Screening for *Aspergillus flavus* Resistance in Almond

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Abstract. *Aspergillus flavus* Link. is a filamentous fungus affecting almond [*Prunus dulcis* (Mill.) D.A. Webb] kernels in the field and during storage. This fungus can produce aflatoxins (carcinogenic and immunosuppressive mycotoxins), which prevent the marketability of almond kernels. *Aspergillus flavus* resistance has not been an objective in conventional almond breeding programs. Because the importance of this disease is increasing, evaluations of cultivar susceptibility are being performed. In this study, the screening for *A. flavus* resistance in 40 almond genotypes has been carried out in controlled inoculation conditions at 26 °C. Eighteen days after the inoculation, kernels of all the almond cultivars assayed showed susceptibility to *A. flavus*. Nevertheless, differences among cultivars in the percentage of kernel surface colonized by the fungus were observed. The Spanish cultivar Ramillete was the least susceptible. Susceptibility was not related to the geographic origin of the cultivar.

Aspergillus flavus Link. is a filamentous fungus affecting almond kernels. Both in the orchards and during storage it produces aflatoxins (carcinogenic and immunosuppressive mycotoxins) (Phillips et al., 1976, 1979; Schatzki and Ong, 2000, 2001), that are dangerous to animals and humans (Dienert et al., 1987).

Preliminary *A. flavus* resistance has not been an objective in conventional almond breeding programs. However, due to the importance of this disease for the commercialization of almond kernels, studies to assess almond susceptibility to this fungus have been initiated. The aflatoxin limit allowed for almond exportation is typically 4 ppb (Hua et al., 1998; Stoloff, 1976). The disease is more important in California cultivars with their paper shells than in European cultivars with hard well-sealed shells (Gradziel and Wang, 1994; Schade et al., 1975).

Sources of resistance to *A. flavus* have been described in peanuts (*Arachis hypogaea* L.) and groundnut (Mehan, 1989; Mixon and Rogers, 1973), cotton (*Gossypium hirsutum* L.) (Amalraj and Meshram, 1981), and maize (Wallin, 1986). In the almond breeding program at the University of California–Davis, the studies of *Aspergillus* resistance in California almond cultivars revealed that, while significant differences were detected, most tested cultivars were susceptible to the fungus (Gradziel and Wang, 1994; Gradziel et al., 2000).

Resistance to *A. flavus* has been reported to be associated with fatty acid composition and metabolism in groundnuts and maize (*Zea mays* L.) (Reding and Harrison, 1994; Zeringue et al., 1996). Nevertheless, in almond no correlations were detected between *A. flavus* aflatoxin production or mold growth and kernel oil composition (Gradziel et al., 2000).

In this study, 40 almond genotypes from different geographical origins (Mediterranean area, Central Europe, California, and Australia) were evaluated for resistance to *A. flavus* in controlled inoculation conditions in petri dishes at 26 °C.

Materials and Methods

Almond cultivars. Forty almond cultivars were assayed (Table 2). They include diverse cultivars from Spain (24), France (5), Italy (2), Portugal (1), Tunisia (1), Ukraine (1), United States (5), and Australia (1), and 'S3056', a bitter genotype obtained in CEBAS-CSIC (Murcia, Spain).

***Aspergillus flavus* isolate.** The *A. flavus* isolate 'NRRL 6412' from the "Colección Española de Cultivos Tipo" (Spanish Collection of Type Cultures) of the Universidad Politécnica de Valencia (Valencia, Spain) (Dawson et al., 1990) was used for the inoculation.

Resistance evaluation. Resistance of almond cultivars to *A. flavus* was evaluated following controlled inoculation conditions in petri dishes. For each cultivar both whole

kernels with the seedcoat and kernels cut longitudinally between the two cotyledons were assayed. For cut kernels, cotyledons were placed with the cut side facing upward. Both whole and cut kernels were inoculated by spraying 0.6 mL of an *A. flavus* spore solution (2×10^6 spores/mL) to each petri dish.

For each cultivar, three petri dishes with five kernels in each were studied. To maintain high humidity, a piece of wet (0.5 mL water) filter paper was placed on the bottom of each dish. After inoculation, petri dishes were incubated at 26 °C, in darkness. The percentages of kernel surface (25%, 50%, 75%, and 100%) colonized by the fungus and in sporulation were recorded 3 and 18 d after inoculation. Differences among cultivars for both traits, in whole and cut kernels, 3 and 18 d after inoculation were analyzed by means of the nonparametric Kruskal–Wallis test, using the SPSSX statistical package (SPSSX, 1986).

Results and Discussion

The Kruskal–Wallis test showed significant differences between cultivars for the surface colonized by the fungus and the surface in sporulation, in whole and cut kernels, 3 and 18 d after inoculation (Table 1).

Eighteen days after inoculation, kernels of all the assayed cultivars appeared to be susceptible to *A. flavus*, although some differences of susceptibility can be observed (Table 2). At this time a good correlation between the surface colonized by the fungus and the surface in sporulation was observed. This correlation was smaller at 3 d after inoculation. In general, the proportion of surface sporulating was smaller than the proportion of colonized surface.

A strong effect of the seedcoat on the level of infection by *Aspergillus* was observed. For intact kernels with seedcoats, the proportion of surface colonized by the fungus 18 d after inoculation averaged 8.52%, compared to 82.10% for cut kernels.

Three days after inoculation of whole kernels, the proportion of kernel surface colonized did not exceed 15%, being 0% in many cultivars. However, 18 d after the inoculation, the proportion of kernel surface colonized by the fungus increased, and cultivars Achaak, Fournat, and Glorieta showed a higher susceptibility with surface colonization exceeding 25%.

For cut kernels, the proportion of colonized surface three days after inoculation often exceeded 25% in 'Cebas-1', 'Del Cid', 'Desmayo-2', 'Jordi', 'Marcona-2', 'Masbovera', 'Pajarera', 'Peraleja', 'Ramillete', 'Tuono', and 'Wawona' and it exceeded 50% in the very susceptible cultivars Ferragnès, Ardechoise and Genco. Most of the remaining

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Table 1. Kruskal–Wallis test for the percentage of surface colonized by *A. flavus* and in sporulation of whole and cut kernel for 40 almond cultivars, 3 and 18 d after inoculation at 26 °C.

	Days	Colonization			Sporulation		
		H	df	P	H	df	P
Whole kernel	3	90.900	39	0.000	82.810	39	0.000
	18	104.498	37	0.000	97.005	37	0.000
Cut kernel	3	147.725	39	0.000	60.197	39	0.016
	18	177.367	38	0.000	125.397	38	0.000

Table 2. Percentage of surface of whole and cut kernels showing colonization and sporulation by *A. flavus*, for 40 almond cultivars, held at 26 °C, for 3 and 18 d after inoculation.

Cultivar	Origin	Whole kernel				Cut kernel			
		3 d		18 d		3 d		18 d	
		Colonization	Sporulation	Colonization	Sporulation	Colonization	Sporulation	Colonization	Sporulation
Achaak	Tunisia	0.00	0.00	25.00	25.00	35.00	0.00	88.46	84.61
Ardechoise	France	0.00	0.00	3.57	3.57	68.33	0.00	100.0	100.00
Atocha	Spain	0.00	0.00	2.50	2.50	40.00	0.00	100.0	81.67
Bonita	Portugal	1.66	0.00	3.57	1.78	41.67	0.00	95.00	88.33
Carretas	Spain	3.84	0.00	14.28	14.28	25.00	0.00	90.00	65.00
Cebas 1	Spain	0.00	0.00	16.66	6.66	21.67	0.00	88.64	65.91
Chellastone	Australia	3.33	0.00	9.09	9.09	26.67	0.00	93.33	81.67
Colorada	Spain	0.00	0.00	0.00	0.00	25.00	0.00	62.50	57.50
Del Cid	Spain	0.00	0.00	5.00	3.33	15.00	0.00	90.00	78.33
Desmayo L-1	Spain	0.00	0.00	3.57	1.78	28.33	0.00	80.36	67.86
Desmayo L-2	Spain	1.66	0.00	3.84	3.84	23.33	0.00	80.36	78.57
Ferraduel	France	0.00	0.00	0.00	0.00	48.33	0.00	83.33	61.67
Ferragnès	France	8.33	5.00	18.18	13.63	66.67	0.00	93.33	91.67
Fournat	France	13.33	5.00	75.00	50.00	48.33	3.33	96.57	93.75
Garrigues	Spain	0.00	0.00	0.00	0.00	41.67	0.00	93.33	70.00
Genco	Italy	0.00	0.00	---	---	66.67	0.00	100.00	86.67
Glorieta	Spain	0.00	0.00	25.00	12.50	40.00	0.00	84.37	78.12
Guara	Spain	0.00	0.00	7.50	5.00	38.33	0.00	91.67	91.67
I.X.L.	USA	1.66	0.00	4.54	2.27	33.33	0.00	88.33	85.00
Jordi	Spain	0.00	0.00	0.00	0.00	18.33	0.00	60.00	57.50
Lauranne	France	6.66	0.00	3.57	3.57	30.00	0.00	58.33	58.33
La Mona	Spain	3.33	0.00	5.76	3.84	31.67	0.00	86.54	86.54
Malagueña	Spain	3.33	0.00	13.46	13.46	28.33	3.33	91.67	91.67
Marcona-1	Spain	0.00	0.00	1.66	1.66	25.00	0.00	91.67	81.67
Marcona-2	Spain	0.00	0.00	0.00	0.00	16.67	0.00	72.50	72.50
Marcona-3	Spain	0.00	0.00	3.33	1.66	25.00	0.00	100.0	81.67
Masbovera	Spain	6.66	0.00	10.00	5.00	20.00	0.00	70.00	67.50
Mono	USA	1.66	0.00	5.00	5.00	40.00	0.00	77.50	77.50
Pajarera	Spain	3.33	0.00	12.50	7.50	23.33	0.00	94.64	89.29
Peralesja	Spain	1.66	1.66	14.58	14.58	21.67	0.00	65.00	65.00
Planeta-1	Spain	0.00	0.00	11.11	8.33	31.67	0.00	83.93	76.78
Planeta-2	Spain	0.00	0.00	4.16	4.10	30.00	0.00	95.00	95.00
Primorskii	Ukraine	5.00	0.00	---	---	45.00	3.33	---	---
Ramillete	Spain	0.00	0.00	0.00	0.00	21.67	0.00	48.21	42.86
Rumbeta	Spain	0.00	0.00	3.12	3.12	33.33	0.00	85.00	80.00
S3056	Spain	0.00	0.00	0.00	0.00	26.67	0.00	56.67	56.67
Tioga	USA	0.00	0.00	0.00	0.00	36.67	6.67	52.08	50.00
Titan	USA	0.00	0.00	12.50	12.50	41.67	0.00	91.67	75.00
Tuono	Italy	0.00	0.00	4.16	0.00	20.00	0.00	62.50	59.37
Wawona	USA	8.33	0.00	9.09	9.0	21.67	0.00	59.61	59.61

cultivars ranged between these values (25% and 50%). Eighteen days after the inoculation of cut kernels, the proportion of kernel surface colonized by the fungus increased greatly. Only the Spanish cultivar Ramillete did not reach 50% surface colonization. For cultivars, such as ‘Colorada’, ‘Jordi’, ‘Lauranne’, ‘Marcona-2’, ‘Masbovera’, ‘Peralesja’, ‘S3056’, ‘Tioga’, ‘Tuono’, and ‘Wawona’, the percentage of surface colonized ranged between 50% and 75%. No important difference in level of resistance was observed between the bitter selection ‘S3056’ and sweet cultivars. The remaining cultivars exceeded 75% of colonized surface at 18 d after inoculation, with some cultivars (‘Primorskii’, ‘Marcona-3’, ‘Genco’, ‘Atocha’, and ‘Ardechoise’) completely covered by the fungus.

Our results agree with those of Gradziel and Wang (1994) who studied the susceptibility to *A. flavus* in a selection of 21 California cultivars. Among these was ‘Mono’, which performed similarly in our test. They observed a high susceptibility for most of cultivars, and only ‘Ruby’ and ‘Ne Plus Ultra’ were less susceptible. Nevertheless, final colonization in whole kernels was more extensive in our assay,

possibly due to environmental conditions of the trial or to the use of different isolates.

The low level of susceptibility found in the cultivars Colorado, Jordi, Marcona-2, S3056, Tioga, and Ramillete could still be useful due to the low level of *Aspergillus* infection commonly found in the field and the limited period of time in which the kernel can be infected (Schade et al., 1975; Schatzki and Ong, 2000, 2001). In addition, these cultivars show a well-sealed shell that drastically reduces the possibilities of infection by the fungus in field conditions. Furthermore, the usual dry storage conditions are not adequate for the fungus multiplication.

The lack of correlation between *A. flavus* susceptibility and almond kernel oil composition described by Gradziel et al. (2000) may explain the failure to find resistance sources in almond. In other crops, like groundnuts and maize, an association between fatty acid composition, metabolism, and *A. flavus* resistance has been described (Reding and Harrison, 1994; Zeringue et al., 1996), and sources of resistance reported (Mehan, 1989; Mixon and Rogers, 1973; Reding and Harrison, 1994; Wallin, 1986; Zeringue et al., 1996).

Other sources or types of resistance to *A. flavus* might be identified in related *Prunus* species. For example, Gradziel et al. (2001) has recently described several almond lines resulting from the introgression of peach [*Prunus persica* (L.) Batsch] genes in which aflatoxin production following controlled inoculations was reduced as much as 90% relative to susceptible almond cultivars.

In conclusion, in spite of the large variability observed among cultivars, all showed susceptibility to *A. flavus*. The geographical origin of the cultivars was not related to their level of susceptibility. The Spanish cultivar ‘Ramillete’ was the least susceptible, with an additional 10 cultivars, ‘Colorado’, ‘Jordi’, ‘Marcona-2’, ‘Masbovera’, ‘Peralesja’, and ‘S3056’ (Spain); ‘Lauranne’ (France); ‘Tioga’ and ‘Wawona’ (United States); and ‘Tuono’ (Italy), showing lesser levels of susceptibility than the rest.

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