

RESEARCH PAPERS

Incidence and etiology of postharvest fungal diseases of pomegranate (*Punica granatum* cv. Mollar de Elche) in Spain

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Summary. Spain is the largest European Union producer and exporter of pomegranates. More than 85% of Spanish commercial plantings are located in the Elche area (Alicante Province, SE Spain), where cv. Mollar de Elche is the most important cultivar. The incidence and etiology of postharvest pomegranate diseases were determined in local environmental conditions. Commercially-grown pomegranates cv. Mollar de Elche from two orchards were assessed, during two consecutive seasons, for latent and wound pathogens causing postharvest diseases. Healthy pomegranates were either artificially wounded in the rind or surface disinfected and placed in humid chambers at 20°C for up to 15 weeks. Additionally, decay was periodically assessed on commercially-handled pomegranates stored at 5°C for up to 27 weeks. The main causal agents of wound and latent infections were *Penicillium* spp. and *Botrytis cinerea*, respectively. The same pathogens were also the most frequently isolated from cold-stored fruit, but decay at 5°C was only significant after 19 weeks. Another relatively frequent pathogen on fruit incubated at 20°C was *Aspergillus niger*. Among the fungi isolated, *Penicillium expansum*, *P. sclerotiorum*, *P. glabrum* and *Pilidiella granati* were pathogenic on inoculated pomegranates, whereas *P. minioluteum* and *Cytospora annulata* were not. No decay caused by *Alternaria* spp. or *Colletotrichum gloeosporioides* was observed.

Key words: postharvest decay, latent infection, wound infection, cold storage.

Introduction

Spain is the largest European Union (EU) producer and exporter of pomegranates. More than 85% of Spanish commercial plantings are located in the Southeast area of the country (Alicante Province in the Valencia Region), where cv. Mollar de Elche accounts for about 90% of plantings. This is a late-ripening cultivar, commonly harvested from the end of September to the middle of November. Exports are mainly (about 90%) directed to EU markets. The importance of this crop in Spain is increasing due to new markets based on the manufacture of pomegranate-derived nutraceuticals and dietary or health supplements (Faria and Calhau, 2011).

Pomegranate is a nonclimacteric fruit that does not ripen off the tree, even with ethylene treatment, and fruit should be picked when fully ripe (Kader *et al.*, 1984). The Spanish pomegranate industry is interested in prolonging fruit storage life to reach off-season markets. Studies with cv. Mollar de Elche recommended storage at 5°C or warmer and relative humidity (RH) of 90% or greater to avoid weight loss and chilling injury, such as brown discoloration of the rinds and/or superficial pitting (Artés *et al.*, 2000). Storage under these conditions, however, can favour the development of postharvest diseases, especially when the incidence of latent fungal infections at the time of harvest is high, or large proportions of fruit are superficially damaged. For instance, storage life of pomegranates cv. Wonderful significantly increased when the incidence of latent crown infections was low and the fruit were carefully sorted (Hess-Pierce and Kader, 2003).

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Fungi causing pomegranate fruit rot worldwide include *Alternaria* spp., *Aspergillus niger* van Tiegh. and other *Aspergillus* spp., *Botrytis cinerea* Pers.: Fr., *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Coniella* spp., *Nematospora* spp., *Penicillium* spp., *Pestalotiopsis versicolor* (Speg.) Steyaert, *Pilidiella granati* Sacc., *Rhizopus* spp., and *Syncephalastrum racemosum* Cohn (Hebert and Clayton, 1963; Sharma and Jain, 1978; Snowdon, 1990; Bardas *et al.*, 2009a; b; Jamadar *et al.*, 2011; Thomidis and Exadaktylou, 2011; Mirabolfathy *et al.*, 2012). Etiology and incidence of postharvest diseases should be determined for each individual growing area because they depend on local preharvest (e.g. cultivar, climate, growing conditions), harvest (e.g. fruit maturity, incidence of rind wounds), and postharvest (e.g. packinghouse handling, storage conditions) factors. Postharvest decay of pomegranate can be caused by fungi that infect either the fruit through injuries or microwounds on any part of the rind (wound pathogens), or the flowers or calices (crowns) of young fruits in the field. These pathogens remain latent, and cause disease after harvest (latent pathogens). Some pathogens, such as *B. cinerea*, are able to infect stored pomegranates by mycelial spread from infected fruit to adjacent healthy fruit, causing 'nests' of decay (Tedford *et al.*, 2005; Palou *et al.*, 2007a). In any case, the type and amount of fungal infections determine the potential economical losses caused by postharvest diseases, and the most appropriate postharvest handling procedures to minimize such losses. This is especially important in Spain and other EU countries where no conventional chemical fungicides are currently permitted for postharvest treatment of pomegranates.

The objectives of the research reported here were to: 1) identify and quantify latent and wound pathogens causing postharvest diseases on pomegranates cv. Mollar de Elche, ii) assess postharvest decay during cold storage of commercially-handled fruit; and iii) test the pathogenicity and characterize the development at different temperatures of selected fungi isolated from pomegranate. Some preliminary results of this study have been published (Palou *et al.*, 2007b, 2009, 2010; Palou and Del Río, 2009).

Materials and methods

Fruit

For two consecutive harvest seasons (2005 and 2006), pomegranates (*Punica granatum* L.) cv. Mollar

de Elche (synonym: 'Mollar') commercially-grown in two orchards in the Elche area (Alicante, Spain; about 40°N, 100 m elevation) were harvested and transported to a local packinghouse. Both orchards were representative of pomegranate plantings and handling in the area. The first was located in the Municipality of Las Bayas (Lat. 38.22 N, Long. -0.64 W) and was planted in 1995; the second was located in the Municipality of Elche (Lat. 38.17 N, Long. 0.41 W) and was planted in 1997. Trees in both orchards were planted at 5 × 4 m spacings. Average values for fruit quality attributes at harvest (two seasons) were: soluble solids content = 16.8%; titratable acidity (citric acid) = 0.22%; and CIE (Comision Internationale del'Eclairage) skin color parameters: lightness = 68.2, chroma = 42.0, and hue angle = 67.2. One day later, intact fruits were randomly transferred from field bins to commercial 40 × 29 × 27 cm cardboard boxes and transported to the Institut Valencià d'Investigacions Agràries (IVIA) facilities where sound pomegranates of a uniform medium size were selected, further randomized, and used the same day in experiments to assess latent and wound pathogens. Another set of fruit was commercially-handled in the packinghouse and used for assessment of decay during cold storage as described below.

Assessment of disease caused by latent pathogens

Pomegranates from the field were surface-disinfected by immersing them for 1 min in diluted bleach (0.5% sodium hypochlorite), then rinsed thoroughly with tap water, and dried with paper towels. In order to prevent killing possible latent pathogens in the fruit crowns, these were each covered with Parafilm® prior to disinfection. Each disinfected fruit was placed in a sterile 9-cm diameter Petri dish base or lid and the dishes were put inside humid chambers. These consisted of 5 L capacity plastic containers with lids that had been surface-disinfected by spraying with 98% ethanol and allowing them to air-dry at room temperature. To allow gas exchange, a 0.5 cm diam. hole had been made in two opposite walls of each container. Paper towels soaked with sterile water had been placed on the bottom of each container. Closed chambers were incubated at 20°C. Each season, eight replicate humid chambers, each containing four pomegranates, were prepared with fruit from each orchard. The number of fungal infections

in the fruit in each chamber was recorded weekly during time periods that depended on the amount of infected fruit; the evaluation period was 7 in 2005 and 15 weeks in 2006. All fungal infections observed on each single fruit were recorded.

Infections with distinctive symptoms were directly recorded, otherwise, putative causal agents were isolated from decayed fruit by placing 5 mm diam. segments of infected tissue onto potato dextrose agar (PDA) medium in 9 cm diam. Petri dishes and incubating at 25°C. Where necessary, fungal isolates were subcultured on PDA and identified on the basis of macroscopic and/or microscopic morphology. With some exceptions, fungi were identified at genus level. Isolates that were common but could not be identified were submitted to the "Colección Española de Cultivos Tipo" (CECT, Spanish Type Culture Collection, University of Valencia, Valencia, Spain) for identification on the basis of morphology of colonies growing on Czapek yeast extract agar, malt extract agar, 25% glycerol nitrate agar or oatmeal agar, and by the amplification and subsequent sequencing of the ribosomal DNA intragenic spacer regions ITS1 and ITS2 along with the 5.8S rRNA gene (White *et al.*, 1990). In some cases, the region D1/D2 in the 5' end of the 28S rDNA gene was also sequenced. Where necessary, pathogenicity tests were performed, as described below.

Assessment of disease caused by wound pathogens

Pomegranates from the field were each wounded at four equidistant points in the equator with a sterilized stainless steel probe, 1 mm wide and 2 mm in length. Each wounded fruit was placed on a numbered sterile Petri dish base or lid and the dishes were put inside humid chambers and incubated as described above. Each season, eight replicate humid chambers, each containing four pomegranates (16 wounds), were prepared with fruit from each orchard. Incubation of humid chambers, data recording and isolation and identification of uncertain, common isolates were conducted as described above. Pathogenicity tests were performed when necessary.

Assessment of disease during cold storage of commercially-handled fruit

Pomegranates that had been commercially-handled in the packinghouse were brought one

day later to the IVIA and stored in a 40 m³ standard research cold storage room at 5°C and 90% RH for about 7 months. Handling in the packinghouse included only visual fruit inspection, selection and packaging in 50 × 30 × 10 cm lidless cardboard boxes with plastic cavity trays that prevented contact between fruit. No other postharvest treatment was applied. Each season, four replicate commercial boxes, each containing 12 pomegranates, were used with fruit from each orchard (total of 48 fruits per orchard). The number of fungal infections on the fruit in each box was recorded every 2 weeks during a 27 week period. All fungal infections observed in each fruit were recorded. Fungi isolated from decayed fruit were identified as described above.

Pathogenicity tests and characterization of disease development

Fungi isolated from decayed pomegranates that were tested for pathogenicity included *Penicillium expansum* L., *P. sclerotiorum* J.F.H. Beyma, *P. glabrum* (Wehmer) Westling, *P. minioluteum* Dierckx, *Pi. granati* and *Cytospora annulata* Ellis & Everh. Five-mm diam. mycelial plugs from 7-d-old colonies grown on PDA at 25°C were aseptically transferred to 5 mm diam., 2–3 mm deep wounds inflicted in the rind of mature pomegranates cv. Mollar de Elche with a sterile cork borer (one wound per fruit). The fruit had been superficially disinfected by dipping for 2 min in aqueous 0.5% sodium hypochlorite and thoroughly rinsed with deionized water. Inoculated fruit were placed in humid chambers as previously described and incubated at 20°C or cold-stored at 5°C (the usual temperature for commercial cold storage of pomegranates). Four chambers containing four pomegranates were used for each fungus and temperature. Additional chambers with four wounded but uninoculated pomegranates were used as experimental controls. Depending on the fungus and storage temperature, the number of infected fruit, the lesion size, and the number of fruit showing spores were determined every 3 to 7 d during incubation for up to 30 d for 20°C storage or 40 d for 5°C. To fulfill Koch's postulates, re-isolations to PDA plates in aseptic conditions were made after 7 d of incubation at 20°C from inoculated pomegranates showing disease symptoms. The tests were conducted twice.

Statistical analyses

Total disease incidence, i.e. percentage of wounds or fruits infected by any pathogen (designated 'all pathogens') incidence of decay caused by each common pathogen, and incidence of decay caused by sporadic or uncommon pathogens (designated 'other pathogens'), were considered as dependent variables. For each evaluation date and each of the three types of disease assessment, *viz.* disease caused by latent and wound pathogens and disease during cold storage, incidence data were arcsine transformed and two-way analyses of variance (ANOVA), with season and orchard as random factors, were performed using SAS software (SAS Institute Inc., Cary, NC, USA). An analysis of the correlations among the most important dependent variables was also performed. Pearson correlation coefficients (r) were calculated using the SAS CORR Procedure and correlation was considered significant at $P < 0.05$. For each type of disease assessment, the average relative frequency of pathogens causing decay at the end of

each evaluation period was also calculated. Since experiment was a non-significant factor in the ANOVA, average data from two experiments are presented for pathogenicity tests.

Results

Disease caused by latent pathogens

According to the ANOVA performed with incidence data from both seasons after 7 weeks of incubation, significant differences were observed between seasons only for total incidence of latent infections, i.e. disease caused by 'all pathogens', and disease caused by *B. cinerea*. No significant interactions between the factors season and orchard were found. Irrespective of season and orchard, disease caused by 'all pathogens' steadily increased during the incubation period of 7 (2005) or 15 weeks (2006) at 20°C (Figure 1). This pattern was due to the incidence of disease caused by *B. cinerea*, which was the fungus isolated most frequently. With the exception of fruit from orchard 1 in

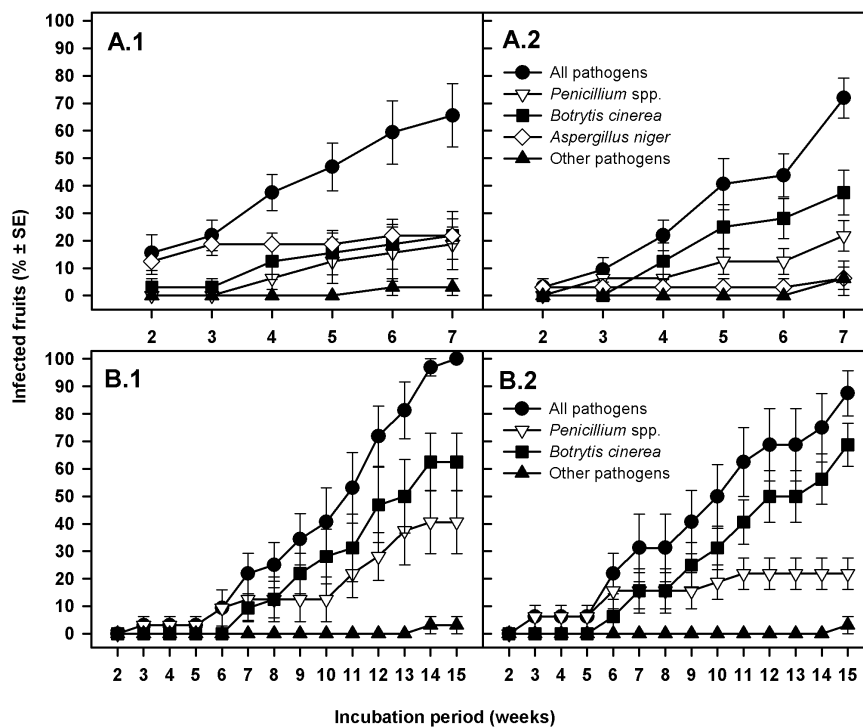


Figure 1. Incidence of postharvest diseases caused by latent pathogens on pomegranates cv. Mollar de Elche from two orchards (1 and 2) surface-disinfected and incubated at 20°C for 7 or 15 weeks. Data from two consecutive seasons, 2005 and 2006 (A and B).

the first season (Figure 1A.1), in which the incidence of disease caused by *A. niger* was high after 3 weeks (about 20%) and remained constant during the incubation period, *Penicillium* spp. were the second most frequently obtained isolates from non-wounded fruit. *Cladosporium* spp., *Aspergillus* spp. (other than *A. niger*) and *Pi. granati* were isolated less frequently. In 2005, after 7 weeks, the total incidence of infections was about 65 and 75% on pomegranates from orchards 1 and 2, respectively; the infections caused by *B. cinerea* in these orchards were 20 and 40%, and the infections caused by *Penicillium* spp. were 20 and 20%. In 2006, after 7 weeks, total incidence on pomegranates from orchard 1 was about 25% and 30% on pomegranates from orchard 2; the infections caused by *B. cinerea* in these orchards were 10 and 15%, and the infections caused by *Penicillium* spp. were also 10 and 15%. In 2006, after 15 weeks, total incidence on pomegranates from orchards 1 and 2 was about 100 and 90%, respectively; the infections caused by *B. cinerea* in these orchards were 65 and 70%, and the infections caused by *Penicillium* spp. were 40 and 20% (Figure 1). It was observed by eye that stamens in the calices of many fruit were infected by *Penicillium* spp. and, in some cases, by *A. niger*.

After 7 weeks of incubation, correlations were highly significant between total incidence of latent infection and the incidence of disease caused both by *B. cinerea* ($P=0.0006$) and *Penicillium* spp. ($P=0.0003$). No significant correlations were found after 15 weeks (Table 1).

In 2005, in fruit from orchard 1, the frequency of disease caused by *B. cinerea* was 38%, by *Penicillium* spp., 21%, by *A. niger*, 38% and by other pathogens, 3%, at the end of the incubation period (7 weeks). In fruit from orchard 2, the frequency of disease caused by *B. cinerea* was 55%, by *Penicillium* spp., 30%, by *A. niger*, 9% and by other pathogens, 6% (Figure 2A). In 2006, these frequencies (after 15 weeks) were: in fruit from orchard 1, disease caused by *B. cinerea*, 63%, by *Penicillium* spp., 34%, by *A. niger*, 0% and by other pathogens 3%; and from orchard 2, disease caused by *B. cinerea*, 72%, by *Penicillium* spp., 22%, by *A. niger*, 0% and by other pathogens 6% (Figure 3A).

Disease caused by wound pathogens

ANOVA of incidence data from both seasons for the variables total incidence of wound infections (decay caused by 'all pathogens'), and disease caused by *Penicillium* spp. after 7 weeks of incuba-

Table 1. Correlations among the incidence of postharvest diseases caused by latent and wound pathogens on pomegranates cv. Mollar de Elche incubated at 20°C for 7 or 15 weeks in humid chambers.

Infection type	Pair of variables ^a	Incubation period (weeks)			
		7		15	
		r ^b	P	r	P
Latent	Pen - Total	0.6020	0.0003*	0.4583	0.0742
	Bc - Total	0.5742	0.0006*	0.4286	0.0977
	Pen - Bc	-0.1455	0.4270	-0.4583	0.0742
Wound	Pen - Total	0.9555	<0.0001*	0.7710	0.0005*
	Bc - Total	0.5099	0.0029*	0.3059	0.2491
	Pen - Bc	0.3524	0.0479*	-0.3366	0.2024

^a Variables are the incidence (percentage of fruits or wounds infected) of disease caused by *Penicillium* spp. (Pen), *Botrytis cinerea* (Bc) and all pathogens (Total). For the 7-week incubation period, data are from two seasons and two orchards. For the 15-week incubation period, data are from one season and two orchards.

^b Pearson's correlation coefficient.

*, Significant correlations ($P<0.05$).

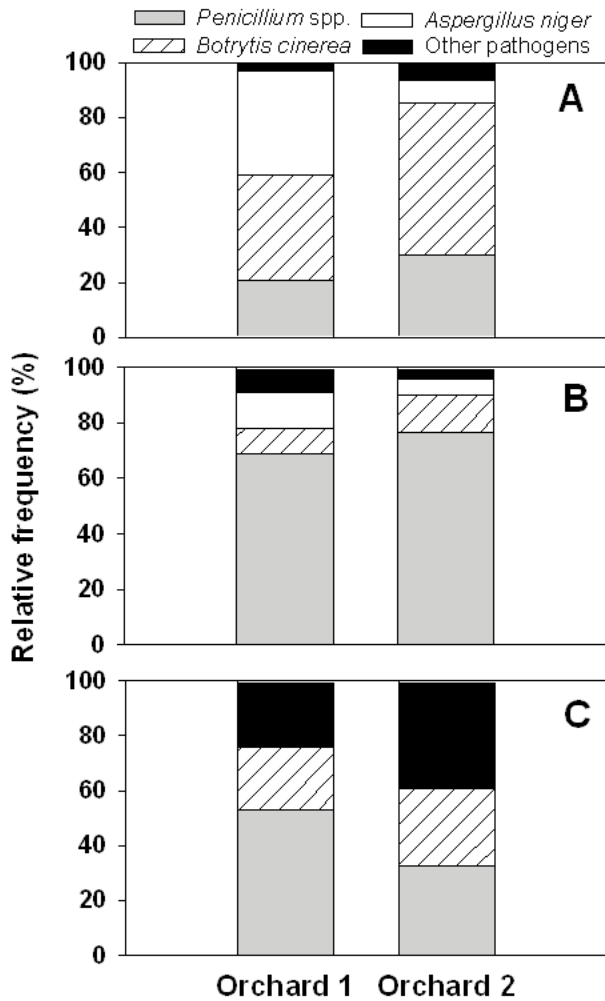


Figure 2. Relative frequency of pathogens causing postharvest diseases on pomegranates cv. Mollar de Elche from two orchards. The fruit were surface-disinfected (A) or artificially wounded (B) and incubated at 20°C for 7 weeks, or stored at 5°C for 27 weeks (C). Data from the 2005 season.

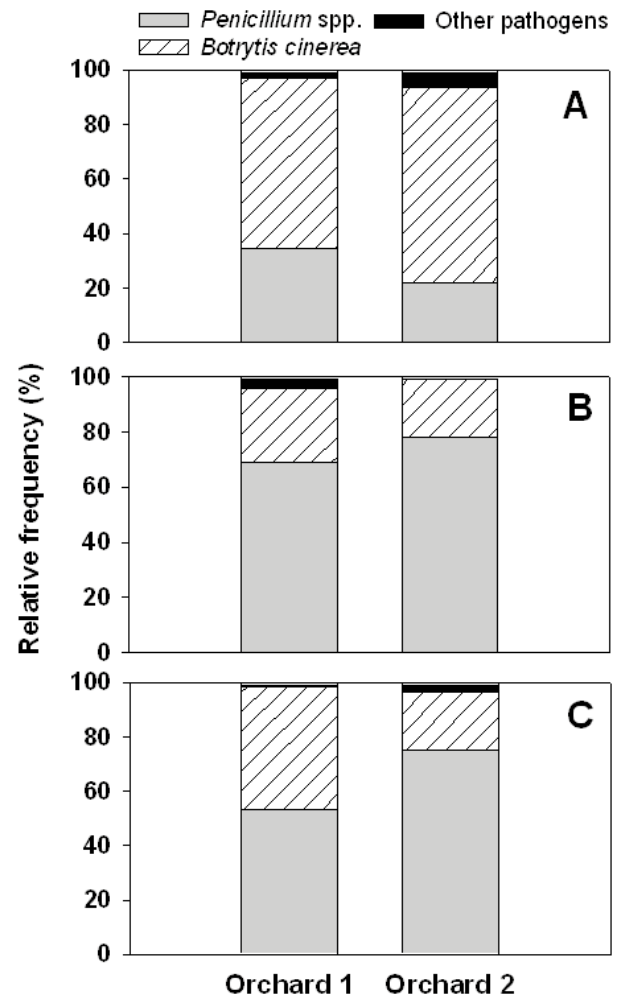


Figure 3. Relative frequency of pathogens causing postharvest diseases on pomegranates cv. Mollar de Elche from two orchards. The fruit were surface-disinfected (A) or artificially wounded (B) and incubated at 20°C for 15 weeks, or stored at 5°C for 27 weeks (C). Data from the 2006 season.

tion showed that there were significant differences between seasons but not between orchards, and the interaction between these two factors was statistically significant. In contrast, no significant differences or interactions were found for the incidence of disease caused by *B. cinerea*. After 3 weeks of incubation at 20°C, total disease incidence on pomegranates from orchard 1 was about 15% in 2005 and 25% in 2006, while incidence in orchard 2 was about 25% in 2005 and 0% in 2006 (Figure 4). After 7 weeks incubation, these values increased, for orchard 1 to 30% in 2005 and 25% in 2006, and for orchard 2 to

60 and 20%. In all cases, *Penicillium* spp. were isolated most frequently from infected wounds, followed by *B. cinerea* and *A. niger*. Of the pathogens isolated sporadically, the most frequent were *Cladosporium* spp., *Aspergillus* spp. other than *A. niger*, *Pi. granati*, *Rhizopus* spp., *Cytospora* spp., and *Alternaria* spp. The incidence of disease caused by *A. niger* was greater in 2005 than in 2006 (Figure 4). Stamens in the calices of many fruit were infected with *Penicillium* spp. or *A. niger*.

The variable total disease incidence was strongly and positively correlated with the incidence of dis-

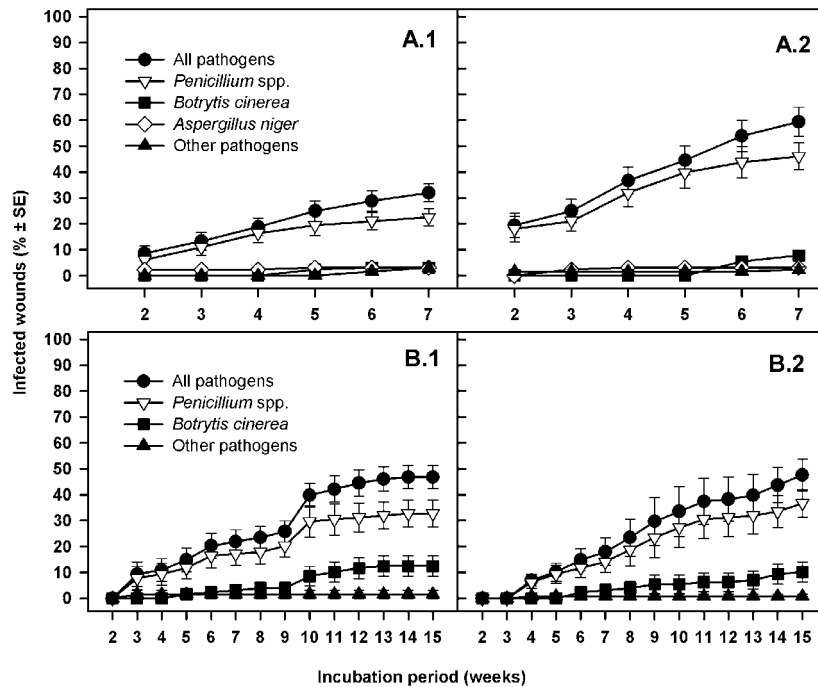


Figure 4. Incidence of postharvest diseases caused by wound pathogens on pomegranates cv. Mollar de Elche from two orchards (1 and 2). The fruit were artificially wounded and incubated at 20°C for 7 or 15 weeks. Data from two consecutive seasons, 2005 and 2006 (A and B).

ease caused by both *Penicillium* spp. ($P < 0.0001$) and *B. cinerea* ($P = 0.0029$) after 7 weeks of incubation, but after 15 weeks only with the incidence of disease caused by *Penicillium* spp. ($P = 0.0005$; Table 1).

In 2005, the relative frequency of infections on fruit from orchard 1 at the end of the incubation period (7 weeks) caused by *Penicillium* spp. was 69%, *B. cinerea* 9%, *A. niger* 13%, and other pathogens 9%. For fruit from orchard 2 the relative frequencies of infections were: *Penicillium* spp. 77%, *B. cinerea* 14%, *A. niger* 5%, and other pathogens 4% (Figure 2B). In 2006, these frequencies at the end of the longer incubation period of 15 weeks were: in fruit from orchard 1, *Penicillium* spp. 69%, *B. cinerea* 27%, *A. niger* 0%, and other pathogens 4%; and in fruit from orchard 2, *Penicillium* spp. 78%, *B. cinerea* 21%, *A. niger* 0%, and other pathogens 1% (Figure 3B).

Disease of commercially-handled fruit during cold storage

In the two-factor ANOVAs performed with data after 19, 23, and 27 weeks of cold storage, no signifi-

cant differences between seasons or orchards were observed for the variable total disease incidence. For the variable incidence of disease caused by *Penicillium* spp., significant differences ($P < 0.05$) were found between seasons only after 27 weeks; for the variable incidence of disease caused by *B. cinerea*, significant differences ($P < 0.05$) were found between seasons after 19 and 27 weeks and between orchards after 27 weeks. The interactions between the factors season and orchard were not statistically significant. Total incidence of decay for all seasons and orchards was 10% or less on cold-stored pomegranates cv. Mollar de Elche during the first 19 weeks of storage at 5°C. Total incidence increased sharply during the last weeks of cold storage to reach 60–75% in 2005, and 90–100% in 2006, after 27 weeks (Figure 5). *Penicillium* spp. and *B. cinerea* were often the most frequent pathogens during and at the end of the cold storage periods. Other pathogens isolated were *Pi. granati*, *Cladosporium* spp., and *Aspergillus* spp. other than *A. niger*. On pomegranates cold-stored for more than 11–13 weeks, *Penicillium* spp., but not *A. niger*, were commonly observed on the stamens.

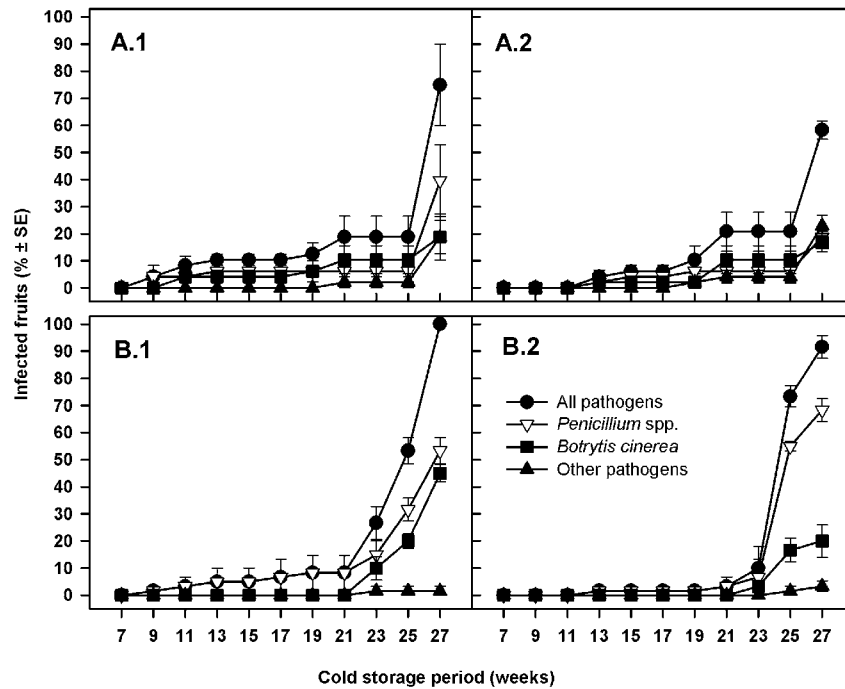


Figure 5. Incidence of postharvest diseases caused by different pathogens on commercially-handled pomegranates cv. Mollar de Elche from two orchards (1 and 2). The fruit were stored at 5°C and 90% RH for 27 weeks. Data from two consecutive seasons, 2005 and 2006 (A and B).

Total disease incidence was always positively correlated ($P < 0.05$) with the variable incidence of disease caused by *Penicillium* spp. It was also positively correlated with the variable incidence of disease caused by *B. cinerea* after 23 ($P = 0.0019$) and 27 ($P = 0.0196$) weeks of storage (Table 2).

In 2005, the relative frequencies of infections in fruit at the end of the 27 week cold storage period were: for orchard 1, *Penicillium* spp. 53%, *B. cinerea* 23%, and other pathogens 24%; and for orchard 2, *Penicillium* spp. 32%, *B. cinerea* 28%, and other pathogens 40% (Figure 2C). In 2006, these frequencies were: for orchard 1, *Penicillium* spp. 53%, *B. cinerea* 45%, and other pathogens 2%; and for orchard 2, *Penicillium* spp. 75%, *B. cinerea* 21%, and other pathogens 4% (Figure 3C).

Identification, pathogenicity and disease development

Penicillium spp. isolated from infected pomegranates were identified by CECT technicians as *P. expansum*, *P. sclerotiorum*, *P. glabrum*, and *P. minioluteum*.

Other isolates identified on the basis both of morphology and DNA sequencing were *Pi. granati* and *C. annulata*. In the pathogenicity tests, disease symptoms were observed on pomegranates cv. Mollar de Elche inoculated with *P. expansum*, *P. sclerotiorum*, *P. glabrum*, or *Pi. granati*, while no decay was observed on control fruit and fruit inoculated with *P. minioluteum* or *C. annulata*.

After 7 d of incubation at 20°C, decay incidence and severity (lesion size) in wounds inoculated with the different pathogens were: *Pi. granati* 100% and 90 mm in diam., *P. expansum* 90% and 12 mm, *P. sclerotiorum* 80% and 10 mm, and *P. glabrum* 20% and 8 mm (Figure 6). After 17 d at this temperature, these values were: *Pi. granati* 100% and 250 mm, *P. expansum* 100% and 25 mm, *P. sclerotiorum* 100% and 20 mm, and *P. glabrum* 75% and 10 mm. In *in vivo* tests at 5°C, all three pathogenic *Penicillium* spp. were able to grow and cause disease, particularly *P. expansum*, with average disease incidence after 28 d of 100% and disease severity of 12 mm in diam. In contrast, pomegranates inoculated with *Pi. granati* did not show disease symptoms after 42 d at 5°C (Figure 6).

Table 2. Correlations among the incidence of postharvest diseases on commercially-handled pomegranates cv. Mollar de Elche stored at 5°C and 90% RH for 19, 23, or 27 weeks.

Pair of variables ^a	Cold storage period (weeks)					
	19		23		27	
	r ^b	P	r	P	r	P
Pen - Total	0.9055	<0.0001*	0.7774	0.0004*	0.8379	<0.0001*
Bc - Total	0.2720	0.3082	0.7146	0.0019*	0.5759	0.0196*
Pen - Bc	-0.1071	0.6930	0.1876	0.4866	0.2506	0.3492

^a Variables are the incidence (percentage of infected fruit) of disease caused by *Penicillium* spp. (Pen), *Botrytis cinerea* (Bc) and all pathogens (Total). Data from two seasons and two orchards.

^b Pearson's correlation coefficient.

*, Significant correlations ($P < 0.05$)

Discussion

Species of the genus *Penicillium* and *B. cinerea* were the most important pathogens causing post-harvest decay of pomegranates cv. Mollar de Elche in the two representative orchards studied in Spain. *Penicillium* spp. were responsible for most of the decay, either caused by wound pathogens or observed on commercially-handled (cold-stored) pomegranates. *Botrytis cinerea* was the most abundant fungus in terms of latent pathogens, and was also very frequently isolated in the trials in which fruit were wounded or cold-stored. In most cases, the incidence of disease caused by these pathogens was positively correlated with total incidence of fruit decay. Irrespective of the type of infection evaluated (latent, wound or on intact cold-stored fruit), however, the pathogens were not correlated with each other, which indicated that infection by one of these pathogens (*Penicillium* spp. or *B. cinerea*) did not predispose the fruit to be infected by the other. Multifactorial ANOVAs showed that, in general, the season was a considerably greater source of variation than the orchard. This was not surprising, considering the general uniformity of the Elche pomegranate growing area in terms of orography, topography and soil characteristics, and the similarity of the two orchards studied, in terms of tree age and planting pattern.

Three out of the four *Penicillium* spp. isolated from decayed fruit were pathogenic on pomegranates cv. Mollar de Elche, as was previously described by Palou *et al.* (2010). Disease development

tests showed that at ambient temperatures *P. expansum* and *P. sclerotiorum* were better adapted than *P. glabrum* to growth on pomegranates cv. Mollar de Elche, whereas at 5°C *P. expansum* was the best adapted species. Other workers have reported *Penicillium* spp. as the cause of pomegranate fruit rot. In some cases the causal agent was not identified at the species level (Kanwar and Thakur, 1972; Artés *et al.*, 2000), but in other cases it was identified as *P. expansum* (Kanwar and Thakur, 1972), *P. glabrum* (Bardas *et al.*, 2009b), *P. digitatum* Sacc. (Salunkhe and Desai, 1984), *P. chrysogenum* Thom (Jamadar *et al.*, 2011), or *P. implicatum* Biourge (Labuda *et al.*, 2004). Labuda *et al.* (2004) also found *P. implicatum*, *P. glabrum*, *P. minioluteum*, and *P. erythromellis* A.D. Hocking on visibly moldy stamens in the crowns of Spanish pomegranates obtained from a market in the Slovak Republic. We also repeatedly observed *Penicillium* spp. on the stamens of pomegranates cv. Mollar de Elche stored at both 20 and 5°C in our trials, but did not identify the species present. Labuda *et al.* (2004) further suggested that moldy stamens could be sources of infection that spread to the fruit following mechanical damage. Since *Penicillium* spp. are typically strict wound pathogens, such type of infection could partially explain the increasing level of penicillium mold that we found in the assessment of latent pathogens and decay on cold-stored pomegranates. *Penicillium* spp. from stamens could contaminate lesions caused by other fungi, infect the fruit through the weakened fruit rinds and overgrow

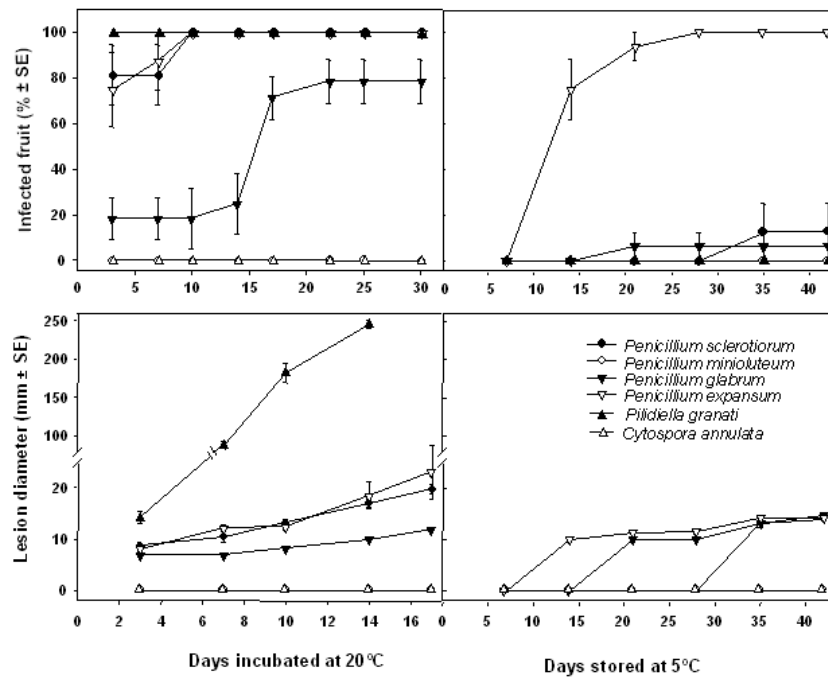


Figure 6. Pathogenicity and disease development tests. Percentage of infected fruit and lesion size on pomegranates cv. Mollar de Elche artificially inoculated with fungi isolated from naturally infected fruit. The inoculated fruits were incubated at 20°C for up to 30 d or stored at 5°C for up to 42 d. Data are means from two experiments.

the other species. Moreover, *Penicillium* spp. could infect microcracks resulting from rind dehydration and/or chilling injury during prolonged incubation at 20°C or after very long cold storage periods. We have noticed a consistent relationship between blue mold incidence on pomegranates cv. Mollar de Elche and fruit weight loss due to dehydration in recent surveys in commercial packinghouses in the Elche area (unpublished data). In any case, it seems that rind condition is an important factor to explain the pattern of fruit decay during cold storage, from the present results and those by other workers (Elyatem and Kader, 1984; Hess-Pierce and Kader, 2003).

Postharvest gray mold of pomegranate, caused by *B. cinerea*, has been reported worldwide (Kanwar and Thakur, 1972; Tedford *et al.*, 2005; Bardas *et al.*, 2009a; Pala *et al.*, 2009). As shown by the present and previous research, the disease can originate both from wound and latent infections. Conidia and mycelia contaminating fruit surfaces can infect the fruit through rind injuries or cracks, but decay originating from latent infections established before harvest is usually more important. Typically, the pathogen

infects the flowers or the crowns of young fruits, remains latent, and after harvest spreads from the crowns to the rest of the fruit, resulting in botrytis crown decay (Palou *et al.*, 2007a). In the present work, no 'nests' of infections were observed because the fruit had been packaged in plastic cavity trays that did not allow direct contact between individual fruits, although botrytis crown decay was the most frequent disease not only in the assessment of latent infections, but also on pomegranates stored at 5°C. Incidence of this disease during the first 4 months of cold storage, however, was less than that reported on pomegranates cv. Wonderful grown in California (Tedford *et al.*, 2005; Palou *et al.*, 2007a). This might be related to varietal susceptibility and/or different environmental conditions in the field that led to latent infection. In California, losses due to gray mold of up to 30% of harvested pomegranates were reported where no postharvest fungicides were applied, seriously jeopardizing the viability of the pomegranate industry during 1999 to 2002, and indicated necessity for the application of postharvest antifungal treatments (Tedford *et al.*, 2005).

Several species of the genus *Aspergillus* have been observed to cause postharvest decay in pomegranates, particularly *A. niger*, *A. flavus* L., *A. niveus* Blochwitz, *A. versicolor* (Vuill.) Tirab, *A. nidulans* (Eidam) G. Winter, and *A. clavatus* Desm. (Kanwar and Thakur, 1972; Pala et al., 2009; Jamadar et al., 2011). In agreement with our results, *Aspergillus* spp. were also found on moldy stamens of pomegranates stored at ambient temperatures (Labuda et al., 2004). *Aspergillus niger* has been typically described as a wound pathogen associated with rind microwounds, cracks or fissures that often causes secondary infections on fruit not stored at low temperatures. Since postharvest treatment with sodium hypochlorite does not kill pathogens in rind wounds (Adaskaveg, 1995), the unexpectedly high incidence of *A. niger* in surface-disinfected fruits from orchard 1 could be due to air-borne conidia that colonized rind microwounds.

Among minor fungi that were less frequently isolated, such as *Aspergillus* spp. (other than *A. niger*), *Cladosporium* spp. or *Rhizopus* spp., we decided to identify and perform pathogenicity tests with *Pi. granati* and *C. annulata* because of their specificity on pomegranates. *Pilidiella granati* was pathogenic on pomegranates cv. Mollar de Elche. Although in our disease development tests the fungus did not grow after 42 d at 5°C, we observed symptomatic pomegranates from both orchards after 25 or 27 weeks of cold storage at 5°C, especially in 2006. Furthermore, variable commercial losses on fruit stored at 5°C for periods longer than 4 months have occurred in Spanish packinghouses during recent seasons due to infection by *Pi. granati* (Palou et al., 2010). It is clear, therefore, that the fungus is able to grow at 5°C, but slowly, and disease symptoms only appear after prolonged storage periods. As was mentioned for penicillium decay, physical and physiological rind condition might be involved. There is some controversy over whether *Pi. granati* and *Coniella granati* (Sacc.) Petr. & Syd. are synonyms. Losses in pomegranate production areas have been attributed to both fungi (Tziros and Tzavella-Klonari, 2008; Michailides et al., 2010; Sharma and Tegta, 2011; Thomidis and Exadaktylou, 2011; Mirabolfathy et al., 2012). However, we have used the name *Pi. granati* in this report as per Mycobank. In contrast to *Pi. granati*, *C. annulata* was not pathogenic in our tests and no growth was observed on inoculated pomegranates cv. Mollar de Elche stored at either 20 or 5°C. We assume that at the time of isolation, the fungus was growing saprophytically in dead pome-

granate tissues, probably caused by primary infections by other fungi, or in surface debris (in the case of unwashed fruit samples). *Cytospora* spp., especially *C. punicae* Sacc. (syn.: *C. punica* Sacc.), have been described as stem, twig and branch pathogens on trees of *P. granatum*, causing cytospora canker (Saccardo, 1884; Porta-Puglia and Mifsud, 2006).

Results from the present study indicate that pomegranates cv. Mollar de Elche were not generally infected by *Alternaria* spp., as very few strains were isolated from wounded pomegranates that had not been surface disinfected. Since *alternaria* black spot and black heart affecting fruit both before and after harvest cause economic losses on pomegranates cv. Wonderful growing in the Elche area (A. Irlles, personal communication), it is possible that varietal susceptibility exists, and that cv. Mollar de Elche is naturally resistant to diseases caused by *Alternaria* spp. This would be significant for the Spanish pomegranate industry because cv. Mollar de Elche account for about 90%, and cv. Wonderful about 5%, of the plantation area in Spain. *Alternaria* spp., including *A. alternata* (Fr.) Keissl., have been described as important pomegranate pathogens in the Mediterranean countries Greece (Tziros et al., 2008) and Israel (Ezra et al., 2010), and in other areas such as California (Michailides et al., 2008) and India (Jamadar et al., 2011). These reports, however, did not identify the pomegranate cultivars affected. Another pathogen that was not isolated in the present study is *C. gloeosporioides*, the cause of pomegranate anthracnose (Jamadar et al., 2011).

This study has demonstrated that blue mold, caused by *Penicillium* spp., and gray mold, caused by *B. cinerea*, were the main postharvest diseases of pomegranate harvested from two orchards in Spain. This information can assist the Spanish pomegranate industry to implement control strategies to minimize economic losses due to postharvest diseases. In the case of gray mold, attention should be devoted to the implementation of field treatments to reduce the incidence of latent infections. In the case of blue mold, nonpolluting, postharvest antifungal treatments should be evaluated to protect the fruit during cold storage and commercial handling.

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Literature cited

- Adaskaveg J.E., 1995. Postharvest sanitation to reduce decay of perishable commodities. *Perishables Handling Newsletter* 82, 21–25.
- Artés F., J.A. Tudela and R. Villaescusa, 2000. Thermal postharvest treatments for improving pomegranate quality and shelf life. *Postharvest Biology and Technology* 18, 245–251.
- Bardas G.A., G.D. Tzelepis, L. Lotos and G.S. Karaoglanidis, 2009a. First report of *Botrytis cinerea* causing gray mold of pomegranate (*Punica granatum*) in Greece. *Plant Disease* 93, 1346.
- Bardas G.A., G.D. Tzelepis, L. Lotos and G.S. Karaoglanidis, 2009b. First report of *Penicillium glabrum* causing fruit rot of pomegranate (*Punica granatum*) in Greece. *Plant Disease* 93, 1347.
- Elyatem S.M. and A.A. Kader, 1984. Post-harvest physiology and storage behaviour of pomegranate fruits. *Scientia Horticulturae* 24, 287–298.
- Ezra D., T. Gat, Y. Skovorodnikova, Y. Vardi and I. Kosto, 2010. First report of *Alternaria* black spot of pomegranate caused by *Alternaria alternata* in Israel. *Australasian Plant Disease Notes* 5, 1–2.
- Faria A. and C. Calhau, 2011. The bioactivity of pomegranate: impact on health and disease. *Critical Reviews in Food Science and Technology* 51, 626–634.
- Hebert T.T. and C.N. Clayton, 1963. Pomegranate fruit rot caused by *Coniella granati*. *Plant Disease Reporter* 47, 222–223.
- Hess-Pierce B. and A.A. Kader, 2003. Responses of 'Wonderful' pomegranates to controlled atmospheres. *Acta Horticulturae* 600, 751–757.
- Jamadar M.M., A.R. Sataraddi, P.V. Patil, R.S. Jawadagi and D.R. Patil, 2011. Status of pomegranate diseases of Northern Karnataka in India. *Acta Horticulturae* 890, 501–507.
- Kader A.A., A. Chordas and S.M. Elyatem, 1984. Responses of pomegranates to ethylene treatment and storage temperature. *California Agriculture* 38, 14–15.
- Kanwar Z.S. and D.P. Thakur, 1972. Post-harvest fungal diseases of pomegranate in Haryana. *Indian Journal of Mycology and Plant Pathology* 2, 189–191.
- Labuda R., K. Hudec, E. Piecková, J. Mezey, R. Bohovic, S. Máteová and S.S. Lukác, 2004. *Penicillium implicatum* causes a destructive rot of pomegranate fruits. *Mycopathologia* 157, 217–223.
- Michailides T.J., D.P. Morgan, M. Quist and H. Reyes, 2008. Infection of pomegranate by *Alternaria* spp. causing black heart. *Phytopathology* 98, S105.
- Michailides T.J., R. Puckett and D.P. Morgan, 2010. Pomegranate decay caused by *Pilidiella granati* in California. *Phytopathology* 100, S83.
- Mirabolfathy M., J.Z. Groenewald and P.W. Crous, 2012. First report of *Pilidiella granati* causing dieback and fruit rot of pomegranate (*Punica granatum*) in Iran. *Plant Disease* 96, 461.
- Pala H., A. Tatli, C. Yilmaz and A.I. Özgüven, 2009. Important diseases of pomegranate fruit and control possibilities in Turkey. *Acta Horticulturae* 818, 285–289.
- Palou L., C.H. Crisosto and D. Garner, 2007a. Combination of postharvest antifungal chemical treatments and controlled atmosphere storage to control gray mold and improve storability of 'Wonderful' pomegranates. *Postharvest Biology and Technology* 43, 133–142.
- Palou L., C. Montesinos-Herrero and M.A. del Río, 2007b. Incidence of postharvest gray mold on 'Mollar de Elche' pomegranates. *Phytopathology* 97, S89.
- Palou L. and M.A. del Río, 2009. Assessment of fungal pathogens causing postharvest decay of pomegranate in South-east Spain. *Acta Horticulturae* 818, 305–311.
- Palou L., A. Guardado and C. Montesinos-Herrero, 2010. First report of *Penicillium* spp. and *Pilidiella granati* causing postharvest fruit rot of pomegranate in Spain. *New Disease Reports* 22, 21.
- Porta-Puglia A. and D. Mifsud, 2006. Fungal and fungal-like plant pathogens of the Maltese Islands. *Petria* 26, 163–256.
- Saccardo P.A., 1884. *Sylloge Fungorum. Ommium Hucusque Cognitorum*. Pata VII. Imp. Friedländer & Sohn, Berlin, Germany, 256.
- Salunkhe D.K. and B.B. Desai, 1984. *Postharvest Biotechnology of Fruits*. Vol. 2. CRC Press, Boca Raton, FL, USA, 168 pp.
- Sharma N.D. and A.C. Jain, 1978. Two new fruit rot diseases of pomegranate (*Punica granatum* L.) caused by *Coniella* spp. *Current Science* 47, 908–909.
- Sharma R.L. and R.K. Tegta, 2011. Incidence of dry rot of pomegranate in Himachal Pradesh and its management. *Acta Horticulturae* 890, 491–499.
- Snowdon A.L., 1990. *A Color Atlas of Post-harvest Diseases and Disorders of Fruits and Vegetables*. Vol. 1: General Introduction and Fruits. CRC Press, Boca Raton, FL, USA, 320 pp.
- Tedford E.C., J.E. Adaskaveg and A.J. Ott, 2005. Impact of Scholar (a new post-harvest fungicide) on the California pomegranate industry. *Plant Health Progress* doi:10.1094/PHP-2005-0216-01-PS. Online, www.plantmanagement-network.org.
- Thomidis T. and E. Exadaktylou, 2011. First report of *Pilidiella granati* on pomegranate with symptoms of crown rot in the prefecture of Xanthi, Greece. *Plant Disease* 95, 79.
- Tziros G.T. and K. Tzavella-Klonari, 2008. Pomegranate fruit rot caused by *Coniella granati* confirmed in Greece. *Plant Pathology* 57, 783.
- Tziros G.T., A.L. Lagopodi and K. Tzavella-Klonari, 2008. *Alternaria alternata* fruit rot of pomegranate (*Punica granatum*) in Greece. *Plant Pathology* 57, 379.
- White T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, ed.), Academic Press Inc., San Diego, CA, USA, 315–322.

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