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DNA damage in *Populus tremuloides* clones exposed to elevated O_3

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Ozone tolerant clones and sensitive Populus tremuloides clones show differences in DNA damage and repair.

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ABSTRACT

The effects of elevated concentrations of atmospheric tropospheric ozone (O_3) on DNA damage in five trembling aspen (*Populus tremuloides* Michx.) clones growing in a free-air enrichment experiment in the presence and absence of elevated concentrations of carbon dioxide (CO_2) were examined. Growing season mean hourly O_3 concentrations were 36.3 and 47.3 ppb for ambient and elevated O_3 plots, respectively. The 4th highest daily maximum 8-h ambient and elevated O_3 concentrations were 79 and 89 ppb, respectively. Elevated CO_2 averaged 524 ppm (+150 ppm) over the growing season. Exposure to O_3 and CO_2 in combination with O_3 increased DNA damage levels above background as measured by the comet assay. Ozone-tolerant clones 271 and 8L showed the highest levels of DNA damage under elevated O_3 compared with ambient air; whereas less tolerant clone 216 and sensitive clones 42E and 259 had comparably lower levels of DNA damage with no significant differences between elevated O_3 and ambient air. Clone 8L was demonstrated to have the highest level of excision DNA repair. In addition, clone 271 had the highest level of oxidative damage as measured by lipid peroxidation. The results suggest that variation in cellular responses to DNA damage between aspen clones may contribute to O_3 tolerance or sensitivity.

1. Introduction

Ozone (O_3) is a major air pollutant and greenhouse gas, and is increasing in concentration in the troposphere along with carbon dioxide CO_2; (IPCC, 2007). In the troposphere, O_3 is a secondary pollutant generated through photochemical reactions between nitrogen oxides and volatile organic compounds in the presence of sunlight. Ozone is an important stressor of over 30% of world forests (Karnosky et al., 2005) and a potentially phytotoxic gas that at certain doses can induce necrosis in plants similar to wounding and pathogen infection (Heath and Taylor, 1997; Pell et al., 1997). Chronic O_3 exposure accelerates leaf senescence, reduces photosynthetic rates, and shifts carbon (C) partitioning (Reich and Amundson, 1985; Pell and Dann, 1991; Karnosky et al., 1996; Pell et al., 1999; Cooley et al., 2001). Carbon dioxide acts oppositely to O_3 in that it generally increases photosynthesis and stimulates growth (Drake et al., 1997; Will and Ceulemans, 1997). Recently, O_3 has been shown to offset gains in photosynthesis and biomass caused by elevated CO_2 (Isebrands et al., 2001; Noormets et al., 2001a,b; King et al., 2005; Kubiske et al., 2007).

Trembling aspen (*Populus tremuloides* Michx.) is the most widely distributed forest tree species in North America. Aspen genotypes have been shown to have considerable variation in responses to O_3 and CO_2 (Karnosky et al., 1996; Kubiske et al., 1998). Elevated CO_2 stimulated growth and photosynthesis in five aspen clones, whereas elevated O_3 decreased growth and photosynthesis in all but one clone (8L), which showed increased growth (Isebrands et al., 2001; Noormets et al., 2001a,b; Kubiske et al., 2007). Clonal variation in foliar symptoms and stress-induced gene expression in response to elevated O_3 have been noted (Karnosky et al., 1999; Wustman et al., 2001). Exposure to elevated CO_2, O_3 or CO_2 + O_3 were also found to alter pest performance (Percy et al., 2002) and influence competitive interactions between clones (McDonald et al., 2002). Interestingly, the same pattern of genotypic variation was reported to carry through open-top chamber, free-air, and ambient gradient studies using common genetic material (Karnosky et al., 2007a). The long-term consequences of variation in response to elevated O_3 and CO_2, alone and in combination, may be changes in stand diversity and structure.

In North America, the best current science, balanced by social, economic, and political considerations, is employed to establish

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ambient air quality standards. The United States and Canada have established the O_3 air quality standard as "the 3-year average of the annual fourth highest daily max. 8-h average O_3 concentration" (Federal Register, 2008; Canadian Council of Ministers of the Environment (CCME) 2000). In the U.S., there is a primary standard (human health-based) and a secondary standard (welfare-based) that can be different or the same. A legally binding, primary standard of 75 ppb O_3 is now used for regulatory purposes, with the secondary standard set the same as the primary standard at this time. In Canada, the form and averaging time are the same as in the U.S., but the level differs. A target value of 65 ppb O_3 (human health-based, not legally binding) has been adopted. Recently, Percy et al. (2007, 2009) have determined that annual 4th highest daily max. 8-h average O_3 concentrations >70 ppb are detrimental to growth in O_3-sensitive aspen clones. Regulatory policy is guided by information on the biological impacts of O_3 on forest species. An understanding of the effects of O_3 on cellular and molecular structures will help to elucidate the effects of this widely-dispersed criteria air pollutant on one of the most important North American trees species, and will contribute to discussions around critical levels.

Ozone enters plant tissues through the stomata and induces the generation of reactive oxygen species (ROS), e.g., superoxide anion radicals and hydrogen peroxide, and triggers oxidative burst (Sharma et al., 1996; Rao and Davis, 1999; Kangasjarvi et al., 2005). Perception of O_3 or ROS triggers several signal transduction pathways involved in responses to oxidative stress. Plant hormones are involved in controlling programmed cell death, ROS formation, and the extent of lesion propagation in response to O_3 exposure. The ROS serve as signaling molecules, and their levels are tightly controlled in plants (Laloi et al., 2004; Mittler et al., 2004; Foyer and Noctor, 2005; Halliwell, 2006; Pitzschke et al., 2006). However, ROS are highly reactive molecules, and uncontrolled production of ROS can damage cellular structures and molecules. Of particular importance are oxidative DNA lesions caused by ROS, as accumulation of high levels of DNA damage can lead to cell death due to blockage of key nuclear functions (e.g., transcription and replication) and to accumulation of deleterious mutations (Britt, 1996; Bray and West, 2005). DNA lesions induced by ROS have been documented to include formation of oxidative adducts, cleavage of bases, and strand breakage (Aust and Eveleigh, 1999; Tuteja et al., 2001; Bjelland and Seeberg, 2003). The ROS also attack lipids, initiating a process of lipid peroxidation that degrades cell membranes and other lipid structures and forms byproducts that damage DNA (Girotti, 1998; Tuteja et al., 2001; Oksanen et al., 2003).

Ozone was determined to be genotoxic to microorganisms, plants, and mammalian cell cultures in studies examining chromosomal aberrations (Janakiraman and Harney, 1976; Victorin, 1992). Chromosomal aberrations have also been shown in root tips of young spruce trees after short-term exposures to O_3 (Muller et al., 1996; Wonisch et al., 1999). Ozone also induces mutations in human cells (Jorge et al., 2002), *Escherichia coli*, and *Saccharomyces cerevisiae* (Victorin, 1992). However, O_3 did not affect mutation rates in tobacco (*Nicotiana* spp.) and *Tradescantia* as measured by the reversion of a chlorophyll and a pink mutation, respectively (Gichner et al., 1992). Another measure of DNA damage is the comet assay. The comet assay measures a wide range of DNA lesions, including single-strand breaks, double-strand breaks, alkali-labile sites (primarily abasic sites), incomplete excision repair sites, and DNA crosslinks (Singh et al., 1988; Collins et al., 1995; Angelis et al., 1999; Menke et al., 2001; Sastre et al., 2001; Collins, 2004; Olive and Banath, 2006). Studies employing the use of the comet assay report that increased DNA damage in plants is associated with periods of the day when O_3 levels are highest and with locations along roadsides with higher levels of air pollution (Restivo et al., 2002; Sriussadaporn et al., 2003). Further investigation is needed to clarify the role of O_3 on genotoxicity in plants.

In this study, we used the comet assay to investigate the effects of elevated O_3 and CO_2, singly or in combination, on DNA in five aspen clones of varying sensitivity to O_3 and grown for 8 years at the free-air Aspen FACE experiment.

2. Materials and methods

2.1. The Aspen FACE experiment

The Aspen FACE experiment in Rhinelander, Wisconsin was established in 1997 to examine the effects of elevated O_3, alone or in combination with elevated CO_2, on northern hardwood species: aspen (*P. tremuloides* Michx.), paper birch (*Betula papyrifera* Marsh.), and sugar maple (*Acer saccharum* Marsh.) (Dickson et al., 2000; Karnosky et al., 2005). The Aspen FACE experiment consists of a full factorial design with twelve 30-m diameter treatment plots: three control plots, three plots with elevated O_3, three plots with elevated CO_2, and three plots with elevated O_3 + elevated CO_2. The plots were planted in late 1997, and treatments ran from budbreak to the end of each growing season during 1998–2007. The eastern half of each plot was randomly planted in two-tree pairs at 1 m × 1 m spacing, with five trembling aspen clones differing in O_3 tolerance (8L = highly tolerant, 271 = relatively tolerant, 216 = less tolerant, 42E = relatively sensitive, and 259 = highly sensitive).

Carbon dioxide and O_3 were delivered during the daylight hours using a computer-controlled system modified from Hendrey et al. (1999), with the target CO_2 being 200 ppm above the daylight ambient CO_2 concentration (347–374 ppm CO_2 during 1998–2005). Ozone was applied at a target of 1.5× ambient. Ozone was not delivered during days when the maximum temperatures were projected to be <15 °C or when leaf surfaces were wet from fog, dew, or rain events, or when wind speeds were <0.4 m/s or >4 m/s. Thus, over the length of the experiment, O_3 was fumigated on only 48.7–51.6% of the potential growing season days (Percy et al., 2006).

Annual growing season O_3 exposure at Aspen FACE during 1998–2006 was calculated using a modified averaging time (annual) for the US EPA National Ambient Air Quality Standard (NAAQS) (Federal Register, 2008) and Canada-Wide Standard for Ozone (CWS) (CCME, 2000). During 2005, growing season mean hourly O_3 concentrations were 36.3 and 47.3 ppb for the ambient and treatment plots, respectively, and 4th highest daily maximum 8-h ambient and elevated ring O_3 concentrations were 79 and 89 ppb, respectively. Ambient CO_2 concentration averaged 374 ppm and elevated CO_2 concentration averaged 524 ppm. Full details about exposure patterns have been published elsewhere (Dickson et al., 2000; Karnosky et al., 2005, 2007b).

2.2. Plant material

The five aspen clones growing at Aspen FACE were sampled on 9 August 2005. The clones had previously been exposed to the four treatments over an 8-year period (1998–2005; growing season range 150–165 days). One tree from each clone was sampled from each replicate FACE ring (n = 3 per treatment). Three fully expanded, green, short-shoot leaves (short shoots comprised 95%+ of canopy leaf area) were removed from each tree in the top of the canopy. Leaves were flash frozen individually in liquid nitrogen in plastic tubes immediately after being detached, transported over dry ice, and stored at −80 °C.

2.3. Nuclei preparation

The comet assay was optimized in early August 2005 using aspen leaves collected from three field trees in Fredericton, NB, Canada. One leaf from each of three trees was sampled at 0900 h, 1300 h and 1700 h, corresponding to 4, 8, and 12 h of daylight exposure.

Nuclei were prepared in a darkroom under dim yellow light according to Gichner and Plewa (1998). A 1 cm × 2 cm segment was cut from a frozen tree leaf and thawed in a 60 mm Petri dish. The segment was placed on 300 μL of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) containing 50 mM EDTA. The thawed leaf segment was sliced with a fresh scalpel blade so that a fringe was produced across most of the leaf area. The fringe was gently squeezed and rinsed with buffer. The Petri dish was tilted and 20 μL of buffer containing nuclei was collected and placed in a microfuge tube.

2.4. Comet assay

A comet assay kit was used (Trevigen, Inc., Gaithersburg, MD, USA) with some changes to the manufacturer's protocol as follows: 200 μL of molten low-melt agarose was added to 20 μL of aspen leaf nuclei and mixed gently, 75 μL of the nuclei mixture was transferred to the sample area of a CometSlide. Slides were placed flat at 4 °C in the dark for 30 min. Nuclei unwinding was done in alkali solution (300 mM NaOH, 1 mM EDTA pH > 13). Unwinding for 30 min was determined to be optimal for detection of comets. Following alkali lysis, nuclei were subjected to electrophoresis at 1 V/cm, 300 mA in the same alkali solution. An electrophoresis time of 30 min was determined to be optimal. Slides were immersed in methanol and air-dried before silver staining. Three leaves from each clone in each treatment ring were assayed.

2.5. Evaluation of comets

Images were captured under dark-field illumination with a microscope at a 20-fold magnification using a digital camera. Comets were scored using the CometScore software (TriTek Corp., Summerduck, VA, USA). Sixty to 100 random nuclei from each leaf were scored, and DNA damage was quantified as "% DNA in tail" according to Collins (2004), and these measurements are plotted for nuclei from each leaf in a histogram to produce DNA damage distributions. The medians of the DNA damage distributions were calculated for each leaf.

2.6. Statistical analysis

The effect of clone and treatment on % DNA in tail measurements (%) was tested for using a two-way general linear model ANOVA (MINITAB, Release 14, State College, PA, USA). Treatment effects were considered fixed and clone effects were considered random. The probability plot of median damage (%) data was not normally distributed ($P = 0.020$) as tested using the Kolmogorov–Smirnov goodness of fit test (Massey, 1951) and data were arcsin-transformed before ANOVA. Differences due to treatment and clone were tested for using ANOVA (GLM). Posthoc analysis (95% confidence interval) was completed using Dunnett's method to compare the mean of each treatment to the control mean.

2.7. Lipid peroxidation assay

Leaves were flash frozen in liquid nitrogen and 0.1–0.5 g tissue were ground to a powder with mortar and pestle. The powder was resuspended in 1 mL of 20 mM Tris, pH 7.4 with 1 mM butylated hydroxyl toluene (from 0.5 M stock in acetonitrile). Homogenates were spun at 12 000 × g for 10 min; supernatants were retained, and protein concentrations were determined using A_{280}. Lipid peroxidation assay was done using a chromogenic reagent that reacts with malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) (Calbiochem, Darmstadt, Germany) according to manufacturer's instructions. Absorbance at 586 nm was used to quantify levels of MDA and HAE. Three leaves were assayed for each clone.

2.8. In vitro DNA repair assay

One-hundred mg of aspen leaves were flash frozen in liquid nitrogen and ground to a powder with a mortar and pestle. Extracts used for *in vitro* repair assays were prepared according to Li et al. (2002) with the following modifications: the homogenate was passed through a Qiashredder column (Qiagen, Mississauga, ON, Canada) at 10 000 rpm for 10 min instead of filtration through nylon meshes. The control DNA substrate was a 4.5 kb linearized plasmid. The repair DNA substrate was pBluescript II SK+ (Stratagene, La Jolla, CA, USA) linearized with Bam HI (3.0 kb), and was oxidatively damaged using methylene blue and light exposure for 30 min to create predominantly 8-oxoG lesions. *In vitro* repair synthesis was done according to Li et al. (2002) with the following modifications: the repair substrate and control DNA were purified using a Qiaquick column (Qiagen, Mississauga, ON, Canada) instead of phenol/chloroform extraction, and vacuum blotting was used instead of capillary blotting. Repair synthesis was detected through incorporation of digoxygenin-labelled dUTP (DIG-dUTP) into the 3.0 kb plasmid substrate. Blots were incubated with a polyclonal sheep anti-digoxygenin antibody (Roche, Laval, QC, Canada) followed by an alkaline phosphatase-conjugated secondary antibody (GE Healthcare Bio-Science, Corp., Piscataway, NJ, USA). The CDP-Star (New England Biolabs, Mississauga, ON, Canada) chemiluminescent alkaline phosphatase substrate was applied to blots followed by exposure of blots to X-ray film.

3. Results

3.1. Diurnal variation of DNA damage in aspen leaves

We used the single-cell gel electrophoresis assay or comet assay to measure DNA damage. There was a range of DNA damage in nuclei from aspen leaves in the ambient atmosphere (Fig. 1). DNA damage was quantified as % DNA in tail. Each leaf had a distribution of nuclei, and the median was used as a measure of the DNA damage in the leaf. The distribution of % DNA in tail for the nuclei of three aspen leaves is plotted in histograms (Fig. 2). Nuclei from an aspen leaf harvested at 0900 h showed a % DNA in tail distribution that was skewed toward higher levels of DNA damage (Fig. 2a). At 1300 h, the distribution was shifted, and there were fewer aspen leaf nuclei having high levels of DNA damage (Fig. 2b). The trend toward lower levels of DNA damage with increasing day length in aspen leaves continued to 1700 h (Fig. 2c).

To further demonstrate the diurnal pattern of DNA damage in aspen leaves, the median of the % DNA in tail distribution was calculated for each of three leaves collected at the three time points. The average % DNA in tail values for the three leaves sampled at each time point are shown in Fig. 2d. Background levels of DNA damage were clearly lowest at 1700 h, the time point used for subsequent sampling of the five aspen clones at Aspen FACE.

3.2. The effect of clone, CO_2, and O_3 on DNA damage

The average amount of DNA damage over the five aspen clones, measured as % DNA in tail, increased in the following order: control (12.03%), CO_2 (23.24%), O_3 (34.06%), and $CO_2 + O_3$ (34.44%). The amount of DNA damage in aspen leaves sampled at 1700 h differed significantly between treatments ($P = 0.002$) and between clones ($P = 0.006$). There was no significant ($P < 0.05$) interaction between treatment and clone. Averaged across the five clones, the level of DNA damage was significantly greater in the O_3 (T-value 3.09; $P = 0.008$) and $O_3 + CO_2$ (T-value 3.182; $P = 0.006$) treatments than in the control (Fig. 3a). Exposure to CO_2 did not (T-value 1.542; $P = 0.293$) increase level of DNA damage relative to the control.

The aspen clones showed high variability in their response to the three treatments (Fig. 3b). In clone 8L, the level of DNA damage under elevated $CO_2 + O_3$ (57.3%) was slightly greater than under O_3 alone (54.6%). Degree of DNA damage was slightly lower under elevated CO_2 (8.4%) than in the control (11.6%). In clone 271, the level of DNA damage was lower under $CO_2 + O_3$ (23.4%) than under O_3 alone (50.4%) (Fig. 3b). The level of damage under CO_2 alone (25.0%) was similar to that under $CO_2 + O_3$. In contrast, clone 216 showed higher levels of DNA damage under elevated CO_2 (51.8%) and $CO_2 + O_3$ (53.9%), than under O_3 alone (26.5%) (Fig. 3b). Clones 42E and 259 did not show the same degree of variability in DNA damage between treatments (Fig. 3b). Level of damage in clone 42E did not exceed 16.8% (CO_2), whereas damage in clone 259 did not exceed 31.9% (O_3) (Fig. 3b).

3.3. Lipid peroxidation

Lipid peroxidation was measured in this study to estimate the level of oxidative damage in plant cells. Lipid peroxidation contributes to the formation of byproducts that damage DNA (Tuteja et al., 2001). The average amount of MDA and HAE lipid peroxidation

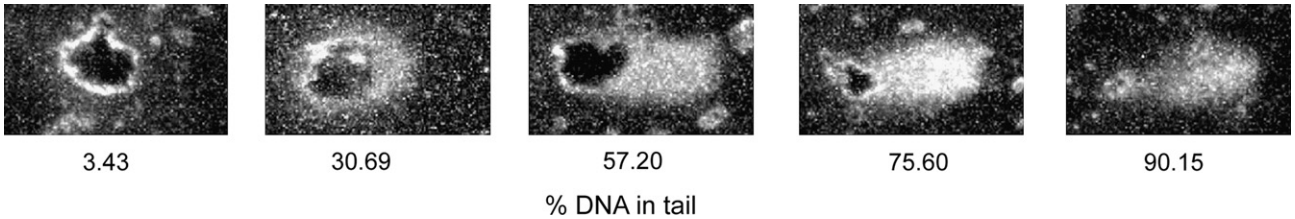

Fig. 1. Comet assay of nuclei from aspen leaves under an ambient diurnal cycle. Damaged DNA migrates out of aspen leaf nuclei under electrophoresis, forming comet tails. DNA damage is quantified as % DNA in tail.

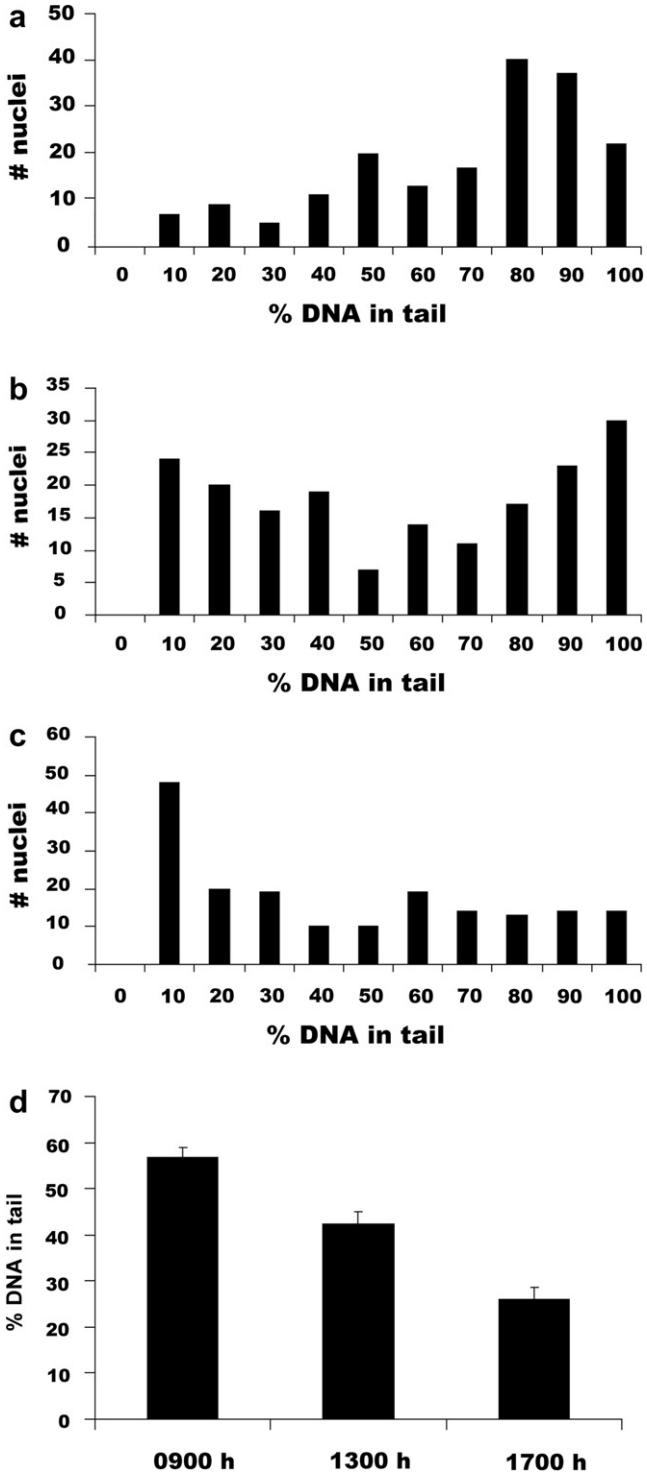

Fig. 2. Diurnal variation of DNA damage in aspen leaves. Percent DNA in tail data for nuclei from each aspen leaves were distributed over 10 intervals from 0 to 100% to generate histograms for leaves collected at a) 0900 h, b) 1300 h and c) 1700 h. d) The median value of the DNA damage distributions for three aspen leaves collected from the greenhouse at 0900 h, 1300 h, and 1700 h is averaged and presented in the graph. Error bars represent standard error of the mean.

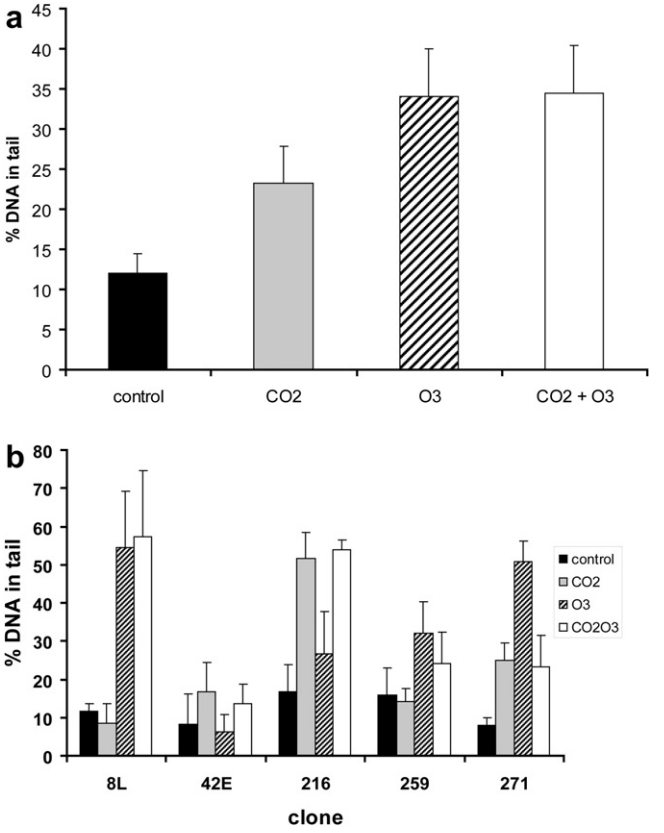

Fig. 3. DNA damage in aspen clones as measured by the comet assay. For each leaf there is a distribution of DNA damage measured as % DNA in tail values. The medians for the distributions for each leaf are determined. a) The combined average of median % DNA in tail values for all leaves collected from all clones in each treatment ring. b) The average median % DNA in tail value for each clone in each treatment. The error bars are standard error of the mean.

products in aspen leaves under ambient air was 4.73 nmol/g protein. The MDA and HAE levels increased with treatment with elevated O_3 (6.41 nmol/g protein) and $CO_2 + O_3$ (6.01 nmol/g protein), but not with CO_2 alone (4.15 nmol/g protein) (Fig. 4a). There was variation between clones in the amount of lipid peroxidation detected under all atmospheric treatments. The MDA and HAE levels ranged in value from 1 to 8 nmol/g protein for all clones for all atmospheric treatments. Clone 271 under elevated O_3 was an exception, where levels of MDA and HAE were much higher, with an average value of 14.39 nmol/g protein (Fig. 4b).

3.4. DNA repair activities

An *in vitro* assay was used to quantify differences in DNA repair activities between clones. Extracts from leaves of the clones in the control ring and the elevated O_3 ring were compared. The repair substrate is a 3.0 kb plasmid containing 8-oxoG lesions which are repaired predominantly by base excision repair (BER). A 4.5 kb undamaged plasmid is used as an internal control (Fig. 5a). The results show a band at 3.0 kb, for clone 8L leaves collected in the O_3 treatment (Fig. 5b), indicating that repair synthesis is detectable for this clone. The excision repair activity under ambient air was also examined and no activity was detected for any of the clones (data not shown).

4. Discussion

As far as we know this is the first application of the comet assay to aspen leaves, and the results suggest that this assay can be used effectively to detect DNA damage in aspen. The comet assay data collected from trees growing under ambient air shows a diurnal pattern of DNA damage in aspen leaves. Damage to DNA was

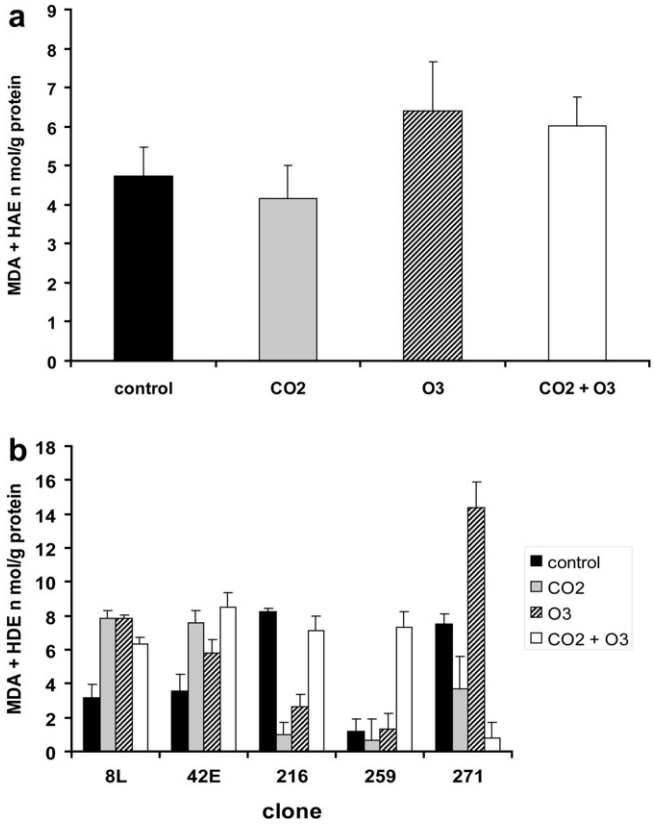

Fig. 4. Lipid peroxidation in aspen clones. Lipid peroxiation is measured by the amount of MDA and HAE nmol/g protein in leaves. a) Combined average MDA + HAE levels for leaves from all clones in each of the treatment rings. b) MDA + HAE levels for each clone in each treatment ring. The error bars are standard errors of the mean.

highest in the sample collected at 0900 h. At 1300 h the DNA damage was reduced and became further decreased at 1700 h. The same diurnal pattern of DNA damage was also observed in *Fagus grandifolia* (beech) leaves from greenhouse grown trees in another experiment (data not shown). The high levels of DNA damage observed at 0900 h may be attributed to high levels of oxidative damage that are observed at the end of the dark period in plants (Bechtold et al., 2004).

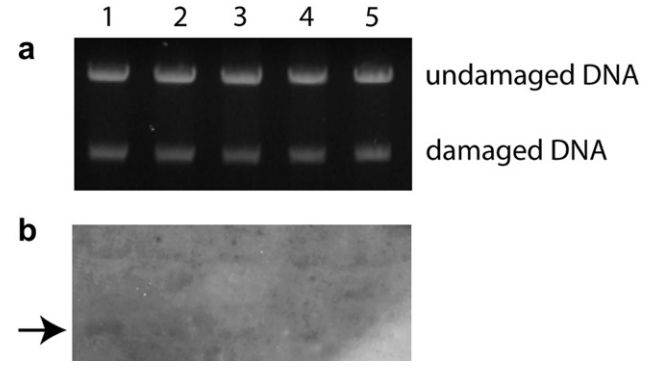

Fig. 5. *In vitro* repair synthesis of damaged plasmid DNA in extracts from O_3-exposed aspen leaves. Control and damaged DNA were incubated with extracts from aspen leaves taken from trees in the O_3 treatment ring. Damaged DNA will undergo repair synthesis and incorporate DIG-labelled dUTP. Repair reactions were run on agarose gels stained with ethidium bromide, transferred to nylon membranes, and repair synthesis is detected with anti-DIG-alkaline phosphatase. A chemiluminescent substrate for alkaline phosphatase is used to detect DIG-labelled repaired plasmid DNA using autoradiography. a) Ethidium bromide stained gel of undamaged (upper band) and damaged (lower band) DNA. b) Autoradiogram of DIG-labelled repaired DNA. The arrow indicates band showing repair synthesis. Lane 1 clone 8L, lane 2 clone 42E, lane 3 clone 216, lane 4 clone 259, and lane 5 clone 271.

This study provides evidence that elevated O_3 is associated with increased DNA damage and concurs with other studies that link O_3 with genotoxicity (Janakiraman and Harney, 1976; Victorin, 1992; Muller et al., 1996; Wonisch et al., 1999; Jorge et al., 2002). Studies on tobacco and roadside plants also show that DNA damage is increased in plants at locations and times during the day associated with elevated O_3 (Restivo et al., 2002; Sriussadaporn et al., 2003).

Our study reveals that the level of DNA damage under elevated O_3 is different between aspen clones. Under elevated O_3, the O_3-tolerant clones 8L and 271 showed increased DNA damage, whereas the less tolerant clone 216 and O_3-sensitive clones 42E and 259 were not as affected. It is important to note that damage to clones 8L and 271 was induced at a growing season 4th highest daily maximum 8-h O_3 concentration of 89 ppb in the year of sampling (2005), preceded by a lower 4th highest daily maximum 8-h O_3 concentration of 77 ppb in 2004. Concentrations within a range of 77–89 ppb O_3 have been measured over extensive areas of the aspen range in Canada and the U.S. (Percy et al., 2007). Our study is one of a few examining adaptation to a genotoxic stress in plants. The results presented suggest that tolerance to elevated O_3 is associated with increased DNA damage in aspen. Although this conclusion is somewhat unexpected, studies of the clones as discussed below shed some light on what biological processes may have led to the DNA damage results observed.

The comet assay not only measures DNA lesions but also DNA nicks associated with repair activities occurring through BER or nucleotide excision repair (NER) (Aust and Eveleigh, 1999; Hosfield et al., 2001; Cadet et al., 2003; Slupphaug et al., 2003). Therefore, repair activities were analyzed separately in an *in vitro* DNA excision repair assay. The DNA excision repair assay results showed that repair activity was not detectable under ambient air for all clones (data not shown). Under elevated O_3, clone 8L showed higher detectable levels of DNA excision repair activity compared to the other clones. Therefore, DNA damage measured by the comet assay in clone 8L can be attributed to DNA repair activities as well as DNA lesions; whereas, DNA damage in the other clones can be mainly attributed to DNA lesions.

Clone 8L growth is stimulated by O_3 exposure up to a 4th highest daily maximum average 8-h concentration (NAAQS, CWS) of 95 ppb O_3, whereas other clones show growth inhibition at much lower O_3 concentrations (Isebrands et al., 2001; Kubiske et al., 2007; Percy et al., 2009). We propose that the increased DNA excision repair activity contributes to the growth advantage observed for clone 8L. Increased genotoxicity has been documented to block plant growth (Jiang et al., 1997; Britt and Fiscus, 2003; Preuss and Britt, 2003). This arrest in growth is dependent on detection of DNA lesions in a process called cell cycle checkpoint. Cell cycle checkpoint leads to cell cycle arrest, DNA repair, and programmed cell death (apoptosis) (Garcia et al., 2000, 2003; Zhou and Elledge, 2000; Preuss and Britt, 2003; Culligan et al., 2004; Kastan and Bartek, 2004; Ishikawa et al., 2006). Increased repair activity can reverse DNA lesions leading to progression through the cell cycle and increased cell proliferation. Although, it is interesting to speculate that variation in growth under elevated O_3 in the aspen clones involves DNA repair responses and cell cycle checkpoint; the integration of cellular level growth control and whole organismal growth is complicated (Beemster et al., 2003) and will require further study. In addition, a number of other factors including O_3 uptake and detoxification processes (Dizengremel et al., 2008; Karnosky et al., 1998; Wustman et al., 2001) and production of plant volatiles (Behnke et al., 2009) should also be considered in determining the mechanisms of O_3 tolerance in trees.

Clone 271 was observed to maintain competitive annual stem volume growth rates under O_3 exposure in the aspen stand from 1998 to 2004 (Kubiske et al., 2007). Under elevated O_3 clone 271 had the highest levels of lipid peroxidation, which was correlated with high levels of DNA damage. These results are consistent with the known role of lipid peroxidation in contributing to oxidative lesions in DNA (Tuteja et al., 2001). Other clones with lower levels of lipid peroxidation under elevated O_3 did not have DNA damage that was significantly different from ambient controls, except clone 8L as discussed above. These results indicate that there may be a threshold level of lipid peroxidation that leads to DNA damage. Levels of ROS that cause lipid peroxidation are partly controlled through production of antioxidants. However, antioxidant enzyme activity for superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase in clones 216, 259, and 271 at Aspen FACE were found to be similar (Wustman et al., 2001). Alternatively, we suggest that high levels of lipid peroxidation and DNA damage in clone 271 may be related to increased tolerance for damage at the cellular level. There is evidence from yeast that DNA damage-arrested cells turn off checkpoints and continue to proliferate in genotoxic environments (Toczyski et al., 1997; Galgoczy and Toczyski, 2001). Continued growth is maintained but high levels of DNA damage and increased mutation rates are sustained. Checkpoint adaptation has not yet been examined in plants, but translesion synthesis that allows for replication of damaged DNA has been demonstrated for plants (Takahashi et al., 2005). A greater understanding of DNA damage and repair mechanisms in plants will be needed to fully interpret the responses of clone 271 to elevated O_3.

A key difference between this and other studies on DNA damage in plants is that long-term adaptive responses to a genotoxic environment was investigated rather than short-term responses. The aspen trees used in this study had been exposed to elevated O_3 for $\geq$8 years and were sampled late in the growing season. The DNA damage observed in our study represents the cumulative balance of the amount of exposure of DNA to damaging agents against the level of cellular responses to reverse or limit DNA damage over the growing season.

The Aspen FACE experiment has shown that CO_2 and O_3, in combination, offset each other, resulting in photosynthesis and growth rates that are similar to ambient air (Isebrands et al., 2001; King et al., 2005; Kubiske et al., 2007). We show here that the average DNA damage, as measured by the comet assay for the five clones when O_3 is combined with CO_2, is increased over the control at the same levels as O_3 alone. Therefore, with respect to DNA damage, elevated CO_2 and O_3 do not offset each other. Carbon dioxide also did not ameliorate the detrimental effects of O_3 on the leaf photosynthetic apparatus (Kull et al., 1996) indicating that CO_2 does not always totally offset adverse effects of O_3. In addition, DNA damage, as measured by the comet assay, is increased under elevated CO_2 alone, although at levels lower than with O_3 alone. Clonal variation in DNA damage under elevated CO_2 and $CO_2 + O_3$ is highly significant. However, it is difficult to draw conclusions concerning CO_2 and DNA damage since it is less clear what mechanism of DNA damage is taking place under elevated CO_2. In contrast for O_3, it has been established that induction of ROS takes place and oxidative DNA damage results (Aust and Eveleigh, 1999; Tuteja et al., 2001; Bjelland and Seeberg, 2003). Future studies are needed to further examine the nature of the DNA damage observed under elevated CO_2.

5. Conclusion

The evidence presented indicates that there is variation at the cellular level in the adaptation strategy used by the clones to survive the genotoxic environment imposed by elevated O_3. In addition, our results provide evidence that elevated O_3 can select for genotypes having higher DNA damage tolerance, which can lead to higher mutation rates and increased mutation load for populations. This will be of increasing importance to the adaptive potential of the species as climate changes.

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