Occurrence and Etiology of Brown Apical Necrosis on Persian (English) Walnut Fruit

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ABSTRACT

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In 1998, a severe fruit drop was observed in Italy, principally on cv. Lara Persian (English) walnut (*Juglans regia*). Dropped fruit showed a brown patch at the blossom end and blackening and rot of inner tissues. The disease, called brown apical necrosis (BAN), was investigated on fruit collected in Italy and France in 1999. In 2000, studies were carried out in three walnut orchards located in Italy and in France to substantiate the etiology of BAN. Isolations performed from inner diseased fruit tissues yielded several fungi, in decreasing frequency of isolation: species of *Fusarium* and *Alternaria*, and one species each of *Cladosporium*, *Colletotrichum*, and *Phomopsis*. However, only *Fusarium* spp. were recovered from stigmas of BAN-affected fruit. The fungi associated with BAN-diseased fruit and species composition differed among locations and over time, confirming results obtained in previous investigations. The species of *Fusarium* used in pathogenicity tests reproduced BAN-disease symptoms when inoculated on fruit, whereas an *Alternaria alternata* isolate caused only limited necrosis of the style. However, the role of the other fungi commonly isolated from BAN-diseased fruit remains to be defined. The walnut blight pathogen, *Xanthomonas arboricola* pv. *juglandis*, occasionally was isolated from BAN-diseased fruit. No correlation was found between the extent of external brown patches and the size of inner lesions. Repeated isolations from and inoculations of fruit demonstrated that BAN can be considered a complex disease, and the inner infections originate from the style of the fruit.

Additional keywords: complex diseases, nut disease

In 1998, in Northern Italy, a severe fruit drop that reduced yield up to 20% was observed on Persian (English) walnut (*Juglans regia* L.) cv. Lara. Cvs. Pedro, Chandler, and Sunland also were affected in decreasing order of severity. The disease, termed brown apical necrosis (BAN), was observed in 1999 in France and again in Italy (4). Symptoms on dropped fruit were a brown to dark-brown patch originating exclusively at the blossom end and a brown to blackish rot of inner tissues. These symptoms differed from those of walnut blight (*Xanthomonas arboricola* pv. *juglandis;* 24), characterized by blackish greasy spots, with or without a yellow halo, which are not restricted to the stigmatic end of the fruit. The disease also differed from anthracnose (*Gnomonia leptostyla* (Fr.:Fr.) Ces. & De Not.), characterized by brown to gray dry spots, often with numerous acervuli. Isolations performed in Italy in 1998 and in both Italy and France in 1999 from BAN-affected fruit yielded species of *Alternaria*, *Fusarium,* and a *Colletotrichum* sp. The fungi associated with this disease and their species composition differed among locations. These preliminary studies suggested that several *Fusarium* spp. were the probable cause of the disease (2,4).

Several *Fusarium* spp. have been reported on *Juglans* spp. The most frequent citations concerned Fusarium cankers on stem and shoots caused by *F. lateritium* Nees:Fr., *F. oxysporum* Schlechtend.:Fr. (13), *F. solani* (Mart.) Sacc. (3,6,21), and *F. sporotrichioides* Sherb. (8). The first association of a brown lesion at the stigmatic end of English walnut fruit with a *Fusarium* sp., namely *F. lateritium*, was reported in 1931 (26). In this report, Wollenweber (26) mentioned that this disease was observed on walnut fruit in France and in Italy. In 1935, *F. torulosum* (Berk. & Curt.) Niremberg (syn. *F. sambucinum* Fuckel) and *F. avenaceum* (Fr.:Fr.) Sacc., together with *F. lateritium,* were reported as causes of an uncommon fruit rot of walnut (15,27). These diseases may be similar to BAN, but none have since been reported.

The objectives of the present research were to substantiate the results obtained with the previous investigations describing the symptoms, identifying fungi associated with BAN lesions, and determining pathogenicity of isolates to walnut fruit. The results of this research can help to further elucidate the disease etiology and epidemiology and provide a basis for control programs of the disease.

MATERIALS AND METHODS

Orchards. Three commercial hedgerow-trained orchards of English walnut cv. Lara were monitored for symptoms of BAN from May to August 2000. One orchard each was located in Rovigo and Treviso provinces in northern Italy, and a third was in St. Livrade sur Lot (Lot-Garonne) in France. The orchards were 8 years old and grown under similar cultural management. The Rovigo orchard was on clay-sand, highly fertile soil; the Treviso orchard was on clay, highly fertile soil; and the French orchard was on a lime, medium-fertile soil. No fungicide sprays were applied on the experimental plots.

Symptoms. Before leaf flushing, several branches were labeled on each of the 2 central trees in each of three 25-tree plots, five rows wide and 5 trees long. Twenty-five fruit were randomly removed from the labeled branches on 22 and 29 May and 12 and 27 June, for a total of 150 fruit per location and per date of sampling. On the basis of external symptoms, fruit were classified as healthy, affected by BAN, or by walnut blight. All fruit were cut in half and the extent of blackening and rot of inner tissues also was recorded.

Isolation and identification from removed fruit. Among the fruit collected from each orchard and for each date of sampling, 25 fruit with BAN symptoms and 5 healthy fruit were randomly chosen for isolation. Fruit were surface disinfected in 1% NaOCl for 1 min, rinsed in sterile distilled water, and dried on sterile blotting paper. Two isolation methods were used for fruit collected in May when stigmas were present. Stigmas, style, and pieces of pericarp tissue cut from lesion margins were cultured on potato-dextrose agar (PDA) in 9-mm-diameter petri dishes. For the second method, fruit were aseptically cut in half, placed onto a wet sterile blotting paper sheet in an aluminum tray (262 by 322 by 40 mm), and sealed in a clear plastic bag (moist chamber). Fruit collected in June were halved and incubated

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in a moist chamber only. Plates and trays were incubated in the dark at 25°C until mycelial colonies developed. Initial colonies that developed on fruit halves were observed under the microscope; after their place of origin was recorded, they were transferred onto PDA with a sterile needle. Fungal isolates were identified based on cultural and microscopic morphology. For identifying *Fusarium* spp., single conidia were subcultured on carnation-leaf agar (CLA) (17). Single conidium cultures of representative isolates of each species were stored on PDA slant tubes at 4°C in the dark.

Isolation and identification of fungi from dropped fruit. Nets were set under the two central walnut trees to collect fallen fruit and prevent them from contacting the soil. A total of 300 fruit per orchard were collected randomly from nets on 12 and 26 June. External and internal symptoms were classified as described above. Isolations were performed on 25 fruit with external BAN symptoms. Colonies present on the outer surface of fruit were transferred onto PDA. When no mycelia were evident, halved fruit were incubated in moist chambers as described above. Subcultures of fungal colonies were made onto PDA amended with or without streptomycin sulfate (250 mg/liter) to control bacterial contaminants.

Pathogenicity tests on young fruit with stigmas. Pathogenicity tests with representative isolates of fungal species isolated from BAN-affected walnut fruit in 1999 were conducted on detached twigs of Lara English walnut, each bearing two young healthy fruit (about 4 to 5 mm in diameter) with turgid stigmas (May). Detached twigs were kept in 50-ml Falcon tubes filled with tap water. Conidial suspensions containing *Alternaria alternata* (Fr.:Fr.) Keissl., *F. equiseti* (Corda) Sacc., *F. graminearum* Schwabe, or *F. semitectum* Berk. & Ravenel at 10^5 conidia/ml were sprayed onto each of 30 fruit of the 15 detached twigs and placed inside a transparent plastic bag to avoid contamination among inocula. Control fruit were sprayed with sterile distilled water. The plastic bags were inflated and sealed to prevent contact between the bag and the inoculated fruit. Fruit were incubated in the greenhouse at 20 to 25°C, bags were removed after 48 h, and symptoms were recorded 5 days after inoculation. Reisolations were performed by placing stigmas and pieces of the style, pericarp, and ovary of each fruit onto PDA and incubating at 25°C in the dark until mycelial colonies developed. The experiment was repeated once.

Pathogenicity tests on fruit before complete shell hardening. Symptomless, detached Lara English walnut fruit of about 35 mm in diameter, just before shell hardening, were inoculated by placing a mycelial mat approximately 2 to 3 mm long on the stigmatic end. Inocula were obtained from aerial mycelia of 10-day-old colonies of *A. alternata*, *F. graminearum*, or *F. semitectum* grown on PDA at 25°C in the dark. Fruit were surface disinfected before inoculation. In all, 24 fruit per fungal species were inoculated with each fungal species, and 24 fruit were treated as previously described without using mycelial mats. Six fruit per rack were enclosed upright in an inflated transparent plastic bag for 48 h and incubated in the greenhouse at 25 to 28°C. Reisolations were performed on PDA 15 days after inoculation by excising pieces of internal tissues from each surface disinfected fruit, following the procedure previously described. The experiment was repeated once.

RESULTS

Symptoms. In 2000, symptoms were first visible in May, and the incidence of BAN increased throughout the season, with full expression in the second half of June to the second half of July. Of a total of 1,800 fruit examined during the growing season, BAN was observed on 19%. External symptoms of BAN appeared at the stigmatic end as a brown to blackish 0.2- to 4-cm patch, sometimes extending over two-thirds of the whole fruit. All fruit affected by BAN showed blackening and rot of internal tissues (Table 1). The extent of the internal lesion increased during the growing season (Table 1). At the end of June, 46.3% of BAN-diseased fruit showed a blackened and rotted ovary and, in 11.5%, the kernel also was blackened. The necrosis of the style always was present on symptomatic fruit, but the extent of external and internal lesions were not correlated.

Isolation from removed fruit. The genera of fungi most commonly isolated from diseased inner tissues, listed in decreasing order of occurrence, were: *Fusarium* spp., *Alternaria* spp., and a species each of *Cladosporium*, *Colletotrichum*, and *Phomopsis* (Table 2). Among fungi isolated with less frequency from removed diseased fruit, *Phomopsis* and *Colletotrichum* spp. were found in the Rovigo orchard and France, respectively. *Phomopsis* sp. was isolated only from diseased ovary and kernel tissues.

Alternaria spp. and a *Cladosporium* sp. were obtained not only from diseased fruit but also from the style, ovary, and kernel of healthy fruit, as was an *Epiccocum* sp., *F. equiseti*, *F. oxysporum*, and *F. proliferatum* (Matsushima) Nirenberg. Other fungi that were incidentally obtained from internal lesions were *Aspergillus* spp., *Epicoccum* sp., *Penicillium* spp., *Periconia* sp., *Rhizopus stolonifer*, *Stemphylium* sp., and *Ulocladium* sp. A few colonies of *X. arboricola* pv. *juglandis* were isolated from the style as well as from the ovary and kernel of both healthy and BAN affected fruit.

Many *Fusarium* spp. were isolated from internal lesions, and the species varied considerably among the three locations (Table 3). *F. avenaceum* was recovered from each of the three orchards, whereas *F. graminearum*, *F. culmorum* (Wm. G. Sm.) Sacc., and *F. sporotrichioides* were isolated only in the French orchard, *F. semitectum* was isolated in Italy (Rovigo) and France, and *F. semitectum* var. *majus* Wollenw. was recovered only in Italy. In particular, *F. semitectum* was frequently isolated early in

Table 1. Symptoms on Persian (English) walnut cv. Lara fruit affected by brown apical necrosis, on each sampling date in 2000

	Diseased fruit (%)[a]			
Symptoms	**22 May**	**29 May**	**12 June**	**27 June**
Necrotic style only	32.2	33.7	51.8	42.1
Necrotic style and pericarp only	58.0	66.2	0.0	0.0
Blackened and rotted style, pericarp, and ovary	9.6	0.0	34.2	46.3
Blackened and rotted style, pericarp, ovary, and kernel	0.0	0.0	13.8	11.5

[a] Percentages are calculated on a total of 62 symptomatic fruit on 22 May, 77 symptomatic fruit on 29 May, 108 symptomatic fruit on 12 June, and 95 symptomatic fruit on 27 June. Fruit were removed from walnut tress in Rovigo and Treviso (Italy), and St. Livrade sur Lot (France) orchards.

Table 2. Incidence of fungi isolated from within fruit affected by brown apical necrosis removed from walnut trees in the three locations in 2000[a]

	Recovery (%)[b]		
Genera	**Rovigo**	**Treviso**	**France**
Alternaria spp.	10.0	14.6	15.7
Cladosporium sp.	1.0	3.6	7.1
Colletotrichum sp.	0.0	0.0	1.4
Fusarium spp.	8.6	14.3	20.7
Phomopsis sp.	2.8	0.0	0.0

[a] Trees were located in Rovigo and Treviso, Italy, and St. Livrade sur Lot, France.
[b] Percentages refer to the number of colonies per each fungal genus out of the total number of colonies isolated from 100 symptomatic fruit analyzed per location.

May, while *F. semitectum* var. *majus* was commonly isolated in June. Only *Fusarium* spp. were isolated from stigmas of BAN-diseased fruit (*data not shown*).

Isolation from dropped fruit. Of the dropped fruit, 25% were affected by BAN. Several genera of fungi colonized the outer and inner tissues of dropped fruit. Most genera isolated from removed fruit were also isolated from dropped fruit. Other fungi occasionally isolated were species of *Gliocladium* and *Trichoderma*. The most commonly isolated genus was *Fusarium*, and *Fusarium* spp. varied according to location. *F. verticillioides* (Sacc.) Niremberg (syn. *F. moniliforme* J. Sheld.) was isolated only from dropped fruit from the Rovigo orchard, whereas *F. semitectum* var. *majus* was only isolated from dropped fruit at the French orchard.

Pathogenicity tests on young fruit with stigmas. *F. semitectum* produced large brown patches on all inoculated fruit, and a few fruit became shriveled. Similar symptoms were caused by *F. equiseti* and *F. graminearum* on 87% of the inoculated fruit. Symptoms were similar to those observed in nature on more mature fruit, though the damage was more severe on the inoculated fruit. All fruit inoculated with *A. alternata* were symptomless. All *Fusarium* spp. used as inoculum were readily reisolated from internal tissues of the style, pericarp, and ovary of inoculated fruit, and very few colonies of *A. alternata* were obtained from asymptomatic inner tissues. Fruit used as control remained symptomless, and only species of *Alternaria*, *Penicillium*, and *Epicoccum* were isolated from inner asymptomatic tissues.

Pathogenicity tests on fruit before complete shell hardening. In these fruit, no external symptoms were evident even after an incubation of 15 days. After cutting nuts in half, blackening of style, ovary, and kernel was evident. More extensive internal lesions were observed when *F. semitectum* and *F. graminearum* were used as inoculum than when *A. alternata* was used. *F. semitectum* and *F. graminearum* caused internal symptoms on the 83.3 and 66.6% of inoculated fruit, respectively. *A. alternata* caused only limited necroses of the style on the 95.8% of inoculated fruit. The fungi were reisolated from diseased tissues. Fruit used as controls remained symptomless, and only *Penicillium* spp. were isolated from inner tissues.

DISCUSSION

In the 2000 investigations, *Fusarium* was confirmed as the most common genus associated with BAN, and *F. avenaceum*, *F. semitectum*, and *F. semitectum* var. *majus* were the most common species of *Fusarium*. Although *Alternaria* spp. were frequently isolated from diseased fruit, they often were present on healthy fruit as well, and results obtained with artificial inoculations performed with an isolate of *A. alternata* did not result in typical symptoms of BAN. Nevertheless, the role of *Alternaria* spp. in BAN disease might be considered and verified with further pathogenicity tests. The complex nature of BAN can be compared to other Fusarium diseases, namely pumpkin fruit rot (9), asparagus decline (10), and wheat foot rot, crown rot, or head blight (7,20), in which several species of *Fusarium* are associated with each of these diseases, and the species composition differs in relation to the area and over time. The causes of changes in fungal frequency and importance are uncertain. In BAN, *F. avenaceum* was dominant in France, whereas *F. semitectum* var. *majus* and *F. semitectum* were dominant in Treviso and Rovigo, respectively. In addition, other fungi of uncertain pathogenicity commonly isolated from diseased fruit may emerge as major factors in the future. *Alternaria* spp., a *Cladosporium* sp., and a *Colletotrichum* sp. might be associated with BAN as in Fusarium fruit diseases of mango, papaya, pineapple (5,19,23), or cranberry fruit rot, in which many different fungal species are involved (18). Also, a *Phomopsis* sp. cannot be excluded as an agent of BAN, even though it was isolated only in the Rovigo orchard, because a *Phomopsis* sp. was reported to be the causative agent of fruit rot on ripening peaches (11,12), and *P. castanea* has been reported as an important storage disease of chestnut (25). Although Koch's postulates were conducted on relatively few fungi, the pathogenicity of three *Fusarium* spp. (*F. equiseti*, *F. graminearum,* and *F. semitectum*) was confirmed.

Most *Fusarium* spp. are soilborne, but some species, including *F. graminearum*, *F. lateritium,* and *F. semitectum*, produce conidia that can be dispersed by wind. Air turbulence produced by a tractor-mounted, high-pressure, high-volume air blast sprayer or by machinery used for walnut harvesting or by other farm equipment could facilitate conidial dispersal. In the Rovigo orchard, *F. semitectum* has been isolated from turf grass, from small necroses on twigs and branches, and from swollen buds of walnut trees (4), suggesting that *F. semitectum* has efficient mechanisms for air dispersal. *F. semitectum* also was reported as the agent of stem and branch cankers of pistachio (16) and necrosis and rot of banana fruit (14).

X. arboricola pv. *juglandis* was isolated from internal tissues of 0.2% of healthy fruit, but also from 10% of BAN-affected fruit. It seems likely that BAN and walnut blight could be present at the same time on the same fruit, and symptoms of both diseases may be observed at the stigmatic end. There have been reports on the association of *Fusarium* spp. with *X. arboricola* pv. *juglandis* (1,22) with no specific mention of walnut fruit.

The present study highlights the fact that BAN has to be considered a complex disease in which several fungi are involved, principally *Fusarium* spp., and that both BAN and walnut blight might coexist in the same diseased area. The brown apical necrosis begins at the stigmatic end, and the style is the key point through which BAN disease expands to the inner tissues of the fruit. Further investigations are needed to test the pathogenicity of the associated fungi and to separate primary pathogens from secondary colonizers.

Control measures to decrease the incidence of fruit drop are under study.

Table 3. Incidence of *Fusarium* spp. isolated from within fruit affected by brown apical necrosis removed from walnut trees in the three locations in 2000[a]

	Recovery (%)[b]		
Species	**Rovigo**	**Treviso**	**France**
F. acuminatum	2.4	8.2	0.0
F. avenaceum	3.3	5.7	14.7
F. chlamidosporum	0.0	1.6	0.0
F. crockwellense	0.0	0.0	0.8
F. culmorum	0.0	0.0	3.3
F. equiseti	1.6	0.0	0.0
F. graminearum	0.0	0.0	4.1
F. lateritium	0.0	0.0	0.8
F. oxysporum	0.8	0.0	1.6
F. proliferatum	1.6	0.0	2.4
F. semitectum	5.0	0.0	5.7
F. semitectum var. *majus*	9.8	18.0	0.0
F. sporotrichioides	0.0	0.0	4.9
F. torulosum	0.8	0.0	0.0
F. tricinctum	0.0	0.8	1.6

[a] Trees were located in Rovigo and Treviso, Italy, and St. Livrade sur Lot, France.

[b] Percentages refer to the number of colonies per each species out of the total number of *Fusarium* spp. colonies isolated from 100 symptomatic fruit analyzed per location.

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