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Induction of phenolics and lipoxygenase in *Arachis hypogaea* in response to crown rot infection

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ABSTRACT

Pseudomonas fluorescens and Bacillus subtilis isolated from rhizosphere of healthy groundnut plants were screened for their antagonistic potential against the crown rot pathogen Aspergillus niger of Arachis hypogaea L. Antagonistic isolates were further challenged for their ability to induce phenolics and lipoxygenase in A.hypogaea seedlings. Maximum accumulation of phenolics (Monophenol - 42 mg g^{-1} FW; Diphenol - 34 mg g^{-1} FW; Total phenol - 261 mg g^{-1} FW) and lipoxygenase (44 Abs mg-1 min-1) occurs in shoots of 30 days old seedlings. GC-MS studies revealed the presence of precursors of hydroperoxides that plays a key role in plant defense mechanism.

Keywords: *Pseudomonas fluorescens*, *Bacillus subtilis*, *Aspergillus niger*, Phenolics, Lipoxygenase.

INTRODUCTION

Safeguarding crop plants from vulnerable attack of phytopathogens using eco-friendly techniques is the core thought of farming community. Understanding the diversity and beneficial activity of the plant-bacterial association is important to sustain agro-ecosystems for sustainable crop production [1].

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 [2].

Pseudomonas fluorescens and *Bacillus subtilis* are root colonizing bacterial biocontrol strains that suppresses soil-borne plant diseases caused by phytopathogenic fungi [3,4,5,6]. These antagonistic bacterial agents induce the immune mechanism of plants to produce significant levels of Plant Defense Enzymes (PDE) [7,8].

The synthesis of phenolic compounds is often enhanced in plant tissue under stress such as mechanical damage [9] or infection by microorganisms [10,11] or oxidative damage [12]. Symptoms such as brown pitting, necrosis, deterioration of mitochondrial activity and cell damage have been associated with increased deposition of phenolic compounds [13].

The synthesis of cell bound phenolics through phenyl propanoid pathway [14] play a major role in defense against pathogen attack [15] and resist cell wall degradation, since they are lignin precursors [16]. The phenolic moieties such as 4-coumariac acid, ferulic acid, sinapic acid and caffeic acid are produced from cinnamate via a series of hydroxylation, methylation and dehydration reactions [17].

Plants express resistance to microbes by mechanism involving metabolism of the poly unsaturated fatty acids (PUFA) such as linoleic acid and linolenic acid via the lipoxygenase (LOX) pathway [18]. Earlier studies demonstrated LOX activation occurs in plants following elicitor treatment or inoculation with pathogens [19]. The products of LOX pathway contributes to defense mechanism by inhibition of pathogen growth and development [20] and induction of phytoalexin accumulation [21].

The activation of Induced Systemic Resistance (ISR) by non-pathogenic rhizobacteria has been associated with the induction of lipoxygenase activity [22,23,24]. The present study was undertaken to analyze and compare the changes that occur in phenolic content and lipoxygenase during infection with *A.niger* and treatment with antagonistic PGPR bacterial strains namely *Pseudomonas fluorescens* 04 and *Bacillus subtilis* 03 in *Arachis hypogaea* L. seedlings.

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 and *Bacillus subtilis* were isolated from rhizosphere of healthy groundnut plants and maintained in laboratory using Kings B and Nutrient agar respectively at 4°C. Antagonistic actions of these bacterial isolates were confirmed by performing Dual Plate Method (DPM).

Procuring Seeds and Raising Seedlings

Surface sterilized groundnut seeds procured from Seed Science Department, TamilNadu Agricultural University (TNAU), Coimbatore, were used in the study. Seeds were sown in pots containing loamy soil and the seedlings were raised in open field condition. After 15 days of germination, the microbial cultures were inoculated in soil (T1-Control; T2-*Aspergillus niger*; T3- *Bacillus subtilis*; T4- *Pseudomonas fluorescens*; T5-Combination of *Pseudomonas fluorescens* and *Bacillus subtilis*) to evaluate the induction of Phenolics and Lipoxygenase in the host plant. After 5^{th}, 10^{th} and 15^{th} day of inoculation, the root and shoot portion of the host plant were utilized in the assay.

Extraction of Phenol

Tissue extracts were prepared by homogenizing 1g of plant tissue with sterilized silica and 5 ml of 80% ethyl alcohol in borate buffer (0.2M, pH 7.6). The homogenate was centrifuged at 10,000 rpm for 5 min at room temperature and the supernatant was used to quantify the phenols [25].

Estimation of Monophenol

The reaction mixture comprised of 100µl phenol extract, 400µl distilled water, 400µl sodium hydroxide (0.5N), 500µl 4-Aamino antipyrene (0.6%), 600µl sodium bicarbonate (9.5M) and 500µl potassium ferrocyanide (2.4%). The resulting color was measured at 520nm using spectrophotometer [26]. Hydroxyl benzene was used as standard and expressed as mg g^{-1} FW (Fresh Weight) of the tissue.

Estimation of Diphenol

The assay mixture includes 50µl phenol extract, 450µl distilled water, 1000µl hydrochloric acid (0.5N), 500µl Arnows reagent (10% sodium nitrite and 10% sodium molybdate in 100 ml distilled water) and 1000µl sodium hydroxide (1N).Absorbance was measured at 525nm [27]. Catechol was used as standard and expressed as mg g^{-1} FW (Fresh Weight) of the tissue.

Estimation of Total Phenol

The reaction mixture contains 20µl phenol extract, 880µl distilled water, 100µl Folin-Ciocalteu reagent (1N) and 2000µl saturated solution of sodium carbonate. The intensity of blue color formed was measured at 660nm using spectrophotometer [28]. Chlorogenic acid was used as standard and expressed as mg g^{-1} FW (Fresh Weight) of the tissue.

Extraction of Lipoxygenase

1 g of plant tissue was homogenized using acetone powder (Acetone subjected to freezing at -20ºC) in the ratio 1:10 (w/v) with 0.2M Tris-HCl (pH -8, 4ºC) for 3 min using a homogenizer at 15,000 rpm. The extract was centrifuged for 1 hr at 15,000 rpm at 4.C and the supernatant was kept over ice until analyzed [29].

Lipoxygenase Assay

LPO activity was assayed using the method described by [30]. The 0.01M substrate stock mixture contained 157.2µl linoleic acid, 157.2µl tween-20 and 10 mL water. Mixing was carried out by filling and ejecting the solution in a Pasteur pipette several times while avoiding air bubbles. Finally the solution was clarified by adding 1 mL of 1N NaoH and made to 50 mL. Prior to assay the, the substrate stock solution was diluted to 200 mL with 0.2M sodium phosphate buffer (pH-7) to give a final concentration of 2.5mM linoleic acid. All glass wares used in the preparation of substrate solution has been wrapped with aluminium foil to exclude light.

For LPO activity assay, 0.3 mL enzyme extract was added to 2.7 mL substrate solution and mixed well. The initial rate of conjugated diene formation was measured from the linear change in absorbance at 234nm using double beam spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that produced a change in absorbance at 234nm. Activity was expressed as Abs234 mg^{-1} min^{-1}.

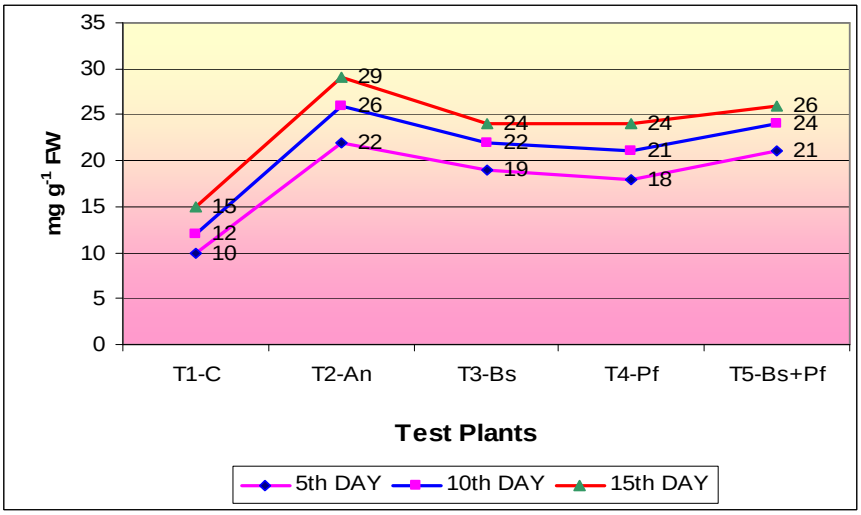

Fig-1: Assay of Monophenol in Roots of Seedlings

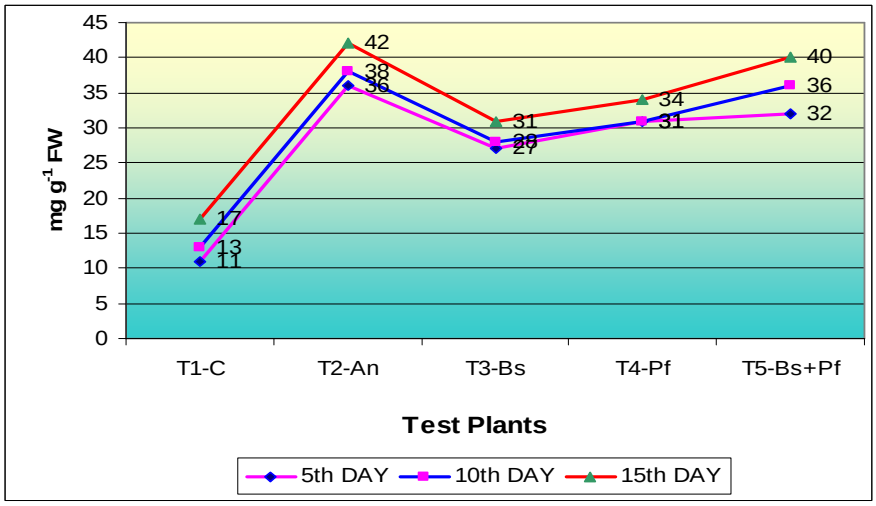

Fig-2: Assay of Monophenol in Shoots of Seedlings

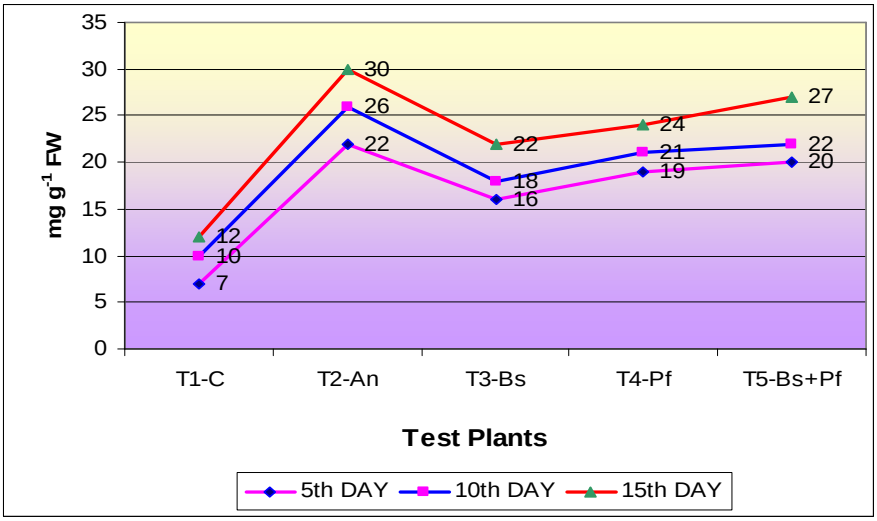

Fig-3: Assay of Diphenol in Roots of Seedlings

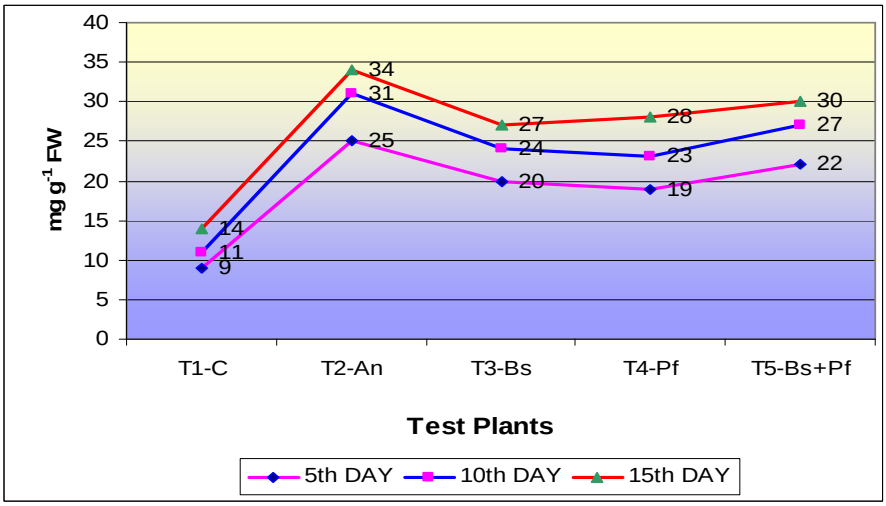

Fig-4: Assay of Diphenol in Shoots of Seedlings

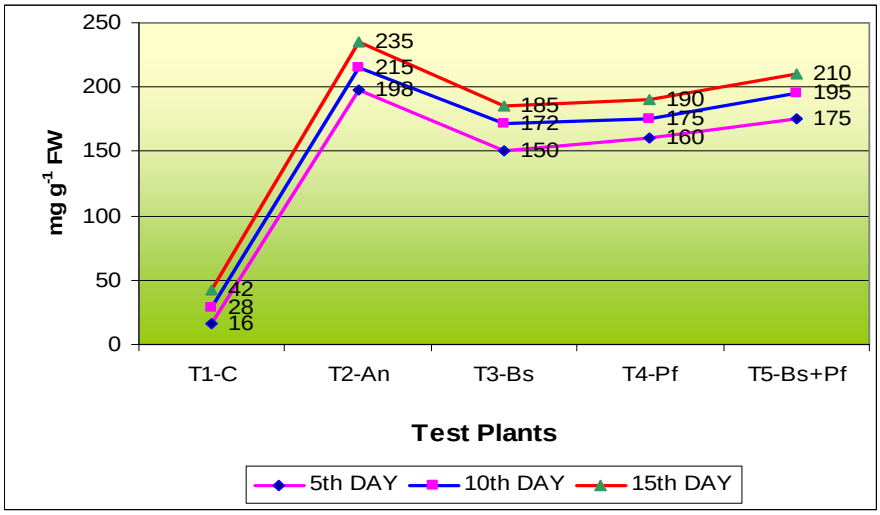

Fig-5: Assay of Total Phenol in Roots of Seedlings

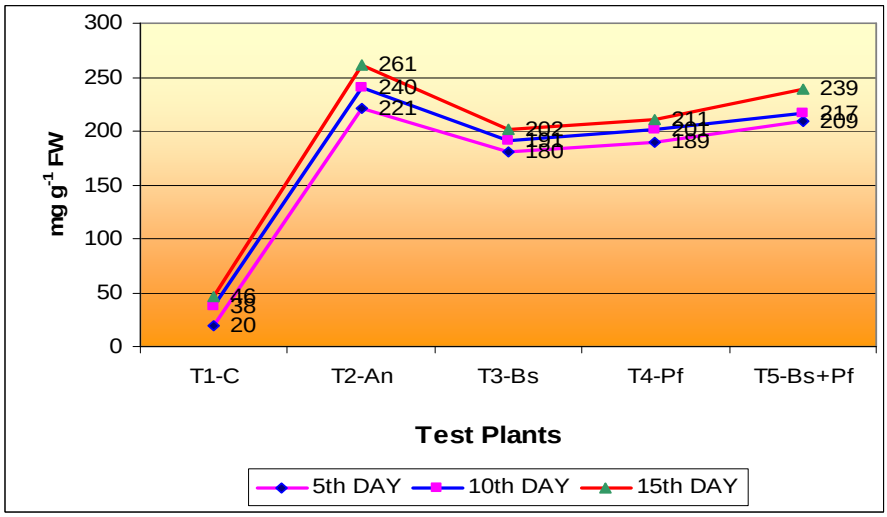

Fig-6: Assay of Total Phenol in Shoots of Seedlings

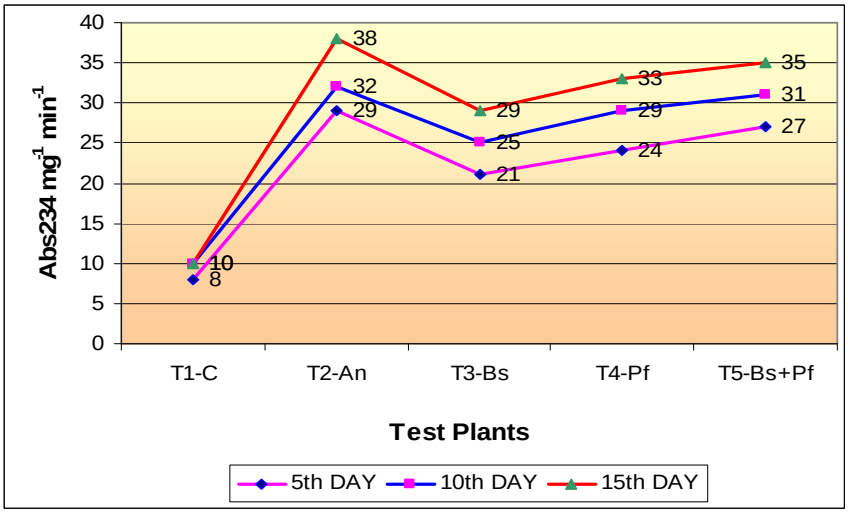

Fig-7: Assay of Lipoxygenase in Roots of Seedlings

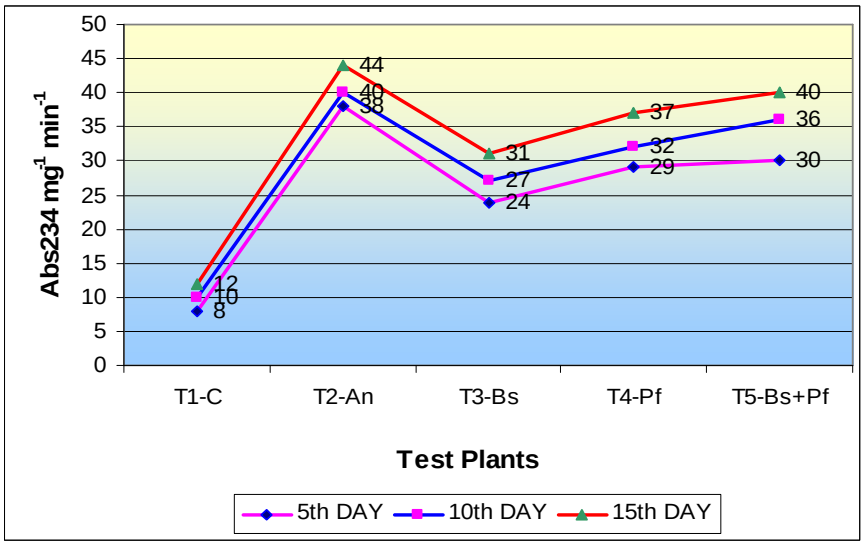

Fig-8: Assay of Lipoxygenase in Shoots of Seedlings

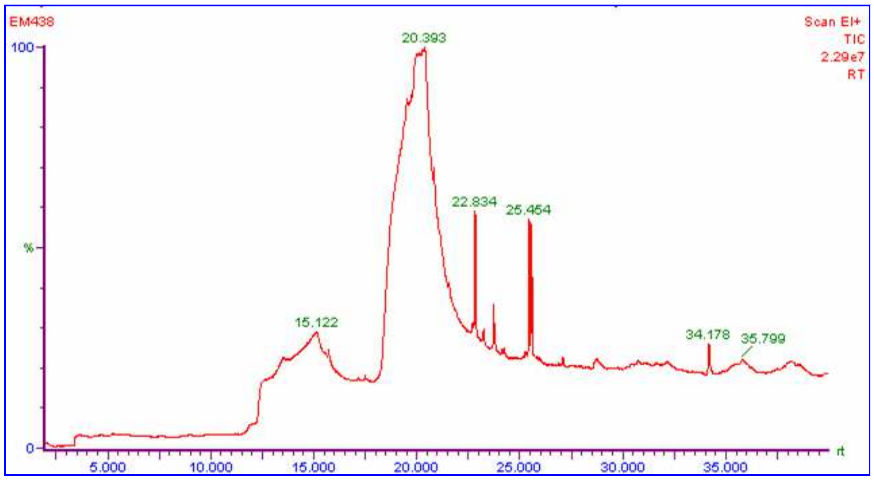

Fig-9: GC-MS of Lipoxygenase products

RESULTS AND DISCUSSION

Plants have inbuilt defense mechanism against phytopathogens. A trigger by a stimulus, prior to infection by a virulent pathogen could reduce disease symptoms. Some plant growth promoting rhizobacteria are able to reduce disease through the stimulation of inducible plant defense mechanisms that render host plant more resistant to further pathogen ingress [31]. This phenomenon is called as Induced Systemic Resistance (ISR).

ISR is contrast to SAR (Systemic Acquired Resistance), where resistance is initiated by the pathogen. ISR is long-lasting, general defense and not specific to a particular pathogen [32]. It gains more importance in integrated pest management strategies [8].

Our investigation in *A.hypogaea* L. seedlings revealed that the phenolic compounds were found to accumulate more in shoots than in roots. Also the concentration of phenolics increases with age of seedlings (Fig-1 to 6). Similar strategy was observed with lipoxygenase too (Fig-7, 8). Maximum accumulation of phenolics (Monophenol - 42 mg g^{-1} FW; Diphenol - 34 mg g^{-1} FW; Total Phenol - 261 mg g^{-1} FW) and lipoxygenase (44 Abs mg^{-1} min^{-1}) occur in the

shoots of experimental set up T2, where the spore suspension is of *A.niger* was inoculated. ISR in terms of phenolics induced by *B.subtilis* and *P.fluorescens* in combinations is considerably good (Fig-1 to 6).

The increase in phenolic contents of the host plant could be correlated as defense mechanism triggered by the interaction with an incompatible necrotizing pathogen [33]. The presence of trace phenolics in control indicate the inherent chemical defense, which get elevated at the time of stress, physical damage or infection by pathogen. The concentration of phenolics is usually higher in resistant varieties than in susceptible varieties [34].

The response of plants to pathogens is characterized by the early accumulation of phenolic compounds at the site of infection, which limit the development of pathogen as a result of rapid cell death [35]. The importance of phenolic compounds in host-parasite interaction is that they act as H donor / acceptors in oxidation/reduction reactions and their involvement in resistance by oxidation to quinines which are more toxic to microorganisms [36]. Apart from protecting plants from pathogens, phenolics are also involved in strengthening the plant cell wall during growth by polymerization in to lignins [37].

Significant level of Lipoxygenase (LOX) accumulate in seedlings (T5) inoculated with combination of *B.subtilis* and *P.fluorescens* (Fig-7, 8). The synergistic action of these PGPR strains could be interrelated for such elevated LOX induction when compared to control. The breakdown products of lipoxygenase were reported to inhibit the growth of *A.niger* [18], since the end products of the pathway alter the phytoalexin metabolism in seedlings and confer protection against the crown rot caused by *A.niger* [38].

Lipoxygenase (LOX) pathway in plants leads to synthesis of various compounds that display antimicrobial activity [39]. Lipoxygenase mediated defense response does not simply result from mechanical wounding of the tissue during infection process but it represent a sequence of interaction between host plant and the microorganism [40]. The major products of LOX pathway are 9S- hydroperoxy linoleic acid and 13S- hydroperoxy linolenic acid.

The 13-monohydroperoxides are precursors of biologically active compounds such as traumatin, jasmonic acid and methyl jasmonate, which have hormone like regulatory and defense related role in plants [41,42]. The 9-monohydroperoxides are reported to posse's antimicrobial properties and induce hypersensitive reaction, a form of programmed cell death, one of the active defense mechanism against microbial invasion [43,44].

GC-MS studies (Fig-9) of the tissue extracts shows the presence of various chemical components of LOX including 9-octa decanoic acid and methyl esters, which serve as precursor for the formation of 13 S-HPODE (13S-Hydroperoxy octadeca dienoic acid) and 13S-HPOTE (13S- Hydroperoxy octadeca trienoic acid), a phenomenal compound associated with plant defense as reported early workers [18,45,46].

CONCLUSION

The outcome from this investigation recommends the combined application of *B.subtilis* and *P.fluorescens*, the antagonistic PGPR strains, which could afford protection against crown rot pathogen *A.niger* in *A.hypogaea* L., by Inducing ISR, phenolic and lipoxygenase pathway, a phenomenon that forms the basis of plant disease control strategies.

REFERENCES

[1] J. J. Germida, S. D. Siciliano, J. R. De Freitas, *FEMS Microbiol, Ecol*., **1998**, 26: 43-50.
[2] J. S. Chohan, Journal *of Research.*, **1973**, 6: 634-640.
[3] K. Manjula, A. R. Podile, *Can. J. Microbiol*., **2001**, 47(7): 618-625.
[4] M. Nagarajkumar, R. Bhaskaran, R. Velazhahan, *Microbiol. Res*., **2004**, 159(1): 73-81.
[5] M. Moataza, M. Saad, *Res. J. Agric. Biol. Sci*., **2006**, 2(6):274-281.
[6] D. Vleesschauwer, M. Javaheri, P. Bakker, M. Hofte, *Plant Physiol*., **2008**, 148: 1996-2012.
[7] D. Saravanakumar, C. Vijayakumar, N. Kumar, R. Samiyappan, *Crop. Prot*., **2007**, 26(4): 556-565.
[8] P. Latha, T. Anand, N. Ragupathi, V. Prakasam, R. Samiyappan, *Biol. Control*., **2009**, 50(2): 85-93.
[9] L. F. Reyes, R. Cisneros-Zevallos, *J. Agric. Food. Chem*., **2003**, 51: 5296-5300.
[10] C. E. Modafar, A. Tantaoui, E. E. Boustani, *J. Phytopathol*., **2000**, 148: 405-408.
[11] S. Seo, H. Seto, S. Koshino, S. Yoshida, Y. Ohashi, *Plant Cell*., **2003**, 15: 863-873.

[12] N. M. Dixon, K. B. Kell, *J. Appl. Bacteriol*., **1989**, 67: 109-136.
[13] L. R. Fukumoto, P. M. Toivonen, P.J. Delaquis, *J. Agric. Food. Chem*., **2002**, 50: 4503-4511.
[14] C. Chen, R. R. Belanger, N. Benhamou, T. C. Paulitz, *J. Physiol. Microbial Plant Pathol*., **2000**, 56 (1): 13-23.
[15] R. A. Dixon, N. L. Pavia, *Plant Cell*., **1995**, **7:**1085-1097.
[16] R. D. Hartley, E. C. Jones, *Phytochemistry*., **1977**, 16: 1531-1534.
[17] A. Podstolski, D. Havin-Frenkel, J. Malinowski, J. W. Blount, G. Kourteva, R.A. Dixon, *Phytochemistry*., **2002**, 61: 611-620.
[18] P. R. Sailaja, A. R. Podile, P. Reddanna, *Eur. J. Plant. Pathol*., **1997**, 104: 125-132.
[19] E. Koch, B. M. Meier, H. G. Eiben, A. Slusarenko, *Plant Physiol*., **1992**, 99:571-576.
[20] T. Namai, T. Kato, Y. Yamaguchi, J. Togashi, *Ann. Phytopathol Soc*., **1990**, 56:26-32.
[21] W.X. Li, O. Kodama, T. Akatsuka, *Agri. Biol. Chem*., **1991**, 55: 1041-1047.
[22] H. A. S. Silva, R. D. S. Romeiro, D. Macagnan, B. D. A. Halfeld-Vieira, M. C. B. Pereira, A. Mounteer, *Biol. Control*., **2004**, 29(2): 288-295.
[23] M. Ongena, F. Duby, F. Rossingol, M. L. Fauconnier, J. Dommes, P. Thonart, *Mol. Plant. Microbe. In*., **2004**, 17(9):1009-1018.
[24] M. Ongena, E. Jourdan, A. Adam, M. Paquot, A. Brans, B. Joris, J. L. Arpigny, P. Thonart, *Environ. Microbiol*., **2007**, 9(4):1084-1090.
[25] S. Keshavkant, S. C. Naithani, *Indian J. Plant. Physi*., **2007**, 12(2): 146-152.
[26] S. Keshavkant, Physiological and biochemical aspects of dieback in sal (*Shorea robusta*) seedlings. Ph.D thesis, Pt. Ravishankar Shukla University, Raipur., **2000**.
[27] A. Mahadevan, Methods in Physiological Plant Pathology. Sivakami Publications, Madras, **1975**.
[28] T. Swain, W. E. Hills, *J. Sci. Food. Agr*., **1959**, 10: 63-68.
[29] M. V. Romero, D. M. Barrett, *J. Food. Sci*., **1997**, 62(4): 696-700.
[30] A. O. Chen, J. R. Whitaker, *J. Agric. Food Chem*., **1986**, 34:203-211.
[31] A. Akram, M. Ongena, F. Duby, J. Dommes, P. Thonart, *BMC Plant Biol*., **2008**, 8**:** 113-125.
[32] C. M. J. Pieterse, S. C. M. Van Wees, J. Ton, J. A. Van Pelt, and L. C. Loon, *Plant Biology*., **2002**, 20(1):1-11.
[33] W. E. Durrant, X. Dong, *Annu. Rev. Phytopathol*., **2004**, 42: 185-209.
[34] K. Arora, D. S. Wagle, *Plant Biochem. Physiol*., **1985**, 180:75-80.
[35] M. R. Fernandez, M. C. Heath, *Can. J. Bot*., **1991**, 69:1642-1646.
[36] Y. K. Arora, N. Mehta, D. P. Thakur, D. S. Wagle, *J. Phytopathol*., **2008**, 116(2): 97-105.
[37] M. Babar Ali, E. J. Hahn, K. Y. Paek, *Plant Physiol. Bioch*., **2005**, 43**:** 449-457.
[38] P. R. Sailaja, A. R. Podile, *Indian J. Exp. Biol*., **1998**, 36:631-634.
[39] J. Shah, *Ann. Rev. Phytopathol*., **2005**, 43: 229-260.
[40] A. E. Maher, N. J. Bate, W. Ni, Y. Elkind, R. A. Dixon, C. J. Lamb, *Plant Biology*., **1994**, 91: 7802-7806.
[41] E. Blee, *Trends. Plant. Sci*., **2002**, **7:**315-322.
[42] I. Feussner, C. Wasternack, *Ann. Rev. Plant. Physiol. Plant. Mol. Biol*., **2002**, 53: 275-297.
[43] M. V. Kolomiets, H. Chen, R. J. Gladon, E. J. Braun, D. J. Hannapel, *Plant Physiol*., **2000**, 124:1121-1130.
[44] C. Gobel, I. Feussner, S. Roshal, *J. Biol. Chem*., **2003,** 278: 52834-52840.
[45] G. B. Burow, H. W. Gardner, N. P. Keller, *Plant Mol. Biol*., **2000**, 42: 689-701.
[46] D. I. Tsitsigiannis, S. Kunze, D. K. Willis, I. Feussner, K. P. Keller, *Mol. Plant. Microbe. In*., **2005**, 18(10): 1081-1089.