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ISSN: 0976-3031

International Journal of Recent Scientific Research
Vol. 6, Issue, 5, pp.4270-4280, June, 2015

International Journal of Recent Scientific Research

RESEARCH ARTICLE

INDUCTION OF PLANT SYSTEMIC RESISTANCE IN LEGUMES *CAJANUS CAJAN, VIGNA RADIATA, VIGNA MUNGO* AGAINST PLANT PATHOGENS *FUSARIUM OXYSPORUM* AND *ALTERNARIA ALTERNATA* – A *TRICHODERMA VIRIDE* MEDIATED REPROGRAMMING OF PLANT DEFENSE MECHANISM

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ARTICLE INFO

Article History:

Received 2nd, April, 2015
Received in revised form 10th, April, 2015
Accepted 4th, May, 2015
Published online 28th, May, 2015

Key words:

Antioxidant enzymes, Defense enzymes, Reactive oxygen species (ROS), Scavenging activity, Systemic induced resistance, *Trichoderma viride*.

ABSTRACT

Legumes rich in protein are affected by *Fusarium oxysporum* and *Alternaria alternata* causing vascular wilt and blight respectively. Exploitation of potent antagonistic microflora is now highly encouraged in effectively controlling and managing plant diseases. In the present study, an attempt is made to reprogram defense mechanism in legumes (*Cajanus cajan*, *Vigna radiata*, *Vigna mungo*) to reduce disease incidence by using *Trichoderma viride*. Significant plant systemic resistance was achieved in the above legumes against two plant pathogens. Legume seeds treated with *T. viride* showed 7.52 – 15.40% and 15.20 – 60.00% decrease in disease incidence against *F. oxysporum* and *A. alternata* respectively with highest decrease (60%) in *V. radiata* against pathogen *A. alternata.* This study clearly demonstrates the physiological stress contributed by amplification of reactive oxygen species (ROS) levels in diseased plants leading to death. However, the prior application of *T. viride* elevated the levels of ROS, which subsequently alleviate the levels of defense enzymes, antioxidant enzymes and phenols to counter the pathogen infection. This mechanism plays an important role in mitigating pathogen-induced oxidative stress in legumes.

INTRODUCTION

Legumes are a group of economically important plants that form the third largest food crop cultivated in semi-arid tropics of India. Legumes are high in protein (18-40%), folic acid, potassium, iron, magnesium, and photo-chemicals. The percent yield loss due to pests, bacteria, fungi, and virus is 40 – 60%, out of which fungal pathogens alone account for 10-25%. Legumes are affected by a number of fungal diseases; among them the major diseases are vascular wilt and blight caused by *Fusarium oxysporum* and *Alternaria alternata* respectively. Fungicides are available to manage the pathogens; however, due to increased environmental concern, fungicide resistance among pathogens and the development of oncogenic risks, the exploitation of potential antagonistic microflora is now highly encouraged in disease management. Potential antagonists such as plant growth promoting rhizobacteria, *Pseudomonas flouroscens* and fungi such as *Trichoderma*, *Gliocladium*, *Ampelomyces*, *Candida* and *Coniothyrium* are called Biological control agents. Among these potential antagonists, *Trichoderma* spp. (Contreras – Cornejo *et al*., 2009; Radjacommare *et al*., 2010; Solanki *et al*., 2011), *T. harzianum* (Singh *et al*., 2011; Haggag and Sedera, 2005; Elad *et al*., 1980; Vinale *et al*., 2008 & 2009; Akrami *et al*., 2011; Ozbay *et al*., 2004; Lo *et al*., 1997), *T. hamatum* (Haggag and Sedera, 2005), *T. koningii* (Haggag and Sedera, 2005), *T. atroviride* (Vinale *et al*., 2008 & 2009) and *T. asperellum* (Akrami *et al*., 2011) play a vital role in the biological control of soil borne plant pathogens.

Trichoderma is a secondary opportunistic invader, that colonizes the root surface, makes cause substantial changes in plant metabolism, increase nutrient availability, promote plant growth, enhance disease resistance and improve crop production (Harman *et al*., 2004). Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy. *Trichoderma* spp. was found to be effective biological inducers to induce plants own defense mechanism in coconut (Karthikeyan *et al*., 2006,) cucumber (Yedidia *et al*., 1999; 2003), cumin (Haggag and Sedera, 2005), tomato (Solanki *et al*., 2011; Vinale *et al*., 2008; Ozbay *et al*., 2004; Christopher *et al*., 2010), bean (Elad *et al*., 1980), chickpea (Raju *et al*., 2008), blackgram (Christopher *et al*., 2007; Surekha *et al*.,

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2013 & 2014), lentil (Akrami *et al*., 2011), sunflower (Singh *et al*., 2011), banana (Kavino *et al*., 2008), canola (Vinale *et al*., 2008) and pepper (Ezziyyani *et al*., 2007) against pathogens *Fusarium oxysporum* (Haggag and Sedera, 2005; Akrami *et al*., 2011; Ozbay *et al*., 2004; Christopher *et al*., 2010; Raju *et al*., 2008), *Rhizoctonia solani* (Solanki *et al*., 2011; Singh *et al*., 2011; Elad *et al*., 1980), *Botrytis cinerea* (Vinale *et al*., 2008; Brunner *et al*., 2005), *Sclerotium rolfsii* (Elad *et al*., 1980), *Phytophtora capsici* (Ezziyyani *et al*., 2007), *Pythium* (Lo *et al*., 1997) and *Macrophomina phaseolina* (Christopher *et al*., 2007).

However, very few studies have been reported on application of *T. viride* on coconut, black gram, tomato and sunflower to induce resistance against *F. solani*, *Ganoderma lucidum* and *Macrophomina phaseolina*. Hence, there is a need for application of *T. viride* on legumes (*Cajanus cajan* (red gram), *Vigna mungo* (black gram) and *Vigna radiata* (green gram)) to examine if *T. viride* reprograms defense mechanism to induce and enhance disease resistance. *T. harzianum* in an aseptic hydroponic system (Yedidia *et al*., 1999), *Trichoderma* species (*T. harzianum, T. hamatum* and *T. koningii*) in peanut haulms compost (Haggag and Sedera, 2005), *Trichoderma, T. virens, T. viride* in talc (Christopher *et al*., 2007 & 2010; Brunner *et al*., 2005) and *T. harzianum* in maize-cob (Singh *et al*., 2011) are the various formulations used to induce the self defense mechanisms in plants.

Responses activated in self defensive mechanism include generation of ROS, defense enzymes, antioxidant enzymes and phenolic substances. Increase in ROS levels (Singh *et al*., 2011), defense enzymes (Yedidia *et al*., 1999; Raju *et al*., 2008; Singh *et al*., 2009 & 2010; Surekha *et al*., 2014), antioxidant enzymes (Singh *et al*., 2009 & 2010; Surekha *et al*., 2013), phenolic substances (Shoresh *et al*., 2008; Surekha *et al*., 2014) are the potential markers observed by various groups during the enhancement of disease resistance against pathogens. One of the limitations in the above studies is that the potential markers are partly addressed as individual factor by different groups and there is no study report on all the markers which will act in a cascade mechanism when a plant reprograms its defense.

In the present study we investigated the role of *T. viride* in inducing the plant systemic resistance in legumes *C. cajan*, *V. radiata*, *V. mungo* against plant pathogens *F. oxysporum* and *A. alternata* for assessment of the interactions between host legumes and plant pathogens, evaluation of the potential role of *T. viride* to manage fusarial wilt and blight in legumes and understanding the role of *T. viride* to enhance disease resistance mechanisms in legumes against plant pathogens.

MATERIALS AND METHODS

Fungal culture and maintenance

Cultures of filamentous fungi - *Trichoderma viride* (NCIM 1053) and two virulent cultures of *Fusarium oxysporum* (NCIM 1072) and *Alternaria alternata* (NCIM 718) causing wilt and blight in legumes were obtained from National collection of Industrial microorganisms (NCIM), National chemical laboratory (NCL), Pune, India. These fungi were cultured on Potato dextrose agar slants and maintained in an environmental chamber set at 28 ± 2°C, 90% relative humidity and 16:8 h light: dark regime. An aqueous conidial suspension of 10^6 condia/ml containing 105 mg/ml Tween 80 was prepared from 14 days old culture (Nageswara Rao Reddy, 2006). Conidial viability was tested and used for further experiments if the conidial germination was more than 95%. Cultures were initiated by inoculating 1 ml of conidial suspension containing 10^7 conidia in 250 ml of Potato dextrose broth for further experiments.

Mass multiplication of biocontrol agent

The mass inoculum of *T. viride* was raised in Erlenmeyer flasks (250 ml) containing groundnut husk in distilled water and autoclaved for two consecutive days for 1 h at 121°C. Each flask was inoculated separately with the fungal strain and incubated at 28±2°C for 7 days. The conidia of *T. viride* was separated and quantified using a hemocytometer and the population was set to 1×10^6 spores/ ml to be used for the seedling treatments.

Assessment of the role of T. viride to enhance disease resistance in legumes against plant pathogens

Seeds of *Cajanus cajan* (L.) var LRG 41, *Vigna radiata* var LGG 460, *Vigna mungo* LBG 623 were surface sterilized with ethanol (70% v/v) for 5 min, followed by mercuric chloride (0.1%) for 4 min and rinsed five times with sterile distilled water. Sterilized seeds were left overnight for soaking in sterile distilled water. The overnight soaked seeds were pretreated with *T. viride* spore suspension (10^6conidia/ml) for 30 min, blot dried and sown in seedling trays (70 plugs), with each plug measuring 4×4.5×2.5 cm. Equal number of seeds were sown as controls without pretreatment with *T. viride.* The seeds were left for germination and growth for one week. One week old plants with and without pretreatment with *T. viride* were exposed to *F. oxysporum* spores (with 10^6conidia/ml at the root of the plant) and *A. alternata* spores (with 10^6 conidia/ml on plant) and observations were noted down. Seeds without pretreatment with *T. viride* which were exposed to *F. oxysporum* and *A. alternata* are observed to assess the interactions between host legumes and plant pathogens. The legume seeds pretreated with *T. viride* followed by exposure to *F. oxysporum* and *A. alternata* are observed for the role of *T. viride* to enhance disease resistance in legumes against plant pathogens. VI and DI; antioxidant enzymes and scavenging activity of ROS by antioxidant enzymes and defense enzymes are the parameters used to assess the interactions between host legumes and plant pathogens and the role of *T. viride* to enhance disease resistance in legumes against plant pathogens.

Assessment of Vigour Index (VI) and Disease Incidence (DI)

VI and DI were used to assess the wilt and blight disease respectively. Plant growth parameters namely root length (cm),

shoot length (cm), germination percentage was used to calculate the VI and DI. VI was calculated using the formula: Root length (cm) + Shoot length (cm) x germination percentage. DI was calculated using the formula (n/N) x 100, where n= number of diseased plants and N= Total number of plants investigated (Singh *et al*., 2011).

Assessment of antioxidant enzymes and their scavenging activity

Antioxidant enzymes activity

One week old plants from various treatments were collected for assaying antioxidant enzyme activity. One gram of leaf was homogenized in 10 ml ice-cold 50 mM potassium phosphate buffer (pH 7.8) in pre-chilled mortar and pestle. Further homogenate was centrifuged at 5,000 rpm for 10 min at 4°C in a refrigerated centrifuge. The supernatant was used as an enzyme source within 12 h of extraction for assaying Superoxide dismutase (SOD), Catalase (CAT) and Ascorbic acid oxidase (AOX).

SOD was estimated as per the procedure of Fridovich (1997). The reaction mixture consists of 3 ml 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 0.1 mM ethylene diamine tetra acetic acid (EDTA), 75 µM nitroblue tetrazolium (NBT) and 100µlof crude enzyme extract. A blank (without enzyme and NBT) and a reference control having NBT but no enzyme were setup to calibrate the spectrophotometer. All the tubes were exposed to 400W bulbs (4×100W bulbs) for 15 min and the absorbance was immediately read at 560 nm using a spectrophotometer. The percentage inhibition is calculated and 50% inhibition of the reaction between riboflavin and NBT in the presence of methionine is taken as 1 unit of SOD activity. The enzyme activity was expressed as units mg^{-1} of protein. CAT was estimated according to the method of Radhakrishnan and Sarma (1964). The reaction mixture consists of 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.5) and 2.5 ml of 0.9% (v/v) Hydrogen peroxide (H_2O_2). To this mixture 0.5 ml of enzyme was added and incubated at 28^oC for 3 min. The reaction was then arrested by adding 0.5 ml of 2 N Sulphuric acid (H_2SO_4) and the residual H_2O_2 was titrated with 0.1 N Potassium permanganate ($KMnO_4$) solution. A blank experiment was carried out similarly with boiled enzyme extract. Unit of CAT activity was expressed as ml of 0.1 N $KMnO_4$ equivalent of H_2O_2 decomposed/min/mg of protein.

AOX was estimated by spectrophotometric method given by Oberbacher and Vines (1963). To 3 ml of substrate (8.8 mg of ascorbic acid in 300 ml phosphate buffer, pH-5.6) solution, add 0.1 ml enzyme extract and measure the absorbance change at 265 nm in 30 sec intervals for 2 min. Ascorbic acid has an $E_{1cm}$of 760 at 265 nm and absorbance of 4.4 per µmole in 3 ml volume. One enzyme unit (0.81µmole ½ oxygen per min) is equivalent to absorbance change of 3.58 per min.

Scavenging activity by antioxidant enzymes

Scavenging activity by antioxidant enzymes for superoxide ($O_2^{\cdot-}$) radical, H_2O_2 radical, hydroxyl radical ($OH^{\cdot}$) radical and lipid peroxidation were estimated.

One week old plants from various treatments were collected for assaying scavenging activity by antioxidant enzymes. One gram of leaf was thoroughly cleaned, homogenized with 10ml of 80% ethanol at 4°C, centrifuged for 2min at 10,000rpm and supernatant was used as crude extract for further experiments.

$O_2^{\cdot-}$ radical scavenging activity was estimated according to the method of Sabu and Ramadasan (2002). The reaction mixture consists of 1 ml of 125 mM sodium carbonate, 0.4 ml of 25 µM NBT and 0.2 ml of 0.1mM EDTA. Reaction was initiated by adding 0.4 ml of 0.1mM hydroxylamine hydrochloride, 0.5 ml of plant extract and incubated for 5 min at room temperature. The absorbance was measured at 560 nm against a blank sample in a spectrophotometer. The percentage of O^{2-} scavenging is calculated as: % scavenged $O_2^{\cdot-}$= (A_c-A_t/A_c) x 100, where A_c is absorbance of control and A_t is absorbance of test.

H_2O_2 radical scavenging activity was assessed according to the method of Ruch *et al* (1989). To 100µl plant extract, 1 ml of H_2O_2 (40mM H_2O_2 is prepared in 50mM phosphate buffer, pH 7.4) is added. After 10 min, absorbance at 230 nm is determined against a blank solution containing phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging is calculated as: % scavenged H_2O_2 = (A_c-A_t/A_c) x 100, where A_c is absorbance of control and A_t is absorbance of test.

$OH^{\cdot}$ radical scavenging activity was assessed according to the method of Kunchandy and Rao (1990). The reaction mixture (1 ml) consists of 500 µl of plant extract, 100 µl of 28 mM deoxyribose in 20 mM KH_2PO_4-KOH buffer (pH 7.4), 200 µl of premixed solution (200 µM ferrous ammonium sulfate and 1.04 mM EDTA (1:1 v/v)), 100 µl of 1.0 mM ascorbic acid, 100 µl of 1.0 mM H_2O_2 and incubated at 37^0C for 1hr. To the above mixture, 1 ml of 2.8% trichloroacetic acid (TCA) and 1.0 ml of 1.0 % aqueous solution of thiobarbituric acid (TBA) are added. The samples were then vortexed and heated in water bath at 100^0C for 20 min to develop the color. The absorbance is measured at 532 nm against an appropriate blank sample. The percentage of $OH^{\cdot}$ scavenging is calculated as: % scavenged $OH^{\cdot}$ = (A_c-A_t/A_c) x 100, where A_c is absorbance of control and A_t is absorbance of test. Lipid peroxidation was assessed according to the method of Heath and Packer (1968). Plant extract of 1.0 ml was incubated with 4.0 ml of 20% TCA containing 1% TBA for 30 min at 95°C. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at 10,000g for 15 min. The reaction product was measured at 532nm and the concentration of malondialdehyde (MDA) was determined using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol ml^{-1}.

MDA equivalents (nmol.ml^{-1}) = 1000[$(A_{532}- A_{600})$/155]

Where A_{532} represents the maximum absorbance of the TBA-MDA complex, A_{600} is the absorbance correction for non-specific turbidity.

Plant defense enzymes and total phenols

The effect of the induction of plant defense system was evaluated by estimating peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and total phenols

(TP). Leaves of four plant samples from each replicate of all legumes were collected after one week from various treatments and used for analysis. Leaf samples (1gm fresh wt.) were washed under running tap water, dried gently and ground with a mortar and pestle in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0). Sample was centrifuged at 12,000 rpm for 15 min at 4^{o}C and the supernatant was used as the enzyme source.

PO activity was assessed according to the method of Hammerschmidt *et al* (1982). The reaction mixture consists of 1.5 ml of 0.25 percent (v/v) guaiacol in 0.01 M sodium phosphate buffer (pH 6.0) and 0.5 ml of 0.1 M H_2O_2. Enzyme extract (0.1 ml) was added to initiate the reaction and the changes in absorbance at 420 nm were recorded at 30 s intervals for 3 min and boiled enzyme preparation was used as blank. The enzyme activity was expressed as the changes in absorbance min^{-1} mg^{-1} protein.

PPO activity was assessed according to the method of Mayer *et al* (1965). To 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) 0.2 ml of the enzyme extract was added. Reaction was initiated by adding, 0.2 ml of 0.01 M catechol and the activity was expressed as the change in absorbance at 495 nm min^{-1} mg^{-1} of protein.

PAL activity was assessed according to the method of Ross and Sederoff (1992). The reaction mixture consists of 100 µl of enzyme, 500 µl of 50 mM Tris hydrochloric acid (pH 8.8), 600 µl of 1 mM L-Phenylalanine. Reaction mixture was incubated for 60 min and the reaction was arrested by adding 2 N hydrochloric acid (HCl). Later, 1.5 ml of toluene was added, vortexed for 30 sec, centrifuged at 1000 rpm for 5 min and toluene fraction containing trans cinnamic acid was separated. The toluene fraction was measured at 290 nm against the blank of toluene. Standard curve was drawn with graded amounts (µg) of cinnamic acid in toluene. The enzyme activity was expressed as in µg of cinnamic acid min^{-1} mg^{-1} protein. TP were assessed according to the method of Singelton *et al* (1999). Fresh leaf sample of 0.5 g is homogenized in 10-times volume of 80 % methanol (v/v), vortexed for 15 min and homogenate was centrifuged at 10,000 rpm for 20 min. Supernatant was then dried, dissolved in 5 ml of distilled water. To 0.5 ml supernatant, 3 ml of water, 0.5 ml of Folin-Ciocalteu reagent was added and incubated for 3 mins. To this, 2 ml of 20% sodium carbonate was added, mixed thoroughly and boiled in a boiling water bath for one min. Absorbance of the developed blue color was measured at 650 nm and were calculated from catechol standard graph (µg). The amount of phenolics is expressed as µg catechol.

Statistical Analysis

The sample data was collected for the effect of the survival of plants; interactions between host legumes and plant pathogens; role of *T. viride* to enhance disease resistance in legumes against plant pathogens. A minimum of three plants were evaluated for each replicate. The data of estimates of VI, DI, antioxidant enzymes (SOD, CAT and AOX), total antioxidant activity, scavenging activity by antioxidant enzymes for $O_2^{\cdot-}$ radical, H_2O_2 radical, $OH^{\cdot}$ radicals, lipid peroxidation PO, PPO, PAL and TP was used to estimate mean and standard error. The data were analyzed by one way analysis of variance (ANOVA) to assess the significance of the various effects.

RESULTS

Assessment of the effect of plant pathogen stress on host legumes

Defense enzymes, antioxidant enzymes and scavenging activity are the parameters taken into consideration for evaluating the effect of pathogens stress on survival of plants.

Defense enzymes (PO, PPO, PAL) and phenol content increased above normal levels in plants which were exposed to pathogens when compared to unexposed plants. Antioxidant enzymes (AOX, CAT, SOD) and scavenging activity of ROS species ($O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$) decreased. Whereas, lipid peroxidation increased in plants exposed to pathogens when compared to unexposed plants (Table I). All the genotypes used in this study showed the same response with all activities.

Table 1 Effects of various treatments based on parameters: survival rate, defense enzymes, phenol, antioxidant enzymes, scavenging activity of ROS species and lipid peroxidation to establish the role of *T. viride* in enhancing disease resistance (p – value < 0.001).

Legumes	Parameters		F
V. radiata	Survival rate	Disease Index	192.86
	Defense enzymes	PO Activity	255.92
		PPO Activity	1001.46
		PAL Activity	11186.02
		Total phenol	1316.43
	Antioxidant enzymes	AOX Activity	99.19
		CAT Activity	359.01
		SOD Activity	234.50
	Scavenging activity	$O^{\bullet}_2$ scavenging	19662.16
		H_2O_2 scavenging	2910.24
		OH radical scavenging	5598.15
		Lipid peroxidation	4932.29
V. mungo	Survival rate	Disease Index	13730.32
	Defense enzymes	PO Activity	312.16
		PPO Activity	1711.64
		PAL Activity	10995.43
		Total phenol	709.03
	Antioxidant enzymes	AOX Activity	349.97
		CAT Activity	1898.93
		SOD Activity	518.21
	Scavenging activity	$O^{\bullet}_2$scavenging activity	2897.71
		H_2O_2 scavenging	1526.08
		OH radical scavenging	1898.93
		Lipid peroxidation	1082.91
C. cajan	Survival rate	Disease Index	7506.16
	Defense enzymes	PO Activity	110.69
		PPO Activity	2990.40
		PAL Activity	15040.95
		Total phenol	1739.68
	Antioxidant enzymes	AOX Activity	1277.93
		CAT Activity	4.36.95
		SOD Activity	586.55
	Scavenging activity	$O^{\bullet}_2$scavenging activity	2448.09
		H_2O_2 scavenging	3401.45
		OH radical scavenging	5915.25
		Lipid peroxidation	4186.64

Increase in defense enzymes, lipid peroxidation and decrease in antioxidant enzymes and their scavenging activities in plants exposed to pathogens when compared to unexposed plants revealed the plant pathogen stress on host.

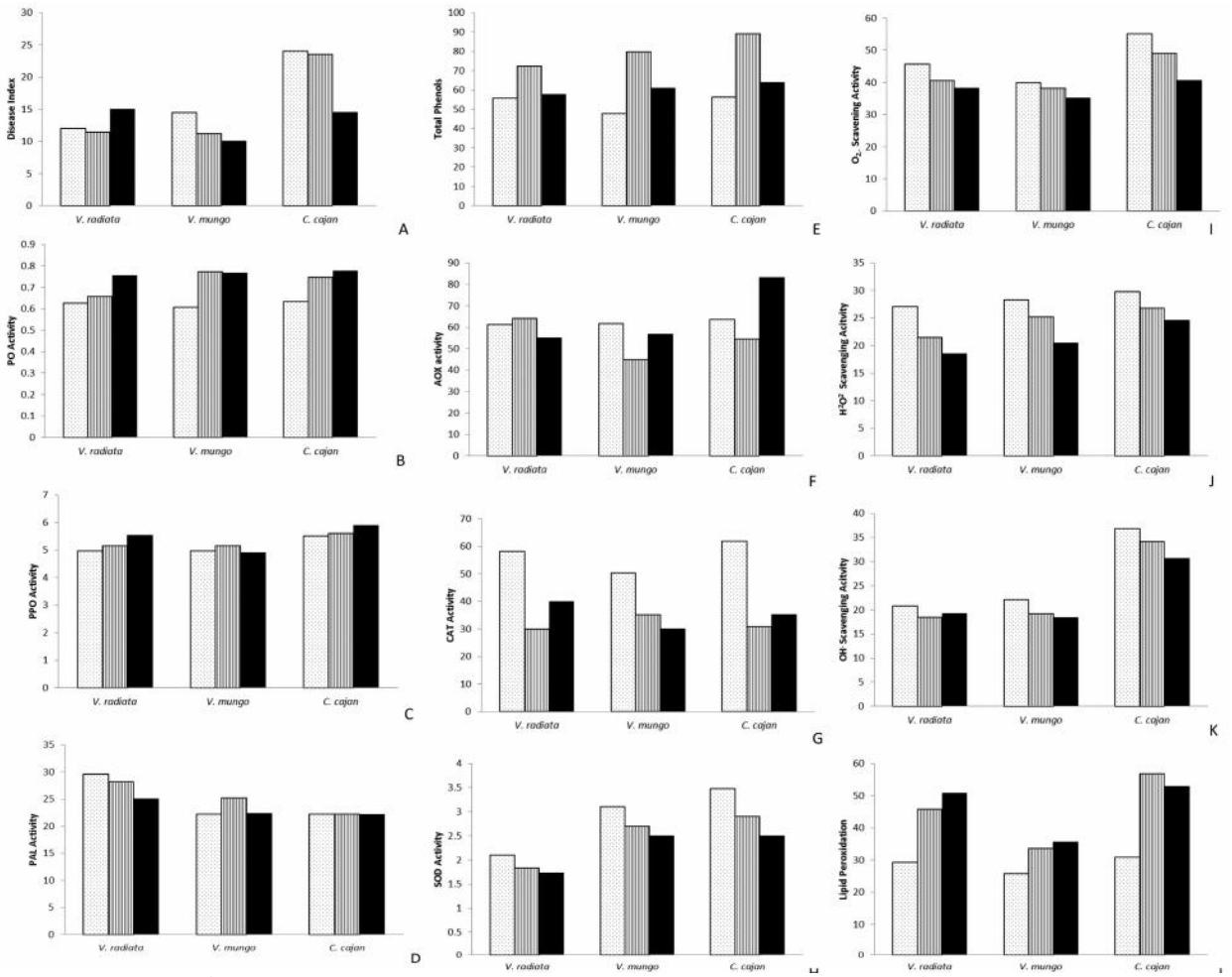

Figure 1 Survival rate (A), defense enzymes (B-D), phenols (E) antioxidant enzymes (F-H), scavenging activity of ROS species (I-K) and lipid peroxidation (L) of plants *C. cajan*, *V. radiata*, *V. mungo* are exposed to pathogens *F. oxysporum* and *A. alternata* and unexposed (control) .

Effects of T. viride treatment on the survival of plants

Survival rate was more in healthy plants when compared to plants that are exposed to pathogens *F. oxysporum* and *A. alternata*. At the same time, survival rate was also more in plants which were pretreated with *T. viride*, followed by exposure to pathogens when compared to plants that were directly exposed to pathogens. VI and DI were the parameters taken into consideration for evaluating the effect of treatment with *T. viride* on survival of plants. Gradual increase in VI and decrease in DI (excepting DI of *V. radiata* with *A. alternata* and *C. cajan* with *F. oxysporum*) was observed over a period of 15 days (Table I).

VI increased and DI decreased in healthy plants than those which are exposed to both the pathogens in different percentages. VI was high and DI was low in all *T. viride* pretreated plants when compared to their controls with highest VI and lowest DI in *V. mungo.* DI decreased in all *T. viride* pretreated plants on 7^{th} day and 14^{th} day of infection with more decrease on 14^{th} day. Decrease in VI when healthy plants are exposed to pathogens and increase in VI when plants were pretreated with *T. viride* and exposed to pathogens support the role of *T. viride* to enhance disease resistance in legumes against plant pathogens. Increase in DI when healthy plants are exposed to pathogens and decrease in DI when plants were pretreated with *T. viride* when exposed to pathogens confirm the role of *T. viride* to enhance disease resistance in legumes against plant pathogens.

Assessment of the role of T. viride to enhance disease resistance in legumes against plant pathogens

Defense enzymes, antioxidant enzymes and scavenging activity of the antioxidant enzymes are the parameters taken into consideration for evaluating the role of *T. viride* to enhance disease resistance in legumes against plant pathogens.

Defense enzymes (PO, PPO, PAL), phenol content, antioxidant enzymes (AOX, CAT, SOD), scavenging activity of ROS species ($O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$) by antioxidant enzymes were high and lipid peroxidation levels were low in plants which were treated with *T. viride* when compared to non-treated (healthy) plants (Table I). This provides evidence for the role of *T. viride* in enhancing defense in legumes against plant pathogens.

There is decrease in levels of defense enzymes, phenol content, antioxidant enzymes and increased scavenging activity along with increased lipid damage in case of *T. viride* pretreated plants which are exposed to pathogens when compared to *T. viride* treated healthy plants.

The present study clearly indicates that *T. viride* induces disease resistance in legumes against pathogens by reprogramming oxidant and antioxidant metabolites, oxidant proteins and defense enzymes.

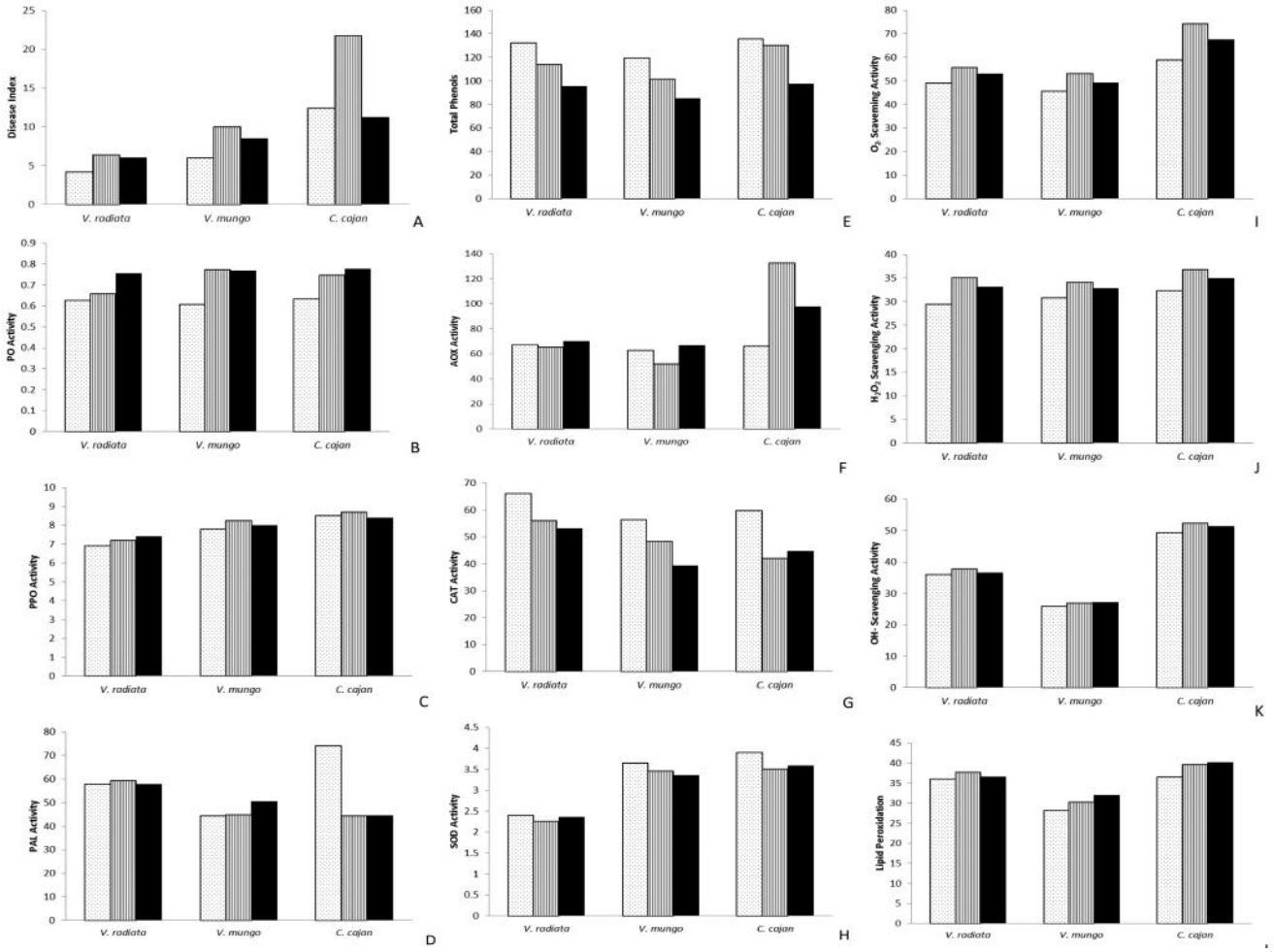

Figure 2 Survival rate (A), defense enzymes (B-D), phenol (E) antioxidant enzymes (F-H), scavenging activity of ROS species (I-K) and lipid peroxidation (L) of plants *C. cajan*, *V. radiata*, *V. mungo* are treated with *T. viride* and then exposed to pathogens *F. oxysporum* and *A. alternata* and unexposed (control) .

Plants which are pretreated with *T. viride* and exposed to pathogens showed increase in levels of defense enzymes, phenol content, antioxidant enzymes, scavenging activity by antioxidant enzymes and lipid peroxidation activities when compared to plants that were directly exposed to pathogens (Table I). The above observations further corroborate the role of *T. viride* to enhance disease resistance in legumes against plant pathogens.

DISCUSSION

Inducing systemic resistance in plants by prior application of BCAs is a novel plant protection strategy (Singh *et al*. 2010; Kashyap and Dhiman 2009). Little information is available on BCAs enhancing defense in host against pathogen by modulating oxidant and antioxidant metabolites, oxidant proteins, defense enzymes and antimicrobial peptides.

Assessment of the effect of plant pathogen stress on host legumes

The interaction between host and pathogen includes recognition of the host, attack and subsequent penetration and killing by secretion of cell wall degrading enzymes (CWDE) that hydrolyze the cell wall of the host. The host recognition of pathogen derived elicitors cause a rapid depolarization of the electrical potential of the plasma membrane. This depolarization is associated with an efflux of K^+ ions and influx of proteins, leading to alkalization of the extracellular space. Influx of another ion Ca^{2+} in cytoplasmic spaceis connected with activation of calmodulin. Flux of ions generate oxidative burst of ROS such as $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ radicals from plasma membrane bound NADPH oxidase (Bruxelles and Roberts 2001).

Ion fluxes as well as ROS are connected to the regulatory process of protein phosphorylation by specific protein kinases and of protein dephosphorylation by specific protein phosphates. The reversible phosphorylation allows fast and specific signal transduction mechanism of extracellular stimuli to the cytosol and nucleus.

the cells of diseased plants i.e. increased ROS levels and decreased scavenging activity in all legumes when treated with both pathogens. The decrease in scavenging activity by antioxidant enzymes and overproduction of ROS amplifies ROS levels in the cells of the host. This ROS may cause oxidative damage, leading to lipid peroxidation and damage of

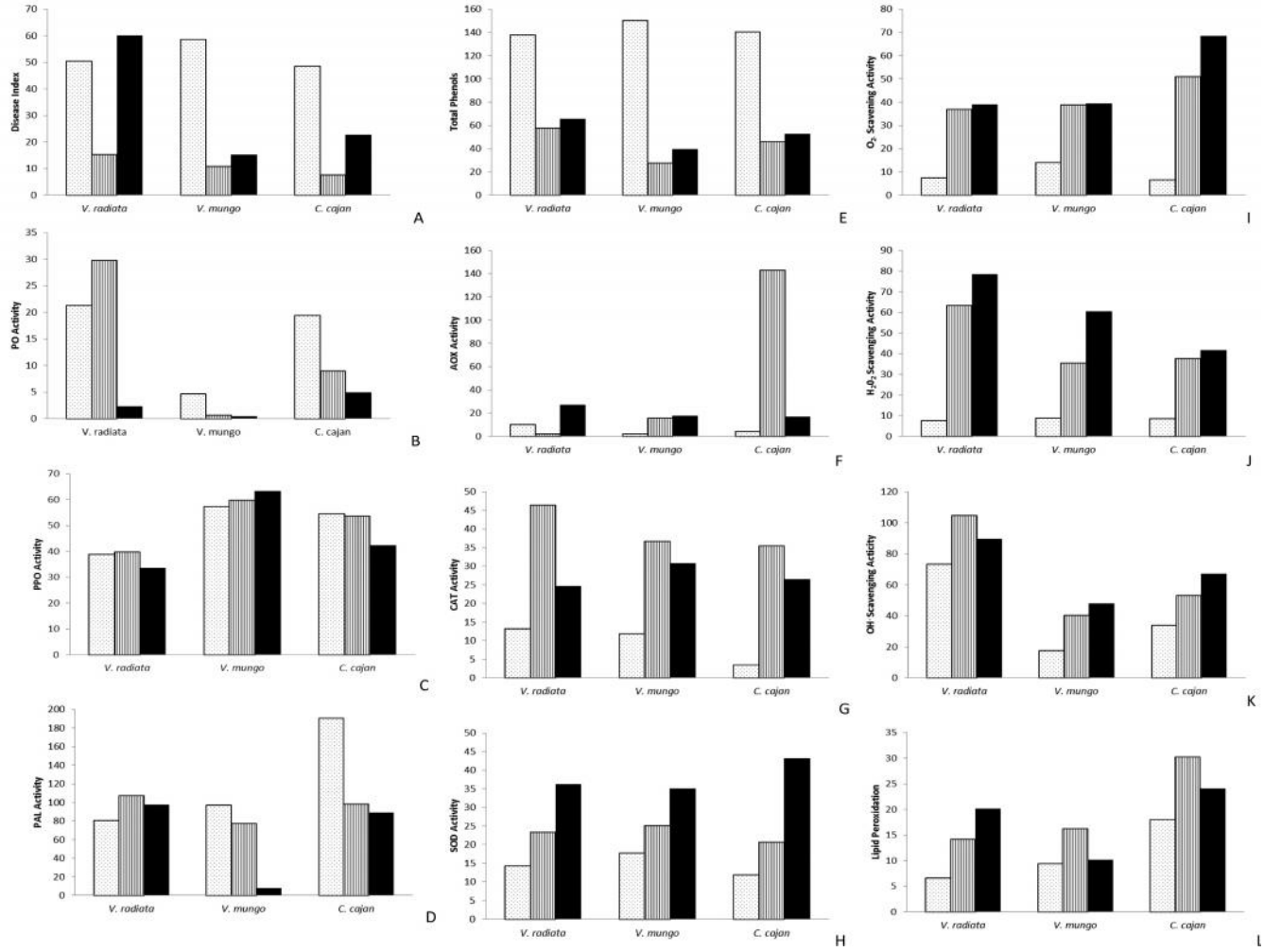

Figure 3 Changes in survival rate (A), defense enzymes (B-D), phenol (E), antioxidant enzymes (F-H), scavenging activity of ROS species (I-K) and lipid peroxidation (L) of plants *C. cajan*, *V. radiata*, *V. mungo* are treated with *T. viride* and then exposed to pathogens *F. oxysporum* and *A. alternata* and unexposed (control) .

One particular signal transduction mechanism activates mitogen activated protein kinase (MAPK) cascade (Zhang and Klessig 2001; Morris 2001). Plants possess an array of antioxidant enzymes (CAT, SOD, GPx, APx) and defense enzymes (PO, PPO, PAL) that can protect the cells from oxidative damage and pathogen. The activation of MAPK leads to phosphorylation of transcription factors, which in turn, activate gene expression of antioxidant enzymes and defense enzymes.

Antioxidant enzymes are produced by host plant, which work together with defense enzymes to promote the scavenging activity of ROS (Brunner *et al*. 2005; Singh *et al*. 2009) thereby inducing resistance against pathogen. Generally, an appropriate intracellular balance is reported between ROS generation and scavenging activity in the cells of healthy plants (Singh *et al*. 2011). In the present study, we report intracellular imbalance between ROS generation and scavenging activity in

such as pigments, proteins, nucleic acids & carbohydrates (Brunner *et al*. 2005; Singh *et al*. 2009), affecting the integrity of cell membrane and inactivating key cellular functions (Singh *et al*. 2009).

Defense related enzymes (PO, PPO, PAL) and phytoalexins (phenols) are induced against pathogen attack to protect host. Of these, the early induction of PAL is more important, since it is the first enzyme in the phenylpropanoid pathway that leads to production of phenolic substances such as phytoalexins, furanocoumarins, phytoanticipins and structural barriers like callose deposition (Shoresh *et al*. 2010). PPO itself act as inhibitory, and oxidize phenolic compounds to quinones which are often more toxic to microorganisms than original phenols (Vidhyasekaran 1988). Phenolic compounds act as antimicrobial, structural barriers, growth inhibitors of invaders,

modulators of pathogenicity and activators of plant defense genes (Singh *et al*. 2010; Surekha *et al*., 2014).

Phenolic monomers form a highly branched heterogenous polymer lignin in the presence of PO which is insoluble and rigid found in the secondary cell wall of plants, providing excellent physical barrier against invading pathogen. Phytoalexins (isoflavonoid like compounds) disrupt pathogen metabolism or cellular structure of pathogens. Furanocoumarins (flavonoid like compounds) are activated by ultraviolet light and can be highly toxic, contributing to rapid cell death (Freeman and Beattie 2008).

Hypersensitive response due to overproduction of ROS in plants challenged by pathogens creates imbalance between ROS generation and scavenging activity which was able to overcome slightly increased activity of defense related enzymes and phenolic substances. Thereby inducing rapid and localized death of plants tissues at the site of infection – necrosis (Brunner *et al*. 2005; Nanda *et al*. 2010) finally leading to the death of the plant.

Effects of T. viride treatment on the survival of plants

Legumes seeds coated with *T. viride* showed 7.52 – 15.4% decrease in DI against *F. oxysporum* and 15.2 – 60% decrease in DI against *A. alternata*. Highest decrease in DI was 60% in *V. radiata* against pathogen *A. alternata.* This is a significant achievement when compared to other studies conducted till date, as we have achieved induction of plant systemic resistance by using *T. viride* in three legumes - *C. cajan, V. radiata, V. mungo* against two plant pathogens - *F. oxysporum, A. alternata*. Similar results were achieved by many groups ranging from 20 – 79%, but *T. viride* was initially grown on special sources or made with formulation like chitin (Solanki *et al*. 2011; Christopher *et al*. 2007), maize cob (Singh *et al*. 2011), peanut haulms (Haggag and Sedera 2005), water-oats-vermiculite (Kavino *et al*. 2008), coir and rock wool (Ozbay *et al*. 2004). Though there was little information on inducing systemic resistance in *V. radiata, V. mungo* (Raju *et al*. 2008; Christopher *et al*. 2007; Surekha *et al*., 2013 &2014) and controlling *F. oxysporum* (Haggag and Sedera 2005; Akrami *et al*. 2011; Ozbay *et al*. 2004; Christopher *et al*. 2010; Surekha *et al*., 2013 &2014), to our knowledge this was the first case study conducted on inducing systemic resistance in *C. cajan* using *T. viride* to control pathogens.

Assessment of the role of T. viride to enhance disease resistance in legumes against plant pathogens

The probable biocontrol mechanism that *T. viride* utilizes is mycoparasitism (Papavizas 1985; Harman and Kubicek 1998; Howell 2003). The host recognition of *T. viride* elicitors initiates early signaling events such as protein phosphorylation / dephosphorylation, ion flux and accumulation of ROS. Increased levels of antioxidant enzymes (CAT, SOD, GPx, APx), defense enzymes (PO, PPO, PAL) and phenols (phytoalexins, furanocoumarins, lignin) are produced by host plant against *T. viride*. *T. viride* establishes itself as an endophyte in plant acting as PGPF (Mandal *et al*. 2009). Plant growth promoting fungi (PGPF) act as a biofertilizer stimulating growth hormones and help in nutrient acquisition to promote plant growth (Almeida *et al*. 2007). Consequences of the interaction between *T. viride* and plants help legumes to grow stronger and be well prepared for pathogen attack.

When plants that are pretreated with *T. viride* are exposed to pathogens, lignin that is already induced against *T. viride* may act as the first structural physical barrier against pathogen. ROS and other phenolic substances such as phytoalexins, furanocoumarins which are already present in the host may contribute to death of the invading pathogen. *T. viride* when recognizes the pathogen attack, penetrates and kills pathogen by secreting cell wall degrading enzymes (Radjacommare *et al*. 2010; Lorito *et al*. 1993), hydrolyzing the cell wall of the fungus the phenomenon which is known to be mycoparasitism (Kubicek *et al*. 2001). These interactions trigger an array of events such as increase in oxidant metabolites (ROS), antioxidant enzymes and antioxidant compounds (Ramamoorthy *et al*. 2002). In the present study we report an increase in oxidant metabolites (ROS), antioxidant enzymes and antioxidant compounds. Oxidant metabolites (ROS) and antioxidant compounds counter pathogen, and antioxidant enzymes scavenge the excess ROS in the host to maintain the balance.

In the present study we also report intracellular imbalance between ROS generation and scavenging activity (i.e. increase in scavenging activity of antioxidant enzymes) in the cells of plants pretreated with *T. viride* and exposed to pathogens when compared to other treatments making the host less susceptible to pathogens. Thus, *T. viride* upon interaction with legume promotes plant growth, induce defense molecules and subsequently amplify defense molecules in the host when it encounters pathogen.

CONCLUSION

The findings of this study suggest that legume seeds treated with *T. viride* induce systemic resistance by reprogramming defense mechanisms in legumes. Reprogramming alleviated the levels of defense enzymes (PO, PPO and PAL), ROS ($O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$), antioxidant enzymes (CAT, SOD), scavenging activity of antioxidant enzymes in response to oxidative stress induced by *F. oxysporum* and *A. alteranata*. This mechanism helps in developing resistance in plants and thereby protect from pathogens.

Future Work

Legume seeds treated with *T. viride* induce antimicrobial peptides, phenolic substances (phytoalexins, furanocoumarins, lignins) along with defense enzymes, ROS, antioxidant enzymes (CAT, SOD) in the plants, contributing to death of the invading pathogen. Establishing the levels of antimicrobial peptides, phytoalexins, furanocoumarins and lignins in response to pathogen attack and their role leading to the death of the pathogen is to be unraveled.

Acknowledgments

GSR, NNRR and CHS are thankful to GITAM University for providing all the necessary facilities to carry out the research. The authors also thank Prof. I. Bhaskar Reddy and Dr.M.Rama Rao for his constant support throughout the research work. We profusely thank Dr. D. Govinda Rao for his language help, stringent review and critical comments which really helped us to improve our data presentation and discussion of the manuscript.

Conflict Of Interest

There is no actual or potential conflict of interest including financial, personal or other relationships with other people or organizations.

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How to cite this article:

Ch. Surekha *et al*., Induction of Plant Systemic Resistance in Legumes Cajanus Cajan, Vigna Radiata, Vigna Mungo Against Plant Pathogens Fusarium Oxysporum and Alternaria Alternata – a Trichoderma Viride Mediated Reprogramming of Plant Defense Mechanism. *International Journal of Recent Scientific Research Vol. 6, Issue, 5, pp.4270-4280, June, 2015*

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