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Induction of Resistance in Rice against *Xanthomonas oryzae* pv. *oryzae* by Foliar Application of Cow Dung Water Extract

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Pretreatment of rice leaves with 20 per cent cow dung water extract (CDWE) was found to reduce bacterial blight disease of rice to about 50 per cent. The suppression of the disease by the extract was not due to direct inhibition of the pathogen. Pretreatment of rice leaves with cow dung water extract as foliar spray induced the accumulation of phenolics and enhanced the activities of peroxidase, chitinase and ß-1,3-glucanase. The induced defense mechanisms might have played a role in reducing the disease.

Keywords: Cow dung water extract, bacterial leaf blight, chitinase, glucanase, peroxidase, phenolics.

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Uyeda and Ishiyama) Dye *(Xoo)* is the most important bacterial disease of rice and is a serious constraint for rice production in irrigated and rainfed lowland ecosystems throughout Asia, Northern Australia, Africa, the Southern part of the United States and Latin America (Mew, 1987). Losses in yield from different countries have been reported to be about 30 per cent in Japan (Suwa, 1962), less than 30 per cent in Indonesia and Phillipines (Rao and Kauffman, 1977), and 4.9 to 9.0 per cent in China (Teng, 1986).

Research on management of BLB is mainly focussed on host plant resistance, chemical control and biological control. Seed treatment with mercuric compounds (Tagami and Mizukami, 1962; Srivastava and Rao, 1964), antibiotics (Devadath and Padmanabhan, 1970) and by hot water (Sinha and Nene, 1967) have been reported to reduce the disease. In addition, foliar application of rice plants with copper fungicides (Jain et al., 1965), antibiotics (Mary and Mathew, 1983), phenazine (Devadath and Premalatha Dath, 1970), ammonium sulphate (Hoa et al., 1984), root dip of seedlings in copper fungicides (Schure, 1953) and antibiotics and seed treatment combined with foliar application of ferrous sulphate and potassium nitrate (Manmeet and Thind, 2001) have also been attempted with varying degrees of success. But the pathogen population dynamics is highly variable in its sensitivity to antibiotics. The existence and development of drug resistant strains also pose serious problem in formulating efficient management strategies.

In this era of environmental awareness, use of botanicals (Bora and Bhattacharyya, 2000; Zeller and Laux, 2001), biocontrol agents (Vidhyasekaran et al., 2001) and development of transgenic plants expressing antibacterial proteins (Norelli et al., 1994; Ko et al., 2002) for the management of plant bacterial diseases is well recognized. Several bacterial

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diseases are reported to be managed by the application of plant extracts. Management of black rot of cabbage (Bora and Bhattacharyya, 2000) and fire blight of *Cotoneaster salicifolius* (Zeller and Laux, 2001) using extracts of *Terminalia chebula* and *Hedera helix* respectively has been reported. In the present study, we explored the possibility of utilizing cow dung water extract (CDWE) for the management of BLB. Foliar application of CDWE induced defense mechanisms in rice and controlled BLB.

Materials and Methods

Plants

Seeds of rice cultivar Taichung Native-1 (TN-1) were obtained form the Paddy Breeding Station, Tamil Nadu Agricultural University (TNAU), Coimbatore. Plants were raised in pots filled with rice field soil under greenhouse conditions. The soil was applied with calculated amount of fertilizers to supply 100:50:50 kg of NPK per hectare and the plants were watered regularly.

Pathogen

X. oryzae pv. *oryzae* was isolated from leaf blight affected rice leaves collected from the Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. The inoculum of the pathogen was prepared by mixing 48 h old bacterial culture grown on Potato Sucrose Agar (PSA) medium with sterile distilled water to obtain an optical density of 0.1 at 620 nm which corresponds to 10^9 cells/ml (Király, 1974).

Preparation of cow dung water extract (CDWE)

Fresh cow dung was collected from the Dairy farm, Tamil Nadu Agricultural University, Coimbatore and 20 per cent extract was prepared with distilled water. Then the slurry was kept overnight at room temperature (28±2 °C). The supernatant was taken the next day and used for spray treatment.

In vitro testing of cow dung water extract against X. oryzae pv. oryzae

In vitro bioassay of CDWE against *Xoo* was carried out in Wakimoto's semi synthetic Potato Sucrose Agar (PSA) (Wakimoto, 1960) medium by filter paper disc diffusion method (Vincent and Vincent, 1944). Bioassays were done with raw cow dung water extract, boiled (50 °C for 10 min) extract, autoclaved extract (121 °C for 20 min) and filter sterilized (using 0.2 m filter) extract.

Pre-inoculation application of CDWE on rice plants

Twenty per cent CDWE was sprayed on 45-day-old rice plants. One day after pretreatment, plants were inoculated with cell suspension of Xoo (containing 10^9 cfu/ml) by leaf clipping method (Kauffman et al., 1973), under greenhouse conditions. Pre-inoculation sprays of streptomycin sulphate (0.06%) + copper oxychloride (0.25%) was used for com-

parison. Plants sprayed with sterile distilled water served as control. Five replications were maintained for each treatment. The disease intensity was recorded 15 days after inoculation according to the Standard Evaluation System for rice (IRRI, 1980).

Studies on induction of systemic resistance in rice plants after spray application of CDWE

Forty five-day-old rice plants were sprayed with 20% CDWE. Plants treated with sterile distilled water served as control. All these treatments were followed by challenge inoculation with the pathogen after 24 h. From the day of inoculation of the pathogen, leaf samples were collected at different time intervals and various analyses were made. Another set of samples were collected from uninoculated plants but treated with CDWE/sterile water alone.

Estimation of total phenols

Leaf samples (200 mg) were extracted in 7.5 ml of 80% ethanol. The ethanolic extract was heated over a boiling water bath for 10 min. After cooling, the extract was centrifuged at 10,000 g for 10 min. The volume of the supernatant was made up to 7.5 ml with 80% ethanol (Lee and Tourneau, 1958) and used for estimation of total phenols.

Total phenolic content was estimated following the procedure of Bray and Thorpe (1954). To 1 ml of the ethanolic extract, 5 ml of distilled water and 250 µl of 1N Folin-Ciocalteau reagent were added. After incubating the mixture at 25 °C for 3 min, 1 ml of saturated solution of sodium carbonate was added and the mixture was further incubated for 15 min. The reaction led to the development of blue colour, the intensity of which was read at 725 nm using Hitachi double beam spectrophotometer. A blank containing all the reagents minus the extract was used to adjust the absorbance of the spectrophotometer to zero. The phenolic content was expressed as mg/g tissue in catechol equivalents.

Assay of peroxidase

Assay of peroxidase activity was carried out as per the procedure described by Hammerschmidt et al. (1982). Leaves (1 g) were ground in 5 ml of 0.01 M sodium phosphate buffer (pH 6.0) in chilled pestle and mortar. The homogenate was taken and centrifuged at 13,000 g at 4 °C for 20 min and the supernatant was taken as enzyme extract. The reaction mixture consisted of 0.25% (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide (Ridge and Osborne, 1970). Enzyme extract (0.1 ml) was added to initiate the reaction, which was followed colorimetrically at 470 nm using Hitachi double beam spectrophotometer. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. The boiled enzyme extract served as control. Enzyme activity was expressed as the increase in absorbance at 470 nm/min/g fresh tissue.

Assay of chitinase

The colorimetric assay of chitinase was carried out according to the procedure described by Boller and Mauch (1988).

Rice leaves (1 g) were homogenized in 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 4 °C for 10 min at 10,000 g. For the colorimetric assay, 10 µl 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml Eppendorf tube. After 2 h at 37 °C, the reaction was stopped by centrifugation at 10,000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl of 3% (w/v) desalted snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl of 1 M sodium borate buffer, pH 9.8. The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice-water bath. After addition of 2 ml p-dimethylaminobenzaldehyde (DMAB) reagent, the mixture was incubated for 20 min at 37 °C. Immediately thereafter, absorbance at 585 nm was measured using a Beckman DU-64 spectrophotometer. N-acetylglucosamine (GlcNAc) was used as a standard. Chitinase activity was expressed in nmol/min/g of tissue.

Assay of ß-1,3-glucanase

Rice leaves (100 mg) were extracted with 1 ml of 0.05 M sodium acetate (pH 5.0) buffer by grinding at 4 °C in a pestle and mortar. After centrifugation at 10,000 g at 4 °C for 15 min, the supernatant was used as enzyme source.

Total ß-1,3-glucanase activity was colorimetrically assayed by the laminarin-dinitrosalicylate method (Pan et al., 1991). Crude enzyme extract 62.5 µl was added to 62.5 µl of 4% laminarin (Sigma) and then incubated at 40 °C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of water, vortexed and its absorbance at 500 nm was recorded. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. Enzyme activity was expressed as µg of glucose equivalents/min/g fresh weight.

Results

Pretreatment of rice leaves with 20% CDWE before challenge inoculation with *Xoo* significantly reduced the disease when compared to the control *(Table 1).* The effect of CDWE was found to be on par with foliar application of streptomycin sulphate (0.06%) + copper oxychloride (0.25%).

None of the treatments *viz.,* raw cow dung extract, boiled extract, autoclaved extract and filter sterilized extract could inhibit *Xoo in vitro.* This showed that cow dung extract has no direct inhibitory effect on the pathogen.

Total phenolic content in rice leaves was found to increase due to inoculation with *Xoo* or treatment with CDWE. The quantity of total phenols in plants sprayed with CDWE followed by inoculation with the pathogen was found to be significantly higher than all the

Table 1

Effect of pre-inoculation foliar spray of cow dung water extract (20%) on bacterial blight development

Treatment	Per cent disease intensity	Mean grade	Per cent reduction over control
Cow dung water extract (20%)	15.11[b] (22.85)	1.36[b]	49.26
Streptomycin sulphate (0.06%) + copper oxychloride (0.25%)	12.44[b] (20.61)	1.12[b]	58.23
Control	29.78[a] (33.04)	2.60[a]	

Leaves of 45-day-old rice plants were sprayed with 20% cow dung water extract and 24 h after pretreatment, leaves were inoculated with cell suspension of *Xoo* (10^9 cfu/ml) by clipping method. The disease intensity was recorded 15 days after inoculation using a 0–9 scale.

Values are means of five replications.

Values in parentheses are arcsine transformed.

In a column, means followed by common letter do not differ significantly (P=0.05) by DMRT.

other treatments throughout the sampling period. When compared to pathogen inoculated control plants, there was about two-fold increase in total phenols in plants sprayed with CDWE followed by inoculation with the pathogen after 24 h *(Fig. 1A).*

Peroxidase activity increased in rice leaves due to inoculation with *Xoo* and reached the maximum after 48 h of inoculation with the pathogen. In plants treated with CDWE followed by challenge inoculation with the pathogen, peroxidase activity was found to be significantly higher during 24, 48, 72 and 96 h of post-inoculation. At 120 h, it was found to be on par with that of plants treated with CDWE alone. After 48 h of pathogen inoculation, plants pretreated with CDWE showed about 1.3 fold increase in peroxidase activity when compared to plants inoculated with pathogen alone *(Fig. 1B).*

Chitinase activity in rice leaves was found to increase due to inoculation with *Xoo* or treatment with cow dung extract throughout the sampling period. At 24, 48 and 72 h after pathogen inoculation, there was no significant increase in chitinase activity in plants sprayed with CDWE, when compared to plants inoculated with pathogen alone. But at 96 and 120 h after pathogen inoculation, there was a significant increase in chitinase activity in plants treated with CDWE than that with pathogen alone *(Fig. 2A).*

Significant increase in the activity of ß-1,3-glucanase was also observed in rice leaves in response to treatment with CDWE or inoculation with Xoo *(Fig. 2B).* In plants treated with CDWE followed by inoculation with the pathogen, maximum ß-1,3-glucanase activity was observed 96 h after inoculation and it remained constant at 120 h also. At 24, 48 and 96 h after inoculation, the enzyme activity was found to be significantly higher in plants treated with CDWE and challenge inoculated with the pathogen after 24 h.

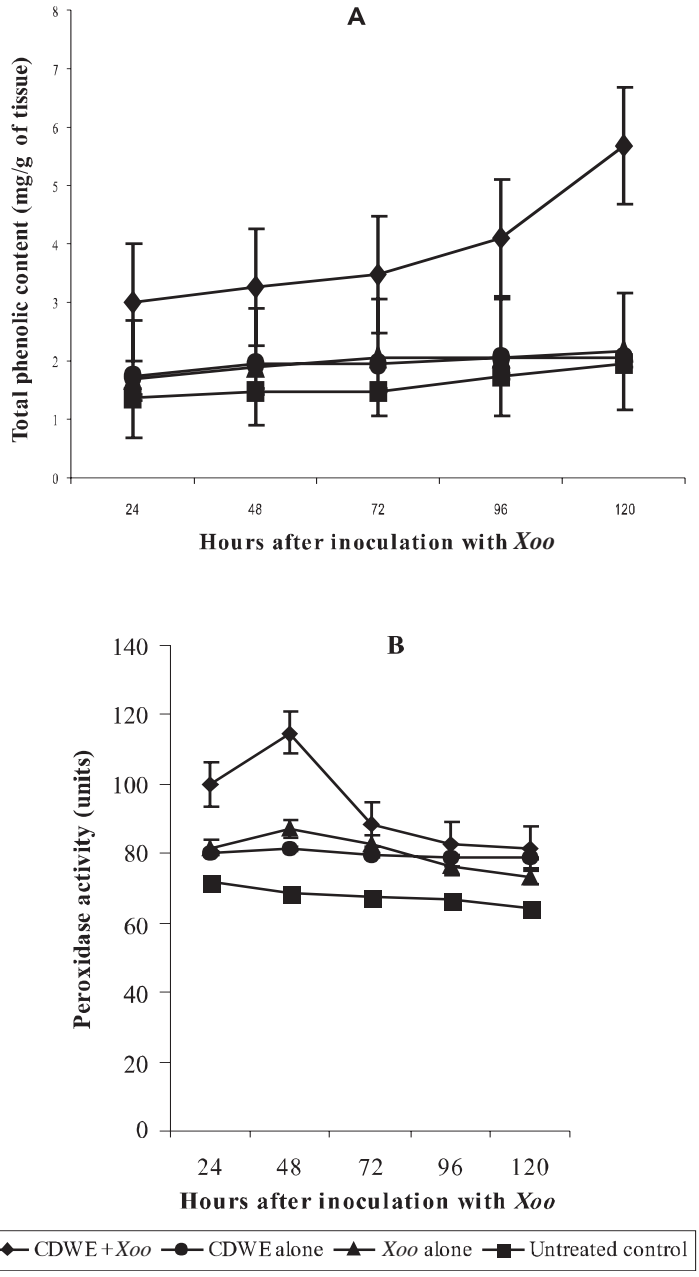

Fig. 1. Changes in phenolics (A) and activity of peroxidase (B) in rice plants by foliar application of CDWE and challenge inoculation of *Xoo*

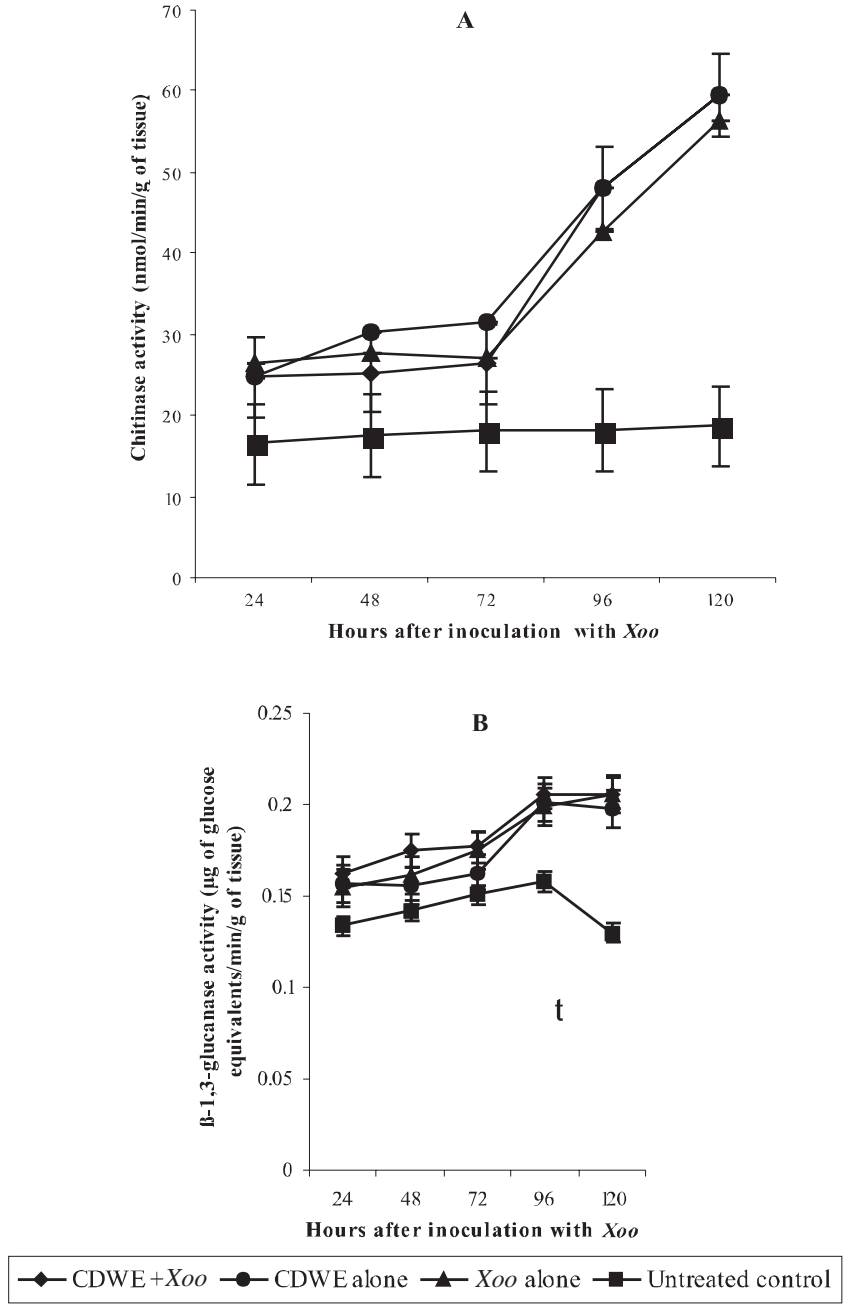

Fig. 2. Changes in activities of chitinase (A) and ß-1,3-glucanase (B) in rice plants by foliar application of CDWE and challenge inoculation of *Xoo*

Discussion

It is well known that animal wastes and compost induce resistance in plants against fungal and bacterial pathogens (Szczech and Brzeski, 1994; Zhang et al., 1998; Pharand et al., 2002). Zhang et al. (1998) reported that a biocontrol agent-fortified compost mix induced SAR in cucumber against anthracnose caused by *C. orbiculare* and in Arabidopsis against bacterial speck caused by *Pseudomonas syringae* pv. *maculicola.* Dipping cabbage roots in a mixture of clay and vermicompost decreased infection by *Plasmodiophora brassicae* (Szczech et al., 1993). Potato plants grown in soil amended with vermicompost were less susceptible to *P. infestans* and the post harvest diseases of tubers decreased compared with the untreated controls (Kostecka et al., 1996). Weltzien (1989) demonstrated the control of powdery mildew of barley following treatment with water extract from vermicompost. Pharand et al. (2002) observed a considerable decrease in crown and root rot caused by *F. oxysporum* f. sp. *lycopersici* in tomato plants grown in medium containing peat moss amended with compost. In our present study, pre-inoculation spray of rice leaves with 20% CDWE significantly decreased the BLB incidence. The absence of inhibition zone in *in vitro* bioassay revealed that no bactericidal principle might be present in cow dung water extract. This finding indicates that pre-inoculation treatment with CDWE induces resistance in rice against subsequent infection by *Xoo.*

The high population of biocontrol agents, mainly the bacterial strains, present in compost amended potting mixtures have been suggested to be the reason for induction of resistance (Liu et al., 1995; Wei et al., 1996). Various bacterial biocontrol agents are reported to induce systemic resistance against bacterial diseases of crop plants. These include *Pseudomonas fluorescens* S97 against halo blight of bean (Alström, 1991), *Pseudomonas fluorescens* W163 and WP95 against bacterial wilt of potatoes (Kempe and Sequeira, 1983), *P. putida* WCS358 against bacterial speck of tomato (van Wees et al., 1997), *P. putida* 89B-27 and *Serratia marcescens* 90-166 against bacterial wilt of cucumber (Kloepper et al., 1993) and *P. putida* 89B-27, *S. marcescens* 90-166 (Liu et al., 1995), *B. pumilus* INR7, *Curtobacterium flaccumfaciens* ME1 and *B. subtilis* GB03 (Raupach and Kloepper, 2000) against bacterial angular leaf spot of cucumber and *S. marcescens* 90–166 against wild fire of tobacco (Press et al., 1997). Vidhyasekaran et al. (2001) reported the induction of systemic resistance by *P. fluorescens* Pf1 against *X. oryzae* pv. *oryzae* in rice leaves. Hence, the presence of such biocontrol agents or some unidentified chemical factors in the CDWE might have played a role in inducing resistance in rice against *Xoo.*

In the present study, it was also observed that foliar application of CDWE increased the phenolic content and peroxidase activity in rice leaves. The presence of phenolic compounds in plants or their synthesis in response to infection has been mostly associated with disease resistance (Farkas and Király, 1962). Horino and Kaku (1989) reported that leaves of resistant rice cultivar had greater amounts of phenolic compounds toxic to *Xoo* compared to leaves of susceptible rice cultivar. Evidences strongly suggest that esterification of phenols to cell wall materials is a common theme in the expression of

resistance (Fry, 1987). Cross linking of such phenylpropanoid esters leads to the formation of lignin like polymers (Fry, 1986). Lignin and other phenolic polymers serve as physical barriers and as such are thought to prevent fungal penetration into host cells (Tiburzy and Reisener, 1990; Hammerschmidt et al., 1982; Ride, 1983; Kerby and Somerville, 1989; Dalisay and Küc, 1995; Manandhar et al., 1999; Chen et al., 2000). Reimers and Leach (1991) have shown that the race-specific resistance of rice carrying the *Xa-10* gene to *X. oryzae* was correlated with the deposition of lignin at the site of infection. The accumulation of higher amounts of total phenols in rice plants following treatment with CDWE might have offered resistance against *Xoo.*

Peroxidase is known to catalyse the oxidation of many mono-, diphenols and aromatic amines to toxic quinones in the presence of hydrogen peroxide (Bonner, 1950). Peroxidases have been implicated in the last enzymatic step of lignin biosynthesis, that is, the oxidation of hydroxy cinnamyl alcohols into free radical intermediates, which subsequently are coupled into the lignin polymer. Peroxidase is also implicated in suberization (Mohan and Kolattukudy, 1990). Increased activity of peroxidase is associated with increased lignin biosynthesis in many host-pathogen interactions (Hammerschmidt et al., 1982; Küc, 1990; Kandan et al., 2002).

Lignin or the lignification process can act in plant defense against infection by establishing mechanical barriers to pathogen growth, chemically modifying cell walls to be more reisistant to cell-wall degrading enzymes, increasing the resistance of walls to the diffusion of toxins from the pathogen, production of toxic precursors and free radicals, and the actual lignification and entrapment of the pathogen (Ride, 1978). The role of peroxidases in conferring resistance to rice against *Xoo* has been reported previously by Rajappan and Vidhyasekaran (1994). Vidhyasekaran et al. (2001) reported enhanced levels of peroxidase in rice plants treated with *P. fluorescens* Pf1 and challenge inoculated with *Xoo.* Elfstrand et al. (2002) have recently demonstrated that tobacco plants transformed with Norway spruce *spi2* gene that encodes a defense related cationic peroxidase displayed high peroxidase activity and increased resistance to *E. carotovora.* The increased peroxidase activity in the CDWE treated rice leaves might have involved in the lignin biosynthesis, which in turn might have contributed for BLB resistance.

Several studies have shown that pathogenesis-related (PR) proteins are induced in plants in response to infection by pathogens, treatment with elicitors, biocontrol agents, ethylene, and wounding (Datta and Muthukrishnan, 1999). Based on the amino acid sequences, serological relationship and/or enzymatic or biological activity, these PR-proteins have been classified into 14 families (van Loon and van Strien, 1999). PR-proteins like chitinase (PR-3) and ß-1,3-glucanase (PR-2) have the potential to hydrolyze chitin and ß-1,3-glucan respectively, the major components of the cell wall of many fungi. Hence these PR-proteins are thought to be implicated in the defense systems of plants (Boller, 1988; Muthukrishnan et al., 2001). Robert et al. (2002) reported the expression of *Vvchit1a* (class I chitinase) and *VvchitIII* (class III chitinase) genes in grape leaves when inoculated with a non-pathogen, *P. syringae* pv. *tabaci* (Schneider and Ullrich, 1994). So the enhanced activity of chitinase in CDWE treated rice plants may also be considered as the host defense mechanism against *Xoo.* Few of the plant chitinases have been reported to

possess lysozyme activity (Tata et al., 1983; Kombrink et al., 1988; Mauch et al., 1988) by which they hydrolyze the peptidoglycan bonds which occur in the murein of bacterial cell walls.

Foliar application of CDWE also induced the ß-1,3-glucanase activity in rice plants. Zhang et al. (1998) reported the enhanced activity of ß-1,3-glucanase in cucumber plants when they were grown in compost mix and inoculated with *C. orbiculare.* However, the role of ß-1,3-glucanases in conferring bacterial disease resistance is not fully understood. The present studies suggest that foliar application of CDWE can enhance defense-related activities in rice conferring resistance against *Xoo.*

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