RIBOFLAVIN INDUCES RESISTANCE IN RICE AGAINST *RHIZOCTONIA* SHEATH DISEASES BY ACTIVATING SIGNAL TRANSDUCTION PATHWAYS LEADING TO UPREGULATION OF RICE CATIONIC PEROXIDASE AND FORMATION OF LIGNIN AS A STRUCTURAL BARRIER

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INTRODUCTION

Plants, like animals, are continually exposed to pathogen attack and have developed an innate defence mechanism that enables them to rapidly response to infection by pathogens. The key differences between the susceptible and resistant interactions are the timely recognition of pathogen attack and the rapid, appropriate expression of defence responses. The defence responses can be mediated by SA, JA, and ethylene which are plant-derived substances with important physiological roles and great potential as mediators of resistance signal transduction in plants. These three compounds affect a variety of processes in plants, including biotic and abiotic stresses.
Riboflavin (vitamin B2) is a water-soluble vitamin, which is involved in vital metabolic processes in the cells, and is necessary for normal cell functions. Small amounts of riboflavin are present in most of animals, plants, and microbes and act as a coenzyme in many physiological processes of the cells. This vitamin is involved in antioxidation and peroxidation; both processes affect the production of reactive oxygen species (ROS). Induction of systemic resistance by foliar application of riboflavin has been reported in some dicots against different pathogens e. g., in *Arabidopsis thaliana* infected with *Peronospora parasitica* and *Pseudomonas syringae* pv. *tomato*, and tobacco infected with *Tobacco mosaic virus* (TMV) and *Alternaria alternata*. Riboflavin-induced resistance (IR) in dicot plants has been shown to be correlated with protein kinase signalling mechanisms and a functional NIM1/NPR1 gene, which is a key regulator of salicylic acid (SA)-mediated systemic resistance. Accumulation of SA is not required, however, as in NahG plants which are deficient in SA accumulation, application of riboflavin still induced systemic resistance against different pathogens (1). The role of riboflavin as an elicitor of systemic resistance and a plant defence activator in rice as an important monocot plant was demonstrated in the present study. We conducted studies of the mechanism of riboflavin-IR and defence responses in rice against *Rhizoctonia* sheath diseases which are among the most important fungal diseases of rice, causing more than 50% yield losses in the world every year. We found that riboflavin-IR can be linked to the induction of defence pathways leading to formation of structural barriers such as lignin in rice plants. Our findings proved that using riboflavin as a plant defence activator can be a new, simple, and environmentally safe strategy to control *Rhizoctonia* sheath diseases of rice.

MATERIALS AND METHODS

Plant materials and chemical treatments. Rice plants (*Oryza sativa* L. cv. IR-64) were grown in potting compost under greenhouse conditions at 30 ± 4°C with a 16/8 h light/dark photoperiod. Four-week-old plants were used for the experiments.

Pathogen maintenance and inoculation. Inoculum consisted of toothpicks, 2 cm in length, that had been sterilized and inoculated with isolates of *R. solani* or *R. oryzae-sativae* (4). After an incubation period of 8 days, one colonized toothpick was placed into the lowest inner sheath of the main tiller, 10 cm above the soil surface. For each isolate, eight replicate rice plants were inoculated in a completely randomized experimental design and the experiment was repeated twice.

Effect of riboflavin on pathogen growth. *R. solani* and *R. oryzae-sativae* were cultured on PDA medium containing 0 to 2 mM riboflavin at 28°C. After 6 days the diameter of the colonies were measured.

Responses of plants. Rice plants were sprayed with riboflavin at different concentrations (0, 0.01, 0.1, 2 mM) and the leaves detached 3 days post treatment (dpt) to investigate for possible phytotoxicity by riboflavin. Detached leaves were stained with trypan blue in lactophenol, cleared with chloral hydrate solution and observed under microscope. Rice plants infected with *R. solani*, as a necrotrophic pathogen causing cell death, were used as positive control.

Evaluation of pathogen infection and disease resistance. Plants were sprayed with riboflavin (0.01, 0.1, 0.2, 2 mM) or mock (0.05% tween 20) and inoculated with
R. solani or *R. oryzae-sativae* at 3 dpt. Control plants which were sprayed with water were added to each experiment to investigate the possible effect of mock treatment on progress of rice sheath diseases. Disease evaluation was done 4 days post inoculation (dpi) by measuring the lesion length.

Duration of the control period. Plants were sprayed with riboflavin (0.01 mM), and inoculated with *R. solani* at 3, 5, 9, 13, 20 dpt. Disease was evaluated at 4 dpi.

Systemic translocation of riboflavin-mediated defence signals. Riboflavin (0.01 mM) was sprayed on 2 lower leaves of rice. Inoculation was done 5 dpt either systemically on untreated upper leaf sheath or locally on the treated leaf sheath. Disease was evaluated at 1, 3, 5, 7 dpi.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was prepared from rice leaves by Trizol reagent (Invitrogen). After treatment with Turbo DNA-free kit (Ambion) to remove genomic DNA, the RNA was quantified spectrophotometrically. cDNA was synthesised with oligo-dT and SuperScript Reverse Transcriptase. Gene-specifc PCR primers for cationic rice peroxidase gene (POC-1) were used in study. Rice actin gene was used as control.

Histochemical detection of H_2O_2. 3,3-diaminobenzidine (DAB) solution (1mg ml^{-1}, PH 3.8) was infiltrated into the leaf segments collected at 0, 6, 12, 18, 24, 48 hpi and cleared in absolute ethanol. DAB solution supplemented with 50 mM ascorbic acid (a H_2O_2 scavenger) was used as control.

Lignin detection. Lignified structures were visualized using the phloroglucinol/HCl test (3).

RESULTS AND DISCUSSION

The lowest concentration of riboflavin tested (0.01 mM) had the best effect on induction of resistance against *R. solani* and *R. oryzae-sativae*, causal agents of sheath blight and aggretate sheath spot of rice, respectively (Figure 1) . Treatment of rice plants with riboflavin locally and systemically reduced the lesion length when inoculated with *R. solani* (Figure 2). The disease protection by riboflavin lasted up to 20 days after treatment. Riboflavin did not have any direct effect on the growth of fungi in vitro. Also, at concentrations necessary for induction of resistance (0.01 to 2 mM), no macroscopic or microscopic cell death in rice was observed. Therefore, riboflavin is able to activate resistance mechanisms in rice, like dicots, in a hypersensitive response (HR)-independent manner. Production of hydrogen peroxide was detected at 12 h after inoculation using DAB staining method. Expression of a cationic rice peroxidase, POC1, was induced at 18 h after inoculation in riboflavin treated rice plants (Figure 3). The expression in control plants was lower than that observed in treated plants. A correlation was found between induction of resistance by riboflavin and upregulation of POC1 gene. A variety of roles have been proposed for the involvement of peroxidases in the defence response (2). One possible role is the generation of reactive oxygen species (ROS) by peroxidase-oxidative activity. The fact that the production of hydrogen peroxide was upstream of induction of POC1 gene expression, ruled out the possibility of involvement of POC1 in the generation of ROS in these interactions. Another possible function of peroxidases is the formation of structural barriers such as cell wall enhancement and deposition of cell wall appositions, both of which can be involved in the polymerization of lignin or suberin, the cross-linking of wall glycoproteins or polysaccharides, and the dimerization of antimicrobial phenols. Lignin formation was investigated using phloroglucinol/HCl test (3), and lignin was detected in riboflavin treated plants. Therefore, riboflavin-induced resistance can be linked to the induction of defence pathways leading to formation of structural barriers in rice plants.

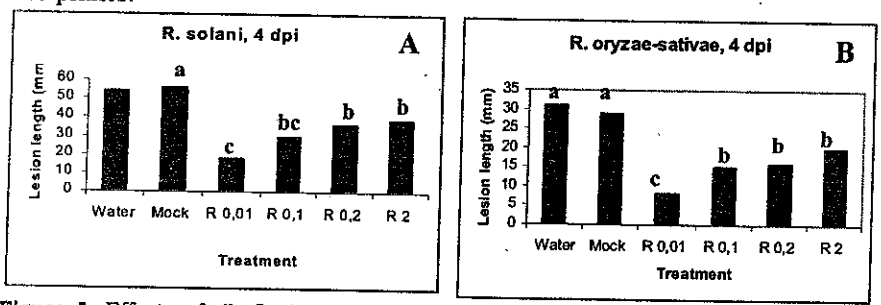

Figure 1. Effects of riboflavin application (0.01 to 2 mM) on disease progress in rice (*Oryza sativa* cv. IR-64) 4 days post inoculation (dpi); A, infected with *R. solani*, and; B, infected with *R. oryzae-sativae*.

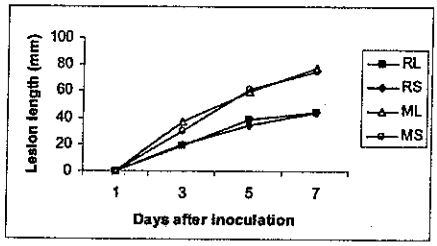

Figure 2. Sheath blight progress in rice, treated with riboflavin or mock. Plants were sprayed with riboflavin (0.01mM) or mock, and inoculated 5 dpt, either (locally) on treated leaf sheaths or (systemically) on untreated upper leaf sheaths. R: Riboflavin, M: Mock, L: Locally, and S: Systemically.

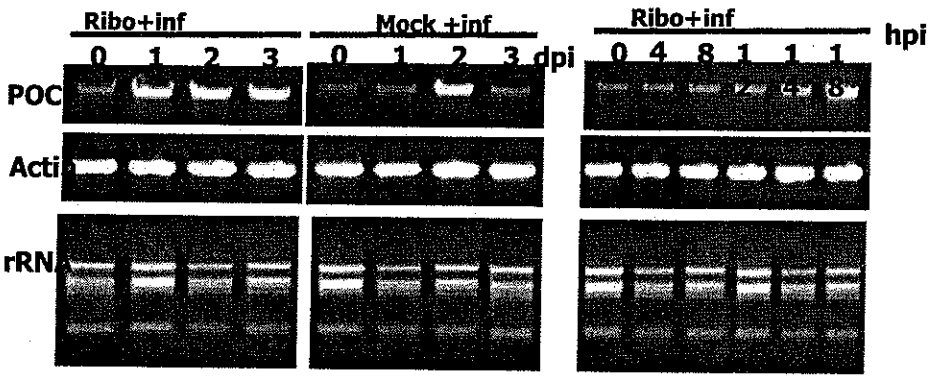

Figure 3. Expression pattern of POC1 gene in riboflavin or mock treated rice plants inoculated with *R. solani* at 5 dpt using RT-PCR.

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