CHANGES IN GLUTATHIONE S-TRANSFERASE ACTIVITY AND ZEARALENONE CONTENT IN SUSCEPTIBLE AND TOLERANT WHEAT HEADS INOCULATED WITH *FUSARIUM GRAMINEARUM,* THE CAUSAL AGENT OF FUSARIUM HEAD SCAB

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Abstract

Glutathione S-transferase (GST) activity pattern was determined in tolerant (cv. Sumai#3) and susceptible (cv. Falat) wheat heads inoculated with *Fusarium graminearum,* the causal agent of head scab disease (FHB), during various developmental stages. GST specific activity exhibited a transient pattern in Sumai#3 reaching a maximum level at the milk stage and declining thereafter. GST level in Sumai#3 was three times greater than that in the susceptible cultivar. Thin layer chromatography and densitometry were used to measure zearalenone (ZEN) content in FHB-inoculated wheat heads. Zearalenone concentration reached a maximum level in Sumai#3 at the milk stage and thereafter it declined. ZEN content in the susceptible wheat heads continued to increase and reached a maximum level at the ripening stage. There seems to be a correlation between the increased GST activity and significantly lower ZEN production in the tolerant wheat heads.

Introduction

Fusarium head blight (FHB) is one of the most devastating diseases of wheat in tropical and subtropical regions of the world. FHB had been occurring sporadically in different regions of Iran [1]. However, due to favorable environmental conditions and the cultivation of wheat varieties in Northern Iran, the disease has become highly endemic and has caused severe yield losses in wheat growing areas [16].

F. graminearum produces a wide spectrum of mycotoxins depending on the environmental conditions and the host plant. These include zearalenone, zearalenol and some derivatives of trichothecene such as nivalenol and deoxynivalenol (DON) [26,27]. Trichothecenes have caused mycotoxicosis in animals

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and Alimentary Toxin Aleukia (ATA) in humans in Russia and Akakabi in Japan [31]. High DON concentrations have been detected in wheat heads severely infected with FHB in wheat growing regions of Canada [35] and USA [13]. A recent report from Iran shows high contents of both zearalenone and DON in FHB-infected wheat heads [36].

The disease resistance mechanism against FHB in wheat is complex and depends on a number of varietal factors such as flowering habit, plant tallness and defense reactions that are themselves influenced by environmental conditions [8,28]. A few studies have indicated that wheat plants possess a biochemical system of detoxifying fungal toxins [25,26]. Wang and Miller [48] have reported a high degree of tolerance in wheat against DON without any disruption in plant growth. This type of resistance has been attributed to a relatively lower rate of membrane binding with DON in corn protoplasts [7].

In higher plants, one of the detoxifying enzymes is glutathione S-transferase (GST). GSTs are multi-functional dimeric proteins that catalyze conjugation of glutathione with a wide array of hydrophobic compounds with electrophilic centers. Conjugation of glutathione results in detoxification of potential cytotoxic and genotoxic compounds [22]. GSTs possess two important active sites that may function independent of each other. The glutathione conjugation site seems to be more specific than site reacting with a large number of toxic substances [6]. Studies have indicated that glutamyl functional group is involved in the conjugation of glutathione in this site [5]. Since GSTs are widespread in bacteria, fungi, animals and plants, their crucial role becomes more evident. GSTs are known to be as homo- or heterodimers comprised of seven isozymes that are specifically induced by xenobiotic compounds [22]. GST subclasses have been shown to be induced during pathogenic attack, oxidative stress, heavy metal toxicity and following treatments with auxin, plant secondary metabolites and herbicides [9,12,15,16,20,22]. Herbicide detoxification catalyzed by GST is achieved through conjugation with glutathione. In bean suspension cells, GST activity was induced after treatment with mycelial walls of *Colletotrichum lindemuthianum* [11]. GstA1 gene was induced at the transcriptional level in wheat leaves inoculated with *Erysiphe graminis* f.sp. *hordei* [23].

The main objectives in this study were to investigate GST specific activity in susceptible and tolerant wheat heads inoculated with *F. graminearum* and whether it is related to zearalenone content and disease resistance mechanism.

Materials and Methods

Pathogenicity Test

Five isolates of *F. graminearum* were obtained from the Cereal Pathology Lab. at the Plant Pest and Disease Research Institute, AREEO, Tehran, Iran. Fungal isolates that had been collected from wheat fields in Golestan province were purified and subsequently identified. Each isolate was separately cultured on wheat straws for spore production. Wheat heads at anthesis were sprayed with conidial suspension at 1×10^6 spores/ml twice, 24 h apart. To maintain humidity, inoculated plants were covered with moist plastic bags. Three days after inoculation, symptoms appeared as white scabs on heads.

Toxin Production Test

Ten flasks each containing 100 g of moist corn kernels in 45 ml sterile distilled water were autoclaved for two consecutive nights and then inoculated with 1×10^6 spores/ml from each isolate in two replicates. Flasks were incubated at 27°C for 4 weeks. Colonized corn seeds were dried at 40°C for 24 h and then powdered in a blender [3].

Inoculum Preparation

Five pathogenic and toxigenic isolates of *F. graminearum* were used in the inoculum preparation based on the method of Wegener [34]. Ten grams of shredded wheat straw was placed in a 500 ml flask containing 250 ml distilled water and then autoclaved for two consecutive nights. Five mycelial disks representing an individual fungal isolate were added to each flask and incubated on an orbital shaker at 25°C for spore production. After five days of incubation, the fungal culture was filtered through a cheesecloth and using hemocytometer, conidial density was adjusted at 4000 spores/ml.

Plant Growth Conditions and Inoculation

Seeds from Falat and Sumai#3 cultivars were surface-sterilized with 2% (v/v) sodium hypochlorite for 3-5 min, washed in sterile distilled water several times, for five minutes each, and placed on a moist sterile filter paper for germination to occur. After 4-5 days of incubation at 16°C, the germinated seeds without contamination were transplanted in plastic pots (four seedlings/pot) containing pasteurized soil. Pots were kept at 18°C under controlled greenhouse conditions of 16 h light:8 h dark [24]. Wheat heads were inoculated with *F. graminearum* conidial suspension at anthesis according to Wang and Miller [32]. Five microliters of spore suspension (1×10^6 spores) was injected into the middle inflorescence using a sterile micropipette. This was carried out twice, 24 h apart. Control plants were injected with sterile distilled water. Sampling was done

at flowering, milk, dough and ripening stages. *F. graminearum* was reisolated and purified from the diseased heads. Samples were kept in freezer bags and stored at -20°C until use.

Tissue Extraction

One gram of head tissue was homogenized in an ice-cold mortar containing 2 ml of 50 mM sodium phosphate (pH 7.0). The homogenate was centrifuged first at 6,500×g and then at 13,000×g, 5 min each, and the supernatant was used for protein and enzyme assays.

Protein Determination

Total soluble protein was measured according to Bradford [4]. Bovine serum albumin (BSA) fraction IV was used as a standard.

GST Assay

Glutathione-S-transferase activity was measured following the method of Habig [18]. One ml of reaction mixture contained 50 µl of 100 mM sodium phosphate buffer (pH 6.5), 400 µl dd water and 1.0 mM reduced glutathione (GSH). The reaction was initiated by adding 1.0 mM 1-chloro-2,4-dinitrobenzene (CDNB), as the substrate, dissolved in ethanol. The change in absorbance at 340 nm was measured for 4 min using a double beam Shimadzu UV-3100 spectrophotometer. The blank cuvette contained all the compounds except CDNB. Both GSH and CDNB were freshly prepared each time. The enzyme kinetic was measured from the slope of the straight line during the first minute. One unit of enzyme activity is defined as one nmol CDNB conjugated/min/mg protein.

Extraction of Fusariotoxins

Wheat heads inoculated with *F. graminearum* were extracted for fusariotoxins according to Bottalico *et al.* [2]. Fifteen grams of shredded wheat head tissue was homogenized in 100 ml methanol:water (45:55, v/v) mixture containing 1% (w/v) NaCl in a blender at 1200 rpm. The homogenate was filtered out through a Whatman No. 1 paper and the filtrate was extracted out twice with 50 ml *n*-hexane for removing lipid materials and three times with 30 ml methylene chloride for purifying toxins. The final extract was first concentrated to a final volume of 10 ml with a rotary evaporator under vacuum and then dried out under nitrogen gas.

Thin Layer Chromatography

Fusariotoxins extracted and purified from wheat heads were identified by comparing the R_f values of unknown samples with those of standard toxins using TLC with developing solvents of benzene:acetone

(7:12) and toluene:ethyl acetate:formic acid (1:3:6) and a mixture of sulfuric acid-methanol (2% v/v) as a chemical reagent. The TLC plates were then scanned by a densitometer and a standard curve was prepared. The standard fusariotoxins were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Results

GST specific activity in heads of tolerant wheat (cv. Sumai#3) followed a transient pattern after the inoculation with *F. graminearum* conidia, reached a maximum rate at the milk stage and declined thereafter (Fig. 1). GST activity level in heads of susceptible wheat (cv. Falat) did not increase significantly during the head development as compared to the control plant. GST relative activity (%control) in heads of tolerant Sumai#3 cultivar was 1.5 times greater than that in heads of susceptible Falat cultivar during the milk stage (Fig. 2).

TLC analysis showed the presence of fusariotoxins including zearalenone in the inoculated heads of susceptible as well as tolerant wheat cultivars at various growth stages. Zearalenone co-migrated with the standard sample and exhibit a R_f value of 0.49 (Fig. 3). Toxigenic study revealed that all five *F. graminearum* isolates produced significant amounts of zearalenone. Three isolates, J4, E5 and E4 produced deoxynivalenol as well. Isolates F130 and F13 produced trace amounts of deoxynivalenol. It appears that the isolate E5 was capable of producing 3-acetyldeoxynivalenol (data not shown).

Zearalenone level increased in both susceptible and tolerant wheat heads following the inoculation with *F. graminearum* conidial suspension (Table 1). In tolerant Sumai#3, ZEN accumulation followed a transient pattern, reaching a maximum level at the milk stage and declining thereafter. In Falat cultivar, however, ZEN continued to increase through the ripening stage, reaching a level more that eleven times greater than that in the tolerant cultivar.

Discussion

The plant biochemical disease resistance mechanism constitutes the production of a wide array of chemicals as pathogenesis-related proteins, phytoalexins, detoxifying enzymes and oxygen-free radicals following microbial attack [21]. As a de-toxifying enzyme, glutathione-S-transferase activity has been studied in a number of plant-pathogen interactions [11,22,23].

In this study, we observed a significant increase in GST specific activity in tolerant wheat heads as compared to that in the susceptible cultivar during the
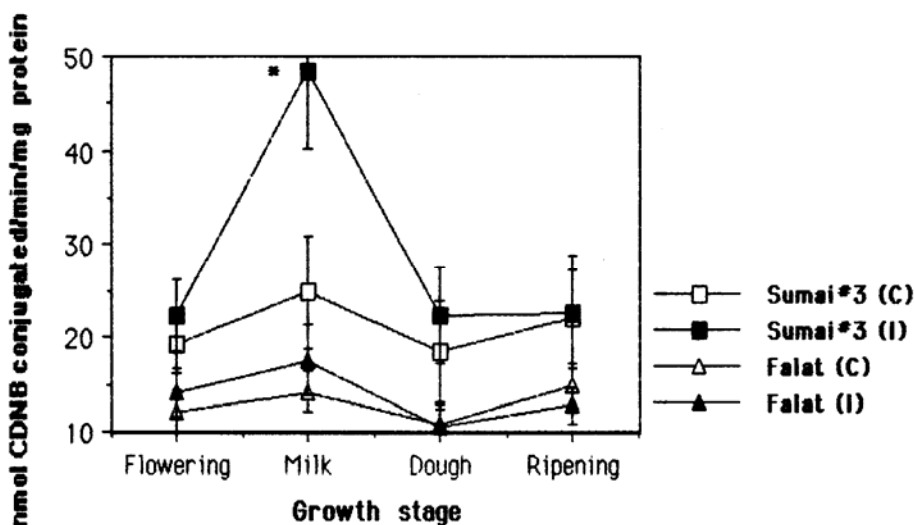
Figure 1. GST specific activity in heads of tolerant (cv. Sumai#3) and susceptible (cv. Falat) wheat cultivars at different growth stages following the inoculation with *F. graminearum* conidia (I) or sterile distilled water (C). The experiment was repeated three times and the results represent the mean of three replicates from one experiment +/–SD. Means were compared using Duncan's multiple range test. Asterisk signifies the difference in mean ∂=1%.
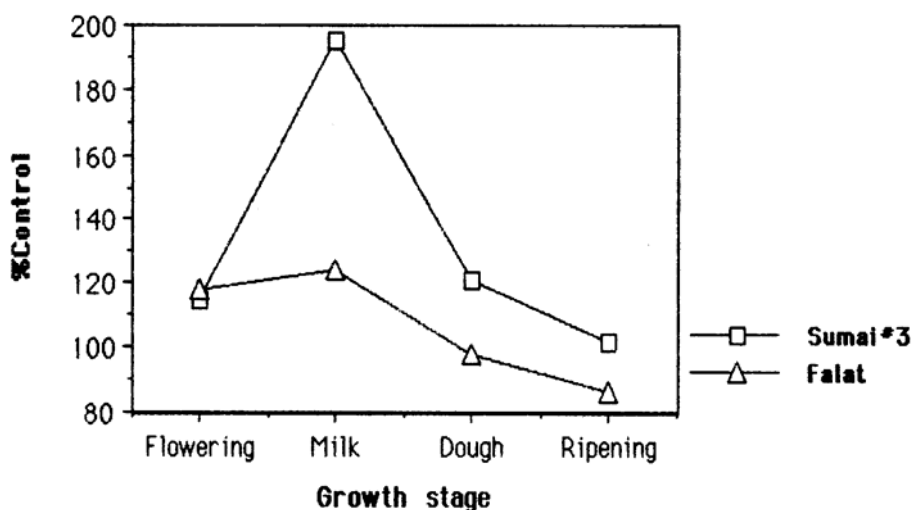

Figure 2. GST relative activity (%control) in heads of tolerant (cv. Sumai#3) and susceptible (cv. Falat) wheat cultivars.

milk stage (Fig. 1). Further, GST relative activity followed a transient pattern similar to that of ZEN, suggesting that GST activity induction might be in response to ZEN accumulation in the tolerant wheat heads. In susceptible heads, ZEN continued to accumulate, whereas GST relative activity declined during both dough and ripening stages (Table 1). Increased GST level has been shown in bean suspension cells 8 h after the inoculation with mycelial walls of *Colletotrichum lindemuthianum* [11]. Mauch and

Dudler [23] have also reported activation of GST specific transcripts in wheat leaves inoculated with *Erysiphe graminis* f.sp. *tritici* or *E. graminis* f.sp. *hordei*. However, this increase was similar in both compatible and incompatible combinations. On the other hand, Gullner *et al.* [17] found induced GST activity in tobacco leaves following inoculation with tobacco necrosis virus or treatment with salicylic acid.

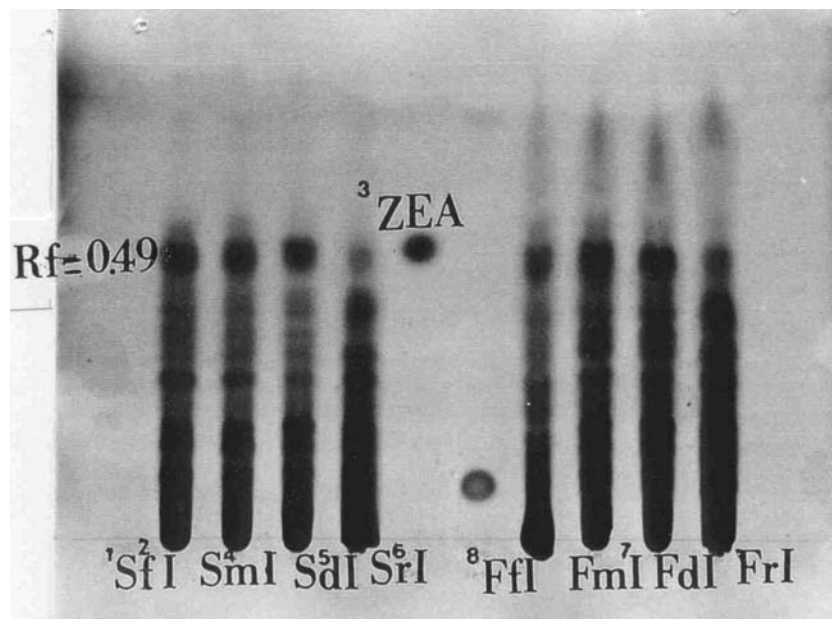GST is known to catalyze conjugation of reduced glutathione tripeptide with toxigenic compounds thus

Figure 3. TLC analysis showing fusariotoxins including zearalenone (ZEA) in the extracts of wheat heads tolerant (Sumai#3) or susceptible (Falat) to *F. graminearum* at various growth stages. S, Sumai#3; F, Falat; f, flowering; m, milk; d, dough and r, ripening.

Table 1. Zearalenone concentration (μg/g tissue fresh wt.) in wheat heads at different growth stages after inoculation with *F. graminearum*

Growth stage	Treatment		
	Sumai#3	Falat	×Higher
Flowering	0.049	0.067	1.37
Milk	0.63	0.78	1.24
Dough	0.45	0.81	1.80
Ripening	0.10	1.16	11.6

converting them to detoxified polar glutathione conjugants. It is possible that ZEN biosynthesis may signal the induction of GST gene expression in tolerant wheat heads thereby suppressing its own accumulation during the dough and ripening stages. GST has been shown to detoxify the herbicide atrazine in resistant plants [9,20,30]. Since both ZEN accumulation and GST relative activity followed a similar transient pattern, it is possible that GST may play a detoxifying role in tolerant wheat heads. This, however, requires to be seen through further experimentation.

It is worth mentioning that deoxynivalenone (DON) produced by *F. graminearum* is a phytotoxin which possess more phytotoxicity than ZEN [29]. We were unable to detect DON in infected wheat heads using TLC. Further, it is possible that a GST-like detoxification system may exist in wheat heads that is capable of degrading or catalyzing glutathione conjugation with DON more efficient that with ZEN [25,26,33]. However, the precise role of GSTs in plant defense mechanism *vis à vis* pathogens remains obscure, although it has been shown to act both as an antioxidant and detoxifying enzyme [17].

References

1. Bamdadian, A. and Torabi, M. Important disease of wheat and barley. Plant Pest and Disease Research institute Press, AREEO, the Ministry of agriculture, Tehran, Iran, 67 p., (1984).
2. Bottalico, A., Lerario, P. and Visconti, A. Mycotoxins occurring in *Fusarium*-infected maize ear in the fields of some European countries. *Proc. Int. Symp. On Mycotoxins.* Pub. Sci. Dept. NIDOC, Cario, 375-382, (1983).
3. Bottalico, A., Lerario, P. and Visconti, A. Production of zearalenone, trichothecenes and moniliformin by *fusarium* species from cereals, in Italy. In: Kurata and Ueno (Eds.) Toxigenic fungi, their toxins and health hazard. Elsevier Sci. Pub. Ltd. Pp. 100-208, (1984).
4. Bradford, M. M. A rapid and sensitive method for the

quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72**, 248-254, (1976).

5. Casini, A. F., Pompella, A. and Comporti, M. Liver glutathione depletion induced by bromobenzene, iodobenzene and diethylmaleat poisoning and its relation to lipid peroxidation and necrosis. *Am. J. Pathol.,* **118**, 225-237, (1985).

6. Chen, W. J., Graminski, G. F. and Armstrong, R. N. Dissection of the catalytic mechanism of isoenzyme 4-4 of glutathione-S-transferase with aternative substrate. *Biochem.,* **27**, 647-654, (1988).

7. Cossette, F. and Miller, J. D. Phytotoxic effect of deoxynivalenol and Gibberella ear rot resistance of corn. In: *Toxins,* **3**(5), 383-388, (1995).

8. Couture, L. receptivity of spring cereal cultivars to contamination of grain in the inflorescence by *Fusarium* spp. *Can. J. Plant Sci.,* **62**, 29-34, (1982).

9. Dean, V. J., Gronwald, J. W. and Eberlin, C. V. Induction of glutathione-S-transferase isozymes in sorghum by herbicide actidotes. *Plant Physiol.,* **92**, 467-473, (1990).

10. Dudler, R., Hertig, C., Rebmann, G., Bull, J. and Mauch, F. A Pathogen-induced wheat gene encodes a protein homologous to glutathione-S-transferase. *Mol. Plant-Microbe Interact.,* **4**, 14-18, (1991).

11. Edwards, R. and Dixon, R. A. Glutathione-S-cinnamoyl transferase in planta. *Phytochem.,* **30**, 79-84, (1991).

12. Edwards, R. and Owen, W. J. Regulation of glutathione-S-transferase of zea mays in plants and cell culture. *Planta,* **175**, 99-106, (1988).

13. Eppley, R., Truckness, M., Nesheim, S., Thrope, C., Wood, G. and Pohland, A. Deoynivalenol in winter wheat, thin layer chromatographic method and survey. *J. Assoc. chem.,* **67**, 43-45, (1984).

14. Golzar, H. Distribution of *fusarium* head scab of wheat in Gorgan and Gonbad region and the extent of susceptibility of commercial wheat cultivars. *Abstract in the Proc. Of 11th Iranian Plant Protection Congress,* Rasht, Iran. 36 p. (1993).

15. Gronwald, J. W., Fuerst, E. P., Eberlin C. V. and Egli, M. A. Effect of herbicide antidotes on glutathione content and glutathione-S-ransferase activity of sorghum shoots. *Pest. Biochem. Physiol.,* **29**, 66-67, (1987).

16. Guddewar, M. B. and Dauterman, W. C. Purification and properties of a glutathione-S-transferase from corn which conjugates triazine herbicides. *Phytochem.,* **18**, 735-740, (1995).

17. Gullner, G., Fodor, J. and Kiraly, L. Induction of glutathione-S-transferase activity in tobacco by tobacco necrosis virus infection and by salicylic acid. *Pestic. Sci., (UK),* **45**, 290-291, (1995).

18. Habig, E. H., Pabst, M. J. and Jakoby, W. B. Glutathione-S-transferase. *J. Biol. Chem.,* **249**, 7130-39, (1974).

19. Hatton, P. A., Cummins, I., Price, L. J., Cole, J. C. and Edwards, R. Glutathione transferases and herbicide detoxification in suspension-cultured cells of giant foxtail

(*Setaria faberi*). *Pestic. Sci. (UK),* **53**, 209-216, (1998).

20. Hunaiti, A. A. and Bassam, R. A. Glutathione-S-transferase from oxadiazon treated chickpea. *Phytochem.,* **29**, 2431-35, (1990).

21. Kombrink, E. and Somssich, I. E. Defense responses of plants to pathogens. *Adv. Bot. Res.,* **21**, 1-34, (1995).

22. Marrs, K. A. The functions and regulation of glutathione-S-transferases in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* **47**, 127-158, (1996).

23. Mauch, F. and Dudler, D. Different induction of distinct glutathione-S-transferase of wheat by xenobiotics and by pathogen attack. *Plant Physiol.,* **102**, 1193-1201, (1993).

24. Mesterhazy, A. Comparative analysis of artificial inoculation methods with *Fusarium* spp. On winter wheat varieties. *Phytopathol. Z.,* **93**, 12-25, (1978).

25. Miller,, J, D, and Arnison, P. G. Degradation of deoxynivalenol by suspension cultures of the Fusarium head blight resistant wheat cultivar Frontana. *Can. J. Plant Pathol.,* **8**, 147-150, (1986).

26. Miller, J. D., Young, J. C. and Sampson, D. R. Deoxynivalenol and fusarium head blight resistance in spring cereals. *Phytopathol. Z.,* **113**, 359-367, (1985).

27. Miller, J. D., Young, J. C. and Treuholm, L. H. Fusarium toxins in field corn: I. Parameters associated with fungal growth and production of deoxynivalenol and other mycotoxins. *Can. J. Bot.,* **61**, 3080-87, (1983).

28. Schroeder, H. W. and Christensen, J. J. factors affecting the resistance of wheat to scab caused by *Gibberella zeae.* *Phytopathol.,* **53**, 831-8, (1963).

29. Snijders, C. H. A. the phytotoxic action of deoxy-nivalenol and zearalenone on wheat seedlings. *Proc. Japan. Assoc. Mycotoxical. Suppl.,* **1**, 103-4, (1988).

30. Timmerman, K. P. Molecular characterization of corn glutathione-S-transferase isozymes involved in herbicide detoxification. *Physiol. Plant.,* **77**, 465-471, (1989).

31. Uneo, Y. Trichothecenes-chemical, biological and toxicological aspects. Elsevier Sci. Publ. Amsterdam. 313 p. (1983).

32. Wang, Y.-Z. and Miller, J. D. Screening techniques and sources of resistance to fusarium head blight. In: wheat production constrains in tropical environments. *Proc. Int. Conf. CIMMYT,* Mexico. pp. 239-250, (1987).

33. Wang, Y.-Z. and Miller, J. D. Effects of *Fusarium graminearum* metabolites on wheat tissue in relation to fusarium head blight resistance. *J. Phytopathol.,* **122**, 118-125, (1988).

34. Wegener, M. Optimierung Von Saatgutpillierungen mit mikrobiellen antagonisten zur biologischen Biologischen Bekampfung Von *Fusarium culmorum* (W. G. SM) Sacc. In Weizen. Diplomarbeit, Universitat Gottingen. (1992).

35. Williams, B. Mycotoxins in food stuffs. In: Scott, Trenholm and Sutton (Eds.), Mycotoxins: a Canadian perspective NRCC, 22848. Ottawa, 49-53 pp. (1985).

36. Zamanizadeh, H. and Khoursandi, H. *Fusarium* spp. And their mycotoxins in wheat fields of Mazandaran province. *Iran. J. of Plant Dis.,* **37**, 23-31, (1996).