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Extracellular reactive oxygen species production by lichens

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Abstract: This review discusses the production of reactive oxygen species (ROS) by lichens and their possible roles. All organisms produce ROS, and production is increased by many abiotic and biotic stresses. Intracellular ROS production is generally considered to be harmful, and a variety of enzymic and non-enzymic scavenging systems exist to detoxify them. However, extracellular ROS formation has been suggested to play 'positive roles', particularly in the response of organisms to stress. Given their high stress tolerance, it is rather surprising that studies on extracellular ROS production by lichens have just started. Surveys of a wide range of lichens have shown that constitutively high rates of extracellular superoxide production occur in the Suborder *Peltigerineae*, but production appears to be absent in other groups. In some members of the *Peltigerineae* ROS production is stimulated by desiccation and wounding. It seems probable that the enzymes that produce the superoxide are laccases, based on first the types of substrates that lichens can break down, and second the dependence of the breakdown of these substrates on pH, temperature and the presence of inhibitors. While much more work is needed, we suggest that physiological roles of extracellular ROS production will be found to include defence against pathogens, melanization, and lignin breakdown.

Key words: laccase, lichens, peroxidase, reactive oxygen species, superoxide

Introduction

One of the paradoxes of life on our planet is that oxygen, the molecule supporting all aerobic life, is involved in many degenerative processes and illnesses (Marx 1985; Skulachev 2000, 2002). The high oxidizing ability of oxygen, necessary for its role in respiration, allows it to participate in other chemical reactions that form 'reactive oxygen species' or ROS. Once the atmosphere of the Earth had become oxygenated as a by-product of photosynthesis, organisms needed to develop effective ways to prevent the toxic action of ROS. However, recently it has become apparent that these molecules also play positive roles in the physiology of organisms. Given the importance of ROS in many biological processes, it is perhaps surprising how little we know about their metabolism in lichens. The first part of this review briefly summarizes the literature data on ROS formation, toxicity, and possible beneficial effects in higher plants and free-living fungi. The second part of the review discusses our recent findings on extracellular ROS metabolism by lichens, specifically, which lichens readily form them, and the enzymes responsible. The review concludes with some suggestions for the physiological significance of ROS formation in lichens, and ideas for future research.

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Biological ROS formation

Free radicals are atoms or molecules with an unpaired electron. This unpaired electron is readily donated, and as a result, most free radicals are highly reactive. Oxygen radicals include superoxide ($O_2\cdot^-$), the hydroxyl radical (·OH) and the nitric oxide radical (NO·). Together with singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), which are not free radicals but are nevertheless highly

reactive, these molecular species are termed ROS. Reactive oxygen species are produced as by-products of oxygen-dependent reactions during normal metabolism, although most biotic and abiotic stresses enhance their production. The brief outline below summarizes the most important pathways that produce ROS, and the general reactivity of the various species. Full details of these reactions can be found in Kranner and Lutzoni (1999).

Singlet oxygen is formed inside cells, particularly in the photosynthetic apparatus. Light energy trapped by chlorophyll molecules can be transferred to $^3O_2$ (triplet oxygen, ground state oxygen), forming singlet oxygen ($^1O_2$, reactions 1 and 2). Singlet oxygen can react directly with polyunsaturated fatty acid side chains to form lipid peroxides (Halliwell 1987).

$$P + \text{light} \rightarrow P^{\star} \text{ (excited pigment)} \quad (1)$$

$$P^{\star} + {}^3O_2 \rightarrow {}^1O_2 + P \quad (2)$$

The superoxide anion is formed by the capture of an electron by oxygen, and its formation is an unavoidable consequence of aerobic respiration (Møller 2001). Normally, when the terminal oxidases, cytochrome c oxidase and the alternative oxidase, react with oxygen, four electrons are transferred and water is the product (reaction 3).

$$^3O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \quad (3)$$

However, triplet oxygen can also react with other electron transport components from which only one electron is transferred, thus forming $O_2\cdot^-$. Up to 4% of the oxygen consumed by cells can be converted to $O_2\cdot^-$ (Møller 2001). In addition, enzymes such as nitropropane dioxygenase, galactose oxidase, and xanthine oxidase catalyse oxidation reactions in which a single electron is transferred from the substrate onto oxygen to produce $O_2\cdot^-$ (reaction 4).

$$^3O_2 + e^- \rightarrow O_2\cdot^- \quad (4)$$

Autooxidation of some reduced compounds (e.g., flavins, pteridines, diphenols, and ferredoxin; reaction 5) can also transfer a single electron to oxygen to produce $O_2\cdot^-$ (Halliwell 1987; Halliwell & Gutteridge 1999).

$$\text{Ferredoxin}_{\text{reduced}} + {}^3O_2 \rightarrow \text{Ferredoxin}_{\text{oxidised}} + O_2\cdot^- \quad (5)$$

In the cell wall and plasma membrane NAD(P)H oxidases, peroxidases, poly- and diaminooxidases and laccases can form extracellular superoxide by reaction 4 (Bolwell *et al.* 1998; Martinez *et al.* 2000; Guillén *et al.* 2000; Delannoy *et al.* 2003). Compared to $^1O_2$ and the ·OH radical, $O_2\cdot^-$ is less reactive, having a half-life of 2–4 μs, and a low cellular concentration ($<10^{-11}$ M). It cannot react directly with membrane lipids to cause peroxidation, and cannot cross biological membranes (Vranová *et al.* 2002). Most $O_2\cdot^-$ formed in biochemical systems reacts with itself non-enzymatically or enzymatically (catalysed by superoxide dismutase) to form H_2O_2 (reaction 6).

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + {}^3O_2 \quad (6)$$

Hydrogen peroxide formed by the previous or other reactions and $O_2\cdot^-$ can react together to form the hydroxyl radical (·OH, reaction 7) in the iron-catalysed Haber–Weiss reaction.

$$H_2O_2 + O_2\cdot^- \rightarrow {}^3O_2 + \cdot OH + OH^- \quad (7)$$

In the cell wall, Liszkay *et al.* (2003, 2004) speculated that this reaction can be catalysed by apoplastic peroxidases via $O_2\cdot^-$-dependent conversion of native ferric enzyme (-FeIII) to Compound III (-FeII-O_2, -FeIII-$O_2\cdot^-$). The ·OH radical is the most reactive and aggressive species known to chemistry, having a half-life of 1 ns.

In addition to $O_2\cdot^-$ dismutation, H_2O_2 can be formed by oxidases such as glycolate oxidase, glucose oxidase, urate oxidase, oxalate oxidase (the so-called 'germin-like' oxidase), and amino acid oxidases (Halliwell

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1987; Halliwell & Gutteridge 1999; Rea *et al.* 2002) (reaction 8).

$$^3O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \quad (8)$$

In higher plants, extracellular peroxidases appear to be able to produce H_2O_2 with $O_2\cdot^-$ as an intermediate (Elstner 1982).

Harmful and beneficial effects of ROS on plants

Intracellularly produced ROS can cause considerable damage to cells by attacking nucleic acids, lipids and proteins, and in healthy organisms a multilevel antioxidant system keeps ROS at safe levels (Elstner & Osswald 1994). However, more recently it has become apparent that extracellularly produced ROS play important roles in some normal biological processes. Higher plants use extracellular H_2O_2 in lignification (Ros Barceló 1997; Boudet 1998; Potikha *et al.* 1999; Pomar *et al.* 2002), while some fungi use $O_2\cdot^-$ to degrade lignin (Hammel *et al.* 2002). Oxygen radicals play key roles in the processes of ripening and natural ageing of plant tissues such as leaves, flowers, fruits (Leshem 1988). The phytohormone ethylene is considered to be an ageing trigger, a compound that promotes lipid degradation and intensifies oxidation induced by free radicals (Kende & Hanson 1977; Rustin *et al.* 1984). Interestingly, biogenesis of ethylene is tightly bound to free radical processes (Pirrung *et al.* 1998). ROS are also involved in the metabolism of the hormone auxin (Kawano 2003), and evidence suggests that ROS act as secondary messengers in signal transduction in plants (Neill *et al.* 2002; Vranová *et al.* 2002). Moreover, the formation of an 'oxidative burst' comprising large amounts of ROS is a common response of plants to many abiotic or biotic stresses (Mika *et al.* 2004). While the existence of the oxidative burst is well documented, the precise physiological significance of the burst remains unclear. Apart from the ROS being directly toxic to invading pathogens (Murphy *et al.* 1998), it is likely that the ROS participate in lignification and suberization processes that reinforce the cell wall (de Bruxelles & Roberts 2001). In addition, they may induce defence-related genes (Minagawa *et al.* 1992; Medentsev *et al.* 1999).

ROS production by lichens

While extracellular ROS formation is an important part of the response of organisms to stress, until recently almost nothing was known about ROS production in lichens. This is perhaps surprising, because many lichens are highly tolerant to a wide range of stresses (Kappen 1973). Methods for determining rates of extracellular ROS are relatively simple. For example, electrons readily donated by $O_2\cdot^-$ can be trapped using the drug epinephrine to form adrenochrome, which can be estimated spectrophotometrically (Misra & Fridovich 1972; Takeshige & Minakami 1979). Tetrazolium-based dyes such as NBT (nitrobluetretrazolium) and XTT (sodium,3′(-1-[phenylamino-carbonyl-carbon]-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene-sulphonic acid hydrate) are also widely used for $O_2\cdot^-$ determination (Able *et al.* 1998). Spectrophotometry can also be used to sensitively detect another ROS, H_2O_2, following reaction with xylenol orange (Gay & Gebicki 2000). Beckett *et al.* (2003) gave full details of how to use these techniques with lichens, and how to confirm their specificity.

Survey of extracellular $O_2\cdot^-$ production in different lichen species

Minibayeva & Beckett (2001) and Beckett *et al.* (2003) reported the results of surveys of extracellular $O_2\cdot^-$ production in 34 species of lichens from different taxonomic groupings and contrasting habitats. Interestingly, all 21 species in the survey from the Suborder *Peltigerineae* produce $O_2\cdot^-$ at high rates, while production is absent in the other 13 lichens. Rates of $O_2\cdot^-$ production are highest in lichens growing in the wettest microhabitats, as judged first by visual inspection of the collection sites, and second by thallus water contents at full turgor

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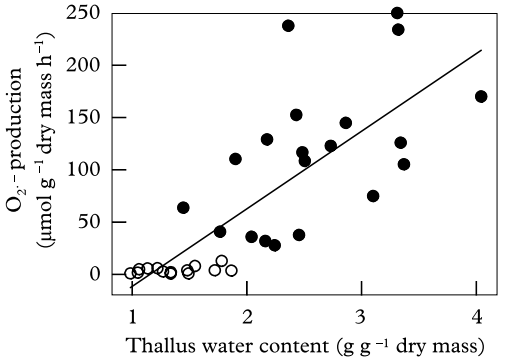

FIG. 1. Rates of extracellular $O_2 \cdot^-$ production are directly correlated with thallus water content at full turgor ($P<0{\cdot}001$) in a range of lichens. In this and the following graphs, $O_2 \cdot^-$ was estimated by the rate of oxidation of 1 mM epinephrine (dissolved in distilled water with the pH adjusted to 7) to adrenochrome over 15 min. Adrenochrome formation was assayed by measuring A_{480} (molecular extinction coefficient is $4{\cdot}02\ mM^{-1}\ cm^{-1}$). Each symbol denotes a single species; solid circles indicate lichens in Suborder *Peltigerineae*, and open circles lichens from other Suborders. Modified from Beckett *et al.* (2003).

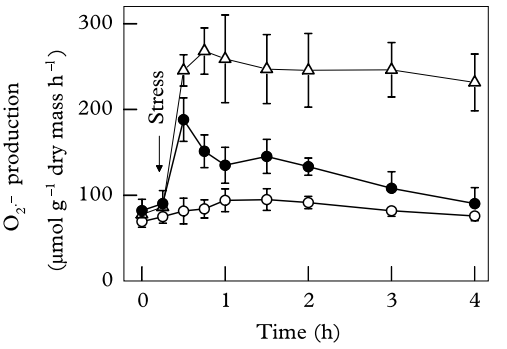

FIG. 2. Desiccation and wounding stresses stimulate extracellular $O_2 \cdot^-$ production in *Peltigera canina*. Open circles indicate material incubated in deionized distilled water throughout the experiment. The arrow indicates the time that material was stressed, either by cutting disks in half (closed circles), or by desiccating disks to a relative water content of 0·05 over 2·5 h then suddenly rehydrated, $O_2 \cdot^-$ production being measured during rehydration (open triangles). Error bars indicate the standard deviation, $n=5$. Modified from Beckett & Minibayeva (2003).

(Fig. 1). Rates of $O_2 \cdot^-$ production are positively correlated to reported concentrations of ergosterol. This correlation is interesting, because (for reasons not fully understood) the concentration of this compound is positively correlated to steady-state respiration rates in lichens (Sundberg *et al.* 1999). Furthermore, comparisons of growth rates also suggest that lichens in Suborder *Peltigerineae* grow relatively quickly (Hale 1973; Rogers 1990; Gilbert 2000; Palmqvist & Sundberg 2000). To summarize, rates of $O_2 \cdot^-$ production are highest in lichens from moist microhabitats with high indices of metabolic activity.

Factors affecting rates of $O_2 \cdot^-$ production and cellular location

Constitutive extracellular ROS production by lichens is unusual, but similar production has been occasionally reported in other organisms, for example of H_2O_2 in higher plant tissues in which secondary cell walls are differentiating (Potikha *et al.* 1999). Little is known about the factors that control the rate of $O_2 \cdot^-$ production from lichens. However, Beckett *et al.* (2003) showed that in some members of the *Peltigerineae* desiccation followed by rehydration induces a large increase in $O_2 \cdot^-$ production. Beckett & Minibayeva (2003) further showed that wounding can increase $O_2 \cdot^-$ production (Fig. 2).

Production rates do not seem to depend on the substratum on which lichens are growing. Beckett *et al.* (2003) collected pairs of seven species of lichens, one collection comprising lichens growing on mosses, and the other on soil or stone. Two way analysis of variance showed that substratum had no significant effect on $O_2 \cdot^-$ production, while the differences between species were highly significant.

The enzymes that produce the $O_2 \cdot^-$ are probably fairly tightly bound to the cell wall or plasma membrane, as only a small proportion can be released by shaking lichens in distilled water. Assays show that a solution in which disks of *Peltigera* have been incubated for 1 h produces $O_2 \cdot^-$ at only 15% of the rate of fresh disks. Interestingly, activity in the incubation solution increased to 50% following wounding, simulated by cutting thalli. More work is needed to confirm

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that wounding stimulates the secretion of $O_2\cdot^-$ producing enzymes into solution, or whether cytosolic leakage causes the increased activity. However, in higher plant roots good evidence now exists for wounding-induced secretion of $O_2\cdot^-$ producing peroxidases (Minibayeva *et al.* 2001, 2003).

Identity of the $O_2\cdot^-$ producing enzymes

As discussed above, cell surface enzymes that produce $O_2\cdot^-$ include NAD(P)H oxidase activities, peroxidases and laccases. Cyanide and diphenylene iodonium (DPI) are often used as so-called 'specific' inhibitors to distinguish between these enzymes. Cyanide is a well known peroxidase and laccase inhibitor (Ellis & Dunford 1968; Kiiskinen *et al.* 2002), and DPI is a flavin analogue, which probably inhibits the catalytic subunit (gp91phox, flavocytochrome *b*) of the phagocyte NADPH oxidase complex (O'Donnell *et al.* 1993, 1994). In lichens, cyanide but not DPI inhibits $O_2\cdot^-$ production (Beckett *et al.* 2003), tentatively suggesting that the enzymes are either peroxidases or laccases. Despite repeated attempts, we have never been able to demonstrate that lichen leachates can break down guaiacol, a substrate that higher plant extracellular peroxidases can readily metabolize (Minibayeva *et al.* 2003). In addition to peroxidases, in free living fungi, another group of cell wall enzymes capable of producing $O_2\cdot^-$ are the laccases (Guillén *et al.* 2000). Laccases are a family of 'blue-copper' oxidase proteins containing four copper ions, found in some higher plants, fungi and prokaryotes. These enzymes reduce dioxygen to two molecules of water and simultaneously perform one-electron oxidation of many aromatic substrates (Thurston 1994). In free-living fungi, laccases may catalyse the conversion of hydroquinones (possibly derived from lignin breakdown products) into semiquinones (reaction 9, Guillén *et al.* 2000).

$$4QH_2 + O_2 \rightarrow 4Q\cdot^- + 2H_2O + 4H^+ \quad (9)$$

Autoxidation of the semiquinones will lead to $O_2\cdot^-$ production (reaction 10).

$$Q\cdot^- + O_2 \leftrightarrow Q + O_2\cdot^- \quad (10)$$

It seems likely that a plasma membrane quinone reductase uses NAD(P)H to regenerate the hydroquinones (Morré 2004; reaction 11).

$$2NAD(P)H + Q \rightarrow 2NAD(P)^+ + QH_2 \quad (11)$$

This redox cycle will provide a supply of $O_2\cdot^-$. In our recent work we tested for the presence of laccases in lichens by examining their ability to metabolize classic laccase substrates such as the synthetic compounds 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Min *et al.* 2001) and *o*-tolidine (Miller *et al.* 1997), and also the natural compound syringaldazine (Medeiros *et al.* 1999). Our preliminary results showed that some lichens possess significant laccase activity, but interestingly only species within the *Peltigerineae*. Using *Pseudocyphellaria aurata* as a test species, we assayed laccase activity using ABTS, syringaldazine and tolidine as substrates. Table 1 summarizes the basic properties of the enzymes. All the results we have obtained so far are consistent with the properties of laccases reported in the literature (Thurston 1994; Mayer & Staples 2002). Furthermore in a survey of 42 species, $O_2\cdot^-$ production was positively correlated to laccase activity (Fig. 3). Our preliminary electrophoretic analysis of the laccase from *Peltigera malacea* suggests that the active form is a tetramer with a molecular mass of 340 kDa and an isoelectric point of 4·7. Although more work is needed, it seems likely that laccases are the enzymes responsible for superoxide production in lichens in Suborder *Peltigerineae*.

Stoichiometry of ROS production

The $O_2\cdot^-$ radical has a half-life of just a few μs (see above), and the radicals that lichens of the *Peltigerineae* produce will be

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TABLE 1. *Properties of released laccases in* Pseudocyphellaria aurata *measured using different substrates*

	ABTS*	Syringaldazine†	*o*-Tolidine‡
Optimum pH	5·0	6·5	5·0
Optimum temperature (°C)	40	30	7
K_M (μmol)	21	14	116
V_{max} (μmol product g^{-1} dry mass h^{-1})	2·24	3·63	—
Concentration of NaN_3 for 50% inhibition (μM)	5	20	5
Concentration of KCN for 50% inhibition (μM)	1000	750	750

*For methods, see legend to Fig. 3.

†Lichen leachates incubated in 10 μM syringaldazine, pH 6·5 for 15 min at 25°C. The extinction coefficient of the product at A_{525} is 65 mM^{-1} cm^{-1}.

‡1 mM *o*-tolidine dissolved in 25 mM acetate buffer, pH 5·0 for 15 min at 20°C. The product was measured at A_{630}.

— Cannot be calculated, as extinction coefficient of product unknown.

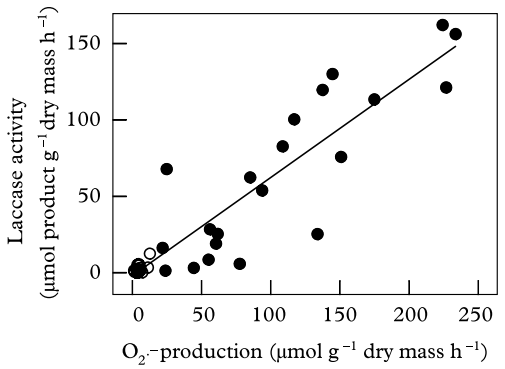

FIG. 3. Rates of metabolism of the laccase substrate ABTS in 42 species of lichens (21 in Suborder *Peltigerineae* and 21 from other Suborders) are positively correlated to rates of $O_2\cdot^-$ production ($P<0{\cdot}001$). Lichens incubated in 1 mM ABTS dissolved in 25 mM phosphate buffer, pH 5 for 15 min. The extinction coefficient of the product measured at A_{420} is 36 mM^{-1} cm^{-1}. Each symbol denotes a single species; solid circles indicate lichens in Suborder *Peltigerineae*, and open circles lichens from other Suborders.

rapidly converted to H_2O_2, either spontaneously or by the action of the enzyme superoxide dismutase (SOD). However, to our surprise, we found that unstressed lichens produce almost no H_2O_2, and that desiccation induces only a small short-lived peak of H_2O_2 production. We hypothesized that lichens can rapidly break down H_2O_2 produced from the dismutation of $O_2\cdot^-$. Consistent with this, we found that incubating *c.* 15 mg of *Peltigera* tissue in 5 ml of 10 mM H_2O_2 reduces the concentration to 2·5 mM within 1 h. Breakdown of H_2O_2 is clearly enzymic, because the process has a high Q_{10}, and autoclaving inhibits enzyme activity. The most common enzymes that break down extracellular H_2O_2 in plants and fungi are class II and III peroxidases. A few reports of extracellular catalases exist in higher plants and fungi (e.g. Salguero & Böttger 1995; Garre *et al.* 1998; Vanacker *et al.* 1998). However, in *Peltigera* the enzymes involved appear to be peroxidases, because while the catalase inhibitor ATZ slightly stimulates H_2O_2 breakdown, breakdown is inhibited by the peroxidase inhibitors cyanide and azide.

We further hypothesised that the rates of H_2O_2 breakdown might be higher in *Peltigerineae* than in other lichens, because of their need to protect themselves from the toxic effect of the $O_2\cdot^-$. Although we plan to test more species, our preliminary results show that all lichens that we have examined so far can breakdown H_2O_2 quickly. Rates tended to be higher in the *Peltigerineae*, but were not significantly different from other groups, and rates of break down were not significantly correlated to rates of $O_2\cdot^-$ production (Fig. 4). We are currently studying the mechanism of H_2O_2 breakdown; preliminary results suggest that rates can be very high. Presumably, the ability to rapidly break down H_2O_2 protects lichens from

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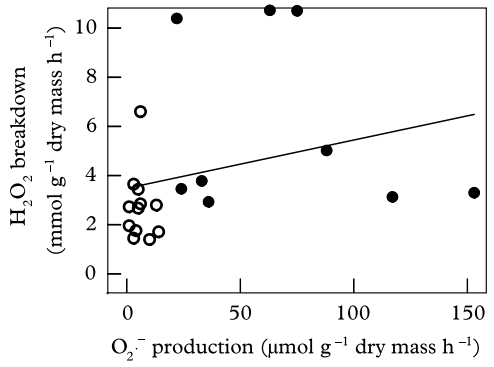

FIG. 4. Rates of H_2O_2 breakdown from a 10 mM solution in a range of lichens are not related to rates of $O_2\cdot^-$ production (correlation is not significant, $P>0{\cdot}05$). Concentrations of H_2O_2 were determined by reaction with xylenol orange. Each symbol denotes a single species. Solid circles indicate lichens in the Suborder *Peltigerineae*, and open circles lichens not belonging to Suborder *Peltigerineae*.

damage potentially resulting from their own oxidative burst. In addition, cell wall peroxidases may also indirectly help to remove ROS produced intracellularly as a result of various stresses in all lichens. Many intracellular free radical scavenging reactions produce H_2O_2, and as it freely diffuses across membranes (Allan & Fluhr 1997) breakdown in the apoplast may be the final phase of cellular detoxification. Rapid break down in the apoplast probably explains why we cannot detect H_2O_2 production by lichens in the *Peltigerineae*, and will complicate future studies on the stoichiometry of the production of different ROS.

Physiological significance of ROS production

Based on our current knowledge of ROS production in lichens and their roles in other organisms, it seems most likely that in lichens ROS are involved in defence against attack by pathogens, decomposition of organic compounds, and possibly melanin biosynthesis, and these aspects are discussed below. However, it now seems certain that in higher plants ROS play roles in signal transduction (Neill *et al.* 2002), and they may have the same roles in lichens.

Defence against pathogens

As discussed above, higher plants defend themselves against pathogens using extracellular ROS production. ROS are either directly toxic to pathogens, or induce an array of other defence mechanisms, collectively termed systemic acquired resistance (SAR). It is tempting to suggest that lichens in the *Peltigerineae* use extracellular ROS in the same way. In higher plants, it is known that ROS production can also be induced by abiotic stresses that leave plants vulnerable to bacteria or fungi, for example mechanical disruption or UV light (Murphy *et al.* 1998). Some lichens produce an oxidative burst following wounding or during rehydration following desiccation (Beckett & Minibayeva 2003), and this may offer protection at a time when they are vulnerable to attack by pathogens.

It is possible that two different strategies for defence against pathogens exist in lichens. Lichens in the *Peltigerineae* produce $O_2\cdot^-$, tend to have high indices of metabolic activity (Beckett *et al.* 2003), and contain low concentrations of secondary metabolites (Huneck & Yoshimura 1996). Conversely, lichens outside the *Peltigerineae* do not produce $O_2\cdot^-$, grow in more stressful habitats, have slower growth rates, and apparently deter pathogens by accumulating high concentrations of secondary metabolites (Rundel 1988). There are no clear explanations for the apparent existence of these two contrasting strategies. However, Grime (1979) proposed that in highly stressed environments, selection will favour species with low inherent growth rates that match low resource availability. In such species, Coley *et al.* (1985) suggest that a given rate of damage by pathogens or herbivores (e.g. for lichens grams of thallus lost per day) represents a larger fraction of annual net production, and therefore selection may favour species with large investments in deterrents. This may explain why stress tolerant lichens growing in xeric habitats have abundant and diverse lichen secondary metabolites. Rather than accumulate secondary metabolites, lichens in Suborder

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Peltigerineae may be protected by extracellular $O_2\cdot^-$ production. Coley *et al.* (1985) suggest that this kind of defence mechanism will be rare in long-lived, slow growing plants, because the continued metabolic costs, for example for re-synthesising enzymes, summed over a life time would be larger than a fixed investment in immobile defences. The energy required to maintain enzyme pools may explain why extracellular $O_2\cdot^-$ production is absent in other lichen groups. A further consideration is that many non-Peltigerineaen lichens allocate a considerable part of their carbon budget to secondary metabolites, which may accumulate to as much as 30% of their dry mass (Huneck & Yoshimura 1996). As most members of the *Peltigerineae* are nitrogen fixing species, a protein-based defence using laccases may be energetically preferable to one based on carbon-containing lichen substances.

Decomposition

Laccases are one of the main enzymes involved in delignification by white rot and other fungi (Thurston 1994). Together with lignin peroxidase and manganese peroxidase, by producing ROS they can remove the lignin from wood and plant litter (Hammel *et al.* 2002). The precise mechanism of ROS production by laccases remains uncertain, but evidence exists that laccases operate with quinone oxidoreductase as outlined in reactions 9, 10 and 11 above. If this mechanism is correct, one consequence will be that $O_2\cdot^-$ is produced via quinone radicals rather than directly by the laccase enzymes, meaning that the $O_2\cdot^-$ can be produced in pores of decomposing cell walls too small for laccases to penetrate. In addition, the laccases are less likely to suffer damage by the radicals that they themselves produce. Quinone radicals can have half-lives in the range of milliseconds (Everett *et al.* 2001), long enough to diffuse away from where they are produced. Furthermore, H_2O_2 produced by the dismutation of $O_2\cdot^-$ could produce ·OH radicals (reaction 7), possibly catalysed by chelated Fe as suggested by Hammel *et al.* (2002). A combination of $O_2\cdot^-$ and ·OH could effectively break down lignin (Hammel *et al.* 2002). Although fungi do not directly derive energy from this process, they expose cellulose, which other enzymes can break down in energy producing reactions (Blanchette 1991; Boddy & Watkinson 1995). Certainly, *Peltigera* possesses β-1,4-glucanases that can break down cellulose exposed by lignin decomposition (de los Ríos *et al.* 1997). Interestingly, laccases are actively produced by some leaf decomposing fungi that grow in similar habitats to *Peltigera* (Leontievsky *et al.* 1997; Heinzkill *et al.* 1998; Tagger *et al.* 1998; Dedeyan *et al.* 2000). Given their high laccase activity, it is possible that lichens in Suborder *Peltigerineae* may play an important role in decomposition.

Melanin synthesis

In free living fungi, ROS produced by laccases are important in melanin synthesis (Williamson *et al.* 1998). While melanins are present in Peltigeralean lichens such as *Lobaria pulmonaria* (Gauslaa & Solhaug 2001), they are also present in non-Peltigeralean lichens without laccase activity such as *Cetraria* (Nybakken *et al.* 2004). We assume that, as for fungi (Butler & Day 1998), various mechanisms of melanin synthesis exist in lichens, some involving laccases, and some other oxidases.

Prospects

Future work is needed to characterize the $O_2\cdot^-$ producing laccases in lichens, for example with respect to molecular mass and substrate specificity. Our preliminary work indicates that the enzymes can be readily secreted, making them relatively easy to isolate and purify. Eventually, it will be interesting to sequence the proteins and construct cladograms to determine the phylogenetic position of lichen laccases within those produced by other fungi. Determining the physiological significance of extracellular ROS production will be harder. A role in defence against pathogens

seems likely, and it would be interesting to test whether fungal parasites of *Peltigera* can induce an 'oxidative burst' similar to that observed during rehydration following desiccation. Future work should also assess the role of laccases in decomposition and melanin biosynthesis.

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References

Able, A. J., Guest, D. I. & Sutherland, M. W. (1998) Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with avirulent zoospore of *Phytophthora parasitica* var *nicotianae*. *Plant Physiology* **117:** 491–499.

Allan, A. C. & Fluhr, R. (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* **9:** 1559–1572.

Beckett, R. P. & Minibayeva, F. V. (2003) Wounding induces a burst of extracellular superoxide production in *Peltigera canina*. *Lichenologist* **35:** 87–89.

Beckett, R. P., Minibayeva, F. V., Vylegzhanina, N. V. & Tolpysheva, T. (2003) High rates of extracellular superoxide production by lichens in the Suborder *Peltigerineae* correlate with indices of high metabolic activity. *Plant, Cell and Environment* **26:** 1827–1837.

Blanchette, R. (1991) Delignification by wood-decay fungi. *Annual Review of Phytopathology* **29:** 381–398.

Boddy, L. & Watkinson, S. C. (1995) Wood decomposition, higher fungi, and their role in nutrient redistribution. *Canadian Journal of Botany* **73:** S1377–S1383.

Bolwell, G. P., Davies, D. R., Gerrish, C., Auh, C-K. & Murphy, T. M. (1998) Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiology* **116:** 1379–1385.

Boudet, A. M. (1998) A new view of lignification. *Trends in Plant Science* **3:** 67–71.

Butler, M. J. & Day, A. W. (1998) Fungal melanins: a review. *Canadian Journal of Microbiology* **44:** 1115–1136.

Coley, P. D., Bryant, J. P. & Chapin, F. S. (1985) Resource availability and plant antiherbivore defense. *Science* **230:** 895–899.

de Bruxelles, G. L. & Roberts, M. R. (2001) Signals regulating multiple responses to wounding and herbivores. *Critical Reviews in Plant Sciences* **20:** 487–521.

de los Ríos, A., Ramírez, R. & Estévez, P. (1997) Production of several isoforms of β-1, 4-glucanase by the cyanolichen *Peltigera canina*. *Physiologia Plantarum* **100:** 159–164.

Dedeyan, B., Klonowska, A., Tagger, S., Tron, T., Iacazio, G., Gil, G. & Le Petit, J. (2000) Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. *Applied and Environmental Microbiology* **66:** 925–929.

Delannoy, E., Jalloul, A., Assigbetse, K., Marmey, P., Geiger, J. P., Lherminier, J., Daniel, J. F., Martinez, C. & Nicole, M. (2003) Activity of class III peroxidases in the defense of cotton to bacterial blight. *Molecular Plant-Microbe Interactions* **16:** 1030–1038.

Ellis, W. D. & Dunford, H. B. (1968) The kinetics of cyanide and fluoride binding by ferric horse-radish peroxidase. *Biochemistry* **7:** 2054–2062.

Elstner, E. F. (1982) Oxygen activation and oxygen toxicity. *Annual Review of Plant Physiology* **33:** 73–96.

Elstner, E. F. & Osswald, W. (1994) Mechanisms of oxygen activation during plant stress. *Proceedings of the Royal Society of Edinburgh* **102:** 131–154.

Everett, S. A., Naylor, M. A., Barraja, P., Swann, E., Patel, K. B., Stratford, M. R. L., Hudnott, A. R., Vojnovic, B., Locke, R. J., Wardman, P. & Moody, C. J. (2001) Controlling the rates of reductively-activated elimination from the (indol-3-yl)methyl position of indolequinones. *Journal of the Chemical Society, Perkin Transactions* **2:** 843–860.

Garre, V., Tenberge, K. B. & Eising, R. (1998) Secretion of a fungal extracellular catalase by *Claviceps purpurea* during infection of rye: putative role in pathogenicity and suppression of host defense. *Phytopathology* **88:** 744–753.

Gauslaa, Y. & Solhaug, K. A. (2001) Fungal melanins as a sun screen for symbiotic green algae in the lichen *Lobaria pulmonaria*. *Oecologia* **126:** 462–471.

Gay, C. & Gebicki, J. M. (2000) A critical evaluation of the effect of sorbitol on the ferric-xylenol orange hydroperoxide assay. *Analytical Biochemistry* **284:** 217–220.

Gilbert, O. L. (2000) *Lichens*. London: HarperCollins.

Grime, J. P. (1979) *Plant Strategies and Vegetation Processes*. Chichester: Wiley.

Guillén, F., Muñoz, C., Gómez-Toribio, V., Martínez, A. T. & Martínez, M. J. (2000) Oxygen activation during oxidation of methoxyhydroquinones by laccase from *Pleurotus eryngii*. *Applied and Environmental Microbiology* **66:** 170–175.

Hale, M. E. (1973) Growth. In *The Lichens* (V. Ahmadjian & M. E. Hale, eds): 473–492. London: Academic Press.

Halliwell, B. (1987) Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Chemistry and Physics of Lipids* **44:** 327–340.

Halliwell, B. & Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*. 3rd edition. Oxford: Oxford University Press.

Hammel, K. E., Kapich, A. N., Jensen, K. A. & Ryan, Z. C. (2002) Reactive oxygen species as agents of wood decay by fungi. *Enzyme and Microbial Technology* **30:** 445–453.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P. & Anke, T. (1998) Characterization of laccases and peroxidases from wood-rotting fungi (family Coprinaceae). *Applied and Environmental Microbiology* **64:** 1601–1606.

Huneck, S. & Yoshimura, I. (1996) *Identification of Lichen Substances*. Berlin: Springer-Verlag.

Kappen, L. (1973) Response to extreme environments. In *The Lichens* (V. Ahmadjian & M. E. Hale, eds): 311–380. New York: Academic Press.

Kawano, T. (2003) Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Reports* **21:** 829–837.

Kende, H. & Hanson, A. D. (1977) On the role of ethylene in aging. In *Proceedings the 9th International Conference on Plant Growth Substances: Plant Growth Regulators* (P. E. Pilet, ed.): 172–180. Berlin: Springer-Verlag.

Kiiskinen, L.-L., Viikari, L. & Kruus, K. (2002) Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*. *Applied Microbiology and Biotechnology* **59:** 198–204.

Kranner, I. & Lutzoni, F. (1999) Evolutionary consequences of transition to a lichen symbiotic state and physiological adaptation to oxidative damage associated with poikilohydry. In *Plant Response to Environmental Stress: from Phytohormones to Genome Reorganisation* (H. R. Lerner, ed.): 591–628. New York: Dekker.

Leontievsky, A. A., Vares, T., Lankinen, P., Shergill, J. K., Pozdnyakova, N. N., Myasoedova, N. M., Kalkkinen, N., Golovleva, L. A., Cammack, R., Thurston, C. F. & Hatakka, A. (1997) Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiology Letters* **156:** 9–14.

Leshem, Y. Y. (1988) Plant senescence processes and free radicals. *Free Radicals in Biology and Medicine* **5:** 39–49.

Liszkay, A., Kenk, B. & Schopfer, P. (2003) Evidence for the involvement of cell wall peroxidases in the generation of hydroxyl radicals mediating extension growth. *Planta* **217:** 658–667.

Liszkay, A., van der Zalm, E. & Schopfer, P. (2004) Production of reactive oxygen intermediates ($O_2\cdot^-$, H_2O_2 and ·OH) by maize roots and their role in wall loosening and elongation growth. *Plant Physiology* **136:** 3114–3123.

Martinez, C., Baccou, J.-C., Bresson, E., Baissac, Y., Daniel, J.-F., Jalloul, A, Montillet, J.-L., Geiger, J.-P., Assigbetsé, K. & Nicole, M. (2000) Salicylic acid mediated by the oxidative burst is a key molecule in local and systemic responses of cotton challenged by an avirulent race of *Xanthomonas campestris* pv *malvacearum*. *Plant Physiology* **122:** 757–766.

Marx, J. L. (1985) Oxygen free radicals linked to many diseases. *Science* **235:** 529–531.

Mayer, A. M. & Staples, R. C. (2002) Laccase: new functions for an old enzyme. *Phytochemistry* **60:** 551–565.

Medeiros, M. B., Bento, A. V., Nunes, A. L. L. & Oliveira, S. C. (1999) Optimization of some variables that affect the synthesis of laccase by *Pleurotus ostreatus*. *Bioprocess Engineering* **21:** 483–487.

Medentsev, A. G., Arinbasarova, A. Y. & Akimenko, V. K. (1999) Regulation and physiological role of cyanide-resistant oxidase in fungi and plants. *Biokhimia* **64:** 1457–1472.

Mika, A., Minibayeva, F., Beckett, R. P. & Lüthje, S. (2004) Possible functions of extracellular peroxidases in stress-induced generation and detoxification of active oxygen species. *Phytochemistry Reviews* **3:** 173–193.

Miller, R., Kuglin, J., Gallagher, S. & Flurkey, W. H. (1997) A spectrophotometric assay for laccase using *o*-tolidine. *Journal of Food Biochemistry* **21:** 445–459.

Min, K.-L., Kim, Y.-H, Kim, Y. W., Jung, H. S. & Hah, Y. C. (2001) Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. *Archives of Biochemistry and Biophysics* **392:** 279–286.

Minagawa, N., Koga, S., Nakano, M., Sakajo, S. & Yoshimoto, A. (1992) Possible involvement of superoxide anion in the induction of cyanide-resistant respiration in *Hansenula anomala*. *FEBS Letters* **302:** 217–219.

Minibayeva, F. & Beckett, R. P. (2001) High rates of extracellular superoxide production in bryophytes and lichens, and an oxidative burst in response to rehydration following desiccation. *New Phytologist* **152:** 333–343.

Minibayeva, F. V., Gordon, L. K., Kolesnikov, O. P. & Chasov, A. V. (2001) Role of extracellular peroxidase in the superoxide production by wheat root cells. *Protoplasma* **217:** 125–128.

Minibayeva, F., Mika, A. & Luthje, S. (2003) Salicylic acid changes the properties of extracellular peroxidase activity secreted from wounded wheat (*Triticum aestivum* L.) roots. *Protoplasma* **221:** 67–72.

Misra, H. R. & Fridovich, I. (1972) The univalent reduction of oxygen by reduced flavins and quinones. *Journal of Biological Chemistry* **247:** 188–192.

Møller, I. M. (2001) Plant mitochondria and oxidative stress. Electron transport, NADPH turnover and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology* **52:** 561–591.

Morré, D. J. (2004) Quinone oxidoreductases of the plasma membrane. *Methods in Enzymology* **378:** 179–199.

Murphy, T. M., Asard, H. & Cross, A. R. (1998) Possible sources of reactive oxygen during the

oxidative burst in plants. In *Plasmamembrane Redox Systems and Their Role in Biological Stress and Disease* (H. Asard, A. Bérczi & R. J. Cauberg, eds): 215–246. Dordrecht: Kluwer Academic Publishers.

Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D. & Hancock, J. T. (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. *Journal of Experimental Botany* **53:** 1237–1247.

Nybakken, L., Solhaug, K. A., Bilger, W. & Gauslaa, Y. (2004) The lichens *Xanthoria elegans* and *Cetraria islandica* maintain a high protection against UV-B radiation in Arctic habitats. *Oecologia* **140:** 211–216.

O'Donnell, V. B., Smith, G. C. M. & Jones, O. T. G. (1994) Involvement of phenyl radicals in iodonium compound inhibition of flavoenzymes. *Molecular Pharmacology* **46:** 778–785.

O'Donnell, V. B., Tew, D. G., Jones, O. T. G. & England, P. J. (1993) Studies on the inhibitory mechanism of iodonium compounds with special references to neutrophil NADPH oxidase. *Biochemical Journal* **290:** 41–49.

Palmqvist, K. & Sundberg, B. (2000) Light use efficiency of dry matter gain in 5 macro-lichens—relative impact of microclimate conditions and species-specific traits. *Plant Cell and Environment* **23:** 1–14.

Pirrung, M. C., Cao, J., Chen, J. (1998) Ethylene biosynthesis: processing of a substrate analog supports a radical mechanism for the ethylene-forming enzyme. *Chemistry and Biology* **5:** 49–57.

Pomar, F., Caballero, N., Pedreño, M. A. & Ros Barceló, A. (2002) H_2O_2 generation during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis. *FEBS Letters* **529:** 198–202.

Potikha, T. S., Collins, C. C., Johnson, D. I., Delmer, D. P. & Levine, A. (1999) The involvement of hydrogen peroxide in the differentiation of secondary walls in cotton fibers. *Plant Physiology* **119:** 849–858.

Rea, G., Metoui, O., Infantino, A., Federico, R. & Angelini, R. (2002) Copper amine oxidase expression in defense responses to wounding and *Ascochyta rabiei* invasion. *Plant Physiology* **128:** 865–875.

Rogers, R. W. (1990) Ecological strategies of lichens. *Lichenologist* **22:** 149–162.

Ros Barceló, A. (1997) Lignification in plant cell walls. *International Review of Cytology* **176:** 87–132.

Rundel, P. W. (1978) The ecological role of secondary lichen substances. *Biochemical Systematics and Ecology* **6:** 157–70.

Rustin, P., Dupont, J. & Lance, C. (1984) Involvement of lipid peroxy radicals in the cyanide-resistant electron transport pathway. *Physiologie Végétale* **22:** 643–663.

Salguero, J. & Böttger, M. (1995) Secreted catalase activity from roots of developing maize (*Zea mays* L.) seedlings. *Protoplasma* **184:** 72–78.

Skulachev, V. P. (2000) Mitochondria in the programmed death phenomena; a principle of biology: 'it is better to die than to be wrong'. *International Union of Biochemistry and Molecular Biology Life* **49:** 365–373.

Skulachev, V. P. (2002) Programmed death phenomena: from organelle to organism. *Annals of the New York Academy of Science* **959:** 214–237.

Sundberg, B., Ekblad, A., Nasholm, T. & Palmqvist, K. (1999) Lichen respiration in relation to active time, nitrogen and ergosterol concentrations. *Functional Ecology* **13:** 119–125.

Tagger, S., Perissol, C., Gil, G., Vogt, G. & Le Petit, J. (1998) Phenoloxidases of the white-rot fungus *Marasmius quercophilus* isolated from an evergreen oak litter (*Quercus ilex* L.). *Enzyme and Microbial Technology* **23:** 372–379.

Takeshige, K. & Minakami, S. (1979) NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. *Biochemical Journal* **180:** 129–135.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology* **140:** 19–26.

Vanacker, H., Carver, T. L. W. & Foyer, C. H. (1998) Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. *Plant Physiology* **117:** 1103–1114.

Vranová, E., Inzé, D. & van Breusegem, F. (2002) Signal transduction during oxidative stress. *Journal of Experimental Botany* **53:** 1227–1236.

Williamson, P. R., Wakamatsu, K. & Ito, S. (1998) Melanin biosynthesis in *Cryptococcus neoformans*. *Journal of Bacteriology* **180:** 1570–1572.

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