



The *Aspergillus flavus* Susceptibility of Hazelnut Varieties (*Corylus avellana* L.) in Laboratory Conditions

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ABSTRACT

Iran has suitable conditions for cultivating high-quality varieties of hazelnuts (*Corylus avellana* L.). Most of hazelnut orchards in Iran have been established by planting native genotypes. *Aspergillus flavus* Link. (AF) is a filamentous fungus affecting hazelnut kernels in orchards and during storage conditions. The most widely explored strategy for reducing aflatoxin contamination is the development of host resistance. The relative susceptibility of 12 native and exogenic hazelnut varieties including Soorii, Gerd-e-Eshkevarat, Gerd-e-Shouk, Navan 1, Fertil du Cortard, Pashmineh 89, Rasmi, Gerje, Ronde du pimount, Shastak, Negret, Gerdooii, to AF contamination was evaluated as *in vitro* by the kernel screening assay. Hazelnut kernels were surface sterilized and then inoculated with spore suspension (1×10^6 spores/ml) of AF by dipping method. Experiments were done in a completely randomized design with four replications. After five days of inoculation and incubation at 28 °C, the criteria of the AF growth (sporulation density, SD %) and sporulation rate (SR, spores/ml) on whole (intact) and wounded (cut) kernels of hazelnuts were measured. Results revealed statistical significant differences among hazelnut varieties for the SD% and SR parameters ($p \leq 0.05$). The Averages SD% and SR of *A. flavus* on intact and wounded kernels ranged from 16.50-75.14%, 48.25-100% and 14.6×10^5 - 16.7×10^6 , 12.6×10^7 - 47.6×10^7 , respectively. The highest and lowest SD% on whole and wounded kernels was related to hazelnut varieties Pashmineh 89 and Gerde-Eshkevarat, respectively. The cultivated variety Gerde-Eshkevarat was recorded as an important potential source of resistance to AF.

Introduction

Hazelnut (*Corylus avellana* L.) belongs to the Betulaceae family and is a popular tree nut in Iran and throughout the world. Among tree nut species, hazelnut plays a major role in human nutrition and health due to their very special nutritional value.

Hazelnut is extremely important horticultural crop in Iran, constituting large proportion of the export market. Iran, with about 20000 ha hazelnut growing area and about 18000 t annual production is the sixth

hazelnut producer in the world. Guilan province (northern Iran) is the main hazelnut growing region with about 16000 ha and annual production 11000 t in Iran. Iranian hazelnut varieties are classified according to fruit quantitative and qualitative characteristics (Hossein Ava and Pirkhezri, 2010). The local hazelnut populations in Iran were originated from the European hazelnut and were adapted to the country's climatic conditions (Pop *et al.*, 2010).

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In Iran, most of the studies on the cultivars have been focused on vegetative and fruit characteristics including fruit dimension, fruit and kernel shape index, fruit weight (Hossein Ava *et al.*, 2006; Soleimani *et al.*, 2011; Salimi and Hossein Ava, 2012). Because hazelnuts prefer regions with mild, moist winters and cool summers, *Aspergillus* spp. contamination can be found. Preliminary, *A. flavus* susceptibility has not been an aim of conventional hazelnut breeding program. No information exists about the *A. flavus* resistance in hazelnut varieties in Iran and either source of resistance to *A. flavus* have not been described in hazelnuts. In the breeding programs, the main objectives were generally to introduce higher yielding varieties for both the in-shell and kernel markets. Hazelnut is potentially attacked by *Aspergillus* spp. The *A. flavus* affecting hazelnut kernels in orchards, during storage and drying, produces aflatoxins that are a group of the most potent and dangerous mycotoxins (Baltaci *et al.*, 2011, Milhome *et al.*, 2014). Aflatoxins can occur in hazelnut as well as in legumes, peanuts, corn, wheat, other crops, and some spices. In the hazelnut growing regions, such as those of the state of Guilan in the north of Iran, the frequency of post-harvest mold infection caused by the *A. flavus* represents a qualitative concern (Houshyarfard, Unpublished).

In Guilan province (northern Iran), hazelnuts are generally dried under the sun by small producers. The problem is that the weather in the Guilan region is very humid and the harvesting season can be wet. Drying may prolong to 2–3 weeks due to rain, which increases the risk of mould contamination and consequent formation of aflatoxins. The impact of potential aflatoxin contamination on hazelnuts as regards to food safety (FAOSTAT, 2011; Amiri *et al.*, 2013; Hedayati *et al.*, 2016). The aim of this study was to evaluate the susceptibility of 12 native and exogenic hazelnut varieties to the *A. flavus* growth and sporulation.

Materials and Methods

Hazelnut varieties

Twelve hazelnut varieties (*C. avellana* L.) were assayed (Table 1). These included diverse varieties from Turkey, Italy, France, Iran and, Spain. The toxigenic *A. flavus* strain Z 2210, a very potent strain for production of aflatoxin (Houshyarfard *et al.*, 2014), was obtained from the Fungal Collection of Plant Pathology Laboratory, Faculty of Agriculture, University of Mashhad.

Total oil content of hazelnut kernels was determined by extracting a known weight of hazelnut sample with diethyl ether, using a Soxhlet apparatus (AOAC 2000; Köksal *et al.*, 2006; Silvia *et al.*, 2007; Cristofori *et al.*, 2008). Nitrogen content was determined by the Kjeldahl method, described by Parvaneh (1998) and the protein content was calculated as total N \times 6.25 (Köksal *et al.*, 2006).

Inoculation and incubation

The *A. flavus* pathogenicity confirmed on fresh hazelnut kernels of Iranian local variety known as Navan following Koch's postulates. Stock culture was maintained at 2-5 °C on slant PDA in screw cap test tube. Fifty hazelnut fruits were surface-disinfested with 0.5% sodium hypochlorite and aseptically shelled for analysis of infection by *A. flavus*. The culture of the toxic mold was grown on corn meal agar (CMA) for 5-6 days at 25 °C until well sporulated. Five mL of 1% solution of Triton X100 (w/v) was added into each plate, and then spores were rubbed from the CMA surface. After that, the high-density spore suspension was prepared by passing through two layers of cheese cloth. The suspension was quantified using a hemocytometer and then diluted to obtain $\times 10^6$ spores/mL. For each variety, 250-300 g of the whole kernel was randomly collected from Fresh hazelnut fruits without visible insect damage and surface sterilized with 0.5% hypochlorite sodium followed by three times thoroughly rinsed in sterile distilled water. After that, the one-half of whole

hazelnut kernels (nutmeat) were cut between the two cotyledons with a sterile knife. For each sample (variety), 4-6 pieces from both whole and cut (wounded) kernels were then transferred to a 250-ml Erlenmeyer flask containing 50 ml *A. flavus* spore suspension (1×10^6 spores/ml) for 10 min (dipping method). After that, five whole and/or wounded inoculated kernels with *A. flavus* were placed in a 15 cm diameter plates containing two layers of filter paper moistened with about 1 ml of sterile distilled water. Then, the petri plates were incubated for five days at 28 °C. The experiments were conducted using a randomized complete design (RCD) with 10 replications. Five days after inoculations, the sporulation rate of *A. flavus* (SR, spores/ml) was evaluated by adding 50 ml saline solution (sterile 0.85% NaCl), shaking vigorously at 280 rpm so that and the spores totally washed off the kernel surface of hazelnut and counting the spores with the hemocytometer and light microscope.

The rate of *A. flavus* growth (surface colonization area %, sporulation density%, SD%) on wounded and whole hazelnut kernels was visually scored from 1-4 (colonized surface of kernel, 1: from no visible sporulation to low sporulation with colony formation in less than %25, 2: moderately sporulation with colony formation from 26 to 50%, 3: heavy

sporulation with colony formation from 51 to 75%, and 4: very heavy sporulation with colony formation in more than >75%) based on the following equation (Joosten *et al.*, 2001):

$$\text{Sporulation density (kernel colonization, \%)} = \frac{\sum n}{4 \times N} \times 100$$

n: scoring number

N: the total number of kernels

Statistical analysis

The spore concentration and colonization % were log and arcsin transformed for statistical analysis. The means were separated using Duncan's Multiple Range Test at $P < 0.05$.

Results

The selection of new commercial varieties for the hazelnut industry, and also resistant to *A. flavus* growth and contamination, has been developed in our study with 12 hazelnut varieties. The hazelnut varieties showed protein and oil content in a range from 9.62-16.43% and 47.12-68.32%, respectively (Table 1).

Table 1. The mean chemical composition of hazelnut varieties (*Corylus avellana* L.)

Variety	Oil content (% Dry weight)	Raw protein (% Dry weight)	Origin
Soorii	61.17	12.67	Turkey
Gerd-e-Eshkevarat	47.12	9.62	Iran
Gerd-e-Shok	59.94	16.33	Iran
Navan-1	52.71	14.88	Iran
Fertil du Cortard	57.23	14.86	Spain
Pashmineh 89	68.32	14.83	Iran
Rasmi	53.74	14.61	Iran
Gerjeh	61.38	13.62	Iran
Ronde du pimount	65.72	14.27	Italy
Shastak	62.54	14.71	Iran
Negret	63.82	16.43	Spain
Gerduii	62.33	15.37	Iran

Analysis of variance showed that there were significant differences among hazelnut varieties in the SR and SD% of *A. flavus* on the kernel of hazelnut variety. Table 2, describes the values of SD% and SR during the analyzes of whole and wounded kernels of hazelnut varieties. We found that SD% and SR on wounded (cut) kernels were more than whole (intact) kernels (Table 2). The fungal colonization (%) levels

were found in the range of 16.50-75.14 and 48.25-100% for whole and wounded kernels, respectively (Table 2). The variety Pashmineh 89 was superior for SD% (75.14 and 100 % in whole and wounded kernels) as compared to other hazelnut varieties. Although, the Pashmineh 89 was highly susceptible (over 75%), the Gerde-Eshkevarat was less (or minimally, 16.50%) susceptible (Table 2).

Table 2. Mean comparison of sporulation density (surface colonization area, %) and Log sporulation rate (Log spores/ml) by *Aspergillus flavus* on whole (intact) and wounded (cut) kernels from different 12 hazelnut varieties on the fifth day after inoculation at 28 °C

Variety	Sorii	Gerd-e-Eshkevarat	Gerde-e-Shok	Navan-1	Fertil du Cortard	Pashmineh 89	Rasmi	Gerjeh	Ronde du pimount	Shastak	Negret	Gerduii
whole (intact) kernel												
Sporulation density (%)	30.53 d	16.50 g	29.20 de	24.15 f	27.35 ef	75.14 a	23.15 f	34.21 cd	51.23 b	36.75 c	38.75 c	35.60 c
Sporulation rate (spores/ml)	6.19 b	6.16 b	6.18 b	6.17 b	6.18 b	7.22 a	6.17 b	6.21 b	7.06 a	6.23 b	7.05 a	6.22 b
wounded (cut) kernel												
Sporulation density (%)	68.60 cd	48.25 e	68.35 cd	66.70 d	66.75 d	100 a	50.65 e	69.55 bcd	74.10 b	72.50 bc	73.40 bc	72.35 bc
Sporulation rate (spores/ml)	8.37 cde	8.10 f	8.25 def	8.20 ef	8.21 ef	8.68 a	8.10 f	8.39 cd	8.62 ab	8.44 c	8.59 b	8.43 c

Means followed by the same letter in each column are not statistically different at (P=0.05)

From the inoculations of the *A. flavus* using whole (intact) kernels of hazelnut varieties, four susceptibility categories (minimally susceptible, moderately susceptible, susceptible and highly susceptible) could be distinguished based on the SD% (<25%, 26-50%, 51-75%, >75%). Based on the SD%, the 'Pashmineh 89' (75.14%) and 'Gerde-Eshkevarat' (16.5%) were highly and minimally susceptible hazelnut varieties, respectively. Hazelnut variety showing 50-75% surface colonization area, including Ronde du pimount (51.23%), made up a third susceptibility category with noticeably increased susceptibility. Kernels of the remaining varieties including Gerde-shok, Sorii, Gerjeh, Shastak, Gerduii, Negret and Fertil du Cortard were intermediate in their susceptibility (SD%=26-50). By the fifth day, samples of all wounded hazelnut kernels (varieties),

were divided into three susceptibility categories (moderate, susceptible, highly susceptible), 'Pashmineh 89' (SD%=100) and Gerde-e-Eshkevarat (SD%=48.3) were highly and moderately susceptible varieties, respectively. Kernels of the remaining hazelnut varieties were assigned in the susceptible category (Sorii, Gerde-shok, Navan, Fertil, Rasmii, Gerjeh, Ronde, Shastak, Negret and Gerduii).

The SR of the *A. flavus* ranged from 14.6×10^5 to 16.7×10^6 and 12.6×10^7 to 47.6×10^7 for whole and wounded kernels of hazelnut varieties, respectively.

In the present study, regression analysis was carried out to decide correlations among SD% or SR of *A. flavus* and kernel oil content (% W/W) or raw protein (% W/W) of hazelnut varieties. The formulas are as follow: Where, X1 is the oil content, X2 is the raw protein of hazelnut kernel, Y1 is the SD% and Y2

is the SR of *A. flavus* on whole (A) and wounded (B) kernel of hazelnut variety.

A:

$$Y1 = 0.084 X^2 - 7.9866 X + 208.35, R2=0.65$$

$$Y2=, Y2=0.0027X2-0.2795X+13.327, R2=0.41$$

B:

$$Y1 = 1.5675 X1 - 25.175, R2=0.63, \\ Y2=0.0261x+6.7907, R2=0.74$$

$$Y2=3.2868X+22.101, R2=0.22$$

We also assessed the susceptibility of hazelnut varieties in relation to *A. flavus* under laboratory conditions and classified them based on SR value. The hazelnut varieties were classified into two and six groups of susceptibility on the basis of the SR of *A. flavus* on whole and wounded kernels, respectively. In fact, hazelnut varieties were classified into one of the two resistance classes (i.e., low susceptible or highly susceptible). However, ‘Pashmineh 89’ was consistently grouped in the highly susceptible group and ‘Gerde-Eshkevarat’ and ‘Rasmi’ in the less susceptible group (Table 2).

Discussion

Commodities, such as hazelnuts, may be contaminated in the orchard or after harvest during storage, processing or transport, resulting in not only a public health hazard, but also a financial loss (Molyneux *et al.*, 2007; Nakai *et al.*, 2008). Post-harvest infection of hazelnut by the *A. flavus* and subsequent aflatoxin contamination is one of the food safety factors that most severely impairs hazelnut production and affects human health, especially in humid areas such as Guilan province.

In this study, susceptibility to kernel mold for some hazelnut varieties with natural post-harvest susceptibility and/or resistance to the *A. flavus* colonization (SD %) and sporulation (SR) have been identified through laboratory kernel screening assay (KSA). The nut kernel is the primary target of toxigenic fungi such as *A. flavus*. Products, such as hazelnuts, may be contaminated in the orchard or after harvest during storage, processing or transport,

resulting in not only a public health hazard, but also a financial loss (Nakai *et al.*, 2008). Therefore, kernel-based susceptibility represents the core objective of hazelnut host susceptibility. However, little is known about the *A. flavus* resistance and/or susceptibility mechanisms in hazelnut varieties. Hazelnut is characterized by the high percentage of carbohydrates and oil content that make it susceptible to infection by the opportunistic pathogen *A. flavus* (Kosalec and Pepeljnjak, 2005). We supposed that the differences in the chemical compositions of different varieties of hazelnuts can be depended on the susceptibility and/or resistance to the *A. flavus* growth and sporulation. In this study, we determined oil contents of hazelnuts and the relationship between oil contents and SD% and/or SR of *A. flavus* in the hazelnut susceptible varieties with *A. flavus* infection. The results showed that wounded and high oil content of hazelnut kernel in each variety could affect SD% and SR of the *A. flavus*. These results were in agreement with that of Doyle *et al.*, (2001). Kabirian *et al.* (2011) reported a relationship between oil content and/ or protein and a susceptibility to *A. flavus* in pistachio, peanut and corn crops. Wounding of the kernel (cut kernel) increased the possibility of a rapid invasion of the *A. flavus* on it. Of course, factors such as the date of harvest, storage humidity and temperature and environment influenced the extent of the *A. flavus* molding. Based on Table 4, SD% showed the greatest variation among hazelnut varieties. Our results also showed that SD% of the *A. flavus* on the kernel of hazelnut is a valid criterion for pre-screening of the *A. flavus* susceptibility and infection. The SD% varied with variety and physical health of hazelnut kernel. These results indicated that SD% of the *A. flavus* may offer a real possibility of simple, rapid, and reliable methods for the early screening of *A. flavus* susceptibility in hazelnut. In addition, this work elucidated if the kernel pericarp is wounded the barrier against the invasion of the *A. flavus* is disrupted leading to kernel infection. Resistance factors have thus been identified in the pericarp of the

hazelnut kernel (ICMFS, 2001). A few studies have shown that fatty acid composition had direct or indirect effects on the SD%, SR and aflatoxin production of the *A. flavus* (Passi *et al.*, 1984; Doehlert *et al.*, 1993; Fabbri *et al.*, 1993; Burrow *et al.*, 1997). Our findings have not indicated a correlation between protein content (%) and susceptibility to the *A. flavus*. Moghaddam *et al.* (2006) found that when pistachio varieties were inoculated with a spore suspension of the *A. flavus*, the susceptibility of them differed in kernel colonization and aflatoxin production. It was assumed that hazelnut testa of variety Gerde-Eshkevarat was capable of serving as a barrier against the *A. flavus* infection. This was in agreement with Mohammadi Moghadam and Hokmabadi (2010) findings where they studied the effect of the pistachio testa on the reduction of the *A. flavus* growth and aflatoxin B1 production. In addition, the chemical composition of the kernel of hazelnut varieties would affect the *A. flavus* growth rate. Bayman *et al.* (2002) and Campbell *et al.* (2003) have stated the hard shell of nuts was a good barrier against bacterial and fungal contaminations. This study provides new insights into the understanding of the susceptibility to pre- or post-harvest the *A. flavus* molding in hazelnut and can contribute developing hazelnut varieties with resistance to aflatoxin contamination. The results revealed that native (local) variety Gerde-Eshkevarat may be moderately susceptible to the *A. flavus* infection in comparison with exotic varieties such as Fertil and Negret. Certainly, additional studies required to investigate, the relationship between the oil content and protein in the more hazelnuts varieties and mineral elements such as zinc, manganese, iron, copper etc. Sources of the resistance (kernel infection, and aflatoxin production by the *A. flavus*) have not been reported in cultivated hazelnut. In addition, resistance to kernel infection remains a key issue in the breeding of hazelnut for resistance to aflatoxin contamination. The strength of this research was in testing the relative importance of oil content of

hazelnut kernel and physical injury affecting the *A. flavus*-hazelnut interaction. In this study, some hazelnut varieties with natural pre- and/or post-harvest resistance to *A. flavus* growth have been identified through laboratory screenings. The KSA of hazelnuts confirmed sources of resistance among 12 hazelnut varieties tested in this study, thus demonstrating that the KSA can be used to rank hazelnut kernel for its preliminary susceptibility to the *A. flavus* growth and contamination. Therefore, the SD% and KSA can be *in vitro* valuable complements to pre-screening and standard breeding practices for initial evaluation of hazelnut genotypes. Some hazelnut varieties with natural pre- and/or post-harvest resistance to the *A. flavus* growth have been identified through laboratory screening. In this research, some of the hazelnut varieties were grouped on the basis of *A. flavus* SD%. This study showed that no sporulation predictive equations could be developed for hazelnut susceptibility based on SR of *A. flavus*. However, little is known about the resistance or susceptibility mechanisms, which has slowed the incorporation of resistance and/or susceptibility into hazelnut cultivars with a commercially acceptable genetic background. Thus, storage and orchard experiments are necessary to evaluate the degree of susceptibility and final confirmation of resistance.

Conclusions

The *A. flavus* growth and contamination in northern Iran are strongly influenced by both weather and genetic resistance of the varieties, which suggests that control of the *A. flavus* on hazelnut kernels can be improved by using less susceptible varieties. Gerde-Eshkevarat variety was consistently grouped in the less susceptibility and 'Pashmineh 89' in the highly susceptible group.

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