



Leaf phenolic compounds in red clover (*Trifolium pratense* L.) induced by exposure to moderately elevated ozone

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Concentrations of antioxidant phenolic compounds from red clover can be influenced by elevated ozone.

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ABSTRACT

Red clover (*Trifolium pratense* L.), an important feed crop in many parts of the world, is exposed to elevated ozone over large areas. Plants can limit ozone-induced damages by various defence mechanisms. In this work, changes in the concentrations of antioxidant phenolic compounds induced by slightly elevated levels of ozone were determined in red clover leaves by high-performance liquid chromatography and mass spectrometry. 31 different phenolics were identified and the most abundant isoflavones and flavonoids were biochanin A glycoside malonate (G-M), formononetin-G-M and quercetin-G-M. Elevated ozone (mean 32.4 ppb) increased the total phenolic content of leaves and also had minor effects on the concentrations of individual compounds. Elevated ozone increased the net photosynthesis rate of red clover leaves before visible injuries by 21–23%. This study thus suggests that the concentrations of phenolics in red clover leaves change in response to slightly elevated ozone levels.

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1. Introduction

Tropospheric ozone (O₃) is considered as the main phytotoxic air pollutant in most parts of the world capable of causing more damages to crop plants than all other known air pollutants (Ashmore, 2005; Feng and Kobayashi, 2009). Globally, the present ozone-caused yield losses of major crops are 7–12% (Van Dingenen et al., 2009), but additional losses are expected in future, as background levels of ozone increase due to industrialization and traffic. Today the background levels are 20–45 parts per billion (ppb) in Europe and US, and they are predicted to increase to 42–84 ppb by the year 2100 due to climate change (Vingarzan, 2004). Potential benefits of rising carbon-dioxide on increasing crop yield may be lost due to detrimental effect of increasing surface ozone under future climate conditions (e.g. Booker and Fiscus, 2005).

The phytotoxicity of ozone is due to its very high oxidative capacity to generate reactive oxygen species (ROS) in exposed plant tissue, as ozone rapidly degrades into various ROS species at the cell wall interface (Apel and Hirt, 2004; Kangasjärvi et al., 2005). Plant exposure to short periods of high ozone doses cause visible injuries,

cell death and many physiological changes in plant metabolism (Dizengremel et al., 2008), while low doses over a long term affect physiological processes, such as changes in rubisco activity, reduced photosynthesis and changes in proteolytic enzymes leading to accelerated senescence in plants (Scebba et al., 2003; Gielen et al., 2007; Puckette et al., 2008). Alterations in plant metabolism may lead to reduced crop yield and quality, directly or indirectly by exposing plants susceptible to biotic or abiotic stress factors.

Plants can limit ozone-induced damages by various protective mechanisms. Active defence systems against ozone-induced stress involve the accumulation of diverse protective enzymes (Kangasjärvi et al., 1994), and rapid activation of several signaling pathways, enabling the function of multiple mechanism to minimize the toxicity of ROS species (Samuel et al., 2000; Kangasjärvi et al., 2005). Phenolic compounds are well-known defence compounds against the ozone damages (Kangasjärvi et al., 1994). Increased accumulation of phenolic compounds, especially flavonoids in leaves of forest trees in response to ozone exposure has been reported in numerous studies (Saleem et al., 2001; Kontunen-Soppela et al., 2007; Häikiö et al., 2009). Further, increased levels of transcription of genes involved in flavonoid biosynthesis was found in ozone resistant leguminous cultivars (Puckette et al., 2008), suggesting that a large number of transcription factors and signaling genes regulated differently enable resistant plants to adapt rapidly to ozone stress. In leguminous plants, transcriptional

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activation of isoflavonoids may be a key protective function against oxidative stress induced cell damages (Treutter, 2006).

Red clover (*Trifolium pratense* L.) contains high concentrations of isoflavonoids, compounds widely distributed in the Leguminosae family (Dixon, 2004). Isoflavonoids are secondary metabolites that can be divided into isoflavones and pterocarpanes. The main isoflavones in red clover are biochanin A and formononetin, which are both abundantly found in leaves (Saviranta et al., 2008). Other isoflavones found in leaves include daidzein, genistein, pratensein, prunetin, pseudobaptigenin, calycosin, methylrobool, afrormosin, texasin, irilin B and irilone (Wu et al., 2003; Klejdus et al., 2003), and also flavonoids such as quercetin are present (Swinny and Ryan, 2005). These compounds have been found as aglycones and their conjugated forms such as glycosides and glycoside malonates, conjugated forms being predominant (Klejdus et al., 2001; Polasek et al., 2007). In addition, clovamide and phenolic acids, such as phaseic acid are found (Tazaki et al., 2002; Oleszek et al., 2007) (Table 1). Even though these compounds are synthesized mainly constitutively, their concentrations can be influenced when plant is exposed to biotic or abiotic stresses such as ozone, UV light, and pathogen and herbivore attacks (Keen and Taylor, 1975; Lozovaya et al., 2004; Swinny and Ryan, 2005). Also some elicitors cause an increase in these compounds (Sivesind and Seguin, 2006).

Red clover, which is an important feed crop in many parts of the world, is exposed to elevated ozone over large areas. There is some information available on the physiological changes of red clover upon ozone exposure, but no information about how ozone affects the protective phenolic compounds in red clover. The background levels of tropospheric ozone have been predicted to further increase in future climate change conditions (e.g. Sitch et al., 2007), and therefore this study was designed using ozone exposure experiment systems which simulate the future conditions, and can therefore be used to predict the changes in plant secondary metabolites.

The aim of this work was to determine the effect of elevated ozone (1) on different phenolic compounds, especially isoflavones and (2) photosynthetic efficiency in red clover leaves in different growth stages.

2. Materials and methods

2.1. Red clover leaf samples

Red clover (cv. Bjursele) was grown at Kuopio University Research Garden, eastern Finland in the summer of 2007. Seeds were planted in 1.5 L pots containing B2 peat (Kekkilä Oy, Tuusula, Finland) and sand (1:1, v:v). Seeds were covered with a thin soil layer and doused with water. Plants were irrigated daily for the first two weeks and after that when necessary. After 12 days, 20 seedlings were left in each pot. Leaves were collected at the age of 3, 6 and 9 weeks. All leaves from 7 pots or 5 pots (3 weeks old and 6/9 weeks old plants, respectively) from each ring were pooled (one sample per ring). Samples were placed in ice until being dried in a ventilated oven at 40 °C for 24 h and ground to a fine powder in an A10 mill (IKA® Werke GmbH & Co. KG, Staufen, Germany). Powders were stored in 15 mL plastic tubes at –20 °C.

2.2. Ozone exposure

Ozone treatments were conducted in an open-field ozone exposure system, which has been technically described in detail by Karnosky et al. (2007). Plants were exposed to ambient (control) and elevated ozone in a system containing four treatment rings and four control rings. Ozone was produced from pure oxygen with an ozone generator (G21, Pacific Ozone Technology Inc., Brentwood, CA, US), and ozone-enriched air was injected into exposure rings at the vertical vent pipes from the upwind direction. Ozone concentrations were continuously monitored with ozone analysers (Dasibi 1008-RS, Dasibi Environmental Corp., Glendale, CA, US and O342, Environment S.A, Bobespierre, Poissy, France) at the height of 0.7 m (ozone exposure rings) or 1.8 m (ambient air). Ozone analysers were calibrated against the reference standard UV Photometer SRP#11 (National Institute of Standards, USA) at the Finnish Meteorological Institute, Helsinki. The fumigations were carried out diurnally between 8:00 am and 10:00 pm except during very low (<0.1 m s⁻¹) wind speed or rain. Wind speed and direction was measured at the centre of each exposure ring. The target set for elevated ozone concentrations was 1.5 times the ambient ozone level, based on predicted near-future concentrations for Finland. The average ozone concentrations (8:00 am–10:00 pm), based on hourly mean values were 25.7 ppb for control rings and 32.4 ppb for elevated-ozone rings. The maximum daily ozone concentrations were 38.8 ppb for control rings and 56.6 ppb for elevated-ozone rings. AOT40 (Accumulated Over a Threshold of 40 ppb) values were 0.1 ppm h for control rings and 4.3 ppm h for elevated-ozone rings.

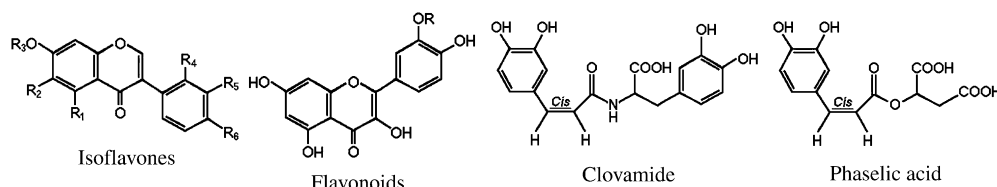
2.3. Net photosynthesis measurements and visible leaf injuries

In order to reveal the impact of moderately elevated ozone on red clover growth physiology, measurements of the net photosynthesis rate were performed at the age of 40 (A) and 61 (B) days, using a Hand Held Portable Photosynthesis System (CI-510, CID, Inc., Camas, Washington, USA) with ambient (386 ± 5.9 (SD) and 398 ± 7.0 (SD)

Table 1

Structures of main phenolics found in red clover leaves (modified from He et al., 1996; Foo et al., 2000; Tazaki et al., 2002; Klejdus et al., 2001, 2003; Oleszek et al., 2007).

Isoflavones	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
Afrormosin	H	OCH ₃	H	H	H	OCH ₃
Biochanin A	OH	H	H	H	H	OCH ₃
Calycosin	H	H	H	H	OH	OCH ₃
Daidzein	H	H	H	H	H	OH
Formononetin	H	H	H	H	H	OCH ₃
Genistein	OH	H	H	H	H	OH
Irilin B	OH	OCH ₃	H	OH	H	H
Irilone	OH	–O–	–CH ₂ –	H	H	OH
Methylrobool	OH	H	H	H	OCH ₃	OH
Pratensein	OH	H	OH	H	OH	OCH ₃
Prunetin	OH	H	CH ₃	H	H	OH
Pseudobaptigenin	H	H	H	H	–O–	–OCH ₂ –
Texasin	H	OH	H	H	H	OCH ₃
Flavonoids	R					
Kaempferol	H					
Quercetin	OH					



ppm for A and B, respectively) CO₂ in the leaf chamber, saturating light (photosynthetically active radiation, PAR 1188.8 ± 6.6 and 1224.8 ± 6.4 μmol m⁻² s⁻¹ for A and B, respectively) and an air temperature of 23.6 ± 1.0 and 24.9 ± 0.9 °C for A and B, respectively. Measurements were performed when the net photosynthesis rate (*P_n*) had reached steady conditions. Ten randomly selected leaves were measured from each control ring, and ten randomly selected leaves without visible injuries from each ozone exposure ring. In addition, the net photosynthesis was measured from 12 leaves (in total) showing visible injuries. All measurements were made between 6:30 am and 1:30 pm.

2.4. Extraction of plant material

Isoflavones and other phenolics for high-performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses were extracted as follows. A 300 mg sample of powdered leaf was extracted three times with 10 mL of 632.8 g L⁻¹ methanol for 40 min with vigorous shaking. Between extractions the solvent was separated by centrifugation (2000 × g, 10 min), the extracts were combined, and the volume was adjusted to 30 mL. Four parallel extracts were taken from both treatments (one per ring) at each growth stage, and all the extracts were done during 1 day.

2.5. HPLC and MS analyses of isoflavones and other phenolics

Isoflavones and other phenolics were analyzed by HPLC (Agilent, Series 1100, Germany), consisting of a binary pump (G1312A), a thermostated autosampler (G1329A), thermostated column oven (G1316A) and a diode array detector (G1315B). The phenolic metabolites were separated by using Hypersil ODS, 4.6 × 60 mm HPLC-column (Agilent Technologies, USA). Samples were eluted (flow rate 2 mL min⁻¹) using the methanol:water-gradient (see, Julkunen-Tiitto and Sorsa, 2001). Samples were filtered before analysis (syringe filters with 0.45 μm pore size), the autoinjection volume was 20 μL and all runs were performed at +30 °C. Phenolic metabolites were identified by comparing their retention times and UV spectra with those of standards, and by using HPLC/API-ES mass spectrometry. Individual compounds were quantified against references at 270 nm. Daidzein (Sigma Chemical Co., St Louis, MO, USA), genistein (Sigma Chemical Co.), formononetin (Fluka BioChemika, Buchs, Switzerland), biochanin A (Sigma Chemical Co.) and quercetin (Sigma Chemical Co.) were used as standards. The concentrations of glycosides and glycoside malonates were calculated by using the standard curve of corresponding aglycone. The concentrations of

isoflavones without standards were calculated by using the standard curve of biochanin A, the most abundant isoflavone in red clover leaves (Saviranta et al., 2008), and concentrations of other phenolics by using the standard curve of quercetin. Ions produced by HPLC/API-ES are listed in Table 2. Conditions for HPLC/API-ES are described by Julkunen-Tiitto and Sorsa (2001).

2.6. Total phenolics

The content of total phenolics in red clover samples were determined by using a method by Singleton and Rossi (1965) with slight modifications. Total of 0.2 mL of diluted extract was mixed with 1.0 mL of 1:10 diluted Folin-Ciocalteu's phenol reagent and 0.8 mL of 7.5% (w/v) sodium carbonate. Absorbance was measured at 765 nm after 1 h. Gallic acid was used as a standard.

2.7. Validation

Recoveries of individual reference compounds were determined by extracting them according to the same method as red clover samples, mimicking average concentrations, and they were 98% for daidzein, 98% for genistein, 101% for formononetin, 98% for biochanin A and 99% for quercetin. The relative standard deviations (RSDs) of repeatabilities were in the ranges 0.1–5.6% (*n* = 3), aglycones possessing the lowest (ave 1.1), and glycosides the highest (ave 1.6) RSDs. HPLC-system suitabilities were in the ranges 0.0–3.7% (*n* = 3). Aglycones possessed the lowest (ave 0.4), and glycosides the highest (ave 1.0) RSDs.

2.8. Statistical analyses

Statistical analyses were performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, US). Data on concentrations of phenolic compounds were subjected to two-way analysis of variance (ANOVA); logarithmic values were used to make concentration distributions more symmetric, and when variances were not equal, independent samples *t*-test with unequal variances was used for each growth stage. Photosynthesis data were subjected to mixed model analysis (comparison between control and elevated ozone; dependent variable: *P_n*, fixed effects: growth stage and treatment, random effect: ring at ozone system) (Brown and Prescott, 2006) and independent samples *t*-test (comparison between leaves with and without visible injuries for each growth stage and comparison between hare-damaged leaves and control).

Table 2
Identification of phenolics in red clover leaves using HPLC-MS.

Peak	<i>t_R</i> (min)	<i>λ_{max}</i> (nm)	[M + H] ⁺	[M + Na] ⁺	[2M + Na] ⁺	Tentative identification
1	12.04	300, 325		319	615	Phaelic acid (<i>cis/trans</i>)
2	12.33	300, 325		319	615	Phaelic acid (<i>cis/trans</i>)
3	16.58	289, 320	360	382		<i>cis</i> -Clovamide
4	17.44	250, 300	417	439		Daidzein-G
5	19.48	250, 260, 287	447	469		Calycosin-G
6	21.52	260, 320	433	455		Genistein-G
7	22.32	262, 334	463	485		Pratensein/Iriline B/Methylorobol-G
8	22.92	260, 330	519	541		Genistein-G-M
9	23.53	250, 300	503	525	1027	Daidzein-G-M
10	24.37	255, 353	465	487		Quercetin-galactoside
11	24.72	255, 353	465	487		Quercetin-glucoside
12	25.04	250, 287	533	555		Calycosin-G-M
13	25.29	250, 295	431	453	883	Formononetin-G
14	25.71	255, 353	551	573		Quercetin-G-M
15	26.14	250, 300	517	539	1055	Formononetin-G-M
16	26.71	265, 350	449	471		Kaempferol-G
17	27.31	260, 330	519	541	1059	Genistein-G-M
18	27.78	263, 340	549	571		Pratensein/Iriline B/Methylorobol-G-M
19	28.98	260, 285, 330	549	571		Pratensein/Iriline B/Methylorobol-G-M
20	30.16	250, 260, 290	531	553	1083	Pseudobaptigenin-G-M
21	30.92	250, 300	517	539	1055	Formononetin-G-M
22	31.45	285, 310	533	555		Maackiain-G-M
23	31.71	260, 330	533	555	1087	Prunetin/Texasin-G-M
24	32.58	270, 340	547	569		Iriline/Afrormosin-G-M
25	33.34	260, 330	271	293		Genistein ^a
26	35.00	262	301	323		Pratensein/Iriline B/Methylorobol
27	36.10	285, 310	285	301		Maackiain
28	36.58	260, 325	533	555	1087	Biochanin A-G-M
29	37.58	250, 302	269	291	559	Formononetin
30	42.45	260, 325	285	307		Prunetin/Texasin ^a
31	43.84	260, 325	285	307		Biochanin A

G = glucoside or galactoside, M = malonate.

^a Sample from leaves damaged by hares.

3. Results

3.1. Isoflavones and other phenolics in red clover leaves

In red clover leaves, a total of 31 different phenolics were identified (Table 2). The most abundant isoflavones and flavonoids were biochanin A glycoside malonate (G-M), formononetin-G-M and quercetin-G-M. Concentrations of these compounds were in the ranges 5.35–11.91, 5.03–7.94 and 3.40–6.71 mg g⁻¹ dry weight (DW), respectively. In addition, leaves were also rich in phaselic acid (6.85–9.77 mg g⁻¹ DW). Effects of ozone exposure on concentrations varied between individual compounds and growth stages (Fig. 1). Ozone treatment increased the concentrations of phenolics in leaves collected at the age of three weeks (ave +9%). In older plants (six and nine weeks) ozone exposure still increased the concentrations of flavonoids and other phenolics (+12% and +0.4% for six and nine weeks, respectively), but decreased the concentrations of isoflavones (-5% and -11% for six and nine weeks, respectively). The age of the plant had a greater effect on the concentrations of phenolics, compared to ozone exposure. Concentrations of biochanin A-G-M, formononetin-G-M and quercetin-G-M were the highest in leaves collected at the age of six weeks.

During the growing period, a part of control plants was partly damaged by hares. In these plants, the concentrations of aglycones (formononetin, biochanin A and prunetin/texasin) were up to 10 times higher compared with trace amounts in non-damaged plants, whereas the concentrations of other compounds were reduced by on average 50% by hare damage.

3.2. Total phenolics

The content of the total phenolics in red clover leaves was increased by ozone exposure by 5–12% (Fig. 2). The highest content was detected in ozone exposed plants at the age of six weeks. Hare damages caused a decrease of 36% in the total phenolic content of the remaining leaves.

3.3. Visible leaf injuries and net photosynthesis

Visible ozone injuries, brown, dry and necrotic lesions on leaves were observed during the fourth week after the initiation of the

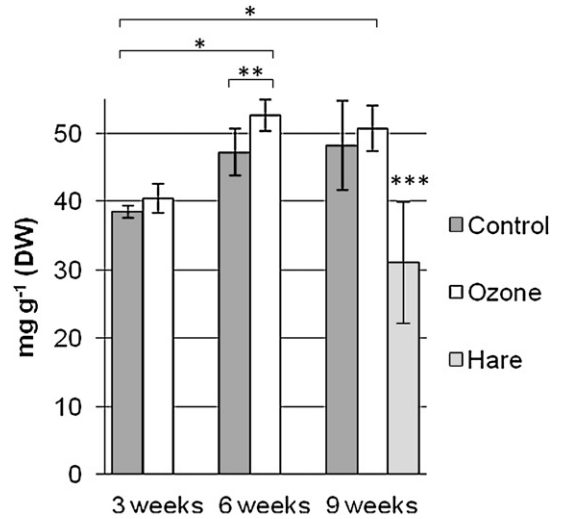


Fig. 2. Total phenolics (mean ± SD) in red clover leaves of control plants, ozone exposed plants and hare-damaged plants. *P < 0.05 between growth stages, **P < 0.05 between treatments, ***n = 2, no statistical analysis performed.

exposure. Elevated ozone increased the net photosynthesis rate of red clover by 21–23% when no visible damage was observed (P < 0.05) (Fig. 3). The effect was neutralized or reversed when visible damage appeared (P < 0.05). The net photosynthesis rate was increased by 110% in the remaining leaves of plants damaged by hares (P < 0.05).

4. Discussion

4.1. Phenolics from red clover leaves

In this study, a total of 31 phenolic compounds were tentatively identified in red clover leaves. Predominant isoflavones and flavonoids were biochanin A, formononetin, and quercetin and their conjugates. Our results are consistent with the findings by Wu et al. (2003), where a total of 31 isoflavones in red clover leaves were detected. The leaves of other *Trifolium* species, *Trifolium repense* L.,

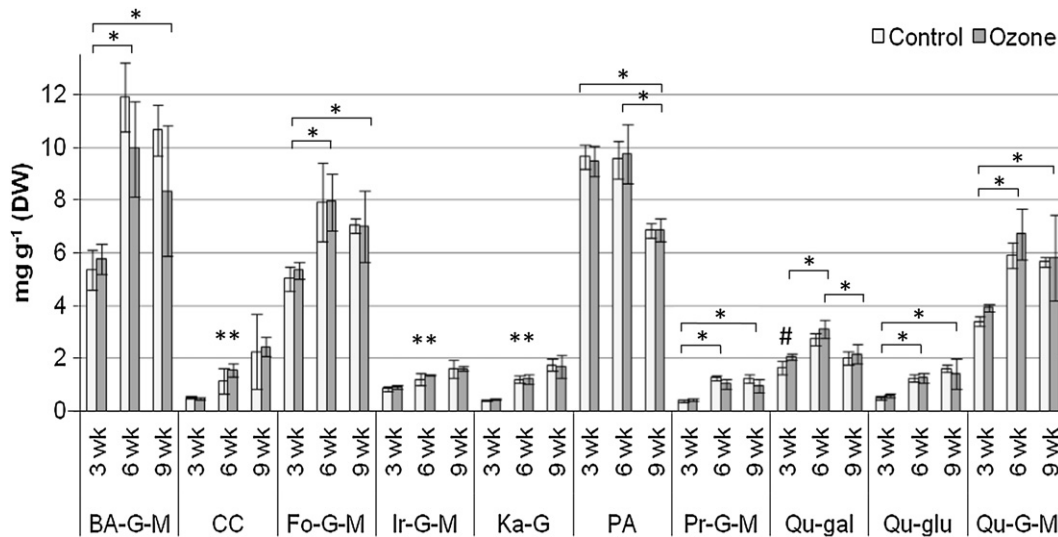


Fig. 1. Concentrations (mean ± SD) of the most abundant isoflavones and other phenolics in red clover leaves exposed to ambient (control) and elevated ozone in different growth stages. BA = Biochanin A, CC = Cis-clovamide, Fo = Formononetin, Ir=Irilone/Afromosin, Ka = Kaempferol, PA = Phaselic acid, Pr=Prunetin/Texasin, Qu = Quercetin, G = Galactoside (gal) or glucoside (glu), M = Malonate. *P < 0.05 between individual growth stages, **P < 0.05 between all growth stages, #P < 0.05 between treatments.

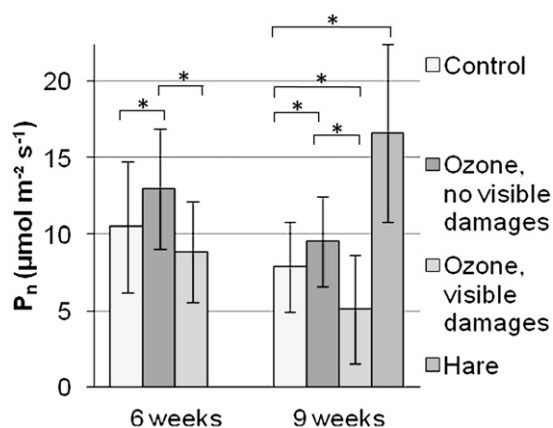


Fig. 3. The net photosynthesis rate (P_n) in red clover leaves of control plants, ozone exposed plants and hare-damaged plants. * $P < 0.05$.

Trifolium hybridum L. and *Trifolium campestre* Schreber, contain fewer isoflavones compared to red clover; 8, 11 and 4 isoflavones, respectively (Wu et al., 2003). In another recent work, the concentrations of four major groups of phenolics, isoflavones, phenolic acids, clovamide and flavonoids in the aerial parts of 57 *Trifolium* species were determined (Oleszek et al., 2007). Red clover was moderately rich in isoflavones, phenolic acids and clovamide, but not in flavonoids in that investigation. Concentrations of these compounds were 39.5, 10.1, 4.3 and 4.8 mg g⁻¹ DW, respectively. Among other species, *Trifolium heldreichianum* contained the highest amount of isoflavones (88.4 mg g⁻¹ DW), *Trifolium spumosum* of phenolic acids (18.1 mg g⁻¹ DW), *Trifolium pallidum* of clovamide (12.9 mg g⁻¹ DW) and *Trifolium leucanthum* of flavonoids (32.4 mg g⁻¹ DW). Concentrations of different groups of phenolics in our samples were similar to the levels obtained in the studies by Oleszek et al. (2007) and Winters et al. (2008). In terms of individual compounds, our results are in line with the previous work of Tsao et al. (2006) and those of Saviranta et al. (2008), where biochanin A and formononetin were found to be the most abundant isoflavones in red clover leaves.

4.2. The effect of elevated ozone on leaf phenolics

In this study we demonstrated that exposure of red clover to slightly elevated ozone (1.26 times the ambient level) increased the total phenolic content of leaves, but had only minor effects on concentrations of individual compounds. In three weeks old plants the concentrations of almost all phenolics were slightly higher in ozone exposed plants, but later the effects were different among isoflavones compared to other phenolics. Interestingly, ozone exposure seems to decrease the concentrations of isoflavones in plants older than three weeks, whereas the concentrations of other phenolics were increased. These results are in line with those of Saleem et al. (2001), where increased amounts of flavonoids and phenolic acids in ozone exposed birch were detected. In open-top chamber experiments with spring wheat, the total phenolic content was also significantly higher in elevated ozone exposed plants than in controls, but variable effects on flavone content were observed to depend on grain filling stage (Li et al., 2008) supporting the observation that plant concentrations of phenolics under ozone stress may depend on plant developmental stages.

Previously, Saleem et al. (2001) detected an increase of 16.2% in total phenolics in plants exposed to elevated ozone. They also observed a correlation between increased phenolic content and impaired growth, indicating that the carbon allocation is changed towards the formation of defence phenolics in ozone exposed

plants. Phenolic acids, (+)-catechin and conjugated forms of myricetin and quercetin were affected by ozone exposure. Many of these phenolic compounds are known to be effective antioxidants (Rice-Evans et al., 1996), which have protective properties against ROS (Wang et al., 2006; Dorta et al., 2008). Among other phenolics, especially isoflavones from red clover have also been shown to possess antioxidative properties (Kroyer, 2004; Occhiuto et al., 2009), suggesting that the increased accumulation of isoflavones demonstrated in this study in ozone exposed plants may be a consequence of antioxidant defence response. This explanation is supported by recent molecular data, where increased levels of transcription of genes involved in flavonoid and isoflavonoid biosynthesis were found in ozone resistant leguminous plants (Puckette et al., 2008), and suggests that an active phenolic defence system may play a key role in the rapid adaptation of leguminous plants to ozone stress.

Some red clover plants were partly damaged by hares with the consequence that the total phenolic content decreased by 36%, whereas ozone exposure caused an average increase of 7%. Concentrations of aglycones were increased up to 10-fold in hare-damaged plants. These results are in line with previous studies by Agrell et al. (2004), where a substantial increase of about 80% in apigenin aglycone concentration in herbivore-damaged alfalfa was detected in fields where plants were grown in an elevated CO₂. It is assumed that this strong increase in aglycones occurs simultaneously with the decrease of corresponding conjugated forms, and it has been suggested, that conjugated forms of isoflavones are hydrolyzed to more active aglycones during stress (Tebayashi et al., 2001). It will be important to address whether ozone can prime plant defence activation towards pathogen or herbivores. Pre-treating plants with the plant defence compound (BTH) before ozone fumigation leads to plants more tolerant to ozone than untreated controls (Iriti et al., 2003). The rapid response of red clover to hare herbivory suggests that isoflavone aglycones may have a role in plant defence.

4.3. The effect of elevated ozone on net photosynthesis

The net photosynthesis rate of leaves increased by over 20% by the moderately elevated ozone levels used in this work before the formation of visible injuries. In a study by Scebbba et al. (2003), acute ozone exposure caused necrotic damages and decreased the net photosynthesis rate in red clover leaves, but the effects of chronic ozone exposure on red clover had not been demonstrated earlier. Chen et al. (2009) recently suggested that acute and chronic ozone exposures do not induce identical mechanisms of ozone damage to photosynthesis processes in soybean, and therefore different fumigation methods are needed to understand the full range of mechanisms of ozone responses. Effects of ozone on forest trees have been widely studied, and although negative impact on photosynthesis is usually found, Oksanen (2003) also detected an increased net photosynthesis in ozone exposed birch in July–August under low-stress conditions. Increased leaf-level photosynthetic rate has been interpreted as a short-term compensation reaction for reduced leaf area and/or photosynthetically active leaf tissue via higher concentration/activity of Rubisco or chlorophyll, when nitrogen availability is sufficient (e.g. Lütz et al., 2000; Oksanen, 2003). It is also plausible, that increased carbon sink strength for production of phenolics may contribute to the high photosynthesis. As moderately elevated ozone did not affect the net photosynthesis rate negatively, it may also suggest that ozone stimulated carbon fluxes from primary growth to the secondary metabolic defence compounds does not entail significant allocation costs. Although Rubisco and chlorophyll were not determined in our study, compensation reaction was also likely explaining the 110% increases

in photosynthesis rates of remaining leaves in hare-damaged control plants (Fig. 2). However, the increase may also be explained by the younger age and thus the greater activity of the remaining leaves.

There are no data available on the differences between red clover cultivars in the susceptibility to elevated ozone. In terms of different *Trifolium* species, Pihl Karlsson et al. (1995) found red clover to be less sensitive to ozone than its relatives *Trifolium subterraneum* L. and *T. repens* L., whereas in the study by Scebba et al. (2003) red clover was more sensitive to ozone exposure than *T. repens* L. Inconsistent data thus suggest that the variation between cultivars might be substantial, and therefore the scale of ozone-induced effects in different growing locations is strongly dependent on the cultivar best-adapted to each climate and soil.

5. Conclusion

The data presented in this paper provide new information about red clover leaf phenolics as determined by HPLC and mass spectrometry, and their responses to slightly elevated ozone levels under open-field system.

Elevated ozone affected the concentrations of red clover leaf phenolics. The total phenolic content was increased due to ozone, whereas in terms of individual compounds isoflavones responded differently compared to flavonoids and other phenolics. Thus, relatively low levels of elevated ozone used in this study indicate that some changes in the concentrations of phenolics present in red clover leaves are to be expected. This may have effects on forage quality, and these changes have to be taken into account when using red clover as a raw material in different biotechnological applications.

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