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Original Research Article

Comparative Biochemical Analysis of Enzymatic Scavengers and Defence Signaling Molecules after *R. solani* Infection in Rice and Barley

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A B S T R A C T

Rhizoctonia solani is an economically important disease of many agricultural crops throughout the world. The biochemical processes underlying the expression of resistance to *R. solani* infection were investigated and compared in cultivated rice and non-host barley. All the inoculated plants were found to had increase changes in the activities of catalase, polyphenol oxidase and phenylalanine ammonia lyase activity after infection. It was found that 2-3 fold increase activity for catalase and other defence enzymes activity in *R. solani* inoculated leaf sheaths of IR42. Maximum enzymes activities were recorded in between the 48 h to 96 h after inoculation in all the host and non-hosts. The enzyme activity analysis of catalase revealed 2-3 isoform of catalase were induced in IR42 and barley var. NDB1445. In contrast upto six peroxidase isozyme isoforms were induced in rice var. IR42 upon infection by *R. solani*. After infection with *R. solani*, few new protein bands appeared ranging from 85 kDa±2, to 26±2 kDa by the hosts and non-host. The greater enzymes activity and more isoforms induction of catalase and peroxidase in cultivated rice var. IR42 may be manifestation of resistance against *R. solani*. Defence regulatory pathways operate in cultivated rice, and barley upon infection with *R. solani*.

Keywords

Antioxidant enzymes, defence mechanism, enzyme activity

Introduction

Rhizoctonia solani infects at least 200 plant species and is one of the most common soil-borne pathogen of agricultural crops (Khandaker *et al.,* 2008). Semi-saprophytic nature of *R.solani* has made it as wide range pathogen that have uncharacterized pathogenecity mechanisms. *R. solani* also causes a wide range of economically important diseases in different plant species; there is a lack of knowledge concerning genes and their function in relation to pathogenicity (Lubeck, 2004). Sheath blight pathogen survives from one crop season to another through sclerotia and mycelia in plant debris and by weed hosts in tropical environments (Kobayashi *et al.,* 2006). Sheath blight (ShB), causes significant yield loss and reduction in grain quality for rice

(*Oryza sativa* L.) in the southern U.S. and other regions of the world (Rush, 1996). *Rhizoctonia* root rot in wheat and barley, caused by the soil-borne fungal pathogen *R. solani* AG-8, was first diagnosed as a problem in direct-seeded wheat and barley in the mid-1980s. In rice, only partial resistance to rice sheath blight has been identified, as evidenced by a survey of 6000 rice cultivars from 40 countries from which no cultivar exhibiting a major gene for rice sheath blight resistance was identified (Jia *et al.,* 2007). The ShB pathogen produces several cell wall degrading enzymes (CWDEs) in plants resulted in breaking of sheath cell, organelles, and also in cell wall cracking (Zhang *et al.,* 2005). CWDEs include polygalacturonase (PG), cellulase (Cx), pectin methylgalacturonase (PMG), and polygalacturonic acid trans-eliminase (PMTE) in improved Marcus's medium of which the activity of PG, Cx, and PMG were significantly higher than PGTE and PMTE. These CWDEs play an important role in lesion formation and expansion (Chen *et al.,* 2006). Plants under stress conditions, such as pathogen attack, are induced to increase the production of reactive oxygen species (Mahmood *et al.,* 2006). The production of active oxygen species (AOS) such as superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) are one of the earliest responses of the plants to attempted infection by pathogens (Grant and Loake, 2000). Active oxygen species may act as second messengers for the activation of a variety of defence genes (Lamband Dixan, 1997). Scavengers of active oxygen species like catalase (CAT) (which catalyzes the decomposition of H_2O_2), superoxide dismutase (SOD) (which scavenges O_2) and ascorbate peroxidase (AP) (which is a scavenger of H2O2) suppress the oxidative burst (Magbanua *et al.,* 2007) and inhibit tissue necrotization. Sareena *et al.,* (2006) studied diverse defence responses in transgenic Pusa Basmati1 rice lines engineered with rice chitinase gene (*chi11*) against the *R. solani*. Enhancement of phenylalanine ammonia lyase, peroxidase, and polyphenoloxidase enzyme activities in response to the pathogen challenge under controlled conditions in transgenic rice lines was found in compared to non-transgenic. There are several studies showing that protein pattern changes that accompanied by the biological changes, which makes the organism more fit to altered environment (Hurkman *et al.,* 1988). The antioxidatve systems of plants during pathogen infection have been well documented (Aggrawal *et al.,* 2002; Diaz-Vivancos *et al.,* 2008; Ghosh *et al.,* 2017), however very few studies have investigated the relationship of these changes to host and non-host resistance. To the best of our knowledge, information about the changes in the antioxidative system and with concerned of ROS in the leaves of cultivated rice and non-host (barley) during the infection process by *R. solani* is not available till date. Therefore, the present study aimed to determine the comparative study of antioxidative, scavenger enzymes and defence signaling molecules of resistance to sheath blight.

Materials and Methods

Plant materials, experimental condition fungal isolates and inoculation

Seeds of cultivated rice (IR42) and was procured from International Rice Research Institute, Mannila, Philippines with the collaboration of National Beuro of Plant Genetic Resource, New Delhi, India, whereas barley var. NDB1445 was collected from the Department of Genetics and Plant Breeding, N.D. University of Agriculture and Technology, Kumarganj, Faizabad India. A multinucleate compatible, highly virulent strain of *R. solani*, D-14 belonging

to AG-1 IA anastomosis group was obtained from the Rice Pathology Laboratory, G.B. Pant University of Agriculture and Technology, Pantnagar, India. This isolate was grown on PDA (potato dextrose agar) media at 28 ± 1°C for 5 days and used as inoculum.

The inoculations were carried out in the humidified chamber in polyhouse of Plant Molecular Biology and Genetic Engineering, N.D. University of Agriculture and Technology, Kuamrganj, Faizabad, India during both the years 2010-11 and 2011-12. The different hosts (rice and wild rice accessions and non-host plants (35 days old) were inoculated with *R. solani* by immature sclerotia, between sheath and culm just above the water level (Jia *et al.*, 2007). At five different time intervals 24 HAI, 48 HAI, 96 HAI and 120 HAI (HAI, hours after inoculation) the plants samples were collected for the biochemical analysis and compared with the control condition.

Assay of catalase (CAT)

The enzyme extract for measuring catalase activity in different samples [Fresh control and infected leaves samples (24 HAI, 48 HAI, 72 HAI 96 HAI and 120 HAI)] were prepared by homogenizing leaves (1 g) samples in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4 ^{0}C. The homogenate was centrifuged at 15,000 x g at 4 ^{0}C for 30 min., pellets were discarded and supernatant was used as enzyme source.

The standard reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 15 mM H_2O_2. A 100 µl sample of the enzyme extract was added to 2.9 ml of the standard reaction mixture to initiate the reaction (Chance and Maehly, 1955). Catalase units were defined as mmol oxygen released min^{-1}g fresh weight^{-1}.

Assay of polyphenol oxidase (PPO)

One g of control and different treated samples were homogenized in 2 ml of 0.1 M phosphate buffer, pH 7.0 at 4 ^{0}C. The homogenate was centrifuged at 16,000 x g at 4^{0}C for 15 min and the supernatant was used as enzyme source.

The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at room temperature (28±2 ^{0}C).

The changes in absorbance at 420 nm were recorded at 30 sec. intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min^{-1} mg^{-1}protein (Hammerschmidt *et al.*, 1982).

Assay of phenylalanine ammonia lyase (PAL)

Leaves samples (1 g) of control and inoculated were homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 m*M* of 2-mercaptoethanol and 0.1 g of insoluble poly venyl pyrrolidone (PVPP). The extract was filtered through cheese cloth and the filtrate was centrifuged at 16,000 x g for 15 min.

The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of Lphenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al.*, (1984). Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min. at 30^{0}C. The amount of trans-cinnamic acid synthesized was calculated. Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} protein.

Catalase and peroxidase activity gel analysis

For the analysis of catalase activity, homogenate extract (control, 24 HAI, 48 HAI and 96 HAI) in 50 mM sodium phosphate buffer (pH 7.5) was centrifuged at 12,000 x g at 4^0C for 20 min. 250 mg of protein samples of (control and inoculated) were separated on 8 % non-denaturing polyacrylamide gels at 120 V for 12 h at 4^0C. Gels were incubated in 0.003% H_2O_2 for 10 min. and developed in a 1% (w/v) $FeCl_3$ and 1% K_3Fe (CN6) (w/v) solution for 10 min as described by Vitoria *et al.,* (2001). Peroxidase activity gel analysis of the homogenate supernatant [control and infected leaves samples (24 HAI, 48 HAI and 96 HAI)] containing 250 µg of protein (Lowery *et al.,* 1951) was subjected to polyacrylamide gel electrophoresis (8 % native PAGE) under non-denaturing condition. Electrophoresis was carried out at constant current of 100 V for 8 h at 4°C with vertical gel electrophoresis unit (Bio-Rad, USA). Gels were stained for peroxidase in 0.2 M acetate buffer (pH 4.5) containing 0.05 % benzidine and 0.03 % H_2O_2. After staining, the gels were immersed in 7 % acetic acid for 3 min. and washed with distilled water (Nadolny and Sequeira, 1080).

Protein isolation and electrophoresis of proteins

Fresh leaves of control and infected plants (24 HAI, 48 HAI and 96 HAI) were homogenized in pre-chilled pestle and mortar in extraction buffer [100 mM Tris–HCl pH 6.8, 5 mM PMSF, 4 % SDS, 30 % glycerol, 200 mM dithiothreitol (DTT)]. The homogenates were centrifuged at 10,000 x g at 4^0C for 15 min. After centrifugation, clear supernatants were collected, and protein content in each sample was determined (Lowery *et al.,* 1951). 250 µg protein of each samples were loaded in each wells and separated on 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970) using vertical electrophoresis system (Bio-Rad, USA) with molecular weight markers (Merck Pvt. Limited, India).

Experimental design and statistical analysis

All experiments of enzymatic and defence signaling assay were performed in triplicates For the enzyme analysis. The data obtained for each enzyme were analysed separately. Paired t test was performed in all the cases using GraphPad PRISM software (Motulsky, 1999). Pearson correlation coefficients and P values were used to show correlations and their significance. Differences of $P<0.05$ were considered significantly.

Results and Discussion

The moderately resistant rice var. IR42 and non-host barley var. NDB1445 plants expressed higher amounts of defence enzymes after inoculated with *R. solani* with the different time of interval.

Catalase activity

The content of catalase in infected rice var. IR42 and barly var. NDB1445 increased after *R. solani* infection at 24 HAI to 48 HAI of inoculation and was significantly higher (up to two fold) in infected plants than the susceptible line and control plants. Although catalase content in infected susceptible plants of both hosts and non-hosts increased from 24 HAI to 48 HAI, and were significantly lower than those of control plants. Furthermore, the time course of change in the content of catalase in the

infected rice and non-host barley var. NDB 1445 was similar up to some level of induction. The rice var. IR42 showed higher level of catalase induction after 24 HAI to 120 HAI of inoculation compared to barley (Fig. 1 a and 1 b).

Phenylalanine ammonia lyase (PAL) activity

PAL activity was increased after 24 HAI to 72 HAI of inoculation in cultivated rice var. IR42 at 96 HAI followed by non-host barley var. NDB1445. Compared to control condition, rice var. IR42 and barley var. NDB1445 showed 3-5 fold significant higher PAL activity (Fig. 2 a and 2 b).

Poly phenol oxidase (PPO) activity

PPO activity significantly increased in all the hosts and non-hosts after 24 HAI of inoculation and maximum increase was observed at 72 HAI of inoculation at which, 3-fold increase in PPO activity was recorded. The cultivated rice var. IR42 and non-host barley var. NDB1445 showed significant increased PPO activity. Highest fold increase (3-4 folds) found in resistant cultivated rice var. IR42 after *R. solani* inoculation. Subsequently the PPO activity declined but remained significantly at higher level than the control (Fig. 3 a and 3 b).

Enzyme activity of catalase and paeroxidase

Catalase activity among rice var. IR42 and non-hosts barley var. NDB1445 were determined in control and after infection with *R. solani*. The higher catalase activities in infected rice var. IR42 were observed followed by and non-host barley var. NDB1445 in comparision to control. Moderately resistant rice var. IR42 and non-host barley var. NDB1445 showed only two catalase isoforms (CAT-1 and CAT-2). After 48 HAI, the CAT-2 isoform was induced in NDB1445, whereas after 72 HAI the moderately resistant rice var. IR42 induced other catalase isoform (Fig. 4 a, and 4 b). Peroxidase activitiy in inoculated plants were found to be increased after *R. solani* infection in rice var. IR42 and barley var. NDB1445. Five peroxidase isoforms (PO-1 to PO-5) were induced by moderately resistant rice var. IR42 and barley var. NDB1445 after 48 HAI to 96 HAI of inoculation. The other moderately resistant rice var. IR42 and non-host barley var. NDB1445 showed increased peroxidase activity as seen by different induced isoforms of peroxidase. However, compared to control; significantly higher peroxidase activity was observed even at different inoculation periods in resistant plants (Fig. 5 a and 5 b). Induction and comparison of induced proteins after *R. solani* infection Different protein bands, ranging from 85±2 kDa to 26±2 kDa molecular weight were induced or diffused from rice var. IR42 and barley var. NDB1445 in control and after infection (Fig. 6 a and 6 b). Generally, infection with *R. solani* induced the appearance of new protein bands in inoculated plants. Protein profiling of control and inoculated plants of non-host barley var. NDB1445, cultivated rice var. IR42 showed that after infection by *R. solani*. It was obsrved that intensity and induction of new protein bands were more prominent in non-host barley var. NDB1445 (30kDa, 45±2 kDa and 85±2 kDa), whereas the cultivated rice var. IR42 induced only two protein bands of 30±2 kDa and 45±2 kDa after 48 HAI of inoculation. In contrast with the term of intensity and down-regulation of the protein the 85±2 kDa protein bands was showed very less intensity in compassion to control, whereas the two other bands (30±2 kDa and 45±2 kDa) disappeared after 48 HAI of inoculation.

Fig.1 Catalase activity (CAT) of the (a) rice var. IR42 and non-host barle var. NDB1445. Statistical differences at *P*= 0.05 have been calculated for each test in all the assays [0-fresh leaves samples, 24-24 HAI leaves sample, 48-48 HAI leaves sample, 72- 72 HAI leaves samples, 96-96 HAI leaves samples and 120-120 HAI leaves samples]

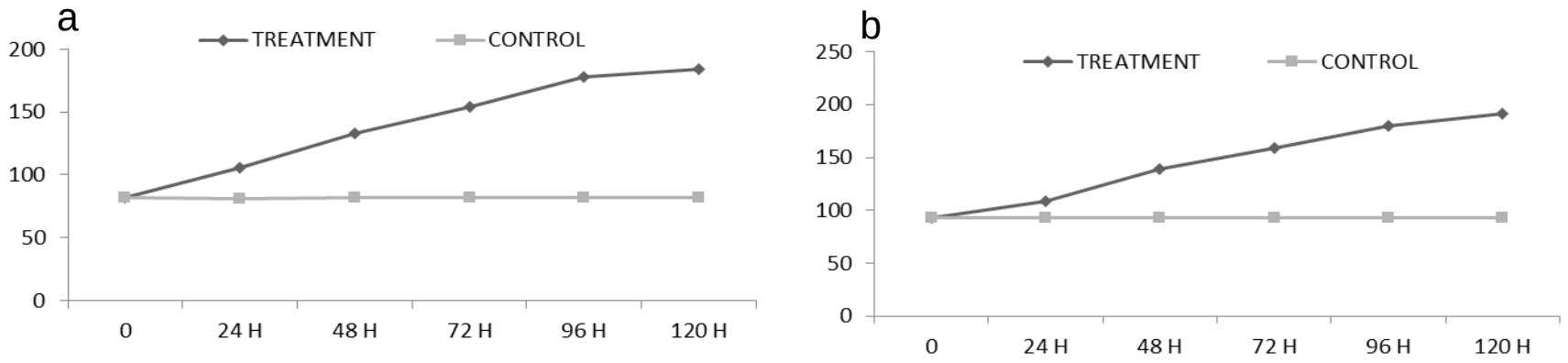


Fig.2 Phenylalanine ammonia lyase (PAL) activity of the (a) rice var. IR42 and non-host barley var. NDB1445. Statistical differences at *P*= 0.05 have been calculated for each test in all the assays [0-fresh leaves samples, 24-24 HAI leaves sample, 48-48 HAI leaves sample, 72- 72 HAI leaves samples, 96-96 HAI leaves samples and 120-120 HAI leaves samples]

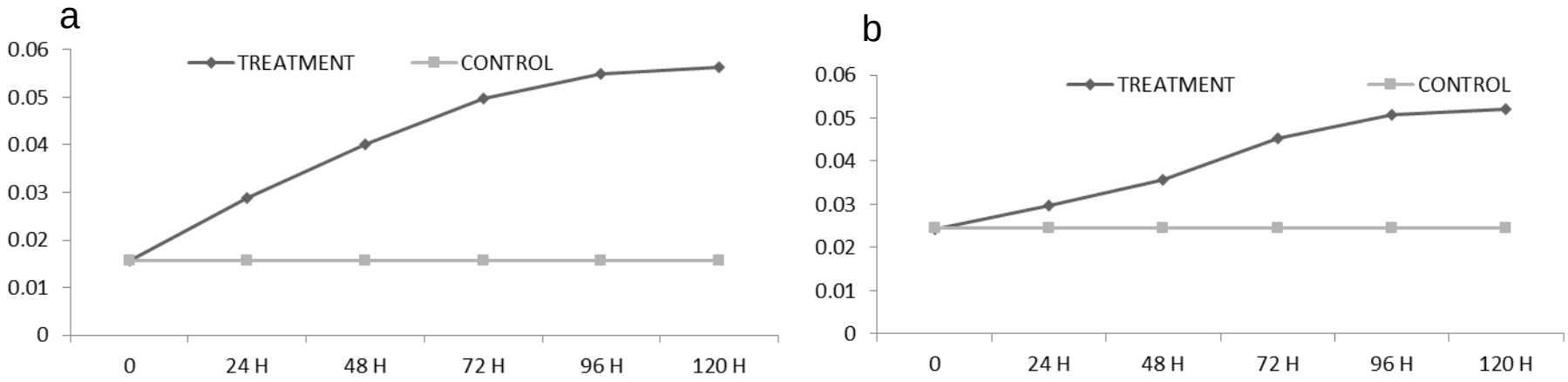


Fig.3 Polyphenol oxidase (PPO) activity of the (a) rice var. IR41 and non-host barle var. NDB1445. Statistical differences at *P*= 0.05 have been calculated for each test in all the assays [0-fresh leaves samples, 24-24 HAI leaves sample, 48-48 HAI leaves sample, 72-72 HAI leaves samples, 96-96 HAI leaves samples and 120-120 HAI leaves samples]

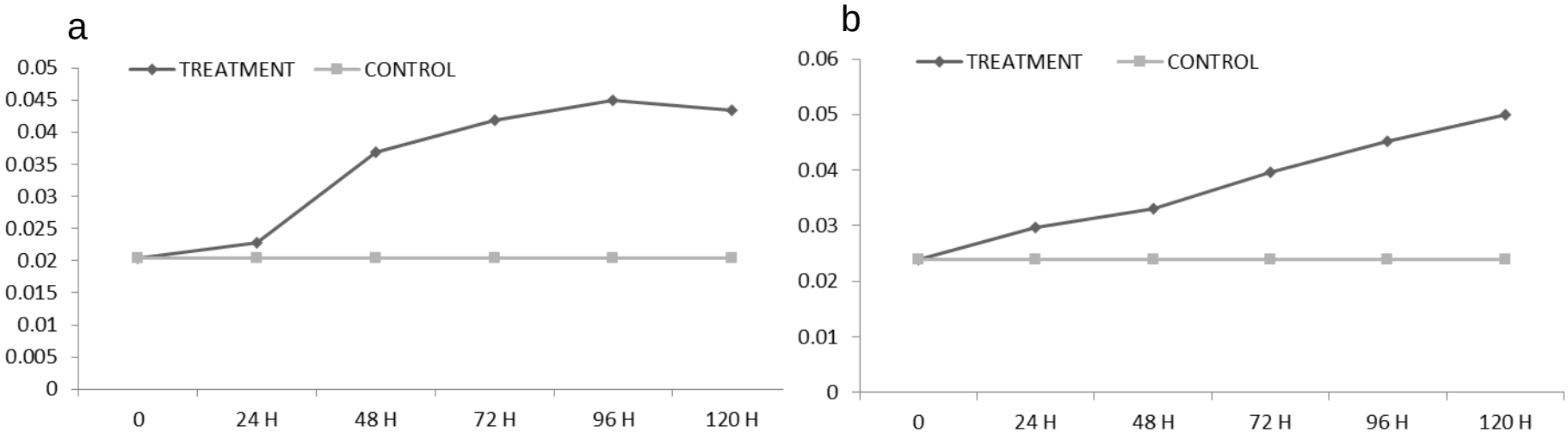


Fig. 4 Comparative native PAGE analysis of extract crude plant samples stained for catalase activity of different time infection, CAT-1, CAT-2 and CAT-3 presented different catalase isoforms **(a).** Rice var. IR42, and **(b).** Non-host barley var. NDB1445) [C-control, 24-24 HAI leaves sample, 48-48 HAI leaves sample, 72- 72 HAI leaves samples, 96-96 HAI leaves samples and 120-120 HAI leaves samples]

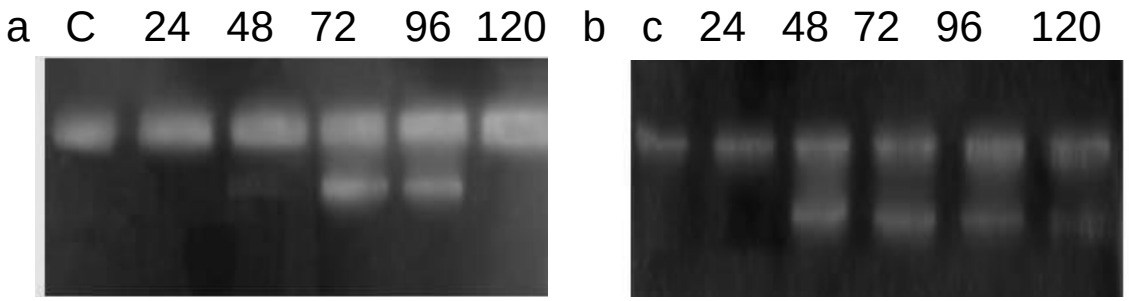


Fig.5 Comparative native PAGE analysis of extract crude plant samples stained for peroxidase activity of different time infectionPO-1, PO-2, PO-3, PO-4 and PO-63 presented different peroxidase isoforms **(a).** Rice var. IR42, and **(b).** Non-host barley var. NDB1445 [C-control, 24-24 HAI leaves sample, 48-48 HAI leaves sample, 72- 72 HAI leaves samples, 96-96 HAI leaves samples and 120-120 HAI leaves samples

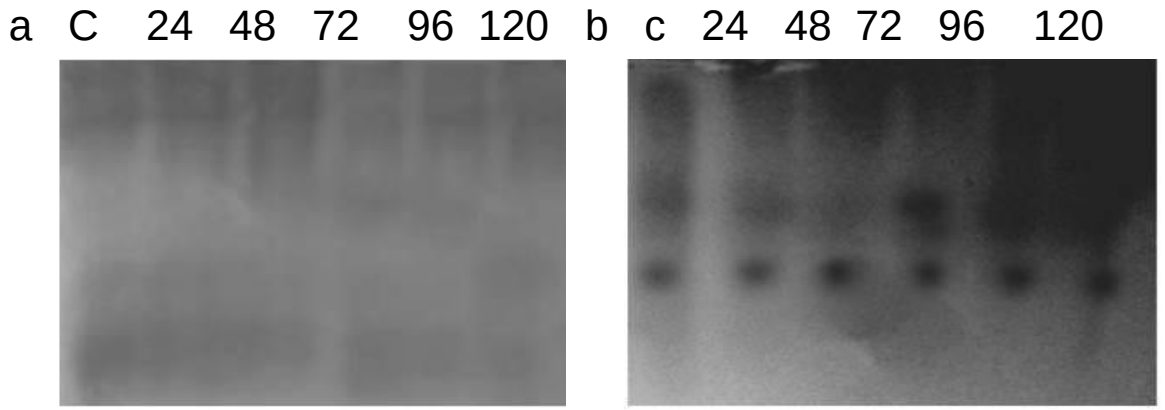
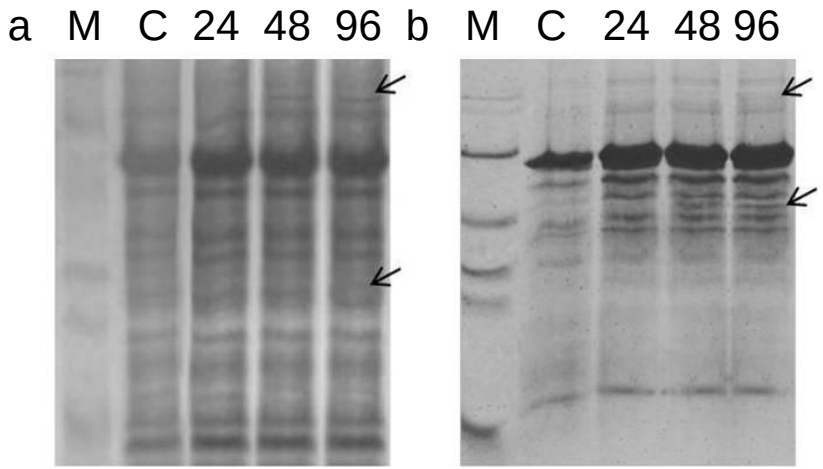


Fig.6 Comparative protein profiling of the different hosts and non-hosts (a-rice var. IR42 and (b). Non-host barley var. NDB1445) on 12 % SDS-PAGE [M denotes marker, C control, 24- 24 HAI samples, 48- 48 HAI samples, 96-96HAI samples]



Plants have equipped with the endogenous defence mechanisms that can be induced after attacking by insects and pathogens. Inducing the plant's own defence mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy against the pathogens (Ramamoorthy *et al.,* 2002). During infection by pathogens, several reactive oxygen species are produced and these active oxygen species have been shown to be associated with the hypersensitive response in plants (Grant and Loake, 2000). The results of the present study indicated that catalase activity significantly increased in rice and barley leaf sheaths after inoculation with *R. solani.* Plants have developed non-enzymatic and enzymatic mechanism to remove the excess ROS generated during the host-pathogen interaction (Scandalios, 2011). Superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase are the some common enzymes involved in the host defence against oxidative stress (Dixon *et al.,* 2010). Plants can control the ROS level through sophisticated mechanisms such as scavenging them by antioxidant defence proteins (Mahmood *et al.,* 2006).

A number of studies suggested that PPO may participate in defence reactions and confer hypersensitivity to plants resistant to diseases. Significant increases of PPO activity in all the hosts and non-hosts leaves after 24 HAI of inoculation were observed. After 72 HAI, a 3-fold increase in PPO activity was recorded. Thipyapong and Steffen (Thipyapong and Steffens, 1997) observed that PPO has a wide range of responses to multiple induction signals such as infection of bacteria or fungi, physiological stress, mechanical damage and signal molecules (methyl jasmonate, salicylic acid, ethylene and cAMP). Other defence enzymes include pathogenesis related proteins (PRs) such as β -1, 3-glucanases (PR-2 family) and chitinases (PR-3 family) which degrade the fungal cell wall and cause lysis of fungal cell. These experimental treatments can induce PPO gene expression and strengthen the stability of the corresponding mRNA (Sommer *et al.,* 1994). PPO is also involved in the oxidation of polyphenols into quinones using molecular oxygen as an electron acceptor and lignification of plant cells during microbial infection (Chittoor *et al.,* 1999).

Peroxidase and catalase isozyme analysis on native PAGE clearly demonstrated that, PO-4, PO-5 and PO-6 and CAT-2 and CAT-3 were induced upon infection by *R. solani.* Although a role for POs in defence responses was not clearly demonstrated, increases in PO activity were correlated with infection in plants (Flott *et al.,* 1989). These enzymes isoforms intensity was also increased 48 HAI of inoculation. Earlier increases in the activities of catalase, ascorbate peroxidase and superoxide dismutase in plants have been correlated with increased susceptibility to pathogens (Durner and Klessig, 1995).

It was found that intensity and induction of new protein bands were more prominent in non-host barley var. NDB1445. Upon pathogen or insect attack, many defence-related proteins are inducible by the signaling compounds salicylic acid, jasmonic acid, or ethylene, whereas other plant hormones, such as abscisic acid, can modulate expression. Pathogenesis-related (PR) and similar proteins have been found to be inducible by infection with various types of pathogens in many plant families and have been classified into 17 families. Proteins showing differential expression between treatments may have important roles in plant-stress responses (Van Loon *et*

al., 2006). The induction of new molecular proteins by rice var. IR42 and non-host barley var. NDB1445 after *R. solani* infection showed that these proteins might be the responsible for the resistance. Induction of defence proteins makes the plant resistant to pathogen invasion. There has been some progress in the understanding of natural defence reactions in rice as a response to attack of *R. solani* (Lee *et al.,* 2006; Zhao *et al.,* 2008; Ghosh *et al.,* 2017).

Culley *et al.,* (1995) reported that disease resistance response protein 206 (4E7) is a nonhost resistance protein in pea. Matsubayashi *et al.,* (2006) also reported that five proteins were included after Xoo infection in rice related to signal transduction: phytosulfokine receptor precursor, G protein, phosphatidylinositol 3-kinase-related protein kinase, CHP-rich zinc finger protein and auxin-regulated protein (U46). In the other proteomic analysis, several other works also reported that after pathogen infection protein patterns were changed in rice (Katso *et al.,* 2001; Liscum and Reed, 2002). The induced proteins might be belonging to ascorbate peroxidase, glutathione S-transferase and mitochondrial chaperonin. As this types of proteins were previously were up-regulated by Xoo infection, which may act together to fight against the ROS produced under the pathogen invasion (Shigeoka *et al.,* 2002).

Different hosts and non-host may induce a similar resistant response and activate different signal transduction pathways with different defence responses when *R. solani* pathogen was infected. As a general conclusion it seems that protein changes in rice var. IR42 and non-host barley var. NDB1445 are somehow related to increased resistance level against *R. solani*, and for this purpose, mechanisms related to different metabolic pathways need further studies.

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