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NADH-stimulated, cyanide-resistant superoxide production in maize coleoptiles analyzed with a tetrazolium-based assay

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Abstract. Using the tetrazolium salt XTT (Na,3′-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate) as a sensitive and physiologically compatible probe for the determination of superoxide ($O_2^{\cdot-}$) production in vivo, we have shown that maize (*Zea mays* L.) coleoptiles possess the capacity of generating $O_2^{\cdot-}$ in the apoplastic space. Our results are in agreement with the notion that this activity is localized at the plasma membrane and can be attributed to an $O_2^{\cdot-}$-synthesizing enzyme with catalytic and kinetic properties similar to that of the NADPH oxidase of mammalian phagocytes, with the important exception that it utilizes NADH instead of NADPH as electron donor. When applied to the apoplastic space, NADH strongly increased the $O_2^{\cdot-}$-producing activity of coleoptiles. The maize NADH-dependent $O_2^{\cdot-}$-synthase activity could clearly be differentiated from peroxidase-mediated $O_2^{\cdot-}$-synthesizing activity by its insensitivity to cyanide and azide, as well as by its much higher affinity to O_2. Formation of $O_2^{\cdot-}$, and concomitantly appearing H_2O_2, was preferentially localized in the outer epidermis of the coleoptile. The physiological significance of $O_2^{\cdot-}$ and H_2O_2 production in relation to the growth-controlling function of the epidermal cell wall is discussed.

Key words: Coleoptile (maize) – Hydrogen peroxide – NAD(P)H oxidase – Peroxidase – Superoxide radical – *Zea* (superoxide production)

Introduction

Plants are capable of producing and secreting superoxide radicals ($O_2^{\cdot-}$) and derived reactive oxygen species under certain physiological conditions, particularly in response to pathogen infection (Doke et al. 1996; Low and Merida 1996; Lamb and Dixon 1997; Ogawa et al. 1997; Wojtaszek 1997). The biochemical mechanism of this reaction, that may play a central role in the inducible pathogen defense system of plants, is at present a matter of intense research and controversial debate. Circumstantial evidence fosters the view that plant cells have at their disposal a plasma-membrane oxidase catalyzing the one-electron reduction of molecular oxygen to $O_2^{\cdot-}$ at the expense of NAD(P)H, similar to the inducible NADPH oxidase (NADPH: dioxygen oxidoreductase) complex in the plasma membrane of mammalian phagocytes (Doke et al. 1996; Low and Merida 1996; Lamb and Dixon 1997; Wojtaszek 1997). Activation of the phagocyte enzyme by chemical signals originating from pathogenic invaders causes a sudden upsurge in O_2 consumption and an extracellular accumulation of H_2O_2 derived from $O_2^{\cdot-}$ by dismutation. A strikingly similar 'oxidative burst' can be observed in plants as an early response to pathogen attack (Low and Merida 1996; Wojtaszek 1997). However, so far the existence of a functional NADPH oxidase of the phagocyte type has not been demonstrated in plants, although proteins with sequence homologies to the mammalian enzyme have been discovered in rice and *Arabidopsis* (Groom et al. 1996; Desikan et al. 1998; Keller et al. 1998). The phagocyte NADPH oxidase is characterized by a nearly absolute specificity to NADPH as the electron donor (K_m = 30–50 μM, with the binding site for NADPH exposed to the interior of the cell), a low K_m for O_2 (5–30 μM), a high sensitivity to inhibition by DPI (diphenyleneiodonium; K_i = 5–10 μM), and resistance to inhibition by catalase and respiratory inhibitors such as cyanide and azide (Cross and Jones 1991; Morel et al. 1991; O'Donnel et al. 1993; Henderson and Chappell 1996).

Abbreviations: DAB = 3,3′-diaminobenzidine; DIECA = Na-diethyldithiocarbamate; DPI = diphenyleneiodonium; Mn-DFA = manganese desferal (Mn-deferoxamine complex); NBT = nitroblue tetrazolium chloride; SOD = superoxide dismutase (EC 1.15.1.1); XTT = Na,3′-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)bezenesulfonic acid hydrate

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A different enzymatic mechanism for the apoplastic generation of $O_2^{\cdot-}$ from O_2 is provided by the NAD(P)H oxidizing activity of peroxidases localized in the cell wall or at the plasma membrane of plant tissues (Vianello and Macri 1991). Operationally, peroxidases can be discriminated from the phagocyte-type NADPH oxidase by (i) equally accepting NADH and NADPH as electron donors, (ii) a relatively high K_m for O_2 (150 μM), and (iii) a high sensitivity to inhibition by catalase, cyanide and azide (Bolwell et al. 1995; Frahry and Schopfer 1998a). Contrary to previous assertations, peroxidases can also be inactivated by DPI, discounting this inhibitor as an unequivocal diagnostic tool for discriminating the mammalian-type NADPH oxidase from the NAD(P)H-oxidizing activity of peroxidases, at least at the higher concentrations (20–100 μM) generally used in experiments in vivo (Bolwell et al. 1998; Frahry and Schopfer 1998b). On account of the sensitivity to cyanide and azide the 'oxidative burst' induced by pathogen infection in *Phaseolus* plants (Bolwell et al. 1995) or elicitor-treated tomato cells (Vera-Estrella et al. 1992) has been attributed to the NAD(P)H oxidase activity of apoplastic peroxidases.

Investigations with cell-free systems, e.g. purified plasma-membrane vesicles, aimed at the elucidation of the $O_2^{\cdot-}$-producing enzyme activity in plants, have resulted in the partial characterization of a bewildering variety of reactions demonstrating either features of a phagocyte-type enzyme, a peroxidase-type enzyme, or an activity that could be incorporated in neither one of these categories (Morré and Brightman 1991; Vianello and Macri 1991; Pinton et al. 1994; Qiu et al. 1995; Kiba et al. 1997; Mithöfer et al. 1997; Ros Barceló 1998; Van Gestelen et al. 1998). The functional relationship of these membrane activities, if any, to the 'oxidative burst' response remains obscure. An elicitor-dependent NADPH oxidase activation, accompanied by the incorporation of proteins immunologically related to subunits of the phagocyte NADPH oxidase, has been demonstrated in plasma membranes isolated from tomato cells (Xing et al. 1997). Van Gestelen et al. (1997) described the isolation of a cyanide-insensitive, NADPH-dependent $O_2^{\cdot-}$ synthase from plasma membranes of *Phaseolus* seedlings which, however, lacks the low-potential cytochrome *b* typical of the phagocyte NADPH oxidase.

Taken together, the experimental information presently available creates the impression that several different enzyme activities can potentially be responsible for the generation of apoplastic $O_2^{\cdot-}$ in plants, depending on the particular physiological context and the particular plant species under investigation (Bolwell et al. 1998; Papadakis and Roubelakis-Angelakis 1999). On the other hand, differences in the analytical methods utilized by different workers might at least partly account for the unclear picture emerging from these investigations. This suspicion is fostered by a recent comparative methodological study demonstrating that the test reactions commonly used for the assay of $O_2^{\cdot-}$ production by plant cells or cell fractions suffer from one or another shortcoming and can lead to divergent results even if applied to the same experimental systems (Murphy et al. 1998).

This unsatisfactory situation could be improved by the recent development of a novel tetrazolium-based assay for the quantitative determination of $O_2^{\cdot-}$ in solution. The customary tetrazolium reduction assay utilizes nitroblue tetrazolium chloride (NBT) that, upon reduction by $O_2^{\cdot-}$, is converted into a coloured, highly insoluble formazan product. This test reaction is therefore very useful for the histochemical visualization of $O_2^{\cdot-}$ production in situ (e.g. Ogawa et al. 1997), but does not permit a reliable quantitative photometric determination of $O_2^{\cdot-}$ in solution. This problem has now been overcome by the introduction of the tetrazolium compound XTT that differs from NBT by forming a water-soluble, coloured formazan that is not adsorbed to plant tissues and allows a sensitive quantitative photometric determination of $O_2^{\cdot-}$ in vivo (Sutherland and Learmonth 1997). Similar to the NBT reaction, the specificity of the XTT reaction for $O_2^{\cdot-}$ can be demonstrated by adding $O_2^{\cdot-}$ scavengers such as superoxide dismutase (SOD) or manganese desferal = Mn-deferoxamine complex (Mn-DFA; Able et al. 1998). In the present contribution we have explored the potential of the XTT reduction assay for the demonstration and quantitative determination of the apoplastic $O_2^{\cdot-}$ production by growing coleoptiles of maize seedlings and for a first characterization of the enzyme activity underlying this in-vivo reaction.

Material and methods

Plant material and growth conditions

Seedlings of maize (*Zea mays* L., cv. Perceval; from Asgrow, Buxtehude, Germany) were grown on damp vermiculite for 4.5 d at 25.0 ± 0.3 °C in darkness interrupted after 3 d by a 10-min red light pulse (Kutschera and Schopfer 1985). Coleoptiles were harvested including the upper 2 cm of the mesocotyl and abraded with polishing cloth (Schopfer 1993). Experiments were performed under normal laboratory light with intact coleoptiles or 1-cm coleoptile segments dissected 3 mm below the coleoptile tip.

Determination of $O_2^{\cdot-}$ production

The reduction of XTT (from Polyscience Europe, Eppelheim, Germany) was continuously measured at 25.0 ± 0.1 °C using the flow-through system (2 ml min^{-1}) described by Frahry and Schopfer (1998a) which was adapted to a Uvikon 940 spectrophotometer (Kontron Instruments, Neufahrn, Germany). Ten intact coleoptiles were placed upside-down in a short test tube with 1.5 ml aerated reaction mixture covering about 50% of the coleoptile's surface. Alternatively, 12 coleoptile segments were floated on 1.5 ml solution. The reaction mixture (total volume 3.5 ml) contained 500 μM XTT in Na-citrate buffer (10 mM citrate) and various additions (SOD and catalase from Boehringer Mannheim, Germany; DPI, dissolved in dimethylsulfoxide, from Biomol, Hamburg, Germany; digitonin from Serva). Manganese desferal (green complex) was prepared from deferoxamine mesylate (Sigma) and MnO_2 as described by Beyer and Fridovich (1989). The absorbance at 470 nm was continuously recorded and transformed into molar concentration using an extinction coefficient of 2.16×10^4 $l\ mol^{-1}\ cm^{-1}$ (Sutherland and Learmonth 1997). Data are based

on tissue fresh mass determined at the end of the measurement in order to reduce statistical variations between samples. For adjusting different O_2 concentrations the reaction mixture was extensively purged with defined air/N_2 gas mixtures (70 ml min^{-1}) as described by Frahry and Schopfer (1998a).

Determination of NADH oxidation

The decrease in NADH absorbance was continuously measured at 340 nm in the flow-through system as described above. Absorbance changes by coleoptiles in the absence of NADH were negligible.

Determination of H_2O_2 production

The H_2O_2-dependent, peroxidase-catalyzed oxidation of scopoletin (Sigma) in the presence of horseradish peroxidase (Boehringer) was continuously measured in the flow-through system adapted to a fluorescence spectrophotometer (type LS-3B; Perkin-Elmer, Überlingen, Germany) as described by Frahry and Schopfer (1998a) using 10 intact coleoptiles or 12 coleoptile segments in a total volume of 3.5 ml assay solution.

Histochemical assays

Up to 10 fresh, hand-cut cross-sections (approximately 200 μm thick) were incubated in 2 ml of appropriate assay solutions in 10 mM citrate buffer (pH 6.0) at 25 °C on a shaker. Photographs were taken (identical optical conditions and exposure times) with Kodak Ektachrome 64T film using a Stemi SV6 microscope (Zeiss).

Statistics

Data are means of 4–6 independent experiments ± standard error.

Results and discussion

Determination and biochemical characterization of $O_2^{\cdot-}$ production with the XTT assay

Superoxide represents a relatively short-lived form of reactive oxygen, the production of which in vivo can only be analyzed reliably by incubating the tissue directly in an assay mixture containing a non-toxic detector reagent at physiological conditions. These experimental constraints are difficult to satisfy and have hampered the quantitative determination of $O_2^{\cdot-}$ production by plant tissues considerably in the past. For example, the occasionally used $O_2^{\cdot-}$ assay based on the luminescence of lucigenin (Auh and Murphy 1995; Papadakis and Roubelakis-Angelakis 1999) involves the incubation of living cells at a non-physiological pH of 9.0 and is, moreover, compromised by the fact that it can produce false-positive results due to the production of $O_2^{\cdot-}$ by autooxidation (Linochev and Fridovich 1997; Vásquez-Vivar et al. 1997; Murphy et al. 1998), except at very low concentrations of lucigenin (Li et al. 1998). The classical photometric assay based on the SOD-inhibitable reduction of cytochrome *c* (McCord and Fridovich 1968) is not subject to these artifacts but cytochrome *c* (12 500 Da) does not easily penetrate the cell wall to reach the site of $O_2^{\cdot-}$ generation at the plasma membrane and thus often lacks sufficient sensitivity for the determination of $O_2^{\cdot-}$ production in intact plant tissues. Using this procedure in preliminary experiments we could demonstrate that abraded maize coleoptile segments secrete an agent that reduces cytochrome *c* with a rate of the order of 0.5 nmol min^{-1} (g fresh mass)$^{-1}$ at pH 5.5. However, the absorbance changes measured in these experiments were small and, moreover, saturating concentrations of SOD inhibited the reaction only by 23%. Thus, the specificity of this assay for $O_2^{\cdot-}$ could not be clearly established.

Replacing cytochrome *c* by the tetrazolium salt XTT provided a remedy to this dilemma. The criteria for a sensitive, cell-wall-permeating and physiologically compatible probe for the determination of apoplastic $O_2^{\cdot-}$ in plant tissues are satisfied by XTT (675 Da), that has recently been used for demonstrating the production of $O_2^{\cdot-}$ in the pathogen-induced oxidative burst of tobacco cells (Sutherland and Learmonth 1997; Able et al. 1998). The apoplastic generation of $O_2^{\cdot-}$ by intact maize coleoptiles determined by the continuous measurement of XTT formazan production is shown in Fig. 1. The assay reaction produces a linear increase in absorbance for at least 15 min, the rate of which can be strongly increased by NADH. There is very little background reaction in the absence of tissue, both in the absence and presence of NADH. Abrasion of the cuticle proved to be an essential prerequisite for making the apoplastic space accessible to the reagent.

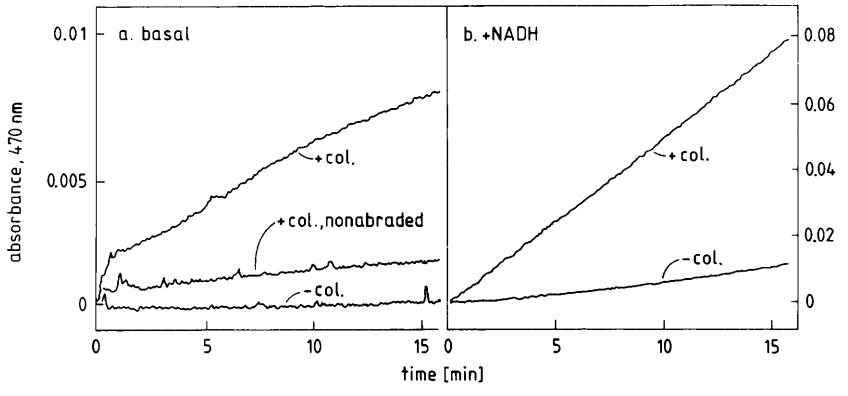

Fig. 1a,b. Kinetics of formazan production from XTT by maize coleoptiles. Ten intact, abraded coleoptiles were incubated in citrate buffer (10 mM, pH 6.0) containing 500 μM XTT in the absence (**a**) or the presence (**b**) of 200 μM NADH. Controls show the background reaction in the absence of coleoptiles (-col.) and with nonabraded coleoptiles

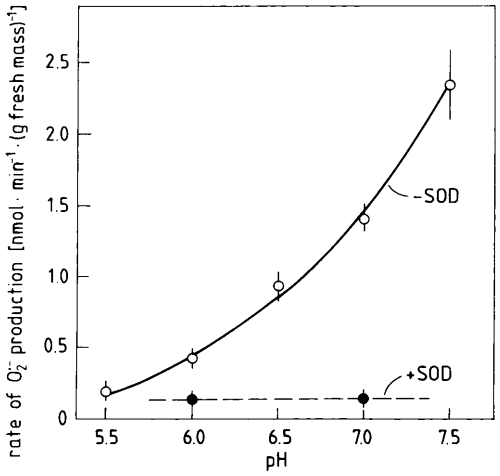

Fig. 2. Effect of pH on $O_2^{\cdot-}$ release by maize coleoptiles determined with the assay illustrated in Fig. 1a at variable pH. The rate of $O_2^{\cdot-}$ production was calculated from the increase in XTT formazan absorbance between 5 and 10 min after the start of the reaction. Superoxide dismutase (50 μg ml^{-1}) was added to the assay mixture together with XTT

Figure 2 shows that the rate of $O_2^{\cdot-}$ release by maize coleoptiles is strongly affected by the pH of the incubation medium. The efficiency of the assay reaction increases by a factor of 12 when the pH is raised from 5.5 to 7.5, in agreement with the steep increase in $O_2^{\cdot-}$ stability at higher pH (Fridovich 1978). The $O_2^{\cdot-}$ scavenger SOD effectively inhibits XTT formazan formation both at pH 6.0 and 7.0, demonstrating the specificity of the test reaction for $O_2^{\cdot-}$. Moreover, since $O_2^{\cdot-}$ is poorly diffusible across the plasma membrane and the action of SOD is restricted to the space outside the plasma membrane, the inhibition by SOD provides evidence for an apoplastic site of O_2 reduction determined by the XTT assay in maize coleoptiles. A medium pH of 6.0 was used in subsequent experiments in order to remain close to the natural, slightly acidic pH in the apoplast.

Table 1 shows the effect of some diagnostic inhibitors and other reagents on the $O_2^{\cdot-}$-producing activity of maize coleoptiles assayed in the absence and presence of NADH. The major results from these experiments can be summarized as follows. (i) Manganese desferal, a potent $O_2^{\cdot-}$ scavenger (Rabinowitch et al. 1987; Able et al. 1998), inhibits formazan production more effectively than SOD, presumably because of better permeation through the cell wall (Able et al. 1998). It has been reported that Mn-DFA acts as a low-molecular-weight mimic of SOD (Darr et al. 1987; Beyer and Fridovich 1989) although there is also evidence that it scavenges $O_2^{\cdot-}$ in a stoichiometric reaction (Weiss et al. 1993). The Mn-DFA concentration for 50% inhibition of $O_2^{\cdot-}$ formation in maize coleoptiles is approximately 80 μM (Fig. 3). (ii) Inhibitors of peroxidase-mediated $O_2^{\cdot-}$ production such as catalase, cyanide and azide do not inhibit $O_2^{\cdot-}$ production by maize coleoptiles. The slight promotive effect of cyanide may be due to the inhibition of endogenous SOD by this agent. (iii) Production of $O_2^{\cdot-}$ by maize coleoptiles is sensitive to DPI, an inhibitor of the NADPH oxidase of phagocytes (O'Donnell et al. 1993). (iv) All tested inhibitors affect the basal and the NADH-stimulated $O_2^{\cdot-}$ production in a similar fashion. However, NADH cannot be replaced by NADPH. (v) Cutting segments, i.e. wounding, has no significant effect on $O_2^{\cdot-}$ production and its measurement, although the inhibitory effect of SOD is less than in unwounded tissue. Thus, the contribution of a SOD-insensitive side-reaction cannot be definitely excluded under these conditions. (vi) Digitonin, a membrane-reactive deter-

Table 1. Effect of various treatments on apoplastic $O_2^{\cdot-}$ production by intact maize coleoptiles or coleoptile segments. Reagents were added to the assay mixture (pH 6.0) together with XTT and the $O_2^{\cdot-}$ production rate calculated from the increase in XTT formazan absorbance between 5 and 10 min after the start of the reaction. The concentration of NADH was 200 μM. Results of experiments where the tissue was boiled for 5 min before the assay are included to demonstrate the heat lability of the $O_2^{\cdot-}$-producing activity

Reagents added	Rate of $O_2^{\cdot-}$ production [nmol min^{-1} (g fresh mass)$^{-1}$]	
	Basal	+ NADH
Intact coleoptiles		
None	0.42 ± 0.05 (100%)	2.4 ± 0.2 (100%)
Mn-DFA (500 μM)	0.03 ± 0.01 (7%)	0.6 ± 0.1 (25%)
SOD (50 μg ml^{-1})	0.14 ± 0.03 (33%)	0.5 ± 0.1 (19%)
Catalase (30 μg ml^{-1})	0.43 ± 0.03 (102%)	2.3 ± 0.3 (96%)
KCN (1 mM)	0.51 ± 0.10 (120%)	2.7 ± 0.4 (116%)
KCN (10 mM)	0.49 ± 0.08 (116%)	2.7 ± 0.3 (113%)
NaN_3 (1 mM)	0.42 ± 0.03 (99%)	2.5 ± 0.2 (106%)
NaN_3 (10 mM)	0.38 ± 0.04 (90%)	2.5 ± 0.2 (107%)
DPI (100 μM)	0.15 ± 0.01 (36%)	0.9 ± 0.1 (38%)
NADPH (200 μM)	0.35 ± 0.05 (83%)	–
Coleoptile segments		
None	0.43 ± 0.04 (100%)	2.3 ± 0.2 (100%)
SOD (50 μg ml^{-1})	0.18 ± 0.03 (42%)	1.0 ± 0.1 (43%)
Digitonin (100 μM)	1.06 ± 0.06 (264%)	–
NADPH (200 μM)	0.44 ± 0.05 (102%)	–
Boiling	0.08 ± 0.05 (19%)	0.10 ± 0.10 (5%)

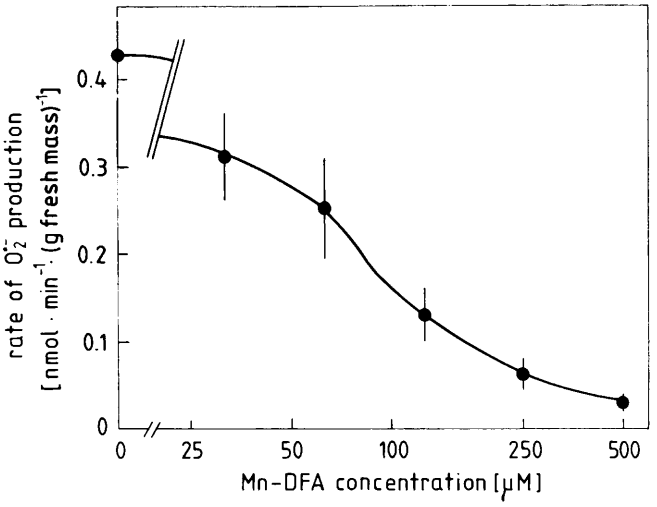

Fig. 3. Effect of Mn-DFA on $O_2^{\cdot-}$ release by maize coleoptiles. Production of XTT-formazan was determined at pH 6.0 in the presence of Mn-DFA as in Fig. 2

Table 2. Demonstration of H_2O_2 production by intact maize coleoptiles or coleoptile segments. Reagents were added to the assay mixture (pH 6.0) together with the H_2O_2 probe scopoletin in the presence of horseradish peroxidase. The H_2O_2 production rate was calculated from the decrease in scopoletin fluorescence between 10 and 15 min after the start of the reaction

Reagents added	Rate of H_2O_2 production [nmol min^{-1} (g fresh mass)$^{-1}$]
Intact coleoptiles	
None	0.38 ± 0.01
Coleoptile segments	
None	0.38 ± 0.02 (100%)
SOD (50 μg ml^{-1})	0.37 ± 0.01 (96%)
$CuCl_2$ (1 mM)	0.40 ± 0.01 (104%)

gent stimulating the phagocyte NADPH oxidase (Cohen and Choraniec 1978) as well as the $O_2^{\cdot-}$-producing activity in plant leaves and protoplasts (Doke and Chai 1985) also increases $O_2^{\cdot-}$ production in maize coleoptiles.

Superoxide represents a rather instable species of reactive oxygen which can rapidly be converted to H_2O_2 by dismutation at pH <7 even in the absence of SOD (Fridovich 1978). Therefore, the production of $O_2^{\cdot-}$ by plant and animal cells is generally accompanied by the appearance of H_2O_2. Table 2 shows that this rule can also be confirmed in maize coleoptiles. Catalysts promoting the dismutation of $O_2^{\cdot-}$ to H_2O_2 such as SOD or Cu^{2+} (Zancani et al. 1995) had no effect on the release of H_2O_2 from coleoptile segments. Unfortunately, however, the interesting question of how H_2O_2 production is affected by cyanide or azide could not be investigated because these inhibitors interfere with the peroxidase-dependent assay reaction for H_2O_2.

The enzymatic reaction responsible for $O_2^{\cdot-}$ production in phagocytes is characterized by a relatively high affinity to O_2 (K_m = 5–30 μM; Cross and Jones 1991). A similarly low K_m (approximately 10 μM) with respect to O_2 has been reported for the peroxidase-independent H_2O_2 production by soybean roots (Frahry and Schopfer 1998a). For determining the O_2 dependence of the $O_2^{\cdot-}$-producing reaction in maize coleoptiles we used freshly cut segments that were incubated in assay solutions of defined O_2 concentrations. In order to eliminate possible contributions by side reactions not involving $O_2^{\cdot-}$, the measurements were performed both in the absence and presence of a saturating concentration of SOD. The SOD-inhibitable fraction of the formazan-forming reaction calculated from these measurements is plotted as a function of O_2 concentration in Fig. 4. It is evident that this activity is absolutely dependent on O_2 and exhibits a half-maximal rate at approximately 10 μM O_2 both in the absence and presence of NADH.

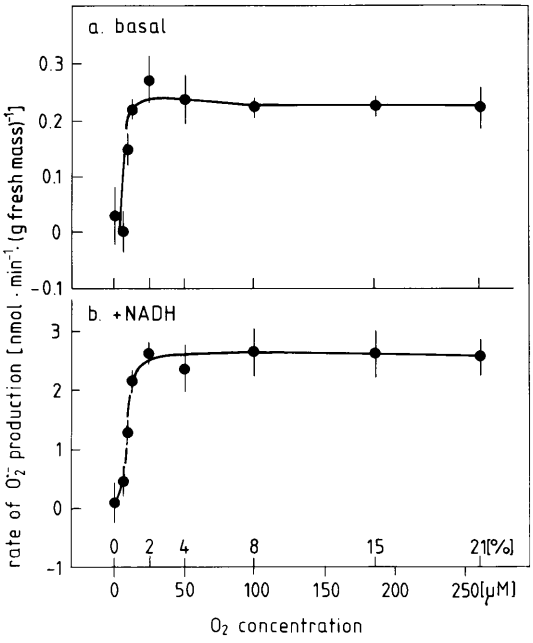

Fig. 4a,b. Effect of O_2 concentration on $O_2^{\cdot-}$ release by maize coleoptile segments in the absence (**a**) or in the presence (**b**) of NADH (200 μM). The data represent the SOD-inhibitable fraction of $O_2^{\cdot-}$ release at pH 6.0 calculated from measurements performed in the absence and presence of SOD (50 μg ml^{-1}). Oxygen concentrations in the assay mixtures (μM) were adjusted by equilibration with air/N_2 gas mixtures adjusted to appropriate O_2 concentrations (Vol %). Other experimental details as in Fig. 2

Localization of $O_2^{\cdot-}$ production in the maize coleoptile

The classical histological NBT assay was used for demonstrating the localization of $O_2^{\cdot-}$ production in the coleoptile at the cellular level. Cross-sections of living coleoptiles were incubated at pH 6.0 with NBT in the presence of various additives (Fig. 5a–e). Blue reaction product was detected primarily in the vascular bundle and the outer epidermis whereby the protoplasts rather than the cell walls became stained (Fig. 5a). The reaction was enhanced by Na-diethyldithiocarbamate (DIECA), an inhibitor of SOD (Ogawa et al. 1996, 1997), indicating that $O_2^{\cdot-}$ is normally to a large extent converted to H_2O_2 by enzymatic dismutation (Fig. 5b). The occurrence of SOD at the apoplastic face of the plasma membrane has been documented in spinach mesophyll cells (Ogawa et al. 1996). The effect of DIECA shown in Fig. 5b is reminiscent of observations in hypocotyl sections of spinach seedlings where $O_2^{\cdot-}$ production could in fact only be demonstrated in the presence of SOD inhibitors (Ogawa et al. 1997). The $O_2^{\cdot-}$ scavenger Mn-DFA inhibited NBT formazan formation both in the absence (not shown) and in the presence (Fig. 5c) of DIECA. Finally, as expected from the experiments with XTT (Fig. 1), staining was strongly enhanced by NADH (Fig. 5d) and this reaction was likewise inhibited by Mn-DFA (Fig. 5e).

For comparison, the localization of H_2O_2 production in these coleoptile sections is illustrated in Fig. 5f–i, using 3,3′-diaminobenzidine (DAB) as a histochemical reagent that is converted into an insoluble brown oxidation product by H_2O_2 in the presence of endogenous peroxidase (Thordal-Christensen et al. 1997). With this assay, H_2O_2 production was shown to occur

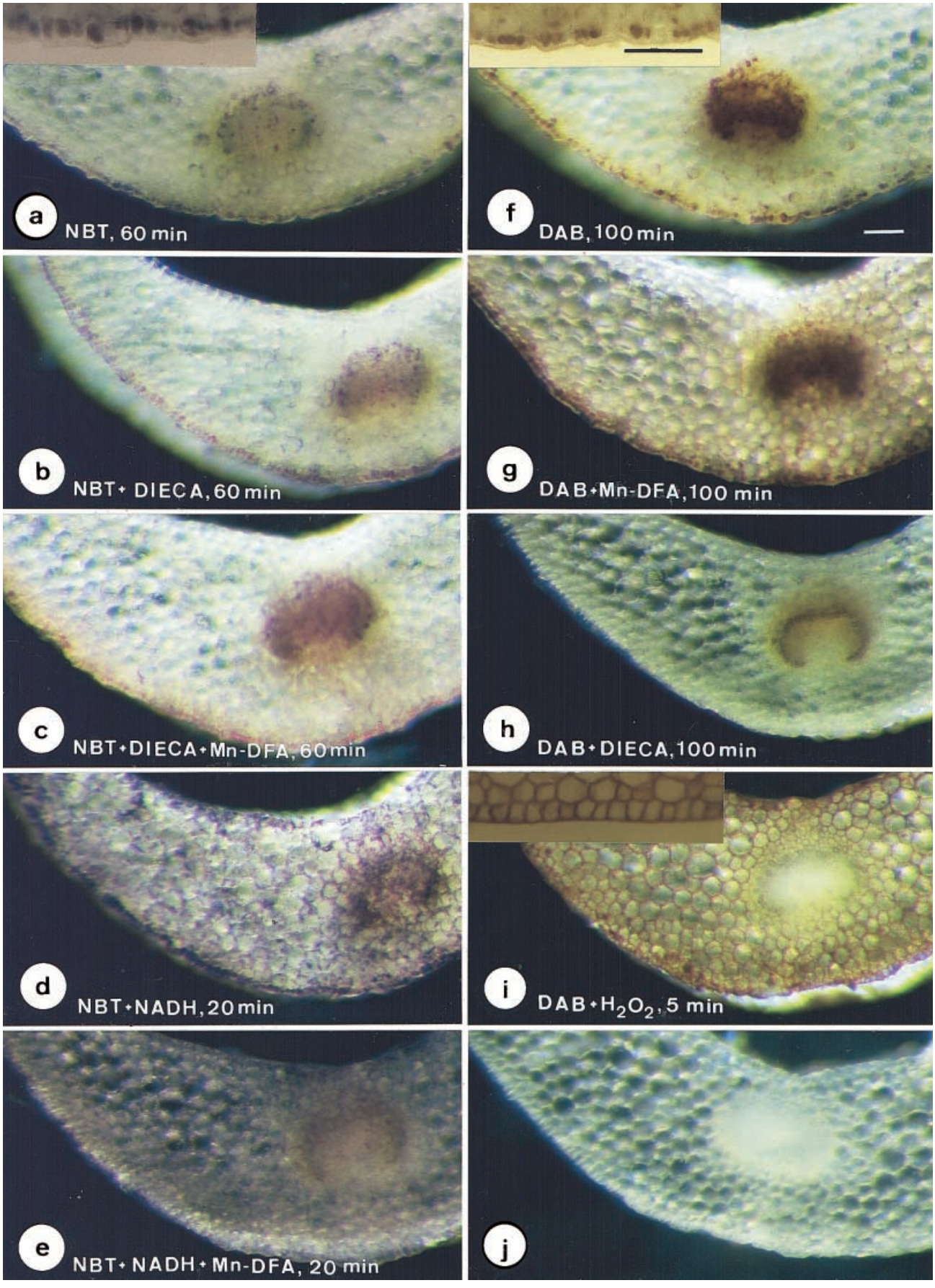
a NBT, 60 min
f DAB, 100 min
b NBT + DIECA, 60 min
g DAB + Mn-DFA, 100 min
c NBT + DIECA + Mn-DFA, 60 min
h DAB + DIECA, 100 min
d NBT + NADH, 20 min
i DAB + H_2O_2, 5 min
e NBT + NADH + Mn-DFA, 20 min
j

Fig. 5a–j. Histochemical localization of $O_2^{\cdot-}$ production (*left*) and H_2O_2 production (*right*) in cross-sections of maize coleoptiles. $O_2^{\cdot-}$ production was visualized by incubating freshly cut sections in citrate buffer (10 mM, pH 6.0) for the indicated periods of time in 500 μM NBT ± 1 mM DIECA, 500 μM Mn-DFA, 200 μM NADH (**a–e**). All assay solutions contained 10 mM NaN_3 for reducing background staining. Production of H_2O_2 was visualized by replacing NBT by 2.5 mM DAB, omitting NaN_3 (**f–h**). Peroxidase activity was localized by an incubation in 2.5 mM DAB + 50 μM H_2O_2 (**i**). Control: section incubated in buffer (**j**). Note: The effect of NADH on H_2O_2 production could not be investigated because of interference with the assay reaction. The insets (**a, f, i**) show stained outer epidermal cells at higher magnification. The NBT formazan appears blue in reflecting light (**a–e**) and brown-black in transmitting light (inset in **a**). Bars = 100 μm

primarily in the vascular bundle and in the outer epidermis where the stain accumulated selectively at the protoplasts (Fig. 5f). The oxidation of DAB was promoted by Mn-DFA (Fig. 5g) in agreement with the idea that it enhances the dismutation of $O_2^{\cdot-}$ to H_2O_2 similarly to SOD (Darr et al. 1987; Beyer and Fridovich 1989). The SOD inhibitor DIECA inhibited the reaction both in the presence (not shown) and in the absence (Fig. 5h) of Mn-DFA. However, since DIECA may also react directly with H_2O_2 (Bolwell et al. 1998), this finding cannot unequivocally be interpreted in terms of an inhibition of SOD. Finally, Fig. 5i illustrates the pattern of DAB oxidation induced by exogenous H_2O_2, indicating the distribution of peroxidase activity. This enzyme appears to be particularly active in the cell walls without detectable preference for the epidermis. In contrast to $O_2^{\cdot-}$ and H_2O_2 (Fig. 5a–g) there is no indication of peroxidase activity in the epidermal protoplasts (Fig. 5i, inset). Methodologically, this comparison provides a critical test for the spatial resolution of the histochemical staining reactions.

Taken together, the experiments compiled in Fig. 5 qualitatively support the results obtained in the quantitative measurements of $O_2^{\cdot-}$ and H_2O_2 production. Moreover, it is shown that the generation of these reactive oxygen species in the maize coleoptile is chiefly a function of the outer epidermal cells, presumably localized at the outer surface of the plasma membrane.

Effect of XTT on NADH oxidation by peroxidase

The data presented so far demonstrate that the apoplastic production of $O_2^{\cdot-}$ by maize coleoptiles does not show the characteristic features of a peroxidase-catalyzed reaction even in the presence of added NADH. This is surprising since maize coleoptiles do contain high activities of peroxidase in their cell walls (Fig. 5i). In comparable experiments with soybean roots treated with NADH, apoplastic peroxidase has been shown to operate in the NADH-oxidase mode (Frahry and Schopfer 1998a). Why is this reaction not detectable in maize coleoptiles? This apparent paradox can be resolved by investigating the effect of XTT on the oxidation of external NADH by coleoptile segments. Table 3 shows that XTT inhibits NADH oxidation to

Table 3. Effect of XTT on the oxidation of NADH by maize coleoptile segments in the absence and presence of KCN. The XTT (500 μM) and KCN (1 mM) were added to the assay mixture (pH 6.0) together with NADH (200 μM). The rate of NADH oxidation was calculated from the linear decrease in NADH absorbance at 340 nm between 5 and 10 min after the start of the reaction

Reagents added	Rate of NADH oxidation [nmol min^{-1} (g fresh mass)$^{-1}$]
NADH	3.3 ± 0.1 (100%)
NADH + XTT	1.8 ± 0.1 (55%)
NADH + KCN	1.7 ± 0.1 (52%)
NADH + XTT + KCN	1.6 ± 0.3 (49%)

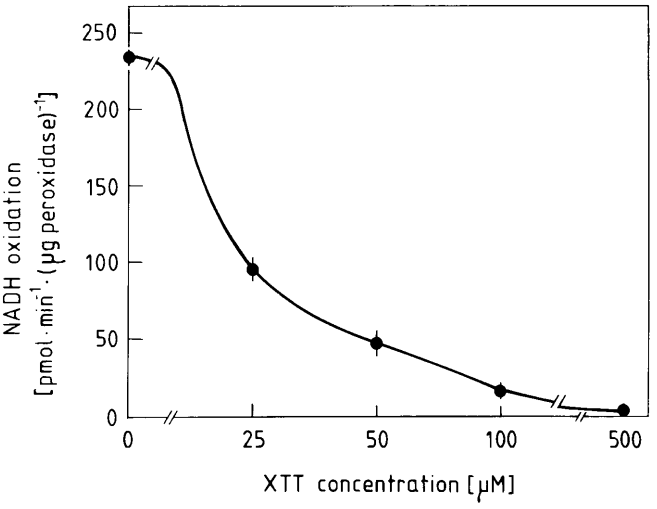

Fig. 6. Effect of XTT on NADH oxidation by horseradish peroxidase. The rate of NADH oxidation was calculated from the decrease in NADH absorbance at 340 nm in a reaction mixture containing citrate buffer (10 mM, pH 6.0), horseradish peroxidase (6 μg ml^{-1}), NADH (600 μM) and various concentrations of XTT

the same extent as cyanide, but has no significant effect on the cyanide-insensitive component of NADH oxidation. This indicates that XTT specifically impairs the peroxidase-catalyzed NADH oxidase reaction. Indeed, the oxidation of NADH by peroxidase in vitro was completely inhibited by XTT at the concentration of 500 μM used in the $O_2^{\cdot-}$ assay with maize coleoptiles (Fig. 6). The inhibition of the peroxidase reaction by XTT is presumably a consequence of the elimination of $O_2^{\cdot-}$, and thus H_2O_2, needed to initiate NADH oxidation by peroxidase. This reaction can be very similarly inhibited by removing H_2O_2 with catalase (Halliwell 1978). An important methodological implication emerging from this finding is that the XTT assay reaction will lead to false-negative results if applied to a $O_2^{\cdot-}$-generating reaction system involving peroxidase.

General conclusions

Growing coleoptiles of maize seedlings have been shown to produce $O_2^{\cdot-}$, and its dismutation product H_2O_2, in the apoplastic space. An in-vivo assay based on the

formation of a soluble formazan dye has been used for demonstrating this reaction and elucidating some characteristic properties of the underlying enzymatic mechanism. The $O_2^{\cdot-}$-producing activity appears to be localized at the plasma membrane and is particularly abundant in the outer epidermis of the coleoptile. The enzyme responsible for this activity can accept electrons from NADH experimentally supplied to the apoplastic side of the plasma membrane, a feature reported previously for other NAD(P)H-consuming reactions at the plasma membrane (Lin 1982; Doke 1983; Doke and Chai 1985; Vianello and Macri 1991; Hicks and Morré 1998; Minibayeva et al. 1998). This does not exclude the possibility that the enzyme normally accepts NADH from the cytoplasmic side of the membrane.

The $O_2^{\cdot-}$-producing enzyme activity of maize coleoptiles can be discriminated from a peroxidase-mediated oxygenase activity by its insensitivity to cyanide and azide, and by its high affinity to O_2 which exceeds the affinity of peroxidase to O_2 by one order of magnitude. In contrast to the functionally similar oxidase of mammalian phagocytes the maize enzyme is characterized by a high specificity for NADH, rather than for NADPH, as an electron donor and qualitatively differs, in this respect, from the $O_2^{\cdot-}$-producing enzyme engaged in the oxidative burst observed in potato tubers (Doke et al. 1996) and tomato cells (Xing et al. 1997).

Superoxide and H_2O_2 can serve as substrates for the formation of hydroxyl radicals ($OH^{\cdot}$) in a peroxidase-catalyzed reaction (Chen and Schopfer 1999). This reaction can be used to cleave cell-wall polysaccharides in vitro (Schweikert et al. 2000). Extension growth of the maize coleoptile is controlled by the tensile strength of the outer epidermal cell wall (Hohl and Schopfer 1992). Thus, all conditions theoretically necessary for a $OH^{\cdot}$-mediated wall-loosening reaction leading to extension growth are satisfied in the coleoptile. Collecting experimental evidence for the actual functioning of this mechanism in the growing organ remains a task for future work.

In agreement with published evidence (Ogawa et al. 1997) the vascular tissues of the coleoptile also demonstrate a striking capacity for $O_2^{\cdot-}$ and H_2O_2 production (Fig. 5). This observation has previously been interpreted in terms of reactions responsible for H_2O_2-mediated lignin formation in the secondary walls of xylem elements (Ogawa et al. 1997). Another, not necessarily alternative, possibility would be that $O_2^{\cdot-}$ and H_2O_2 produced by living cells in the vascular bundle are involved in the programmed cell death of transiently fuctional protoxylem vessels and in the subsequent loosening of their primary walls. There is indeed increasing evidence that reactive oxygen species act as mediators of cell death in plants and animals (Jabs 1999). Ultrastructural evidence indicates that the protoxylem walls in growing plant organs undergo extensive degradation and modification for facilitating their passive extension during elongation (Ryser et al. 1997).

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