Induced Systemic Resistance and Their Implications in Host Resistance to Physic Nut against Leaf Blight Disease

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Abstract The aim of this work was to study the Induced systemic resistance and their implications in host resistance to physic nut against leaf blight disease. In pot culture experiment plant treated with Mancozeb (0.2%) and Propiconazole (0.1%) thrice starting from 30 days after planting with an interval of 15 days was significantly superior over all the treatments with the PDI of 18.44 and 19.00 respectively. The plant height was significantly increased in all treated plants ranging from 99.80 cm to 148.25 cm. Plants treated with \textit{Pseudomonas fluorescens} (Pf1) induces more phenyl alanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), \(\beta\)-1,3 glucanase, super oxide dismutase (SOD) and phenol activity in treated physic nut plant followed by \textit{Bacillus} EPCO16 treated plants.

Keywords Bio control agents; \textit{Curvularia clavata}; Defence enzymes; Leaf blight; Micro nutrients; Physic nut; Plant products

1 Introduction

Physic nut (\textit{Jatropha curcas} L.) is an important commercial bio-diesel plant species and is being advocated for development of waste land and dry land. In India, the diesel consumption alone was about 38 million tones and the subsidies offered by the Central Government were Rs. 9,130 crores. Diesel is the most widely used fuel for transportation purpose apart from other industrial uses. Therefore, if physic nut oil based bio-diesel can be used as an extender for mixing with diesel (about 20 per cent), a huge savings can be made in terms of not only money value (both on spending and foreign exchange), but also on the renewable resource and decrease our dependency on foreign sources for oil (Paramathma et al., 2006). Among the several constraints in physic nut cultivation, diseases play a major role in the yield reduction and it is affected by many fungal and viral diseases. Among the foliar diseases, leaf blight disease caused by \textit{Curvularia clavata} is a major fungal disease.

Considering the importance of the crop, destructive nature of the disease and availability of limited information on this pathogen in this crop, the present studies were undertaken for inducing systemic resistance against leaf blight disease by the application of plant oils, micro nutrients and bio agents.

1 Results

1.1. Effect of plant oil, micronutrient, bio control agents and fungicides against leaf blight disease under glass house conditions

The results of the pot culture experiment under glass house conditions showed that foliar application of Mancozeb (0.2%) and Propiconazole (0.1%) thrice starting from 30 days after planting with an interval of 15 days was significantly superior over all the treatments with the PDI of 18.44 and 19.00 respectively on 60 days after planting. Hexaconazole was the next best which recorded PDI of 22.27 followed by Tricyclazole (24.83 PDI). Among the bio control agents, Pf1 (0.2%) sprayed pots recorded the PDI of 26.81 while in control the PDI was 56.73. The plant height was significantly increased in all treated plants ranging from 99.80 to 148.25 cm whereas in control the plant height was 56.73 (Table 1).

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Table 1 Efficacy of plant oil, bio control agents, micronutrient and fungicides against leaf blight disease under glasshouse conditions

| S. No | Treatments          | *Per cent disease index | Plant height (cm) |
|-------|---------------------|-------------------------|-------------------|
| 1     | Mancozeb (0.2%)     | 18.44(25.42)            | 148.25            |
| 2     | *Pseudomonas* *fluorescens* (0.2%) | 26.81(31.17)          | 130.20            |
| 3     | Bacillus sp EPCO16 (0.2%) | 30.32(33.40)          | 127.40            |
| 4     | Lemon grass oil (0.1%)  | 34.24(35.80)           | 120.20            |
| 5     | Copper sulphate (0.1%)  | 36.00(36.86)           | 99.80             |
| 6     | Control             | 56.73(48.87)           | 87.50             |

Note: *Values are the mean of four replications; Values in parentheses are arcsine transformed. Means followed by a same letter are not significantly different at the 5 % level by DMRT

1.2 Induction of defense mechanism in plants

1.2.1 Changes in the phenylalanine ammonia lyase (PAL) activity in physic nut plants treated with plant oil, bio control agents and micronutrient

The phenylalanine ammonia lyase (PAL) activity gradually increased in physic nut leaves following treatments with *P. fluorescens*, *Bacillus* EPCO16, Copper sulphate and Lemon grass oil which reached maximum level at 5th day after treatment when compared to control. However the enzyme activity declined subsequently. Among them, Pf1 recorded maximum PAL activity (22.303 n mol of transcinamic acid min⁻¹g⁻¹ of fresh tissue) followed by *Bacillus* EPCO16 (21.030 n mol of transcinamic acid min⁻¹g⁻¹ of fresh tissue), Copper sulphate (19.160 n mol of transcinamic acid min⁻¹g⁻¹ of fresh tissue) and Lemon grass oil (17.710 n mol of transcinamic acid min⁻¹g⁻¹ of fresh tissue) on 5th day and thereafter declined. The plants treated with pathogen alone recorded (11.210 n mol of transcinamic acid min⁻¹g⁻¹ of fresh tissue) only slight increase in PAL activity, while the healthy plant recorded lesser PAL activity (8.230 n mol of transcinamic acid min⁻¹g⁻¹ of fresh tissue) (Figure 1A).

1.2.2. Changes in the peroxidase (PO) activity in physic nut plants treated with plant oil, bio control agents and micronutrient

The activity of peroxidase gradually increased in physic nut leaves following treatments with *P. fluorescens*, *Bacillus* EPCO16, Copper sulphate, Lemon grass oil reached maximum level at 5th day after treatment when compared to control. The enzyme activity subsequently declined. Among them, Pf1 recorded maximum PO activity (3.790 changes in A420 min⁻¹g⁻¹ of fresh tissue) followed by *Bacillus* EPCO16 (3.210 changes in A420 min⁻¹g⁻¹ of fresh tissue), Copper sulphate (2.210 changes in A420 min⁻¹g⁻¹ of fresh tissue) and Lemon grass oil (1.970 changes in A420 min⁻¹g⁻¹ of fresh tissue) on 5th day and thereafter declined. The plants treated with pathogen alone recorded (1.850 changes in A420 min⁻¹g⁻¹ of fresh tissue) only slight increase in PO activity. The healthy plant recorded lesser PO activity (1.570 changes in A420 min⁻¹g⁻¹ of fresh tissue) (Figure 1B).

1.2.3. Changes in the polyphenol oxidase (PPO) activity in physic nut plants treated with plant oil, bio control agents and micronutrient

The polyphenol oxidase (PPO) activity gradually increased in physic nut leaves following treatments with *P. fluorescens*, *Bacillus* EPCO16, Copper sulphate and Lemon grass oil reached maximum level at 5th day after treatment when compared to control. The enzyme activity subsequently declined. Among them, Pf1 recorded maximum PPO activity (2.820 changes in A490 min⁻¹g⁻¹ of fresh tissue) followed by *Bacillus* EPCO16 (2.570 changes in A490 min⁻¹g⁻¹ of fresh tissue), Copper sulphate (2.210 changes in A490 min⁻¹g⁻¹ of fresh tissue) and Lemon grass oil (1.970 changes in A490 min⁻¹g⁻¹ of fresh tissue) on 5th day and thereafter declined. The plants treated with pathogen alone recorded (1.850 changes in A490 min⁻¹g⁻¹ of fresh tissue) only slight increase in PPO activity. The healthy plant recorded lesser PPO activity (1.570 changes in A490 min⁻¹g⁻¹ of fresh tissue) (Figure 1C).

1.2.4. Changes in the β-1, 3 glucanase activity in physic plants treated with plant oil, bio control agents and micronutrient

The β-1, 3 glucanase activity gradually increased in physic nut leaves following treatments with *P. fluorescens*, *Bacillus* EPCO16, Copper sulphate and
Figure 1 Induction of defence enzymes like PAL, PO, PPO, β-1,3 glucanase SOD and Phenol activity in physic nut plants treated with plant oil, biocontrol agents and micronutrient against C. clavata

Note: A- Assay of PAL; 1B-Assay of PO; 1C- Assay of PPO; 1D- Assay of β-1,3 glucanase; 1E-Assay of SOD; 1F- Phenol
Lemon grass oil reached maximum level at 5th day after treatment when compared to control. The enzyme activity subsequently declined. Among them, Pf1 recorded maximum glucanase activity. The maximum β-1, 3 glucanase activity was observed in plants treated with Pf1 (29.220 µg of glucose min⁻¹ g⁻¹ of fresh tissue) followed by Bacillus EPCO16 (25.790 µg of glucose min⁻¹ g⁻¹ of fresh tissue), Copper sulphate (24.220 µg of glucose min⁻¹ g⁻¹ of fresh tissue) and Lemon grass oil (24.070 µg of glucose min⁻¹ g⁻¹ of fresh tissue) on 5th day and thereafter declined. The plants treated with pathogen alone recorded (20.410 µg of glucose min⁻¹ g⁻¹ of fresh tissue) only slight increase in β-1, 3 glucanase activity. The healthy plant recorded lesser β-1, 3 glucanase activity (15.780 µg of glucose min⁻¹ g⁻¹ of fresh tissue) (Figure 1D).

1.2.5. Changes in the Super Oxide Dismutase (SOD) activity in physic nut plants treated with plant oil, bio control agents and micronutrient

The Super oxide Dismutase activity gradually increased in physic nut leaves and following treatments with P. fluorescens, Bacillus EPCO16, Copper sulphate and Lemon grass oil, reached maximum level at 5th day after treatment when compared to control. The enzyme activity subsequently declined. Among them, Pf1 recorded maximum SOD activity (11.640 unit min⁻¹ g⁻¹ of fresh tissue) followed by Bacillus EPCO16 (10.120 unit min⁻¹ g⁻¹ of fresh tissue), Copper sulphate (9.360 unit min⁻¹ g⁻¹ of fresh tissue) and Lemon grass oil (7.110 unit min⁻¹ g⁻¹ of fresh tissue) on 5th day and thereafter declined. The plants treated with pathogen alone recorded (6.210 unit min⁻¹ g⁻¹ of fresh tissue) only slight increase in super oxide dismutase (SOD) activity. The healthy plant recorded lesser super oxide dismutase (SOD) activity (4.440 unit min⁻¹ g⁻¹ of fresh tissue) (Figure 1E).

1.2.6. Changes in the phenolic content in physic nut plants treated with plant oil, bio control agents and micronutrient

The phenolic activity gradually increased in physic nut leaves following treatments with P. fluorescens, Bacillus EPCO16, Copper sulphate and Lemon grass oil reached maximum level at 5th day after treatment when compared to control. The enzyme activity subsequently declined. Among them, Pf1 recorded maximum phenolic activity (15.390 µg of catechol g⁻¹ of fresh tissue) followed by Bacillus EPCO16 (14.320 µg of catechol g⁻¹ of fresh tissue), Copper sulphate (13.880 µg of catechol g⁻¹ of fresh tissue) and Lemon grass oil (12.470 µg of catechol g⁻¹ of fresh tissue) on 5th day and after declined. The plants treated with pathogen alone recorded (11.510 µg of catechol g⁻¹ of fresh tissue) only slight increase in phenolic content. The healthy plant recorded lesser phenolic content (9.450 µg of catechol g⁻¹ of fresh tissue) (Figure 1F).

1.2.7. Detection of different isozymes by gel electrophoresis

1.2.7.1. Isoform pattern of peroxidase (PO)

The results showed that plants treated with Pf1 and Bacillus EPCO16 showed three isofoms viz., PO1, PO2 and PO3. The other treatments viz., Copper sulphate and Lemon grass oil showed two isofoms viz., PO1 and PO2. The result indicates that the expression of PO3 peroxidase in physic nut plants may be due to the treatment with Pf1 and Bacillus EPCO16 which expressed additional peroxidase activity. The other treatments including control revealed only one isoform with less intensity (Figure 2A).

1.2.7.2. Isoform pattern of poly phenol oxidase (PPO)

The results showed that plants treated with Pf1 and Bacillus EPCO16 showed three isofoms viz., PPO1, PPO2 and PPO3. The other treatments viz., Copper sulphate and Lemon grass oil showed two isofoms viz., PPO1 and PPO2. The result indicated that the expression of PPO3 poly phenol oxidase in physic nut plants may be due to the treatment with Pf1 and Bacillus EPCO16 which expressed additional poly phenol oxidase activity. The other treatments including control revealed only one isoform with less intensity (Figure 2B).

2. Discussion

2.1. Induction of defense mechanism in plants

2.1.1. Phenylalanine ammonia lyase (PAL)

Early induction of PAL is very important as the biosynthesis of lignin originate from L-phenylalanine.
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General phenylpropanoid metabolism is defined as the sequence of reactions involved in the conversion of L-phenylalanine to activated cinnamic acids. The first enzyme of this pathway is PAL that catalyzes the trans elimination of ammonia from L-phenylalanine to form trans cinnamic acid which in turn enters different biosynthetic pathways leading to lignin. Similarly studies in cucumber revealed that PAL is a key enzyme in the production of phenolic and phytoalexin. Systemic increase in the PAL activity upon treatment with PGPR has been reported in several crops including pigeon pea and rice (Meena et al., 2000). Induction of PAL by fluorescent pseudomonads was reported in cucumber against *P. aphanidermatum* (Chen et al., 2000), Tomato against *F. oxysporum* f.sp. *lycopersici* (Ramamoorthy et al., 2002) and in mango against *C. gloeosporioides* (Vivekanadhan et al., 2004). Present study showed that PAL activity was gradually increased in physic nut leaves and following treatments with *P. fluorescens*, *Bacillus* EPCO16, Copper sulphate and Lemon grass oil reached maximum level at 5th day after treatment when compared to control. The enzyme activity subsequently declined. Among them, Pf1 recorded maximum PAL activity (22.303 n mol of transcinnamic acid min⁻¹g⁻¹ of fresh tissue) followed by *Bacillus* EPCO16 (21.030 n mol of transcinnamic acid min⁻¹g⁻¹ of fresh tissue).

2.1.2. Peroxidase (PO)
The present study results are supported by several authors including peroxidase is involved in lignification leading to disease resistance. Polymerization of cinnamyl alcohols to lignin is catabolised by PO. For lignifications specific cell wall peroxidases are thought to be required to generate H₂O₂ and monolignol radicals. Constabel et al. (1998) proved that PO could be induced upon induction by Jasmonic acid. The bacteria and the pathogens induced one acidic peroxidase isozyme in the roots. Two other isozymes induced by the pathogen were not found to be induced by PGPR (Chen et al., 2000). Karthikeyan et al. (2009) reported that soil and foliar application of *P. fluorescens* (Pf1) induced the accumulation of phenolics and enhanced the activities of peroxidase, phenylalanine ammonia lyase and polyphenol oxidase in black gram against *Urdbean leaf crinkle virus* (ULCV) in blackgram. New isoforms of peroxidase and polyphenol oxidase were also induced by the treatment.

2.1.3. Poly phenol oxidase (PPO)
The present study revealed that the poly phenol oxidase (PPO) activity gradually increased in physic nut leaves and following treatments with *P. fluorescens*, *Bacillus* EPCO16, Copper sulphate and Lemon grass oil reached maximum level at 5th day after treatment when compared to control. Poly phenol oxidases (PPO) are enzymes which use molecular oxygen to catalyse the oxidation of monophenolic and orthophenolic compounds. PPO usually accumulated upon wounding in plants. Similarly increased activation of PPO could be detected in the cucumber leaf in the vicinity of lesions caused by some foliar pathogens. Moreover PPO can be induced through octadecanoid defense signal pathway and specific isoforms of PPO were induced in plants treated with the PGPR formulation after challenge inoculation with
Pathogens and their expressions were prominent when compared to untreated control. Similarly, Thaler et al. (1996) observed that induction of PPO in tomato plant by caterpillar feeding. Radjacommare et al. (2000) reported that Pf1 strain induced PPO isozymes in rice against sheath blight and leaf folder. Harish (2005) reported the higher induction of PPO enzymes in PGPR treated banana plants challenge inoculated with viruliferous aphids carrying Banana bunchy top virus. Mathiyazhagan et al. (2009) reported that application of Pseudomonas strains BSCBE4, PA23 and ENPF1 increased the defense related enzymes such as peroxidase, polyphenol oxidase, chitinase and β-1, 3 glucanase in P. amaranthus up to ten days after challenge inoculation with C. cassicola. Native gel electrophoretic analysis revealed that challenge inoculation of pathogen with PA23 induced both peroxidase and polyphnol oxidase isoforms.

2.1.4. β-1, 3 glucanase
The current results are in agreement with several workers pathogenesis related (PR) proteins are host coded proteins induced by different pathogens and abiotic stresses (Van Loon, 1997). Synthesis and accumulation of PR proteins have been reported to play important role in plant defense. β-1, 3 glucanase are classified under PR-2 group of PR proteins have been reported to be associated with plant resistance against fungal pathogens (Maurhofer et al., 1994). In Tobacco induction of β-1, 3 glucanase was noticed due to application of P. fluorescens isolate CHAO in response to infection by Tobacco necrosis virus (TNV) (Maurhofer et al., 1994). Induction of hydrolytic enzymes was also reported in pea against P. ultimum and F. oxysporum f. sp pisi (Benhamou et al., 1996). Govindappa et al. (2010) reported that the higher activity of peroxidase, phenylalanine ammonia-lyase, chitinase, polyphenol oxidase and β-1, 3-glucanase in P. fluorescens and T. harzianum treated safflower plants after challenge inoculation with M. phaseolina.

2.1.6. Superoxide dismutase (SOD)
In this study SOD activities were more in the Physic nut plants treated with Pf1 than the uninoculated control. Plants produce active oxygen species (AOS) such as superoxide anion (O2-), hydrogen peroxide (H2O2) and hydroxyl radical (OH) as one of the earliest responses to attempted infection pathogens (Grant and Loake, 2000). Scavengers of AOS like catalase (which catalyzes the decomposition of H2O2) (Scandalios, 1994), SOD (which scavenges O2-) suppresses the oxidative burst and inhibit tissue necrotization. Peroxidase are of particular interest because of their role in binding salicylic acid which plays an important role in induced resistance. Production of reactive oxygen species (ROS) particularly H2O2 has repeatedly been associated with diverse plant pathogen. Systemic H2O2 production has been observed in response to wounding in several plant species. H2O2 accumulates in inter cellular spaces near vascular bundles and can move in the plant tissues by diffusion. It functions as the second messenger to activate the membrane bound NADPH complex which leads to defence genes induction (Orozco- Cardens et al., 2001).

2.1.4. Phenol
The present study revealed that the phenolic activity gradually increased in physic nut leaves and following treatments with P. fluorescens, Bacillus EPCO16, Copper sulphate and Lemon grass oil reached maximum level at 5th day after treatment when compared to control. The enzyme activity subsequently declined. Similar findings were reported in sugarcane against C. falcatum (Viswanathan and Samiyappan, 1999), groundnut against C. personata (Meena et al., 2000), rice against R. solani (Radjacommare et al., 2004), and turmeric against P. aphanidermatum. Cherif et al. (2007) reported that phenolics compounds were shown to accumulate both in roots and shoots in response to various fungal infection and elicitors, and their levels significantly increase in plants pre- treated with antagonistic microorganisms.

3. Materials and Methods
3.1. Isolation of pathogen and maintenance of bio control agents
The leaf blight pathogen Curvularia clavata was isolated from coleus plants showing typical root rot symptoms and pure cultures of the pathogen were obtained by the single hyphal tip method (Rangaswami 1972). The bio control agents
Pseudomonas fluorescens and Bacillus EPCO16 were obtained from the culture collection section, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, India.

3.2. Glass house experiments
A pot culture experiment was laid out in completely randomized design to test the efficacy of fungicide (Mancozeb), bioagents Pseudomonas fluorescens, Bacillus EPCO16 and plant oils Lemon grass oil and Copper sulphate. Potting medium (red soil: Cow dung: manure at 1:1:1 w/w/w) was autoclaved for 1h for two consecutive days and filled in pots. The culture of C. clavata was inoculated by wound inoculation method at leaf surface in all the treated plants. The pathogen alone inoculated served as control. Four replications were maintained at each treatment and the pots were arranged in a randomized manner. The leaf blight disease incidence of Curvularia clavata was recorded at 30, 60 and 90 days after planting and expressed as percentage of disease incidence. All the treatments were applied as foliar spray viz., Mancozeb 75%WP @ 0.2%; Foliar spray of Pseudomonas fluorescens (Pf1) Bacillus EPCO16 and @ 0.2%; Lemongrass oil @ 0.1%; Copper sulphate @ 0.1% and Healthy Control.

3.3. Studies on induction of defence mechanism
3.3.1. Sample collection and enzyme extraction
Six key defence enzymes viz., phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), β-1, 3 glucanase and super oxide dismutase (SOD) were estimated. Leaf samples were collected from the following treatments (T1 - Foliar spray of Lemongrass oil @ 0.1%; T2 - Foliar spray of Copper sulphate @ 0.1%; T3 - Foliar spray of Pseudomonas fluorescens (Pf1) @ 0.2%; T4 - Foliar spray of Bacillus EPCO16 @ 0.2%; T5 – Inoculated control; T6 – Healthy Control) at bottom portion of the plant at two day interval up to nine days.

3.3.2 Assay of phenylalanine ammonia lyase (PAL) activity
PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. Enzyme activity was expressed in fresh weight basis as nmol trans-cinnamic acid min⁻¹ mg⁻¹ of sample (Dickerson et al., 1984).

3.3.3. Assay of peroxidase activity
Fresh plant leaves (1 g) were homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) in a prechilled mortar and pestle. The homogenate was centrifuged at 18000 rpm at 58°C for 15 minutes and supernatant was used within two to four hours which 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 H₂O₂ solution were added and mixed well. The absorbance was recorded at 420 nm using spectrophotometer. The enzyme activity was expressed as changes in absorbance min⁻¹ g⁻¹ of fresh tissue (Hammerschmidt and Kuc, 1982).

3.3.4. Assay of polyphenol oxidase (PPO) activity
The polyphenol oxidase activity was determined as per the procedure given by Mayer et al. (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M Catechol was added and the activity was expressed as change in absorbance min⁻¹ g⁻¹ of protein.

3.3.5. Assay of β-1, 3-glucanase activity
The enzyme extracts was prepared by homogenizing 1 g tissue of the leaf in 5 ml of 0.05 M sodium acetate buffer (pH 5.0) at 4°C. The homogenate was centrifuged at 20000 rpm at 4°C for 10 min and the supernatant was used as enzyme source. The crude extract of 62.5 ml was added to 62.5 ml of laminarin (4 per cent) and then incubated at 40°C for 10 min and the reaction was stopped by adding 375 ml of dinitrosaliclycic 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 laminarin with zero time incubation served as blank. The activity was expressed as µg equivalent of glucose min⁻¹ g⁻¹ of fresh tissue (Kavitha et al., 2005).
3.3.6. Assay of super oxide dismutase (SOD) activity
The enzyme extracts were prepared by homogenizing 1 g tissue of the leaf in 2 ml of 0.2 m citrate phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 15000 rpm at 4°C for 30 min. The supernatant served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT (Giannospolitis and Ries, 1977). The assay mixture (3 ml) contained 50mM sodium phosphate buffer (pH 7.8, 13 mM methionine, 75µM NBT, 2 µM riboflavin, 0.1mM EDTA 100 µl of the enzyme extract and the riboflavin was added at the end. Tubes were shaken and placed under a 40-W fluorescent at 25°C. The reaction was initiated and terminated by turning the light on and off respectively. The absorbance at 560 nm was measured against identical non illuminated in parallel to the sample tubes for blank. Each extract was substracted from the blank and multiplied by 100 to obtain the percentage inhibition of NBT-photoreaction. The SOD activity was expressed in SOD units g⁻¹ tissue (50 per cent NBT inhibition=1 unit) (Belid et al., 1993)

3.3.7. Assay of phenol activity
One gram of the leaf samples was ground in a pestle and mortar in 10 ml of 80 per cent methanol. The homogenate was centrifuged at 10000 rpm for 20 minutes. The supernatant was evaporated to dryness and the residue was dissolved in five ml of distilled water. From this, 0.2 ml was taken and the volume was made up to three ml with distilled water. To that 0.25 ml of (1N) Folin-Ciocalteau reagent was added. After three minutes, one ml of 20 per cent sodium carbonate was added and mixed thoroughly. Thus the tubes were placed in boiling water for one minute and cooled. The absorbance was measured at 725 nm against a reagent blank. The phenol activity was expressed as µg of catechol g⁻¹ of plant tissue (Zieslin and Ben-Zaken, 1993).

3.3.8. Activity gel electrophoresis
3.3.8.1. Peroxidase (PO)
To study the expression pattern of different isoforms of peroxidases in different treatments activity gel electrophoresis was carried out. One gram of physic nut leaf tissue was homogenized in 2 ml of 0.01 M potassium phosphate buffer (pH 7.0), centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was used as enzyme source. For native anionic polyacrylamide gel electrophoresis resolving gel of 8 per cent acrylamide concentration and stacking gel of 4 per cent acrylamide concentration were prepared. After electrophoresis the gels were incubated in a solution containing 0.15 per cent benzidine in 6 per cent NH4Cl for 30 min in darkness. Then a few drops of 30 per cent H2O2 were added with constant shaking until the appearance of bands. After staining the gel was washed with distilled water and photographed (Sindhu et al., 1984).

3.3.8.2. Polyphenol oxidase (PPO)
One gram of leaf tissues were homogenized in 2 ml of 0.01 M potassium phosphate buffer (pH 7.0) centrifuged at 10000 rpm for 15 min at 480C and the supernatant was used as an enzyme source. After native electrophoresis the gel was equilibrated for 30 min in 0.1 per cent p-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in the appearance of dark brown discrete enzyme bands. After staining the gel was washed with distilled water and photographed (Jayaraman et al., 1984).

3.4. Statistical analysis
The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). Prior to statistical analysis of variance (ANOVA) the percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels (P < 0.05 and P < 0.01) and means were compared by Duncan’s Multiple Range Test (DMRT).

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