Turkish Journal of FIELD CROPS

Turk J
Field Crops
2019, 24(1), 21-27
DOI: 10.17557/tjfc.562634

BIOLOGICAL CONTROL OF AFLATOXIGENIC FUNGI ON PEANUT: FOR THE PRE-HARVEST APPROACH

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Received: 11.12.2018

ABSTRACT

This study was carried out to determine the efficacy of different applications of a biopesticide for reduction of aflatoxin contamination in peanut. The biopesticide, afla-guard, delivers a nontoxigenic *Aspergillus flavus* to the field where it competes with naturally occurring toxigenic fungus. Biocontrol treatments included: (ı) soil application during sowing, (ıı) multiple application during sowing and 40 days after planting, (ıı) foliar application at 60 days after planting (ıv) control (untreated plots). Biopesiticide was applied to peanut plots in 2015 and 2016 in Randomized Complete Block Design with four replications. Peanuts were collected from control and treated plots at harvest-drying-pre-storage periods and analysed for aflatoxins. Aflatoxin concentrations were generally quite low in 2015, also the aflatoxin concentration in treated samples (from 0.04 to 0.71 µg/kg) was reduced by 97.38 to 99.82% compared with controls (from 21.84 to 27.12 µg/kg). In 2016, reductions were also noted for all biocontrol treatments (from 89.07 to 92.39%) compared with controls. In conjunction with the reductions in aflatoxin contamination, biocontrol treatments produced significant reductions with biopesticide in peanut. Therefore, it can be said that a biological control method is a promising approach for controlling aflatoxin.

Keywords: Aflatoxin, *Aspergillus flavus*, *Aspergillus flavus* NRRL 21882, Biological control, Peanuts

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is a one-year plant belonging to the Fabaceae family and contains a high level of fat in its seed (Arıoglu, 2000). The crop is widely consumed in Turkey, as a rich source of protein and vitamins. However, fungal contamination is a main problem in peanut production. Fungi are the main spoilage agents both various plant pathogens and food. Fungal contamination caused plant infection not only seed contamination with mycotoxins but also results in a decrease of crop yield and significant economic losses of a quality (Makun et al., 2010).

Some fungal species make mycotoxins that are toxic secondary metabolites (Richard, 2007; Russell et al., 2010). *Aspergillus flavus and Aspergillus parasiticus* are the major aflatoxin producing species on crops (Yu et al., 2004). Aflatoxins are known to be the most carcinogenic among all of the mycotoxins (Singh et al., 2018). Therefore, aflatoxin exposure can be in serious health conditions such as cancer and liver cirrhosis, weakened immune systems (Wu and Khlangwiset, 2010). The more common toxins groups are aflatoxin B_1, B_2, G_1 and G_2; among them aflatoxin B_1 is themost toxic. International cancer studies are classified by the agency as group 1 carcinogen (IARC, 1993).

Peanuts are the main sources of human exposure to aflatoxin because it is immensely consumed worldwide (13.3 million tons of peanuts were use up in 2001-2003 and expected consumption of 16.32 million tons in 2030) and unfortunately are the most susceptible crop to aflatoxin contamination (Waliyar et al., 2009; Mutegi, 2010; Wu and Khlangwiset, 2010). For this reason, exposure to aflatoxin in peanut represent a serious risk to economy and health for many countries (Kumar et al., 2008; Guo et al., 2009).

A. flavus and *A. parasiticus* are caused aflatoxin contamination on peanuts. These fungi are contacted developing peanut pods to grow and increase in the soil. When the peanut pods are exposed to drought conditions, they become available to contamination. A method of biological control has used for reducing aflatoxin contamination which nontoxigenic *A flavus* is applied to

peanut soil to deport the toxigenic strain. A biopesticide, afla-guard(®), has been developed for controlling aflatoxin in peanuts (Isakeit et al., 2010). This biopesticide supplies of introducing a competitive and nonaflatoxigenic strain of *Aspergillus flavus* into soils. This commercial product is contained of the nontoxigenic strain of *A. flavus* conidia, which is applied to peanut fields during the cultivation season. After the conidia germination, growing, and sporulating, increasing population of the nontoxigenic strain in the soil (Dorner, 2004, 2005; Dorner and Lamb, 2006). Therefore, it is seen that biological control is efficient for both pre and post-harvest for aflatoxin contamination.

Even though several studies have researched the effects and performance of afla-guard in different countries, any studies on afla-guard haven't been done in Turkey. Particularly, there is no dearth of information about the suitability and adaptability of afla-guard by peanut in Turkey (Lavkor and Bicici, 2015; Lavkor et al., 2017). The purpose of this study was conducted to evaluate the efficacy of three different treatments of nontoxigenic *Aspergillus flavus* NRRL 21882 to decrease preharvest aflatoxin contamination of peanuts.

MATERIALS AND METHODS

Materials

This study has been performed in 2015 and 2016 at the fields of Cukurova University located in Adana, Turkey as a second crop peanut. Halisbey variety belonging to Virginia market type was used as a plant material in this experiment.

Methods

Aflatoxigenic A. flavus and inoculum preparation: A. flavus isolated from peanut stores in Osmaniye in 2011, which produced 22.67 µg/L AFB_1, 1.06 µg/L AFB_2 and 23.73215 µg/L aflatoxin. Isolates were maintained on Czapekagar slants at 4°C.

A modified inoculation method according to Denizel and Kosker (1972) was carried out. Aflatoxigenic *A. flavus* was inoculated on Czapek agar media and incubated at 24°C for 4 days. Following which, grains (300 g) were soaked in 50 ml of distilled water, and they autoclaved for 30 minutes in a 1000 ml flask. After cooling, the grains were incubated for 7 days at 25°C with aflatoxigenic strain to allow colonization for further growth and sporulation. The product was colonized with an aflatoxigenic isolate in a flask and blended gently shaking it. Conidial spores were removed from the flask with a long-stemmed sterile spatula. Then spore suspensions of the aflatoxigenic isolate were prepared in 0.1% Tween 80, and adjusted the concentration of conidia 10^7 per ml using a hemocytometer. Then, the strain mixture was inoculated with 100 seeds in the flask. At last, aflatoxin producing *A. flavus* isolate was artificially inoculated with peanut seeds and then sown in the field plots.

Field Plots: Experiment was designed at Randomized Complete Block with four replications with four plots in each block. Each plot consisted of 3 rows 5.0 m long and 70 cm apart. Furthermore, the seeds were sown by hand on 18 June April 2015 and 17 June 2016 with 70 x 10 cm distance. In the experiments, 25 kg/da diammonium phosphate (DAP) was used before planting. Also, 30 kg/da ammonium nitrate (33%N) was applied two times; before first (flowering period) and second (pod formation) irrigation in each years. After, inoculated seeds with aflatoxigenic *A. flavus* of conidial suspension ($1x10^7$ conidia/ml) were sown.

Applications of Afla-guard (Aspergillus flavus NRRL 21882*):* An aqueous conidial suspension of the nontoxigenic *A. flavus* was applied in three different treatments in the experiment and included; (ı) afla-guard applied to soil during sowing at 907 g/da; (ıı) afla-guard applied to soil during sowing (455 g/da) and 40 days after planting (455 g/da); (ııı) afla-guard applied to foliar at 60 days after planting (907 g/da); (ıv) Control (untreated plots) (Table 1) (Anonymous, 2014). The experiment also included untreated controls with inoculation of aflatoxigenic *A flavus*, but not applied afla-guard. The suspension was applied soil and foliar as a spray when good soil moisture is available. This can be soon after a rain or shortly before (if a high probability of rain exists). Even in the absence of rain, good growth of the fungus can take place in the warm, humid environment under the plant canopy if there is good protection from direct sunlight. Afla-Guard® (contains 0.0094% active ingredient with a minimum of 1.2 x 10^8 CFU/lb) is a registered trademark of a Syngenta Group Company.

Table 1. Application details of Afla-guard (g/da)

Biopesticide	**Soil Application (g/da)**		**Foliar Application (g/da)**
	I. Application	**II. Application**	
Aspergillus flavus NRRL 21882% (active ingredient %0.01) 1.2 x10^8 cfu/lb (Afla-guard)	Soil application during sowing 907 g/da	Soil application during sowing 455 g/da Soil application at 40 days after planting 455 g/da	Foliar application at 60 days after planting 907 g/da

Harvest: The peanut samples were collected at three different periods which included harvest, drying, and pre-storage. Peanut plots were harvested on 06 November 2015 and 04 November 2016. After each plot was harvested, the pods were dried in the naturally field conditions for 6-10 days. Then, the peanuts were

eliminated from soil for the pre-storage period. Later, the peanuts were transferred to storage. About 5 kg peanuts samples were divided into a paper bag and about 1 kg of peanut subsamples were collected for aflatoxin analysis. The shells were removed manually. Samples composed of 1 kg each were manually separated from the shell and were retained at +4°C for aflatoxin analysis (Lavkor, 2013).

Aflatoxins Analyses: The aflatoxins were analyzed by using immunoaffinity columns and aflatoxins B_1, B_2, G_1, and G_2 were affected by High-Performance Liquid Chromatography (HPLC) method in accordance with Arzandeh and Jinap (2011). Peanut samples weighing 50 g were weighed together with 5 g of sodium chloride (NaCl) and shaken. Methanol: water (125 ml) in a ratio of 70:30 was added to the jar and the sample was mixed with 2-3 minutes. The mixture sample poured onto a filter paper. Filtered extract of the 15 ml was diluted with water (30 ml). Afterwards, a 1 ml of methanol was eluted at column and the elute was collected in a vial. Solvent flow column rates of 1 ml/min. The Agilent 1100 HPLC system was used. Excitation and emission wavelengths of 360 and 440 nm was used for fluorescence detector system. HPLC system consisted of C18 column (R-Biopharm Rhône) with a mobile phase of water/acetonitrile/methanol (600:200:300, v/v/v). Flow rate was 1 ml/min; injection volume was 100 ml. The HPLC column was maintained at fix temperature (T=25°C). All the data were shown as a µg/kg.

Climate Conditions: The average temperature, soil temperature, 10 cm top soil temperature and relative humidity of the experimental site during the 2015-2016 growing period were given in Table 2.

As can be seen in the Table 1, the climate data were collected during the growing seasons of 2015 and 2016 in experimental area. The average temperature data during the growing periods in 2015 and 2016 were ranged from 15.20 mm to 30.10 ºC, respectively. 10 cm soil temperature during the growing periods in 2015 and 2016 were between 15.60 ºC and 35.16 ºC, respectively. Soil temperature was ranged from 6.33 ºC to 30.10 ºC in 2015 and 15.20 ºC to 29.88 ºC in 2016. The relative humidity was ranged from 50.48% to 69.81% in 2015 and 51.75% to 67.50% in 2016. The total rainfall was between 0.00 mm and 65.00 mm during the growing periods in 2015 and 2016, respectively.

Table 2. Average temperature, soil temperature, 10 cm top soil temperature and relative humidity of the experimental site during the 2015-2016 growing period (Anonymous, 2017)

Months	Average temperature		Soil temperature		10 cm soil temperature		Relative humidity		Total rainfall	
	(^{0}C)						(%)		(mm)	
	2015	**2016**	**2015**	**2016**	**2015**	**2016**	**2015**	**2016**	**2015**	**2016**
June	25.15	27.29	18.24	20.17	28.28	29.95	69.11	63.79	1.60	9.12
July	28.45	29.50	22.30	23.32	34.57	35.16	69.81	67.50	0.40	0.20
August	30.10	29.88	23.52	23.82	35.13	35.05	62.32	67.39	5.45	8.20
September	28.72	26.15	20.82	19.16	32.05	29.19	63.55	59.92	65.00	7.96
October	22.43	22.90	15.65	13.77	23.73	24.18	65.12	55.16	4.59	0.00
November	16.93	15.20	6.33	4.27	16.58	15.60	50.48	51.75	3.50	3.97

Statistical Analysis: The data were evaluated to analysis of variance (ANOVA). Duncan test ($P < 0.05$) was compared with the means (Gomez and Gomez, 1983). Statistical analysis was carried out using the statistical package MSTAT-C (1991).

RESULTS AND DISCUSSION

In the field experiments carried out in two years, three different biocontrol treatments were reduced aflatoxin level signally; also all treatments usually were seemed to result in less aflatoxin in 2015 and 2016 compared with untreated controls. There were statistically expressive differences ($P \leq 0.05$) compared groups but no statistically significant ($P \leq 0.05$) of all treated plots between each other.

According to the results of the plot experiments, total aflatoxin concentration of peanut samples from harvest, drying, and pre-storage periods showed a difference ranging between 0.04 and 0.71 µg/kg, in connection to the results of treated plots in 2015. Afla-guard effects of treated plots were found to be effective between 97.38 and 99.82% according to Abbot formula (Table 3, Figure 1). In 2015, there were significant ($P \leq 0.005$) effects on aflatoxin contamination by three periods (harvest, drying and pre-harvest) and all biocontrol treatments compared with control plots. When data of aflatoxin concentrations for peanuts in harvest, drying and pre harvest periods are analyzed together, each treatment produced significant reductions compared with the control.

In the second year of the field experiment performed, the effectiveness of plot treatment was different from the control statistically. As in 2015, there was significant ($P \leq 0.005$) effect on aflatoxin contamination from the plot treatments under harvest, drying and pre-harvest periods in 2016. In 2016, of the total aflatoxin in treated plots was between 1.79 and 2.87 µg/kg while the total aflatoxin in control plots was between 23.48 and 26.25 µg/kg in harvest, drying, and pre-storage periods. The aflatoxin concentration decreasing the effect of treated plots was found between 89.07 and 91.64% (Table 3, Figure 2).

Three different biocontrol treatments were applied under field conditions in Turkey to determine the effectiveness of afla-guard in mitigating aflatoxin contamination of

peanut. In general it was demonstrated that the biocontrol treatments investigated in this study reduced the aflatoxin in field experiments. This study corroborates previous studies demonstrating the biological control of aflatoxin contamination in peanuts by competitive exclusion (Dorner et al., 1992, 1998; Dorner and Cole, 2002; Pitt and Hocking, 2006; Dorner and Horn; 2007).

Table 3. Effect of biological control treatments on aflatoxin contamination of peanuts in harvest, drying, pre-storage periods in 2015 and 2016

Treatment	Harvest	Drying	Pre-storage	Harvest	Drying	Pre-storage
	Total aflatoxin (µg/kg)			% Effect (Abbott)		
			2015			
Soil[1]	0.04b*	0.16b	0.04b	99.82	99.41	99.82
Multiple[2]	0.07b	0.26b	0.17b	99.68	99.04	99.24
Foliar[3]	0.34b	0.71b	0.24b	98.44	97.38	98.93
Control	21.84a	27.12a	22.49a	-	-	-
			2016			
Soil	2.41b	2.60b	1.96b	90.29	90.10	91.64
Multiple[2]	2.14b	2.45b	1.79b	91.38	90.66	92.39
Foliar[3]	2.48b	2.87b	2.05b	90.02	89.07	91.29
Control	24.81a	26.25a	23.48a	-	-	-
			2015-2016			
Soil[1]	1.23b	1.38b	1.00b	95.06	94.76	95.73
Multiple[2]	1.11b	1.36b	0.98b	95.53	94.85	95.82
Foliar[3]	1.41b	1.79b	1.15b	94.23	93.23	95.11
Control	21.33a	26,69a	22,99a			

[1]Afla-guard applied to soil during sowing at 907 g/da
[2]Afla-guard applied to soil during sowing (455 g/da) and 40 days after planting (455 g/da)
[3]Afla-guard applied to foliar at 60 days after planting (907 g/da)
*Means within column followed by different letters are significantly different (P≤0.05) according to Duncan multiple range test

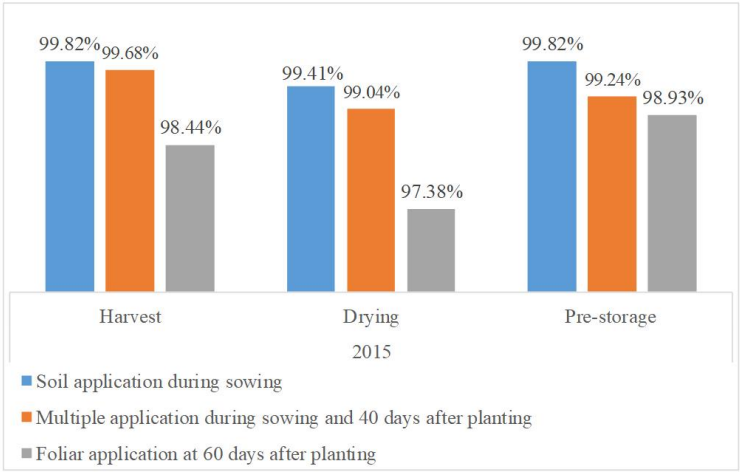

Figure 1. Effect of biological control treatments on aflatoxin contamination of peanuts in harvest, drying, pre-storage periods in 2015

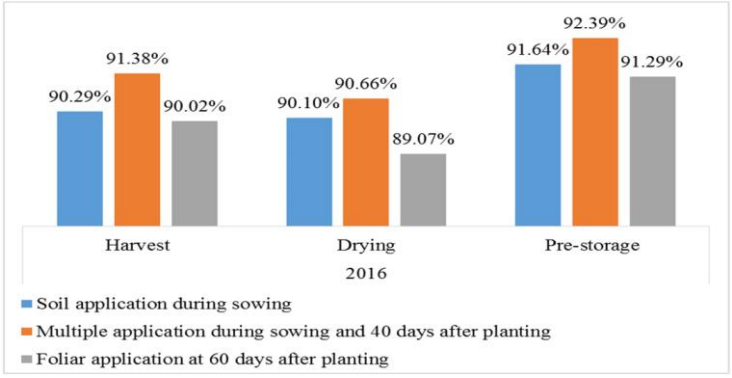

Figure 2. Effect of biological control treatments on aflatoxin contamination of peanuts in harvest, drying, pre-storage periods in 2016

Our results showed that three biocontrol treatments were as effective in reducing aflatoxin contamination. Nevertheless, three biocontrol treatments had similarly affected on the levels of aflatoxin observed. This is the first study to demonstrate that biopesticide has been used to decrease aflatoxin contamination of peanut under field conditions. Also, with this results have been reached solution that address common and serious problem of aflatoxin contamination in peanut field in Turkey.

The reduction of aflatoxin contamination in peanut with applying afla-guard in this study (from 89.07 to 99.82%) is similar with different research results. This conclusion is obvious from an examination of the aflatoxin data, particularly for 2015 and 2016, during which significant differences in aflatoxin contamination were not observed in treated plots. With closer examination of all data shows that the various treatments with the nontoxigenic *A. flavus* had a reducing effect on aflatoxin contamination in the treated peanut. There was also no difference in total aflatoxin contamination of peanut among biocontrol treatments. In a previous, similar study testing different biocontrol formulations in peanuts, significant differences were found between controls and treatments for aflatoxin contamination. Also, biocontrol treatments were significantly reduced aflatoxin contamination by 91.6% in 1997, 89.5% in 1999, 98.2% in the first harvest in 2002 and 98.4% of the second crop harvest in 2002 in the USA (Dorner, 2004).

For this reason, in our study demonstrated the potential for biocontrol of aflatoxin in peanut, and it did so with application rates that were practically optimum for commercial use. With the current study sought for biocontrol in peanut by using commercially available afla-guard was applied at an economically practical rate (907 g/da). It was also approved the efficacy of three different modes of application.

Moreover, as overall years in 2015 and 2016, aflatoxin data for each biocontrol treatments are given in Table 3. Plot treatments produced significant ($P \leq 0.05$) reductions in aflatoxin compared with control plots. Also, treated plots were found to be effective between 93.23 and 95.82% (Table 3). The mean concentration of aflatoxins in peanut from control and treated plots in 2015 and 2016 are shown in Figure 3. Significantly, ($P \leq 0.05$) aflatoxins decrease was achieved from treated plots. The mean aflatoxin concentration between 0.98 and 1.79 ppb from treated plots in 2015 and 2016 represented a reduction of compared with control plots between 21.33 and 26.69 µg/kg (Table 3, Figure 3).

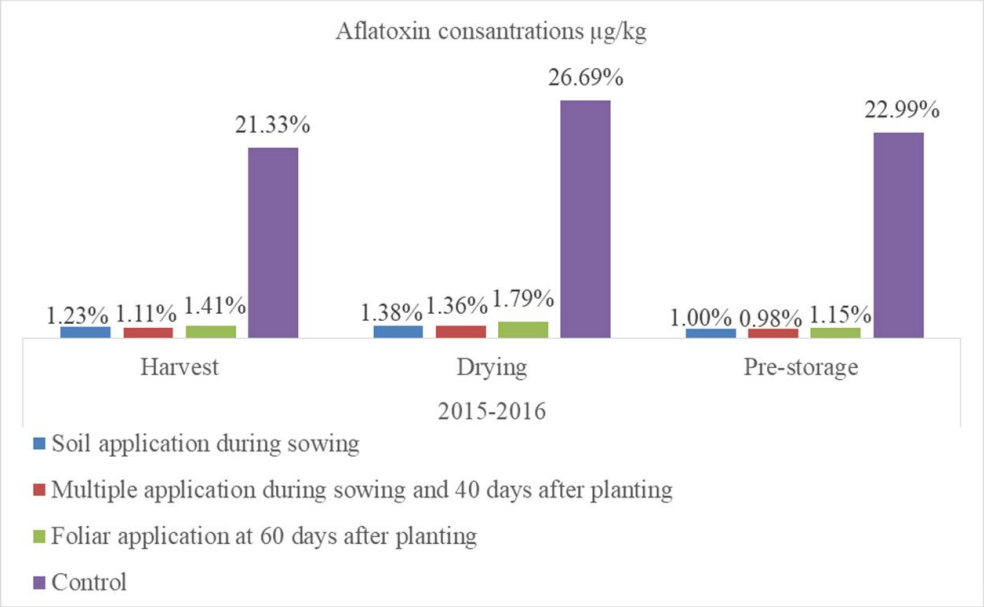

Figure 3. Mean aflatoxin concentrations (µg/kg) of biological control treatments in control and treated plots of peanuts in harvest, drying, pre-storage periods in 2015-2016

Regardless of the conditions experienced in the two years, the overall aflatoxin reductions by 97% in 2015 and 90% in 2016 were similarly. These reductions are also similar to the reduction of 85% produced in peanuts in efficacy study conducted in 2004 (Dorner and Lamb, 2006). Furthermore, other fields and plot studies using the competitive exclusion concept for biological control of aflatoxin contamination have demonstrated aflatoxin reductions ranging from 92% (Dorner et al., 2003). Similar results were reported by some other researchers (Dorner, 2004; Dorner and Horn, 2007; Dorner, 2008).

In addition to having demonstrated that competitive exclusion could reduce preharvest aflatoxin contamination, we studied to determine the potential for reductions in contamination that occur during peanut drying and pre-storage periods. Peanuts from control plots were exposed a significant increase in aflatoxin production during drying and pre-storage periods (from

22.99 to 26.69 µg/kg) in this study between 2015 and 2016, while aflatoxin levels of peanuts from treated plots contained from 0.98 to 1.79 µg/kg under drying and pre-storage periods. Moreover, biocontrol treatments were effectively reduced aflatoxin contamination between 93.23 and 95.82% under drying and pre-storage periods.

The treatment to the soil of nontoxigenic strains of *Aspergillus* both decrease levels of preharvest aflatoxin contamination on peanuts (Cole et al., 1989; Dorner et al., 1992; Dorner et al., 1998; Dorner, 2004; Dorner, 2005), and also has a carry-forward impact, decreasing aflatoxin contamination that would be occur during storage (Dorner and Cole, 2002; Dorner, 2009). Dorner (2004) reported that plots of treated and not treated with nontoxigenic strains in 1998 peanut field research were stored in a warehouse and exposed to the storage conditions that could be contaminated with aflatoxin. At the end of this study, peanuts from untreated plots were caused at increasing aflatoxin level during storage (from 0.0 to 78.0 µg/kg), while aflatoxin level 1.4 µg/kg in peanuts were detected in treated plots, 98% reduction of aflatoxin contamination. Thus, reduction of aflatoxin contamination in peanut not only preharvest but also postharvest stages have been a promising approach to the biological control (Dorner et al., 1998; Dorner and Cole, 2002; Dorner, 2010).

Furthermore, the biocontrol applications are based on environmental conditions as a soil temperature (Yin et. al., 2008; Chepsergon et. al, 2014**)**. Soil temperature can major impress both growth and sporulation of the nontoxigenic fungus. *A. flavus* sporulates at temperatures under 10 °C on medium in the laboratory, but field experiments displayed that establishment of biocontrol isolates did not consist of easily when soil temperature under 20 °C (Pitt and Hocking, 2006). The results point out that application of nontoxigenic isolates to soil should be held up until soil temperature reaches at least 20 °C. In Arizona, USA, later April and early June are the appropriate time for application of the nontoxigenic biocontrol strain. A lot of studies performed in Georgia, the biocontrol strain NRRL21882 was applied between 50 and 70 day after planting of peanuts (Dorner et al., 1992; 1998; Dorner, 2004). A similar relationship holds for our region.

As a result, this study is conducted for the first time in Turkey show that biological control methods may have no adverse efficacy on the human and environment health and are efficient options for aflatoxin contamination. Furthermore, afla-guard, which suppresses the contamination of aflatoxin, has been identified for the first time in our country by this study to be applied in peanut crop cultivation. For this purpose, it has been determined that biopesticide, which has been applied in three treatment in the cultivation of peanuts, decreased aflatoxin contamination in the rates ranging from 89.1% to 99.8%. Therefore, our research study has shown that this biological control strategy can produce reductions in aflatoxin contamination. This study also showed that the displacement of toxigenic strains by nontoxigenic strains that occurs in the field prevents significant aflatoxin contamination when conditions during harvest, drying and pre-storage periods facilitate *A. flavus* growth. As a result, it has been determined that afla-guard is an effective ingredient biopesticide, and can be used to prevent aflatoxin contamination on peanuts. With this research result, scientific data on biological control and prevention of aflatoxin contamination has been obtained for integrated pest management programs to be applied in peanut cultivation.

ACKNOWLEDGMENTS

This study (Project No 115O007) was financial supported by The Scientific Technological Research Council of Turkey (TUBITAK).

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