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ENHANCED RESISTANCE TO *Aspergillus flavus* IN TRANSGENIC PEANUT HAVING RICE CHITINASE *RCG*3 GENE

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The herbicide resistance (*bar)* and rice chitinase (*rcg*3) genes were incorporated in the commonly cultivated varieties of peanut (BARD-479 and Golden) through *Agrobacterium* mediated transformation. The role of genetically transformed peanut in controlling aflatoxin contamination by *Aspergillus flavus* was investigated by HPLC and TLC methods. In case of detection by HPLC the herbicide resistance *bar* gene had no affect on the aflatoxin content and the aflatoxin B1 found in non transgenic seeds was also present in the transgenic seeds. Transgenic seeds having *rcg*3 gene controlled the contamination of aflatoxin B1 that appeared in the non transgenic counter parts of the same variety. Whereas in case of aflatoxin determined by TLC method the plants having *rcg*3 gene also showed the control of *Aspergillus flavus* in mature peanut seeds.
Keywords: Aflatoxins, *rcg*3 gene, *bar* gene, peanut

INTRODUCTION

The impact of genetically modified organisms (GMOs) on the environment is very diverse and needs to be investigated very carefully. GM crops have a profound impact on society, including livelihoods, trade, production systems, culture and ethics. There are broad-spectrum safety issues concerning the commercialization of different transgenic plants in different parts of the world that may be evaluated on case to case basis before release of any transgenic plant. The concerns described by OECD (2000) are: genetic stability of the transgene; transfer of genetic information to related and unrelated organisms; effects on non- target organisms and weediness potential.

Aflatoxins are toxic compounds produced by *Aspergillus flavus.* There are four major aflatoxins: B1, B2, G1, G2 and two additional metabolic products, M1 and M2 that are of significance as direct contaminants of grains. Aflatoxins are carcinogenic, teratogenic and immune suppressants that cause growth retardation in children, immune-suppression and decreased human and animal productivity (Groopman *et al*., 2005). In general aflatoxins are known to degrade the quality of oil seed crops including peanut. Aflatoxin contamination of peanuts is an industry-wide problem affecting peanut growers, manufacturers and consumers. Shelled peanuts having greater than 20 ppb (parts per billion) of aflatoxin, are known as potent human carcinogen (FAO, 2004).

Genetic Engineering of crops may affect the entire behavior of the host plant and ultimately its response to different biotic and abiotic stresses. Reddy *et al*. (2007) found increased incidence of contamination by aflatoxins in glyphosate resistant (GR) maize as compared to non GR maize. The contamination was attributed to the differences in the crop management practices rather than the insertion of GR gene. Chriscoe (2008) also reported that the expression of oxalate oxidase in peanut did not cause an increase in aflatoxins contamination of peanut seeds. Parasad *et al*. (2012) transformed rice chitanase gene in peanut as antifungal gene. The three progenies of these events showed significant negative correlation between the chitinase activity and the infection frequencies of Late leaf spot (LLS), rust and *A. flavus* infection ($P< 0.01$). These results indicated that the transgenic events with high chitinase activity showed lower disease incidence and vice versa thereby indicating that the levels of resistance might be improved by pyramiding resistance genes from diverse sources to provide transgenic protection to peanut against infection by aflatoxin-producing fungi (Sharma *et al*., 2006). The authors concluded that the improved resistance against the pathogen was a consequence of enhanced chitinolytic active activity and other defense-related mechanisms being triggered by the presence of chitinase.

Peanut is very important food and cash crop in the potohar region of Pakistan. Contamination by aflatoxins poses a significant threat that decreases the economic returns to the producer. Genetic transformation of the Peanut to herbicide and disease resistance is supposed to increase the farm level production. In the present study the effect of genetic transformation of peanut having *bar* and *rcg*3 on the aflatoxin content was investigated and the mean values were also compared with their non transgenic counterparts using two different detection methods.

MATERIAL AND METHODS

A comparative approach was used for determination of aflatoxin content in the transgenic and non transgenic seeds using two different techniques (HPLC and TLC).

***Collection of samples*:** The genetically transformed peanut seeds having herbicide resistance *bar* gene and antifungal *rcg*3 gene resistant to tikka disease was sown at Barani Agricultural Research Station, Chakwal in Randomized Complete Block Design. In the field transgenic seeds were surrounded by non transgenic counterpart for the comparison of aflatoxin contamination. The Seeds were harvested and packed in airtight polythene bags and stored for analysis at room temperature.

***Determination of aflatoxins using HPLC*:** Peanut seeds were ground using 50M sieve size**.** Aflatoxins were extracted from the seed samples (25 g) using 84 % acetonitrile (100ml) having 5 g sodium chloride. For complete extraction samples were subjected to shaking in orbital shaker for one hour. The samples were filtered and for purification extract was passed through Mycosep cleanup column. Then sample were derivatized with TriFloure Acetic acid and analyzed for aflatoxin contamination by HPLC-FLD (Agilent 1100 series, USA). Mixed standard for aflatoxin having B1, B2, G1 and G2 was obtained from Supelco, USA (Abbas *et al.*, 2012).

***Determination of total Aflatoxin by TLC*:** For the determination of total Aflatoxin contents protocol described by AOAC (1984) was followed. Ground seed sample of 25g was added into 250ml Erlenmeyer flask. In the flask 100ml of 60:40 Methanol/water (v/v) was added and after covering with aluminum foil blended at high speed for 3 minutes. The extract was filtered into a sample jar with a funnel using Whattman No. 1filter paper. The extract was diluted by using PBS (Phosphate buffer pH=7.4) and the AflaStar Fit-Immunoaffinity column was placed onto an adapter. The diluted extract was applied and allowed to pass completely through the column. The column was rinsed after the diluted extract was completely passed through the column. Then syringe barrel was removed from the column and labeled test tube was placed under the column for the collection of elute. After elution, evaporated whole residue by Romer EvapSystem, re-dissolved the residue in 400µl 95:5/Tol: AcN (v/v) and vortexed for 30 seconds. Then spotted 40µL of sample along with standard solution on a silica gel TLC plate and after spotting, developed TLC plate in 9:1/Chloroform: Acetone (v/v). The plate was dipped in 10% H_2SO_4 in Methanol (v/v) and was observed under long wave UV light at 365 nm. For aflatoxin determination the yellow fluorescent spots of sample were compared with the aflatoxin standard spot.

***Statistical analysis*:** The data were analysed by Microsoft Excel for calculation of mean and standard deviation.

RESULTS

Aflatoxin levels were not significantly different among transgenic and non transgenic peanut seeds having *bar* gene and the analysis of both transgenic and non transgenic seeds showed only the peaks of aflatoxin B1 (Fig. 5); however, the values were far below the reference range. In case of seeds having *rcg*3 gene in transgenic seeds no peak for aflatoxin appeared indicating that the rice chitanase gene retarded the production of aflatoxin B1 (Fig.3). Similar trend was observed when the total aflatoxins were determined by the TLC method. Aflatoxins B1 and G1 were <0.1 ppb in the transgenic seeds having *bar* gene but values for the seeds having *rcg*3 gene were below detection limit (Table 1). In case of aflatoxin B2 and G2 the values for *bar* gene were <0.5 while no contamination was observed for the seeds having *rcg*3 gene. Beside the little variations all the observed values were far below the limit for low concentration (<20 ppb) outlined by AOAC.

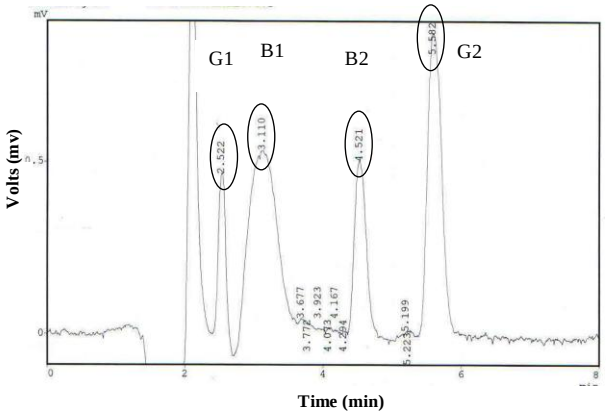

Figure 1. Chromatogram of standard for aflatoxin

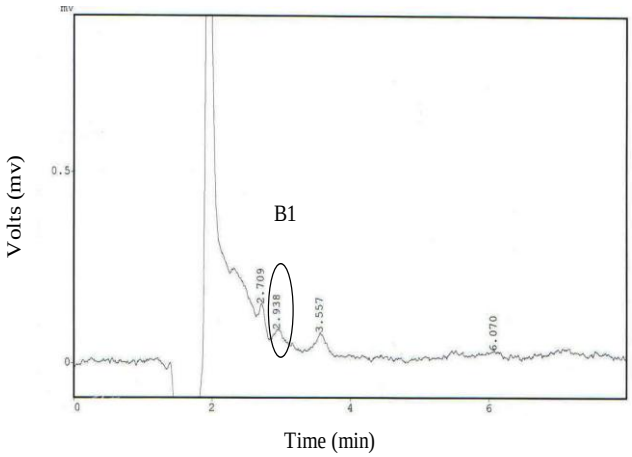

Figure 2. Chromatogram of peanut seeds control for *rcg*3 gene

Table 1. Total aflatoxin contents determined by TLC methods

Aflatoxins (ppb)	Transgenic seeds having *rcg*3 gene	Control for *rcg*3 gene	Transgenic seeds having *bar* gene	Control for *bar* gene
Aflatoxin B1 & G1	-	< 0.1±.001	<0.1±.005	<0.1±.005
Aflatoxin B2 & G2	-	<0.5±0.1	<0.5±0.1	<0.5±0.1

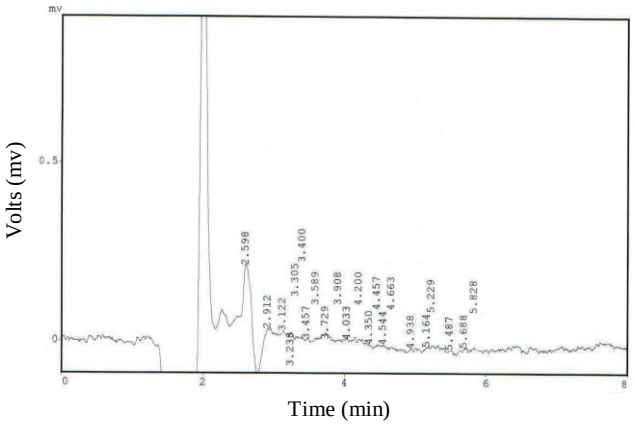

Figure 3. Chromatogram of Peanut seeds having *rcg*3 gene

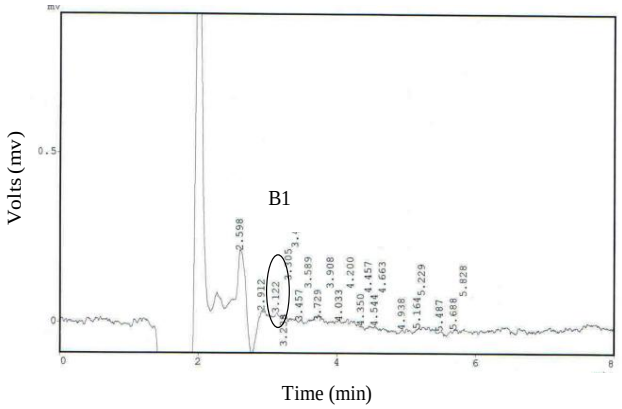

Figure 4. Chromatogram of peanut seeds having bar gene

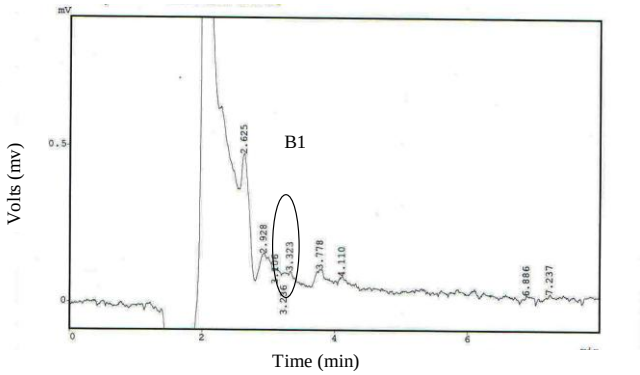

Figure 5. Chromatogram of peanut seeds control for *bar* gene

DISCUSSION

Fungus known as *Aspergillus flavus* or *A. fumigates* normally produce aflatoxin in the peanut kernels in the post harvest period. As peanut seeds are directly consumed by end users after roasting and without further processing therefore, the contamination triggers the quality issues. From the results obtained there is no indication that the insertion of *bar* or *rc*g3 gene enhanced the level of aflatoxin in the seeds rather insertion of *rcg*3 decreased the incidence of aflatoxin as compared to non transgenic control determined by HPLC and TLC methods. Small variations found were far below the toxic limit. Although non transgenic seeds had no significant contamination of aflatoxins in this study but it is interesting that the aflatoxin contamination declined in the seeds having *rcg*3 gene.

Similarly Chriscoe (2008) also reported that insertion of oxalate oxidase gene in peanut did not increase the aflatoxin level in the seed in consecutive three year of research. The advances in genetic engineering and transformation with chitinase genes from different origins resulted in a quantitatively enhanced fungal disease resistance in several crop species including banana (Sreeramanan *et al*., 2009), Italian rye grass (Takahashi *et al.,* 2005) and cucumber (Kishimoto *et al*., 2002). Different genes used for control of fungal diseases are barley oxalate oxidase (Livingstone *et al*., 2005), tobacco chitinase (Rohini and Rao, 2001), tobacco β-1,3-glucanase (Sunderesha *et al*., 2009),Similar results were reported by Parasad *et al*. (2012). These results indicated that the transgenic events with high chitinase activity showed lower disease incidence and vice versa thereby indicating that the levels of resistance might be improved by pyramiding resistance genes from diverse sources to provide transgenic protection to peanut against infection by aflatoxin-producing fungi (Sharma *et al*., 2006). The authors concluded that the improved resistance against the pathogen was a consequence of enhanced chitinolytic active activity and other defense-related mechanisms being triggered by the presence of chitinase. Rohini and Rao (2001) also reported positive correlation between resistance to early leaf spot and increased chitinase activity .

It is very useful finding as aflatoxin is very important issue in the peanut growing areas and its control through insertion of antifungal gene increased the value of stored grain along with enhancing the yield through the control of fungal disease.

Conclusion: It can be concluded from the current study that the insertion of rice chitinase gene in genetically transformed peanut not only controlled the tikka disease caused by *Cercospora* sp. but also controlled the aflatoxin contamination caused by *Aspergillus* sp. The promoisng transgenic lines will undergo further testing for response to other fungal diseases in the field conditions under different agro ecological zones. We believe that the combination of this transgenic technology and conventional breeding programme will offer durable control for fungal disease infection.

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REFERENCES.

Abbas, M. A., M. Khan, M. R. Asi and J. Akhtar. 2012. Pervasiveness of aflatoxin in peanuts growing in the area of Pothohar, Pakistan. World Academy of Science, Engineering and Technology 69: 624-627.

AOAC. 1984. Official methods of analysis. 14th Edn. Association of Official Analytical Chemists. Washington DC.

Chriscoe, S.M. 2008.Characterization of transgenic peanuts expressing oxalate oxidase for governmental approval of their release for control of sclerotinia blight. MSc. Thesis Blacksburg, VA.

FAO. 2004. FAO/WHO Regional Conference on Food Safety for Asia and Pacific. Aflatoxin contamination in foods and feeds in the Philippines. Seremban, Malaysia, 24-27 May 2004.

Groopman, J.D. and T. Kensler. 2005. Role of metabolism and viruses in aflatoxin-induced liver cancer. Toxicology and Applied Pharmacology, 206:131-137.

Kishimoto, K., Y. Nishizawa, Y. Tabei, T. Hibi, M. Nakajima and K. Akutsu. 2002. Detailed analysis of rice chitinase gene expression in transgenic cucumber plants showing different levels of disease resistance to gray mold (*Botrytis cinerea*). Plant Sci. 162: 655-662.

Livingstone, D.M., J.L. Hampton, P.M. Phipps and A.G. Elizabeth. 2005. Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. Plant Physiol. 137: 1354-1362.

OECD. 2000. Report of the working group on harmonisation of regulatory oversight in biotechnology, C (2000)86/ADD2. Organisation for Economic Co-operation and Development (OECD), Paris, 65 pp. Available on line with updates at http://www.olis.oecd.org/olis/2000

Parasad, K., B. Mathur, F. Waliyar and K.K. Sharma. 2012. Overexpression of a chitinase gene in transgenic peanut confers enhanced resistance to major soil borne and foliar fungal pathogens. J. Plant Biochem. Biotech. Available on line with updates at http://dx.doi.org/10.1007/s13562-012-0155-9

Reddy, K.N., H.K. Abbas, R.M. Zablotowicz, C.A. Abel and C. H.Koger. 2007. Mycotoxin occurrence and *Aspergillus flavus* soil propagules in a corn and cotton glyphosate-resistant cropping systems. Food Additives Contaminants 24: 1367-1373.

Rohini, V.K. and K.S. Rao. 2001. Transformation of peanut (*Arachis hypogaea* L.) with Tobacco chitinase gene: variable response of transformants to leaf spot disease. Plant Sci. 160: 889–898.

Sharma, K.K., F. Waliyar, P.L. Kumar, S.V. Reddy, R.K. Reddy, S. Muthukrishnan, M. Lavanya, S.N. Nigam, R. Aruna and D. Hoisington. 2006. Development and evaluation of transgenic groundnut expressing the rice chitinase gene for resistance to Aspergillus flavus. In International Conference on Groundnut Aflatoxin Management and Genomics, 5–10 November 2006, Gungdon Hotel, Guangzhou, China.

Sreeramanan, S., M. Maziah and R. Xavier. 2009. A protocol for *Agrobacterium* mediated transformation of banana with a rice chitinase gene. Emirates J. Food Agric. 21: 18–33.

Sundaresha, S., K.A. Manoj, S. Rohini, S.A. Math, E. Keshamma, S.C. Chandrashekar and M. Udayakumar. 2009. Enhanced protection against two major fungal pathogens of groundnut, *Cercospora arachidicola* and *Aspergillus flavus* in transgenic groundnut over-expressing a tobacco β1-3 glucanase. Eur. J. Plant Pathol. 126: 497- 508.

Takahashi, W., M. Fujimori, Y. Miura, T. Komatsu, Y. Nishizawa, T. Hibi and T. Takamiz. 2005. Increased resistance to crown rust disease in transgenic Italian ryegrass (*Lolium multiflorum* L.) expressing the rice chitinase gene. Plant Cell Rep. 23: 811-818.