Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Annals of Biological Research, 2010, 1 (1) : 199-207
(http://scholarsresearchlibrary.com/archive.html)

Antifungal metabolites of *Pseudomonas Fluorescens* against Crown Rot Pathogen of *Arachis Hypogaea*

[1]Anand R. and [2]Kulothungan S.

[1, 2]Postgraduate and Research Department of Botany & Microbiology, A. V. V. M. Sri Pushpam College, Poondi, Tanjore, Tamilnadu

---

Abstract

Pseudomonas fluorescens isolated from rhizosphere of healthy groundnut plants were screened for their antagonistic activity towards the crown rot pathogen *Aspergillus niger* in *Arachis hypogaea* L. Invitro assay revealed that 3% (5/60) of the isolates were antagonistic in nature and they were tested for their ability to produce mycolytic enzymes namely protease, lipase and secondary metabolites such as Hydrogen cyanide (HCN), Salicylic acid (SA) and iron chelating Siderophores using standard protocols. Maximum production of mycolytic enzymes (Protease - 48.7U/ml; Lipase – 4.2U/ml) and secondary metabolites (HCN – 0.0.8 Abs_{625}; SA – 6.14 mg/ml; Siderophores – 4.92 µ mol benzoic acid/ml) were found with *Pseudomonas fluorescens* 04, an assert for biocontrol of phytopathogens, when put in to practice.

Keywords: *Pseudomonas fluorescens*, Protease, Lipase, HCN, SA, Siderophores.

Introduction

Understanding the diversity and beneficial activity of the plant-bacterial association is important to sustain agro-ecosystems for sustainable crop production [1]. Several bacteria thrive on abundant nutrients in the rhizosphere and some of these possess antagonistic action, which safeguard plants from pathogens and stimulate growth [2].

Arachis hypogaea [groundnut], an annual legume is known as peanut, earthnut, monkeynut and goobers. It is the 13th most important food crop and 4th most important oilseed crop of the world. Crown or collar rot caused by *Aspergillus niger* in groundnut leads to “patchy” crop stand and ultimately reduce the yields. Collar rot reported to cause 40 per cent loss in yield in India [3].

With increasing awareness of possible deleterious effects of fungicides on the ecosystem and growing interest in pesticide free agricultural products biological control now appears to be a promising strategy for managing diseases in a range of crops [4,5]. The search for alternatives to chemical control of pathogens, such as biological control has gained momentum in the recent years due to the emergence of fungicide resistance in pathogens besides increased health concerns for the producer and the consumer [6].

Biological control is a potent means of reducing the damage caused by plant pathogens [7]. Biological control of plant disease can occur through different mechanisms, which are generally classified as; antibiosis, competition, suppression, direct parasitism, induced resistance, hypovirulence and predation. The antagonistic activity has been associated with the production of secondary metabolites [8,9].

Ecofriendly biological control was given high priority in the Integrated Disease Management (IDM). Understanding the mechanisms through which the biocontrol of plant diseases occurs is critical to the eventual improvement and wider use of biocontrol methods [10].

Pseudomonas fluorescens, the root colonizing bacterial biocontrol strain suppresses soil-borne plant diseases caused by phytopathogenic fungi [11-16]. Production of secondary metabolites like antibiotics, Fe-chelating siderophores and cyanide is most often associated with fungal suppression by fluorescent pseudomonads [17]. The present study demonstrates the ability of *P.fluorescens* to produce lytic enzymes and secondary metabolites that serve as biochemical weapons against phytopathogens.

Materials and Methods

Isolation and Characterization of Phytopathogen

The rotted seedlings with black mass of spores in collar were collected from a farm and brought to the laboratory for further studies. The fungal pathogen namely *Aspergillus niger* was isolated from the collar of *Arachis hypogaea* L, using PDA and further characterized based on macroscopic and microscopic observations (LPCB staining).

Isolation and Characterization of Bacterial Antagonist

Pseudomonas fluorescens was isolated from rhizosphere of healthy groundnut plants and maintained in laboratory using Kings B at 4°C. Antagonistic actions of these bacterial isolates were confirmed by performing Dual Plate Method (DPM). To evaluate the mycolytic enzymes producing ability of the antagonistic strain, the King's B broth was amended with autoclaved mycelium of the pathogen, *Aspergillus niger*.

Mycolytic Enzymes Produced by *P.fluorescens*

Protease

For protease activity, a reaction mixture containing 1.0 ml of 1% soluble casein in 0.05M citrate phosphate buffer (pH 6.5) and 1.0 ml of supernatant of the culture filtrate was added. The reaction mixture was incubated for 1 h at 37ºC, then stopped by adding 10 % trichloroacetic acid (TCA), kept for another 20 min at the same temperature, followed by centrifugation at 4000 rpm for 20 min. Samples of 75µl were removed and tyrosine was determined according to Lowry

[18]. One unit of the enzyme activity was defined as the amount of enzyme required for the formation of 1.0 µM of the product/min of the reaction, under the standard assay conditions.

Lipase

Modified method of Elad [19] was followed for the estimation of lipases secreted by the antagonistic culture. 2 ml of the crude enzyme (culture supernatant) was diluted with 8 ml of distilled water and mixed well with 0.5 ml of vegetable oil. The reaction mixture was incubated at 37ºC for 2 hrs in a rotary shaker at 200 rpm. Ethanol was added to obtain a final concentration of 30%. Free fatty acids formed were extracted with pure petroleum ether and the extractant was evaporated in a rotary evaporator. The free fatty acids were dissolved in 15 ml of neutralized ethanol containing phenolphthalein at 60ºC. Each sample was titrated with ethyl alcohol containing 0.5N NaOH. Free fatty acids were neutralized and one lipolytic unit (LU) was defined as micromoles of NaOH/mg protein/hr.

Secondary Metabolites Produced by *P.fluorescens*

Hydrogen Cyanide

P.fluorescens isolates were grown at room temperature (28±2ºC) on a rotary shaker in King's B broth. Filter paper (Whatman No.1) was cut in to uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed inside the conical flask in a hanging position. After incubation at 28ºC for 48 hours, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. Placing the filter paper in a clean test tube containing 10 ml distilled water eluted the color and the absorbance was measured at 625 nm [6].

Salicylic acid

P.fluorescens isolates were grown at room temperature (28±2ºC) for 48 hours on a rotary shaker in 250 ml conical flask containing 50 l of the King's B broth. Cells were then collected by centrifuging at 10,000 rpm for 10 minutes and 4 ml of cell free culture filtrate was acidified with 1N HCl to pH 2.0 and salicylic acid was extracted with chloroform (2x2 ml). To the pooled chloroform extracts, 4 ml of distilled water and 5 ml of 2M $FeCl_3$ were added. The absorbance of the purple iron-salicylic acid complex, which was developed in the aqueous phase, was read at 527 nm in a spectrophotometer [20]. A standard curve was prepared with salicylic acid dissolved in King's B broth. The quantity of salicylic acid in the culture filtrate was expressed as mg ml^{-1}.

Siderophores

P.fluorescens isolates were grown at room temperature (28±2ºC) on a rotary shaker in King's B broth for 3 days and centrifuged at 10000 rpm for 10 min and the supernatant was collected. The pH of the supernatant was adjusted to 2.0 with 1N HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. Five ml of ethyl acetate fraction was mixed with 5ml of Hathway´s reagent (The reagent was prepared by adding 1ml of 0.1 M ferric chloride in 0.1N HCl to 100ml of distilled water, and to this 1ml of 0.1 M potassium ferricyanide was added). The absorbance for dihydroxy phenols was read at 700nm in a spectrophotometer [21]. A standard curve was prepared using dihydroxy benzoic acid. The quantity of siderophore synthesized was expressed as µ mol benzoic acid ml^{-1} of culture filtrate.

Results and Discussion

Interest in biological control has recently intensified because of imminent bans of chemical controls such as methyl bromide, widespread development of fungicide resistance in pathogens [22] and a general need for a more effective and safer alternative disease control strategies [23]. Plant Growth Promoting Bacteria (PGPB) can benefit plant growth by different mechanisms [24]. Any biocontrol agent, which acts as nutrient mobiliser in the rhizosphere, would be an added advantage [25] that is applicable for *P.fluorescens*. It can induce physiological changes throughout entire plants, making them more resistant to pathogens [26,27].

Among the different strains tested *P.fluorescens* was found to be potent producer of mycolytic enzymes and secondary metabolites. It produces a maximum of 48.7U/ml protease and 4.2U/ml of lipase in King's B broth supplemented with autoclaved mycelium of the pathogen (2g/100ml). Mycoparasitism by *P.fluorescens* using scanning electron microscope (SEM), revealed the attachment of *Pseudomonas* to fungal hyphae, followed by penetration on to fungal cell wall causing deterioration of fungal mycelium and cell wall [28,29], possibly due to the secretion of extracelluar mycolytic enzymes. In *Pseudomonas fluorescens* CHA0, mutation of *aprA* gene encoding for protease resulted in reduced biocontrol [30], which substantiate the antagonistic effect on protease.

HCN, a volatile metabolite is thought to play a major role in biological control of some soil borne diseases [31]. Exposing plants to the volatile metabolites of antagonist causes a significant increase in peroxides activity, which may contribute to induction of disease resistance [32]. In our study *P.fluorescens* 04 was found to produce HCN at the rate of 0.0.8 $Abs_{625.}$ The production in King B broth is better than reported by earlier workers [33] in Trypticase soy agar. In addition to HCN *Pseudomonas* spp. were reported to produce antibiotics like 2, 4 - diacetyl phloroglucinol and pyoluteorin [20,34] that has profound action through antibiosis [35].

Salicylic acid is known to play a critical role in the activation of plant defense response [36,37,38] after pathogen attack [39,40,41] and induces the expression and accumulation of pathogenesis related proteins in the leaves [42,43]. A maximum concentration of 6.14 mg/ml of salicylic acid was produced by *P.fluorescens* 04 in our investigation. It acts as endogenous signal for the activation of plant defense including hypersentive cell death [44,45].

Siderophores are low molecular weight (0.5 to 1.5KDa), high specificity Fe^{3+} chelating agents [46]. The present study revealed varying levels of siderophore production by test *Pseudomonas* spp. ranging from 1.0 to 4.92 µ mol benzoic acid/ml. Siderophores produced by *P.fluorescens* including salicylic acid, chelates ion and other metals; also contribute to disease suppression [47,48,49] through the induction of the biosynthesis of other antimicrobial compounds [22,26]. Also the water soluble fluorescent pigment produced by fluorescent pseudomonads was reported to mimic siderophore characters [51,52].

One of the pre-requisites for the efficiency of biological control agents in controlling the plant diseases is their capacity to survive in the target sites [53,54,55]. *Pseudomonas* has been shown to reduce soil borne fungal diseases when applied as seed, soil or root inoculants [56,57].

Conclusion

Since *P.fluorescens* strains possess multiple mechanism of antagonism and stable production of antifungal agents under variable growth conditions to sustain antagonism during plant root development, it can be explored as one among the best biocontrol agent against phytopathogens.

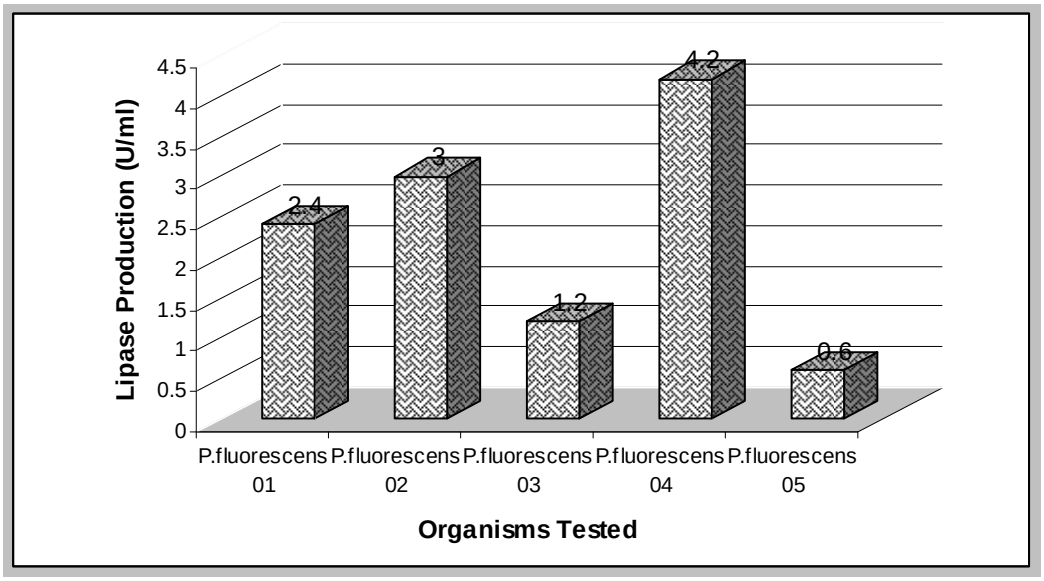

Fig-1: Production of Lipase by isolates of *P.fluorescens*

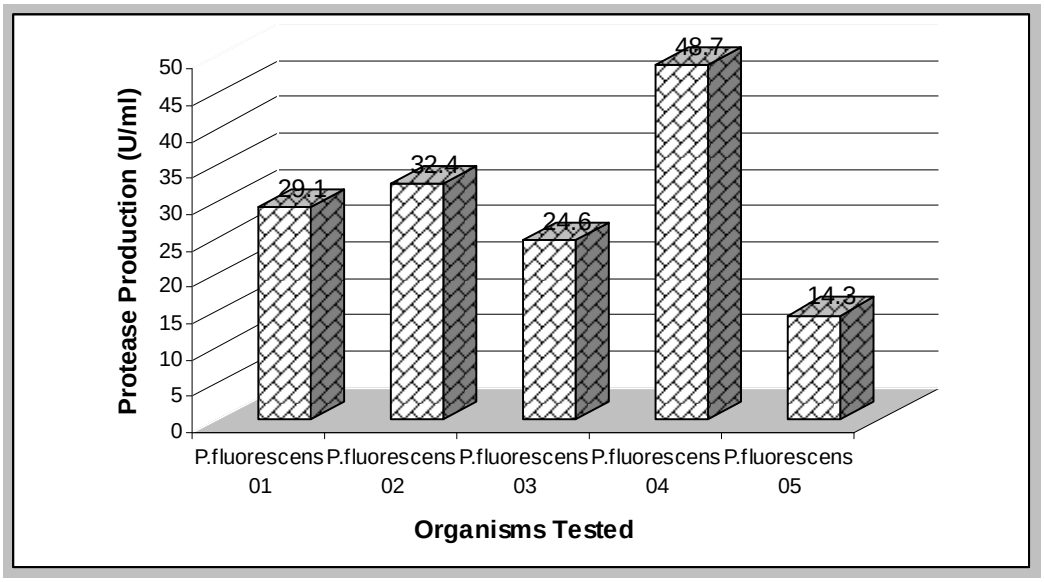

Fig-2: Production of Protease by isolates of *P.fluorescens*

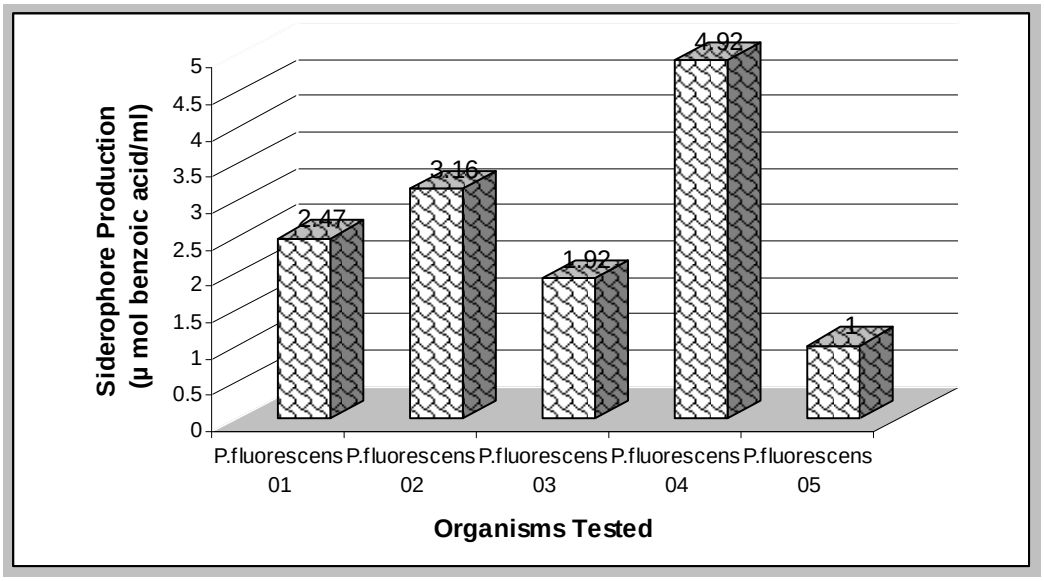

Fig-3: Production of Siderophores by isolates of *P.fluorescens*

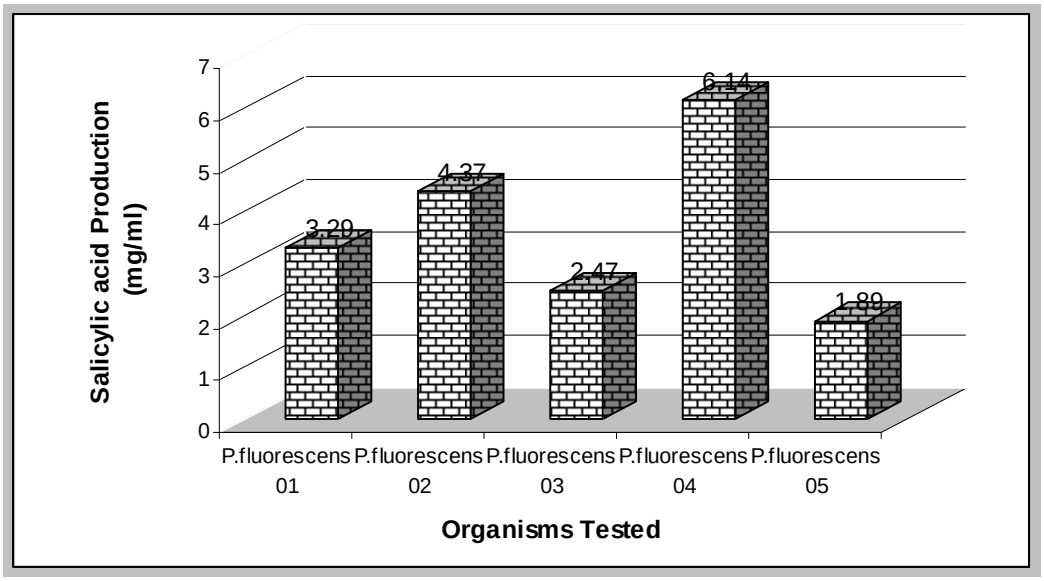

Fig-4: Production of Salicylic acid by isolates of *P.fluorescens*

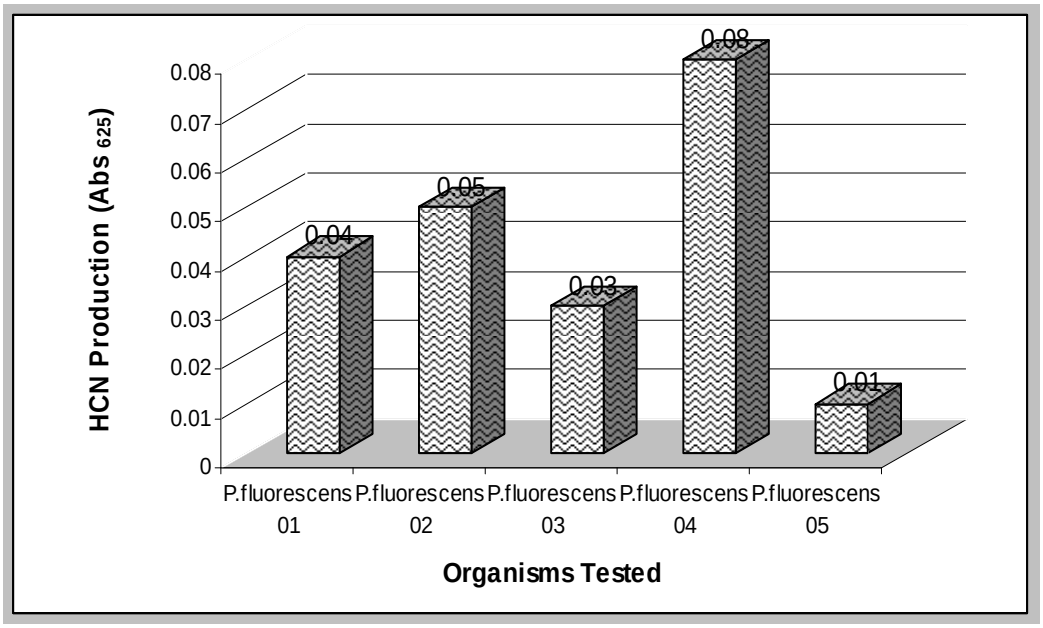

Fig-5: Production of HCN by isolates of *P.fluorescens*

References

[1] J.J. Germida, S.D. Siciliano, J.R. De Freitas. *FEMS Microbial Ecology*, **1998;** 26: 43-50.
[2] E.J. Gray, D.L. Smith. *Soil Bio. Biochem.*, **2005;** 37:395-410.
[3] J.S. Chohan. *J. Res.*, **1973;** 6:634-640.
[4] R. Deborach. *Nato Advance Research Workshop Biological Control of Plant Diseases*, **1991;** 9-24.
[5] B.V. David. *Ind. Phytopathol.*, **2008;** 1(1):1-5.
[6] B.P. Reddy, K.R.N. Reddy, M. Subba Rao, K.S. Rao. *Cur.Trends. Biotechnol. Pharma.*, **2008;** 2(1):178-182.
[7] R. Jayarajan, S. Nakkeeran. *Biocontrol potential and its exploitation in Sustainable Agriculture*, *Biocontrol*, **2000;** 1:95-116.
[8] R. Velzahan, R. Samiyappan, P. Vidyasekaran. *J. Pl. Dis.Protec.*, **1999;** 106:244-250.
[9] J.W. Kloepper, C.M. Ryu, S. Zhang. *Phytopathol.*, **2004;** 94:1259-1266.
[10] D. Paul, Y.R. Sharma. *Arch. Phytopathol. Pl. Protec.*, **2006;** 39(0):1-6.
[11] J.W. Kloepper, M.N. Schroth. *Phytopathol.*, **1981;** 71:1020-1024.
[12] D.M. Weller, W.J. Howie, R.J. Cook. *Phytopathol.*, **1988;** 78:1094-1100.
[13] P. Diby, A. Kumar, M. Anandraj, Y.R. Sarma. *Ind. Phytopathol.*, **2001;** 54(4):515.
[14] M.S. Khan, A. Zaidi. *Ann. Pl. Protect. Sci.*, **2002;** 10(2): 265-27.
[15] S. Moataza. *Res. J. Agri. Biol. Sci.*, **2006;** 2(6):74-281.
[16] D. Vleesschauwer, M. Javaheri, P. Bakker, M.Hofte. *Pl. Physiol.*, **2008;** 148:1996-2012.
[17] B. Lovic, C. Heck, J.J. Gallian, A.J. Anderson. *J. Sugar Beet. Res.*, **1993;** 30:169-184.
[18] O. Lowry, N. Rosebrouh, A. Farr, R. Randall. *J. Biol. Ceh.*, **1951;** 139:265.
[19] Y. Elad, I. Chet, Y. Henis. *Can. J. Microbiol.*, **1982;** 28:719-725.
[20] J.M. Meyer, P. Ajelvandre, C. Georges. *Biofactors*, **1992;** 4:23-27.
[21] M. Reeves, L. Pine, J.B. Neilands, A. Bullows. *J. Bacteriol.*, **1983;** 154: 324-329.

[22] B.K. Duffy, G. Defago. *Appl. Envtl. Microbiol.*, **1999;** 65(6): 2429-2438.
[23] D. Fravel. *Ann. Rev. Phytopathol.*, **2005;** 43: 337-359.
[24] Y. Bashan, L.E. De-Bashan. *Encyclopedia of Soils in the Environment, 1, Elsevier*, **2005;** 103-115.
[25] P. Diby, K.A. Saju, P.J. Jisha, Y.R. Sarma, A. Kumar, M. Anandraj. *Ann. Microbiol.*, **2005;** 55(2): 129-133.
[26] G.D. Meyer, M. Hofte. *Phytopathol.*, **1997;** 87(6): 588-593.
[27] L.C. Van Loon, P.A. Bakker. *Springer, Berlin,* **2003;** 297-330.
[28] Haggag, M. Wafaa. *Arab. J. Biotechnol.,* **2002;** 5:151-164.
[29] E.H.E. Ziedan, R.S.R. El-Mohamedy. *Res. J. Agri. Biol. Sci.,* **2008;** 4(5): 346-353.
[30] I.A. Siddiqui, D. Haas, S. Heeb. *Appl. Envtl. Microbiol.*, **2005;** 71(9): 5646-5649.
[31] I.A. Siddiqui, S.S. Shaukat, I.H. Sheikh, A. Khan. *W. J. Microbiol. Biotechnol.,* **2006;** 22: 641-650.
[32] F.M. Wangi, R. Hauschild, R.A. Sokora, E. Mutitu. *Challenges to Organic farming and Sustainable land use in the Tropics and subtropics, Deutschea Tropentag, Witzenhausen,* **2002;** October 9-11.
[33] L.R. Freeman, P. Angelini, G.J. Silverman, C. Merritt. *Appl. Microbiol.*, **1975;** 29(4): 560-561.
[34] G. Defago, C.H. Berling, U. Burger, D. Haas, G. Khar, C. Keel, C. Voisard, P. Wirthner, B. Wuthrich. *Biological Control of Soil borne Plant Pathogens*, **1990;** 93-108.
[35] C.R. Howell, *Trichoderma **and** Gliocladium*, **1998;** 2:173-184.
[36] M. Maurhofer, C. Reimman, P. Sachere, S. Heep, D. Hass, G. Defago. *Phytopathol.*, **1998;** 88:678-684.
[37] P.A. Bakker, L.X. Ran, C.M.J. Pieterse, L.C. Van Loon. *Can. J. Pl. Pathol.,* **2003;** 25:5-9.
[38] I.A. Siddiqui, S.S. Shaukat. *J. Pl. Pathol.*, **2005;** 4(1): 21-25.
[39] J. Draper, *Tre. Pl .Sci.,* **1997;** 2:162-165.
[40] K. Shirasu, H. Nakajima, K. Rajashekar, R.A. Dixon, C. Lamb. *Pl. Cell.*, **1997;** 9:261-270.
[41] D.F. Klessig, J. Durner, R. Noad, D.A. Navarre, D. Wendehenne, D. Kumar, J.M. Zhou, H. Silva. *Proceedings of National Academy of Sciences*, **2000;** 16:8849-8855.
[42] L. Sticher, B. Mauch-Mani, J.P. Metraux. *Ann. Rev. Pl. Pathol.*, **1997;** 35: 235-270.
[43] B. Nandi, N.C. Sukual, N. Banerjee, S. Sengupta, P. Das, P.S. Babu. *Mediter. Phytopathol.*, **2002;** 41: 39-44.
[44] M.V. Rao, J.R. Koch, K.R. Davis. *Pl. Mol. Bio.*, **2000;** 44:345-358.
[45] M.V. Rao, H. Lee, R. Davis. *The Plant Journal*, **2002;** 32: 447-456.
[46] K. Barbeau, G. Zhang, D.H. Live, B. Alison. *J. Amer. Chem. Soc.*, **2002;** 124:378-379.
[47] J.E. Loper, M.D. Henkels. *Appl. Envtl. Microbiol.*, **1997;** 63:99-105.
[48] Haggag, M. Wafaa, S.A. Abo Sedera. *Egypt. J. Phytopathol.*, **2000;** 28:1-16.
[49] B.P. Dave, H. Dube. *Ind. Phytopathol.*, **2000;** 53:97-98.
[50] J.M. Meyer, M.A. Abdallah. *J. Gen. Mirobiol.*, **1978;** 107:319-328.
[51] J. Misaghi, J. Stowell, R.G. Grogan, L.C. Spearman. *Phytopathol.*, **1982;** 72(1): 33-36.
[52] D. Haas, C. Keel. *Annual Review of Phytopathol.*, **2003;** 41:117-153.
[53] S. Chatterton, J.C. Sutton, G.J. Boland. *Biological Control*, **2004;** 30:360-373.
[54] R. Vimala, M. Suriachandraselvan. *J. Biopes.*, **2008;** 1(2):130-133.
[55] Y. Wang, W.H. Tang, L.O. Zhang. *8th Congress of Plant Pathology, 2-7 Feb., Christchurch, Newzealand,* **2003;** 28.

[56] E.H. Ziedan, M. Saad, R.S. El-Mohamedy, S. Farrag. *Afr. J. Mycol. Biotechnol.,* **2005;** 13(3):19-36.
[57] M.N. Nielsen, J. Sorensen, J. Fels, H.C. Pedersen. *App. Envtl. Microbiol.,* **1998;** 64(10):3563-3569.