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Induction of systemic resistance in cowpea plants treated with *Pseudomonas fluorescens* and neem cake

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Abstract
An eco-friendly management strategy for this disease, *Pseudomonas fluorescens* and neem cake were tested individually and in combination against *Macrophomina phaseolina* under pot culture conditions. Among the various treatments tested, the combination treatment (T_6) involving *P. fluorescens* as seed and soil application (@10 gm/kg of seed and 2.5 kg/ha) plus neem oil cake as soil application @ 0.25 t/ha recorded induction of higher levels of defense enzymes and phenol than other treatments confirming the role of ISR in the disease suppression.

Keywords: Systemic resistance, cowpea plants, *Pseudomonas fluorescens,* neem cake

Introduction
Cowpea is affected by many diseases caused by viruses, bacteria, fungi and nematodes (Emechebe and Lagoke, 2002) [13]. Among the diseases, charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goid is an important fungal disease that significantly reduces growth and yield in arid regions of the world (Marroni, 2015) [30]. In India, cowpea and other pulse crops are mostly cultivated under rainfed condition accounting above 78% of area and being a tropical environment, favours the disease incidence. *M. phaseolina* attacks crop plants at different stages of plant growth and causes complex disease syndromes like root rot, seedling blight, charcoal rot, ashy stem blight, wilt, collar rot, dry rot, pod rot and seed rot in several crops (Ma *et al.*, 2010) [29].

The most common method for control this pathogens is the use of fungicides. The existence and development of drug-resistant strains also poses serious problems in formulating fool-proof control agents. Hence, much attention is being focused on the alternative methods of pathogen control which are eco-friendly and enhance crop yield. Thus, crop improvement and disease management have to be achieved with the use of bio-resources such as antagonistic microbes, plant based products *etc*. by replacing chemical pesticide and fertilizers. Biological control of plant pathogens is a potential non-chemical means and known to be cheap and effective eco-friendly method for the management of crop diseases (Harman, 1991) [19]. Among the biological methods, the use of plant growth-promoting rhizobacteria (PGPR) would be an attractive alternative to decrease the use of chemical fungicide which also effect environmental pollution (Ali *et al.*, 2010) [2]. In the context of the international concern for food and environmental quality, PGPR's have been applied to various crops to suppress pathogens, enhance seedling emergence, crop growth and yield (Minaxi and Saxena, 2010; Misk and Franco, 2011) [34, 35]. Several authors have reported about the induction of defense enzymes in crop plants treated with biocontrol agents and challenged with the pathogen (Jayalakshmi *et al.*, 2009; Loganathan *et al.*, 2010; Ardebili *et al.*, 2011) [20, 28, 4].

Besides the use of PGPR's plants and their constituents are generally less phytotoxic, systemic, easily degradable and do stimulate host metabolism. It has been demonstrated that many plant products and plant essential oils are effective antimicrobials against various pathogens (Grayer and Harborne 1994; Lawson and Kennedy, 1998) [15, 27] and especially, some plant products have shown antifungal activity and induce the systemic resistance against *M. Phaseolina* and suppressed root rot disease (Karthikeyan *et al.*, 2006; Dhingani *et al.*, 2013) [24, 10]. Several studies were reported that plant based oil cakes *viz*., cotton cake (Ehteshamul-Haque *et al.*, 1995) [12], pungam cake (Karthikeyan *et al.* 2006) [24], mustard cake and cotton cake (Anis *et al*., 2010) [3] and neem cake (Meena *et al*., 2014) [33] and onion bulb extract (Savaliya *et al*., 2015) [42] against *M. phaseolina* causing root rot of various crops has also been well documented.

Therefore, the present study was undertaken to investigate the effect of combined application of *P. fluorescens* and neemcake on induction of systemic resistance in cowpea plants for managing root rot disease.

Materials and methods

Sterilized soil (1.0 kg) was mixed with the pathogen inoculums @ five per cent level (multiplied on sand maize medium) and filled in 15 x 30 cm dia. earthen pots. Liquid formulation of the antagonist was applied to the soil 10 days before sowing as per the treatment schedule. In addition the organic amendment neem oil cake is added to the treatments in order to provide a food base for the antagonists applied through dual delivery systems.

The treatment schedule followed is mentioned below.

T1: Seed treatment with *P. fluorescens* (Pf_2) @ 10 gm/kg of seeds
T2: Soil application with *P. fluorescens* (Pf_2) @ 2.5 kg/ha
T3: T1+T2
T4: Soil application with Neem oil cake @ 0.25 t/ha
T5: T2 + T4
T6: T3 + T4
T7: Seed treatment and soil drenching of Carbendazim 50 WP 2g/kg and 0.1%)
T8: Inoculated control
T9: Healthy Control

The experiment was conducted in a randomized block design and replicated thrice. Soil drenching with carbendazim @ 0.1% and seed treatment with carbendazim @ 4 g/kg of seeds was used for comparison and pathogen alone inoculated pots served as control. The treated seeds were sown in pathogen inoculated soil @ 5 seeds per pot and maintained with need based irrigation following all standard agronomic practices.

Collection of plant samples

The cowpea seeds pre-treated with bioformulation were sown in earthen pots. At 15 DAS the pots were challenge inoculated with the pathogen and also treated with the biocontrol agents. The samples were collected starting from zero to nine days after challenge inoculation of the pathogen. Four plants were sampled from each replication of the treatment separately and used for analysis.

Enzyme extraction

One g of root sample was homogenized with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4oC. The homogenate was centrifuged for 20 min. at 10,000 rpm. Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used for the estimation of Peroxidase (PO), Polyphenol Oxidase (PPO), and Phenylalanine Ammonia Lyase (PAL).

Peroxidase (PO)

Peroxidase activity was assayed as per the procedure described by Hammerschmidt *et al*. (1982) [17]. The reaction mixture consisted of 2.5 ml of a mixture containing 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. Enzyme extract (0.1 ml) was added to initiate the reaction, which was followed colorimetrically at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. the boiled enzyme preparation served as blank. Activity was expressed as changes in absorbance at 470 nm min-1g-1 of fresh tissue.

Polyphenol oxidase (PPO)

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, 0.01 M catechol was added and the activity was expressed as changes in absorbance at 470 nm min-1 g-1 of fresh tissue.

Phenylalanine Ammonia Lyase (PAL)

The PAL activity was assayed as per the method described by Ross and Seder off (1992). The assay mixture containing 100 µl of enzyme, 500 µl of 50 m MTris-HCl (pH 8.8) and 600 µl of 1 m M L-phenylalanine was incubated for 60 min. and the reaction was arrested by adding 2 N HCl. Later 1.5 ml of toluene was added, vortexed for 30 sec. centrifuged (1000 rpm, 5 min.) and toluene phase was measured at 290 nm against the blank of toluene. Standard curve was drawn with graded amounts of cinnamic acid in toluene as described earlier. The enzyme activity was expressed as n moles of cinnamic acid min^{-1} g^{-1} of fresh tissue.

Phenol

Phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993) [45]. One g of fresh tissue was homogenized in 10 ml of 80 per cent methanol and agitated for 15 min at 70˚C. One ml of the methanol extract was added to 5 ml of dist. water and 250 µl of Folin-Ciocalteau reagent (1N) and the solution was kept at 25˚C. After three min. one ml of saturated solution of Na_2Co_3 and one ml of dist. water was added and the reaction mixture was incubated for 1h. at 25˚C. The absorption of the developed blue colour was measured using a spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reagent with a phenol solution (C_6H_5OH) and expressed as catechol equivalents g-1 of fresh tissue.

Statistical analysis

The statistical analysis of the experiment results was performed employing the computer software package 'SPSS' by Duncan Multiple Range Test (DMRT) and the values are expressed as mean (Gomez and Gomez, 1976) [46].

Result

Among the various treatments, the combination treatment (T_6) involving *P. fluorescens* as seed and soil application (@10 gm/kg of seed and 2.5 kg/ha) plus neem oil cake as soil application @ 0.25 t/ha recorded higher peroxidise (1.64), Polyphenol oxidase (0.96) and phenylalanine ammonia lyase (186.72) activities on 12^{th} day when compared with other treatments. This was followed by T_3 (1.46, 0.91 and 175.65), T_5 (1.32, 0.86 and 164.44) and T_1 (1.20, 0.80 and 140.70) treatments in the decreasing order of merit. The treatments T_2 (1.14, 0.75 and 126.56) and T_4 (1.13, 0.78 and 119.89) also showed statistically similar results in inducing the defense enzymes activity respectively. The maximum induction of enzymes activity was observed on the 12^{th} day in all the treatments and thereafter a gradual decrease was observed. The healthy control plants also showed slight increase in enzymes activity up to nine days and thereafter showed decline in all the treatments (Fig. 1,2 and 3).

With regard to phenol accumulation, the results revealed increased activity of phenolics due to treatment with *P. fluorescens* and neem oil cake and challenge inoculation with the pathogen. Among the treatments, treatment T_6 recorded

higher phenolics activity (178.80) on 12th day when compared to other treatments. This was followed by T_3 (172.75), T_5 (154.67) and T_1 (145.63) treatments in the decreasing order of merit. The treatments T_2 and T_4 also showed statistically similar results in inducing the phenolics activity (113.69 and 116.59). The maximum phenylalanine ammonialyase activity was observed on the 12th day in all the treatments and thereafter a gradual decrease was observed. The healthy control plants also showed slight increase in phenolics activity up to nine days and thereafter showed decline (Fig. 4).

Discussion

Induced resistance as technique of phyto-immunity has received great attention and inducing the plants own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy (Ramamoorthy *et al.*, 2001) [39]. Plants are bestowed with various defense related genes. It is well known that the defense genes are sleeping genes and appropriate stimuli or signals are needed to activate them. Various types of biological agents, plant extracts and crude extract of bio-agents which are not considered as fungicides are used as inducers for induction of resistance in various crops (De cal and Metagarejo, 2001; Salim *et al.*, 2011; Karthiba, 2012) [9, 41, 22].

In the present study, the plants treated with seed and soil application of *P. fluorescens* (@10 gm/kg and 2.5 kg/ha) plus soil application of neem oil cake @ 0.25 t/ha (T_6) and challenged with the pathogen recorded the maximum activity of defense related enzymes *viz.*, peroxidase (PO), polyphenoloxidase (PPO), phenylalanine ammonia lyase (PAL) and phenols. Similar to the present study, Akila *et al*. (2011) [1] positively correlated the treatment with combination of botanicals and bacterial antagonist such as *P. fluorescens* and *B. subtilis* on the induction of defense enzymes against *Fusarium* wilt of banana.

Various studies reported that *P. fluorescens* and certain plant products induced peroxidase in response to pathogen attack (Chen *et al.*, 2000; Kagale *et al.*, 2004) [30, 8]. High level of expression of defense enzymes was reported in *P. fluorescens* treated tomato plants challenged with *F. oxysporum* f.sp. *lycopersici* (Ramamoorthy *et al.*, 2002) [38] and banana plant challenged with *F. oxysporum* f.sp. *cubense* (Harish, 2005) [18]. Soil application of neem and sunflower cake considerably enhanced the activity of PO, PPO and PAL in cowpea after challenge inoculated *with M. phaseolina* (Dubey *et al*., 2009; Bharadwaj and Sahu, 2015) [11, 6]. Increased activity of peroxidase elicited by PGPR's in plants such as groundnut (Meena *et al.*, 2000) [32], sugarcane (Viswanathan and Samiyappan, 2001) [44], black gram (Karthikeyan *et al*., 2003) [23], safflower (Govindappa *et al.*, 2011) [14] and chillies (Kavitha *et al.*, 2005; Sundaramoorthy *et al.*, 2012) [25, 43] have also been reported. These reports corroborate with the present findings. Likewise, increased induction of PO, PPO and PALwas observed in *P. fluorescens* treated safflower plants challenge inoculated with *M. phaseolina* (Karthikeyan *et al*., 2006; Govindappa *et al*., 2011) [24, 14].

Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou *et al.*, 1996) [5] and in tomato (M' Piga *et al.*, 1997) [36]. Induction of enzymes such as PAL and PO leading to the accumulation of phenolics and lignin can occur in response to insect and pathogen attack (Ramamoorthy *et al.*, 2001) [39]. In the present study also, higher level of accumulation of phenolics occurred in cowpea plants treated with *P* seed and soil application of *P. fluorescens* (@10gm/kg and 2.5 kg/ha) plus soil application of neem oil cake @ 0.25 t/ha against the root rot pathogen. Similar increased level of accumulation of phenolics were reported in rice (Meena *et al.*, 2000) [32], in tomato and hot pepper against *P. aphanidermatum* (Ramamoorthy *et al*., 2002) [38], in blackgram against *M. phaseolina* (Karthikeyan *et al*., 2006) [24] and in safflower against *M. phaseolina* (Govindappa *et al.*, 2011) [14]. These earlier reports are in line and add value to the present findings. Thus, the ISR mediated through *P. fluorescens* and neem cake as observed in the present study resulted in enhancement of lignification with increased activities of enzymes involved in phenyl propanoid pathway and PR protein synthesis (Boller and Mauch, 1988; Hammerschmidt and Kuc, 1995; Akila *et al.*, 2011) [7, 16, 1] which could be attributed as the reason for the enhanced suppression of root rot incidence and increased plant growth parameters of cowpea.

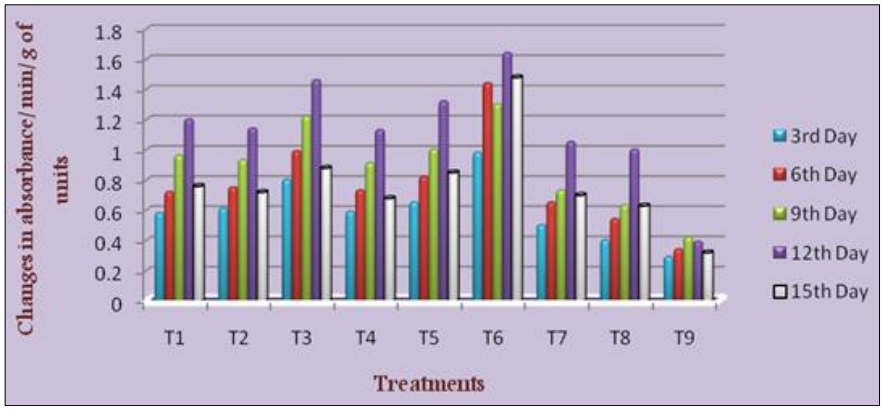

Fig 1: Induction of peroxidase (PO) activity in cowpea crop treated with Pf_2 with neem oil cake and challenge inoculated with *M. phaseolina* (MP_5)

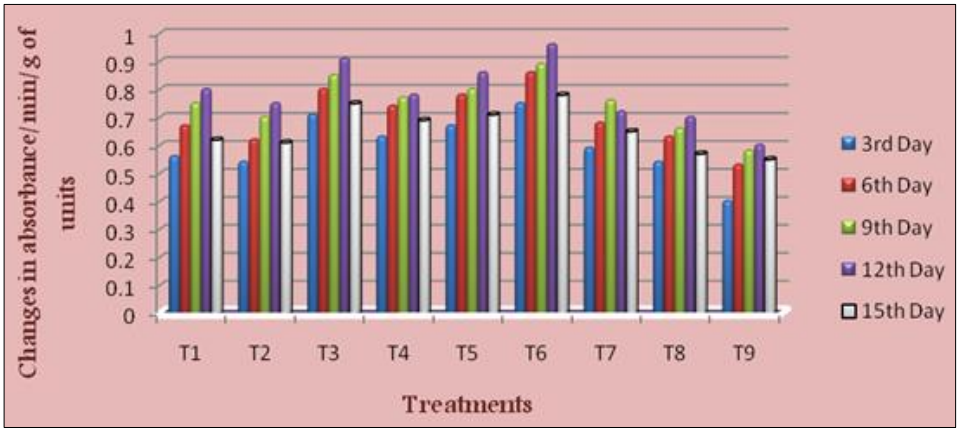

Fig 2: Induction of Polyphenol oxidase (PPO) activity in cowpea crop treated with Pf_2 with neem oil cake and challenge inoculated with *M. phaseolina* (MP_5)

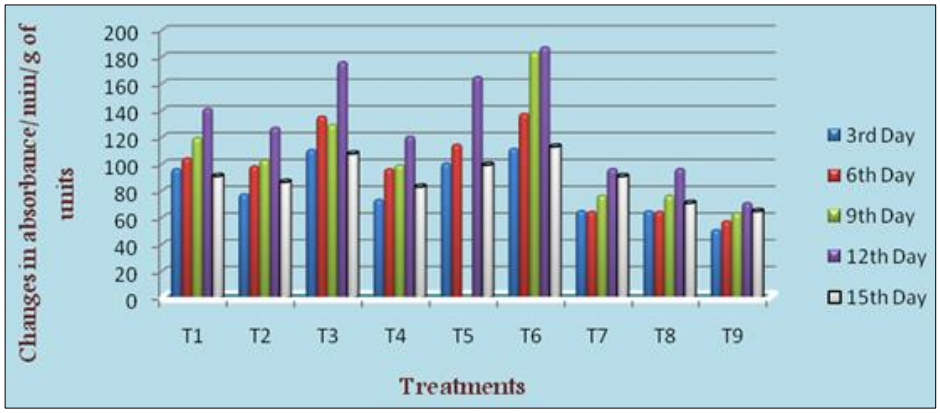

Fig 3: Induction of phenylalanine ammonia lyase (PAL)activity in cowpea crop treated with Pf_2 with neem oil cake and challenge inoculated with *M. phaseolina* (MP_5)

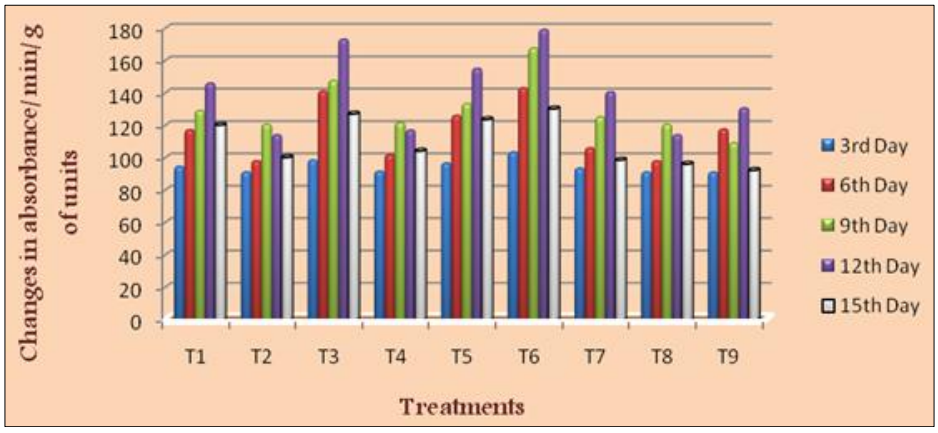

Fig 4: Phenolic content in cowpea crop treated with Pf_2 with neem oil cake and challenge inoculated with *M. phaseolina* (MP_5)

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