Diurnal fluctuations of antioxidative systems in leaves of field-grown beech trees (*Fagus sylvatica*): responses to light and temperature. Physiol Plant., 111: 158–164.

Polle A, P Schwanz, C Rudolf, 2001. Developmental and seasonal changes of stress responsiveness in beech leaves (*Fagus sylvatica* L.). Plant Cell Environ., 24: 821–829.

Polle A. D Peltzer, P Schwanz, 2001. Resistance against oxidative stress in leaves of young beech trees grown in model ecosystems with different soil qualities, elevated CO_2 and lachnid infestation. Forstw. Cbl., 120: 1–7.

Pullen K, S Cunningham, 2010 Measuring area of colour patches (e.g. dead or damaged areas) on leaf surfaces. In: PrometheusWiki. http://www.publish.csiro.au/prometheuswiki/tiki-pagehistory.php?page=Measuring area of colour patches (e.g. dead or damaged areas) on leaf surfaces&preview=5

Stefanova N, S Georgieva, S Anev, P Dimitrova-Mateva, N Tzvetkova, M Zhiponova, G Chaneva, 2016. Changes of the antioxidative enzymes in the beech leaves after *Orchestes fagi* infestation. XXVI Scientific conference, Stara Zagora, 2-3 June, 2016, p. 64.

Tausz M, A Wonisch, D Grill, D Morales, MS Jimenez, 2003. Measuring antioxidants in tree species in the natural environment: from sampling to data evaluation. J. Exp. Bot., 54(387): 1505–1510.

Thaler JS, MJ Stout, R Karban, SS Duffey, 2001. Jasmonate mediated induced plant resistance affects a community of herbivores. Ecol. Entomol., 26: 312–324.

War A, M Paulraj, T Ahmad, A Buhroo, B Hussain, S Ignacimuthu, H Sharma, 2012. Mechanisms of plant defense against insect herbivores. Plant Signal Behav., 7(10): 1306–1320.