Postharvest Fruit Rots of Apple in Greece: Pathogen Incidence and Relationships Between Fruit Quality Parameters, Cultivar Susceptibility, and Patulin Production

S. Konstantinou, G. S. Karaoglanidis, and G. A. Bardas, Laboratory of Plant Pathology, and I. S. Minas, Laboratory of Pomology, Aristotelian University of Thessaloniki, School of Agriculture, Thessaloniki, Greece; and E. Doukas and A. N. Markoglou, Agricultural University of Athens, Laboratory of Pesticide Science, Athens

Abstract

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The incidence of pathogens associated with postharvest fruit rots on the four most extensively cultivated apple cultivars (Red Delicious, Golden Delicious, Granny Smith, and Fuji) in Greece was surveyed during two consecutive storage periods (2008–09 and 2009–10) in five packinghouses located in northern Greece. The fungi isolated were identified based on their morphological characteristics and internal transcribed spacer gene sequencing. In the four cultivars sampled, *Penicillium expansum* and *Botrytis cinerea* were the predominant pathogens, accounting for averages of 44.2 and 23.6%, respectively, of the pathogens isolated from the sampled fruit. Two other important rot pathogens were *Alternaria tenuissima* and *Mucor piriformis*, accounting for 16.1 and 6.6%, respectively, of the diseased apple fruit. Other pathogens such as *Monilinia laxa*, *M. fructigena*, *Botryosphaeria obtusa*, *Geotrichum candidum*, *Fusarium avenaceum*, and *F. proliferatum* were isolated at low frequencies and are considered of minor importance. Measurements of the resistance level of the four apple cultivars

to fruit rot caused by *P. expansum* and *Botrytis cinerea* revealed that Golden Delicious was the most susceptible to blue mold while Fuji was the most susceptible to gray mold infections. Susceptibility to gray mold was negatively correlated with flavonoid and phenol concentration as well to fruit antioxidant activity, while susceptibility to blue mold was negatively correlated with fruit firmness and phenol concentration. Patulin production was significantly higher in Red Delicious and Golden Delicious fruit than in Granny Smith and Fuji fruit and was negatively correlated with the acidity of the fruit. The high incidence of *P. expansum* and *A. tenuissima* along with the presence of *F. avenaceum* and *F. proliferatum*, all of which are potentially mycotoxin producers, emphasize the risk for mycotoxin contamination of apple fruit juices and by-products. Furthermore, information on the distribution of the pathogens on the main cultivars may be useful for the implementation of strategies to control the diseases and minimize the threat of mycotoxin contamination on each cultivar.

More than 90 fungal species can cause fruit rot diseases on apple fruit (9). Gray mold caused by *Botrytis cinerea* Pers. and blue mold caused by *Penicillium expansum* Link are considered to be the major postharvest diseases on pome fruit. These pathogens, along with several species of *Alternaria*, *Monilinia*, *Mucor*, *Rhizopus*, *Fusarium*, and *Botryosphaeria*, have been estimated to cause 5 to 25% losses of total yield in apple during postharvest storage and commercialization (8).

Postharvest diseases of apple are the result of either latent infections that occur in the field during the growing season or infections from wounds during harvest and handling operations. Consequently, fungal pathogens associated with postharvest rots of apple can be separated into two main groups: “latent infection” and “wound” pathogens (9,16). *Alternaria* spp., *Fusarium avenaceum*, *Glomerella cingulata*, *Pezizula malicortitis*, and *Sphaeropsis pyriputrescens* belong in the former group (9,10,26). *B. cinerea*, *Penicillium expansum*, *Monilinia* spp., and *Mucor piriformis* belong in the latter group (8,17,20).

Losses caused by postharvest rots of apple are not only quantitative due to the developing decay of the fruit but also qualitative due to possible mycotoxin contamination. This is particularly important because large amounts of apple fruit are used for juice and, often, fruit designated to be used for processing and not for direct consumption are of lower quality due to fungal infections (20). Some of the fungi that infect apple produce mycotoxins which can lead

to various human health problems, including teratogenic, cancerogenic, neurotoxic, oestrogenic, and immunosuppressive effects (7). None of the species of the major fruit rot genera on stored apple fruit (e.g., *Botrytis*, *Monilinia*, and *Mucor*) are known to produce a mycotoxin, whereas other species of fungal genera such as *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* are capable of producing a wide range of mycotoxins (13).

Patulin is by far the most important mycotoxin contaminant of apple juice and other apple products and is produced mainly by *P. expansum* (7). Due to its cytotoxic, immunotoxic, and neurotoxic effects on animals, the Joint Food and Agricultural Organization and World Health Organization Expert Committee on Food Additives has established a provisional maximum tolerable daily intake of 0.4 $\mu\text{g kg}^{-1}$ of body weight for patulin that was endorsed by the European Union Scientific Committee for Food in 2000. The maximum permitted levels of patulin in Europe are 10 $\mu\text{g kg}^{-1}$ for fruit baby food, 50 $\mu\text{g kg}^{-1}$ for fruit juice, and 25 $\mu\text{g kg}^{-1}$ for solid apple products (18). Factors affecting patulin accumulation in fruit juices include temperature, storage conditions, fruit qualitative parameters, and fungicide treatments (19,21,25).

The factors that affect yield losses caused by postharvest pathogens have been reviewed by Coates and Johnson (4) and include pathogen species, commodity type, cultivar susceptibility, storage environment, qualitative characteristics of the fruit, and the treatments used for disease control. Despite the fact that the reports on the causal agents of apple fruit rot are numerous, only limited information has been provided for the relative frequency and incidence of causal agents causing postharvest rot and internal fruit spoilage (10,29,32). Furthermore, the importance of each fungal pathogen can vary from country to country. Accurate knowledge of the incidence of each pathogen on the cultivars cultivated in each country along with information regarding their susceptibility to fungal pathogens is a prerequisite for the implementation of appropriate disease management strategies.

Corresponding author: G. S. Karaoglanidis,
E-mail: gkarao@agro.auth.gr

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Despite the fact that apple is one of the most important tree crops in Greece, little is known regarding the incidence of pathogens causing postharvest rots on apple fruit and the susceptibility of the main apple cultivars to postharvest pathogens. Therefore, a study was initiated to (i) identify the causal agents and determine the prevalence and incidence of postharvest fruit rot diseases of apple fruit of the main cultivars in Greece, (ii) determine the levels of susceptibility of these apple cultivars to the main pathogens causing postharvest rots and to correlate the susceptibility with the qualitative characteristics of the fruit, and (iii) investigate patulin accumulation in *P. expansum*-inoculated fruit.

Materials and Methods

Sources and sampling of fruit. The apple fruit in this study were obtained from five packinghouses located in the region of Imathia, in northern Greece, one of the main apple-cultivating regions of the country. Samples were taken at 15-day intervals from November to March (apple storage period in Greece) for 2 consecutive years (2008–09 and 2009–10). Fruit from four apple cultivars were sampled (Golden Delicious, Red Delicious, Fuji, and Granny Smith), representing the main apple cultivars of the country. In each sampling, 15 to 20 fruit per cultivar, showing decay symptoms, were arbitrarily collected from the selection line during the packing operations. In total, 1,075 rotted fruit were collected during the two sampling periods.

Pathogen isolation and identification. The sampled fruit were transported to the laboratory for fungal isolation. Isolations were carried out from surface-disinfected fruit (they were drenched for 1 min in a 1% sodium hypochlorite solution) by removing small fruit pieces at the margin of diseased/healthy tissue and transferring them to petri dishes containing acidified potato dextrose agar (PDA; Oxoid, Unipath Ltd., Basingstoke, UK). Petri dishes were incubated at 22°C in an incubator with a 12-h photoperiod provided by fluorescent lights. After 3 to 5 days of incubation, the emerging fungal colonies were transferred to fresh PDA medium to obtain pure cultures of the isolated fungi.

Fungal identification was based on colony appearance and morphological features of fruiting bodies and spores. Known fruit rot pathogens were identified to species based on fungal descriptions (2), while nonsporulating isolates on culture media or isolates that could not be readily identified based on morphological characteristics were identified at the species level using analysis of the nucleotide sequences of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). In the latter group belonged fungal species of the genera *Alternaria*, *Fusarium*, *Botryosphaeria*, *Phomopsis*, and *Stemphylium*.

All isolates subjected to molecular identification were single-spore isolates. DNA was extracted from mycelium using Qia Puregene Core Kit A (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. ITS1 and ITS2 regions, including the ribosomal 5.8S RNA gene, were amplified using the universal ITS-1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS-4 (5-TCCTCCGCTTATTGATATGC-3) primers that anneal to the flanking 18S and 28S rRNA genes. Polymerase chain reaction (PCR) products of each isolate were purified using the Qiaquick PCR Purification Kit (Qiagen GmbH). The purified products were subjected to sequencing (Lark Technologies Inc., Essex, UK) in both directions using primers ITS-1 and ITS-4. Sequences were aligned using the computer software package Clustal W 2.0.9 (12). The sequences obtained were compared with sequences in the National Center for Biotechnology Information database using BlastN 2.2.18.

Fruit quality and compositional assays. Fruit of the four cultivars that were monitored for pathogen incidence were screened for their susceptibility to gray mold and blue mold and patulin production. Prior to the artificial inoculation with the pathogens and the measurement of patulin production, several fruit qualitative parameters were measured. The fruit were obtained from a commercial storage room (0.0 ± 0.5°C, 90% relative humidity) at the end of October 2009 after a short storage period of 10 to 30 days, depending on the cultivar. Fruit were selected on a basis of size and uni-

formity of shape and absence of any wound. Thirty fruit without defects and with similar weight (170 ± 15 g) from each cultivar, separated arbitrarily into three replicate samples, were used to assess the quality parameters and the compositional status. The sampling of the fruit was repeated 1 week later for an independent replication of the measurements.

Tissue firmness was determined on two opposite sides of each fruit. A 1-mm-thick disk of skin was removed from each side of the fruit and the force in N, required to insert a Chatillon penetrometer fitted with a probe of 12 mm in diameter, was recorded. Soluble solids content (SSC) and titratable acidity (TA) were assessed in juice obtained from three replicate samples of 10 fruit per cultivar. Percent SSC was determined with a refractometer (Atago PR-1; Atago Co. Ltd., Tokyo) and TA by titration with NaOH at 0.1 mol liter⁻¹ to pH 8.2 and expressed as percentage of malic acid.

Total carotenoids were determined according to the method of Kuti (11), with slight modifications. From each replicate sample, 2.5 g/cultivar was extracted with 20 ml of hexane/acetone/ethanol (50:25:25, vol/vol) and centrifuged (6,500 rpm, 5 min, 5°C). The lipophilic phase was recovered and transferred to a 10-ml volumetric flask, and the volume adjusted with hexane. The absorbance of the hexane solution was measured at 450 nm with a UV-Vis spectrophotometer (model UV-1700 Pharmaspec; Shimadzu, Kyoto, Japan) and total carotenoids were calculated using the value 2,592 as a specific extinction coefficient of β -carotene. The results were expressed as milligrams of β -carotene equivalents (β -CE) 100 g⁻¹ fresh weight (f.w.).

Fruit from each replicate sample per cultivar were homogenized and 2.5 g of tissue was extracted with 20 ml of 70% acetone, containing 0.5% acetic acid, for 24 h at 5°C under darkness. The supernatant was filtered in a Buchner funnel with Whatman no. 1 filter paper into a volumetric flask and the total volume was adjusted to 25 ml with extraction solution. The extract, kept at 5°C in the darkness, was used for the determination of total phenol, total flavonoids, and its antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl method (DPPH; 3).

The amount of total phenolics in apple extracts was determined according to the Folin-Ciocalteu procedure (27). Briefly, 0.25 ml of extract was mixed with 2.5 ml of 1:10 diluted Folin-Ciocalteu's phenol reagent, followed by 2 ml of 7.5% (wt/vol) Na₂CO₃. After 5 min at 50°C, samples were allowed to cool and absorbance was measured at 760 nm with a UV-Vis spectrophotometer (model UV-1700 Pharmaspec; Shimadzu). Phenolic content was estimated from a standard curve of gallic acid and results were expressed as milligrams of gallic acid equivalents (GAE) 100 g⁻¹ f.w.

The determination of total flavonoids was based on the color caused by the binding of Al³⁺ ions in flavonoids. The reaction was completed in 11 min and then the absorption was determined at 510 nm (5). Briefly, 1 ml of extract and 2.7 ml of distilled H₂O were added to 150 ml of 5% NaNO₂, 5% and, after 5 min, 150 ml of 10% AlCl₃ · 6H₂O, were added followed by stirring in a vortex. Then, 6 min later, 1 ml of NaOH at 1 mol liter⁻¹ was added. The final sample was mixed by vortexing and, after 90 min, the absorbance was determined at 510 nm with a UV-Vis spectrophotometer. For quantification, a standard curve was constructed (0 to 100 μ g) with standards of catechin. Results were expressed as catechin equivalents (CE) mg 100 g⁻¹ f.w.

The DPPH method, used to monitor antioxidant activity, was conducted according to the modified procedure of Brand-Williams and co-workers (3). In its radical form, DPPH[•] has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Briefly, 50 μ l of extract plus 2,950 μ l of a solution of free DPPH at 0.3 mmol liter⁻¹ in methanol (MeOH) was placed in darkness at room temperature (20°C). After 30 min, the decrease in absorption of the DPPH solution at 517 nm was measured with a UV-Vis spectrophotometer. The antioxidant activity was expressed as milligrams of ascorbic acid equivalents antioxidant capacity (AEAC) 100 g⁻¹ f.w.

Cultivar susceptibility measurement. One isolate each of *P. expansum* and *B. cinerea*, randomly selected from the source of the

identified isolates, was used for the cultivar susceptibility measurement. Inoculum for both pathogens was produced on PDA in 9-cm petri dishes. Conidia were collected by adding 10 ml of sterile distilled water to each dish and gently rubbing the sporulating colonies with a L-shaped bent glass rod. Spore concentration was adjusted with the aid of a hemacytometer to obtain a suspension containing 1×10^5 spores ml^{-1} . Prior to inoculation, fruit were surface disinfested for 5 min by drenching them in a 1% NaOCl solution. After removing them from the disinfestant solution, they were rinsed three times with sterile deionized water and subsequently air dried. The fruit were wound inoculated at the equator. Two wounds per fruit, 3 mm in depth, were made using a 2-mm-diameter nail head which was inserted into the fruit. On each wound, 20 μl of a conidial suspension was placed with a pipette. Fruit wounded similarly but treated with sterile water were used as noninoculated controls. In all, 40 fruit per pathogen and cultivar combination were inoculated. The fruit were placed on wire mesh platforms (10 fruit per box) in plastic boxes (23 by 31 by 10 cm [length by width by height]). Water (30 ml) was added in each box and the box covered to maintain high relative humidity. In total, 20 of the inoculated fruit per pathogen and cultivar combination were incubated for 7 days at 22°C and the remaining 20 fruit were incubated for 30 days at 2°C. After the end of the incubation period, the infections were scored by measuring lesion diameter. The experiment was performed two times.

Patulin extraction, purification, and measurement. Patulin production measurement was conducted on the 20 apple fruit per cultivar inoculated with *P. expansum* and incubated at 22°C for 7 days. After the end of the incubation period and the measurement of disease severity, the diseased fruit were separated into four samples of five fruit each and ground in a blender with 75 ml of methanol/water (60:40) for 5 min and the supernatant was filtrated through cheesecloth. The residual extract was partitioned with 30 ml of chloroform and, after evaporation to dryness, the extract was redissolved in 5 ml of methanol and filtered through a polyvinylidene difluoride Teflon filter. Patulin was detected on thin-layer chromatography (TLC) plates by spraying them with 3-methyl-2-benzothiazolinone hydrazone hydrochloride, according to a method previously described (15). The quantitative analysis of patulin was performed by a Shimadzu high-performance liquid chromatography system equipped with a binary solvent pump (LC-10ADvp), DGU-14A degasser, CTO-10Avp column oven, SIL-10ADvp autosampler with volume injection set at 2 μl , and a UV diode array detector (SPD-M10Avp). Data acquisition and processing were performed with the liquid chromatography–mass spectrometry solution Ver. 3.0 Workstation. The chromatographic separation was performed on a Discovery reversed-phase C_{18} narrow-bore column (250 by 4.6 mm i.d., 5- μm particle size), thermostated at 40°C. The mobile phase was water/acetonitrile (90:10, vol/vol), delivered isocratically at a flow-rate of 0.7 ml min^{-1} for 15 min. The calibration plot was linear for the UV diode array

detector over the range of 0.01 to 5 $\mu\text{g ml}^{-1}$, and the detectable limit (log of the likelihood ratio) of the analytical method was approximately 2.5 ng ml^{-1} . The recoveries from the apple samples at fortification levels of patulin at 10, 50, and 100 ng g^{-1} were 87 to 98.6%, with precision values (relative standard deviation) of 7.6 to 12.5%. The experiment was performed two times.

Data analysis. Data from the two independent trials on fruit qualitative parameters of the four cultivars used in the experiments, lesion diameter caused by the two pathogens on the fruit of the cultivars tested, and patulin production on fruit artificially inoculated with *P. expansum* were tested for homogeneity of variance using the Levene test. In these analyses, data of the two independent trials were considered block treatments and the replications within each trial were used as subsamples. The data of the two independent trials fulfilled the criteria for homogeneity of variance ($P > 0.05$) and, therefore, they were combined and subjected to analysis of variance. Mean values of each parameter tested were compared using Fisher's protected least significant difference test at $P = 0.05$. Pearson correlation coefficients were estimated between mean values of fruit qualitative parameters, mean lesion diameters, and patulin production. Statistical analyses were conducted using SPSS (version 17.0; SPSS, Chicago) and graphs were created using Prism (v5.01; Graph Pad Inc., San Diego, CA).

Results

Fungal pathogen incidence. During the 2-year survey, more than 10 different fungi were isolated from decayed apple fruit of the four cultivars sampled (Table 1). *P. expansum*, *B. cinerea*, *Alternaria tenuissima*, and *M. pyriformis* were four major fruit rot pathogens isolated from all cultivars, accounting for 44.2, 23.6, 16.1, and 6.6% of the decayed fruit, respectively (Fig. 1). These four pathogens were isolated, although at different frequencies, from the four cultivars in both years of the study. Blue mold caused by *P. expansum* was the most common disease on Red Delicious, Granny Smith, and Golden Delicious during both storage periods; however, blue mold on Fuji fruit was the most common disease in 2008 but not in 2009 (Table 1). *B. cinerea*, causal agent of gray mold, was isolated at frequencies of 26.9 to 38.4% from fruit of Red and Golden Delicious during both years of the study. However, the frequency of gray mold on Granny Smith and Fuji varied between 2008 and 2009. During 2008, frequency of gray mold was low, accounting for only 1.9 and 3.5% of the decayed Granny Smith and Fuji fruit, respectively, compared with 21.5 and 34.2% in 2009 (Table 1). *Alternaria* rot caused by *A. tenuissima* was the third most prevalent fruit rot disease in fruit of Red Delicious, Granny Smith, and Golden Delicious at frequencies of 7.5 to 16.8%. Interestingly, *Alternaria* rot incidence was higher in Fuji fruit, accounting for 33 and 34.2% of the rotted fruit during 2008 and 2009, respectively (Table 1). The frequency of *Mucor* rot ranged from 0.8% on Red Delicious in 2008 to 22.8% on Granny Smith in 2008. The incidence of other diseases, including brown

Table 1. Frequency of recovery of postharvest fungal pathogens on naturally infected fruit of four apple cultivars, sampled from packinghouses in northern Greece during 2008 and 2009

Postharvest pathogens	Frequency of recovery (%)							
	Red Delicious		Granny Smith		Golden Delicious		Fuji	
	2008	2009	2008	2009	2008	2009	2008	2009
<i>Penicillium expansum</i>	46.5	43.2	54.2	58.7	44.0	35.1	49.0	20.7
<i>Botrytis cinerea</i>	30.2	38.4	1.9	21.5	36.5	26.9	3.5	34.2
<i>Alternaria tenuissima</i>	8.9	10.4	10.4	7.5	8.4	16.8	33.0	34.2
<i>Mucor pyriformis</i>	5.0	0.8	22.8	4.1	2.1	1.4	12.2	4.5
<i>Monilinia laxa</i>	0.7	0.0	0.0	0.8	0.0	0.0	0.0	3.6
<i>M. fructigena</i>	0.7	1.6	1.9	0.0	5.3	3.8	0.0	1.9
<i>Fusarium avenaceum</i>	0.0	3.8	1.1	2.8	0.0	1.4	0.0	0.0
<i>F. proliferatum</i>	0.0	1.0	0.9	0.5	0.0	0.0	0.0	0.0
<i>Geotrichum candidum</i>	4.6	0.0	4.7	4.1	3.2	0.0	2.3	0.0
<i>Botryosphaeria obtusa</i>	2.7	0.8	1.9	0.0	0.0	0.0	0.0	0.0
Other ^z	0.7	0.0	0.2	0.0	0.5	14.6	0.0	0.9

^z *Stemphylium vesicarium*, *Phomopsis mali*, *Diaporthe ambigua*, *Botryosphaeria dothidea*, and some unidentified yeast genera.

rot caused by *Monilinia laxa* and *M. fructigena*, black rot caused by *Botryosphaeria obtusa*, sour rot caused by *Geotrichum candidum*, and wet core rot caused by *Fusarium avenaceum* and *F. proliferatum*, varied widely by cultivar and year. In general, these latter pathogens occurred sporadically in decayed apple fruit at frequencies of 0 to 5.3% and, thus, are considered minor diseases.

Fruit quality, phytochemicals, and antioxidant activity. Granny Smith and Fuji apple fruit were significantly firmer (66.39 and 64.72 N, respectively) compared with the other cultivars (Table 2). Fuji apple fruit had the higher SSC levels, followed by Red Delicious (14.2 and 13.2%, respectively). Granny Smith was the most acidic cultivar (pH value of 3.6). The acidity content (0.90% malate acid) of this cultivar was almost twofold higher compared with the other cultivars. Golden Delicious had the highest carotenoid levels (0.25 mg β -CE 100 g⁻¹ f.w.) and Granny Smith had the lowest (Table 2). Total flavonoid content was almost 1.5 times higher on Red Delicious and Granny Smith apple fruit (40.65 and 40.26 mg CE 100 g⁻¹ f.w., respectively) compared with the other cultivars. The highest amount of total phenolics was recorded on Red Delicious followed by Granny Smith (125.16 and 116.98 mg GAE 100 g⁻¹ f.w.). The antioxidant activity of the apple extracts, as estimated by the DPPH method, was higher for Red Delicious than

on Granny Smith, but both had significantly higher antioxidant activity than Golden Delicious and Fuji (Table 2).

Cultivar susceptibility to gray mold and blue mold. The average growth for lesions caused by *P. expansum* on fruit incubated at 2 and 22°C ranged from 0.4 to 0.9 and 3.9 to 5.1 mm/day, respectively. Similarly, for *Botrytis cinerea*, the average growth on fruit incubated at 22°C ranged from 2.6 to 4.9 mm/day whereas, at 2°C, the average lesion growth ranged from 0.55 to 1.0 mm/day. Measurements of lesion diameter on the inoculated fruit showed that Golden Delicious was the most susceptible to blue mold at both temperatures, while Fuji was the most susceptible cultivar to gray mold when fruit were incubated at 22°C (Table 3). At 2°C, Fuji and Golden Delicious fruit were equally ($P > 0.05$) susceptible (Table 3). Granny Smith and Red Delicious were the most resistant cultivars to blue mold at both incubation temperatures, with lesion diameters significantly lower ($P < 0.05$) than those in Fuji and Golden Delicious (Table 3). Red Delicious was significantly ($P < 0.05$) more resistant to gray mold than Golden Delicious and Fuji, while susceptibility of Granny Smith fruit was intermediate at both incubation temperatures and similar ($P > 0.05$) to that of Red Delicious or Golden Delicious and Fuji fruit (Table 3).

Calculations of Pearson correlation coefficients among the qualitative characteristics of fruit used in the artificial inoculation assays and the lesion diameter caused by both pathogens on fruit incubated at 22°C showed that susceptibility to gray mold was negatively correlated with flavonoid ($r = 0.91$) and phenolic concentration ($r = 0.89$) as well with the fruit antioxidant activity ($r = 0.91$) (Table 4). None of the remaining parameters measured was correlated ($P > 0.05$) with the susceptibility to gray mold. Susceptibility to *P. expansum* was negatively correlated ($P < 0.05$) to the fruit firmness and phenolic concentration, with r values of 0.73 for both parameters (Table 4).

Patulin production. Initial studies for the patulin production, using TLC plates, showed that patulin was present in the four cultivars. Patulin production was significantly higher in Red Delicious and Golden Delicious fruit than Granny Smith and Fuji (Fig. 2). Calculation of Pearson correlation coefficients showed that patulin production in decayed apple tissues was negatively correlated with the firmness and the acidity of the fruit, with r values of 0.93 and 0.83, respectively (Table 4). In contrast, none of the remaining fruit qualitative parameters (carotenoids, flavonoids, phenolics, and antioxidant activity) tested were correlated ($P > 0.05$) to the patulin production.

Discussion

In this study, at least 14 different pathogens were associated with postharvest rot of apple in Greece, confirming previous reports that various fungal pathogens cause fruit rots on stored apple fruit (9). The pattern of pathogen distribution on the four cultivars was, to a great extent, similar during both years of the study. Among them, *P. expansum*, *B. cinerea*, *A. tenuissima*, and *Mucor pyriformis* were the most common and were found in the cultivars during both

Fig. 1. Incidence (percentage of rotted fruit) of postharvest fungal pathogens of apple fruit collected from packinghouses in Greece during 2008–09 and 2009–10 storage periods.

Table 2. Quality parameters, chemical composition, and antioxidant activity of Red Delicious, Granny Smith, Golden Delicious, and Fuji apple fruit determined after harvest and before the artificial inoculation of the fruit[†]

Cultivar	Firmness (N)	SSC (%) ^u	pH	TA (malate acid, %) ^v	Carotenoids (mg β -CE 100 g ⁻¹ f.w.) ^w	Flavonoids (mg CE 100 g ⁻¹ f.w.) ^x	Phenolics (mg GAE 100 g ⁻¹ f.w.) ^y	Antioxidant activity (mg AEAC 100 g ⁻¹ f.w.) ^z
Red Delicious	42.70 b	13.32 b	3.67 a	0.39 c	0.22 b	40.65 a	125.16 a	186.95 a
Granny Smith	66.40 a	11.15 d	3.26 c	0.90 a	0.07 c	40.26 a	116.98 b	161.22 b
Golden Delicious	35.01 c	11.85 c	3.42 b	0.48 b	0.25 a	27.58 b	91.82 c	121.22 c
Fuji	64.72 a	14.20 a	3.46 b	0.50 b	0.23 b	24.72 c	93.90 c	125.53 c

[†] Mean values of pooled data after testing homogeneity of variance of the two independent trials using Levene test ($P > 0.05$). Within columns, values followed by the same letters did not differ significantly according to Fisher's protected east significant difference test ($P = 0.05$); f.w. = fresh weight.

^u Soluble solid content.

^v TA = titratable acidity.

^w β -CE = β -carotene equivalents.

^x CE = catechin equivalents.

^y GAE = gallic acid equivalents.

^z AEAC = ascorbic acid equivalents antioxidant capacity.

years of the study. These results are somewhat similar to two previously published reports, suggesting that blue and gray mold were the two most prevalent postharvest diseases of apple fruit in Washington State, United States, and British Columbia, Canada (10,29). However, we found significant differences in the incidence of *Alternaria* spp. and *M. pyrifomis* between our study and those previously mentioned. *Alternaria* spp. were identified only as a minor pathogen in both Washington State and British Columbia, whereas the incidence of *M. pyrifomis* was low in Washington State but relatively high in British Columbia (10,29). Such differences are probably related to the fact that apple fruit rot is a dynamic disease complex in which different fungal species predominate at different locations and that frequencies can vary greatly over time.

One of the most interesting differences observed among our results on pathogens incidence and those previously reported was the relatively high incidence of *Alternaria* spp. (10,29). Currently, *Alternaria* rot on apple fruit is considered to be relatively unimportant while leaf blotch caused by *A. mali* has received great attention (6). The taxonomy of the plant-pathogenic *Alternaria* spp. affecting several hosts is not clear. In our study, the small-spored *Alternaria* sp. isolated from the apple fruit was identified as *A. tenuissima* based on the ITS sequence of the isolates. However, because *A. tenuissima* is considered to be mostly a species group rather than a single species, further research is required for more accurate identification of the isolates based on molecular differences and differences on the metabolite profile (1). Several previous studies reported *Alternaria* spp. as a fruit rot pathogen but they did not provide accurate identification at the species level (10,29,32). Our identification matches two recent reports suggesting that isolates of *Alternaria* spp. obtained from apple flowers, leaves, or fruit belong in the *A. tenuissima* species group and have the potential to cause core rot in apple fruit (1). As mentioned, *A. tenuissima* was isolated from the four cultivars sampled during both sampling periods but, interestingly, in Fuji fruit, the incidence of *A. tenuissima* was significantly higher than that recorded for the

remaining three cultivars. *Alternaria* spp. are considered to be mainly a core rot pathogen and, consequently, require fruit with an open sinus to enter the fruit. The high frequency of *A. tenuissima* observed in Fuji fruit may be related to the higher number of fruit with open sinus in Fuji compared with that in the remaining other cultivars tested (31).

The incidence of *F. avenaceum* and *F. proliferatum* found in this study was low. However, to the best of our knowledge, this is the first report of postharvest rot of apple fruit caused by these two *Fusarium* spp. in Greece. *F. avenaceum* and *F. proliferatum* are two of the most commonly encountered *Fusarium* spp. in small grains and maize (13); however, recently, a relatively high incidence of *F. avenaceum* on apple fruit was reported in Slovenia (28). Furthermore, Tournas and Uppal-Memon (32) reported that 3% of apple fruit showed internal spoilage associated with *Fusarium* spp. Although the mycotoxigenic potential of *A. tenuissima*, *F. avenaceum*, and *F. proliferatum* isolated from apple fruit was not tested in this study, it is well known from previous studies that they may pose a mycotoxin risk (1,30).

Information on the relative resistance of apple cultivars toward the most important postharvest diseases of apple fruit is limited (10,31) and data were contradictory. This may be due, in part, to the absence of a common experimental design and similar cultivars. The results of our study showed that there was not a consistent pattern toward resistance or susceptibility to blue mold or gray mold in any of the four cultivars evaluated, suggesting that rot resistance among the four cultivars tested was nonspecific. Similarly, Spotts et al. (31) found no differences in gray mold and blue mold disease susceptibility among cultivars. However, the range of cultivars evaluated was different from ours and, consequently, a direct comparison of the results of these two studies is not possible.

Resistance of apple tissues to fungal decay is affected by several biochemical parameters. In this study, phenolic content of the fruit tissue was negatively correlated with the development of both diseases. In addition, gray mold development was negatively correlated with the flavonoid content and antioxidant activity of the fruit. The phenolic composition of apple fruit is highly variable and cultivar is one among the several factors that regulate phenolic contents (22). The negative correlation between disease severity and phenolic content observed in our study may be correlated with the production of phytoalexins, which are low molecular weight compounds of phenolic nature constituting an important part of the plant defense mechanisms. Such a hypothesis is consistent with the findings of a recent report which showed that exogenous application of several phenolic compounds resulted in lower *P. expansum* growth and patulin production (26). Similarly, preformed or induced flavonoids play a major role in postharvest resistance of fruit (33). Ambient pH is considered to be one of the most important factors regulating the secretion of cell-wall-degrading enzymes associated with the aggressiveness of fruit rot-associated fungi. Previous studies have shown that both *B. cinerea* and *P. expansum* are more aggressive in low-pH environments, which induce the expression of polygalacturonases (23,24,34). However, the results of our study showed that fruit pH values were not correlated with

Table 3. Susceptibility (lesion diameter) of four apple cultivars to blue and gray mold diseases caused by *Penicillium expansum* and *Botrytis cinerea*, respectively^y

Cultivar	Lesion diameter (mm) ^z			
	Blue mold		Gray mold	
	22°C	2°C	22°C	2°C
Red Delicious	29.8 ab	16.5 ab	18.5 a	16.5 a
Granny Smith	27.9 a	14.8 a	24.3 ab	23.0 ab
Golden Delicious	36.0 c	26.2 c	28.5 b	29.8 b
Fuji	30.9 b	20.1 b	34.4 c	30.0 b

^y Mean values of pooled data after testing homogeneity of variance of the two independent trials using Levene test ($P > 0.05$). Within columns, values followed by the same letter do not differ significantly according to Fisher's protected least significant difference test ($P = 0.05$).

^z Measurements of lesion diameter on the artificially inoculated fruit were carried out 7 and 30 days postinoculation on fruit incubated at 22 and 2°C, respectively.

Table 4. Pearson correlation coefficients among apple fruit quality and compositional parameters, cultivar susceptibility (determined as lesion diameter) to blue and gray mold, and patulin production by *Penicillium expansum*^a

Fruit quality and compositional parameters	Lesion diameter at 22°C		
	<i>Botrytis cinerea</i>	<i>Penicillium expansum</i>	Patulin production
Firmness	0.34	-0.73*	-0.93*
Soluble solid content	0.30	-0.02	0.22
Titrateable acidity	-0.04	-0.53	-0.83*
pH	-0.38	0.09	0.79*
Carotenoids	0.27	0.73*	0.73
Flavonoids	-0.91*	-0.67	0.01
Phenolics	-0.89*	-0.73*	0.07
Antioxidant activity	-0.91*	-0.67	0.19

^a Asterisks (*) indicate r value significant at $P = 0.05$.

the disease severity caused by either of these pathogens, suggesting that other physiological factors and their changes during the ripening process may also contribute to the interactions among the two pathogens and the apple tissue.

Measurements of patulin accumulation in the juice of the decayed tissue after artificial inoculation with *P. expansum* and incubation for 10 days at 20°C revealed marked differences among the four cultivars tested. Although patulin accumulated at high concentrations in the four cultivars, it was significantly higher in Red and Golden Delicious fruit than Granny Smith and Fuji. The finding that Golden Delicious fruit accumulated higher concentration of patulin than Fuji fruit at 20°C is in agreement with the findings of a previous study comparing patulin accumulation in these two cultivars (14,25) but contradicts the findings of another study (19). Thus, it can be concluded that the cultivar itself is not the determinant factor for patulin accumulation. However, qualitative characteristics of the fruit, which are not affected only by the cultivar, may play a crucial role. In our assays, the Granny Smith fruit were more acidic and had lowest patulin concentration, while growth of *P. expansum* remained unaffected. Such a finding is in agreement with those of Marin et al. (14), who suggested that fruit acidity is an important factor affecting patulin production.

In conclusion, identification of *Fusarium* spp. and *Alternaria* spp. as causal agents of postharvest rot in apple fruit in Greece, along with the confirmed prevalence of the patulin-producer fungus *P. expansum* and the atoxigenic pathogen *B. cinerea*, is of significant importance because they may be responsible for high quantitative yield losses. Furthermore, the toxigenic species can contribute to qualitative deterioration of the stored apple fruit considering that high amounts of apple juices and other by-products are consumed by a vulnerable population such as infants and young children (32). The results of the study can be used for making decisions for implementation of control strategies aiming at the reduction of yield losses and increasing food safety by decreasing mycotoxin contamination of apple fruit by-products.

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Fig. 2. Patulin production in artificially inoculated fruit of four apple cultivars by *Penicillium expansum*. Measurements were made after 10 days of incubation at 22°C. Data were pooled after testing homogeneity of variance of the two independent trials using Levene test ($P > 0.05$). Bars on the columns indicate standard error of the mean.

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