Effect of ascorbate and its oxidation products on H₂O₂ production in cell-suspension cultures of *Picea abies* and in the absence of cells

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**Abstract**

Dehydroascorbate and traces of ascorbate were present apoplastically in living spruce (*Picea abies*) twigs. Since the proposed apoplastic ascorbate degradation pathway contains several steps that possibly generate H₂O₂, the effects of ascorbate and some of its degradation products were tested on apoplastic H₂O₂ concentrations in a cell culture of *P. abies* as a model and on non-enzymic H₂O₂ production in vitro. Ascorbate scavenged H₂O₂ in the culture medium of lignin-producing *Picea* cells and in spent and boiled spent medium; in the presence of Cu²⁺ or fresh medium, ascorbate led to the non-enzymic generation of H₂O₂. Preparations of dehydroascorbate (the initial oxidation-product of ascorbate), and diketogulonate (the hydrolysis-product of dehydroascorbate) induced H₂O₂ accumulation both non-enzymically and enzymically in *Picea* cell-suspensions. Paper electrophoresis showed that the dehydroascorbate and diketogulonate preparations contained several degradation products; some of these probably contributed to H₂O₂ production and/or scavenging in these experiments, and would also do so in *vivo*. These results indicate a complex ability of apoplastic ascorbate, dehydroascorbate, diketogulonate, and further products to modulate H₂O₂ concentrations, with potential consequences for the control of growth, development and lignification.

**Key words:** Apoplast, ascorbate, dehydroascorbate, diketogulonate, hydrogen peroxide, reactive oxygen species.

**Introduction**

Vitamin C, L-ascorbic acid (AA), is a well-known antioxidant that efficiently scavenges free radicals, for example reactive oxygen species, formed in cellular metabolism or derived from the atmosphere (reviewed by Noctor and Foyer, 1998; Horemans et al., 2000; Smirnoff, 2005). In addition to its protective role against oxidative stress, AA is a co-factor of many enzymes and has a role in plant cell division and elongation (Chinoy, 1984; Asard et al., 2004).

In many plant tissues AA is present at mM concentrations, predominantly as the singly ionized form (pK_\text{a1} / 25.4.2). A few per cent of the total of the plant’s AA is found in the apoplast (the liquid permeating the cell walls; Castillo et al., 1987; Polle et al., 1990; Takahama, 1993a; Otter and Polle, 1994; Sánchez et al., 1997; Horemans et al., 2000; Hernandez et al., 2001; Veljovic-Jovanovic et al., 2001; Burkey et al., 2003; Córdoba-Pedregosa et al., 2003; Pedreira et al., 2004; Hu et al., 2005), making it an important antioxidant in the plant cell wall as well (Pignocchi and Foyer, 2003; Sanmartín et al., 2003; Jaspers et al., 2005). Many abiotic and biotic stresses lead to rapid changes in the concentration and/or redox status of the apoplastic AA, for example, wounding (Takahama, 1993a), salt stress (Hernandez et al., 2001), ozone treatment (Burkey et al., 2003; Jaspers et al., 2005), and water deficit (Hu et al., 2005). AA concentration also changes during the diurnal cycle (Burkey et al., 2003) and with normal development (Sánchez et al., 1997; Córdoba-Pedregosa et al., 2003; Pedreira et al., 2004).

AA is oxidized either enzymically or non-enzymically to L-dehydroascorbate (DHA) via the monodehydro-L-ascorbate
radical (Noctor and Foyer, 1998; Deutsch, 2000; Smirnoff, 2005). In the symplast an efficient enzymic regeneration of AA from DHA occurs via the AA–glutathione cycle (Foyer and Halliwell, 1976; Noctor and Foyer, 1998), making AA a major redox buffer in plants. Ascorbate oxidase (AAO), a cell wall-localized enzyme, regulates the redox status of AA in the apoplast: it oxidizes AA with the concomitant reduction of O₂ to water (Pignocchi and Foyer, 2003; Sanmartin et al., 2003; Smirnoff, 2005). No clear evidence for an apoplastic AA regeneration system exists. However, different plasma membrane-associated carrier systems for AA and DHA translocation have been reported in several plant species (reviewed by Horemans et al., 2000). Preferential uptake of the oxidized form (DHA) into the cells for re-reduction has been described (Horemans et al., 2000). Alternatively, as DHA is unstable in aqueous solutions, it can be further metabolized in the cell wall. The first step is the hydrolysis of DHA to form 2,3-diketo-L-gulonate (DKG), which is thought to be unstable in aqueous solutions, it can be further metabolized to form the polymer, lignin (Boerjan et al., 2003; Ralph et al., 2003). Oxidative coupling of ferulates to form dehydrodiferulates in primary cell walls (and to some extent intra-protoplasmically: Fry et al., 2000) of gramineous monocots and of certain dicots (e.g. Chenopodiaceae) and of tyrosine residues of cell wall glycoproteins to form diisodityrosine and related products are possibly mediated by peroxidases (Kerr and Fry, 2004; Fry, 2004, Encina and Fry, 2005). All these cross-linking reactions in the cell wall are likely to lead to the restriction of cell elongation. In this context, it is interesting that a negative correlation was found between apoplastic H₂O₂ levels and the growth rate of pine hypocotyls, and that there was a positive correlation between the AA/H₂O₂ ratio and the growth rate (Pedreira et al., 2004).

AA interferes with the peroxidase-mediated oxidation of phenolics by reducing phenolic radicals back to the original phenolics, thereby scavenging H₂O₂ (Takahama, 1993a, b; Otter and Polle, 1994; Sanchez et al., 1997). It would therefore be predicted that, to allow lignification or dimerulate formation to occur in the cell wall, apoplastic AA would first need to be completely oxidized to DHA. The reduced form (AA), however, only partially inhibited phenolic oxidation by covalently bound cell wall peroxidases from stems of Kalanchoe daigremontiana (Takahama, 1993a), young stems of Vigna angularis (Takahama, 1993b) or from lignifying needles of Picea abies (Otter and Polle, 1994). H₂O₂ content was shown to increase and AA content to decrease in the apoplastic fluid of pine hypocotyls with increasing hypocotyl age and along the hypocotyl axis, correlating with the reduction in growth (Pedreira et al., 2004).

In addition to its antioxidant character, the pro-oxidant nature of AA is well established. In the presence of transition metal ions, AA generates H₂O₂ non-enzymically (Dekker and Dickinson, 1940). H₂O₂ can then be used to generate extremely reactive hydroxyl radicals (•OH) via the Fenton reaction (Fry, 1998; Tabbi et al., 2001; Vreeburg and Fry, 2005). Hydroxyl radicals are hypothesized to have a role in cell-wall loosening by non-selectively breaking bonds within cell-wall polysaccharides, for example during root elongation (Schopfer, 2001; Rodriguez et al., 2002, 2004; Liszkay et al., 2004) or fruit ripening (Fry et al., 2001; Dumville and Fry, 2003; Vreeburg and Fry, 2005). In this connection, it is interesting that Pedreira et al. (2004) found a small increase in irreversible extension in response to exogenous 5 mM H₂O₂ in auxin-untreated pine hypocotyl segments (P <0.01 according to the data plotted in Fig. 2a of Pedreira et al., 2004). Likewise, Córdoba-Pedregosa et al. (2003) found higher apoplastic H₂O₂ concentrations in the apical 2 cm of onion roots (i.e. the portion containing the growing zone) than in the region 2–6 cm back from the tip. The apical 2 cm also had a lower AA/(AA+DHA) ratio in the apoplast than did the non-growing parts further back from the tip. Thus rapid cell expansion was associated here with a relatively oxidizing apoplastic environment (Córdoba-Pedregosa et al., 2003).

The present paper presents observations on AA, DHA, DKG, and apoplastic H₂O₂ production in cells of a conifer, the Norway spruce (Picea abies). Needles of Picea are among the plant materials already reported to possess both AA and DHA in the apoplast (Castillo et al., 1987; Polle et al., 1990; Otter and Polle, 1994), as are hypocotyls of another conifer, Pinus (Sánchez et al., 1997; Pedreira et al., 2004). Since the proposed apoplastic AA degradation pathway contains several steps that possibly generate H₂O₂ (Green and Fry, 2005a, b), the effects of AA and some of its degradation products were tested on apoplastic H₂O₂ levels. A cell culture of Picea abies that produces extracellular lignin (Simola et al., 1992; Brunow et al., 1998; Kärkönen et al., 2002) was used in in-vivo assays for H₂O₂. Peroxidases have an important role in the activation of monolignols for subsequent polymerization in this cell culture as the removal of endogenous H₂O₂ significantly decreased the extracellular lignin formation (Kärkönen et al., 2002; Koutaniemi et al., 2005). Non-enzymic responses in in-vitro assays were monitored by the use of fresh culture medium, cell-free spent culture medium, boiled spent culture medium, and CuSO₄ (1 µM) solution. As some AA degradation products were observed to induce H₂O₂ generation in vivo, the endogenous AA and DHA

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content of the spent medium of spruce cell-suspensions and of the apoplast of spruce twigs were investigated during xylem differentiation. In this paper the two opposing effects of AA on H₂O₂ levels are confirmed: first, the scavenging of H₂O₂ in the culture medium of lignin-producing Picea cells and in spent and boiled spent medium; secondly, the non-enzymic generation of H₂O₂ in the presence of Cu²⁺ or fresh medium. The existence of degradation product(s) of AA that induce efficient H₂O₂ production (i) in a cell-dependent way, and (ii) in a non-enzymic reaction independent of cells is also reported. The data are discussed with reference to the biochemistry of the plant cell wall.

Materials and methods

Chemicals

AAO, catalase, DHA, and xylenol orange (XO) were obtained from Sigma-Aldrich. AAO was dissolved (1 U μl⁻¹) in 50 mM succinate (Na⁺, pH 5.6) supplemented with 0.05% BSA. DKG was prepared from the commercial DHA by alkali treatment (White and Krupka, 1965; Green, 2003): a stock of DHA (50 mM) was prepared in water, a 1.3-fold excess of NaOH was added and the mixture incubated at 20°C for 6 min. The reaction was stopped by the addition of L-tartaric acid to a final pH (checked by pH paper) of ~3.5–4.0.

Cell suspensions of spruce

Cultured cells of Picea abies Karst. (kindly provided by Professor emerita LK Simola, Professor TH Teeri, and Professor IA Kilpeläinen, University of Helsinki) were used as a model system for in vivo studies of H₂O₂ production. Callus was maintained as previously described (Simola et al., 1992; Kärkönen et al., 2002). For the experiment, c. 3 g callus (c. 2 weeks after subculturing) was transferred into 150 ml of liquid medium ‘5’ (Simola and Santanen, 1990) modified by omission of amino acids (except glycine), but supplemented into 150 ml of liquid medium ‘5’ (Simola and Santanen, 1990) and buffered to pH 4.5 with 10 mM Na₂EDTA, 250 μM FeCl₃, 100 μM CuSO₄, 250 μM (NH₄)₂SO₄. However, because AA interferes with the XO assay (probably by reducing Fe³⁺ back to Fe²⁺), all AA-containing samples were treated with AAO (1 U 100 μl⁻¹) for 5 min before addition of the XO reagent; in control experiments, AAO did not affect H₂O₂ during a 5 min treatment (data not shown). All sample/XO mixtures were incubated for 40 min at room temperature before measurement of A₅₆₀ against a blank prepared with 100 μl water+1 ml of XO mixture. Because of some darkening of the XO reagent mixture with time, and so that the background of the medium or CuSO₄ solution could be subtracted, the A₅₆₀ given by fresh nutrient medium (or 1 μM CuSO₄)+XO at the beginning and at the end of the experiment was plotted against time. A correction factor obtained was subtracted from the A₅₆₀ values given by samples+XO. CuSO₄ was observed not to interfere with the XO assay. Experiments were performed at least in duplicate, with each treatment in triplicate. The pattern of the response was similar in each repetition, and the figures show the results of one representative experiment.

For comparison of independent experiments, a standard curve was prepared with different concentrations of H₂O₂ in the fresh nutrient medium or CuSO₄ solution. A dilution series of H₂O₂ was prepared in water and a portion of this added to nutrient medium or CuSO₄ immediately before addition to XO reagent.

Search for the active component in the nutrient medium responsible for induction of non-enzymic H₂O₂ production after DHA or DKG addition

Since commercial DHA and the DKG preparation induced rapid non-enzymic H₂O₂ production when added to fresh nutrient medium, the nutrient components were tested individually to indicate which one(s) induced DKG-dependent H₂O₂ production. Each nutrient salt was dissolved in water [at the same concentration as in medium ‘5’ (Simola and Santanen, 1990) and buffered to pH 4.5 with 10 mM succinate (Na⁺, pH 5.6)] and was added to 1 ml of the buffered solution and incubated for 60 min at 25°C, after which 100 μl of the solution was added to XO reagent.

Catalase treatment

Catalase (100 μg; 1100 U) in 10 μl water was added to 5 ml of cell suspension or medium at the same time as any AA, DHA, DKG, or elicitor (= time 0) when indicated.

UV absorption spectrum of the DKG preparation

The absorption spectrum of the DKG preparation (0.5 mM), without or with AAO treatment (4 U ml⁻¹), in 50 mM succinate (Na⁺, pH 5.6) was scanned over the range of 190–390 nm with an Agilent 8453 spectrophotometer.
Analysis of the purity of DHA and DKG

DHA and DKG are unstable in aqueous solutions (Kimoto et al., 1993; Deutsch and Santhosh-Kumar, 1996; Nishikawa et al., 2001). For checking of the purity of these compounds, 5–200 μg portions of each compound were subjected to high-voltage electrophoresis at pH 6.5 (3.5 kV, 30 min) on Whatman 3MM paper as described by Fry (2000). The electrophoretogram was stained with silver nitrate.

Extraction of AA and DHA

For assessment of the physiological significance of the biochemical findings, AA and DHA were assayed in the apoplastic space of developing Picea wood and in the spent medium of Picea cell suspensions. Extraction of the extracellular washing fluid (EWF) was conducted according to Castillo et al. (1987) and Polle et al. (1990). The current year’s twigs, with developing wood, were collected in Ruotsinkylä, South Finland, in mid-July (c. 09:30–10:00 h on a hot, sunny day) and transported to the laboratory on ice. Twigs of three individual trees were collected on two dates (one day between each) so the results are from six individuals. The bark and phloem were peeled off by hand and the secondary xylem was used for AA extraction. Twigs were cut into c. 1.0–1.5-cm lengths and briefly stored in distilled water. The material was blotted dry with paper tissue, weighed, and infiltrated for 10 min (2×5 min) with 40 mM acetate (Na+, pH 4.5), supplemented with 100 mM KCl in a water-flow vacuum. After blotting with tissue paper, the plant material was packed in 5-ml syringe barrels, which were installed into 15-ml centrifuge tubes already containing 50 μl 10% metaphosphoric acid (HPO₃) to stabilize AA. EWF was eluted by centrifugation (5 min, 1000 g, 4 °C) and used for AA determination immediately.

After elution of the EWF, the remaining twigs were frozen in liquid nitrogen and stored at −80 °C until the next day when protoplasmic AA was extracted. For this, twigs were ground in liquid nitrogen in a mortar with a pestle. Four volumes of 5% HPO₃ were added to the powder and the samples kept on ice for c. 20 min. The cell-wall debris was removed by centrifugation (5 min, 15 000 g, 4 °C), and the supernatants were transferred to new tubes and centrifuged for 5 min before the AA analysis.

In assays for AA and DHA in the culture medium of Picea cells, non-buffered spent medium after 1 d in liquid culture was mixed with HPO₃ (to 5%) and used for the AA determination.

Determination of AA and DHA

AA analysis was conducted with an AAO method modified from Takahama and Onuki (1992). AA has an absorbance maximum at 265 nm at neutral pH, whereas DHA has negligible absorbance in this region. The assay mixture contained (final volume 1 ml) 90 mM phosphate (Na⁺, pH 6.8), 38 mM succinate (Na⁺, in order to neutralize the sample containing HPO₃), and 30 μl of extract, EWF or spent medium in 5% HPO₃. After assay of the initial A₂₆₅, AAO (2 U) was added to oxidize AA to DHA and A₂₆₅ was remeasured. DHA was reduced back to AA by incubation with dithiothreitol (DTT, freshly prepared, to 14.8 mM; final pH 5.8) for 10 min at 20 °C and A₂₆₅ was assayed again. With pure AA, 15 min was sufficient for complete back-reduction (data not shown). A standard curve was prepared with a dilution series of AA in 5% HPO₃. From the changes in A₂₆₅ value, the concentrations of AA and DHA were calculated.

Results

Effect of ascorbate on H₂O₂ production

Traces of Cu²⁺ and other transition metals promote H₂O₂ production in the presence of AA (Dekker and Dickinson, 1940; Fry 1998). It was confirmed that an aerated solution of AA (250 μM) containing 1 μM CuSO₄ generated H₂O₂ (at least 90 μM) in the absence of cells (Fig. 1). The effect of catalase verified the product as H₂O₂. Ten minutes’ preincubation of AA [250 μM (Fig. 1) or 1 mM (data not shown)] with AAO totally abolished its effectiveness, indicating that AA itself was the active species in H₂O₂ generation, and that its initial oxidation product, DHA, was unable to mimic it.

Fresh nutrient medium contains 1 μM Cu²⁺ and might therefore be expected to give yields of H₂O₂ in the presence of AA comparable to those shown in Fig. 1. When added to fresh nutrient medium, 100 μM AA rapidly induced some net production of H₂O₂ (Fig. 2) but much less than in pure 1 μM CuSO₄ solution (Fig. 1). After the first 15 min, the

Fig. 1. Non-enzymic generation of H₂O₂ by commercial ascorbate, commercial dehydroascorbate or a 2,3-diketogulonate preparation in the presence of Cu²⁺. Concentrations of additives were 250 μM AA, 1 mM DHA, and 1 mM DKG. All solutions contained 10 mM tartrate (Na⁺, pH 4.5) and 1 mM CuSO₄. The effects of catalase (220 U ml⁻¹; added at the same time as AA) and a 10-min pretreatment of the additives with ascorbate oxidase (AAO, 2 U per 5 ml) are also shown. A solution of 1 μM CuSO₄ with no AA, DHA, or DKG was used as a control for the XO assay and therefore gave 0 μM H₂O₂ by definition. The lower graph shows the same data on a different γ-axis scale. Note concerning the figures: where possible the following convention has been used throughout this paper—squares (□, □, □, etc.) = ascorbate; circles (●, etc.) = dehydroascorbate; triangles (▲, ▲, etc.) = diketogulonate; diamonds (♦, etc.) = exogenous H₂O₂; hexagons (○, etc.) = no added oxidants/antioxidants.
H$_2$O$_2$ reached a steady-state concentration of 0.5–1.0 µM (Fig. 2 and data not shown). These results indicate that fresh medium contains a factor which, in the presence of AA, destroys H$_2$O$_2$; this destruction competed with the (AA+Cu$^{2+}$)-induced formation of H$_2$O$_2$. Possibilities could include the 25 µM iodide and 125 µM EDTA present in fresh medium. In spent or in boiled spent medium, AA functioned as an antioxidant, accelerating the removal of the endogenous H$_2$O$_2$ until it too reached a steady-state concentration of 0.5–1.0 µM (Fig. 2).

When AA (5–100 µM) was added to non-elicited spruce cell suspensions, the response was similar to that observed in spent or boiled spent medium: the steady-state extracellular H$_2$O$_2$ concentration routinely observed in water-treated control cultures (3.6±0.7 µM; mean ±SD of five experiments) was diminished by AA treatment (Fig. 3). This effect was probably partly due to the ability of AA to scavenge the free radicals ($\Phi$–O$^\cdot$) formed from apoplastic phenolic compounds ($\Phi$–OH) by peroxidase +H$_2$O$_2$ (Takahama 1993a, b), thus regenerating the phenolics and constantly restoring their ability to react with any remaining H$_2$O$_2$. AA also promoted the disappearance of H$_2$O$_2$ from boiled spent medium about as effectively as in unboiled spent medium (Fig. 2). However, this was probably due to the heat-resistant peroxidase activity (c. 20% of the initial; data not shown; cf. Otter and Polle, 1994) still present after 5 min boiling. It was confirmed that coniferyl alcohol was secreted by these cells (data not shown) and that the addition of AA to cell suspensions caused exogenous H$_2$O$_2$ to disappear rapidly (Fig. 4). This scavenging effect was dependent on the AA concentration used (Fig. 3); at lower concentrations (5 µM AA), the H$_2$O$_2$ concentration returned to the value observed in water-treated control cell-suspensions, probably because 5 µM AA was fully oxidized during the 3-h experimental period, allowing H$_2$O$_2$ concentrations subsequently to build back up to their steady-state levels.

![Fig. 2](https://academic.oup.com/jxb/article-abstract/57/8/1633/525164)

Fig. 2. Generation of H$_2$O$_2$ by ascorbate, and longevity of exogenous H$_2$O$_2$, in non-elicited Picea cell-suspensions and their media. H$_2$O$_2$ levels in the medium were assayed after addition of AA (100 µM) or H$_2$O$_2$ (25 µM) to live cell-suspensions of Picea abies, to fresh nutrient medium (FM), to cell-free spent medium (SM), or to boiled spent medium (BSM). The lower graph shows the same data on a different y-axis scale.

![Fig. 3](https://academic.oup.com/jxb/article-abstract/57/8/1633/525164)

Fig. 3. Effect of ascorbate on the H$_2$O$_2$ concentration in the media of non-elicited and elicited Picea cell-suspensions. The AA was added at 5, 25, and 100 µM; the elicitor, when present, was at $\sim$5 µg ml$^{-1}$. 

Apoplastic ascorbate modulates H$_2$O$_2$ levels
In the absence of AA, the Heterobasidion elicitor preparation induced a large burst of extracellular H$_2$O$_2$ after a brief lag period (Fig. 3). The peak occurred at 60–90 min, after which the H$_2$O$_2$ concentration started to decrease. In elicited cell-suspensions, 5 μM AA did not affect the timing or extent of this burst of H$_2$O$_2$ production (Fig. 3), probably because the large amounts of H$_2$O$_2$ formed were easily able to titrate out the limited amounts of AA. With 25 μM AA, however, there was evidence for an ~8 min increase in the lag period (Fig. 3), comparable to the time taken for 25 μM H$_2$O$_2$ to be destroyed by AA in the presence of cells (Fig. 4). With 100 μM AA, the lag period for elicitation of net H$_2$O$_2$ production was increased to c. 30 min, possibly representing the time required to produce sufficient H$_2$O$_2$ to oxidize this high dose of AA; interestingly, H$_2$O$_2$ concentrations thereafter continued rising until at least 180 min post-elicitation (Fig. 3), suggesting that an oxidation-product of AA was able to enhance H$_2$O$_2$ production in vivo. The effects of two specific AA breakdown products were therefore tested: DHA and DKG.

**Effect of dehydroascorbate on H$_2$O$_2$ production**

When commercial DHA (1 mM) was incubated with 1 μM CuSO$_4$ (Fig. 1) or with fresh nutrient medium in the absence of cells (Fig. 5), a gradual generation of H$_2$O$_2$ was observed. The formation of an XO-reactive product in the presence of commercial DHA was prevented by catalase, confirming the identity of H$_2$O$_2$ (Fig. 5). A 10-min pretreatment of the DHA with AAO abolished the subsequent non-enzymic formation of H$_2$O$_2$ in the presence of Cu$^{2+}$ (Fig. 1). Since AAO-treated AA (i.e. *in-situ* prepared DHA) did not drive H$_2$O$_2$ production (Fig. 1), it is suggested that it was not DHA itself that induced H$_2$O$_2$ formation but that the commercial DHA preparation contained a small proportion of AA as an H$_2$O$_2$-generating impurity. The presence of an AA-like compound in the DHA preparation was also suggested by the promotion of H$_2$O$_2$ destruction when commercial DHA was added to spent medium (Fig. 5).

Nevertheless, commercial DHA (1 mM) did promote H$_2$O$_2$ accumulation when added to cell suspensions (Fig. 6), whereas AA had the opposite effect (Figs 2, 3). This observation indicates that a compound other than AA was responsible for the induction of H$_2$O$_2$ generation.
after DHA addition to the cells, and shows that the cells have an active role in using (commercial) DHA to produce 
H$_2$O$_2$. The latter conclusion was supported by that fact that 
DHA induced more H$_2$O$_2$ in the presence of cells (Fig. 6) 
than in cell-free 1 μM CuSO$_4$ (Fig. 1) or fresh medium (Fig. 
5). Boiled spent medium already contained some XO-
reactive material (data not shown), whose identity as H$_2$O$_2$ 
was indicated by catalase sensitivity. Against this back-
ground of endogenous H$_2$O$_2$, it was not possible to detect 
any (additional) formation of H$_2$O$_2$ in boiled spent medium 
when supplied with commercial DHA (data not shown).

The process by which commercial DHA (1 mM) caused 
spruce cells to produce H$_2$O$_2$ was partially characterized. It 
required a short lag period (Fig. 6). Since the process did 
not occur in spent medium (Fig. 5), it clearly required the 
cells, not merely the enzymes of the spent medium.

Accumulation of the XO-reactive product was blocked by 
catalase, but not by boiled catalase, confirming its identity 
as H$_2$O$_2$ (data not shown). NaN$_3$ (100 μM) totally inhibited 
the DHA-induced increase in H$_2$O$_2$ concentration, and diphenylene iodonium (DPI; 5 μM) reduced it by ∼60–
70% (Fig. 6), suggesting that the H$_2$O$_2$ production was enzymic, possibly catalysed by peroxidases and/or plasma membrane-bound NADPH oxidase. However, the inhibitors used are unspecific, and further work is being carried out to characterize the enzymic systems involved (A Kärkönen, LK Simola, SC Fry, unpublished data).

In elicited cell suspensions, commercial DHA usually 
had little quantitative effect on the H$_2$O$_2$ burst, although in 
some experiments (e.g. Fig. 6) the duration of the elicited 
peak of H$_2$O$_2$ accumulation was prolonged by the DHA. 
However, DHA did have a qualitative effect: 100 μM azide 
did not completely block (DHA+elicitor)-induced H$_2$O$_2$ 
accumulation (Fig. 6), unlike the situation with H$_2$O$_2$ 
production induced by elicitor alone (which was completely 
inhibited even by 20 μM azide; A Kärkönen, LK Simola, 
SC Fry, unpublished results) or by DHA alone (Fig. 6). 
DPI also appeared to be less inhibitory to H$_2$O$_2$ pro-
duction induced by DHA+elicitor than to that induced 
by DHA alone (Fig. 6). These observations indicate 
that a mixture of DHA+elicitor was able to induce H$_2$O$_2$ 
production by a mechanism different from that (or those) 
evoked by either the elicitor or the DHA alone. The nature 
of the (DHA+elicitor)-induced mechanism remains unknown.

**Effect of a diketogulonate preparation on 
H$_2$O$_2$ production**

Addition of DKG to fresh medium evoked a rapid pro-
duction of H$_2$O$_2$ (Fig. 5). Each component of the fresh 
culture medium (except Fe, sucrose, inositol, and growth 
regulators) was tested individually (data not shown):

CuSO$_4$ was the only one that catalysed non-enzymic 
H$_2$O$_2$ production after DKG feeding, as detected by the 
XO assay (Fig. 1). (The XO assay was not used in solutions 
containing Fe$^{3+}$ since this is the species that reacts with 
XO; Gay *et al.*, 1999.)

Addition of DKG to spent medium or boiled spent 
medium (Fig. 5), or to a live cell suspension (Fig. 7) also 
induced a rapid increase in H$_2$O$_2$ concentration. This was 
partly due to the fact that the DKG preparation already 
contained some H$_2$O$_2$ (XO assay; data not shown). 
However, the pre-existing H$_2$O$_2$ was strongly exceeded 
by that produced during the incubation with fresh or boiled 
spent medium. When added to fresh medium, DKG 
induced intense non-enzymic H$_2$O$_2$ formation (Fig. 5), 
which was clearly additional to any H$_2$O$_2$ already present 
in the DKG preparation. The identity of the H$_2$O$_2$ pro-
duced was verified by catalase addition (data not shown).

DKG had much the same effect in boiled spent me-
dium as in fresh medium (Fig. 5). In non-boiled spent 
medium (i.e. with secreted *Picea* enzymes still active), 
the high concentration of initially formed H$_2$O$_2$ 
gradually decreased (Fig. 5), probably because of the 
peroxidase activity and monolignols present in the spent 
medium (Kärkönen *et al.*, 2002). The decrease was more 
rapid in some experiments than in others (data not shown), 
possibly because of variation in endogenous phenolic com-
 pounds or other H$_2$O$_2$ scavengers.

In fresh medium, the non-enzymic production of H$_2$O$_2$ 
by DKG (Fig. 5) was very rapid and had plateaued by 
20 min, while in 1 μM CuSO$_4$ it kept gradually rising 
almost linearly up to 90 min (Fig. 1). The plateauing is

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**Fig. 6.** Generation of H$_2$O$_2$ by dehydroascorbate and/or elicitor in *Picea* cell-suspensions, and the effect of metabolic inhibitors. Concentrations 
were DHA, 1 mM; *Heterobasidion* elicitor (E), ∼5 μg ml$^{-1}$; sodium 
azide, 100 μM; diphenylene iodonium (dpi), 5 μM.
unexplained: it was probably not due to a steady state (where production=decomposition) since exogenous H₂O₂ was stable in the presence of fresh medium (data not shown).

The DKG preparation had a prominent peak of UV absorbance at 271–272 nm (at pH 5.6; ε=780 M⁻¹ cm⁻¹ with respect to DKG molarity) probably attributable to an unidentified DKG degradation-product (Fig. 8). Interestingly, a 10 min pretreatment of DKG with AAO (before the addition of CuSO₄) led to higher initial H₂O₂ concentrations than were obtained with non-AAO-treated DKG (Fig. 1). A dramatic decrease (removal of the peak) in the A₄7×₁ of the DKG preparation was observed during AAO treatment (Fig. 8), probably indicating that the A₄7×₁ compound was being converted to a new (non-UV-absorbing) compound that was responsible for this initial H₂O₂ production. The initial difference in the H₂O₂ concentrations formed by AAO-treated and -untreated DKG had disappeared by 90 min (Fig. 1), suggesting that the gradual non-enzymic degradation of AAO-untreated DKG had generated the same stimulatory metabolite.

In the case of non-elicited cell-suspensions, the immediately DKG-induced H₂O₂ (possibly H₂O₂ pre-existing in the DKG preparation) was transient, returning to control levels in 30–60 min (Fig. 7). The cell suspension’s behaviour thus resembled that of spent medium (Fig. 5), suggesting that soluble extracellular materials played the major role in destroying H₂O₂ rather than cell-surface-located components.

When elicitor and DKG were added simultaneously, the immediately DKG-induced H₂O₂ was again seen; it was followed by a prolonged peak of H₂O₂ that remained high even when the H₂O₂ levels in elicitor-only controls had started to come down and those in DKG-only controls had returned to control values (Fig. 7). Catalase treatment confirmed the identity of H₂O₂ as the product formed in the presence of DKG+elicitor (data not shown). Thus, elicitor+DKG appeared to cause H₂O₂ production by a mechanism different from that/those triggered by either elicitor or DKG alone.

**Active component(s) in dehydroascorbate and diketogulonate preparations inducing H₂O₂ production**

It took at least 30 min to dissolve DHA (a 50 mM stock) in water. DHA is known to be unstable in water and non-enzymically hydrolyses to DKG, which is itself unstable (Kimoto et al., 1993; Deutsch and Santhosh-Kumar, 1996).
This lability was confirmed by paper electrophoresis followed by silver staining (Fig. 9). The DHA preparation contained several breakdown products, DKG among others (paper electrophoretograms in other pHs show that it is not only DKG that co-migrates with the big spot); and the DKG preparation contained several further breakdown products including some that were not detectable in the DHA preparation, probably including the unknowns referred to by Green and Fry (2005) as ‘C’ and ‘E’ (Fig. 9). These compounds were formed non-enzymically in aqueous solution at physiological pH, and will therefore probably also be generated from DHA and DKG in vivo. Work is underway to characterize the active compound(s) which contributed to H$_2$O$_2$ generation.

**Natural occurrence of apoplastic ascorbate in Picea**

For evaluation of the physiological significance of these biochemical findings, the AA and DHA content of developing *Picea* wood was assayed. Consistent results were obtained from six different trees. In general, the developing wood yielded c. 90 μg g$^{-1}$ FW EWF, representing the xylem sap plus an eluate of the aqueous solution that permeates the cell walls. About 1.5% of the total (AA+DHA) was detected in the EWF, almost all in the oxidized form (ratio AA/(AA+DHA)=0.06). There was therefore little AA in the cell wall to interfere with lignification. The concentration of DHA in the EWF was ~0.15 mM (i.e. 13.5±1.8 nmol g$^{-1}$ FW tissue). In the protoplasm, by contrast, the majority of the AA+DHA was in the reduced form, the average ratio AA/(AA+DHA) being 0.77.

Spent medium of *Picea* cell suspensions contained no detectable AA or DHA (limit of detection ≈1 μM), indicating that these compounds were either not secreted or were rapidly reabsorbed by the cells or metabolized in the medium to further degradation products (e.g. those shown in Fig. 9; Green and Fry, 2005a, b).

**Discussion**

**Endogenous apoplastic ascorbate and dehydroascorbate**

The concentrations of apoplastic AA+DHA observed here in lignifying spruce twigs (14.2±1.6 nmol g$^{-1}$ FW tissue) are comparable with the levels previously reported in some other tissues. For example, apoplastic total AA+DHA in the current year’s needles of spruce was 26 nmol g$^{-1}$ FW (Polle et al., 1990). Apoplastic fluid extracted from lignifying spruce needles contained a 5-fold lower AA+DHA concentration and a higher proportion of DHA than that from mature needles (1.17 mM and 5.26 mM with AA/(AA+DHA) ratios of 0.62 and 0.91, respectively; Otter and Polle, 1994). In *Pinus* hypocotyls, apoplastic AA was highest (2.8 nmol g$^{-1}$ FW) up to 13 d after germination, decreasing to ~1 nmol g$^{-1}$ FW thereafter (Pedreira et al., 2004).

Most investigators have found a much lower AA/(AA+DHA) ratio in the apoplast than in the symplast (Takahama, 1993a, 1998; Kollist et al., 2001; Veljovic-Jovanovic et al., 2001), although an exception was the report that apoplastic AA contributed about 80–90% of total apoplastic AA+DHA in young *Pinus* hypocotyls, decreasing to 65–70% after the growth of the hypocotyls had ceased (Sánchez et al., 1997). The present results with spruce twigs [AA/(AA+DHA)=0.06] are in this respect fairly typical. However, the possibility exists that during extraction of EWF some oxidation of AA occurred (Moldau et al., 1996; Sanmartin et al., 2003). It can be concluded that AA is secreted into the cell wall of developing xylem of spruce and a relatively high proportion of it is oxidized to DHA (as expected in a system with active lignin formation) and, in all probability, additional degradation products.

It was not possible to detect endogenous AA or DHA in the spent medium of spruce cell suspensions. Further work will be required to determine to what extent this observation is due to rapid turnover of a very small pool of AA, DHA and further degradation products. If cells of leaves, twigs, and roots secrete AA, it is likely that cultured cells also do, although such secreted AA would immediately be diluted into a large volume of culture medium making it difficult to detect.
Dehydroascorbate and other degradation products of ascorbate

Very soon after DHA has been dissolved in a neutral aqueous solution, some hydrolysis to DKG occurs (Bode et al., 1990; Kimoto et al., 1993). The appearance of a small proportion of AA (less than 1%) has also been demonstrated (Kimoto et al., 1993). It was found that DKG itself is also relatively unstable, yielding a range of incompletely characterized products. It might be suggested that since the preparations of DHA and DKG used in the present work were impure (Fig. 9), the present data do not establish the ability of these substances to produce and/or scavenge H$_2$O$_2$ in biological systems. However, it is likely that many or all of the products formed non-enzymically from DHA and DKG during the laboratory preparation of these substances would also be generated non-enzymically in the apoplast in vivo. Therefore, the data presented here can be assumed to provide information on the potential of AA, DHA, and DKA, and/or their respective degradation products, to contribute to the production and scavenging of H$_2$O$_2$ in the apoplast.

Although many of the reactions noted in the present work were non-enzymic, they were readily demonstrated at physiologically relevant pH values (4.5, typical of the apoplast in metabolically active plant cells) and room temperature. On this basis, these results do indeed support an extremely complex ability of apoplastic AA, DHA, DKG, and their further products to influence profoundly both the production and scavenging of H$_2$O$_2$, both in the presence of spruce cells and in cell-free culture media.

The production of H$_2$O$_2$ by AA, DHA and DKG at pH 4.5, was observed under certain circumstances DHA and DKG have previously been observed to accelerate the peroxidation of linoleic acid in neutral, but not in slightly acidic solutions (Takagi et al., 1988). A superoxide-scavenging agent, Tiron, suppressed linolate peroxidation; catalase had no effect, suggesting that the superoxide radical was the active species initially generated during incubation with DHA and DKG. H$_2$O$_2$ generation was observed in a reaction mixture buffered to pH 4.5 even though DHA is thought to be more stable at more acidic conditions. Although H$_2$O$_2$ was the species detected in the present work, the possibility remains that a different reactive oxygen species, for example superoxide, was its precursor, forming H$_2$O$_2$ by dismutation.

Biological relevance of AA and its degradation products in the cell wall

AA’s role as the major low molecular weight antioxidant in the apoplast is well established. The present results with AA are in accordance with the previous data and confirm the concentration-dependent antioxidant effect of AA in extracellular lignin-forming cell suspensions of spruce. The efficient removal of H$_2$O$_2$ in the culture medium by AA (Fig. 3) would interfere with peroxidase-mediated phenolic oxidation, thus reducing lignin formation, a situation similar to that when H$_2$O$_2$ was scavenged by KI (Nose et al., 1995; Kärkönen et al., 2002). Interestingly, the data about partial inhibition of phenolic oxidation by covalently bound cell wall peroxidases (Takahama, 1993a, b; Otter and Polle, 1994) suggest that some lignin polymerization could occur even in the presence of AA.

It was observed that further degradation products of AA can generate H$_2$O$_2$ in vivo (Figs 6, 7). Reported apoplastic concentrations of AA are in the millimolar range, with a higher percentage oxidation in the apoplast than in the cytoplasm. Even though DHA can be transported into the cytoplasm for re-reduction (Horemans et al., 2000), it is probable that some of it gets further degraded in the cell wall either enzymically or non-enzymically with the concomitant generation of H$_2$O$_2$ (Green and Fry, 2005a, b). This could have an effect, for example, during the growth of leaves or roots, since H$_2$O$_2$ can generate •OH radicals, which possibly have a role in cell wall loosening (Fry, 1998; Fry et al., 2001; Schopfer, 2001; Dumville and Fry, 2003; Rodríguez et al., 2002, 2004; Liszkay et al., 2004).

In addition, further DHA degradation products include a compound that inhibits peroxidase activity in vitro (A Kärkönen, SC Fry, unpublished results). The build-up of this inhibitory compound in the cell wall would decrease peroxidase-catalysed H$_2$O$_2$ scavenging and thus lead to the accumulation of H$_2$O$_2$ into the apoplast. As H$_2$O$_2$ is a signalling molecule mediating changes in gene expression or inducing direct physiological effects (Desikan et al., 2005) this can have a profound effect on the subsequent fate of the cells.

Future work will investigate the nature of the incompletely characterized degradation products of AA, DHA, and DKG, and their individual abilities to generate H$_2$O$_2$ in biological and biomimetic systems.

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