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Short communication

Inhibition of pre-penetration development of blast fungus during the infection of resistant rice cultivars

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Spore germination and appressorium formation of the rice blast fungus, *Magnaporthe grisea*, were compared during infection of rice cultivars differing for their resistance to strain H5-3. On leaves of the susceptible cv. Sha-tiao-tsao neither spore germination nor appressorium formation were inhibited, in comparison to a control germination test in water on polystyrene. On the resistant cv. Tadukan, both spore germination and appressorium development were strongly inhibited whereas on the partially resistant cv. Shimokita a slight inhibition of germination and significant delay of appressorium formation was observed. Inhibitors of germination were not produced in leaf diffusates of the susceptible cultivar Sha-tiao-tsao. On the contrary, such compounds were excreted by leaves of the two resistant cultivars. The fungitoxicity of diffusate of cv. Tadukan was strong from 5 to 24 h after inoculation. The diffusate of cv. Shimokita was less toxic at 5 h but at 24 h it reached the same level of toxicity as diffusate of cv. Tadukan. Such antifungal plant metabolites accumulating in drops of inoculum may inhibit the pre-penetration development of the rice blast fungus and presumably contribute to disease resistance, including the partial resistance.

Plant resistance to fungal diseases might be associated with the inhibition of pathogen development in host tissues during early stages of the infection. The rice blast fungus *Magnaporthe grisea* does not penetrate readily into leaves of some resistant rice cultivars (Heath et al., 1990; Koga and Kobayashi, 1982) or cannot spread over the first invaded cells (Arase et al., 1983; Peng et al., 1988; Kaur et al., 1984). Inhibition of spore germination and appressoria formation were observed during inoculation of leaf fragments from completely resistant rice cultivars (Lapikova and Dzhavakhiya, 1987). This may be due to plant defence responses (syntheses of lignin, phytoalexins and pathogenesis-related proteins etc.) which may liberate fungitoxic products into the extracellular space.

Previously, we found that the partially resistant cultivar Shimokita in combination with strain H5-3 manifests a reduced infection frequency, smaller lesions and a reduced rate of spore production per lesion (Pasechnik et al., 1995), phenomena typical for partial resistance (Roumen et al., 1993). The pre-penetration development of the rice blast fungus on such rice cultivars is not well documented.

The aim of the present work was to assess whether fungal development is affected on the leaf surface of cv. Shimokita. The susceptible cv. Sha-tiao-tsao and the completely resistant cv.Tadukan were taken as controls and intact plants rather than leaf fragments were used for the assessment.

We used strain H5-3 (race 007) of the rice blast fungus, *Magnaporthe grisea* (Herbert) Barr. The rice (*Oryza sativa* L.) cultivars differ in their resistance to race 007: the susceptible cv. Sha-tiao-tsao develops numerous lesions, on the completely resistant cv. Tadukan no visible symptoms are produced and cv. Shimokita exhibits partial resistance with the rare occurrence of compatible-type lesions. Plants were grown in a climatic chamber up to the fourth fully expanded leaf stage (Lapikova et al., 1994). The fourth leaf was used for the analyses. The central part of the leaf was fixed horizontally in an inoculation chamber (Lapikova et al., 1995) and a plastic ring (ID 3 mm, height 1 mm) was placed on the leaf. One drop (10 μl)

of spore suspension (10^5 ml^{-1}) was deposited in the ring. Inoculated plants were incubated for 24 or 48 h at 23 °C in a dark humid chamber.

The drops were transferred from the leaves to microscopic slides. The leaf segments (about 1 cm length) that contained inoculation sites, were cut off and stained with aniline blue (Koch-Light Laboratories Ltd.) in lactophenol: ethanol (1: 3, v/v). Before observation, the plant material was cleared by water chloral hydrate (2.5 g ml^{-1}) (Merck) (Lapikova and Dzhavakhiya, 1987). From each cultivar, five leaves and five drops were collected per time point. The number of germinated spores and formed appressoria were counted and expressed as percentage of the total number of spores observed on the leaf and in the drop which was set at 100%. As a control, we assessed the development of the fungus in five drops placed on a polystyrene surface and kept under the same conditions as the leaves.

The changes in the ability of leaf diffusates to affect spore germination were assessed as follows. Drops containing a spore suspension or water were collected from the leaves 5 or 24 h after application; in the case of cv. Shimokita samples were also taken after 0.5, 2 or 4 h. The spores were removed from the drops by filtration, and the filtrate, called 'diffusate', was collected. A freshly prepared suspension of spores of strain H5-3, race 007 was added to the diffusates to a final concentration of 3.5×10^4 spores ml^{-1} and incubated for 5 h at 23 °C in 96-well plates ('Titertek'). After incubation, 100 μl of spore suspension was fixed with ethanol, and spore germination was counted under an inverted microscope.

Early stages of the fungal development (spore germination and appressorium formation) were not inhibited on leaves from the susceptible cultivar Sha-tiao-tsao (Table 1). In the case of the completely resistant cultivar Tadukan, both spore germination and appressorium formation were significantly inhibited during the two first days of infection. On cv. Shimokita a weak inhibition of spore germination, and a strong inhibition of appressorium formation was found on the first day after inoculation. At 48 h, the percentage of germinated spores was similar to that found on the susceptible cultivar; and the appressorium formation was not longer inhibited. Apparently, this cultivar delayed appressorium formation for about one day. In the susceptible cv. Sha-tiao-tsao many infection pegs were found under the appressoria but in leaves of resistant cv. Tadukan they were rare and, if present, they were thin and unbranched. The partially resistant cv. Shimokita was intermediate with respect to intracellular development of the fungus.

The resistance-dependent inhibition of pre-penetration development of the fungus may be caused by fungitoxic exometabolites of the host plant. Previously, we reported that diffusates collected from the leaves of cv. Sha-tiao-tsao or other susceptible cultivars two days post-inoculation inhibited spore germination and appressorium formation only weakly. This was in contrast with diffusates of resistant cultivars, such as Tadukan, which inhibited both these stages markedly (Lapikova and Dzhavakhiya, 1987; Lapikova et al., 1994; Pasechnik et al., 1995). However, the strong inhibition of spore germination caused by leaf diffusates of cv. Shimokita (Pasechnik et al., 1995) was not consistent with an almost complete lack of the inhibition *in vivo* that we found in this study (Table 1). We hypothesised that antifungal effects on the leaf surface depend on the rate of accumulation of toxicants in the inoculation drop. To test this, we compared the inhibition of germination by diffusates obtained from drops incubated on the leaves for 24 h, as was done previously (Pasechnik et al., 1995), and for shorter periods.

As expected, leaf diffusates obtained from the susceptible cultivar Sha-tiao-tsao were not toxic neither after 5 h nor after 24 h. The leaf diffusates obtained from non-infected resistant cv. Tadukan were weakly toxic after five hours and moderately toxic after 24 h but diffusates from infected Tadukan leaves were highly toxic as early as 5 h after inoculation. In the case of cv. Shimokita, 30-min incubation of water drops on leaves was sufficient to obtain diffusate weakly toxic to spore germination but afterwards (within 5 h) it did not change significantly (Figure 1). Diffusates from infected Shimokita leaves were slightly more toxic, even 1 h after inoculation. Five hours is the time required for 95% germination of spores on a polystyrene surface in water. However, diffusates collected from non-infected Shimokita leaves at 24 h after inoculation inhibited germination. This inhibition was stronger with diffusates from infected Shimokita leaves and similar to values obtained with diffusates from leaves of resistant cv. Tadukan.

Our results demonstrate that leaves of the partially resistant cultivar Shimokita excrete fungitoxic exometabolites less rapidly than those of the completely resistant cultivar Tadukan. Because there is a low concentration of toxicants during the first 5 h after inoculation of Shimokita, most spores have germinated, and suppression of fungus development becomes only

Table 1. Spore germination and appressorium formation of *M. grisea* (strain H5-3, race 007) on the 4th leaves of intact plants of different rice cultivars within two days after inoculation

	Rate of spore germination*		Rate of appressorium formation*	
	in 24 h	in 48 h	in 24 h	in 48 h
Polystyrene	78 ± 4	81 ± 5	43 ± 11	34 ± 2
Leaves of rice				
cv. Sha-tiao-tsao (susceptible)	87 ± 5	78 ± 12	36 ± 4	30 ± 9
cv. Tadukan (completely resistant)	41 ± 9^3	29 ± 12^3	8 ± 5^3	11 ± 8^2
	(53)**	(63)	(78)	(63)
cv. Shimokita (partially resistant)	78 ± 4^1	85 ± 13^0	11 ± 8^1	46 ± 11
	(12)	(–8)	(70)	(–35)

* Five plants inoculated with one infective drop per plant were used. All spores of the inoculum were examined. Data (means ± SD in these 5 replicates) are expressed as percentages of the total number of spores in one drop.

**The relative inhibition (%) of spore germination and appressorium formation on resistant cultivars compared to the susceptible one are represented in parentheses.

Superscript indexes indicate the level of significance (Student's test) between the susceptible and the resistant cultivars.

[0] differences are insignificant.

[1] differences are significant for P = 0.05.

[2] differences are significant for P = 0.01.

[3] differences are significant for P = 0.001.

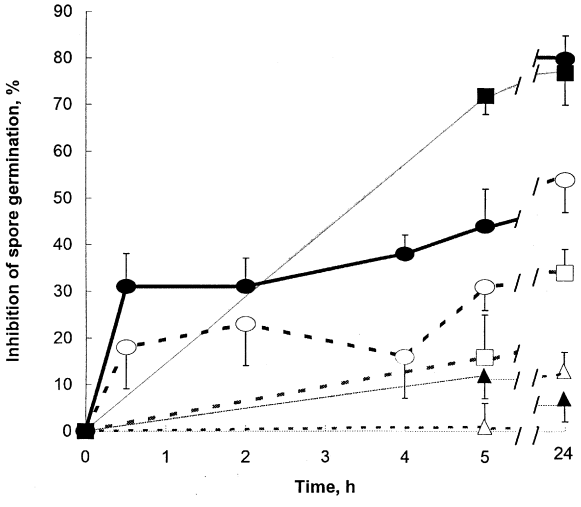

Figure 1. Time-course of rice leaf diffusate toxicity to *M. grisea* during incubation of water drops or drops containing a spore suspension on the fourth leaf of three different rice cultivars. Toxicity was determined as described in the text. The percentage of germinated spores in the water control was about 60–70. At every time point, diffusate was collected from eight plants. Average values and SD were calculated in five series of hundred spores for every time point. Dotted lines and empty symbols represent diffusates from healthy leaves, solid lines and filled symbols represent diffusates from infected leaves. △, ▲ – Sha-tiao-tsao (susceptible); □, ■ – Tadukan (completely resistant); ○, ● – Shimokita (partially resistant).

apparent at later stages. The inhibition of development of the rice blast fungus prior to penetration of rice leaves may contribute to disease resistance, this in addition to post-penetration plant responses.

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