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Research paper

Mistletoe (*Viscum album*) infestation in the Scots pine stimulates drought-dependent oxidative damage in summer

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This study sought to contribute to the understanding of the detrimental effect of the mistletoe (*Viscum album* L.), a hemiparasitic plant, on the mortality of the Scots pine (*Pinus sylvestris* L.). Fieldwork was conducted in the town of Kelkit (Gumushane province, Turkey) from April to October in 2013. Pine needles of similar ages were removed from the branches of mistletoe-infested and noninfested Scots pine plants, then transported to the laboratory and used as research materials. The effects of the mistletoe on the Scots pine during infestation were evaluated by determining the levels of water, electrolyte leakage (EL), malondialdehyde (MDA, being a product of lipid peroxidation) and reactive oxygen species (ROS) such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). In addition, the activities of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) were measured in the same samples. The highest level of drought stress was found in summer (especially in August) as a result of the lowest water content in the soil and the highest average temperature occurring in these months. The drought stress induced by mistletoe infestation caused a regular decrease in water content, while it increased the levels of EL, MDA and ROS (H_2O_2, $O_2^{-\bullet}$ and $^{\bullet}OH$). The infestation also stimulated the activities of CAT and POX, with the exception of SOD. On the other hand, in August, when the drought conditions were the harshest, the levels of EL and MDA, which are two of the most important indicator parameters for oxidative stress, as well as the levels of H_2O_2 and $^{\bullet}OH$, which are two of the ROS leading to oxidative stress, reached the highest values in both infested and noninfested needles, whereas the $O_2^{-\bullet}$ level decreased. For the same period and needles, CAT activity increased, while SOD activity decreased. Peroxidase activity, however, did not exhibit a significant change. Our findings indicate that the increased mortality of the Scots pine may result from the mistletoe-induced very severe drought stress, and that the increase in the capacity of antioxidative enzyme system does not protect the plant against oxidative stress in dry summer seasons.

Keywords: antioxidant enzyme, oxidative stress, *Pinus sylvestris*, reactive oxygen species.

Introduction

Since the beginning of the last century, high mortality rates have been observed in the Scots pine (*Pinus sylvestris* L.) over large areas in Turkey. The decline of Scots pine populations in the forests of Turkey is not a local phenomenon (Catal and Carus 2011). High mortality rates of Scots pine have also been documented from other central European countries such as Italy (Vacchiano et al. 2012), Switzerland (Rebetez and Dobbertin 2004, Dobbertin et al. 2005, Eilmann et al. 2006), Austria (Cech and Perny 2000), Greece (Tsopelas et al. 2004) and France (Thabeet et al. 2009). Such mortality episodes in Scots pine populations have been attributed to a number of different agents, including drought stress (Erbilgin and Raffa 2002, Bigler et al. 2006, Vacchiano et al. 2012, Giuggiola et al. 2013), tree and herbaceous competition (Weber et al. 2008), and mistletoe infestation (Tsopelas et al. 2004, Dobbertin and Rigling 2006). Some researchers have suggested that drought stress is among the most important causes of the Scots pine decline in Europe, although the Scots pine is known to resist drought conditions (Oberhuber et al. 1998, Rigling et al. 2002, Allen et al. 2010).

Bigler et al. (2006) also exerted that the pine mortality risk increased in parallel with multiple years of drought.

Mistletoe (*Viscum album* L.) is an evergreen, perennial and parasitic flowering plant with functional chlorophyll and, therefore, is considered a hemiparasitic plant (Catal and Carus 2011). It has an adverse effect on the height and diameter growth of the host, inducing premature mortality. Furthermore, it also affects the quality and quantity of wood, reduces fruiting and exposes the host to the risk of being attacked by other agents such as insects or fungi (Varga et al. 2012). In connection with this, Reid et al. (1994) and Tsopelas et al. (2004) confirmed that the infested trees had significantly higher mortality than those where mistletoe had been removed. Most mistletoes have lower water use efficiency for photosynthesis than their hosts due to much higher transpiration rates and stomatal conductance, and lower leaf water potential (Mathiasen et al. 2008). Thus, mistletoe decreased the resistance of the host plant to drought stress (Glatzel and Geils 2009, Rigling et al. 2010). In such a case, one of the most unfavorable handicaps is the oxidative damages occurring due to the increase in reactive oxygen species (ROS) (Mutlu et al. 2011).

Reactive oxygen species such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) are also produced during the response of a plant to the stresses it suffers (Apel and Hirt 2004, Mutlu et al. 2009*a*, 2009*b*, 2011, Hu et al. 2010). The toxic levels of ROS cause detrimental metabolic damage ranging from membrane permeability (Dias et al. 2011) to the degradation of DNA, proteins and unsaturated membrane lipids (Bertrand et al. 2011, Kekec et al. 2013). In the scavenging of ROS, the antioxidative system, including enzymatic and nonenzymatic antioxidants, is the most important component. Antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) powerfully ensure the elimination of ROS in cells. Decreasing ROS level under stress has a critical role for living cells, because ROS can seriously disrupt normal metabolism through oxidative damage (Rout and Shaw 2001).

The role of the enzymatic antioxidative system of Scots pine during its response to mistletoe infestation when it is exposed to drought is still unclear due to limited data. This study investigates the effects of mistletoe infestation by evaluating some physiologic parameters such as the water content, electrolyte leakage (EL), malondialdehyde (MDA) and ROS, and the activities of antioxidative enzymes in Scots pine needles.

Materials and methods

Study site

Fieldwork was conducted from April to October in 2013 in the town of Kelkit (39°20′9″E; 40°14′41″N), located 70 km south of the province of Gumushane (Turkey) (Figure 1). The study site is situated at ~1650 m above sea level on a south-exposed slope with 50% incline and within the belt of vegetation

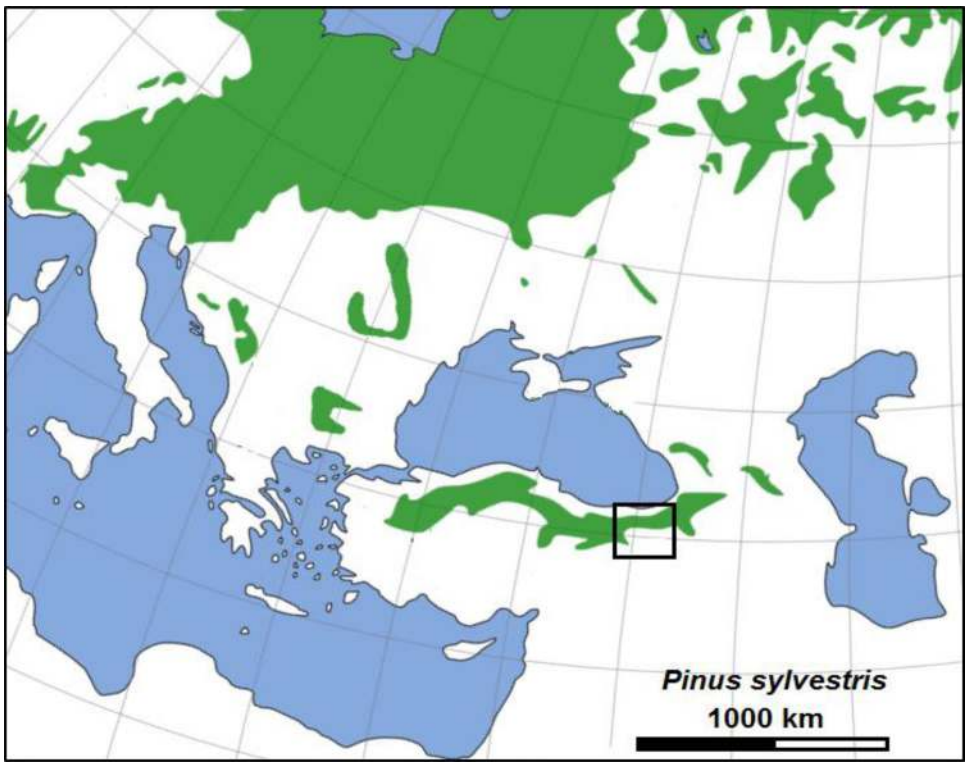

Figure 1. Distribution area of *P. sylvestris* in Europe (http://www.euforgen.org/) and study sites (square) in Gumushane (north of Turkey), near the southern distribution limit of the species.

dominated by *P. sylvestris* and *Quercus infectoria* forests. These forests host trees ranging from noninfested to severely infested by parasitic mistletoe plants.

Plant material and sampling

The collected samples of *P. sylvestris* and *V. album* were checked against the voucher specimens (*P. sylvestris*: 6358 and *V. album*: 4269) deposited in Erzincan University Herbarium at the Department of Biology, Science Faculty, Erzincan, Turkey. For the sampling in March 2013, eight neighboring trees (four noninfested and four mistletoe-infested trees) in the study site were selected (Table 1). We took special care to choose trees of similar age and crown structure, which was estimated by crown transparency assessment. Due to the similarity of the trees, a possible effect of an individual tree on growth response of its branches to mistletoe infection was regarded as negligible. Identical needles (1-year-old) of branches were harvested to determine water content, levels of EL, lipid peroxidation and content of ROS ($O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$), and the activity of antioxidant enzymes (SOD, CAT and POX) in the middle of each month (from April to October), and they were used as research material. For this, pine needles of similar age were removed from the neighboring, noninfested and infested plants, collected in a thermos bottle cooled with dry ice, and then quickly transported to our laboratory, where they were stored in a deep-freeze (−80 °C) until the analysis. All the collected needle samples from sample branches were washed with deionized water before the analysis in order to eliminate superficial contamination by dust. For all the below-mentioned analyses requiring homogenization, the samples were first ground and pulverized in a mortar and pestle in liquid nitrogen because of the fibrous and durable structure of the Scots pine needles, and then the following processes were carried out. In order to avoid an increase in the heat due to friction during the homogenization and centrifugation and any consequential activity loss, we preferred to grind in liquid nitrogen in the mortar and to use cooled centrifuge.

Meteorological data

Kelkit Meteorological Station, located 10 km away from the study site, provided the local air temperature data on a monthly basis (from April to October) in 2013. The water content of the soil was calculated on the basis of monthly mean precipitation data (Willmott et al. 1985).

Determining the water content of needles

Fresh Scots pine needles were sampled randomly and their fresh weights (FW) were recorded. Then, they were dried in an oven for 72 h at 65 °C in order to obtain dry weights (DW). The water content percentage of the needles was calculated using the following equation (Mutlu et al. 2011):

$$\text{Water content of needles (\%)} = \left[\frac{\text{FW} - \text{DW}}{\text{FW}}\right] \times 100$$

As the results of the subsequent analyses are presented as the quantity or activity per gram dry weight (g^{-1} DW), we also used the data attained through this formula during the calculation of each result given for other parameters.

Determining the level of EL

The membrane leakage of cells in the needles was measured according to Lutts et al. (1995). Fresh needles were cut into 2 cm lengths and rinsed in six changes of water to remove cellular proteins from the cut ends. The same weight of needles (0.1 g) was collected and placed into 20 ml of deionized water in a test tube and washed slowly at room temperature to remove solutes from both needle surfaces. The electrical conductivity (EC) of the samples was measured at various times using an EC meter. Samples were boiled for 30 min and then incubated at room temperature for 24 h in order to measure the maximum level of electrical leakage (EM) that could be attained through control. The conductivity (ion leakage value) of the solution in each tube was measured at room temperature with an EC bridge. The obtained ion leakage value ($\mu S\ cm^{-1}$) was used to express membrane injury (Mutlu et al. 2013). Electrolyte leakage (%) was expressed as EC/EM × 100.

Determining the level of lipid peroxidation

The level of MDA as a product of lipid peroxidation was measured with the method of Heath and Packer (1968) with slight modifications (Ananieva et al. 2002). Needle material (0.5 g) was homogenized in 3 ml of 0.1% trichloroacetic acid (TCA)

Table 1. Characteristics and infestation condition of the studied Scot pines (noninfested and infested with mistletoe) in different sites.

Site code	Altitude (m)	Pine code	Infestation condition	Mistletoe (number)	Diameter (cm)	Height (m)
S1	1653	Pine 1	Noninfested	1	34.3	17.6
		Pine 2	Infested	24	32.6	16.1
S2	1656	Pine 3	Noninfested	0	27.6	14.9
		Pine 4	Infested	15	26.9	13.8
S3	1650	Pine 5	Noninfested	2	24.1	11.2
		Pine 6	Infested	38	24.1	10.0
S4	1649	Pine 7	Noninfested	0	33.3	18.2
		Pine 8	Infested	19	34.6	17.2

and centrifuged at 15,000*g* for 30 min at 4 °C. One milliliter reagent [0.5% thiobarbituric acid (TBA) in 20% TCA, w/v] was added to a 0.5 ml aliquot of the supernatant. For a negative control, 0.5 ml of 0.1% TCA and 1 ml reagent were added. The test tubes were incubated at 95 °C for 30 min and then quickly cooled in an ice bath. After cooling and centrifugation, the absorbance of the supernatant at 532 nm was read and the value for the nonspecific absorption at 600 nm was subtracted. The level of MDA was estimated by using the mmol l^{-1} extinction coefficient of 155 mmol l^{-1} cm^{-1}.

Determining the content of $O_2^{-\bullet}$

The $O_2^{-\bullet}$ production rate was measured according to the method of Zhao et al. (2008) with slight modifications. One gram of the sample was homogenized in 4 ml of 65 mM potassium phosphate buffer (pH 7.8), and centrifuged at 5000*g* for 10 min. The incubation mixture contained 0.9 ml of 65 mM potassium phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylammonium chloride and 1 ml of supernatant. After incubation at 25 °C for 20 min, 17 mM aminobenzene sulfanilic acid and 7 mM 1-naphthylamine were added to the incubation mixture. After the reaction at 25 °C for a further 20 min, absorbance was read at 530 nm. A standard curve with $NaNO_2$ was used to calculate the production rate of $O_2^{-\bullet}$. The production rate of $O_2^{-\bullet}$ was expressed as ng g^{-1} DW (Esim et al. 2014).

Determining the content of H_2O_2

Hydrogen peroxide content was measured by monitoring the absorbance at 410 nm of the titanium peroxide complex as suggested by He et al. (2005). Supernatant (1 ml) obtained from cold acetone extraction was added to 0.1 ml of 20% titanium reagent and 0.2 ml of 17 mol ammonia solution. The solution was centrifuged at 3000*g* at 4 °C for 10 min and the supernatant was discarded. The pellet was dissolved in 3 ml of 1 mol sulfuric acid. The absorbance of the solution was measured at 410 nm. Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2 (Mutlu et al. 2011).

Determining the content of $^{\bullet}OH$

Hydroxyl radical content was determined as per Halliwell et al. (1987). Pine needles (250 mg) were incubated in 1 ml of 10 mM PO_4^{3-} buffer (pH 7.4) with 15 mM 2-deoxy-D-ribose at 37 °C for 2 h. An aliquot of 0.7 ml from the above-mentioned mixture was added to the reaction mixture (3 ml of 0.5% TBA, w/v, made in 5 mM sodium hydroxide) with a subsequent addition of 1 ml glacial acetic acid. The mixture was heated at 100 °C for 30 min, cooled and centrifuged at 10,000*g* for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for nonspecific absorbance at 600 nm. The content of $^{\bullet}OH$ was determined using $\varepsilon = 155$ mM^{-1} cm^{-1} and expressed as nmol g^{-1} DW (Ahuja et al. 2015).

Extracting the antioxidative enzymes

The fresh needles (0.5 g) were homogenized with a pestle and mortar in 0.2 M phosphate buffer (pH 7). The homogenate was centrifuged at 12,000*g* at 4 °C for 15 min. The supernatant was used to determine the activities of CAT, POX and SOD (Mutlu et al. 2009*b*, 2011).

Determining the enzyme activities

The enzyme activities in fractions were measured spectrophotometrically. Catalase (EC 1.11.1.6) activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) containing 20 mM H_2O_2. One unit of CAT activity was defined as the amount of enzyme that used 1 μmol H_2O_2 per minute (Upadhyaya et al. 1985). The POX (EC 1.11.1.7) activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1 mM guaiacol and 0.5 mM H_2O_2. One unit of POX activity was defined as the amount of enzyme that caused an increase in the absorbance of 0.01 per min (Upadhyaya et al. 1985). The SOD (EC 1.15.1.1) activity was estimated by recording the decrease in the optical density of nitro-blue tetrazolium dye by the enzyme (Beauchamp and Fridovich 1971). Three milliliters of the reaction mixture contained 2 μM riboflavin, 13 mM methionine, 75 μM nitro-blue tetrazolium chloride, 0.1 mM ethylenediaminetetraacetic acid, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.05 ml enzyme fraction. The reaction was started by adding riboflavin solution and placing the tubes under two 30-W fluorescent lamps for 15 min. A complete reaction mixture without enzymes, which gave the maximal color, served as a control. The reaction was stopped by switching off the light and putting the tubes in the dark. A nonirradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as the amount of enzyme that reduced the absorbance reading to 50% in comparison with the tubes lacking enzyme.

Statistical analysis

All experiments were designed in a randomized complete block. The samples were collected from four infested and four noninfested plants. For each experiment, three tissue samples were collected from each individual of both groups (a total of 12 samples for each group per experiment). Then the data obtained from the samples at random were used for statistical analyses ($n = 4$) and the average values are stated in the figures. The data (water content, EL, MDA content, ROS content and activities of antioxidative enzymes) were evaluated using a repeated-measures analysis of variance in SPSS 19.0 for Microsoft Windows. Repeated-measures analysis of variance was used to determine intergroup effects (infested or noninfested), within-subject effects and interactions between groups and time. Sphericity assumption was checked with Mauchly's test and

Gerainhause–Geisser P values were used. Statistical significance for all analyses was set at $P < 0.05$.

Results

In previous studies, the effects of both stresses (drought and mistletoe) were mostly studied separately on plants growing in a natural environment. However, no information, to our knowledge, is available in the literature about the effects of mistletoe infestation on the oxidative and antioxidative systems in the Scots pine under drought conditions. In an attempt to discover the possible effect mechanisms, the endogenous status of some key indicators of stresses such as MDA (an indicator of lipid peroxidation), the levels of EL and ROS ($O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$), and the activities of antioxidant enzymes (SOD, CAT and POX) were determined in mistletoe-infested Scots pines. The water content of the needles was also evaluated along with these parameters.

Effects of seasonal changes on environmental condition

Soil samples were taken from the plant root zones during sampling. The soil water content was measured (Figure 2). Although the soil samples were not collected from several meters deep and did not provide exact information about the water content of the soil, they presented significant clues about the seasonal change in the water level of the soil. The results from the soil demonstrate significant seasonal changes in the water content. The lowest water content in the soil was found in August (Figure 2). The average air temperature of the studied area gradually increased, while soil water content decreased consistently along side this increase. The low water content measured in August is a result of water evaporation in the soil due to the dry weather conditions. Both data showcase the same trend, with the most extreme drought values in August (Figure 2).

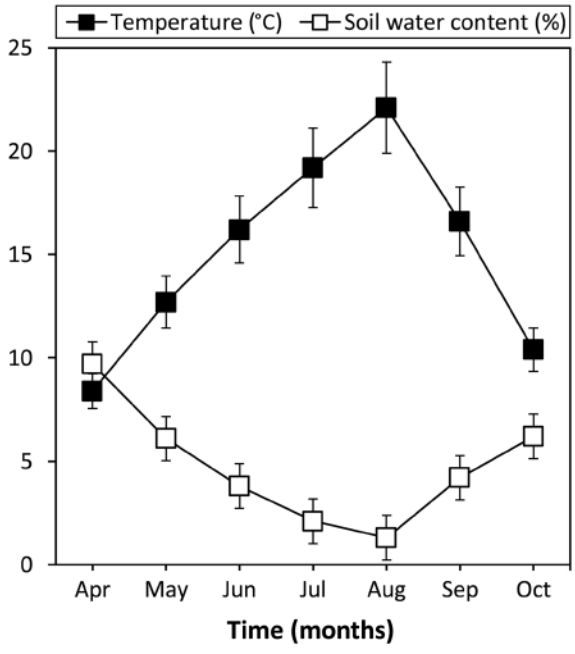

Figure 2. Monthly soil water content and average temperature data for the studied area (data obtained from the Turkish Meteorological Station Network). Data were obtained between April and October in 2013.

The effects of mistletoe-induced drought on needle water content

Average water content of the infested needles decreased ($P < 0.05$) steeply due to increasing water loss caused by the mistletoe. The water content of the infested plant needles decreased along with the deteriorating drought conditions (low soil water content and high air temperature) from May to August, while it increased from August to October (Figure 3). Wet days after a rain (at the beginning of September) increased the water content in the infested plant needles. However, the water content of the noninfested plant needles kept increasing slightly from April to October, with a fall in August (Figure 3). Seasonal changes in the noninfested plants were not statistically significant ($P > 0.05$) in terms of water content.

The effects of mistletoe-induced drought on EL and lipid peroxidation level

The parasitic mistletoe infestation alone increased the EL level in Scots pine needles in all months (from April to October) in comparison with noninfested plants (Figure 3). However, increasing ratios of EL level were statistically more significant ($P < 0.05$) in both noninfested and infested plants. Electrolyte leakage level gradually increased in parallel with the decrease in the water content of the Scots pine needles in summer (from June to August) (Figure 3). Both the MDA levels and the EC in the infested plants were higher in all months (from April to October) in comparison with the noninfested plants (Figure 3). The MDA level in the infested plants gradually increased in parallel with the increase in drought conditions (Figures 2 and 3).

The effects of mistletoe-induced drought on ROS

Superoxide anion content was reduced by the increase in the drought conditions from April to August in both noninfested and infested plants, while it increased from August to October (Figure 4). However, the content of $O_2^{-\bullet}$ in the infested plants was always higher than that in the noninfested plants (Figure 4). The content of H_2O_2 increased along with the increasingly severe drought conditions from May to August in the infested plants, while it decreased from August to October (Figure 4). However, the content of H_2O_2 in the noninfested plants gradually increased in parallel with the increasing drought conditions (from May to October) and it was always lower than the content of infested plants (from April to October) (Figure 4). Hydroxyl radical content of both the noninfested and infested plants gradually decreased inversely to the drought conditions (from May to October). The $^{\bullet}OH$ content of

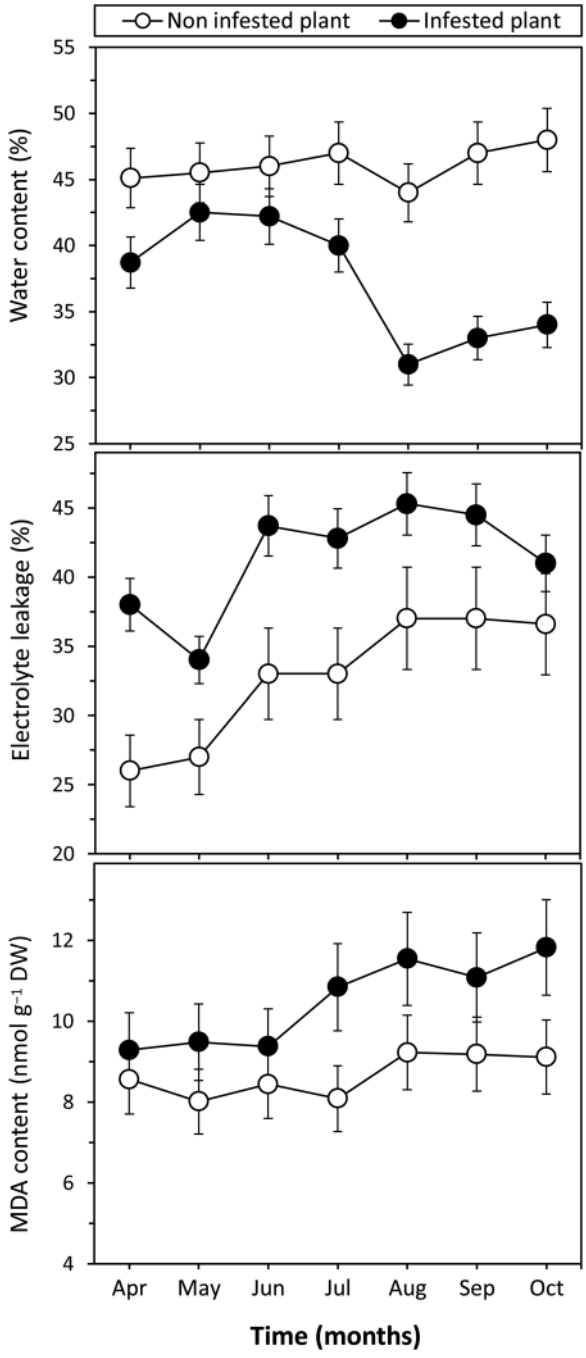

Figure 3. Monthly change in the water content (%), the level of EL (%) and the content of MDA in the needles of infested and noninfested *P. sylvestris*. Data were obtained between April and October in 2013 and are the average of independent samples ($n = 4$). Vertical bars represent standard error.

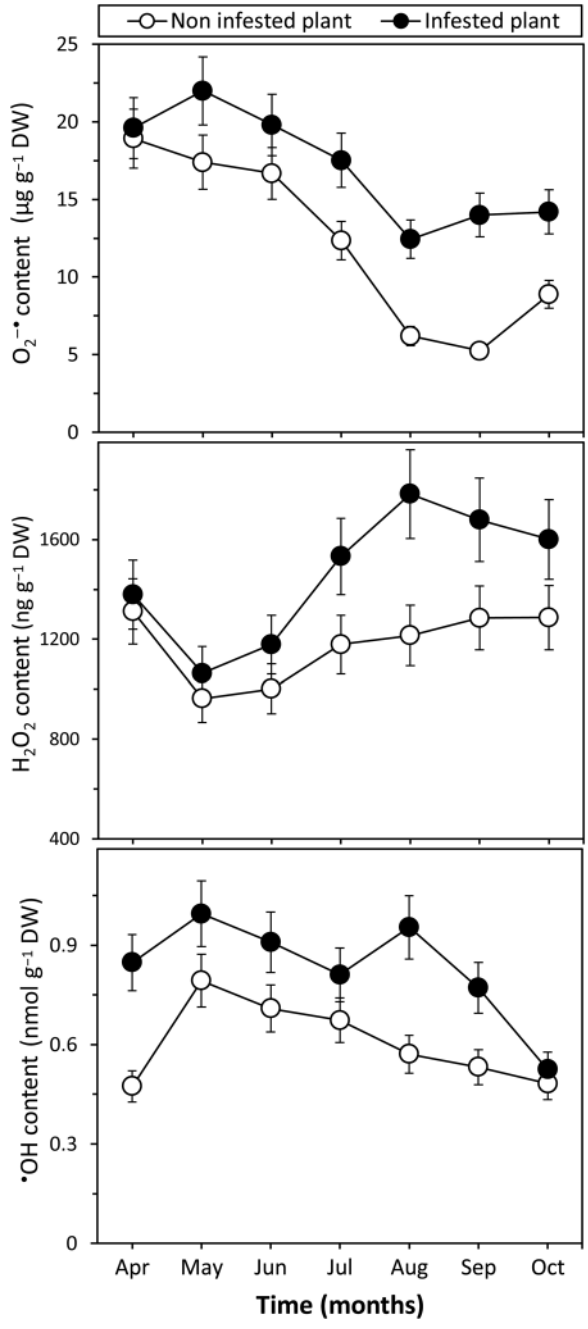

Figure 4. Monthly change in the content of ROS ($O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$) in the needles of infested and noninfested *P. sylvestris*. Data were obtained between April and October in 2013 and are the average of independent samples ($n = 4$). Vertical bars represent standard error.

the infested plants was higher than that of the noninfested plants from May to October (Figure 4). In August, when the drought conditions were the most severe, the levels of H_2O_2 and $^{\bullet}OH$, in parallel with the levels of EL and MDA, were found to reach the highest values in both the infested and noninfested plants (Figures 3 and 4).

The effects of mistletoe-induced drought on antioxidant enzymes

In the noninfested plants, SOD activity gradually decreased inversely to the increasing drought conditions (from May to October), and the activity of SOD in the noninfested plants was higher than in the infested plants (from May to October) (Figure 5). Catalase activity increased from May to August in both types of plant, while it decreased from August to October (Figure 5). However, CAT activity in the infested plants was

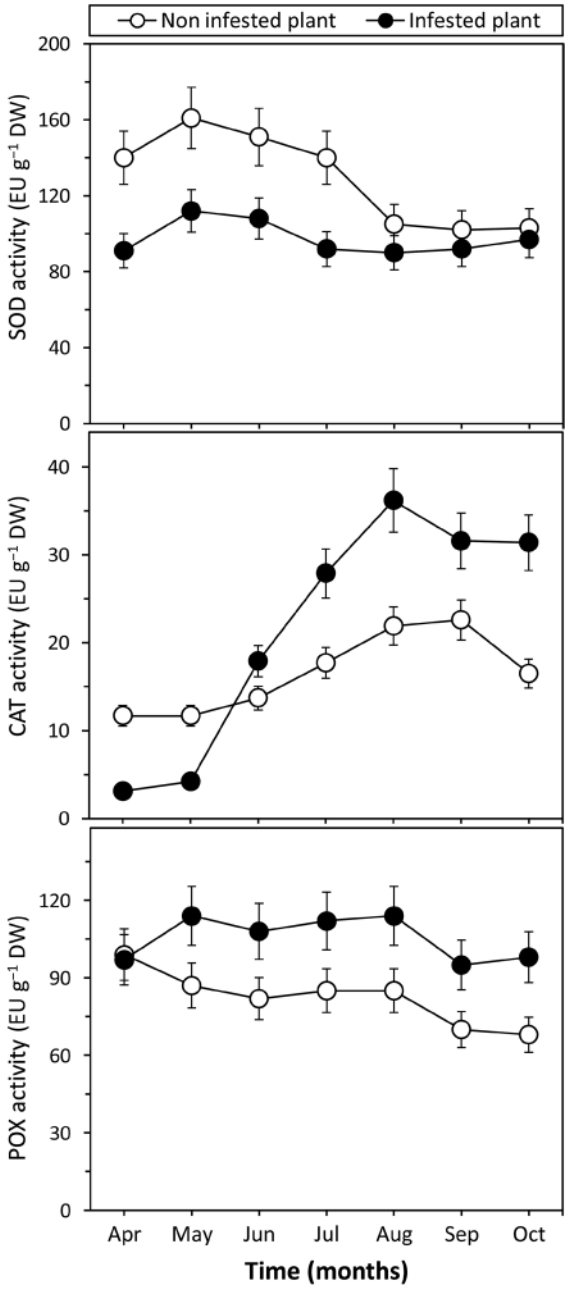

Figure 5. Monthly change in the activity of antioxidant enzymes (SOD, CAT and POX) in the needles of infested and noninfested *P. sylvestris*. Data were obtained between April and October in 2013 and are the average of independent samples ($n = 4$). Vertical bars represent standard error.

higher than in the noninfested plants from June to October (Figure 5). Peroxidase activity reduced inversely to the increasing drought conditions from April to October in the noninfested plants, whereas it increased from April to August and reduced in September and October in the infested plants (Figure 5).

Discussion

Previous studies have shown that parasitic mistletoe plants can affect host plant growth, development and lifespan (Dobbertin and Rigling 2006, Catal and Carus 2011). These studies pointed out that mistletoe infestation had a detrimental effect on growth parameters such as length, radial increment, ramification, needle growth and the number of needles (Rigling et al. 2010), radial growth (Catal and Carus 2011), and some physiological parameters such as CO_2 assimilation, chlorophyll florescence (Strong et al. 2000), stomatal conductance (Zweifel et al. 2012), carbohydrate concentrations (Sangüesa-Barreda et al. 2012), hydraulic conductivity (Reblin and Logan 2015) and water uptake mechanism (Mutlu et al. 2015) in host plants in comparison with noninfested plants. However there are no reports on the effects of mistletoe infestation on oxidative and antioxidative systems of plants under either natural or experimental growth conditions. It is well known that the mistletoe derives considerable amounts of water from infested branches and shows higher transpiration rates and larger sap flow rates than host trees, and thus, it causes severe drought stress in host plants (Ziegler et al. 2009, Sangüesa-Barreda et al. 2013). Although there are many studies on the effect of drought on oxidative and antioxidative systems in plants (Gao et al. 2009, Sangüesa-Barreda et al. 2012), there is no available information in the literature about the effect of mistletoe infestation on these systems in host plants.

Low water content in the needles of stressed Scots pines along with the very low soil water content (Figures 2 and 3) show that these plants are exposed to a severe drought in summer. Similar results were also observed by several authors (Schwanz and Polle 2001, Gao et al. 2009). For example, Gao et al. (2009) attributed the significant decrease in actual water content of needles of some *Pinus* species growing under experimental conditions to drought severity. In this study, stomatal conductance was significantly reduced in studied *Pinus* species after the end of drought treatment (Gao et al. 2009). Low soil water content can interrupt the absorption of water and close the stomata of Scots pine suffering mistletoe-induced drought stress. In a recent study, we found that the mistletoe accumulates the nutrient elements in its structure as a trap (Mutlu et al. 2015). One explanation for the accumulation of potassium that has been proposed is the active uptake of an element important for osmosis and stomatal control (Glatzel and Geils 2009). Stomatal conductance is controlled by the leaf-xylem water potential: when the water supplied by the host xylem is less than that lost, the water potential drops, and this triggers stomatal closure (Brodribb and Holbrook 2003, Brodribb et al. 2003). It is assumed that lowered leaf water potential and, accordingly, decreasing turgor pressures have a strong closing effect on stomata (Buckley et al. 2003, Ewers et al. 2007, Zweifel et al. 2007, 2012, Ache et al. 2010). Essentially, the mistletoe bypasses the homeostatic control of water by the host. As a plant suffering mistletoe-induced drought stress closes its stomata in order to reduce water loss, its CO_2 intake and fixation correspondingly decrease, which potentially reduces the host carbon

assimilation (Glatzel and Geils 2009, Zweifel et al. 2012, Mutlu et al. 2015). However, the plant still has a sufficient concentration of O_2, and photorespiration and Mehler reaction are the most effective mechanisms used by the plant to dissipate the excess energy accumulated within the leaves due to the effect of light. As a result of these two processes, the plant increases the production of ROS significantly and, consequentially, suffers oxidative stress (Baquedano and Castillo 2006). Thus, mistletoe infestation can lead to a decrease in the availability of water and this increases the risk of drought-induced mortality of the host when growing in a xeric environment (Rigling et al. 2010).

Electrolyte leakage is one of the most significant parameters for plants suffering oxidative stress. Mistletoe-induced drought stress increased the level of EL in Scots pine needles. The results of this study (Figure 3) are consistent with previous studies in which drought treatment was found to increase EL in *Pinus densata* (Gao et al. 2009) and *Pinus radiata* (De Diego et al. 2013). Membrane disruption is a result of the peroxidation of polyunsaturated fatty acids or lipids in biomembranes due to ROS that lead to the formation of by-products such as MDA (Singh et al. 2006). Lipid peroxidation-induced disruption of the cellular membrane results in enhanced EL, changed permeability and cellular dysfunction (Nigam and Schewe 2000).

The oxidative stress in living cells was evaluated by measuring the level of MDA content in the needles (Figure 3). Malondialdehyde is a product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. An increase in MDA content leads to an increase in lipid peroxidation level of cell membranes (Xu et al. 2006, Mutlu et al. 2013), and drought stress also increases the generation of ROS including H_2O_2 and $O_2^{-\bullet}$ in plants (Gao et al. 2009). In this study, mistletoe-induced drought stress caused important damage to membrane lipids by increasing the levels of the ROS such as $O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$ (Figure 4). Likewise, our results suggested that such a drought leads to oxidative stress in Scots pine needles. Gao et al. (2009) also found that MDA contents in some *Pinus* species growing in drought conditions were higher than in controls. Therefore, we suggest that this effect of mistletoe-induced drought can disrupt the permeability of the cell membrane structure of the Scots pine.

We found the H_2O_2 content to be parallel with the increase in the CAT activity of the plants (Figures 4 and 5). Superoxide dismutase transforms $O_2^{-\bullet}$ to H_2O_2 by acting as the first line of defense against ROS, and H_2O_2 is also scavenged by POX and CAT to water (Rout and Shaw 2001). One can assert that an increase in CAT activity will lead to a decrease in H_2O_2 content. If H_2O_2 accumulation is not prevented by CAT, oxidative stress can occur (Mutlu et al. 2011). The results of this study are in agreement with those reported earlier by other researchers. Gao et al. (2009) found that H_2O_2 content was significantly high in all studied *Pinus* species exposed to drought in comparison with their respective controls. Mistletoe-induced drought can stimulate the accumulation of ROS such as H_2O_2 in a plant cell. If H_2O_2 accumulation induced by drought is excessive, serious oxidative stress can occur and lead to unrecoverable membrane damage (Mutlu et al. 2011).

In Scots pine needles, the decrease of SOD activity can induce reduction of the amount of the excess $O_2^{-\bullet}$ that is scavenged in plant cells during the mistletoe-induced oxidative stress, suggesting that the reduced SOD activity or the excessive production of $O_2^{-\bullet}$ is one of the fundamental factors in metabolic deterioration during drought stress. Although there are no reports elucidating SOD activity in plants treated with mistletoe infestation, it has been shown that the activity of SOD increased in some *Pinus* species needles with increasing drought stress (Schwanz and Polle 2001, Gao et al. 2009). The result implies that oxidative system can play a significant role in the response to drought conditions by regulating the impaired activity of SOD caused by mistletoe-induced stress in the Scots pine. Conversely, the effect of mistletoe-induced stress on the deactivation of SOD in the Scots pine may facilitate the impairment of membrane structures of the cell, due to the fact that the deactivation of SOD is involved in the activation of the lipid peroxidation processes. Therefore, the activation of antioxidant enzymes induced by drought can contribute to the protection from detrimental effects of this stress (Schwanz and Polle 2001, Gao et al. 2009, Dziri and Hosni 2012).

While CAT activity increases in the noninfested plants depending on the increase in the drought severity, CAT activity increases even more in the infested plants due to the severe drought stress induced by the mistletoe. These data suggest that CAT activity can be significantly affected by both drought stress and mistletoe infestation. The stimulation of CAT activity by biotic and abiotic stresses is a phenomenon that occurs in many different plant species (Hernandez et al. 2000, Schwanz and Polle 2001, Patykowski and Urbanek 2003, Mutlu et al. 2009*a*). Our results suggest that the increase of CAT activity in the plant can result from the scavenging of H_2O_2 produced excessively in cells during mistletoe stress, and CAT is an important H_2O_2-scavenging enzyme leading to the drought tolerance of the plants. Some researchers have reported that exposure to drought caused an increase in CAT activity in some *Pinus* species, which is in parallel with our results. We suggest that higher CAT activity in the plant under drought stress is a result of a more efficient scavenging system, which may result in better protection against ROS during stress.

Compared with the noninfested plants, an increase in POX activity due to mistletoe-induced drought stress was found in the infested plants. Peroxidase is the primary H_2O_2-scavenging enzyme that detoxifies H_2O_2 in plant cells. A recent study has found that drought increased POX activity of some *Pinus* species growing under drought conditions (Gao et al. 2009). It is a well-known fact that POX protects cells against the damaging effects of H_2O_2 during an oxidative-burst response under stress conditions (Levine et al. 1994). However, the increasing effect of drought stress did not have an affect on POX activity in either the

infested or the noninfested plants. Stresses are also known to be effective in the biosynthesis processes of some substances such as lignin and suberin, which strengthen the cell wall, by causing an increase in POX activity (Sakhabutdinova et al. 2004). In addition, the activities of antioxidant enzymes (especially CAT and POX) in the Scots pine are very much lower than the activities of most plants that we have studied recently, such as *Medicago varia*, *Isatis candolleana*, *Astragalus christianus*, *Anchusa leptophylla*, *Euphorbia orientalis*, *Taraxacum androssovii*, *Tragopogon albinervis*, *Crambe orientalis*, *Convolvulus sepium* (Mutlu et al. 2009*a*), *Amaranthus retroflexus*, *Bromus danthoniae*, *Bromus intermedius*, *Chenopodium album*, *Cynodon dactylon*, *Lactuca serriola*, *Portulaca oleracea* (Mutlu et al. 2011), *Triticum aestivum* (Mutlu and Atici 2013) and *Hordeum vulgare* (Mutlu et al. 2016). Therefore, this study indicates that the enzymatic antioxidant capacity of Scots pine is very weak, and severe drought-induced antioxidant systems are not sufficient for the plant to protect itself from oxidative stress.

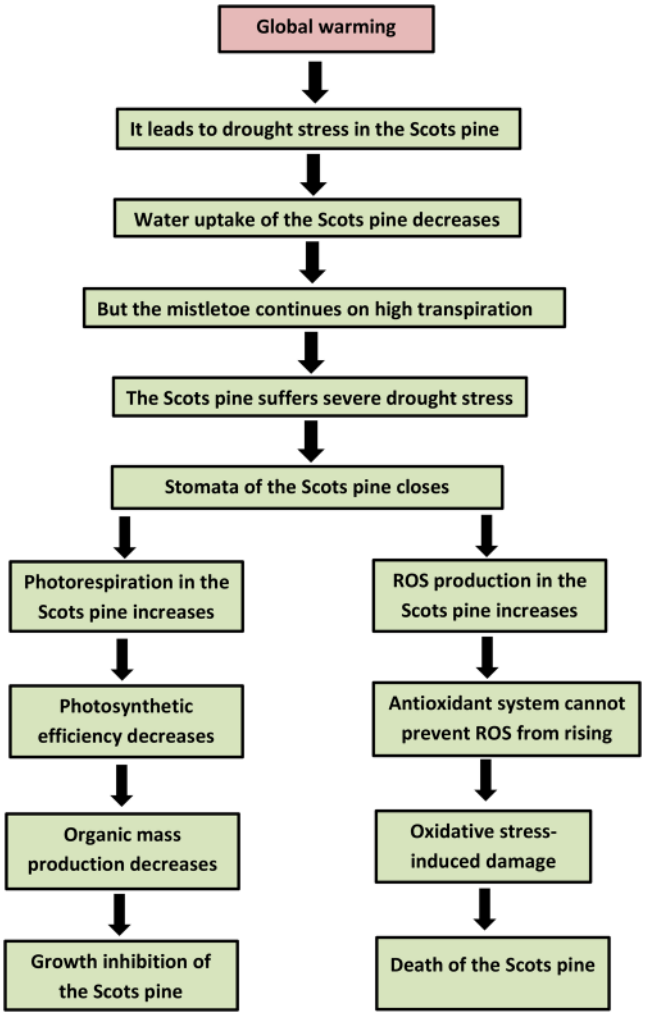

Figure 6. Possible action mechanism of the mistletoe (*V. album*) on mortality of the Scots pine by causing severe drought-dependent oxidative damage in summer seasons.

Conclusion

This study clearly reveals that mistletoe infestation can lead to a decrease in the availability of water, and oxidative stress causes important damage to membrane lipids, which are already shortened due to the chronic drought situation, by increasing the levels of the ROS such as $O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$. In addition, the infestation positively affects some scavenging antioxidative enzymes (CAT and POX) in the plant. In the plants suffering mistletoe-induced drought stress, although the antioxidant enzyme capacity, which is known to play a protective role against this stress, increases, it seems to be insufficient for the plant to avoid oxidative stress. In light of these findings, it is possible to assert that the increased mortality of Scots pine may result from mistletoe-induced very severe drought stress, and the increase in the capacity of the antioxidative enzyme system does not protect the plant against oxidative damage in the dry summer season (Figure 6).

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Conflict of interest

None declared.

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