Metabolic-dependent changes in plant cell redox power after ozone exposure

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REVIEW ARTICLE

Metabolic-dependent changes in plant cell redox power after ozone exposure

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ABSTRACT

The tropospheric level of the phytotoxic air pollutant ozone has increased considerably during the last century, and is expected to continue to rise. Long-term exposure of higher plants to low ozone concentrations affects biochemical processes prior to any visible symptoms of injury. The current critical level of ozone used to determine the threshold for damaging plants (biomass loss) is still based on the seasonal sum of the external concentration above 40 nl·l^{-1} (AOT40). Taking into account stomatal conductance and the internal capacity of leaf defences, a more relevant concept should be based upon the 'effective ozone flux', the balance between the stomatal flux and the intensity of cellular detoxification. The large decrease in the Rubisco/PEPc ratio reflects photosynthetic damage from ozone, and a large increase in activity of cytosolic PEPc, which allows increased malate production. Although the direct detoxification of ozone (and ROS produced from its decomposition) is carried out primarily by cell wall ascorbate, the existing level of this antioxidant is not sufficient to indicate the degree of cell sensitivity. In order to regenerate ascorbate, NAD(P)H is needed as the primary supplier of reducing power. It is hypothesised that increased activity of the catabolic pathways and associated shunts (glucose-6-phosphate dehydrogenase, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, isocitrate dehydrogenase and malic enzyme) can provide sufficient NAD(P)H to maintain intracellular detoxification. Thus, measurement of the level of redox power would contribute to determination of the 'effective ozone dose', serving ultimately to improve the ozone risk index for higher plants.

INTRODUCTION

Tropospheric ozone is a pollutant that harms plants (Matyssek & Innes 1999; Krupa *et al.* 2001; Ashmore 2005; Karnosky *et al.* 2007; Wittig *et al.* 2009). The phytotoxicity of ozone is determined in several successive steps: (i) concentration of the pollutant in the external atmosphere surrounding leaves; (ii) deposition of ozone to soil and plant surfaces, especially leaves; (iii) degree of uptake of ozone within leaves through stomata; and (iv) level of antioxidants counteracting the oxidative attack. Measurement of ambient ozone concentrations in air (step 1) has been used to define different critical levels, based on long-term cumulative exposure. The AOT 40 concept (sum of hourly ozone concentrations above a threshold of 40 n·l^{-1} during daylight hours of the growing season) is the reference parameter used within Europe (Fuhrer *et al.* 1997). Other measures of ozone exposure, such as the SUM06 (sum of all hourly average concentrations over 0.06 ppm) or the sigmoidally weighted W126 exposure index used in North America (Lefohn *et al.* 1988) are also available. Concentration-based concepts, however, ignore the real flux of ozone through stomata, which is influenced by deposition of ozone on different surfaces including leaf cuticles (step 2) and uptake through stomata (step 3), both of which depend on

climatic conditions. The direct harmful effects of ozone on leaves thus depend on the stomatal flux of ozone, which integrates parameters linked to stomatal conductance (Ashmore *et al.* 2004; Grünhage *et al.* 2004; Karlsson *et al.* 2004; Gerosa *et al.* 2009). The degree of stomatal opening is, however, not necessarily the only factor conferring sensitivity or tolerance to ozone stress.

After penetrating leaves (Fig. 1), gaseous ozone dissolves in the apoplasm, giving rise to reactive oxygen species (ROS) such as O_2^-, H_2O_2 and OH. (Byvoet *et al.* 1995). A range of antioxidant metabolites and detoxifying processes that can scavenge ROS (step 4) is present, and apoplastic ascorbate appears the best candidate as a first line of defence (Plöchl *et al.* 2000). The presence of a high level of apoplastic ascorbate, however, is not always sufficient to counteract the toxicity of ROS (Ranieri *et al.* 1999; Conklin & Barth 2004; D'Haese *et al.* 2005). Apoplastic ascorbate must be regenerated *via* intracellular detoxifying pathways, and the rate of regeneration controls the 'effective ozone flux', corresponding to the non-detoxified ozone molecules (Matyssek *et al.* 2004; Musselman *et al.* 2006; Paoletti & Manning 2007; Tausz *et al.* 2007; Wieser & Matyssek 2007; Dizengremel *et al.* 2008). This scenario would be valid if realistic concentrations of ozone are chronically delivered and must be adjusted in the case of short-term acute exposure (Renaut *et al.* 2009). This review highlights ways in which cellular metabolism creates conditions that allow reducing power to play a prominent role in keeping antioxidant levels high.

Effective ozone flux and detoxification power

The 'effective ozone flux' concept is based on a balance between stomatal flux and leaf cellular detoxification. The degree of stomatal opening controls the quantity of ozone molecules entering the leaf and subsequently being converted to ROS. Ozone itself can trigger the activity of a plasma membrane NADPH oxidase delivering the superoxide ion (Langebartels *et al.* 2002). On the other hand, even though representing a small percentage of total superoxide dismutase (SOD), an apoplastic Cu–Zn SOD could scavenge, at least in part, the superoxide ion (Schinkel *et al.* 1998; Srivastava 1999). The main level of defence relies both on the existing content of cellular antioxidants (*e.g.* ascorbate) and intensity of the detoxifying processes regenerating these metabolites. A specific pool of apoplastic ascorbate is thought to react directly with ozone and ROS (Polle *et al.* 1995; Luwe 1996; Turcsanyi *et al.* 2000). However, the apoplasm can be easily and rapidly depleted of ascorbate, allowing the subsequent oxidative action of ROS in foliar cells (Zheng *et al.* 2000; van Hove *et al.* 2001). An efficient protective mechanism would require the transfer of ascorbate from intracellular detoxifying systems to the cell wall. The detoxification processes thus elicited must be able to cope with this increased demand. Increases in antioxidant enzyme activities [SOD, ascorbate peroxidase (APX), glutathione reductase (GR)] have been observed in a large number of ozone fumigation experiments (Tandy *et al.* 1989; Sen Gupta *et al.* 1991; Wingsle *et al.* 1992; Pitcher & Zilinskas 1996; Sehmer *et al.* 1998; Srivastava 1999). These changes are often linked to parallel changes in gene expression (Willekens *et al.* 1994; Gupta *et al.* 2005; Heath 2008). However, while a general increase in antioxidant defences usually follows ozone attack, the degree of sensitivity of foliar cells will depend on equilibrium between the importance of the raised detoxifying barrier and the degree of stomatal ozone uptake (Di Baccio *et al.* 2008). A good indicator of sensitivity would thus be the potential detoxification capacity estimated per unit of ozone influx (Di Baccio *et al.* 2008; Matyssek *et al.* 2008).

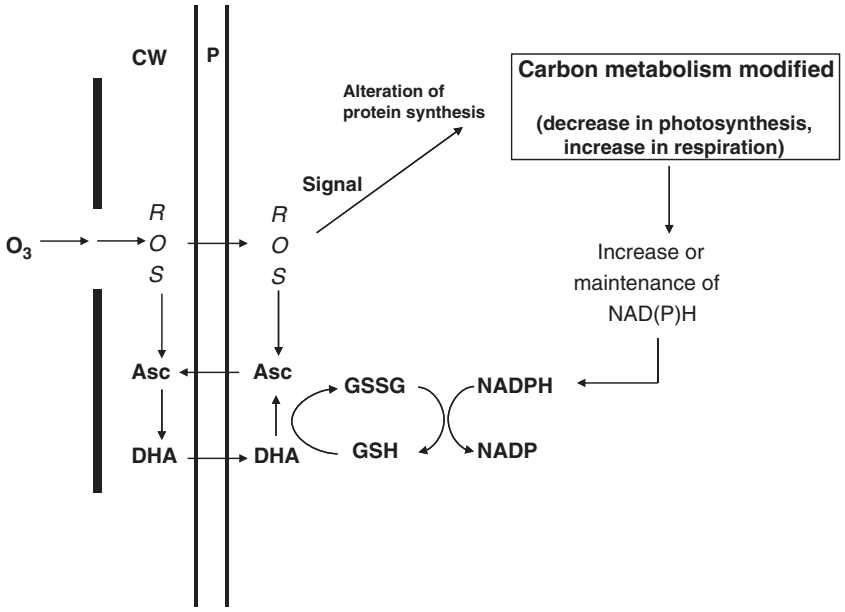

Fig. 1. Dual effect of ozone-induced ROS on the detoxification process and metabolic regeneration of reducing power.
Asc = ascorbate; CW = cell wall; DHA = dehydroascorbate; GSH/GSSG = reduced/oxidised glutathione; P = plasma membrane; ROS = reactive oxygen species.

Regeneration of the reduced forms of ascorbate and glutathione can be provided by enzymes using the reducing power of NAD(P)H. These soluble redox molecules appear to play a central role in establishment of antioxidant defence mechanisms (Noctor 2006; Dizengremel *et al.* 2008).

Ozone uptake and changes in carbon fixation

Plant cells perceive the presence of ozone and associated ROS as an 'oxidative signalling' stimulus. This has been documented to cause carbon metabolism changes and associated alteration in gene expression (Dizengremel 2001; Foyer & Noctor 2005; Heath 2008; Renaut *et al.* 2009). The effect of ozone in decreasing the photosynthetic rate relates to a corresponding decrease in activity of Rubisco, as observed in various plant species (Pell *et al.* 1992; Dizengremel *et al.* 1994; Dizengremel 2001; Gaucher *et al.* 2003; Inclan *et al.* 2005; Wittig *et al.* 2007; Bagard *et al.* 2008). This ozone-induced decrease in Rubisco activity is linked to a decrease in Rubisco quantity, which could involve both an increased rate of protein degradation and an inhibition of protein synthesis (Brendley & Pell 1998; Pell *et al.* 1999; Junqua *et al.* 2000; Dizengremel 2001; Pelloux *et al.* 2001; Gupta *et al.* 2005; Matyssek *et al.* 2006; Bohler *et al.* 2007; Heath 2008). In C_3 plants, the carboxylating enzyme is Rubisco in chloroplasts, while phosphoenolpyruvate carboxylase (PEPc) refixes respiratory CO_2 in the cytosol. In the presence of ozone, Rubisco activity decreases, while a strong increase in activity of PEPc is observed (Landolt *et al.* 1994, 1997; Sehmer *et al.* 1998; Fontaine *et al.* 1999; Lütz *et al.* 2000; Gaucher *et al.* 2003; Inclan *et al.* 2005; Leitao *et al.* 2008; Renaut *et al.* 2009). Table 1 shows the opposite effect of chronic doses of ozone on the ratio between the two carboxylases for several species. The Rubisco/PEPc ratio decreases between three- and twentyfold (Table 1). This contrasting behaviour of the two carboxylases was observed in field studies where needles of declining trees in ozone-polluted areas had a lower Rubisco/PEPc ratio compared with that measured for needles from healthy trees (Wild & Schmitt 1995; Dalstein *et al.* 2002). The increase in PEPc activity is clearly linked to an increase in the quantity of PEPc protein and of the corresponding mRNAs (Dizengremel 2001; Fontaine *et al.* 2003; Matyssek *et al.* 2006). PEPc could, under chronic ozone constraint, contribute to an anapleurotic pathway accounting for the replenishment of the mitochondrial Krebs cycle *via* oxaloacetate (or malate), as previously suggested (Dizengremel 2001).

Table 1. Rubisco and PEPc activities (in nkat mg^{-1} protein) and ratios of the two carboxylases in leaves of trees submitted to chronic ozone exposure in phytotron chambers. (C) control; (O_3) ozone-fumigated.

	Rubisco		PEPc		Rubisco/ PEPc ratio	
	C	O_3	C	O_3	C	O3
Norway spruce[a]						
Gerardmer	12.8	6.0	0.50	5.40	*25.6*	*1.11*
Istebna	13.2	6.2	0.45	2.55	*29.3*	*2.43*
Aleppo pine[b]	13.6	7.5	0.55	1.96	*24.7*	*3.82*
Poplar[c]	17.0	9.1	0.36	1.35	*47.2*	*6.74*
Beech[d]	9.2	4.65	1.33	1.81	*6.9*	*2.56*
Sugar maple[e]	2.56	1.76	0.75	1.73	*3.4*	*1.01*

[a]Antoni 1994. Three-year-old Norway spruce (*Picea abies*; Gerardmer and Istebna clones) submitted to 200 $nl \cdot l^{-1}$ O_3 during the photoperiod for 3 months. Measurements were made at the end of the experiment.

[b]Fontaine *et al.* 1999. Three-year-old Aleppo pine (*Pinus halepensis*) exposed to 200 $nl \cdot l^{-1}$ O_3 during the photoperiod for 3 months. The activities were measured on day 34.

[c]Deschaseaux 1997. Hybrid poplar (*Populus tremula* × *alba*) cuttings exposed to 60 $nl \cdot l^{-1}$ O_3 during the photoperiod for 1 month. Activities were measured after 2 weeks of fumigation.

[d]Lütz *et al.* 2000. Three-year-old beech (*Fagus sylvatica*) exposed for 4 months to 2 × ambient O_3 concentration (up to 110 $nl \cdot l^{-1}$). Measurements were made after 3 months.

[e]Gaucher *et al.* 2003. Forty-five-day-old sugar maple seedlings (*Acer saccharum*) were fumigated with 200 $nl \cdot l^{-1}$ O_3 during the photoperiod for 2 months. Measurements were made at the end of the experiment.

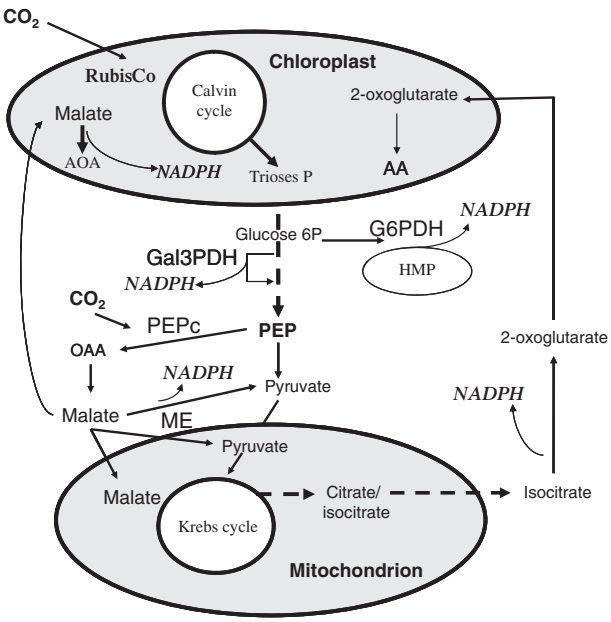

Fig. 2. Enzymes and associated pathways contributing to the supply of NADPH. AA = amino acids; Gal3PDH = NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase; G6PDH = glucose-6-phosphate dehydrogenase; HMP = hexose monophosphate pathway; IDH = -NADP-dependent isocitrate dehydrogenase; ME = NADP-dependent malic enzyme; OAA = oxaloacetic acid; PEPc = phosphoenolpyruvate carboxylase.

Ozone, pathways of carbohydrate breakdown and reducing power

The anapleurotic pathway, after replenishment of the mitochondrial Krebs cycle, provides precursors for amino acid synthesis (Stitt 1999). This pathway uses citrate (or isocitrate) exported from this cycle to produce 2-oxoglutarate in the cytosol through an NADP-dependent isocitrate dehydrogenase (NADP-IDH) delivering NADPH (Chen & Gadal 1990; Fig. 2). Cytosolic NADP-IDH has been suggested to play a role in plant defence against oxidative stress by delivering NADPH (Hodges *et al.* 2003; Leterrier *et al.* 2007). An increase in activity of NADP-IDH has been measured in two tree species under ozone fumigation (Table 2) (Citerne *et al.* 1989; Fontaine 1999), which reinforces this hypothesis. The anapleurotic pathway also serves as a donor of malate directly in the cytosol to produce pyruvate and to provide NADPH through the NADP-malic enzyme (NADP-ME). Cytosolic NADP-ME has been linked to stress responses (Drincovich *et al.* 1998; Müller *et al.* 2008) and an increase in activity of this enzyme has been observed under ozone treatment (Table 2) (Fontaine 1999). Since respiration is generally increased by ozone (Dizengremel & Petrini 1994; Dizengremel 2001), enhancement of both the glycolysis–Krebs cycle and pentose phosphate pathway is expected. The most striking effect of ozone is increased activity of glucose-6-phosphate dehydrogenase (G6PDH) in various tree species, which can contribute to increased NADPH content (Citerne *et al.* 1989; Antoni 1994; Dizengremel *et al.* 1994; Sehmer *et al.* 1998; Fontaine 1999; Gaucher *et al.* 2003). The two main glycolytic enzymes, phosphofructokinase (PFK) and pyruvate kinase (PK), are also involved in a general increase of catabolism (Table 2) (Antoni 1994; Dizengremel *et al.* 1994; Sehmer *et al.* 1998; Fontaine 1999). In mitochondria, fumarase and NAD-malic enzyme (NAD-ME) activities increase with ozone exposure (Table 2) (Citerne *et al.* 1989; Antoni 1994; Dizengremel & Petrini 1994; Dizengremel *et al.* 1994; Gérant *et al.* 1996; Sehmer *et al.* 1998; Fontaine 1999). A set of three cytosolic enzymes (G6PDH, NADP-IDH, NADP-ME) is especially interesting as they provide NADPH in the cytosol, assisting ozone detoxification (Dizengremel *et al.* 2008). Another cytosolic enzyme, the non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-Gal3PDH), has been

Table 2. Effect of chronic ozone exposure on the activities (in nkat.mg^{-1} protein) of enzymes belonging to different catabolic pathways.

	Glycolysis		*HMP Pathway*	*Cytosol*		*Mitochondria*	
	PFK	PK	G6PDH	NADP-ME	NADP-IDH	Fumarase	NAD-ME
Norway spruce (clone Gerardmer)							
OTC[a]			×1.43		×1.06	×2.07	×1.20
Phytotron[b]	×1.80		×5.40			×2.52	
Beech (OTC)[c]	×1.06		×1.80			×1.23	
Loblolly pine (OTC)[d]							
current-year	×1.20		×1.18			×1.06	×1.16
1-year-old	×1.22		×1.28			×1.09	×1.07
Aleppo pine (phytotron)[e]							
current-year		×1.48	×1.25		×1.42		×1.35
1-year-old		×2.70		×2.30			×2.10
1-year-old[f]							×1.31
Sugar maple (phytotron)[g]			×2.05				
Poplar (phytotron)[h]		×3.66		×2.36		×1.96	×1.56

[a]Six-month-old spruce seedlings were fumigated from spring 1985 in open-top chambers (OTC) with ambient ozone concentrations. Measurements were made on current year needles in November 1987 (Citerne *et al.*, 1989).

[b]Three-year-old spruce trees were fumigated in phytotronic chambers for 3 months with 200 nL.L^{-1} ozone. Measurements were made on current-year needles at the end of the experiment (Sehmer *et al.*, 1998).

[c]Five-year-old beech trees were fumigated for 2 years (vegetation period) in OTC with non-filtered + 50 nL.L^{-1} ozone. Measurements were made in summer, at the end of the treatment (Antoni, 1994).

[d]One-year-old loblolly pine trees were fumigated in OTC with 2 x ambient ozone concentration (12h daily mean ozone concentration of 86 nL.L^{-1}). Measurements were made in summer on current-year and 1-year old needles, the third growing season of exposure (Dizengremel *et al.*, 1994; Dizengremel and Pétrini, 1994).

[e]One-year-old Aleppo pine trees were fumigated in phytotronic chambers for 3 months with 200 nL.L^{-1} ozone. Measurements were made at the end of the experiment on current-year and 1-year old needles (Fontaine, 1999).

[f]Three-year-old Aleppo pine trees were fumigated in phytotronic chambers for 3 months with 200 nL.L^{-1} ozone. Measurements were made at the end of the experiment on 1-year old needles (Gérant *et al.*, 1996).

[g]Forty-five-day-old sugar maple seedlings were fumigated in phytotronic chambers for 2 months with 200 nL.L^{-1} ozone. Measurements were made at the end of the experiment (Gaucher *et al.*, 2003).

[h]Two months old poplar seedlings were fumigated in phytotronic chambers during 1 month with 100 nL.L^{-1} ozone (Dizengremel and Jolivet, 2000, unpublished data).

implicated in coping with oxidative stress (Bustos *et al.* 2008) and also needs to be studied (Fig. 2).

Reducing power and threshold for ozone risk to plant cells

In addition to deposition on cuticles, ozone molecules enter leaves through the stomata. Some molecules can then impair membranes of mesophyll cells (the fate of guard cells is still largely debated) while others and/or O_3-derived ROS could be destroyed by apoplastic ascorbate. When ROS formation exceeds the antioxidant capacity of cells, ROS can play a dual role, directly oxidising the detoxifying systems in chloroplasts and cytosol and inducing a chain of signal messengers leading to changes in gene expression (Sandermann *et al.* 1998; Dizengremel 2001; Rao & Davis 2001; Foyer & Noctor 2005; Kangasjärvi *et al.* 2005; Heath 2008). The photosynthetic machinery is one of the first targets of ROS. The Calvin cycle, through the oxidation of the Rubisco protein and the repression of its synthesis, is damaged before any impairment of the light reactions (Dizengremel 2001; Heath 2008). An over-reduction of stromal compounds takes place transiently, with NADPH allowing the functioning of the Halliwell–Asada pathway. A possible transfer of excess NADPH in the cytosol by a malate-oxaloacetate shuttle could also transiently occur (Scheibe 2004). NADPH export stops when the photosystems are damaged.

In parallel with the above reactions, there is a general increase in activity of catabolic pathways, with a particular emphasis on the huge rise in PEPc activity and synthesis (Fontaine *et al.* 2003). An unavoidable lack of carbon compounds, needed for repair and growth, may result. However, before falling inexorably towards death, cells typically activate an antioxidant defence mechanism. A possible signal could be the increase in PEPc activity, which allows the provision of malate through this shunt of glycolysis, and thus starts the anapleurotic pathway. Malate is known to play a central role in plant cell metabolism, and an increase in its quantity could directly provide additional NADPH through the NADP-ME pathway. Another possible role for malate is in continuation of the anapleurotic pathway by transfer of malate to mitochondria, where it participates in the Krebs cycle (Fig. 2). Citrate or isocitrate are subsequently transferred to the cytosol, where 2-oxoglutarate and NADP-IDH are produced (Fig. 2). In both cases, malate stimulates production of NADPH, which is useful for the ascorbate/glutathione reducing system. Finally, a provocative hypothesis is that malate enters the chloroplast, where it is oxidised by malate dehydrogenase (MDH) to produce NADPH to restore (at least in part) intra-chloroplastic reducing power (Fig. 2) Malate could thus play a central role to balance cellular energy supply between compartments (Scheibe 2004).

Other sources of cytosolic NADPH co-exist in the form of NADP-glyceradehyde-3-phosphate dehydrogenase (another shunt of glycolysis) and G6PDH (hexose monophosphate pathway). The observed increases in activity of this set of enzymes with ozone exposure could be related to a modification of the enzyme itself (NADP-Gal3PDH) or activation of transcriptional regulation. The question arises as to whether all these systems equally contribute to the NADPH supply, or whether there is a hierarchical system favouring one of the above systems. New experimental data will, hopefully, allow for a definition of the ratio between reduced and oxidised forms of pyridine nucleotides beyond a value where damage is irreversible. This breaking point should be used in a cellular model of effective ozone flux, which should be integrated into a global model to improve prediction of ozone risk for plant growth. The ultimate goal of this approach is to enhance estimation of the environmental and economic consequences of tropospheric ozone to plants.

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