African Journal of Basic & Applied Sciences 4 (5): 186-191, 2012
ISSN 2079-2034

DOI: 10.5829/idosi.ajbas.2012.4.5.1114

Biochemical Alteration Induced in Tomato (*Lycopersicum esculentum*) in Response to *Fusarium oxysporum* F. Sp. *Lycopersici*

P. Parmar and R.B. Subramanian

B R D School of Biosciences, Sardar Patel University, Post Box no. 39,
Vallbh Vidya Nagar-388120 (Gujarat) India

Abstract: On encounter of pathogen plant interacts with it by expression of proteins which in terns decides the compatible and incompatible reactions. In order to justify the above findings time course study was carried out in the present study with development of biochemical profile for twenty days after inoculation. The findings showed that different markers are expressed at different time and it rises and falls over a period of time without a constant expression, the response is inconsistent as the infection proceeds so it is difficult to discriminate the varieties as resistant or partial entity at particular time period.

Key words: Biochemical markers · *In vivo* · Tomato

INTRODUCTION

Two types of defense response are observed in plants that are passive defense response includes a waxy cuticle or reservoirs of antimicrobial compounds and active responses, induced by pathogen attack [1, 2]. Generation of reactive oxygen species (ROS) is first events towards the recognition of a pathogen by the plant [3]. ROS, is generally being used as a collective term for radicals and other non-radical but reactive species derived from oxygen, which is associated with plant response towards resistance [4, 5]. ROS is also considered to be the cause of lipid peroxidation, which is an early event of HR, results to damage or modify DNA and proteins [6].

There are number of enzymes involved in ROS metabolism. Cell wall peroxidase (POX) [7, 8] and membrane-bound NADPH oxidase, neutrophil-analogous, [9] belong to an important enzyme system generating H_2O_2. It has been thought that a rapid increase in either intra- or extracellular H_2O_2 is associated with induction and/or execution of hypersensitive reaction (HR) [4, 10]. The production of H_2O_2 may also lead to the development of an antimicrobial environment within the apoplast [11]. H_2O_2 is required for the cross-linking of plant cell wall components as a part of the structural defense response [5]. Peroxidase (POX) is also capable of reducing the level of H_2O_2, e.g. during H_2O_2-dependent polymerisation of hydroxycinnamyl alcohols (lignin biosynthesis) [12] and H_2O_2-dependent cross-linking of cell wall proteins such as hydroxyproline rich glycoproteins and proline-rich proteins [13]. These events are followed by a broad spectrum of metabolic changes that represents: (a) stimulation of the phenylpropanoid and fatty acid pathways, (b) production of defense-specific chemical messengers such as salicylic acid (SA) or jasmonates and (c) accumulation of components with antimicrobial activities such as phytoalexins and pathogenesis related (PR) proteins [14].

In addition, plant phenolics, carbohydrates and amino acids also plays critical role in disease resistance. In fact, the metabolic pathways of these substances are interconnected: phenolic and amino acid pathways use products of carbohydrate metabolism as their precursors. During host-pathogen interaction, amino acids may have a fungistatic effect through their involvement in metabolic reactions associated with disease resistance [15, 16] or act as substrate for the pathogen [17]. In present work the time course study of the known resistant and partial varieties were evaluated to disclose the pattern of response towards the pathogen attack.

MATERIALS AND METHODS

Experimental Design: The isolate *Fusarium oxysporum* f. sp. *lycopersici*, maintained on Potato sucrose broth was used to prepare inoculum suspension following the

Corresponding Author: R.B. Subramanian, B R D School of Biosciences, Sardar Patel University, Post Box no. 39, Vallbh Vidya Nagar- 388120 (Gujarat) India. E-mail:asp.fus@gmail.com.

method of Thakur *et al.* [18] for *in vivo* assay. Two varieties of tomato, LA 3042 wild resistant and GT2 (Gujarat tomato-2) partial resistant as per Parmar *et al.* [19] was selected for the present study were grown in pots. The experimental pots were inoculated with 10 ml of 10 days old inoculum suspension containing mycelia and spores, through soil after the plants reached maturity (60 days), while the control pots were devoid of the fungal inoculum (non-inoculated). Leaf samples were collected at regular interval of time for the evaluation of plant response towards the fungal pathogen.

Biochemical Evaluation: Using a chilled pestle and mortar, 1 g of leaf sample was homogenized in 3ml of 0.1 M potassium phosphate buffer (pH 7.0), containing 0.05 mM phenyl methane sulfonyl fluoride (PMSF) to which a pinch of polyvinylpyrollidone (PVP) was added. The homogenate was centrifuged at 8000 g for 15 min at 4°C and the supernatant was used as the enzyme source for the assays.

Enzymes Assay:

Assay of Peroxidase (EC. 1.11.1.7): The reaction mixture consisting of 1.0 ml of 0.01 M O-Dianisidine, 2.4 ml of distilled water, 1.0 ml of 0.1 M phosphate buffer and 0.5 ml of 20mM H_2O_2. The reaction was initiated by 0.2 ml of enzyme extract and the mixture was incubated at room temperature. After 5 minutes reaction was terminated by application of 1.0 ml 2 N H_2SO_4 and absorbance were taken at 430 nm in a Spectrophotometer (Unicam Heëios Alpha & beta, Cambridge, United Kingdom). At the start of enzyme reaction, the absorbance of the control mixture containing 1.0 ml of 0.01 M O-Dianisidine, 2.4 ml of distilled water, 1.0 ml of 0.1 M phosphate buffer and 0.5 ml of 20 mM H_2O_2, 0.2 ml of enzyme extract and 1.0 ml 2 N H_2SO_4 was set to zero at 430 nm in a Spectrophotometer. Peroxidase (PO) activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} ml^{-1} of fresh weight [20].

Assay of Poly Phenol Oxidase (EC. 1.14.18.1): The poly phenol oxidase (PPO) activity was assayed using the modified method of Mayer *et al.* [21]. The standard reaction mixture contained 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0), 0.5 ml of enzyme preparation and 0.5 ml of 0.1 M catechol. The reaction mixture was incubated at room temperature and the absorbance was set to zero at 495 nm. The change in the absorbance was recorded at 30 sec intervals for two min and the PPO activity was expressed as change in absorbance of the reaction mixture ml^{-1} g^{-1} of fresh weight.

β- 1,3 Glucanase (EC. 3.2.1.39): β-1, 3 glucanase activity was assayed by the laminarin Dinitrosalicyclate method [22]. One gram of tomato leaf was homogenized with 3ml of sodium acetate buffer (0.05 M), pH 5.0 at 4°C using a chilled pestle and mortar. The extract was then centrifuged at 10,000 g for 15 min at 4°C and the supernatant was used as crude enzyme extract. The crude enzyme extract (62.5 µl) was added to an equal volume of laminarin (4%) and incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent and boiled them for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance at 500 nm was determined. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µmol min^{-1} $ml^{-1.}$

PAL Activity (EC. 4.3.1.5): PAL activity was determined as the rate of formation of phenylalanine to trans-cinnamic acid at 290 nm as described by Brueske [23]. Tomato leaves (1gm) were homogenized in 3 ml of sodium borate buffer (0.1 M), pH 7.0 containing 0.5 g insoluble polyvinylpolypyrrolidone (PVP) and 0.05 mM phenyl methane sulfonyl fluoride (PMSF). The extract was centrifuged at 8,000 rpm for 15 min at 4°C. The supernatant was used for enzyme assay. Samples containing 0.2 ml of enzyme extract were incubated with 0.5 ml of borate buffer (0.2 M), pH 8.7, 1.3 ml distilled water and 1.0 ml of L – phenylalanine (0.1 M) in the same buffer for 30 min at 30°C. After 30 min the reaction was stopped by adding Trichloroacetic acid (1M). In reference cell, 0.2 ml of enzyme extract was taken along with 0.5 ml of borate buffer, 1.3 ml distilled water, 1 ml of L – phenylalanine and 0.5 ml of Trichloroacetic acid. The amount of Trans-cinnamic acid synthesized was calculated using a standard graph. Enzyme activity was expressed on fresh weight basis as µmol $min^{-1}ml^{-1}gm^{-1}$.

Chitinase Activity (EC. 3.2.1.14): The colorimetric assay of chitinase was carried out according to the procedure developed by Monreal and Reese [24]. One gram of tomato leaf was extracted with 3ml of potassium phosphate buffer (200 mM), pH 6.0 with 2 mM calcium chloride. The homogenate was centrifuged for 15 min at 8,000 g and the supernatant was used as an enzyme source. The reaction mixture consisted of 2 ml of 1.25 % (w/v) colloidal chitin solution and 0.5 ml of enzyme solution was incubated on a rotary platform at a speed sufficient to keep the chitin in suspension at 25°C. The reaction was stopped by placing the vial in a boiling water bath for 5 minutes. An aliquot of the supernatant

(1.0 ml) was pippetted into a glass reagent tube containing 1.5 ml color reagent and incubated for 5 minutes in boiling water bath. After cooling to room temperature, absorbance at 540nm was measured using the Spectrophotometer. N – acetyl glucosamine (GLcNAc) was used as a standard. The enzyme activity was expressed as µmoles GLcNAc gm^{-1} ml^{-1}.

Estimation of Total Phenols: Leaves were collected and total phenolics were estimated by homogenizing 1gm of the tissue with 10 times volume of 80% ethanol, 0.5ml of Folin-Ciocalteau reagent was added and incubated for 3 minutes. Two ml of 20gm/l Na_2CO_3 was added and the absorbance was read at 650nm against a reagent blank in treated varieties along with control using catechol (1 mg/ml) as standard [25].

RESULTS

Time Course Response of Tomato Varieties against Fusarium Pathogen: The study was centered to identify the pattern of the plant response in terms of biochemical marker against the fungal pathogen at regular interval of time to disclose the mechanism of resistance in plant. However, the markers evaluated showed significant response towards the pathogen attack.

Biochemical Studies: Phenol content (Fig. 1) was observed to be highest 76.3 µgm/gm in resistant variety in comparison to partial variety 63.8 µgm/gm, which may be the cause of the resistance against the pathogen. As the interaction proceeds the level gets down which then rises again and falls, at last around 360 hrs it attains the peak of 81.1 µgm/gm greater than the original content. In case of partial variety it also gets down and increases to attain peak at 15 hrs and then falls towards the end by up and downs. Chitinase and polyphenol oxidase pattern (Fig. 2 & 3) in both the varieties showed similar fact of higher activity at zero time in comparison to partial which rises and falls as time proceeds the level was observed to be highest at 216 hrs in resistant and 360 hrs in partial varieties. An interesting observation was that the activity of partial variety out competes the resistant variety. In contrast the glucanase activity (Fig. 4) was found to be lower in resistant variety (0.16 U/ml/min) in comparison to partial variety (0.68 U/ml/min) at zero level which attains peak at 3 hrs and gets up and down towards the end, in case of partial similar pattern was observed with up and downs and peak at 288 hrs. Similar observation was obtained in peroxidase activity (Fig. 5) with higher level of activity in resistant varieties in comparison to partial ones

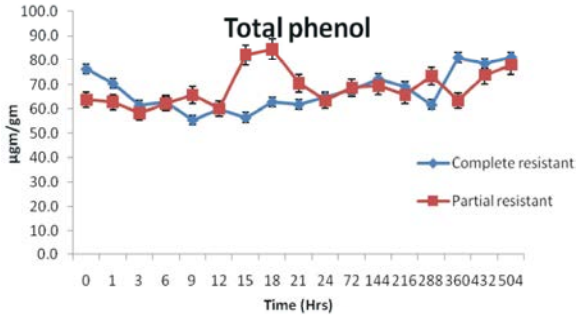

Fig. 1: Time course study of induction of total phenol after inoculation of tomato plant with *Fusarium oxysporu*m f. sp. *Lycopersici.*

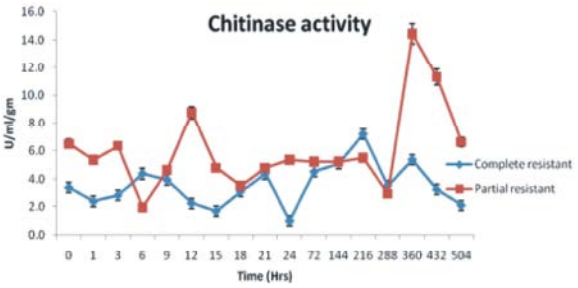

Fig. 2: Time course study of induction of Chitinase activity after inoculation of tomato plant with *Fusarium oxysporu*m f. sp. *Lycopersici.*

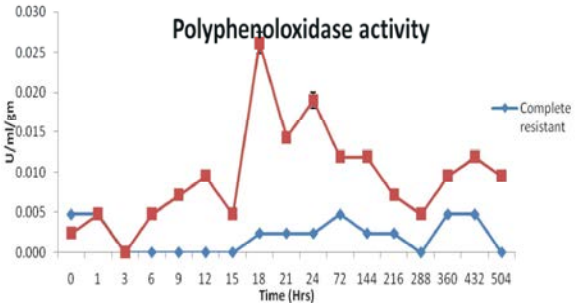

Fig. 3: Time course study of induction of Polyphenoloxidase activity after inoculation of tomato plant with *Fusarium oxysporu*m f. sp. *Lycopersici.*

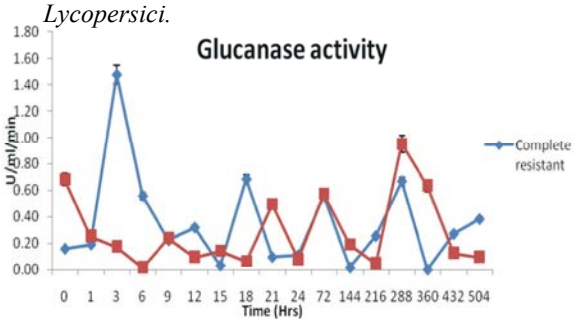

Fig. 4: Time course study of induction of Glucanase activity after inoculation of tomato plant with *Fusarium oxysporu*m f. sp. *Lycopersici.*

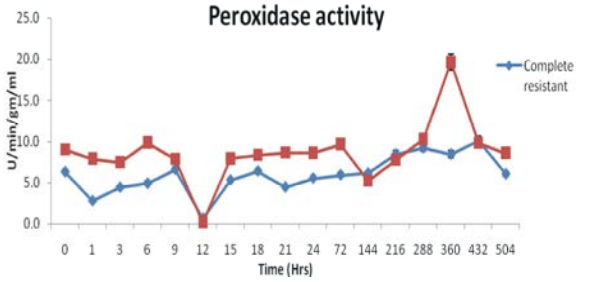

Fig. 5: Time course study of induction of Peroxidase activity after inoculation of tomato plant with *Fusarium oxysporu*m f. sp. *Lycopersici.*

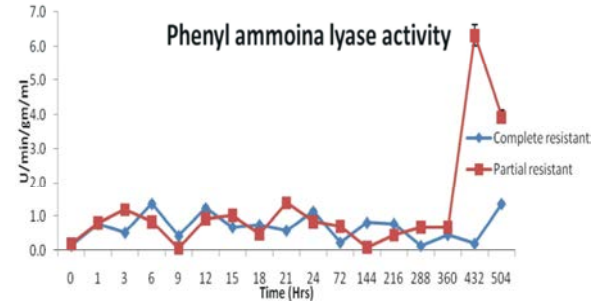

Fig. 6: Time course study of induction of Phenyl ammonia lyase activity after inoculation of tomato plant with *Fusarium oxysporu*m f. sp. *Lycopersici.*

which gets up and down as time proceeds and the partial one out competes the resistant one. On comparing the pattern of phenol content and the glucanase activity it is noticed that at particular time the single factor plays an important role to protect the plant against the disease. The selected parameters showed significant difference in both the partial and resistant varieties except the phenyl ammonia lyase activity (Fig. 6) which showed identical pattern in both the case with negligible response.

DISCUSSION

In this work, consideration was given to some of the general biochemical substances regarded to be involved in resistance, such as phenolics, PAL, chitinase, β-1, 3 glucanase, peroxidase, poly phenol oxidase and catalase. PAL is the first enzyme of phenylpropanoid metabolism in higher plants and it plays a significant role in regulating the accumulation of phenolics, phytoalexins and lignins, the three key factors responsible for disease resistance [26]. A direct role of chitinase and β-1, 3 glucanase in defense of plants against pathogen have been proposed because substrate for these enzymes are major component of the cell walls of many fungi [27, 28]. PPO is a copper containing enzyme which oxidizes phenolics to highly toxic quinines and involved in the terminal oxidation of diseased plant tissue which was attributed for its role in disease resistance [29].

Peroxidases are a large family of enzymes with very diverse functions in plant systems. They often increase in response to stress. One of the principal roles of peroxidases appears to be cellular protection from reactive oxygen species generated by various stresses [30]. It has been reported that peroxidases have an anti-fungal activity against various pathogens [11]. Increased catalase and peroxidase activity in resistance response has been related to lignification, which has an important role in the reduction and blockage of nutrient diffusion from neighboring host cells to the haustoria [31]. Increased activities of the enzymes peroxidase and catalase and lignin deposition are thought to play a major role in local and systemic disease resistance [32]. During the course of pathogens penetrating the plant cell, besides chemical secretion, the pathogens may cause mechanical signal by the physical pressure on the plant cell. Zhao *et al.* [33] took the stress as the mechanical signal elicitor to find the effect of plant resistance induced by stress. Their results showed that appropriate stress stimulation can evidently improve the plant resistance. However, disruption of the plasma membrane-cell wall adhesion will absolutely eliminate this kind of inducement effect, which suggests that the plant resistance induced by stress depend on the adhesion of plasma membrane-cell wall. Also they found that stress stimulation may cause synthesis of lignin and increase the activity of phenylalanine ammonia lyase (PAL) chitinase and beta-1, 3-glucanase obviously. Stress stimulation may not only enhance ability of the plant cell resistance to pathogen penetration but also elicit the accumulation of pathogens suppression or antimicrobial chemical substance in the plant cell. Accumulation of phenolic compounds was also more in highly resistant varieties.

CONCLUSIONS

Compiling the response of the all parameters evaluated in the study, suggest that though the response is varied in partial and resistant varieties but it is insignificant to judge as resistant or partial entity due to up and downs at any particular point in both the selected varieties. The response was inconsistent to reveal the discrimination amongst the variety on the basis of pattern.

ACKNOWLEDGEMENTS

This work was supported by grants for scholarship to meritorious students from University Grants Commission (UGC), Government of India.

REFERENCES

1. Hutcheson, S.W. 1998. Current concepts of active defense in plants, Annu. Rev. Phytopathol., 36: 59-90.
2. Lebeda, A., L. Luhova, M. Sedlarova and D. Jancova, 2001. The role of enzymes in plant–fungal pathogens interactions. J. Plant Dis. Protect., 108: 89-111.
3. Baker, C.J. and E.W. Orlandi, 1995. Reactive oxygen in plant pathogenesis, Annu. Rev. Phytopathol., 33: 299-321.
4. Low, P.S. and J.R. Merida, 1996. The oxidative burst in plant defense: function and signal transduction. Physiol. Plant. 96: 532-542.
5. Lamb C. and R. Dixon, 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol., 48: 251-275.
6. Adam, A.L., C.S. Bestwick, B. Barna and J.W. Mansfield, 1995. Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to *Pseudomonas syringae* pv. *Phaseolicola*. Planta, 197: 240-249.
7. Bolwell, G.P., V.S. Butt, D.R. Davies and A. Zimmerlin, 1995. The origin of the oxidative burst in plants. Free Radic. Res., 23: 517-532.
8. Gross, G.G., C. Janse and E.F. Elstner, 1977. Involvement of malate, monophenols and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib). Planta, 136: 271-276.
9. Levine, A., R. Tenhaken, R. Dixon and C. Lamb, 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell, 79: 583-593.
10. Bestwick, C.S., I.R. Brown, M.H. Bennett and J.W. Mansfield, 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *Phaseolicola*. Plant Cell, 9: 209-221.
11. Peng, M. and J. Kuc, 1992. Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf discs. Phytopathol., 82: 696-699.
12. Monties, B. and Lignins, 1989. Methods Plant Biochemistry. In: Dey, P.M. J.B. Harborne (Eds.), Academic Press, London, I: 113-157.
13. Bestwick, C.S. M.H. Bennett nd J.W. Mansfield, 1995. Hrp mutant of *Pseudomonas syringae* pv. *phaseolicola* induces cell wall alterations but not membrane damage leading to the HR in lettuce (*Lactucasativa*), Plant Physiol., 108: 503-516.
14. Hutcheson, S.W., 1998. Current concepts of active defense in plants, Annu. Rev. Phytopathol., 36: 59-90.
15. Schmelzer, E., S. Kroeger-Lebus and K. Hahlbrock, 1989. Temporal and special patterns of gene expression around sites of attempted fungal infection in parsley leaves. The Plant Cell, 1: 993-1001.
16. Graham, T.L., J.E. Kim and M.Y. Graham, 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. Mol. Plant-Microbe Interact. 3: 157-166.
17. Titarenko, E., J. Hargreaves, J. Keon and S.J. Gurr, 1993. Defense-related gene expression in barley coleoptiles cells following infection by *Septoria nodurum*. In: Fritig, B. and M. Legrand, (Eds.), Mechanisms of plant defense responses. Dordrecht: Kluwer Academic Publishers, pp: 308-311.
18. Thakur, S.M., D.R. Sharma and S.K. Sharma, 2002. In vitro selection and regeneration of carnation (*Dianthus caryophyllus* L.) plants resistant to culture filtrate of *Fuarium oxysporum* f.sp. *dianthi.* Plant Cell Reports, 20: 825-828.
19. Parmar, P.P., V.P. Oza, A.D. Patel, K.B. Kathiria and R.B. Subramanian, 2011. Development of a rapid and reliable bioassay to discriminate between susceptible and resistant cultivars of tomato against fusarium wilt. Int. J. Biosci. Agric. & Technol., 3(2): 6-11.
20. Sumner, J.B. and E.C. Gjessing, 1943. A method for the determination of peroxidase activity. Archive Biochem. 2: 291-293.
21. Mayer, A.M., E. Harel and R.B. Shaul, 1965. Assay of catechol oxidase a critical comparision of methods. Phytochem. 5: 783-789.
22. Pan, S.Q., X.S. Yc, J. Kuc, 1991. A technique for detaction of chitinase, β-1,3 glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocusing. PhytoPathology, 81: 970-974.

23. Brueske, C.H., 1980. Phenyl ammonia lyase activity in tomato roots infected and resistant to the root – knot nematode *Meloidogyne incogneta*. Physiol. Plant Pathol., 16: 409-414.
24. Monreal, J. and E.T. Reese, 1969. Colorimetric estimation of chitinase. Canadian journal of Microbiol., 15: 689-696.
25. Zieslin, N. and R. Ben-zaken, 1993. Peroxidase activity and presence of phenolics substances in peduncles of rose flowers. Plant Physiol Biochem., 31: 333-339.
26. Vidhyasekaran, P., 1988. Physiology of disease resistance in plants. CRC press. Florida, 2: 127.
27. Lim, H., Y. Kim and S. Kim, 1991. *Pseudomonas stutzeri* YLP -1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. Appl. Environ. Microbiol., 57:510-516.
28. Fridlender, M., J. Inbar and I. Chet, 1993. Biological control of soil borne plant pathogens by a β-1,3 glucanase producing *Pseudomonas cepacia*. Soil Biol. Biochem. 25: 1211-1221.
29. Kosuge, T., 1969. The role of phenolics in host response to infection. Ann. Rev. Phytopathol., 7: 195-222.
30. Siegel, B.Z., 1993. Plant peroxidases-an organismic perspective. Plant Growth Regulation, 12: 303-312.
31. Kuc, J. and Preisig, 1984. Fungal regulation of disease resistance mechanisms in plants. Mycologia, 76: 767-784.
32. He, C.Y., T. Hsiang and D.J. Wolyn, 2002. Induction of systemic disease resistance and pathogen defense responses in *Asparagus officinalis* inoculated with non-pathogenic stains of *Fusarium oxysporum*. Plant Pathol., 51: 225-230.
33. Zhao, H., J. Wang, B. Wang and Y. Wang, 2005. Stress stimulation induced resistance of plant. Colloids Surf B Biointerfaces, 43(3-4): 174-178.