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ScienceDirect
Environmental Pollution 146 (2007) 707–714
ENVIRONMENTAL POLLUTION
www.elsevier.com/locate/envpol

Ozone stress and antioxidant substances in *Trifolium repens* and *Centaurea jacea* leaves

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Received 26 December 2005; accepted 4 April 2006

Low leaf ascorbic acid levels are a main cause for visible ozone injuries in Trifolium and Centaurea.

Abstract

Ozone-sensitive (NC-S clone) and resistant plants (NC-R clone) of *Trifolium repens* and *Centaurea jacea* were exposed to moderate ozone concentrations in ambient air. The aim of this study was the investigation of the relation between ozone-sensitivity and leaf concentrations of antioxidants (ascorbic acid, total phenolics and total antioxidant capacity). NC-R clone showed the highest concentrations of antioxidants with 50–70% more ascorbic acid than NC-S. NC-R had about 5 times more ascorbic acid in the young leaves and 9 times more in the old leaves than *Centaurea*. In a fumigation experiment with acute ozone stress (100 nl L^{-1}) the antioxidant levels changed profoundly. The ozone-injured leaves of NC-S had 6–8 times more total phenolics than uninjured leaves. Generally older leaves had lower antioxidant concentrations and were more prone to ozone injury than younger leaves. Ascorbic acid concentrations were closer related to the appearance of visible ozone injury than the other antioxidative parameters.

Keywords: White clover; Brown knapweed; Phenols; Ascorbic acid; Antioxidative capacity

1. Introduction

Surface-level ozone is an important phytotoxic gas, naturally occurring in small amounts in the troposphere due to the chemical equilibrium in the atmosphere between oxygen, volatile organic compounds (VOC) and nitrogen oxides (NO_x; Karlsson, 2003). Although ozone in low concentrations close to background (<30 to 40 nl L^{-1}) does not seem to be harmful for vegetation, there is evidence that at higher levels it causes reductions in photosynthesis rates, accelerated leaf senescence or even necrotic leaf injuries, resulting in yield and growth depressions and eventually in economical losses. Although peak ozone concentrations in industrialized countries are declining, a long-term trend of increasing background concentrations has been observed and may contribute to an alteration of regional biological diversity, favouring ozone resistant species (Krupa et al., 2001; Ashmore, 2005).

Since the discovery of the ozone-hypersensitivity of the tobacco cultivar Bel W3 (Heggestad and Menser, 1962; Heggestad, 1991) bioindicator plants are being used to monitor and study changes in the environment. A bioindicator must be sensitive and show characteristic symptoms to the studied parameter. Nowadays white clover (*Trifolium repens* cv. Regal) and brown knapweed (*Centaurea jacea* L.) have also been standardized as bioindicator plants in international monitoring programmes (Buse et al., 2003; Bassin et al., 2004) for monitoring ozone effects.

The ozone sensitivity of different clover species and clones is well documented (Heagle et al., 1994, 1995). Several studies using clover report that ozone injury is correlated to species, leaf age and/or thickness and exposure dynamics

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doi:10.1016/j.envpol.2006.04.006

(Karlsson et al., 1995a,b, 2003; Pleijel et al., 1994). From the biochemical and physiological point of view not much is known about antioxidants in clover plants. Only few authors studied metabolic changes and cellular damage induced by ozone stress, finding substantial differences between *Trifolium pratense* and *Trifolium repens* (Degl'Innocenti et al., 2003; Scebba et al., 2003).

Centaurea jacea was observed to be most sensitive to ozone when reaching the reproductive stage and much less sensitive at the rosette stage (Bassin et al., 2004). Populations from different countries differed significantly in frequency and extent of O_3 injury, as well as in phenological development. Some ozone-sensitivity studies in relation to soil moisture conditions and plant strategy included *Centaurea* plants (Bungener et al., 1999a,b; Nussbaum et al., 2000) but did not consider the biochemical aspect of ozone-sensitivity in relation to antioxidant status.

Ozone enters the plants through open stomata. Inside the plant it produces several reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$) and hydroperoxyl ($HOO^{\bullet}$) radicals (Turcsányi et al., 2000). These compounds may oxidise plant components and damage membrane structures with enhanced chlorosis and senescence and subsequent formation of lesions and tissue collapse. Antioxidants, as a part of plant defence systems, are a large and diverse group of compounds including vitamins, oligopeptides, phenolic acids, flavonoids and carotenoids that are able to scavenge the ROS. In small phenolic molecules, such as gallic acid, caffeic acid or ferulic acid, the basic structure requirement responsible for antioxidant capacity is the existence of a phenyl ring with one or two hydroxyl groups in *ortho*- or *meta*-positions (Hall and Cuppett, 1997). In more complex molecules such as catechin, quercetin or kaempferol the structure arrangements imparting the greatest antioxidant activity are: (a) *ortho*-3′,4′-dihydroxy moiety in ring B, (b) *meta*-5,7-dihydroxy arrangements in ring A, (c) 2,3-double bound in combination with both 4-keto group and 3-hydroxyl group in ring C (Rice-Evans et al., 1997). The more of these structural characteristics a molecule has, the more powerful it is as an antioxidant.

Ascorbic acid (AA, vitamin C) is considered the most important antioxidant defence line in the apoplast (Burkey and Eason, 2002) and is a substrate for key enzymatic reactions (Smirnoff, 1996; Horemans et al., 2000; Smirnoff and Wheeler, 2000). AA is also required in rapidly growing non-photosynthetic tissues therefore it is transported from the mature leaves to sink tissues through the phloem (Franceschi and Tarlyn, 2002). Studies with AA deficient mutants and clones (Conklin et al., 1996, 2000; Veljovic-Jovanovic et al., 2001) have related the low concentrations of AA in the plant tissue to deficient growth and enhancement of ozone sensitivity. There is substantial evidence of the important role of the ascorbate-glutathione cycle in the defence against oxidative stress (Strohm et al., 2002). A rapid glutathione-mediated reduction of oxidised dehydroascorbate (DHA) provides a constantly renewed reduced ascorbate.

Our study analysed two biotypes of white clover (*Trifolium repens* L.): an ozone-sensitive (NC-S) and an ozone-resistant clone (NC-R) as well as brown knapweed (*Centaurea jacea* L) in concern of the biochemical background of ozone sensitivity. The main objective was to study the relation between visual ozone injury, antioxidative capacity and concentrations of individual antioxidants in the leaves of these species. Additionally, differences between the effects of chronic and acute ozone impacts were investigated.

2. Materials and methods

2.1. Field experiment

Plants of white clover (*Trifolium repens* L. cv. Regal, clones NC-S and NC-R) were placed outdoors after transplanting cuttings into 15-L pots. Visible ozone injury was assessed daily following the ICP-Vegetation scale. Destructive harvests were performed at 28-day intervals (August to October) and samples of different leaf ages with and without ozone injuries were collected. The first fully expanded leaf from the tip of the stolon was considered as a young leaf, while the fourth/fifth leaf from the same stolon was considered as an old leaf.

Brown knapweed (*Centaurea jacea* L.) plants were transplanted to 15-L pots when the seedlings showed 8–10 true leaves but they were only then exposed to ambient conditions when they had about 15 true leaves. The weekly visible ozone-injury assessments started in July and they were performed as described in the ICP-Vegetation protocol until all the plants produced seeds in September. *Centaurea* leaves were harvested considering leaf age classes and ozone-injury. Young leaves were taken from the top and old leaves from the base of the shoot; no rosette leaves were harvested and ozone-injured leaves were collected from the shoot without considering age.

All leaf material was ground and stored in liquid nitrogen until chemical analysis.

2.2. Open-top chamber experiment

At the end of the field experiment, the clover clones (NC-S, NC-R) were submitted to elevated concentrations of ozone (100 ± 20 nl L^{-1}) in small open top chambers during 10 days from 09:00 to 17:00 h. Four chambers were used, two serving as fumigation chambers with ambient air plus additional ozone and two chambers used as reference with ozone concentrations <40 nl L^{-1}. The chambers were 1.40 m high with a diameter of 1.20 m with two air volume exchanges per minute. Temperature regime in the chambers was established at 23 °C during the day and 17 °C during the night. Light was supplemented from 06:00 to 18:00 h as the chambers were installed in a greenhouse. Ozone was produced by electric discharge from pure oxygen (Fischer technology, model 502) and its concentration was monitored by UV absorption detection (Horiba, APOA-350E). Leaf material of different age classes with and without ozone injuries was collected and stored for chemical analysis as explained before.

2.3. Determination of ascorbic acid (AA)

Ascorbic acid (AA) was determined by a fluorometric method (AOAC methods, 1980). This fluorometric method is based on the oxidation of the extracted AA to dehydroascorbic acid, in the presence of activated carbon. The dehydroascorbic acid is reacted with *ortho*-phenylenediamine to produce a fluorophor having activation maximum at 350 nm and a fluorescence maximum at 430 nm. The fluorophor development is prevented by the formation of the boric acid dehydroascorbic acid complex (30 g L^{-1} boric acid in 500 g L^{-1} sodium acetate buffer) instead of *ortho*-phenylenediamine-dehydroascorbic acid complex. Fluorescence of the samples is always measured against a boric acid - dehydroascorbic acid blank.

Ascorbic acid in leaf material (1.50 g) stored in liquid N_2 was extracted once with 50 ml solvent consisting of 30 g L^{-1} (pH 1.4) *meta*-phosphoric acid and 8.5% acetic acid. After ultrasonic extraction (3 min) the mixture was immediately filtered by vacuum. The filtrate was allowed to react