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# *Pseudomonas fluorescens* Modulate *In-vitro* lytic Enzyme Production and Inhibit the Growth of Collar Rot Pathogen (*Aspergillus niger)* in Groundnut (*Arachis hypogaea* L.)

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**Collar rot of groundnut (*Arachis hypogaea* L.) is caused by *Aspergillus niger* (*A. niger*). Ten different isolates of *Pseudomonas fluorescens* (*Pf*-1 to *Pf*-10) were screened for their biocontrol potential against the *A. niger* evaluated through their potential to produce the lytic enzymes during *in-vitro* antagonism. Among the ten isolates, *Pf*-3 exhibited highest *in-vitro* growth inhibition (78.88%) of *A. niger* followed by *Pf*-9 (64.52%) and *Pf*-5 (62.30%). The culture media containing *Pf*-3 isolates along with *A. niger* had higher activities of chitinase and $^{2}$-1,3-glucanase and lower activities of cell wall degrading enzymes such as cellulase and polygalacturonase compared to the other tested isolates. A significant positive association ($p$ = 0.05) between percentage growth inhibition of *A. niger* and specific activity of chitinase was observed where as no association could be established for $^{2}$-1,3-glucanase. However a negative association was established between percentage growth inhibition of *A. niger* and specific activity of polygalacturonase and cellulase. From the findings of the present study, it may be concluded that *Pf*-3 could be the best biocontrol agent against *A. niger*, the collar rot causing pathogen in groundnut.**

**Key words:** Growth inhibition, *in-vitro* antagonism, lytic enzymes, correlation.

Groundnut (*Arachis hypogaea L.*) is an annual legume and a major oilseed crop in India. Besides continuing to be an oilseed crop, groundnut has now established itself as an important supplementary food crop too[1]. In India is mostly grown in rainfed conditions by resource poor farmers; hence the crop productivity is hampered by a number of biotic and abiotic stresses. Collar rot is caused by *Aspergillus niger* (*A. niger*), is one of the important seed and soil borne diseases of groundnut. In 1952, this disease was first reported in India[2]. *A. niger* is an ubiquitous filamentous ascomycete fungus, which has been implicated in opportunistic infections of humans too. Being a soil saprophyte with a wide array of hydrolytic and oxidative enzymes, this fungus is involved in the breakdown of plant lignocelluloses[3]. *A. niger* causes pre emergence rotting of groundnut seeds, sudden wilting followed by shredding of the collar region in young seedlings. Infected collar regions are profusely covered with black masses of mycelium and conidia[4]. Collar rot is a more serious problem in sandy soil of India, where the losses may amount to 40-50% in terms of mortality of plants due to *A. niger*. Management of collar rot disease can be achieved by use of tolerant varieties and adjustment of sowing dates[5]. The extensive use of fungicides for control of this pathogen is not recommended because of its deteriorating effect

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on human health and development of pathogen resistance to fungicide. Biocontrol through microbes has been emerged as an eco-friendly and sustainable method for control of fungal plant diseases. Biocontrol agents from *Pseudomonas ûuorescens (P. fluorescens)* are non-speciûc in their ability to protect plants from soil phytopathogens because of their versatile mode of actions against the plant pathogenic fungus through competition for niches and nutritions, antibiosis, predation and capability to induce plant defense responses[6]. Interestingly, each biocontrol strain can typically act on more than one pathosystem, i.e. protect more than one plant species from often distinct pathogens, provided the rhizosphere is successfully colonized. The use of *P. fluorescens* as inoculants of soil or plants, has been successfully implemented in agronomic ûeld trials[7,8]. Also the use of *P. ûuorescens* as biocontrol agent has thought to have a limited ecological impact on indigenous saprophytic populations and to take place without negative side-effects on rhizosphere functioning[9].

Though collar rot is prevalent in almost all groundnut growing states of India, it is causing more damage in sandy loam and black soils of Gujarat, especially in Saurasthra region. Hence the present study aimed at evaluating some *P.fluorescens* isolates for their antagonistic potential against the *A. niger*, the collar rot causing pathogen in groundnut and to identify the potential isolates to be used as a biocontrol agent for the disease.

## MATERIAL AND METHODS

### *Pseudomonas fluorescens* isolation and maintenance

Ten *Pseudomonas fluorescens* (*P. fluorescens)* isolates used in the present study were previously isolated from different locations of groundnut growing areas of Gujarat and maintained as glycerol stock at the microbiology cell in the food testing laboratory. The isolates were revived in King's B broth before use.

### Isolation and maintenance of *A. niger*

The pure culture of the fungus was isolated as described by Gajera and Vakharia[10] for which the root tissue of collar rot infected groundnut plants were cut into small bits with the help of sterilized blade and were placed on the solidified potato dextrose agar (PDA) medium in petri plates. After four days of incubation at 28 ± $2^0$C, from the typical black mycelium (conidia) growth of *A. niger,* the pure culture was made by hyphal-tip isolation method[11].

### *In-vitro* growth inhibition of *A.niger by P. fluorescens*

To assess the *in-vitro* antagonistic effect of *P. fluorescens* isolates against *A. niger*, both organisms were allowed to grow on King's B + PDA medium (1:1) in the petri plates. The *P. fluorescens* isolates were grown in King's B broth for 72 hrs and *A. niger* was grown in PDA medium for 4-5 days. For antagonism study, each isolates of *P. fluorescens* were placed in round shape by using a sterilized wire loop keeping 1 cm away from the edge of the petri plate and a mycelial disc of 4 mm diameter of *A. niger* was placed in the centre. The petri plate containing only the test fungus was considered as the control. The plates were incubated at 28 ± $2^0$C for 6 days, and observations were taken[12]. Percentage of growth inhibition of *A. niger* by *P. fluorescens* was calculated following the method as described by Zarrin[13] as below,

% Growth Inhibition = {(C- T)/C} X 100, where,
C = colony diameter of pathogen in control and T = colony diameter of pathogen in inhibition plate

### Extraction of Lytic Enzymes

Crude enzyme extract was prepared from the petri plates after six days of incubation containing fungal pathogen and *P. fluorescens* isolates (T1 to T10) as well as the test fungus alone (T11) as a control (C)[10]. For that, 25 ml of 100mM phosphate buffer (pH-5.5) containing 50mM sodium chloride, was added to each petri plates and transferred mycelia mat along with the bacterial growth to conical flask. For lytic enzyme induction, 1% of carboxymethyl cellulose (CMC), sodium polypectate, chitin or casein was added into culture medium of conical flask and pH was adjusted to 5.5[14]. Cultures were then shaken well in orbital shaker at 120 rpm at 28 ± $2^0$C for about 6 hours[15]. After that, the content was centrifuged at 14,000 rpm for 10 min. Supernatant was collected and stored at -$20^0$C until it used for assay of enzymes activities and protein content. The Folin-lowry method[16] was followed for determination of protein content in

the culture supernatant and to calculate the specific activity of the lytic enzymes.

**Cell wall degrading enzymes:**

**Assay of Cellulase activity**

The reaction mixture was composed of 100 µl of the culture supernatant with 400 µl of 100mM sodium citrate buffer (pH 5.2) containing 1 % carboxy methyl cellulose[17]. After incubation at 55$^0$C for 15 min, the released glucose was measured following Nelson somyogi method[18]. Specific activity of cellulase was expressed as unit.mg$^{-1}$ protein and one unit of cellulase activity was defined as the amount of cellulase necessary to produce one mg free glucose per min per ml of enzyme extract.

**Assay of Polygalacturonase (PG) activity:**

The culture supernatants (100 µl) were incubated with 400 µl of 50mM sodium acetate buffer (pH 5.2) containing 0.25 % sodium polypectate[17]. After 1 hour incubation at 37$^0$C, the released galacturonic acid was measured following Nelson-somyogi method[18]. One unit of polygalacturonase was defined as the amount of polygalacturonase necessary to produce one mg free glucose per min per ml of enzyme extract and specific activity of was expressed as unit.mg$^{-1}$ protein.

**Pathogenesis related enzymes**

**Assay of Chitinase activity**

Reaction mixture containing 200 µl of 0.5 % chitin in 10mM sodium acetate buffer (pH 5.2) and 100 µl of culture supernatant was incubated at 50$^0$C for 1 hour[19]. The formation of sugar N-acetylglucosamine was measured following DMAB method[20].The unit of chitinase was expressed as µmol of GlcNAc released per ml per minute and specific activity was expressed as units of enzymes per mg of protein in the enzyme extract.

**Assay of ²-1,3-glucanase activity:**

The reaction system contained 100 µl of 0.2 % laminarin in 50mM sodium acetate buffer (pH 5.2) and 100 µl of culture supernatants[21]. Reactions were carried out at 37$^0$C for 10 min. After incubation, the amount of released glucose by enzyme ²-1,3-glucanase was measured following Nelson somoygyi method[18]. Unit activity was defined as the amount of enzyme necessary to produce one mg of corresponding reducing sugar per min per ml of enzyme extract.

**Statistical analysis**

Analysis of variance (ANOVA) was performed using DSAASTAT 1.1[22] and correlation between different traits was studied using PAST v1.89 software[23].

## RESULT AND DISCUSSION

***In vitro* percent growth inhibition of *A. niger***

*In-vitro* growth inhibition of *A. niger* by *P. fluorescens* isolates at six days after inoculation (DAI) was depicted in (Fig. 1). All the *P. fluorescens* isolates were significantly restrict the *in-vitro* growth of the pathogen (Table 1), however the highest inhibition was observed in T3 (~ 79%), which was followed by T4, T5 and T9 which were at par (61.7 to 63.43%). The lowest inhibition was for T1 followed by T7, T6, T10 and T2 respectively. Hence, it was concluded that among the ten isolates of *P. fluorescens* studied, *Pf-3* was found to be the best antagonist for *A. niger* followed by *Pf-4, Pf-5, Pf-9* and *Pf-2*. The *Pf-1* and *Pf-7* were found to be not efficient enough to control the fungal pathogen. Earlier it was reported that the *P. fluorescens* along with neem cake can reduce the collar rot disease by about seven percent[24]. Further, evaluation of 57 isolates of *P. fluorescens* against *Sclerotium rolfsii* showed that *P. fluorescens* are capable of reducing the mortality rate (55.9%) in groundnut[25].

**Lytic Enzymes**

The first physical barrier which is encounter by most plant pathogens are cell wall of either biocontrol agent *P. fluorescens* or host plants, and thus it must be degraded for successful entry of the hyphae of the pathogens and subsequent tissue colonization. Mostly, necrotrophic fungal pathogens degrade the structural polymers in plant host cell wall and colonize in the intercellular spaces facilitated by the production of cell wall degrading enzymes (CWDEs) such as cellulase, and polygalacturonase. However, as an encounter the production of chitinase and ²-1,3-glucanase by the host plants or the *P. fluorescens* prevent the entry of the pathogens.

**Chitinase activity**

Chitin being the integral cell wall component of many fungal pathogens, biocontrol

agents like *P. fluorescens* inhibit the growth of plant pathogenic fungus through production of chitinase. In the present *in-vitro* antagonism study, a significant difference was observed among the tested *P. fluorescens* isolates for their capability to produce chitinase against *A. niger*. The significant differences were found in different culture medium (Fig. 2a). Among tested isolates of *P. fluorescens*, the specific chitinase activity (U.mg$^{-1}$) was ranged between 2.12 (T1) to 21.32 (T3).

The *P. fluorescens* isolate 3, which was producing the maximum chitinase among all isolates study, also showed the maximum *in-vitro* growth inhibition of the fungus, *A. niger*. The specific activity for chitinase for T6, T8, T9 and T10 were at par (11.36 to 12.64 U.mg$^{-1}$), making the respective isolates equally potential in chitinase production against *A. niger*. It was also reported that chitinase gets induced antagonist treatment by *Trichoderma* against test fungus *A.*

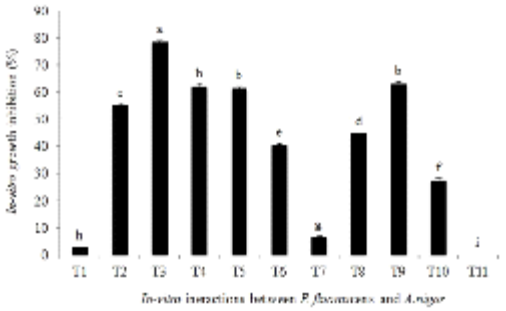


**Fig.1.** *In-vitro* growth inhibition of *A. niger* by *Pseudomonas fluorescens* isolates, the values of each column represent the mean of three independent replications, and the bar value on each column represents the standard error (±SE); treatments having same letter on respective column show no significant difference at p<0.05.

**Table 1.** *In-vitro* growth inhibition (%) of *A. niger* by *P. fluorescens* isolates, the values are mean of three independent experiment ± standard error (SE)

| Treatments | Isolate of *P. fluorescens* used against *A. niger* | *In-vitro* growth inhibition (%)(Mean ± SE) |
|---|---|---|
| T1 | *Pf-1* | 02.72 ± 0.01 |
| T2 | *Pf-2* | 55.27 ± 0.90 |
| T3 | *Pf-3* | 78.77 ± 0.49 |
| T4 | *Pf-4* | 62.24 ± 1.19 |
| T5 | *Pf-5* | 61.71± 0.39 |
| T6 | *Pf-6* | 40.84 ± 0.33 |
| T7 | *Pf-7* | 06.65 ± 0.12 |
| T8 | *Pf-8* | 44.84 ± 0.17 |
| T9 | *Pf-9* | 63.43 ± 0.65 |
| T10 | *Pf-10* | 27.65 ± 0.78 |
| T11 | *A. niger* only, as control | 00.00 ± 0.00 |
| LSD (p<0.05) | | 1.73 |
| C.V. ( %) | | 2.52 |

**Table 2.** Correlation among lytic enzyme production capability and *in-vitro* growth inhibiting potential of *P. fluorescens* isolates against *A. niger*

| Parameters | GI | Chi | ² Gl. | PG | Cell. |
|---|---|---|---|---|---|
| (%)Growth Inhibition (GI) | 1.000 | | | | |
| Chitinase (Chi.) | 0.603* | 1.000 | | | |
| ²-1,3-glucanase (² Gl.) | 0.202 | 0.471 | 1.000 | | |
| Poly galacturonase (PG) | -0.500* | -0.557 | -0.689 | 1.000 | |
| Cellulase (Cell.) | -0.573** | -0.503 | -0.337 | 0.744 | 1.000 |

* refers to significant (P<0.05) and ** highly significant (P<0.01) correlation

*niger* and thereby inhibit the growth of the fungus[10].

**â-1,3-glucanase activity**

The enzyme â-1,3-glucanase produced by the biocontrol agents like *P. fluorescens* have a capacity to hydrolyze the branched ²-1,3-glucans found in the most fungal cell walls. All the *P. fluorescens* isolates produced significant amount of the enzyme against *A. niger*, however the maximum specific activity was found in T6 (116.92 U.mg$^{-1}$protein) followed by T1 (92.50 U.mg$^{-1}$protein) (Fig 2b). The specific activity of â-1,3-glucanase was at par among T7, T8, and T3 and ranged from 77.12 to 78.73 U.mg$^{-1}$protein. The lowest specific activity of the enzymes was found in T2 followed by T7, T10, T4, and T5 respectively. From the above result it was seen that although the *P. fluorescens* isolate-1 produces higher amount of ²-1,3-glucanase, but was not able to inhibit the *in-vitro* growth of the fungus, where as the isolate-3 shower the highest *in-vitro* growth inhibition by producing less amount of the enzyme. Our results for â-1,3-glucanase were not in accordance with the findings of some other researchers[26] which showed signiûcant relationship between the antagonistic activity of *P. ûuorescens* isolates and their level of production of ²-1,3-glucanase

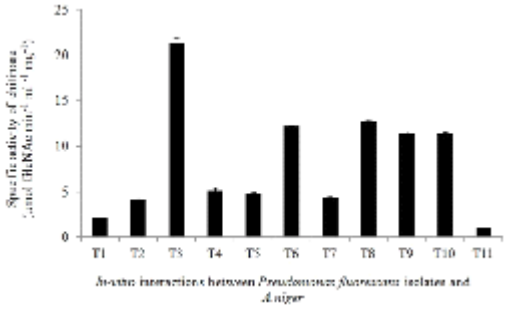


**Fig. 2.a**. *In-vitro* production of chitinase by *Pseudomonas fluorescens* isolates against *A. niger* (T1 to T10, and T11 as control), the values of each column represent the mean of three independent replications, and the bar value on each column represents the standard error (±SE).

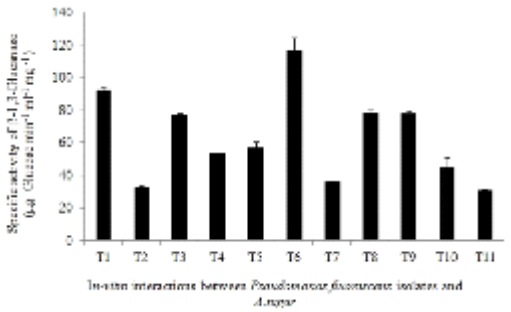


**Fig. 2.b**. *In-vitro* production of ² 1,3-Glucanase by *Pseudomonas fluorescens* isolates against *A. niger* (T1 to T10, and T11 as control), the values of each column represent the mean of three independent replications, and the bar value on each column represents the standard error (±SE).

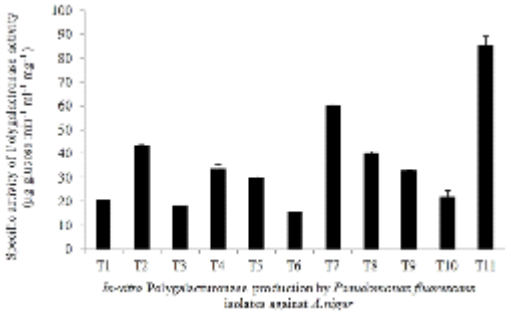


**Fig. 3.a**. *In-vitro* production of Polygalacturonase by *Pseudomonas fluorescens* isolates against *A. niger* (T1 to T10, and T11 as control), the values of each column represent the mean of three independent replications, and the bar value on each column represents the standard error (±SE).

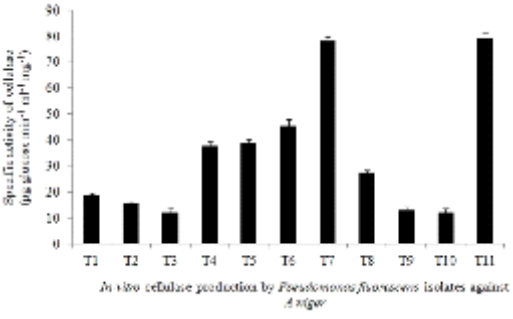


**Fig. 3.b.** *In-vitro* production of Cellulase by *Pseudomonas fluorescens* isolates against *A. niger* (T1 to T10, and T11 as control), the values of each column represent the mean of three independent replications, and the bar value on each column represents the standard error (±SE).

activity. Hence, it may be hypothesized that different isolates of *P. fluorescens* may adapt differently to inhibiting the fungal pathogens by modulating their lytic enzymes production.

**Cellulase activity**

The cellulase is produced by the pathogens and presence of antagonistic *P. fluorescens* inhibits the production of the enzyme, thereby reduces the severity of the fungal diseases. The inhibition of cellulase production of *A. niger* by the tested *P. fluorescens* isolates were significant (Fig. 3a) and ranged between 1.4 (T7) to 84.5% (T10). Specific activity of cellulase was significantly highest in control (79.02 $U.mg^{-1}$ proteins) as no inhibition was made due to the absence of *P. fluorescens* isolates. However, the maximum inhibition of cellulase activity was found in T3 which was at par with T10 and T9. The least inhibition was found in T7 followed by T6. Hence, among the isolates of *P. fluorescens* , *Pf-3* was found to be the best inhibitor of cellulase production by the pathogen, making it the best potential biocontrol against *A. niger.* The cellulose production inhibiting capacity of *Trichoderma* isolates against *A. niger* has suggested its probable role in biocontrol of fungal pathogens[27].

**Polygalacturonase (PG) activity**

*In-vitro* inhibition of PG of *A. niger* by the *P. fluorencens* isolates showed significant differences (Fig. 3b). As expected, the specific activity of PG was significantly higher in control (85.55 $U.mg^{-1}$ protein) because no inhibition was made in absence of the biocontrol agent *P. fluorescens.* The inhibition of PG ativity by the *P. fluorescens* isolates were ranged between 30-82%. The lowest activity of PG was observed in T6 (15.45 $U.mg^{-1}$ protein) and T3 (18.50 $U.mg^{-1}$ protein) followed by *Pf*-1 (20.49 $U.mg^{-1}$ protein) and *Pf*-10 (22.16 $U.mg^{-1}$ protein). Hence, among the *P. fluorescens* isolates, *Pf-6* was identified to be as the best for *in-vitro* inhibition of PG activity (82%) during antagonism between *P. fluorescens* and *A. niger.* The *Pf-3* and *Pf-1* also showed significant inhibition of PG activity of the fungus (78 and 76% respectively). However, in presence of *Pf-7,* the specific activity of PG was highest (59.87 $U.mg^{-1}$ protein), showing the lowest inhibition (30%) among the isolates studied. The present findings were supported by earlier study[28] which has suggested that the *Pseudomonas aeruginosa* GSE 18 and GSE 19 inhibited the *in vitro* activity of plant cell wall degrading enzymes (polygalacturonase and cellulase) of test fungus (*Scelorotium rolfsii*) by 55% and 50% respectively.

**Correlation among lytic enzymes and growth inhibition (%) of fungal pathogen**

From the correlation study among specific activity of lytic enzymes and percent growth inhibition (Table 2), it was found that a highly significant but negative association between percentage growth inhibitions of *A. niger by P. fluorescens* and specific cellulase activity during *in-vitro* study, suggesting the probable role of cellulase inhibition by biocontrol agent during antagonism against fungal pathogens. A similar phenomenon of cellulase activity was reported during *in-vitro* antagonism study between *Trichoderma spp*. and *A. niger* in groundnut[7]. Further, in the present study, a significant negative association was observed between the specific activity of PG and percent growth inhibition, hence it can be hypothesized that the isolates which are capable of inhibition the cell wall degrading enzymes of the fungal pathogens can be better and suitable biocontrol agents. No association was observed for the ability of the *P. fluorescens* isolates to produce $^{2}$-1,3-glucanase and their respective ability to inhibit the fungal growth *in-vitro;* however the ability for chitinase production was shown to have a significant positive association (Table 2).

## CONCLUSION

*P. fluorescens* has been used as biocontrol agent against many fungal pathogens, the present study concluded that certain isolates of *P. fluorescens* can also be used as biocontrol agent against the collar rot pathogen (*A. niger*) of groundnut. Among ten isolates of *P. fluorescens* studied in the present experiment, the isolate 3 (*Pf-3*) was found to be the most suitable biocontrol agent against the test fungus. Further the study revealed the probable roles of lytic enzymes produced by both the *P. fluorescens* and fungal pathogens during *in-vitro* antagonism study. The positive association between chitinase activity and percent growth inhibition during *in-*

*vitro* antagonism confirmed the major role of chitinase in imparting biocontrol ability of *P. fluorescens against* collar rot pathogen *(A. niger)* in groundnut.

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## REFERENCES

1. Bishi, S.K., Lokesh, K., Dagla, M.C., Mahatma, M.K., Rathnakumar, A.L., Lalwani, H.B., Misra J.B. Characterization of Spanish peanut germplasm (*Arachis hypogaea* L.) for sugar profiling and oil quality. *Ind. Crops Prod.*, 2013; **51**: 46–50.
2. Jain, A.C., Nenra, K.G. *Aspergillus* blight of groundnut seedlings. *Sci. Cult.*, 1952; **17**: 348-349.
3. Emmanuel, E.S.C., Rathnavel, S., Maruthamuthu, S. A Survey of collar rot disease of *Arachis hypogaea* by *Aspergillus niger* in certain locations of Madurai district (Tamil Nadu), India. *Plant Archives.*, 2011; **11**: 645-647.
4. Gibson, I.A.S. Crown rot, a seeding disease of groundnut caused by *Aspergillus niger*. II. An anamalous effect of organo-mercurial seed dressings. *Trans. Brit. Mycol. Soc.*, 1953; **36**: 112-119.
5. Ghewande, M.P., Desai, S., Basu, M.S. Diagnosis and management of major diseases of groundnut. *NRCG Bulletin.*, 2002; pp 8-9.
6. Rakh, R.R., Raut, L.S., Dalvi, S.M., Manwar, A.V. Biological controls of *Sclerotium rolfsii*, causing stem rot of groundnut by *Pseudomonas* cf. *monteilii*. *Rec. Res. Sci. Tech.*, 2011; **3**: 26-34.
7. Amein, T., Omer, Z., Welch, C. Application and evaluation of *Pseudomonas* strains for biocontrol of wheat seedling blight. *Crop Protect.*, 2008; **27**; 532–536.
8. Karthikeyan, V., Gnanamanickam, S.S. Biological control of Setaria blast (*Magnaporthe grisea*) with bacterial strains. *Crop Protect*., 2008; **27**: 263–267.
9. Mark, G.L., Morrissey, J.P., Higgins, P., O'Gara, F. Molecular-based strategies to exploit Pseudomonas biocontrol strains for environmental biotechnology applications. *FEMS Microbiol Ecol.*, 2006; **56**: 167–177.
10. Gajera, H.P., Vakharia, D.N. Production of lytic enzymes by *Trichoderma* isolates during *in vitro* antagonism with *Aspergillus niger* the causal agent of collar rot of peanut. *Brazilian J. Micro.*, 2012; 43-52.
11. Sinclair, J.B., Dhingra, O.D. Basic Plant Pathology Method. Florida: CRC Press, Inc. Corporate Blud, 1985; pp 295-315.
12. Reddy, B.P., Reddy, K.R.N., Rao, M.S., Rao, K.S. Efficacy of Antimicrobial Metabolites of *Pseudomonas fluorescens* Against Rice Fungal Pathogens. *Curr. Tre. Biotech. & Pharma.*, 2008; **2**: 178-182.
13. Zarrin, F., Saleemi, M., Zia, M., Sultan, T., Aslam, M., Rehman, R., Chaudhary, M. F. Antifungal activity of plant growth-promoting rhizobacteria isolates against *Rhizoctonia solani* in wheat. *Afri. J. of Biotech*., 2009; **8**: 219–225.
14. Kishore, G.K., Pande, S., Podile, A.R. *Pseudomonas aeruginosa* GSE 18 inhibits the cell wall degrading enzymes of *Aspergillus niger* and activates defence-related enzymes of groundnut in control of collar rot disease. *Aus. Pl. Pathol*., 2006; **35**: 259-263.
15. Sivan, A., Chet, I. Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. *J. Gen. Microbiol*., 1989; **135:** 675-682.
16. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. *J. Biol Chem.* 1951; **193**: 265-75.
17. Collmer, A., Reid, J.L., Mount, M.S. Assay methods for pectic enzymes. In: *Meth. Enzymol*., 1988; **161**: 329-335.
18. Sadashivam, S., Manikam, A. Biochemical methods for agricultural sciences, 3rd edn. New Delhi: New age international publisher, 2008; pp 1-246.
19. Boller, T., Mauch F. Colorimetric assay of chitinase. *Meth. Enzymol.*, 1988; **161**: 431-5.
20. Reissig, J.L., Strominger, J.L., Lefloir, L.F. A modified colorimetric method for the estimation of N-acetyl amino sugars. *J Biol. Chem.*, 1955; **217**: 959-966.
21. Kauffman, S., Legrand, M., Geoffory, P., Fritig, B. Biological functions of 'pathogenesis related' proteins four PR proteins of tobacco have ²-1,3-glucanase activity. *EMBO J.*, 1987; **6**: 3209-3212.
22. Onofri, A. Routine statistical analyses of field experiments by using an Excel extension. In: Proceedings 6th national conference Italian

biometric society: La statistica nelle scienze della vita e dell'ambiente, Pisa, 20–22 June, 2007; pp 93–96.

23. Hammer, O., Harper, D.A.T., Ryan, P.D. PAST–PAlaeontological STatistics, ver. 1.89. *Palaeontol Electronica*., 2001; **4**: 1–9.
24. Sheela, J., Packiaraj. Management of collar rot of groundnut by *Pseudomonas fleuorescens*. *IAN.*, 2000; **20**: 51:53.
25. Manjula, K., Kishore, G.K., Girish, A.G., Singh, S.D. Combined application of *Pseudomonas fluorescens* and *Trichoderma Viride* has an improved biocontrol activity against stem rot in groundnut. *Plant Pathol. J.,* 2004; **20**: 75-80.
26. Nagarajkumar M., Bhaskaran R., Velazhahan R. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. *Microbiological Research*., 2004; **159**: 73–81.
27. Gajera, H.P., Vakharia, D.N. Molecular and biochemical characterization of *Trichoderma* isolates inhibiting a phytopathogenic fungi *Aspergillus niger* Van Tieghem. *Physiol. Mol. Plant Pathol.,* 2010; **74**: 274-282.
28. Kishore, K.G., Pande, S., Podile, A.R. *Pseudomonas aeruginosa* inhibits the plant cell wall degrading enzymes of the *S. rolfsii* and reduces the severity of the groundnut stem rot. *Eur. J. plant pathol*., 2005; **113**: 315-320.