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Full Length Research Paper

Adathoda vasica leaf extract induces resistance in rice against bacterial leaf blight disease (*Xanthomonas oryzae* pv. oryzae)

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Three plants extracts were used for the management of *Xanthomonas oryzae* pv. oryzae *in vitro*. The efficacy of the plant extracts was tested by antibacterial activity and was used as seed treatment to know the enhancement of seed germination and seedling vigour. Beside promising results given, plant extracts were tried under greenhouse studies to test the efficacy in controlling bacterial blight disease incidence. To get the molecular level of evidence and the estimated defense enzymes in plant extracts, plants were treated, after challenge inoculation, with a target pathogen at different intervals. *Adathoda vasica* leaf extract significantly reduced the bacterial leaf blight pathogen, *X. oryzae* pv. oryzae (Xoo) *in vitro*. Seed treatment was found to be effective in reducing the incidence of the disease under greenhouse condition. Physiological observation of *A. vasica* treated plants indicated that restriction of pathogen colonization or disease development in plant tissue was correlated with the pronounced increase of peroxidase, PAL, β-1, 3-glucanase, polyphenol oxidase and phenol activity after challenge inoculation with the target pathogen. This investigation clearly demonstrated that the *A. vasica* leaf extract has the ability to induce the activation of defense enzymes accumulation which can be associated with induction of resistance against rice bacterial leaf blight.

Key words: *Adathoda vasica*, bacterial leaf blight, defense enzymes, induced systemic resistance, rice, *Xanthomonas oryzae* pv. oryzae.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most widely cultivated food crops in the world. Losses due to pests and diseases are important constraints in rice production. Seed-borne bacterial leaf blight (BLB) of rice, caused by *Xanthomonas oryzae* pv. oryzae (Ishiyama) (Swings et al., 1990), is the major limiting factor in rice seed production. In India, the yield loss due to this disease is up to 81.3% (Srivastava, 1967; Sonti, 1998; Gnanamanickam et al., 1999; Veena et al., 2000). In addition, to direct yield loss in the seed production programmes, this disease also adversely affects the seed quality through seed discoloration. This pathogen is known to be seed-borne and seed transmitted (Veena et al., 2000). However, the primary sources of the diseases' inoculums are external and internal contaminated seeds (Srivastava and Rao, 1964). The results of the contaminated seed in poor germination and severe infection affects the plants at tillering and flowering stages, thereby resulting in the formation of chaffy seeds.

Unsuccessful attempts have been made to manage this disease using chemotherapeutics, which prompted us to develop alternative management strategies. Several broad spectrum bactericides have been recommended for the bacterial leaf blight. However, the chemicals are expensive and they also affect the beneficial microorganisms. Plant origin 'biocides' are non-phyto-

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Table 1. Plant species used in the study.

Scientific Name	Family	Parts used
Adhatoda vasica Nees	Acanthaceae	Leaf
Lantana camera L.	Verbenaceae	Leaf
Allium sativum L.	Alliaceae	Bulb

toxic, systemic and easily biodegradable (Mason and Mathew, 1996; Qasem and Abu- Blan, 1996; Singh,1994; Kagale, 2004). The active compounds from plant,act on the pathogen directly (Amadioha, 2000; Ansari,1995) and induce the systemic resistance in growing plants, which in turn reduced the disease development (Narwal et al.,2000; Paul and Sharma, 2002). Induced systemic resistance activates the multiple defense mechanisms which include increased pathogenesis related (PR) proteins (peroxidase, chitinase, etc) (Xue et al., 1998). Phenylalanine ammonia lyase (PAL) and peroxidase are the main enzymes involved in the phenyl-propanoid metabolism (Xue et al., 1998). Therefore, in the present study, an attempt has been made to use plant extracts in place of synthetic chemicals not only to reduce the *X. oryzae* pv. oryzae incidence, but also to improve the seed quality, evaluate the antibacterial activity of three plants under *in vitro* and greenhouse conditions and to study the potential induction of systemic resistance in rice by *A. vasica*.

MATERIALS AND METHODS

Collection and screening of paddy samples

Five seed samples were collected from seed companies and evaluation for the occurrence of *X. oryzae* pv. oryzae (Xoo) were based on direct plating method, liquid assay method (Mortensen, 1992) and seedling symptom test (Veena et al., 2000). Pathogen was confirmed by various biochemical tests and pathogenicity test (Kauffman et al., 1973).

Plant materials

All the three plant species tested for their antibacterial activity were collected from Mysore region, Karnataka, India (Table 1).

Plant extracts preparation

Fresh leaves of *A. vasica* and *Lantana camera* and bulbs of *Allium satium* were harvested and thoroughly washed in tap water 3 to 4 times. 100 g of leaves or bulbs of each were macerated to paste with the help of sterilized mortar and pestle with 100 ml of sterilized distilled water and it was filtered through the muslin cloth. The extract was then centrifuged at 5,000 rpm for 15 min. To obtain solvent extracts, 100 g of finely powdered dry samples were homogenized in 100 ml solvent (methanol) (1/1 w/v). The mixture was kept in room temperature (26 ± 2°C) for 4 h, then it was centrifuged at 5,000 rpm for 10 min. Subsequently, the supernatant was evaporated completely and the residue was dissolved in sterile distilled water to get aqueous extract (1 g/ml) and stored at 4°C for further use. Afterwards, 1:10 dilution was used for all experiments.

In vitro assay of the antimicrobial activity of leaf extracts against Xoo

The modified technique of Nene and Thapliyal (1979) was followed to study the antibacterial activity of leaf extracts. Five drops of Xoo ($1x10^6$ CFU) were spread on Petri plates containing peptone sucrose agar medium (PSA). 5 mm paper disks (sterilized Whatman No. 1) were made and dipped in different concentrations of different plant extracts and placed equidistantly on Xoo spread PSA medium containing plates, followed by incubation at growth chamber (28±2°C). PSA without leaf extract and with either sterile distilled water or streptomycin (100 ppm) served as the control treatments. The observations on the development of waxy, shiny yellow mucoid colonies were recorded within 48 h of incubation and the inhibition zones were measured. Each treatment was replicated 3 times with 5 plates per replication. Three independent experiments were performed with similar results.

Effect of plant extracts on the incidence of Xoo, seed germination and seedling vigour

The seeds were soaked with crude aqueous extracts over night at room temperature and used in direct plating method to know the incidence of Xoo, while paper towel method was used to check the seed germination and seedling vigour. Four replicates of 100 seeds were maintained for each treatment, while distilled water treated seeds served as control.

Effect of plant extracts on the incidence of BLB disease of rice under greenhouse conditions

The various aqueous extracts tested for all the plant species were found to be highly effective in suppressing the *in vitro* growth of Xoo, and hence used to assess their effect on severity of bacterial blight diseases under greenhouse conditions. Treated and untreated seed samples were sown in earthen pot containing 1:1 ratio of sand and soil (25 seeds/pot) and maintained under greenhouse conditions. Inoculum (Xoo) was sub-cultured and the load was prepared by adding the sterile distilled water at $1x10^8$ CFU/ml. Inoculation was done for forty-five days old plant leaves by scissors-dip method, followed by the standard procedure of Kauffman et al. (1973). Pre and post -inoculation applications (two days prior to and after inoculation, respectively) with aqueous extract (1:10 dilution) of the leaves of selected species, streptomycin (100 ppm) and sterile water (control), were made at 45 days after sowing. Observation was made on plants for development of bacterial leaf blight symptom after fourteen days of inoculation. The experiment was repeated thrice and similar results were observed. The severity of bacterial leaf blight disease was done as per the standard evaluation system (SES) for rice (Anonymous, 1980).

Assay of induced enzymes

Tissue collection

The Xoo inoculated leaf portions of rice plants sprayed with leaf extracts of *A. vasica* or streptomycin or water were collected at various time intervals (0, 24, 48, 72, 96 and 164 h) after pathogen inoculation and were quickly frozen in liquid nitrogen and stored at -20°C.

Table 2. Screening of different rice seed samples for incidence of *Xanthomonas oryzae* pv. *oryzae*.

Rice varieties	Direct plating (%)	Seedling symptom test (%)	Liquid assay method (cfu/ml)
Swarna	80	77	92 x10^4
IR-20	80	67	74 x10^4
IR-64	97	80	96 x10^4
Jaya	99	84	99 x10^4
Badami	96	72	92 x10^4

Data based on four replicates of 100 seeds for each sample.

Assay for peroxidase

Fresh plant leaves (1 g) were homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) with pre chilled mortar and pestle. The homogenate was centrifuged at 18,000 rpm at 5°C for 15 min and used within 2 to 4 h. Supernatant was served as an enzyme source. To a spectrophotometric sample cuvette, 3 ml of buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml H_2 O_2 solution were added and mixed well. Absorbance was recorded at 470 nm using spectrophotometer (Hitachi, 2000, Japan). The enzyme activity was expressed as changes in absorbance ($min^{-1}g^{-1}$) of fresh weight (Hammerschmidt et al., 1982). As such, three replicates were maintained for each treatment.

Determination of phenylalanine ammonia lyase (PAL) activity

Leaf tissues (300 mg) from each of the three replicates for each treatment were homogenized in the ice-cold 0.25 M borate buffer (pH 8.7) in an ice bath. The homogenate was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was then centrifuged at 15000 rpm for 15 min at 4°C. As such, the resultant clear yellowish-green supernatant was used as a crude enzyme extract. The reaction mixture contained 1 ml of enzyme extract, 0.5 ml of 0.2 M borate buffer (pH 8.7), 1.3 ml of distilled water and 0.2 ml of 1 M l-phenylalanine. Changes in absorbance at 290 nm were observed using spectrophotometer (Hitachi, Japan, 2000). Reaction mixture without substrate served as control, in that one unit of enzyme activity produced 3.37 nm of cinnamic acid/hour (Singh and Prithiviraj, 1997). However, three replicates were maintained for each treatment.

Assay of polyphenol oxidase (PPO)

One gram fresh weight of *A. vasica* from treated plants and control plants were ground to fine powder in liquid nitrogen and extracted in 1 ml of extraction buffer containing 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 15,000 r/m for 15 min at 4°C and the supernatant was used as enzyme source. The reaction mixer started when 0.2 ml of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm at 30 s interval for 3 min. As such, the absorbance was recorded at 0, 24, 48, 72, 96 and 164 h. The mean change in absorbance was calculated for 1 min and the activity was expressed as changes in absorbance per min mg of protein of the plant sample (Mayer et al., 1965).

-1, 3-glucanase assay

The crude extracts of 62.5 l was added to 62.5 l of lamanarin and then incubated at 40°C for 10 min. Then, the reaction was stopped by adding 375 l of dinitrosalicylic acid and heated for 5 min in a boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with lamanarin amid zero time incubation served as blank. The activity was expressed as g equivalent of glucose/min/mg of protein (Kavitha et al., 2005).

Estimation of phenolic substances

One gram of fresh sample was homogenized with 10 ml of 80% methanol and agitated for 15 ml at 70°C (Zieslin et al., 1993), while 1 ml of the methanolic extract was added to 5 ml of distilled water and 250 l of Folin-Ciocalteau reagent (1N). Consequently, the solution was kept at 25°C. The absorbance of the blue was measured using a spectrophotometer (Hitachi, 2000, Japan) at 725 nm and catachol was used as the standard (Kagale et al., 2004).

Statistical analysis

Percentage data were transformed into arcsine values and the analysis of variance was carried out (ANOVA). Means were compared for significance using Duncan's new multiple range test (DMRT; P = 0.05).

RESULTS

The effect of natural products (plant extracts) in comparison with bactericide and chloramphenicol were selected and evaluated against bacterial leaf blight pathogen, *X.o.pv.oryzae*. In the present study (Table 1), the three plant species belonging to three different families were tested, except *L. camera*, which significantly inhibited the growth of Xoo. The aqueous leaf extract of *A. vasica* was most effective in inhibiting the growth of Xoo as compared to bulb extract of *A. sativum*.

When compared to chloramphinicol, the *A. vasica* significantly suppressed Xoo growth, whereas the leaf extract of *L. camera* showed least antimicrobial activity. Tables 2 and 3 clearly indicate the prevalence of bacterial pathogen on rice seeds and its effect on seed quality, that is, on seed germination and seedling vigour. As such, the five different seed samples showed high incidence of Xoo.

Table 3. Effect of plant extracts on seed germination and seedling vigour of rice.

Plant extracts		Rice varieties				
		Swarna	**IR-20**	**IR-64**	**Jaya**	**Badami**
Adhatoda vasica	Ger.	98	98	98	96	98
	MSL	13.2+1.2	12.9+1.3	12.6+0.8	13.1+2.0	12.8+1.1
	MRL	11+1.3	11.8+1.1	10.3+0.3	11.4+1.5	11.6+1.1
	VI	2371	2420	2293	2352	2391
Allium cepa	Ger.	98	96	96	94	96
	MSL	8.7+0.4	8.1+1.1	8.3+0.7	8.9+1.8	9.2+1.6
	MRL	7.6+1.3	7.5+0.6	7.1+1.1	7.5+1.4	6.5+1.1
	VI	1564	1497	1485	1541	1507
Lantana camera	Ger.	96	94	94	94	94
	MSL	7.1+0.4	9.1+1.1	8.6+1.1	8.5+1.8	8.4+1.6
	MRL	8.6+1.3	7.2+0.6	7.5+1.4	7.8+1.3	7.4+1.1
	VI	1507	1532	1513	1532	1518
Untreated control	Ger.	93	87	82	86	86
	MSL	6.5+0.3	7.1+0.2	6.1+2.4	8.8+0.4	6.3+0.9
	MRL	6.4+0.4	5.3+0.4	6.4+0.3	4.2+0.5	6.1+0.3
	VI	1071	1078	1025	1118	1066

Data based on four replicates of 100 seeds. Ger: germination; MSL: mean shoot length; MRL: mean root length; VI: vigour index.

Table 4. Effect of different plant species aqueous extracts on the growth of Xoo by *in vitro* inhibition assay method.

Plant material	Zone of inhibition (cm)
Adhatoda vasica	4.1c
Allium sativum	1.8b
Lantana camera	1.3a
Streptomycin (100 ppm)	1.4a

Means followed by the same letters are not significantly different at 0.05 levels according to LSD.

Effect of plant extracts on seed germination and seedling vigour in different varieties of rice

Aqueous leaf extracts of *A. vasica* significantly increased the seed germination and seedling vigour of the highly infected variety “Jaya” and it also enhanced the root and shoot length in all the tested varieties. All the plant extracts increased the seed germination and seedling vigour compared to the control. The result (Table 3) depicts that, due to high infection with pathogen, the seed germination and seedling vigour was low. When treated with plant extracts, enhancement was observed. Application of leaf extracts prior to seed germination is found to be better in decreasing the bacterial leaf blight development.

In vitro inhibition activity

Out of the three plant species, *A. vasica* and *A. sativum* significantly reduced the growth of Xoo. The leaf extract of *A. vasica* was more effective in the suppression of Xoo growth in comparison to the control. When compared to bactericide, chloramphenicol the *A. vasica* showed more antibacterial activity against Xoo. However, the leaf extract of *L. camera* showed least antibacterial activity among the three plant species tested (Table 4).

Effect of *A. vasica* extract on severity of bacterial blight

Table 5 depicts reduction of bacterial leaf blight disease under greenhouse condition. Here, three methods were followed separately. As such, pre inoculation followed by post inoculation was found to be the best, whereas seed treatment was the best and was closer to the combined data of pre and post inoculation. Seed treatment with *A. vasica* was the best among all seed treatments.

First, *A. vasica* was found to be decreasing the disease

Table 5. Effect of seed treatment, pre and post inoculation spraying of aqueous extracts on the incidence of bacterial leaf blight of rice var. Jaya under greenhouse conditions.

Treatments	Seed treatment	Pre-inoculation	Post-inoculation
A.vasica	10.4d	11.2d	14.6d
A. cepa	23.8c	26.7c	31.8c
L. camera	28.1b	29.4b	34.3b
Streptomycin (100 ppm)	9.6e	10.8e	12.9e
Untreated control	42.9a	38a	43.6a

Means followed by a common letter within a column are not significantly different (P=0.05) by Duncan's multiple range test. All leaf extracts were used at 1:10 dilution.

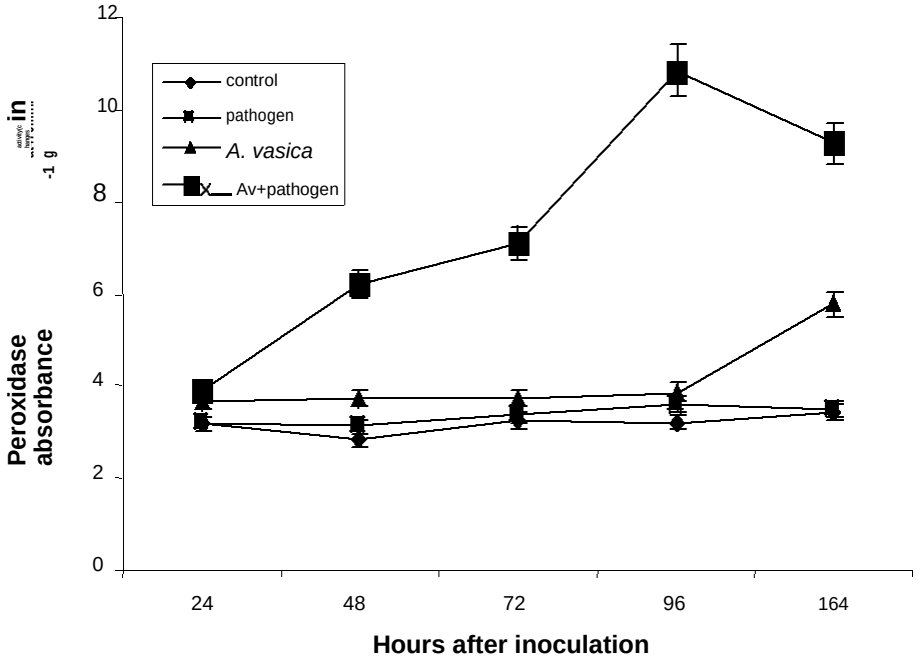

Figure 1. Induction of peroxidase in rice leaves in response to *A. vasica* treatment against bacterial leaf blight.

more than 50%. *A. sativum* and *L. camera* also restrict the bacterial blight incidence, but were not equal to *A. vasica*. Application of seed treatment was found to better restrict the symptom or pathogen establishment than pre or post inoculation and it avoids extra work and restricts the pathogen establishment from seed germination to seed setting. So, we chose *A. vasica* alone for further studies and it showed better antimicrobial activity than other used plant species in both laboratory and greenhouse conditions.

Activity of the defense related enzymes against bacterial leaf blight by *A. vasica* leaf extract

Aqueous extract of *A. vasica* induces resistance against bacterial leaf blight in rice and more than two-fold increase in accumulation of peroxidase, PAL, polyphenol oxidase and β-1,3-glucanase recorded with leaf extract and challenged with pathogen (Figures 1 to 5). The activity began to increase after 24 h of challenge inoculation and remained significantly higher at 96 h in *A. vasica* leaf extract treatment compared to the control. The activities of defense enzymes were decreased after 96 h of challenge inoculation with *X. oryzae* pv. oryzae, and so, the higher PO activity was observed in rice plants treated with *A. vasica* and challenged with Xoo (Figure 1). The activity was high at 96 h after challenge inoculation and it reduced after 96 h, whereas such decline in the activity was not observed in PAL and phenol after 96 h of challenge inoculation with the pathogen. No change was observed in the activity of PR proteins in the control, and as such, similar results were observed with the pathogen or the *A. vasica* leaf extract

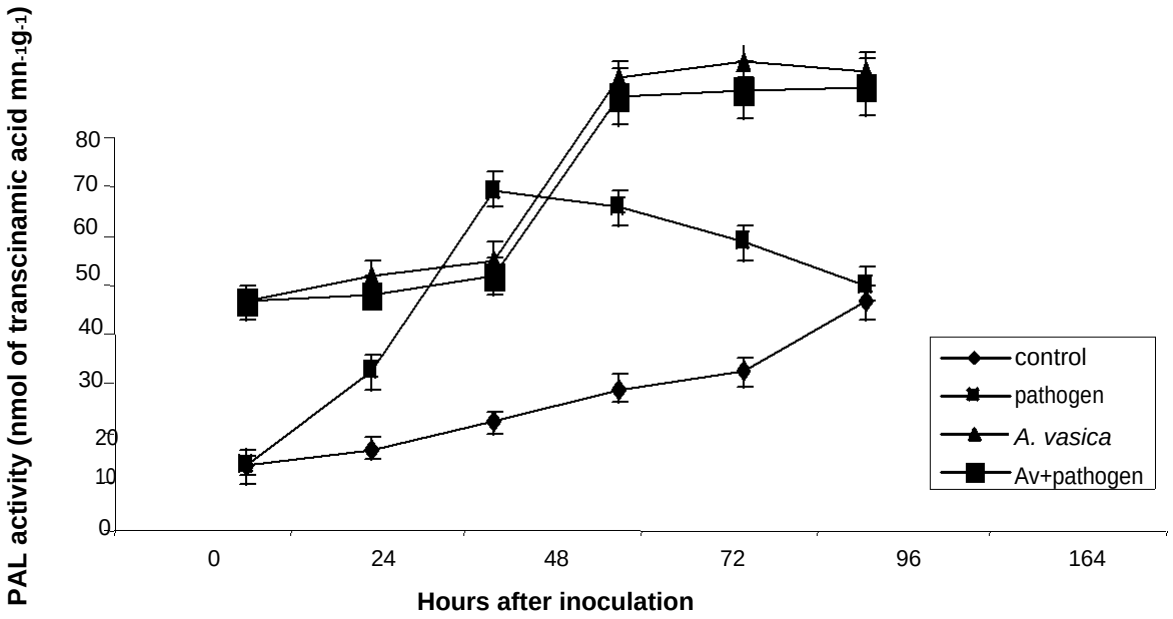

Figure 2. PAL activity in rice leaf in response to leaf extract treatment against bacterial leaf blight.

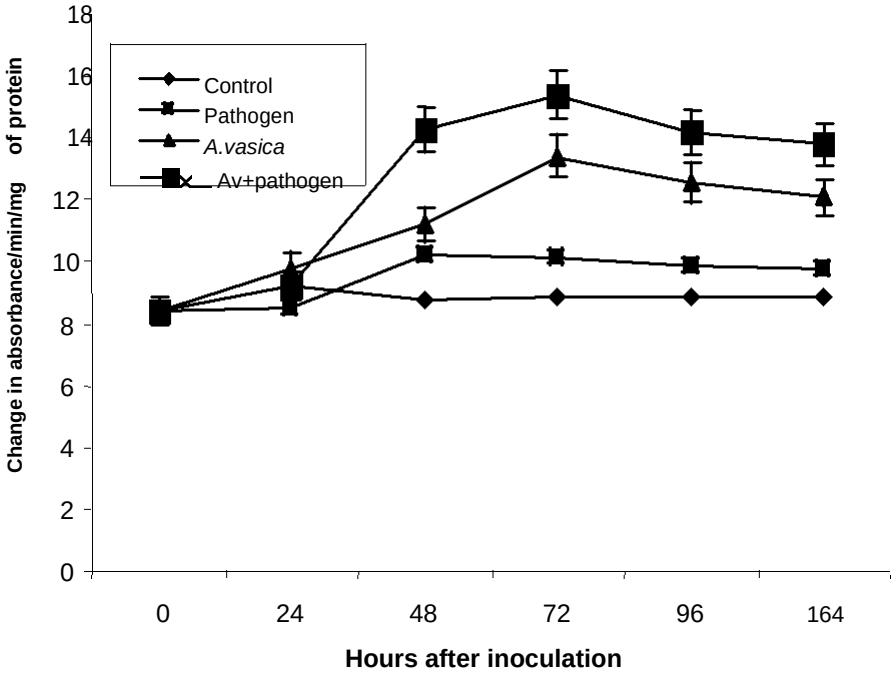

Figure 3. Induction of polyphenol oxidase activity in rice treated with *A. vasica* against blb.

that was sprayed alone (or as a seed treatment alone) and which did not show better enhancement or change in the defense enzymes' accumulation.

Seed treatment with *A. vasica* leaf extract and challenge inoculation by *X. oryzae* pv. oryzae significantly enhanced PAL and phenol substances observed. PAL activity was enhanced and it reached a maximum even after 164 h of challenge inoculation (Figure 2). *A. vasica* treated plants showed maximum PAL at 164 h even after challenge inoculation with Xoo or alone. Increase in the activity of PPO was observed in *A. vasica* treated plants challenge inoculation with the pathogen and remained at a higher level throughout the experimental period (Figure 3). The activity was higher at 72 h after challenge

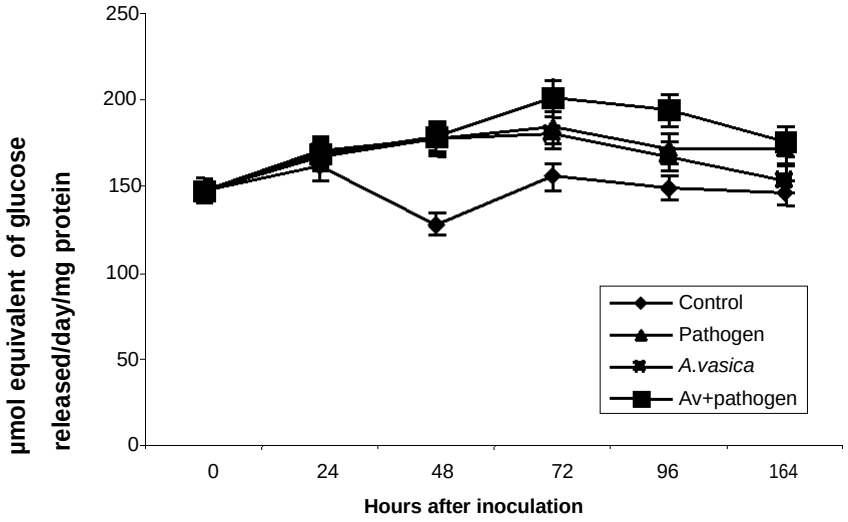

Figure 4. Induction of glucanase activity treated with *A. vasica* against blb.

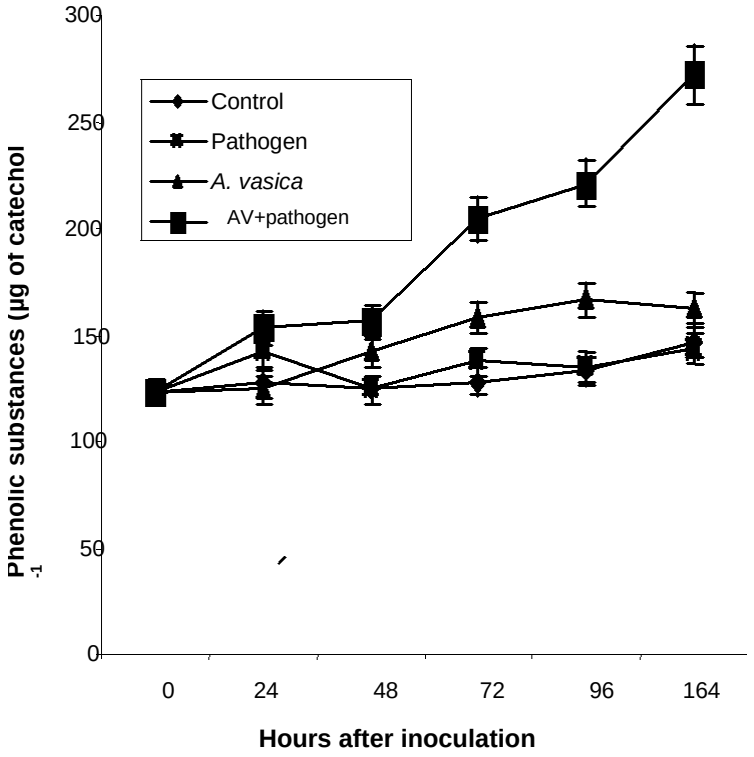

Figure 5. Changes in phenol after treatment with *A. vasica.*

inoculation. Enhancement of the PPO, also observed with *A. vasica* was not followed by challenge inoculation, but was not equal to the treatment with challenge inoculation. As such, a significant increase in the activity of β,1, 3-glucanase was observed in rice seedlings treated with *A. vasica* extract after challenge inoculation (Figure 4). *A. vasica* alone or with inoculated or untreated pathogen only showed changes in their β,1, 3-glucanase activity.

The activity increased 24 h after challenge inoculation and reached a maximum at 72 h. Phenolic substances accumulation was observed after 24 h, but it reached maximum at 16 h in plants treated with *A. vasica* after challenge inoculation (Figure 5). However, control or plant extract alone or pathogen inoculated plants did not show any changes in phenolic substances or PAL activity.

DISCUSSION

In the present study, we tested three different plant species which belong to three different families for antibacterial activity against bacterial leaf blight pathogen, *X. oryzae* pv. oryzae and they are commonly available throughout India. Aqueous leaf extract of *A. vasica* was found to be highly effective in inhibiting the growth of Xoo compared to the leaf extract of *L. camera* and bulb extract of *A. sativum*. Narasimhan et al. (1995) and Kagale et al. (2004) have reported antibacterial activity and management of bacterial diseases with use of different plant extracts in other crops. Various plant extracts were reported to possess differing level of antimicrobial activities *in vitro* against phytopathogenic fungi, yeast and bacteria. However, only few limited data available on antimicrobial activity due to plant extracts in plant pathogenic bacteria. Seed treatment with *A. vasica* was most effective in the reduction of disease incidence compared to control. Significantly, increase in yield was also reported due to plant extracts and was confirmed with antimicrobial activity against some bacteria (Madhiazhagan et al., 2000). In plant-pathogen investigations, problems were often encountered where a number of factors were involved. One of the important ways of defending the host might be by enzymes or metabolites. High factors of peroxidase, polyphenol oxidase, PAL and β-1, 3-glucanase in rice plants reflect the plant response to disease and this increase may be higher around penetration or pathogen establishment site.

In this study, induction of resistance in rice with *A. vasica* was evident from increased accumulation of PR proteins and other related compounds. Schneider and Ullrich (1994) and Kagale et al. (2004) reported that treatment of plant extracts led to an increase in the activities of chitinase, peroxidase, polypehol oxidase, β-1,3-glucanase and PAL. Biological active compounds which are present in plants act as elicitors to induce resistance in host plants resulting in a reduction of disease development (Vidhyasekaran, 1992). Enhanced peroxidase activity, very often, is associated with resistance phenomenon such as lignin production (Reuveni et al., 1992), and as such, PAL plays an important role in biosynthesis of peholic phytoalexins (Daayf et al., 1997). The increase in PAL activity indicates the activation of phenol propanoid pathway. In several host pathogen interactions, increased PAL levels have been shown to be correlated with compatibility (Ralton et al., 1989). The PAL product is a transcinnamic acid which is an immediate precursor for the biosynthesis of SA; and so, it is a signal molecule in the systemic acquired resistance (Klessig and Malamy, 1994).

The present study assessed the plant extract's effect of *A. vasica* on the disease development of Xoo. Using other plant extracts, induction of resistance against wide spectrum of fungal, bacterial and viral pathogens have been studied by Brisset et al. (2000) and Baysal and Zeller (2004). Induction of peroxidase has been mixed up in the production of toxic radicals, such as O^{2-} and H_2O_2. The increased production of both the superoxide radicals and H_2O_2 is a common feature of defense responses to plants challenged by pathogens and elicitors and there is ample evidence indicating that H_2O_2 performs several important functions in disease resistance (Mehdy et al., 1996). Nicholson and Hammerschmidt (1992) have reported that an increase in peroxidase activity can be involved in the formation of lignin and in the inhibition of the pathogen spread in the xylem. Stintzi et al. (1993) have noticed that the enzymatic activities of several PR proteins have been identified PR-2 (β-1, 3-glucanase) to possess direct antimicrobial activity by spreading and degrading the microbial cell wall components. Van Loon et al. (1994) have reported a relation between β -1,3-glucanase synergic activity and the SAR pathway that includes salicylic acid as a signal molecule and which is activated by necrotizing pathogens and chemical inducers. Peroxidase shows affinity to substrates involved in cellular lignification and the products of its activity have direct antimicrobial activity in the presence of H_2O_2 (Ride, 1995). Also, PAL generates precursors of lignifications biosynthesis and other phenolic compounds that are accumulated in the response to infection (Klessig and Malamy, 1994).

In this investigation, we have demonstrated the antibacterial activity of *A. vasica* leaf extract and the involvement of the systemic resistance induction against bacterial leaf blight disease caused by *X. oryzae* pv. oryzae in rice. In conclusion, our study results suggest that *A. vasica* leaf extract has strong *in vitro* inhibition against Xoo and has reduced the BLB disease under greenhouse condition. In contrast, the pronounced recovery growth, productivity and defense enzyme enhance the *A. vasica* leaf extract in minimizing seed-borne bacterial leaf blight disease in rice. Therefore, we recommend the use of *A. vasica* in order to control seed-borne bacteria, Xoo and development of disease, in that the use of *A. vasica* leaf extract is environmentally safe in the management of seed-borne bacterial leaf blight disease in rice.

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