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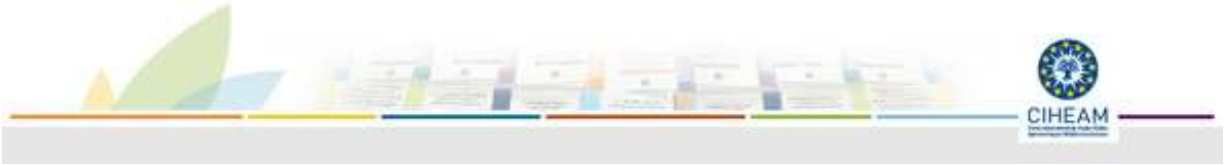

Active oxygen: A possible role for rice resistance to blast

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Abstract. Active oxygen (AO) species are cytotoxic products of aerobic metabolism: hydrogen peroxide, superoxide 2- and hydroxyl OH free radicals. They are chemically detectable on rice leaf surface and in drop diffusates of leaves. In the latter case, AO accounts for the fungitoxicity of diffusates. In this work we compared AO production (detected with chemical and biological tests) and disease resistance. During 3 days after inoculation both AO indexes were significantly higher in vertically resistant cultivars than in susceptible ones. Partially resistant cultivars manifested similar features, but the mechanism of AO generation was apparently different. The susceptible cultivar with heat-induced resistance produced more AO. Enzymes superoxide dismutase and catalase when added to inocula prevented heat-induced disease resistance that indicated the involvement of 2- and H2O2 in its mechanism. Some commercial anti-blast fungicides and several disease controlling redox compounds also stimulated AO production. It is likely that overproduction of AO on leaf surface suppresses the ectophytic phase of fungal development and, therefore, contributes to blast resistance of various nature.

Active oxygen (AO) species are hydrogen peroxide H_2O_2, superoxide O_2^- and hydroxyl •OH free radicals. Now they are realized to be rather trivial products of aerobic metabolism (Afanas'yev,1989). AO species are cytotoxic and cause oxidative damage of lipids, enzymes and nucleic acids primarily. Hydroxyl radical is the most potent oxidant among AO species. Usually, it arises from the Haber-Weiss reaction

$$O_2^- + H_2O_2 \longrightarrow \bullet OH + OH^- + O_2$$

catalyzed by iron or copper ions. The implication of this reaction in many pathologic states is confirmed by antidote effects of substances destroying AO or binding catalyzers. Such substances (for instance, superoxide dismutase, SOD) are normal constituents of any aerobic cell. They carry out antioxidant function minimizing an injury of AO (Halliwell, Gutteridge, 1984).

AO production by higher plants is involved in defence responses against infection. Thus, AO mediate hypersensitive cell death (Doke, Chai, Kawaguchi, 1987) induces synthesis of phytoalexins (Apostol, Heinstein, Low, 1989), participates in lignin (Westermark, 1982) and ethylene (McRae, Baker, Thompson, 1982) synthesis and, possibly, activates resistance genes (Chen, Silva, Klessig, 1993).

The purpose of the present work was to follow the AO involvement in different kinds of rice resistance to blast. We focused mainly on fungitoxic effects of AO and on opposing antioxidant capacity of the blast fungus.

1. Toxicity of plant phenols to causal fungus

The rice blast resistance is based to some extent on fungitoxic effects of plant phenolics.

We revealed superoxide production (measured by SOD-sensitive cytochrome *c* reduction) during autoxidation of some phenols. The toxicity of ferulic acid to *Pyricularia oryzae* spores was diminished by SOD or other antioxidant compounds supposing involvement of AO in the toxic effect. In the mixture peroxidase/H_2O_2 ferulic, caffeic or chlorogenic acid acquired extra-toxicity which was prevented by SOD or hydroxyl radical scavengers (Aver'yanov, Lapikova, 1994).

Therefore, plant phenols can produce AO mediating their fungitoxicity and so participating in plant defence reactions.

2. Varietal resistance

Superoxide production by rice leaves may be measured by SOD-sensitive oxidation of exogenous epinephrine on intact leaf surface or in drop diffusates collected from leaves. The reaction was detected in healthy leaves and usually increased within 1-3 days after their inoculation with blast especially in incompatible combinations. The production of H_2O_2 and •OH by rice leaves was also shown.

The concentration of AO in an infective drop was apparently fungitoxic since drop diffusates of leaves inhibited germination of blast fungus spores. The effect was diminished by SOD, scavenger of O_2^- (tiron), catalase, scavengers of •OH (mannitol, formate, thiourea) or by Fe^{+3}-chelator (desferrioxamine) as shown for the resistant cultivar “Zenith” (Table 2). The toxicity of diffusates, like chemically assayed superoxide production by them, rose after inoculation. Both indexes were significantly higher in vertically resistant cultivars than in susceptible ones (Table 1).

The cultivar “Shimokita” was recognized as partially resistant. Diffusates of its healthy and notably infected leaves also inhibited spore germination. Quantitatively, this fungitoxicity was close to the level of other resistant cultivates. Whereas, there were qualitative distinctions as to effects of some factors on the antifungal action. Catalase, mannitol, formate and desferrioxamine (Table 2) abolished fungitoxicity of both “Zenith” and “Shimokita” diffusates implying the role of •OH and H_2O_2. However, SOD and tiron (and thiourea as well) were protective only with “Zenith”. So, the toxic property of this partially resistant cultivar seems to be independent of superoxide. Furthermore, boiling blocked completely the toxicity of “Zenith” leaf diffusates but was quite indifferent with “Shimokita”.

We think that a relatively high antifungal activity of diffusates of infected leaves is indicative of both kinds of varietal resistance. The effect could be due to hydroxyl radical formed by different ways in different cultivars.

Some other cultivars (CNAB kweichow, China 1039 and Norin 18) with signs of partial resistance (to races 007, 047, 037.3, 137.5 and 137.7) were also tested. In contrast to “Shimokita”, the fungitoxicity of diffusates were slightly diminished by SOD, thiourea or boiling in some combinations. This might be explained by the presence of major resistance genes in addition to genes of partial resistance.

3. Heat-induced resistance

We confirmed that high night temperatures (28°C instead of 23°C) render susceptible rice cultivar somewhat resistant to blast. The warming did not affect the development of the fungus *in vitro* but increased O_2^- production in diffusates of healthy or infected leaves. The fungitoxicity of diffusates enhanced with the temperature up to the level of resistant cultivars and was sensitive to SOD or catalase. The administration of these antioxidant enzymes to the inocula prevented completely the heat-dependent resistance indicating AO involvement in its mechanism (Aver’yanov, Lapikova, Dzhavakhiya, 1993).

4. Chemically-induced resistance

The anti-blast commercial fungicides tricyclazole, fthalide and probenazole, nontoxic to fungus, stimulated O_2^- production in leaf diffusates. In parallel, the diffusates became more fungitoxic still in H_2O_2- and O_2^- -dependent manner. In infected plants these phenomena were observed only with that kind of fungicide application which prevented the disease. Bayleton, ineffective against blast, did not change oxygen activation (Nikolaev, Aver’yanov, Lapikova, Djavakhia, 1994).

Therefore, a stimulation of AO production *in planta* may be tested by the additional mode of action of fungicides. This view was confirmed with several metal-organic complexes. They were found to possess similar properties including some disease control.

5. Antioxidant capacity of a pathogen as a factor of pathogenicity

Since AO may restrict plant invasion, any inhibition of AO production should favor compatibility.

Normal mycelia and spores of *P. oryzae* contain melanin, known factor of penetration (Sisler, Ragsdale, 1987). In addition we found that this pigment had SOD and catalase activities and scavenged ·OH radical. The isolated pigment protected spores from damage by any artificially formed AO species or by leaf diffusates. Spores of melanin-deficient mutants were more vulnerable to diffusates or to model AO-generating systems than that of normally pigmented parental strain. These mutants were non-pathogenous, possibly (at least, partly) due to enhanced sensitivity to oxidative damage. Spores of the pathogenous strain grown in different conditions varied in their aggressiveness. The latter correlated with melanin content and with the spore tolerance to leaf diffusates and artificial AO. Fungicides tricyclazole and fthalide (but not probenazole), inhibitors of melanin biosynthesis, sensitized spores to leaf diffusates and model AO.

Obviously, the melanin is not the only antioxidant compound of *P. oryzae*. Other constituents, in particular enzymes, may play a similar role. The activities of SOD and, in some instances, catalase were relatively low in spores of nonpathogenic pigment mutants and of low-aggressive cultures of the wild type. Fthalide suppressed both enzymes in spores. The inhibitor of SOD, sodium diethylditiocarbamate, added to the nutrient medium did not suppress mycelial growth. Meanwhile, spores from treated culture were not pathogenous probably because of the observed high sensitivity to damage by AO.

The data presented here allow to conclude that plant overproduction of AO species and, in particular, their fungitoxic effects may contribute to various kinds of rice resistance to blast. Opposing antioxidant capacity of the fungus might be a prerequisite of its pathogenicity.

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Annexe

Table 1. Oxidation of exogenous epinephrine in drop diffusates of blast-infected rice leaf fragments and fungitoxicity of these diffusates

Cultivars	Epinephrine oxidation[1)]	Diffusate toxicity[2)]
Resistant		
Tadukan	6.6 ±0.3	75 ± 3
Fukunishiki	6.4 ±0.3	66 ± 4
Carreon	3.0 ±0.2	63 ± 4
IAC-25	6.0 ±0.1	63 ± 5
Zenith	7.3 ±0.1	53 ± 5
BL-1	3.7 ±0.1	53 ± 3
Te-tep	2.8 ±0.1	52 ± 3
Susceptible		
Ishikari-Shiroke	2.0 ±0.1	29 ± 1
Primanychsky	1.0 ±0.1	23 ± 4
Sha-tiao-tsao	1.8 ±0.1	18 ± 3
Tsuyuake	1.6 ± 0	12 ± 5
K-59	1.9 ±0.1	12 ± 5
Kuban'-3	1.9 ±0.1	10 ± 5

1) Optical density (492 nm) of 1 mM epinephrine solution incubated 2 h at 37°C in diffusate with 20 mM potassium-phosphate buffer, pH 8.2. Values were normalized to control (the same mixtures with water instead of diffusate).
2) Relative inhibition (%) of spore germination as compared to water.
Table represents maximum values of each index within two days after inoculation. Inoculum and test-organism were *P. oryzae*, race 137.5.

Table 2. Effect of antioxidant reagents on the fungitoxicity of diffusates of intact rice leaves infected with blast

Media of spore germination	Cultivar Zenith	Shimokita
Leaf diffusate	63 ±7	75 ± 5
The same +100 ug/ml SOD	-5 ± 3	80 ± 6
-»- +1 mM tiron	2 ± 5	77 ± 3
-»- +100 ug/ml catalase	0 ± 8	-8 ± 7
-»- +1 mM sodium formate	21 ±3	13 ± 6
-»- +10 mM mannitol	25 ±7	-4 ± 6
-»- +0.5 mM thiourea	-3 ± 4	80 ± 3
-»- +0.2 mM desferrioxamine	-2 ± 6	5 ± 6
-»- , boiled	-7 ± 3	86 ± 5

Relative inhibition (%) of spore germination as compared to water. Negative values mean stimulation against water control.
Inoculum and test-organism were *P. oryzae*, race 007.