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DIFFERENCES IN PHYTOALEXIN RESPONSE AMONG RICE CULTIVARS OF DIFFERENT RESISTANCE TO BLAST

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Key Word Index—*Oryza sativa*; Gramineae; rice; *Pyricularia oryzae*; rice blast; phytoalexins; flavanone; sakuranetin; momilactone A; oryzalexin S; cultivar variation.

Abstract—The production of both flavonoid and diterpenoid phytoalexins after induction by UV irradiation was studied in five rice genotypes of different susceptibility to the rice blast fungus *Pyricularia oryzae*. Consistent qualitative and quantitative differences were found between the rice cultivars in the phytoalexins produced, and there was a strong correlation between the accumulation of the phytoalexins, sakuranetin, momilactone A and oryzalexin S, and rice resistance to blast. Production of phytoalexins was also investigated in rice genotype Tetep after inoculation with an incompatible race of *P. oryzae*. Similar levels of sakuranetin and oryzalexin E were formed 3 days after both inoculation and UV irradiation of the leaves, but there were different levels of momilactone A and the other oryzalexins. Although a given rice genotype may respond quite differently in its production of phytoalexins depending on whether it has been irradiated or inoculated with a fungus, and in the latter case on whether a compatible race of the pathogen has been used, the present results indicate that genetic differences in phytoalexin response between rice cultivars are likely to play an important role among the many factors that determine differences in blast resistance between different rice genotypes. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The rice blast disease, caused by *Pyricularia oryzae* Cav. (teleomorph: *Magnaporthe grisea*), is the most serious fungal disease of the rice crop, and is capable of causing considerable losses in yield. The fungus is in a state of dynamic co-evolution with its host plant, different strains of the fungus varying in pathogenicity and different cultivars varying in their resistance or susceptibility. Much work has been done on the biochemistry of fungal resistance in rice. Constitutive defence includes high silicate levels in the leaf [1], the production of an antifungal resorcinol in seedlings [2] and the formation of hydroxy and epoxy fatty acids [3]. An induced response involving increases in lignification has been reported, but the major inductive response is the formation of phytoalexins. These are, unusually, formed by two different biosynthetic pathways: terpenoid and phenolic. The terpenoid phytoalexins all have diterpenoid structures and include momilactones A and B, oryzalexins A to F, oryzalexin S and phytocassanes A to D [4–6]. Only one phenolic rice phytoalexin has been found so far, the flavanone sakuranetin (5,4′-dihydroxy-7-methoxyflavanone), which is formed in rice in response to UV irradiation or blast infection [7].

The role of phytoalexins in the resistance of rice to blast is not yet clear. Very little research has been done so far to determine whether resistant rice genotypes produce larger quantities of phytoalexins or different types of these antifungal substances than do susceptible genotypes. We have therefore investigated differences in phytoalexin response between a number of rice cultivars, to see whether we could find a correlation between phytoalexin accumulation and blast resistance. Since inoculation of rice plants with *P. oryzae* in the laboratory is difficult, we chose to use UV irradiation to induce phytoalexin formation in our main experiments. Three of the five rice genotypes used for this study, Tetep, Ta-poo-cho-z and Carreon, were blast resistant, whereas the other two, IR50 and B40, were blast susceptible. For the rice genotype Tetep, the results were compared with those obtained by punch inoculation of the leaves with an incompatible race of *P. oryzae*.

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Table 1. Mean concentration of sakuranetin in rice plants induced by UV irradiation for 3.5 days

Rice cultivar	Sakuranetin (ppm)	Group	*n*
Tetep	19.8 ± 1.9	B	30
Ta-poo-cho-z	18.4 ± 3.3	B	6
Carreon	4.5 ± 1.3	A	6
IR50	4.9 ± 1.3	A	6
B40	0.0	A	7

n, number of replicates. ANOVA: $F = 11.983$, $P < 0.000$. Group A not significantly different. Group B not significantly different. Controls (untreated): no sakuranetin detected.

RESULTS AND DISCUSSION

After about 2 days of incubation after UV irradiation the leaves of most rice cultivars started to show necrosis, showing that a hypersensitive reaction had taken place. After 4 days most of the leaves were more than 90% necrotic, whereas control leaves stayed green for this period. Since preliminary studies indicated that the optimum incubation time for the production of the phytoalexin sakuranetin is 3.5 days [8], this incubation period was used for most experiments.

The amounts of flavanone and diterpenoid phytoalexins produced in the leaves of the five rice genotypes exposed to UV irradiation and 3.5 days of incubation are shown in Tables 1 and 2. Since sakuranetin has a characteristic UV absorption spectrum, it was detected and quantified by means of HPLC coupled with a photodiode array, whereas the levels of the major diterpenoids produced, momilactones A and oryzalexins D, E and S, were analysed by using gas chromatography. The amounts of sakuranetin detected 3.5 days after UV treatment in resistant cultivars Tetep (19.8 ± 1.9 ppm) and Ta-poo-cho-z (18.4 ± 3.3 ppm) appeared to be significantly higher than that recovered from the susceptible cultivars IR50 (4.9 ± 1.3 ppm) and B40 (0.0 ppm) (Table 1). However, in the case of the third resistant cultivar, Carreon, the level of sakuranetin was not significantly different from the amounts produced by the susceptible IR50. The low concentrations of sakuranetin formed in Carreon leaves after UV irradiation may be explained by the fact that this cultivar, in contrast to the four others, produced hardly any lesions after irradiation with UV light. This suggests that the leaves are protected by some physical means, e.g. high silica content, and this rather than phytoalexin production could also be an explanation for the blast resistance of Carreon.

The results of the diterpenoid phytoalexin analyses after UV irradiation are given in Table 2 (momilactones and oryzalexins only; the phytocassanes [6] were reported after our experiments had finished, so they have not been taken into account). The concentration of momilactone A was significantly higher in UV-treated Ta-poo-cho-z (136.83 ppm) than in other UV-treated cultivars. It was detected at 36.16 ppm in UV-treated Tetep and in still lower levels in the other three genotypes (see Table 2). Oryzalexin S was also significantly higher in Ta-poo-cho-z (13.72 ppm) than in the susceptible cultivars. However, the amount of oryzalexin D was significantly higher in B40 (45.88 ppm) and Tetep (43.40 ppm) after UV treatment than that in Ta-poo-cho-z (6.64 ppm), whereas with oryzalexin E there were no significant differences between the UV-treated cultivars. The highest level of the latter phytoalexin was in Tetep at 45.69 ppm.

To compare the phytoalexin response of rice plants after UV irradiation with the response after fungal infection, we carried out experiments using rice genotype Tetep inoculated with an incompatible race of *P. oryzae*. After 3 days of incubation after inoculation, similar levels of sakuranetin (15.8 ppm, see Table 3) were formed in the disease lesions of Tetep as in UV-irradiated tissue (18.4 ppm), whereas the surrounding leaf tissue yielded only 1.2 ppm. The production of this compound is therefore a localized reaction. The levels of oryzalexin E found in the lesions of Tetep after infection were also very similar to those after UV irradiation (47.2 and 45.69 ppm, respectively).

Table 2. Mean concentration of diterpenoids in rice plants induced by UV irradiation for 3.5 days

Rice cultivar	Momilactone A (ppm)	Oryzalexin D (ppm)	Oryzalexin E (ppm)	Oryzalexin S (ppm)	*n*
Tetep	36.16*	43.4†	45.69*	9.02*†	15
Ta-poo-cho-z	136.83†	6.64*	36.53*	13.72†	5
Carreon	22.09*	18.78*†	36.06*	6.92*†	5
IR50	24.01*	15.92*†	17.36*	0.76*	5
B40	13.72*	45.88†	44.50*	0.00*	5
ANOVA					
F	3.446	2.568	0.707	1.928	
P	0.0197	0.0582	0.5938	0.1314	

n, Number of replicates.
* Group A not significantly different.
† Group B not significantly different.

Table 3. Concentrations of phytoalexins produced in Tetep plants inoculated with *P. oryzae**

Time (days)	Sakuranetin (ppm)	Oryzalexin E (ppm)	Momilactone A (ppm)
3	15.8 (1.2)	47.2 (8.0)	417.4 (79.6)
4	4.3 (1.1)	10.2 (5.6)	49.9 (5.3)
4.8	4.2 (1.0)	5.2 (6.0)	18.3 (23.6)
5.8	4.9 (0.8)	7.3 (4.3)	0 (7.4)

* Amounts produced in disease lesions as against the rest of the leaf (in parentheses). Oryzalexin S not detected; oryzalexin D (184.2 ppm) only detected from disease lesions at 4 days.

However, the levels of oryzalexin S were very much lower (0 ppm in inoculated tissues of Tetep and 9.02 ppm in irradiated ones) and those of momilactone A very much higher (417.4 and 36.16 ppm, respectively) after 3 and 3.5 days of incubation. Sakuranetin, oryzalexin E and, especially, momilactone A sharply decreased in concentration when the leaves were incubated for more than 3 days after fungal inoculation. After 5.8 days, hardly any more momilactone A could be detected, indicating that the pathogen may be able to metabolize the phytoalexins further. However, no oryzalexin D could be detected after 3 days of incubation after infection, but a high concentration was present after 4 days (184.2 ppm), which is far higher than the ED_{50} value of oryxalexin D against spore germination of the *P. oryzae* (30 ppm [9]). So perhaps oryzalexin D is effective at a later stage of the infection. Since only the cultivar Tetep was infected by rice blast, it is not possible to conclude what relative roles the different phytoalexins play in the resistance of rice plants to blast. This will furthermore depend on a combination of the amounts produced of each phytoalexin, the speed of their production and their antifungal activity. it is also possible that the various phytoalexins act in a synergistic manner. Table 4 shows the ED_{50} values against spore germination or hyphal growth of a number of rice phytoalexins and related compounds [9–11] which were monitored or tested during the present study. Previously, we found that the flavanone naringenin, which is produced as a biosynthetic precursor of sakurantetin in the elicitation process, is present in rice leaves 2 days after UV irradiation, when levels of sakuranetin are still low [8]. To assess a possible role of naringenin in the early stages of infection, we determined its ED_{50} value against hyphal growth of *P. oryzae*. It appeared to have negligible antifungal activity (Table 4). Similarly, the 4′-glucoside of sakuranetin, sakuranin, which has not previously been detected in rice plants, was not found to exhibit any significant antifungal properties against the pathogen (Table 4).

Table 4. ED_{50} values of phytoalexins against *P. oryzae*

Phytoalexin	v. spore germination	v. hyphal growth	Ref.
Momilactone A	31.4–314*	–	[10]
Oryzalexin D	30	–	[9]
Oryzalexin E	62.5	–	[4]
Oryzalexin S	20	–	[11]
Sakuranetin	15	35	[7], this work
Naringenin		360	This work
Sakuranin		200	This work

* Values obtained for inhibition of germ tube growth.

In summary, we have shown that there are significant and consistent differences between rice cultivars in their phytoalexin response, both qualitatively and quantitatively, when irradiated by UV light, and that phytoalexins are often produced at much higher levels in blast-resistant than in blast-susceptible rice genotypes. Whether this correlation also applies after blast infection remains to be investigated. Phytoalexin production will also depend on whether the plant has been inoculated with a compatible or incompatible strain of the pathogen. Changes in phytoalexin concentrations over time may be also important in the disease resistance of rice plants. Clearly, environmental factors are involved as well, and future experiments need to consider whether both constitutive and induced defence systems work together to provide blast resistance.

EXPERIMENTAL

Growth of rice plants. Seeds of rice cultivars showing a degree of resistance to rice blast [Tetep (Vietnam), Ta-poo-cho-z (Taiwan) and Carreon] and those susceptible to blast [B40 (Indonesia) and IR50] were obtained from the International Rice Research Institute (IRRI), Los Baños, Philippines. The seeds were germinated on Whatman No. 1 filter paper placed on cotton wool soaked in demineralized water in a glass beaker sealed with clingfilm and incubated at 25° in a 12 hr light/12 hr dark regime for 6–10 days. The seedlings were then potted (2 per pot) in John Innes No. 2 compost and the pots were placed in trays filled with water. To prevent iron deficiency, Murphy's sequestrene preparation was applied. Seedlings were grown in a greenhouse (heated in the winter) with natural lighting supplemented with white halide lamps.

UV induction of phytoalexins. Fresh leaves (1–2 g wet weight) were cut from rice plants at first tillering stage (4 weeks post-sowing) to heading stage (10–12 weeks). The leaves were floated adaxial side uppermost in tap water (75 ml) with a drop of Tween 20 in a clear plastic sandwich box. The box was then irradiated with short wavelength UV light (254 nm) for 20 min. After irradiation the box was sealed with clingfilm and incubated at 26° under a 12 hr dark/12 hr light regime, starting with a dark period of at least 8 h, for 3.5 days. The maximum concentration of sakuranetin was previously found to be detected at 3.5 days [8]. The maximum concentration of momilactones and oryzalexins is about 72 hr [12]. Control leaves were incubated under the same conditions.

Extraction of rice leaves. After incubation the water was removed and the leaves cut into small pieces (1–2 cm). Leaf pieces were immersed in 100 ml 80% aqueous MeOH (at boiling point) in a conical flask and boiled for 10 min on a hot water bath. After cooling the flasks were left at room temperature, in the dark, for 24 h. The extract was then filtered through a Whatman No. 1 filter paper and washed through with a further 100 ml 80% aqueous methanol. The extract was concentrated *in vacuo* at 40° until *ca* 2 ml of liquid remained. The concentrated leaf extract was re-extracted three times with 2 ml diethyl ether. The combined ether fractions were evaporated to dryness on a rotary evaporator.

HPLC. Leaf extract was dissolved in 0.5 ml 80% aqueous methanol and filtered through a Gelman Acrodisc filter (0.45 μm). 20 μl extract was used to quantify sakuranetin by means of a reverse phase HPLC with light diode array detection. An isocratic solvent system was used consisting of 40% solvent A (5% aqueous acetic acid) and 60% solvent B (methanol:acetic acid:water, 18:1:1) and run for 20 min. The column used was μ-Bondapak C-18 phenyl, 30 cm × 3.9 mm i.d. with a flow rate of 1 ml min^{-1} and a column temperature of 25°. A light diode array detector was used and sakuranetin was detected at 290 nm (λ_{max}) with a retention time of 9–10 min. This was verified with a standard.

TLC. Leaf extract was re-extracted in 0.5 ml ethyl acetate and the extract spotted in duplicate together with phytoalexin standards on a 20 × 20 cm silica gel TLC plate. The plate was run in 3% ethanol in chloroform (as solvent). After processing, half the plate was sprayed with 1% vanillin in 30% H_2SO_4 in ethanol and heated in an oven at 110° for 5 min. The colour reactions were noted under UV light and compared with those of markers (sakuranetin, oryzalexin D, momilactone A obtained from Dr J. L. Ingham, Plantech, Reading, U.K.). The remaining, duplicate half-plate was used for detecting antifungal activity and was sprayed with spores (5×10^9 spores ml^{-1}) of *Cladosporium herbarum* in a glucose/mineral solution (10 ml 15% glucose solution added to 20 ml salt solution consisting of (g l^{-1}): 7 g KH_2PO_4, 4 g KNO_3, 3 g $NaHPO_4 \cdot 2H_2O$, 1 g $MgSO_4 \cdot 7H_2O$). The half-plate was incubated at 24° for 3 days in a moist chamber. Inhibition zones were noted and compared to reference values and colour reactions obtained with the vanillin spray.

GC. Leaf extract was re-extracted in 1 ml 100% HPLC grade MeOH and filtered through a Gelman Acrodisc filter (0.45 μm). Diterpenoids were quantified using a Pye Unicam Series 204 chromatograph with a split/splitless injector system and a flame ionization detector. A fused silica glass capillary column coated with DB-5 (0.25 mm × 30 m, J & W Scientific) with a temperature programme of 250–310° (4° min^{-1}) was used for the analysis. The method was adapted from [13].

Growth of fungi. Pyricularia oryzae MG8G was obtained from Dr S. Prasad, The Queen's University, Belfast. The culture was originally from South India and is compatible with the rice cultivar IR50. It was maintained on V8 agar (V8 juice 200 ml, demineralized water 800 ml, $CaCO_3$ 3 g, agar 20 g, pH 6.2) at 30°. For long-term storage it was dried on Whatman filter paper No. 1 and stored at −18°. Bioassays against hyphal growth of this fungus were carried out using standard procedures [14].

Inoculation. The punch inoculation method was adapted from [15]. A pair of pliers with a headless, flat-tipped nail glued to the inner jaw and a thin piece of cardboard glued to the inside surface of the opposite jaw was used to crush the leaf surfaces. A suspension of *Pyricularia* MG8G (1×10^5 spores ml^{-1}/mycelial fragments) with a drop of Tween 20 and 1% rice polish as an adhesive, was used.

Inoculated and control rice plants were maintained at 25° in a 16 hr light/8 hr dark regime starting with an initial 24 hr dark period. High humidity (92.5% RH) was obtained at the critical stages by spraying the growth chamber with water at 0, 18, 20 and 22 hr. The pots were stood in trays of water that maintained RH at 90% throughout the incubation period.

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