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RESEARCH ARTICLE

INDUCTION OF SYSTEMIC RESISTANCE AND WILT MANAGEMENT IN CHICKPEA BY ANTAGONISTIC RHIZOBACTERIA CO-INOCULATED WITH *MESORHIZOBIUM CICERIS.*

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Abstract

The use of PGPR as an inducer of systemic resistance in crop plants is relatively novel, cheaper and appropriate method to provide resistance against a broad spectrum of pathogens *via* production of defense related proteins. This study has reported the potential of five native rhizobacterial antagonists (2B, 7B, 28P, 34Pand 38P) alone and in combination with *Mesorhizobium* to control the disease severity and to boost the defense enzymes in chickpea plants (variety GPF-2) against *Fusarium oxysporum* f. sp. *ciceris* under glass house conditions. Selected antagonists were observed for the production of Salicylic and Gibberellic acid. Gibberellic acid production ranged from (242.4- 377.3 µg/ml). Maximum Salicylic (72.9 µg/ml) and Gibberellic acid (377.3 µg/ml) production was shown by 38P. Seed bacterization with 38 P and 34P along with *Mesorhizobium*, efficiently controlled the disease (76.8 % and 75.4 %) than the fungicide treatment (69.2%) and rhizobacterial isolates alone indicating the synergistic effect. Estimation of the Pathogenesis related proteins from the root tissues of chickpea revealed that maximum production of phenolic, Peroxydase and Polyphenol oxidase was also observed in 34P and 38P in combination with *Mesorhizobium*. PR proteins play important role in disease resistance and also help the plant to adapt to the environmental stress. This study reveals that PGPRs co-inoculation with specific rhizobia not only enhance the growth rate or protect the plants from pathogens *via* antagonism, but also help to induce enhanced systemic resistance i.e. plant defence mechanism by the induction of phenolic and several pathogenesis related proteins.

Introduction:-

Management of plant diseases has major economic significance as well as a crucial role in food safety. Use of chemical pesticides is a common practice to control phytopathogens but result in accumulation of toxic chemicals in plants and soil and elimination of beneficial soil organisms (Sharma, 2011). As an environmentally sound alternative, biological control is an attractive method against soil borne diseases (Compant et al., 2005). Bacterization with plant-growth-promoting rhizobacteria (PGPRs) as biocontrol agent in pulse crops is taking a serious turn in achieving crop sustainability (Reino et al., 2008).

Certain plant growth promoting microorganisms could enhance defensive activity and stimulate plant resistance against soil borne pathogens (Whipps et al., 2001). Many rhizosphere colonizing bacteria, including *Azotobacter, Azospirillum, Bacillus, Clostridium,* and *Pseudomonas*, typically produce substances that stimulate plant growth or inhibit root pathogens (Govindarajan et al., 2007)). Among biocontrol agents, root-associated fluorescent *Pseudomonas* spp. and *Bacillus* spp. have received special attention according to some studies because of their

excellent root colonizing ability, potential to produce a wide variety of anti-microbial metabolites, and induction of systemic resistance (Kumari and Khanna, 2014) and can improve plant health through the enhancement of plant resistance/tolerance against biotic stresses (Kavino et al., 2007).

In plants, certain secondary metabolite pathways are induced by infection with microorganisms (Haidar et al., 2012). It is well known that the defence genes are inducible genes and presence of appropriate stimuli or signals like prior application of a biological inducer activate them (Ramamoorthy et al., 2002). This induction of plant defence genes by prior application of inducing agents is called induced resistance (Saravanakumar et al., 2007). The defence gene products include peroxidase (PO), polyphenol oxidase (PPO) that catalyse the formation of lignin and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexins and phenolic synthesis. Other defence enzymes include pathogenesis-related proteins (PRs) such as β-1,3-glucanases (PR-2 family) and chitinases (PR-3 family) which degrade the fungal cell wall and cause lysis of fungal cell (Haidar et al., 2012).

Fusarium wilt of chickpea incited by *Fusarium oxysporum* f. sp. *ciceris* is a serious soil borne disease of chickpea (*Cicer arietinum* L.). It is a major constraint to chickpea cultivation throughout the world and especially in Indian subcontinent where chickpea is a commonly grown pulse crop as it can cause up to 100% yield loss annually (Pande et al., 2010). Several rhizospheric bacteria have the ability to control diseases and promote growth under laboratory and field condition. Earlier studies indicated that seed treatment with *Pseudomonas fluorescens* isolate 63-28 prevented the entry of fusarium wilt pathogen in the vascular tissue by strengthening cell wall structures and accumulation of phenolic substances and chitinases (Nikam et al., 2011).

Induced systemic resistance is an important mechanism of biocontrol that received considerable attention in recent times as it is an enhancement of plant defence capability by PGPR against a wide spectrum of invading pathogens. Several mechanisms are involved in induced systemic resistance including production of salicylic acid, antibiotics and induction of pathogenesis related proteins ie. peroxidase, polyphenol oxidase and other phenolic compounds. Thus, resistance-inducing rhizobacteria offer an attractive alternative, providing a natural, safe, effective, persistent, and durable type of protection. The development of bacterial agents showing biocontrol potential and plant growth promotion traits will help to strengthen the sustainability of agricultural system.

The present study aimed to investigate the potential of five antagonists, previously selected from a batch of 200 isolates to reduce fusarium wilt symptoms in chickpea plants infected by Foc as well as to potentiate defense enzyme activities.

Materials and methods:-

Bacterial isolates:-

Five rhizobacterial isolates isolated from chickpea rhizosphere, screened on the basis of antagonistic traits (Dual culture plate assay, liquid antibiosis, HCN production , Ammonia production, Siderophore production, diffusible and volatile antifungal metabolites production, were identified on the basis of cultural and biochemical characterization (Kumari and Khanna, 2014). *Mesorhizobium ciceris* specific to chickpea was procured from the Department of Microbiology, Punjab Agricultural University, Punjab, India.

Pathogen culture:-

The fungal pathogen *Fusarium oxysporum* f. sp. *ciceris* was obtained from the Department of Plant Breeding and genetics, Punjab Agricultural University, the culture of pathogen was maintained on Potato Dextrose agar slants. Five antagonists screened on the basis of antagonistic traits (Kumari and Khanna, 2014) were further evaluated for Salicylic acid and Gibberellic acid production.

Production of Salicylic acid:-

Salicylic acid (SA) production of the isolated bacterial strains was determined as per the method described by Meyer and Abdallagh (1978). The strains were grown in the standard succinate medium at 28˚C for 48 hrs. Cells were collected by centrifugation at 8000 rpm for 5 min and were resuspended in 1ml of 0.1 M phosphate buffer. A 4ml cell free culture filtrate was acidified with 1 N HCl to 2.0 and SA was extracted in $CHCl_3$. Four ml of water and 5 µl of 2M $FeCl_2$ were added to the pooled $CHCl_3$ phases. The absorbance of the purple iron- SA complex, which was developed in the aqueous phase, was read at 527 nm. A standard curve was prepared with SA dissolved in succinate.

Production of Gibberellic acid:-
The gibberellic acid production by plant growth promoting rhizobacteria was determined as per the method by Borrow et al . (1955).Culture media were filtered, and then samples were acidified to pH 2.5 with HCl and extracted using liquid-liquid (ethylacetate/$NaHCO_3$). Gibberellic acid in the ethyl acetate phase was measured by UV spectrophotometer at 254 nm. The amount of gibberellic acid was calculated from the standard curve.

Glass house experiment:-
A poly-bag (pot) culture experiment was conducted to study the influence of the 5 selected antagonists on the wilt incidence and induction of systemic resistance as enhancement in the production of pathogenesis related proteins viz. Peroxydase, Polyphenol oxidase and phenolic compounds in root tissues of treated chickpea plants (variery GPF-2).

Soil preparation:-
Local chickpea soil was collected from different chickpea locations of Punjab Agricultural University, mixed thoroughly and autoclaved at 121 ^{o}C and 15 psi pressure for one hour and 500 grams sterilized soil was taken per pot.

Pathogen inoculation to soil:-
Polyethylene bags (15 x 10 cm) were filled with sterilized soil inoculated with 1 g fresh weight of fungus in 100 g soil. A chickpea (desi) genotype, GPF-2 (Department of Plant Breeding and Genetics PAU, Ludhiana, India) was selected for glass house observations.

Seed bacterization:-
The seeds of GPF-2 chickpea variety were surface sterilized with 0.1% Mercuric chloride and 70% ethanol and treated with 5 PGPR cultures (10^6 ml $^{-1}$ broth) individually and in combination with Mesohizobium (1:1) for 30-45 minutes before sowing the seeds.

Wilt incidence and systemic resistance induced by rhizobacteria under glass house condition:-
The plants were maintained for 50 days by regular watering and were observed for the wilt incidence at regular intervals (10 days) and induction of pathogenesis or defense related proteins at 50th day. Each treatment was replicated 10 times with 10 seeds/ bag, seeds treated with fungicide captan @ 2 g/kg were sown similarly as a separate treatment and same parameters were recorded at regular intervals. Absolute control was taken as seeds sown without rhizobacterial treatment in non pathogenc soil i.e. sterile soil. Whereas in negative control soil was treated with pathogen but the seeds were sown without any rhizobacterial treatment. The plant growth in terms of fresh weight was also recorded after 50 days of sowing. The statistical analysis was done using CPCS1 software developed by Department of Mathematics, Statistics and Physics, PAU, Ludhiana.

Analysis of Pathogenesis related proteins, induced under glass house conditions:-
After 50 days fresh and healthy plants were uprooted from each treatment. Plant samples were washed thoroughly and then enzymes and phenolics were extracted from the root tissues and were estimated spectrophotometrically.

Estimation of Phenol:-
Phenol production was determined as per the procedure given by (Zieslin and Ben-Zaken, 1993). Root samples (1g) were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70˚C. One ml methanol extract was added to 5ml distilled water and 250µl of Folin-Ciocalteau reagent (1N) and the solution was kept at 25˚C, till a blue colour developed. The absorbance was measured using a spectrophotometer at 725 nm. The amount of phenolic was expressed as µg catechol mg^{-1} of root tissue.

Assay of Peroxidase (PO):-
PO activity was determined as per the procedure given by (Hammerschmidt et al., 1982).Root samples (1g) were homogenized in 2ml of 0.1M phosphate buffer, pH 7.0 at 4˚C. The homogenate was centrifuged at 16000 rpm at 4˚C for 15min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 m pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at room temperature (28±2˚C). The changes in absorbance were recorded at 420 nm at 30 sec intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min^{-1} mg^{-1} of root tissue.

Assay of Polyphenol oxidase (PPO):-

PPO activity was determined as per the procedure given by (Mayer et al., 1965). Roots sample (1g) was homogenized in 2ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16000 rpm for 15 min at 4˚C. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH-6.5). To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min^{-1} mg^{-1} of root tissue.

Results and discussion:-

Production of Salicylic acid

Salicylic acid (SA) is a plant Phenolic and hormone-like endogenous regulator and its role in the defense mechanisms against biotic and abiotic stress has been well documented (Szalai et al., 2000). Salicylic acid enhances the drought and salt stress resistance of plants. Even in some cases it has also been reported to enhance resistance in plants to several pathogens (Tari et al., 2002). Five selected antagonistic rhizobacterial isolates (2B, 7B, 28P, 34P and 38P) were further evaluated for Salicylic acid production in vitro. Salicylic acid production was shown by all the five isolates. Maximum Salicylic acid production was shown by 38P (72.9 µg/ml) followed by 2B (65.6 µg/ml) and 34P (57.6 µg/ml) (Table 1). Some reports are there, revealing the critical role of salicylic acid in the classical Systemic acquired resistance (SAR) in induced cucumber and tobacco plants (Yalpani et al., 1991; Malamy et al., 1990; Metraux et al., 1990). Similarly, SA producing PGPRs treated tobacco plants were found to be resistant to blue mold (Zhang et al., 2002). Bano and Fatima (2009) have reported that inoculation with Pseudomonas maintains the osmotic potential of leaves in maize due to production of salicylic acid that helps to tolerate biotic and abiotic stress conditions.

Production of Gibberellic acid:-

Gibberellic acid (GA) is a plant growth regulator of economic importance (Gelmi et al., 2002). Various gibberellins are available and are associated with several plant growth and development processes, such as seed germination, stem elongation, flowering, and fruit development (Gomi and Matsuoka, 2003). The selected rhizobacterial isolates (2B, 7B, 28P, 34Pand 38P) were also evaluated for Gibberellic acid production. All the five isolates showed the production of Gibberellic acid. Maximum Gibberellic acid was produced by 38P (377.3 µg/ml) followed by 34P (337.8 µg/ml) (Table 1). Evidence of GA production by PGPR is rare, however, Gutierrez-Manero et al. (2001) provide evidence that four different forms of GA are produced by *Bacillus pumilus* and *B. licheniformis* and it is usually used in concentrations between 0.01-10 mg/L by plants. This study indicates the beneficial role of the Gibberellic acid producing rhizobacteria on plant growth under glass house conditions.

Effect of rhizobacteria on wilt incidence and fresh weight of the plants:-

A poly-bag (pot) culture experiment was conducted to study effect of seed bacterization with PGPRs alone and their co-inoculation with *Mesorhizobium* on wilt incidence caused by *Fusarium oxysporum* f. sp. *ciceris* and plant growth in terms of plant weight (Plate 1). Observations revealed that PGPR co-inoculation with *Mesorhizobium* was found to be more effective in controling the wilt incidence in chickpea variety GPF-2. Moreover co-inoculation with *Mesorhizobium* further enhaced the plant growth compared to PGPRs alone.

Plants were observed for the wilt incidence at 10 days interval under glass house conditions for 50 days. Observations were made for the dry, brown and drooped plantlets whereas in non-wilted plants, fresh and green plantlets were observed (Plate2). The percentage wilt recorded at 10 days interval revealed the efficacy of rhizobacterial isolates and their co-inoculation with *Mesorhizobium,* to reduce the incidence of wilt at different stages of chickpea plants. Comparatively lesser disease severity was observed in the treatments where rhizobacterial isolates were co-inoculated with specific *Mesorhizobium* culture. Moreover this was at par to that of effect of fungicide (Table 2). Wilt incidence increased along with the growth of the plants up to 50^{th} day. Wilting was observed after 20^{th} day of sowing. Initially disease severity was recorded 6.9 % in negative control whereas minimum wilt was recorded with 38P (2%) alone and 7B (1%) coinoculated with *Mesorhizobium* at 20^{th} day. Similarly minimum disease severity (or maximum disease suppression) was recorded in 38P treated plants at 30^{th} day 5.8% (94.2%), 40^{th} day 9.8% (90.2 %) and 50^{th} day 31.4 (68.6 %) compared to negative control i.e.11.6 % (88.4%), 23.2 % (76.7%) and 60.4 % (39.6 %) at 30^{th}, 40^{th} and 50^{th} day respectively. Leon et al. (2009) reported antagonistic Pseudomonas sp. significantly increased disease suppression in many crops. Seed bacterization with 38 P and 34P along with *Mesorhizobium,* efficiently controlled the disease at 50^{th} day i.e. 76.8 % and 75.4% , found to be better than rhizobacterial isolates alone indicating the synergistic effect (Plate 3) . Hemissi et. al (2011) reported that rhizobia produce toxic metabolites which have inhibitory effect on soil-borne plant pathogens thus co-

inoculation with *Rhizobium* significantly reduces the disease incidence compared to rhizobacteria alone. In this study similar results were observed.

Plant fresh weight was also recorded at 50th day. Enhanced plant growth was recorded in rhizobacterial treated plants in terms of plant fresh weight compared to negative control. Isolate 2B treated plants were recorded with maximum shoot weight (2.00 g) followed by 38P (1.98) and 34P (1.68 g) that was found to be significantly higher than negative control (0.96 g) and at par the fungicide treatment (1.52 g). Similarly maximum root weight was recorded in 2B (0.81g) bacterized plants compared to negative control (0.46g) and fungicide (0.32g). However, combination of bio-antagonist with *Mesorhizobium* further enhanced the shoot and root weight in the present study and was found to be better than absolute control for some of the isolates co-inoculated with *Mesorhizobium* (Table 3). Seed bacterization with 38 P and 34 P along with *Mesorhizobium* were found to be most effective in terms of enhancing plant weight i.e 3.84g and 3.52 g respectively. Studies also have revealed that treatment with PGPR strains improved growth rate of the plants compared to control in case of rice and maize respectively (Ashrafuzmann et al., 2009). Hahm et al. (2012) also reported in his studies that PGPR treatments resulted in the taller peppers compared to negative control treatment and resulted in enhanced fresh weight of the plants. Pathak et al. (2001) reported that PGPR co-inoculation with *Rhizobium* may have significantly increased the growth and yield of legume crops as it provided more nutrition to the plant. Synergistic effect in use of dual cultures is well documented (Hahm et al., 2012). The same synergistic response was evident in this study.

Induction of Pathogenesis related proteins (PRs):-

The stimulation of Pathogenesis related proteins (PRs) such as peroxidase (PO), polyphenol oxidase (PPO) and other phenolic compounds by rhizobacteria has been associated with induction of systemic resistance in plants known as Induced systemic resistance (ISR). In the green house experiment 5 rhizobacterial isolates alone and in combination with *Mesorhizobium* were inoculated to test their ability to induce the production of phenol, PO and PPO in root tissues of chickpea plants (GPF-2) along with fungicide treatment and negative control. Out of the five isolates tested, 34P and 38P co-inoculation with *Mesorhizobium* significantly reduced the wilt incidence under greenhouse conditions as compared to negative control (Table 2).

Observations revealed higher accumulation of phenolic as compared to PO and PPO. Maximum production of phenol was observed in 28P (408.5 µg/mg) followed by 34P (401.9 µg/mg) and 38P (348.8 µg/mg) as compared to negative control (189.5 µg/mg) in terms of µg of catechol mg^{-1} of protein. Highest PO activity was also recorded in 7B (0.712 Δ**abs min^{-1} mg^{-1}**) followed by 28P (0.674 Δ**abs min^{-1} mg^{-1}**) and 34P (0.644 Δ**abs min^{-1} mg^{-1}**) treated root tissues challenged with the pathogen. Increased activity of PPO was observed in 38 P (9.81Δ **abs** min^{-1} g^{-1}) followed by 34P (7.45 Δ **abs** min^{-1} g^{-1}) bactericized chickpea plants. Interestingly estimation of the defense mechanism by stimulation of PRs in different chickpea treated plants have revealed that combination of *Mesorhizobium* with rhizobacterial isolates further enhanced the production and activity of PRs as compared to PGPR isolates alone (Table 4). Rhizobacterial isolates 34P, 38 P and 7B along with *Mesorhizobium* further enhanced the production of phenolics (404.9 µg/mg, 363.2 µg/mg and 346.2 µg/mg respectively) compared to these isolates alone i.e. (401.3µg/mg), (348.8 µg/mg) and (163.2 µg/mg) and in conformation, activity of PO and PPO was also successfully enhanced by 38P, 34P and 2B isolates when inoculated in combination with *Mesorhizobium* (Table 4). Nandakumar et al. (2001) and Kavino et al. (2007) reported that tomato plants treated with the mixtures of Pf1 + Py15 + Bs16 showed maximum increase in phenolic, PO, PPO, PAL and β-1,3-glucanase activity. They also reported that higher levels of PO, PPO, PAL, β-1, 3-glucanase and phenolic may contribute collectively to induce resistance in tomato plants against early blight pathogen, which eventually resulted in higher yield (Ramamoorthy et al., 2002). The association between higher levels of defence-related enzymes and greater disease resistance has also been reported by these workers. PGPR strains induce systemic resistance by activation of various defence-related enzymes such as PO, PPO, PAL, chitinase and β-1,3-glucanase (Rajendran et al., 2007).

Table 1:- PZoduction of Salicylic acid and Gibberellic acid by rhizobacterial isolates.

Isolates	Salicylic acid production (µg/ml)	Gibberellic acid production (µg/ml)
2B	65.6	242.4
7B	27.0	274.1
28P	39.2	313.2
34P	57.6	337.8
38P	72.9	377.3

Table 2:- Disease severity at 10 days interval under glass house condition (Variety GPF-2).

GPF-2	Wilt incidence (%)			
Treatments	**20thday**	**30thday**	**40thday**	**50thday**
Control	1.7	7.0	9.0	35.0
Negative control(pathogen)	6.9	11.6	23.29	60.4
Captan(fungicide)	2.0	14.6	17.8	30.8
2B	3.0	11.3	13.2	38.8
7B	6.0	8.0	12.0	36.0
28P	4.0	14.2	18.4	40.8
34P	4.0	12.3	14.2	38.7
38P	2.0	5.8	9.8	31.4
2B+*Mesorhizobium*	3.6	4.2	8.5	25.0
7B+ *Mesorhizobium*	1.0	5.8	9.8	31.4
28P+ *Mesorhizobium*	3.8	7.7	11.5	28.9
34P+ *Mesorhizobium*	3.5	3.5	8.8	24.6
38P+ *Mesorhizobium*	3.6	10.7	10.8	23.2

Table 3:- Effect of Bio-antagonist on plant weight under glass house conditions in chickpea variety (GPF-2)

Treatments	Fresh weight		
	Shoot weight / plant (g)	**Root weight/ plant (g)**	**Plant weight**
Negative control(pathogen)	0.96	0.46	1.42
Captan(fungicide)	1.52	0.32	1.84
Absolute control	1.60	0.69	2.29
2B	2.00	0.81	2.81
7B	1.58	0.69	2.27
28P	1.04	0.59	1.63
34P	1.68	0.46	2.14
38P	1.98	0.56	2.54
2B+ *Mesorhizobium*	2.00	0.64	2.64
7B+ *Mesorhizobium*	1.90	0.89	2.79
28P+ *Mesorhizobium*	1.80	0.89	2.69
34P+ *Mesorhizobium*	2.28	1.24	3.52
38P+ *Mesorhizobium*	2.50	1.34	3.84
CD at 5%	1.4	1.2	1.3

Table 4:- Induction of phenolic and PRs by antagonistic rhizobacterial isolates under glass house conditions.

GPF-2 Treatments	Phenolic (µg/mg)	Peroxidase activity (Δ abs min^{-1} mg^{-1})	Polyphenol oxidase activity (Δ abs min^{-1} mg^{-1})
Negative control(pathogen)	189.5	0.220	2.19
Captan(fungicide)	304.7	0.252	3.95
2B	108.8	0.219	6.54
7B	163.2	0.712	4.06
28P	408.5	0.674	.4.11
34P	401.3	0.644	.7.45
38P	348.8	0.324	9.81
2B+ *Mesorhizobium*	108.8	1.863	10.00
7B+ *Mesorhizobium*	346.2	2.334	5.89
28P+ *Mesorhizobium*	371.8	3.262	6.54
34P+ *Mesorhizobium*	404.9	3.811	13.24
38P+ *Mesorhizobium*	363.2	4.651	15.41

Plate 1:- Pot culture experiment conducted under glass house conditions variety (GPF-2)

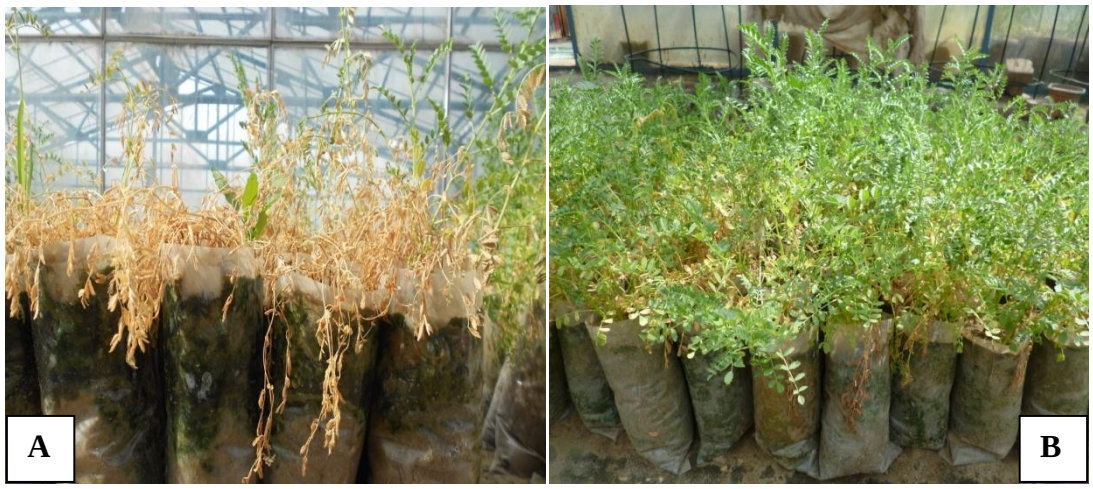

Plate 2:= Wilted chickpea plants (dry, brown and drooped) (A) vs Non-wilted plants (fresh and green) **(B)**

P38 **Negative Control** **P38 +*Mesorhizobium***

Plate 3: Relative symptoms of wilting between culture (P38) and its co-inoculation with *Mesorhizobium* compared to Negative control.

Conclusion:-

The results of the present study indicate that the antagonists 34P and 38P and their co-inoculation with *Mesorhizobium* are not only involved in plant growth promotion, but also as effective as the fungicide (Captan) in reducing the Fusarium wilt symptoms in chickpea with the participation of antifungal compounds i.e. Salicylic acid and the defence enzymes PO, PPO and phenolic compounds which are induced by them, are commonly involved in the host's resistance against fungal diseases.

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