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Induction of Resistance to *Xanthomonas oryzae* pv. *oryzae* in Rice by Benzothiadiazole (BTH)

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Systemic acquired resistance induced by benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) in rice against bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* was studied. Rice plants (IR 50) pre-treated with BTH showed resistance to a challenge infection with *Xanthomonas oryzae* pv. *oryzae.* About 50% reduction in disease intensity was observed in plants treated with BTH at 100 µg a.i./ml. Immunoblot analysis using barley chitinase antiserum revealed the induction of a 35 kDa chitinase in rice in response to treatment with BTH. The results indicate that the BLB resistance can be induced even in genetically susceptible cultivar through application of BTH.

Keywords: Bacterial leaf blight, BTH, chitinase, induced resistance, *Oryza sativa,* pathogenesis-related proteins.

Bacterial leaf blight (BLB) of rice (*Oryza sativa* L.) caused by *Xanthomonas oryzae* pv. *oryzae* is one of the important destructive diseases affecting rice production worldwide (Adhikari et al., 1995). Effective chemical control measures against the disease are lacking and breeding for disease resistance has become the most important approach to manage the disease Lin et al. (1995). Disease resistant cultivars with one or two resistance genes are unable to sustain in the field because of high pathogenic variability. Development of rice cultivars with durable resistance is ideal but success in this regard is limited. Under these circumstances, low molecular weight synthetic signal molecules can be exploited to induce systemic resistance in rice against *Xanthomonas oryzae* pv. *oryzae.*

Abiotic inducers like benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA) have been shown to induce systemic acquired resistance (SAR) in a variety of plants against a wide range of pathogens without possessing direct antimicrobial activity *in vitro* or *in planta* (Kessmann et al., 1994; Ruess et al., 1995; Gorlach et al., 1996; Lawton et al., 1996; Beber et al., 2000; Ramesh Sundar et al., 2001; Sauerborn et al., 2002). The broad-spectrum activity of BTH, conferring protection against bacterial, fungal and viral diseases strongly suggests an indirect mode of action via activation of plant defense mechanisms. In SAR, number of defense pathways are stimulated and diverse defense products are synthesized which

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includes lignin, pathogenesis-related proteins, phytoalexins, thionins and defensins (Kessmann et al., 1994; Broekaert, 1995; Epple et al., 1997; Ramesh Sundar et al., 2001). Schweizer et al. (1999) reported that rice seedlings treated with BTH acquired resistance to subsequent attack by the rice blast fungus *Magnaporthe grisea.* It has been demonstrated recently that sugarcane plants treated with BTH were strongly protected against a challenge inoculation with *Colletotrichum falcatum,* the red rot pathogen (Ramesh Sundar et al., 2001). In the present study, we explored the possibility of inducing systemic resistance in rice against *Xanthomonas oryzae* pv. *oryzae* (Xoo) by application of BTH (CGA 245704; acibenzolar-S-methyl). The induction of PR-proteins in BTH-treated rice plants was also examined.

Materials and Methods

Plant material and BTH treatment

Rice (*Oryza sativa* L.) seeds of the susceptible cultivar IR-50 were obtained from the Paddy Breeding Station, Coimbatore. Twenty-five rice seeds (cv. IR 50) were soaked overnight in water and germinated on wet filter paper in dark. The sprouted seeds were placed over wire meshes. Five hundred ml of Hoagland solution (Hoagland and Arnon, 1938) was taken in a 500 ml beaker and the wire mesh was placed over the beaker so that Hoagland solution touches the wire mesh. The sprouted seeds were allowed to grow for ten days on the Hoagland solution.

Different concentrations (1,10 and 100 µg a.i ml^{-1}) of BTH were added into the Hoagland solution in a 500 ml beaker. The control plants were treated with water. Two days after the addition of BTH, leaves of the rice plants were clip-inoculated with the bacterial (Xoo) suspension containing 109 cfu/ml. The bacterial blight disease incidence in the BTH-treated and the bacteria-inoculated leaves as well as untreated control plants were assessed after 7 days of inoculation with Xoo using 0–9 scale (IRRI, 1980). All data are the means of 25 leaves per treatment. All experiments were performed twice with similar results; the data presented are from one experiment only.

Induction of pathogenesis-related (PR) proteins

Induction of PR proteins in two days and one day BTH-treated (100 µg a.i ml^{-1}) or pathogen-inoculated rice plants were assessed. Proteins were extracted from the leaf samples (1 g) in 2 ml of 0.1 M potassium phosphate buffer (pH 6.5) using a pre-chilled pestle and mortar at 4 °C. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4° C. Protein content of the supernatant was determined according to the method described by Bradford (1976) using bovine serum albumin as a standard.

Proteins (100 µg) in aliquots were subjected to 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using Hoefer Electrophoresis Unit. The electrophoresis was performed at 40 V till the dye reached the separating gel and then increased to 100 V and continued till the blue dye reached the bottom

of the gel. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R 250 (Bio-Rad) for over night. A protein marker with known molecular weight (Sigma. St. Louis, USA) was co-electrophoressed to estimate the molecular weights of the proteins.

Western blotting

The proteins (100 µg) from BTH-treated or pathogen-inoculated rice plants were separated by SDS-PAGE according to Laemmli's method (Laemmli, 1970) and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) using semidry transblot apparatus (Bio-Rad). After electroblotting, the membrane was blocked for 3 h by constant shaking at room temperature in Tris-buffered saline (TBS) (10 mm Tris-HCl, 150 mM NaCl, pH 8.0) containing 0.05 per cent Tween-20 and 2.5 per cent w/v gelatin. The blot was then incubated for 3 h at room temperature with gentle shaking in antiserum containing antibodies raised against barley chitinase (a gift from Prof. S. Muthukrishnan, Kansas State University, USA) diluted to 1:1500 in TBS containing 0.05% Tween 20 (v/v) (TBST). Unbound antibody was removed by four washes with TBST (30 ml) and the blot was incubated for 3 h at room temperature in antirabbit immunoglobulin conjugated with horse-raddish peroxidase (1:1500 dilution). Unbound secondary antibody was removed by four washes with TBST (30 ml) followed by two washes with TBS. The protein bands were visualized using 4-chloro-1-napthol (Bio-Rad). Molecular weight of the proteins was determined using prestained kaleidoscope protein markers (Bio-Rad, USA).

Results and Discussion

It is well known that plants are endowed with various defense mechanisms against pathogens. However, in the susceptible plants the defense mechanism are not induced or suppressed. For the induction of defense mechanisms, signals are needed. The defense mechanisms can be triggered even in the susceptible cultivars by manipulating the signal transduction system (Vidhyasekaran, 1997; Lucas, 1999). One of the benzothiadiazole compounds (BTHs), acibenzolar-S-methyl (CGA 245704) was reported to induce resistance in wheat (Ruess et al., 1996), *Arabidopsis* (Lawton et al., 1996), bean (Siegrist et al., 1997), cucumber (Benhamou and Belanger, 1998; Ishii et al., 1999), rice (Schweizer et al., 1999), sugarcane (Ramesh Sundar et al., 2001), sunflower (Sauerborn et al., 2002) against diverse plant pathogens. In the present study, it was observed that the treatment with BTH induced resistance in rice plants to subsequent challenge with *Xanthomonas oryzae* pv. *oryzae.* All the test concentrations of BTH viz. 1, 10 and 100 mg a.i/ml were effective in inducing resistance to bacterial leaf blight pathogen in terms of reducing the lesion length. However, maximum resistance was observed in plants treated with 100 mg a.i/ml of BTH *(Table 1).* About 50% reduction in disease intensity was observed in 100 mg a.i/ml BTH-treated rice plants. The induction of SAR in response to inducers is correlated with the systemic accumulation of extracellular peroxidase, ß-1,3-glucanase and chitinase (Boller and Metraux, 1988). In the present study, SDS-PAGE analysis

Table 1

Induction of systemic resistance in rice by BTH against *Xanthomonas oryzae* pv. *oryzae*

BTH concentration (µg a.i. ml^{-1})	Disease intensity[a] (in grade values)	Per cent reduction in disease intensity
	72 h	
0	7.4	–
1	7.1	4.05
10	5.4	27.02
100	3.7	50.00

Data followed by the same letter in a column are not significantly (P = 0.05) different from each other according to Duncan's Multiple Range Test (DMRT

[a]Disease intensity was assessed 72 h after challenge inoculation with *Xanthomonas oryzae* pv. *oryzae*

revealed the induction of a protein with a molecular weight of 35 kDa in rice leaves in response to treatment with BTH. Similar induction was not observed, when rice plants were inoculated with the Xoo. However, when BTH-treated rice plants were inoculated with Xoo, the 35 kDa protein was rapidly induced. Another protein with a molecular weight of 40 kDa also induced in rice leaves upon treatment with BTH or inoculation with Xoo. This 40 kDa protein could not be detected in control plants. In control leaves the 35 kDa protein was expressed constitutively at very low level *(Fig. 1).*

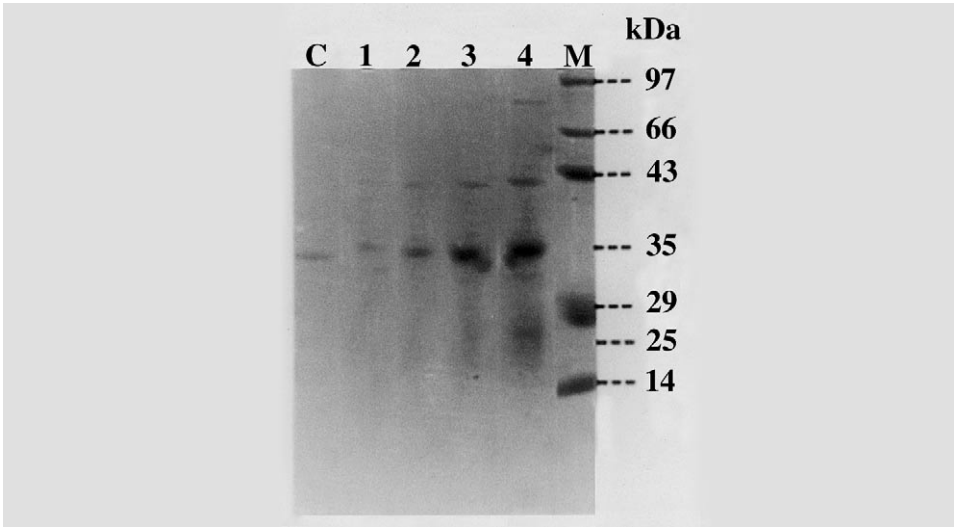

Fig. 1. Coomassie blue stained SDS-PAGE gel showing induction of a 35 kDa chitinase in rice in response to BTH treatment and *Xanthomonas oryzae* pv. *oryzae* inoculation.
C – Control; 1 – Xoo inoculated; 2 – One day after inoculation in 100 µg a.i ml^{-1} BTH-treated plants; 3 – Two days after inoculation in 100 µg a.i ml^{-1} BTH-treated plants; 4 – Two days after Xoo inoculation in 100 µg a.i ml^{-1} BTH-treated plants

Western-blot analysis of BTH treated rice leaves revealed the induction of a 35 kDa cross-reacting protein with barley chitinase antiserum *(Fig. 2).* The 35 kDa chitinase was not found to be induced in rice leaves upon inoculation with the Xoo. However, the inoculation with Xoo rapidly induced the 35 kDa chitinase in plants treated with BTH. It can be concluded that the 35 kDa protein induced in BTH-treated plants may be of chitinase. Since BTH does not exhibit significant direct activity against phytopathogenic fungi and bacteria (Kessmann et al., 1995), reduction in disease intensity might be due to the induction of SAR. The present studies have opened a new avenue for protecting rice plants against bacterial leaf blight by BTH.

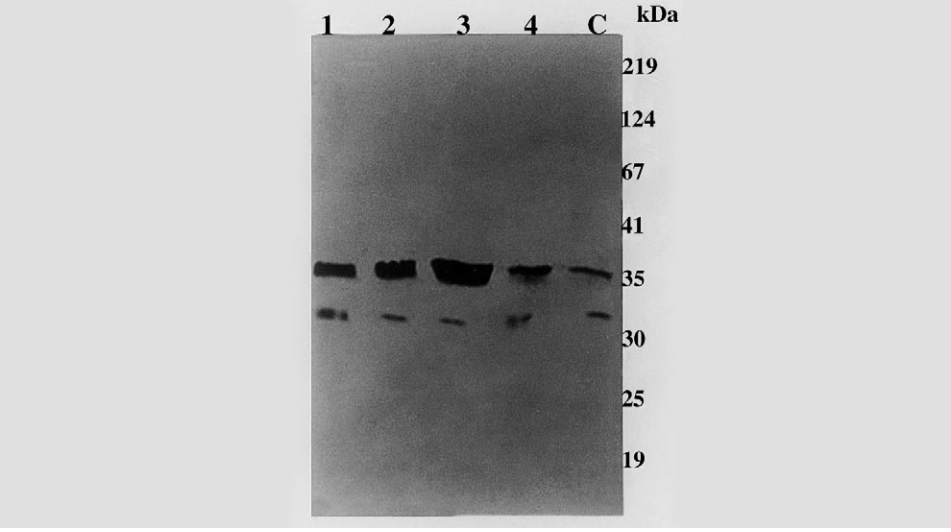

Fig. 2. Induction of a 35 kDa chitinase in rice in response to BTH treatment and *Xanthomonas oryzae* pv. *oryzae* inoculation.
1 – BTH-treated; 2 – One day after inoculation in 100 µg a.i ml^{-1} BTH-treated plants;
3 – Two days after Xoo inoculation in 100 µg a.i ml^{-1} BTH-treated plants;
4 – Xoo inoculated; C – Control

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