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DIFFERENTIAL SENSITIVITY OF RICE PATHOGENS TO GROWTH INHIBITION BY FLAVONOIDS

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Key Word Index—*Oryza sativa*; Gramineae; rice; flavonoids; anthocyanins; antibacterial; antifungal activity.

Abstract—Differential sensitivity of the major pathogens of rice, *Xanthomonas oryzae* pv. *oryzae*, *Pyricularia oryzae* and *Rhizoctonia solani* to inhibition by certain flavonoids was tested using paper disc/liquid culture and spore germination assays. Naringenin, the first intermediate of the flavonoid pathway, displayed growth inhibition of *Xanthomonas* strains and spore germination of *P. oryzae*. On the other hand, no such inhibition was found with *Rhizoctonia solani*. Crude extracts of leaf and pericarp tissues of a fully purple pigmented rice cultivar, Purpleputtu, also showed growth inhibition of *Xanthomonas*. © 1997 Elsevier Science Ltd

INTRODUCTION

Diseases of rice are a major obstacle in achieving sustainable yield targets. This is in spite of multitude of approaches to build durable resistance of the rice plant to major diseases. A different approach to this important problem is to exploit the metabolic pathways, particularly the secondary metabolic pathways leading to the synthesis of molecules that serve as defense chemicals. Flavonoids derived from both malonate and shikimate pathways, are emerging to be important, both as defense and survival compounds in plants [1–3]. The effects of flavonoids and anthocyanins *per se* on fungi, bacteria and viruses are well documented [4–10]. However, not many systematic studies have been conducted to establish the role of the flavonoids in disease resistance in crop plants, particularly in rice. This report deals with the differential sensitivity of major rice pathogens, *Xanthomonas oryzae* pv. *oryzae* (the bacterial blight pathogen), *Pyricularia oryzae* (the fungal blast pathogen) and *Rhizoctonia solani* (the fungal sheath blight pathogen) to growth inhibition by flavonoids.

RESULTS AND DISCUSSION

The toxicity of naringenin (flavanone), dihydroquercetin (dihydroflavonol), kaempferol and quercetin (flavonols) to the rice pathogens has been assayed. Sensitivity to the tested compounds was found to be significantly variable among the pathogens. Naringenin inhibited the growth of all the six strains of *X. oryzae* at 25 μg disc^{-1}. In contrast, dihydroquercetin was effective only against two strains (Table 1). Liquid culture assays with naringenin (10 μg ml^{-1}) showed a ten-fold reduction in the growth of *X. oryzae*.

The antifungal activity of these compounds was assayed by their effects on the growth of spore and mycelium of *Pyricularia*. Naringenin and kaempferol showed a significant inhibition of spore germination of *P. oryzae* from 7 μg onwards (Table 2). The spore morphology studies with naringenin revealed an overall inhibition, i.e. absence of germ tube and appresorium formation. This observation assumes significance since infection begins with the germination of the spore and thereby the spread of the pathogen in the plant. None of the tested compounds had any significant effect either on the mycelial or sclerotial growth of *Rhizoctonia*.

Anthocyanin extracts from the leaf and pericarp of a pigmented rice cultivar, Purpleputtu, were tested on *Xanthomonas*. Growth inhibition was apparent at 100 μg disc^{-1} (data not shown). These extracts have been shown to comprise mainly of cyanidin and peonidin glycosides [11, 12].

These studies revealed the non-specific toxicity of naringenin to both *Pyricularia* and *Xanthomonas*. The tested flavonoids differ in their hydroxylation pattern in the B and the C rings. Naringenin inhibited the spore germination of *Pyricularia* at 7 μg. Earlier studies demonstrated that while naringenin inhibited spore germination of *Pyricularia* at 25 ppm, its 7-*O*-methyl ether, sakuranetin was found to be inhibitory at 5

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Table 1. Growth inhibition of *Xanthomonas oryzae* pv. *oryzae* by flavonoids (average diameter of inhibition zone in mm)*

Flavonoids	*Xanthomonas* strains					
	A3846	A3821	AP19	AP24	AP3843	CN2
Naringenin	10	11	10	10	8	8
Kaempferol	0	0	0	0	0	0
Dihydroquercetin	0	16	0	0	0	15
Quercetin	0	0	0	0	0	0

* Paper disc bioassay was performed on PSA medium. Paper discs were loaded with the flavonoids and 80% methanol (control). The diameter of the inhibition zones were measured in mm after a 24 hr incubation. Data reported are average of three independent experiments. The analysis of variance was performed using the level of significance $\alpha = 0.05$.

Table 2. Effect of flavonoids on spore germination of *P. oryzae*

Flavonoid	Substitution pattern of the flavonoid type	Amount of the flavonoid (μg)	Germination (%)*
Naringenin	5,7,4′-trihydroxyflavanone	14	1.73
		7	9.60
Kaempferol	3,5,7,4′-tetrahydroxyflavone	14	35.0
		7	47.0
Quercetin	3,5,7,3′,4′-pentahydroxyflavone	14	100
		7	98.5
Dihydroquercetin	3,5,7,3′,4′-pentahydroxyflavanone	14	76.0
		7	100

* Spores of *Pyricularia* were incubated in the indicated concentrations of the flavonoids along with the control (methanol) for 5 hr. The percentage of germinated spores were expressed in comparison with the respective controls. Data represented indicate the average of three independent experiments.

ppm [13]. In our studies, naringenin showed complete inhibition of spore germination at 70 ppm. Our data support the finding that different pathogens exhibit different sensitivities towards flavonoids. Further, different strains of the same pathogen may show significant differences in the sensitivity to the same flavonoid. Such variations may reflect differences in membrane permeabilities and genetic backgrounds.

The data seem to indicate that the non-polar flavonoids showed appreciable inhibition (Table 2). Incidentally, most of the phytoalexins are non-polar and lipophilic in nature [14]. Besides, the detoxification of phytoalexins by microbes often occurs through reactions that make them more polar. The toxicity of naringenin and kaempferol to *Pyricularia* is presumably due to their increased lipophilicity. On the other hand, none of the tested compounds was inhibitory to *Rhizoctonia*. Apparently, this fast growing fungus has an ability to detoxify a variety of flavonoids.

Flavonoids have been shown to accumulate in many plants under infection. However, there are few reports in cereal crops showing such accumulation. In *Sorghum*, the accumulation of 3-deoxyanthocyanidins has been shown to be toxic to the pathogen *Colletotrichum graminicola* [15, 16]. In addition, an increase in the flavan 4-ol content was reported in certain mould resistant *Sorghum* lines [17]. Naringenin was detected in rice leaves exposed to UV-B irradiation and also after blast infection [18]. Besides, rice cultivars were shown to exhibit differential phytoalexin response to blast [19].

In our study, the leaf and pericarp anthocyanin extracts showed growth inhibition of *Xanthomonas*. The pigments have already been characterised as cyanidin and peonidin glycosides. These pigments are localised in the epidermis of the leaf. The accumulation of flavonoids in the epidermis has been considered to be a protective mechanism [20].

EXPERIMENTAL

Bacteria and fungi. The rice pathogens *Xanthomonas oryzae* pv. *oryzae* strains (A3846, A3821, AP19, AP24, AP3843 and CN2) *Pyricularia oryzae* (1 isolate) and *Rhizoctonia solani* (1 isolate) were used in this study (courtesy of Dr Sam Gnanamanickam, University of Madras). Cultures of bacteria and fungi were maintained on peptone sucrose agar (PSA) and potato-dextrose agar (PDA), respectively. Sclerotia were aseptically collected from 7-day-grown PDA cultures of *R. solani* and maintained at 4°.

Flavonoids. The flavonoids, naringenin, quercetin, dihydroquercetin and kaempferol were obtained from

Roth Chemicals, Germany. Stock sols of 20 mM of the flavonoids were used for the experiments.

Inhibition assays with flavonoids

Anti-bacterial activity. The antibacterial activity of the flavonoids to *Xanthomonas* was tested by paper-disc or liquid culture bioassays. PSA plates were overlaid with 100 μl of bacterial suspension (10^9 cfu ml^{-1}) mixed with 5 ml of 0.7% molten agar. The test compounds (25–90 μg) along with the solvent control (80% MeOH) were loaded onto paper discs with a minimum of three replicates for each strain. The plates were incubated at 28° for 48 hr and growth-inhibition zones were measured [21]. A direct plate assay also was set up where the compounds were spotted directly on the agar surface. Liquid culture assays were performed with 100 ml of peptone sucrose broth containing 1 mg of naringenin or 80% MeOH and 50 μl of bacterial suspension (10^9 cfu ml^{-1}) that was shaker-incubated at 160 rpm at 28° for 12–24 hr. Cultures were withdrawn at regular intervals and serially diluted. Aliquots of 100 μl of appropriate dilution were plated onto triplicate of PSA plates and incubated for 2 days at 28°.

Anti-fungal activity. Paper-discs loaded with test compounds (25 to 90 μg) or 80% MeOH were placed on PDA spread with conidial suspension (25 000 conidia ml^{-1}) of *P. oryzae*. In another assay, 4-day-old agar-mycelial plugs (10 mm diameter) of *R. solani* or *P. oryzae* were inoculated at three positions on PDA plates with the test compounds placed between the agar-mycelial plugs. After 4 or 7 days incubation the plates were observed for inhibition of radial mycelial growth of the fungi. The sclerotial inactivation test of *Rhizoctonia* was done by placing uniform sized sclerotia onto paper discs containing the test compounds and the solvent. The germination of sclerotia was assessed after 24 hr [22].

A cavity slide assay was performed to study the inhibition of flavonoids on spore germination of *P. oryzae*. 100 μl of spore suspension with MeOH served as solvent control. Flavonoids (7 and 15 μg) were added to the experimental slides. The slides were incubated for 5 hr at 25°. Fifteen microscopic fields were considered for spore counting. The number of germinated spores for each treatment was expressed as a percentage of their respective controls [23].

Plant material, isolation of anthocyanins and antimicrobial testing. Leaf tissue harvested from 40 day old plants and dehusked seeds (2 g each) of puple-puttu, a fully purple coloured indica rice line, were extracted with (25 mg ml^{-1}) of acidified MeOH (1% HCl) for 24 hr at 4° in dark. To 1 ml of the extract, 0.75 ml water and 2 ml $CHCl_3$ were added resulting in Folch partition. The anthocyanin concn in the upper phase was estimated from the A_{525} using a mM extinction coefficient of 31.6. The total vol. of the supernatant was evapd using a Büchi R-124 rotavapour apparatus. The residue was weighed and dissolved in 80% MeOH. Aliquots of 0–200 μg of the extract were tested for inhibition of *Xanthomonas* by the paper disc assay as described above. TLC was carried out on Merck pre-coated cellulose plates (DC-Plastikfolien; layer thickness 0.1 mm). The TLC analysis of the pericarp and leaf extracts in *n*-BuOH–HOAc–H_2O (4:1:5) revealed the presence of two distinct bands corresponding to cyanidin and peonidin glycosides [11].

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