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Characterisation of the fungal population in citrus packing houses

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Abstract The aim of this work was the characterisation of the environmental and superficial mycoflora of equipment and facilities of two citrus packing houses in Sao Paulo State, Brazil, in 2004 and 2005. One of the packing houses packed fruit for the export market (municipality of Matao), the other for the domestic market (municipality of Engenheiro Coelho). The study also identified the presence of isolates of *Penicillium* spp. resistant to thiabendazole and imazalil fungicides in packing houses. The environmental mycoflora was sampled according to the gravimetric method, using Petri dishes containing potato dextrose agar medium opened for 2 min. The superficial mycoflora on equipment and facilities was sampled with Rodac plates. The mycoflora in the environment and on surfaces of the packing houses in Matao were 12.3 and 52.3 cfu/plate, respectively, while these populations for the Engenheiro Coelho packing house were 46.3 and 68.2 cfu/plate, respectively. *Cladosporium* and *Penicillium* were the most prevalent genera of fungi. The contamination levels of clean zones in the packing houses (washing of fruits, packing table, boxes and containers) was not substantially lower than the contamination in dirty zones (reception of fruits and first selection). The percentage of *P. digitatum* isolates in Matao that was resistant to thiabendazole and imazalil was 25.9 and 1.5 in the environment and 30.1 and 16.0 on packing house surfaces, respectively. In Engenheiro Coelho, percentage of resistance to these fungicides was 51.9 and 0.1 in the environment and 39.2 and 0.9 on packing house surfaces, respectively.

Keywords *Citrus* · Fungicide resistance · *Penicillium digitatum*

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Introduction

Fungal diseases represent one of the main causes of post-harvest damage to citrus fruits (Dantas et al. 2003). Several factors related to the fruit itself, the pathogen, weather and post-harvest conditions determine the incidence and severity of diseases (Eckert and Eaks 1989). Among these factors, the quality and quantity of the fungal inoculum are very important. The probability of infection depends on the quantity of inoculum present in a susceptible part of the fruit. This dependence has been documented for pathogens

including *Penicillium digitatum* (Wild and Eckert 1982), *Geotrichum candidum* (Baudoin and Eckert 1982) and *Colletotrichum gloeosporioides* (Brown 1975), showing that the quantity of spores in citrus packing houses decisively influences rot levels, especially rots caused by pathogens that penetrate into damaged fruit parts, such as *P. digitatum* and *P. italicum*, responsible for green and blue mold, respectively. These are post-harvest diseases of high economic importance on citrus around the world (Tuset 1987; Eckert and Eaks 1989; Zhu et al. 2006).

Assessments of the mycoflora in Spanish packing houses during a tangerine harvest season showed the prevalence of the genera *Cladosporium* and *Penicillium*. The populations of *Penicillium* spp. increased during the harvest season, especially on surfaces of processing lines and in cold chambers, where it outnumbered the populations of *Cladosporium* spp. by 40–65% of fungal colonies (Palou et al. 2001a).

Problems deriving from high fungal populational levels in citrus packing houses are increased in the presence of fungicide-resistant pathogen isolates. The use of fungicides favours the selection and proliferation of resistant biotypes. Several packing houses process fruits throughout the year, and the use of fungicides can result in continuous selection pressure on the population of pathogens (Holmes and Eckert 1999). Studies report that isolates of *P. digitatum* and *P. italicum* are resistant to fungicides belonging to the benzimidazole, imidazole and orthophenilphenate fungicide groups and describe not only cases of simple resistance but also cases of cross- and multiple-resistance (Brown 1982; Schmidt et al. 2006; Zhu et al. 2006). Samples from the environment of Spanish packing houses showed that 33% of isolates of *Penicillium* spp. were resistant to thiabendazole, from the benzimidazole group, and 5% to imazalil, from the imidazole group (Palou et al. 2001a). When isolates were sampled from packing house equipment and facility surfaces, 35% and 20% were resistant to thiabendazole and imazalil, respectively. Resistant isolates of *P. digitatum* and *P. italicum* were found, although at low frequencies when compared to other resistant *Penicillium* species.

New concepts of integrated production and alternative control methods have been developed to address concerns about the application of synthetic fungicides to control post-harvest diseases in relation to potential detrimental effects of fungicides on health and the environment. Such methods include fungicide application in the field, treatments to maintain or improve the intrinsic fruit resistance to infection and the correct sanitisation of packing houses (Eckert and Eaks 1989). Identifying and quantifying the fungal contamination in different parts of the packing house, as well as studying the presence of resistant isolates, is important to establish the potential risks and project appropriate control systems. Identifying the areas of higher contamination allows for cleaning and disinfestation programmes to be adjusted to target these areas. It also can raise the interest in constructing new packing houses or in expanding the current infrastructure (Gardner et al. 1986).

The purpose of this work was to identify and quantify the fungal population in the environment, on equipment and on surfaces of facilities in citrus packing houses in Sao Paulo State, as well as to determine the presence of isolates of *Penicillium* spp. resistant to thiabendazole and imazalil fungicides commonly used in post-harvest control.

Materials and methods

Sampling of the mycoflora and isolates of *Penicillium* spp. resistant to the studied fungicides was carried out in two packing houses located in Sao Paulo State, Brazil. In one of them, located in Matao municipality, where fruits of Valencia and Westin sweet oranges and Murcott tangor destined for the international market are packed, sampling was carried out at 14-day intervals, from July to November 2004 and 2005. In the other packing house, located in Engenheiro Coelho municipality, where fruits of Pera, Lima and Natal sweet oranges and Murcott tangor destined for the domestic market are packed, sampling was carried out at 14-day intervals from September to January 2004/05 and 2005/06.

The identification and quantification of the mycoflora and of isolates of *Penicillium* spp. resistant to thiabendazole and imazalil were carried out in the University of Sao Paulo, Piracicaba, Brazil.

Characterisation of the mycoflora in packing houses

The mycoflora of the packing houses were sampled at the following points: reception or fruit arrival from the field, first manual selection of fruits, after fruit

washing, degreening chambers, after the application of wax, packing tables and containers. The following surfaces of the processing lines were sampled: fruit reception conveyor, post-washing conveyor, degreening chambers, degreening bins, post-waxing conveyors, packing tables, packers' gloves and containers. Sampling during the first manual selection of fruits, on degreening bins and chambers, as well as on containers, was carried out only in the packing house servicing the external market (Matao/SP), once such stages were not part of the procedures followed in the packing house where fruits were destined for the domestic market (Engenheiro Coelho/SP). However, in the Engenheiro Coelho packing house, the surface of reusable wooden boxes was sampled. This kind of sampling was not conducted in the packing house where fruits were destined for the external market because such fruits are packed in non-reusable cardboard boxes.

The environmental mycoflora in each zone were sampled according to the gravimetric method. Five 9 cm diam Petri dishes (replicates) containing potato dextrose agar (PDA) medium containing 0.4 g l^{-1} veterinary Pentabiotic (Fort Dodge®) at the molten state were equidistantly distributed through each zone and left open for 2 min to allow fungal spores to set on the culture medium by gravity. When possible, a Petri dish was placed in each extremity and in the middle of the degreening chamber and container. Surfaces were sampled with 5.5 cm diam Rodac (Replicant Organism Direct Agar Contact) plates containing PDA medium added to 0.4 g l^{-1} veterinary Pentabiotic, by contact between the culture medium and the surface, with slight pressure applied to keep spores adhering to the medium. Five Rodac plates (replicates) were used in the degreening bins, degreening chamber and container and three plates were used for the other stages of the processing line (Palou et al. 2001a).

Plates were incubated at 20°C, with a 12 h photoperiod, for 5 to 7 days, after which counts and identification of the fungal colonies were carried out. Fungi were identified to genus, except for *P. digitatum* (Webster 1980; Ellis 1993; Samson et al. 1995). The frequency of each fungal genus was expressed in number of colony forming units (cfu/plate).

For the statistical analysis, frequencies of the most prevailing fungal genera and the total fungal population were transformed to $\sqrt{x+0.5}$ and means were compared by Tukey test ($P<0.05$), using the SAS statistical software package (SAS 1989), in relation to the different packing house zones and surfaces sampled, considering the mean of each sampling procedure as a replicate.

Determination of isolates of *Penicillium* spp. resistant to fungicides

The presence of isolates of *Penicillium* spp. resistant to thiabendazole and imazalil fungicides in the environment and surfaces of the facilities and infrastructure of the packing houses was determined together with the mycoflora sampling. For sample collection, Petri dishes and Rodac plates containing four distinct culture media were prepared by addition of Pentabiotic (0.4 g l^{-1}): PDA, PDA containing 10 mg l^{-1} of the active ingredient (ai) thiabendazole, PDA containing 1 mg ai l^{-1} of imazalil and PDA containing both fungicides at the same rates. The fungicide doses used correspond to the experimentally determined resistant doses, under which only resistant isolates can grow (Brown 1990; Holmes and Eckert 1999; Zhu et al. 2006). Thiabendazole and imazalil, as well as Pentabiotic, were added to the molten PDA medium inside a laminar flow chamber. Sampling and incubation of dishes were carried out as previously described, but using three replicates for the Petri dishes, which remained open for 4 min. Environment and surface sampling were conducted at the arrival of fruits, in the degreening chamber and on the packing table. Three fungal groups were visually identified during the count procedure by means of their colour and pathogenicity to citrus fruits: *P. digitatum*, *P. italicum*, and other *Penicillium* spp. Frequencies of resistant isolates were transformed to $\sqrt{x+0.5}$ and submitted to analysis of variance (ANOVA). Means were separated using Tukey test ($P<0.05$), by SAS statistical package (SAS 1989), in order to determine the differences between the zones and surfaces sampled.

Penicillium digitatum isolates grown on PDA medium containing 10 mg l^{-1} of a.i. thiabendazole (eight isolates from Matao and ten from Engenheiro Coelho) and on PDA medium without fungicides (eight isolates from Matao and six from Engenheiro Coelho) were replicated and purified in dishes containing PDA medium. Petri dishes containing 0, 1, 10, 100 and 1,000 mg l^{-1} of a.i. thiabendazole in PDA were prepared and, after cooling, received a

0.5 cm diam disc of water agar (WA) with approximately 10^4 conidia of *P. digitatum* on its surface. These were incubated at 25°C with a 12 h photoperiod. After 7 days, a perpendicular measurement of colony diameter was carried out, determining the percentage of inhibition for each treatment in relation to the control. The linear regression for the percent inhibition versus the fungicide concentration was calculated and the fungicide concentration necessary to inhibit mycelial growth by 50% (ED_{50}) was determined by interpolation (Holmes and Eckert 1999). Three Petri dishes were used for each culture medium and *P. digitatum* isolate. Differences in the mycelial growth between the *P. digitatum* isolates at the different thiabendazole concentrations were verified through ANOVA (Tukey, $P<0.05$), by SAS statistical package (SAS 1989), using the inhibition percentage data transformed to *arc sin* $\sqrt{x/100}$.

In order to assess the *in vivo* resistance of citrus, eighteen *P. digitatum* isolates presenting distinct growth patterns according to the thiabendazole dose, were inoculated in sweet Pera orange fruits previously disinfested with 70% alcohol and sprinkled with a thiabendazole (1 ml l^{-1}) suspension + silicone-polyester copolymer (1 ml l^{-1}) surfactant. Inoculum was introduced into 0.3 mm deep injuries using a set of three histological sterilised needles below the point where a 40 µl *P. digitatum* conidial suspension (10^6 conidia ml^{-1}) had been previously spread on the medial portion of the fruit to be inoculated (Fischer et al. 2004). For each *P. digitatum* isolate, 20 fruits were used, half of which were not treated with fungicide. Fruits were individualised in plastic trays and kept in a humid chamber at 25°C for 24 h. After removal of the humid chamber , fruits were kept for 5 days at 25°C and 85% relative humidity (RH). The number of fruits showing disease, as well as the perpendicular diameter of injuries, were assessed 6 days after inoculation.

Results

Fungal population in the environment and on surfaces of Matao packing house

The average number of fungal colonies per Petri dish isolated from the Matao packing house environment in 2004 and 2005 was 11.9 and 12.8, respectively. Among the fungal genera isolated from the colonies, 39.3% were *Cladosporium*, 38.1% *Penicillium*, from which 11.6% was *P. digitatum*, 4.5% *Fusarium*, 3.5% *Epicoccum*, 2.2% *Aureobasidium*, 1.7% *Aspergillus*, 1.4% *Alternaria* and the 9.3% remaining genera were *Rhizopus*, *Mucor*, *Geotrichum*, *Trichoderma*, *Humicola*, *Neurospora* and others (data not shown).

The total environment mycoflora (cfu/Petri dish) was significantly higher in the fruit selection zone, when compared to the waxing, arrival, packing table and degreening chamber zones. The washing zone and container showed intermediate values of cfu/Petri dish (Table 1). *Cladosporium* was more frequent (cfu/Petri dish) in the selection zone and container. Although the frequency of *P. digitatum* was statistically similar in the different sampled zones, it was numerically higher in the washing zone, as opposed to other *Penicillium* species, which were more frequently

Table 1 Colonies (cfu/plate) of fungal genera present in the environment of different zones of the Matao (SP) packing house

Zones	Fungal population[a]							
	Cladosporium	*Penicillium* spp.[b]	*Penicillium digitatum*	*Fusarium*	*Aureobasidium*	*Epicoccum*	Others	Total
Arrival	3.0 b	1.9 ab	0.7 a	0.6 a	0.2 a	0.8 a	1.4 a	8.6 b
Selection	12.7 a	2.6 ab	2.7 a	0.8 a	1.1 a	0.6 a	1.9 a	22.4 a
Washing	4.0 b	1.4 b	3.8 a	0.5 a	0.2 a	0.5 a	1.5 a	11.9 ab
Wax	2.5 b	2.2 ab	0.7 a	0.2 a	0.1 a	0.2 a	1.2 a	7.1 b
Table	3.0 b	3.2 ab	0.5 a	0.3 a	0.1 a	0.5 a	1.1 a	8.7 b
Chamber	4.1 b	2.5 ab	1.0 a	1.3 a	0.1 a	0.2 a	1.1 a	10.3 b
Container	4.5 ab	9.0 a	0.6 a	0.2 a	0.1 a	0.2 a	2.4 a	17.0 ab
Mean	4.8	3.3	1.4	0.6	0.3	0.4	1.5	12.3

Means followed by the same letter within columns are not significantly different (Tukey, $P<0.05$)

[a] Means of 14 replicates, ten in 2004 and four in 2005

[b] Species of *Penicillium* spp., with the exception of *P. digitatum*

observed in the container and less frequently observed in the washing zone.

For the surface sampling, the average numbers of fungal colonies per Rodac plate, considering all zones sampled in 2004 and 2005, were 54.5 and 50.1, respectively. Among the fungal genera isolated from the colonies, 64.9% were *Cladosporium*, 14.4% *Penicillium*, from which 2.1% was *P. digitatum*, 4.5% *Fusarium*, 2.9% *Aureobasidium*,, 2.8% *Epicoccum*, 1.5% *Trichoderma*, 1.2% *Geotrichum* and the 7.8% remaining genera were *Aspergillus*, *Alternaria*, *Rhizopus*, *Mucor*, *Humicola*, *Neurospora* and others (data not shown).

The highest number of fungal propagules (cfu/Rodac plate) was isolated from degreening bins and chamber and from the fruit reception zone. The lowest population was sampled from glove surfaces, washing and waxing zones (Table 2). The *Cladosporium* genus was most frequently found in degreening bins, followed by the fruit reception zone, degreening chamber and packing table, while the *P. digitatum* genus, as observed in the environment sampling, did not differ between the zones sampled. The other *Penicillium* species were more frequently found in the container, where their population surpassed the *Cladosporium* population, as well as in the degreening chamber.

The number of fungal propagules in the degreening bins was 30% lower in the second year, because of the change to washing the bins with benzalkonium chloride (0.5 g l^{-1}) before degreening. The bins used for fruit harvest were the same as used in the degreening stage.

Fungal population in the environment and on the surfaces at Engenheiro Coelho packing house

The average number of fungal colonies per Petri dish isolated from the Engenheiro Coelho packing house environment in 2004 and 2005 was 50.9 and 41.7, respectively. Among the fungal genera isolated from the colonies, 37.3% were *Cladosporium*, 50.2% *Penicillium*, from which 13.6% was *P. digitatum*, 2.8% *Fusarium*, 1.5% *Trichoderma*, 1.3% *Epicoccum*, 1.2% *Geotrichum*, 0.7% *Aureobasidium*, while the 5.1% remaining genera were *Alternaria*, *Aspergillus*, *Rhizopus*, *Mucor*, *Neurospora* and others (data not shown).

The total environment mycoflora, as well as the population of *Cladosporium* and *Penicillium* did not differ significantly among the packing house zones sampled (Table 3). *Penicillium (P. digitatum* and other *Penicillium* spp.) were the most common genera in the zones sampled, except for the packing table zone, where the *Cladosporium* genus was more frequently observed.

The average numbers of fungal colonies per Rodac plate isolated from the packing house surfaces, considering all the zones sampled were 76.1 and 60.3 in 2004 and 2005, respectively. Among the fungal genera isolated from the colonies, 55.4% were *Cladosporium*, 15.9% *Geotrichum*, 13.9% *Penicillium*, from which

Table 2 Colonies (cfu/plate) of fungal genera present on the surfaces of different zones of the Matao (SP) packing house

Zones	Fungal population[a]							
	Cladosporium	*Penicillium* spp.[b]	*Penicillium digitatum*	*Fusarium*	*Aureobasidium*	*Epicoccum*	Others	Total
Reception	50.4 ab	4.2 ab	1.4 a	3.4 a	3.4 a	1.3 ab	5.9 a	70.0 ab
Washing	10.2 bc	2.5 b	1.7 a	1.2 a	1.3 a	0.4 b	6.0 a	23.3 bc
Wax	13.7 bc	2.6 b	0.5 a	2.1 a	0.2 a	1.4 ab	4.5 a	25.0 bc
Table	47.4 ab	3.9 ab	1.2 a	2.6 a	1.1 a	2.6 ab	5.1 a	63.9 abc
Chamber	49.6 ab	9.7 ab	1.3 a	2.1 a	0.3 a	0.7 b	6.5 a	70.2 ab
Container	4.8 c	23.1 a	0.9 a	2.9 a	0.1 a	0.2 b	1.8 a	33.8 bc
Bin	92.4 a	3.2 b	1.4 a	3.7 a	5.6 a	4.8 a	5.0 a	116.1 a
Glove	3.0 c	2.4 b	0.3 a	0.9 a	0.3 a	0.1 b	8.8 a	15.8 c
Mean	33.9	6.5	1.1	2.4	1.5	1.4	5.5	52.3

Means followed by the same letter within columns are not significantly different (Tukey, $P<0.05$)

[a] Means of 14 replicates, ten in 2004 and four in 2005

[b] Species of *Penicillium* spp., with the exception of *P. digitatum*

Table 3 Colonies (cfu/plate) of fungal genera present in the environment of different zones of the Engenheiro Coelho (SP) packing house

Zones	Fungal population[a]							
	Cladosporium	*Penicillium* spp.[b]	*Penicillium digitatum*	*Fusarium*	*Trichoderma*	*Epicoccum*	Others	Total
Arrival	17.7 a	14.3 a	8.6 a	0.9 a	0.3 a	0.8 a	2.7 a	45.3 a
Washing	22.2 a	16.6 a	7.6 a	0.8 a	0.3 a	0.8 a	4.9 a	53.2 a
Wax	7.9 a	20.2 a	6.1 a	0.7 a	0.9 a	0.5 a	2.3 a	38.6 a
Table	21.3 a	16.7 a	2.9 a	2.8 a	1.2 a	0.3 a	2.9 a	48.1 a
Mean	17.3	17.0	6.3	1.3	0.7	0.6	3.2	46.3

Means followed by the same letter within columns are not significantly different (Tukey, $P<0.05$)

[a] Means of 11 replicates, five in 2004 and six in 2005

[b] Species of *Penicillium* spp., with the exception of *P. digitatum*

6.8% was *P. digitatum*, 4.0% *Aureobasidium*, 3.1% *Fusarium*, 2.3% *Epicoccum*, 1.2% *Trichoderma* and the 4.2% remaining genera were *Rhizopus*, *Mucor*, *Aspergillus*, *Alternaria*, *Neurospora* and others (data not shown).

The highest fungal population (cfu/Rodac plate) was isolated from wooden packing boxes (internal market), followed by the fruit reception zone. The lowest population was sampled from glove surfaces and waxing zones (Table 4). The *Cladosporium* genus was most frequently found in packing boxes, followed by the fruit reception zone, packing table and washing zone, while the *Geotrichum* genus was most frequently found on gloves and in the after-washing zone. *Penicillium digitatum* populations, as observed in the environment sampling, did not differ between the zones sampled, while the other *Penicillium* species were mainly found in packing boxes.

Isolates of *Penicillium* spp. resistant to fungicides

The average percentages of *P. digitatum* isolates resistant to thiabendazole in the environment and on surfaces of the Matao packing house were 26.7 and 30.7, respectively and the corresponding average frequencies were 0.71 and 0.53 cfu/plate (Table 5). The average percentages of *P. digitatum* isolates resistant to imazalil in the environment and on surfaces were 1.5 and 17.0, respectively, and the corresponding average frequencies were 0.04 and 0.29 cfu/plate. No isolates of *P. digitatum* resistant to both fungicides simultaneously were found. The average percentages of other isolates of *Penicillium* spp. resistant to thiabendazole in the environment and surfaces were 7.0 and 12.5, respectively, while the corresponding figures for imazalil were 57.0 and 68.3. The frequency of isolates of *Penicillium* spp. resistant to the fungicides

Table 4 Colonies (cfu/plate) of fungal genera present on the surfaces of different zones of the Engenheiro Coelho (SP) packing house

Zones	Fungal population[a]							
	Cladosporium	*Penicillium* spp.[b]	*Penicillium digitatum*	*Fusarium*	*Geotrichum*	*Aureobasidium*	Others	Total
Reception	61.9 ab	5.3 ab	7.3 a	3.2 a	9.9 ab	9.1 a	5.6 a	102.3 ab
Washing	26.4 bc	2.0 b	5.1 a	1.6 a	17.8 a	3.3 ab	2.4 a	58.6 bc
Wax	4.9 c	4.2 ab	4.0 a	1.2 a	9.4 ab	0.3 ab	2.3 a	26.3 c
Table	41.7 bc	2.5 ab	3.9 a	1.2 a	4.2 ab	0.7 ab	5.5 a	59.7 bc
Box	82.4 a	14.6 a	2.8 a	1.6 a	0.3 b	2.8 ab	11.1 a	115.6 a
Glove	9.5 c	0.3 c	4.8 a	4.0 a	23.5 a	0.0 b	4.8 a	46.9 c
Mean	37.8	4.8	4.7	2.1	10.9	2.7	5.3	68.2

Means followed by the same letter within columns are not significantly different (Tukey, $P<0.05$)

[a] Means of 11 replicates, five in 2004 and six in 2005

[b] Species of *Penicillium* spp., with the exception of *P. digitatum*

Table 5 Frequency of *Penicillium* spp. resistant to thiabendazole (TBZ) and imazalil (IZ) in the environment and surfaces of the Matao packing house

Penicillium isolates	Treatment (environment)	Frequency (Petri dish)	Stages of the processing line[a]			
			Reception	Table	Chamber	Mean
Penicillium digitatum	PDA	cfu/dish	5.62 a	1.40 ab	0.98 a	2.67
	PDA+TBZ	cfu/dish	1.34 a	0.71 a	0.09 a	0.71
		%	23.8	50.5	9.4	26.7
	PDA+IZ	cfu/dish	0.08 a	0.05 a	0.00 a	0.04
		%	1.4	3.3	0.0	1.5
	PDA+TBZ+IZ	cfu/dish	0.00 a	0.00 a	0.00 a	0.00
		%	0.0	0.0	0.0	0.0
Other *Penicillium* spp.	PDA	cfu/dish	5.67 a	3.80 a	5.45 a	4.97
	PDA+TBZ	cfu/dish	0.31 a	0.42 a	0.32 a	0.35
		%	5.4	11.0	5.9	7.0
	PDA+IZ	cfu/dish	4.70 a	2.20 a	1.60 a	2.83
		%	82.9	57.9	29.4	57.0
	PDA+TBZ+IZ	cfu/dish	0.06 a	0.00 a	0.05 a	0.04
		%	1.0	0.0	0.8	0.7
	(surface)	Rodac dish				
Penicillium digitatum	PDA	cfu/dish	1.71 a	1.61 a	1.84 a	1.72
	PDA+TBZ	cfu/dish	0.65 a	0.90 a	0.04 a	0.53
		%	37.8	55.8	2.0	30.7
	PDA+IZ	cfu/dish	0.14 a	0.37 a	0.37 a	0.29
		%	8.1	22.9	20.1	17.0
	PDA+TBZ+IZ	cfu/dish	0.00 a	0.00 a	0.00 a	0.00
		%	0.0	0.0	0.0	0.0
Other *Penicillium* spp.	PDA	cfu/dish	4.08 a	4.90 a	5.00 a	4.66
	PDA+TBZ	cfu/dish	0.66 a	0.98 a	0.11 a	0.58
		%	16.2	20.0	2.2	12.5
	PDA+IZ	cfu/dish	3.50 a	2.60 a	3.45 a	3.18
		%	85.8	53.1	69.0	68.3
	PDA+TBZ+IZ	cfu/dish	0.12 a	0.07 a	0.07 a	0.09
		%	2.9	1.4	1.4	1.9

Means followed by the same letter within rows are not significantly different (Tukey, $P<0.05$)

[a] Means of nine replicates, five in 2004 and four in 2005

tested did not differ between the zones and surfaces sampled in the Matao packing house (Table 5).

The average percentages of *P. digitatum* isolates resistant to thiabendazole in the environment and on surfaces of the Engenheiro Coelho packing house were 50.1 and 38.4, respectively, and the corresponding average frequencies were 4.51 and 2.36 cfu/plate (Table 6). The average percentages of *P. digitatum* resistant to imazalil in the environment and on surfaces were 0.1 and 1.0, respectively, and the corresponding average frequencies were 0.01 and 0.06 cfu/plate. No isolates of *P. digitatum* resistant to both fungicides simultaneously were found. The average percentages of other isolates of *Penicillium* spp. resistant to thiabendazole in the environment and on surfaces were 16.1 and 61.6, respectively, while the corresponding figures for imazalil were 67.4 and 43.2. The frequency of other *Penicillium* spp. resistant to thiabendazole was higher in the environment and on the surfaces of packing tables when compared to the fruit reception zone. Higher frequencies of other isolates of *Penicillium* spp. resistant to imazalil were observed on the conveyor surfaces in the fruit reception zone, when compared to the surfaces of the packing tables.

Isolates of *P. italicum* resistant to either fungicide were very seldom observed. Only two isolates of *P. italicum* resistant to thiabendazole were found in each packing house and one isolate resistant to imazalil

Table 6 Frequency of *Penicillium* spp. resistant to thiabendazole (TBZ) and imazalil (IZ) in the environment and surfaces of the Engenheiro Coelho packing house

Penicillium isolates	Treatament (environment)	Frequency (Petri dish)	Stages of the processing line[a]		
			Reception	Table	Mean
Penicillium digitatum	PDA	cfu/dish	12.21 a	5.81 a	9.01
	PDA+TBZ	cfu/dish	5.40 a	3.62 a	4.51
		%	44.3	62.2	50.1
	PDA+IZ	cfu/dish	0.02 a	0.00 a	0.01
		%	0.2	0.0	0.1
	PDA+TBZ+IZ	cfu/dish	0.00 a	0.00 a	0.00
		%	0.0	0.0	0.0
Other *Penicillium* spp.	PDA	cfu/dish	20.01 a	18.21 a	19.11
	PDA+TBZ	cfu/dish	1.62 b	4.54 a	3.08
		%	8.1	24.9	16.1
	PDA+IZ	cfu/dish	13.62 a	12.15 a	12.88
		%	68.1	66.7	67.4
	PDA+TBZ+IZ	cfu/dish	0.07 a	0.02 a	0.05
		%	0.4	0.1	0.3
	(surface)	Rodac dish			
Penicillium digitatum	PDA	cfu/dish	7.73 a	4.54 a	6.14
	PDA+TBZ	cfu/dish	2.75 a	1.96 a	2.36
		%	35.6	43.2	38.4
	PDA+IZ	cfu/dish	0.08 a	0.05 a	0.06
		%	1.0	1.0	1.0
	PDA+TBZ+IZ	cfu/dish	0.00 a	0.00 a	0.00
		%	0.0	0.0	0.0
Other *Penicillium* spp.	PDA	cfu/dish	4.96 a	6.23 a	5.60
	PDA+TBZ	cfu/dish	1.34 b	5.55 a	3.44
		%	27.0	89.1	61.6
	PDA+IZ	cfu/dish	3.86 a	0.97 b	2.41
		%	77.8	15.5	43.2
	PDA+TBZ+IZ	cfu/dish	0.14 a	0.00 a	0.07
		%	2.7	0.0	1.2

Means followed by the same letter within rows are not significantly different (Tukey, $P<0.05$)

[a] Means of nine replicates, four in 2004 and five in 2005

was found in the Engenheiro Coelho packing house (data not shown).

Isolates of *P. digitatum* classified as resistant and subjected to different doses of thiabendazole showed a mycelial growth inhibition <70% at 10 mg l^{-1} (ED_{50}=30.3±28.4) (Table 7). Among isolates collected from fungicide-free medium, the six most sensitive ones showed >70% mycelial growth inhibition under fungicide doses as low as 1 mg l^{-1} (Table 8). The other *P. digitatum* isolates, although collected from fungicide-free medium, showed similar behaviour to resistant isolates and showed a mycelial growth inhibition < 50% at 10 mg l^{-1} (ED_{50}=38.7±16.0).

The six *P. digitatum* isolates most sensitive to thiabendazole (Table 8) showed average fruit disease incidence of 26.7% and 5.6 cm diam lesions when inoculated in Pera sweet oranges previously treated with the fungicide. The other twelve isolates classified as resistant showed average fruit disease incidence of 94.2% and 8.1 cm lesions. All *P. digitatum* isolates caused 100% incidence of green mold in fruits not treated with thiabendazole, inducing lesion diameters >8.0 cm 6 days after inoculation.

Discussion

Results must be analysed taking the sampling methods into consideration. Different rates of germination and mycelial growth of the spores collected may mask the

Table 7 Percent inhibition by thiabendazole of the mycelial growth of *Penicillium digitatum* strains isolated from PDA+ thiabendazole medium

Isolates[a]	Doses of thiabendazole (mg l^{-1}) in PDA				ED_{50}
	1	10	100	1000	
M-1	47.9 a	68.1 a	93.2 ab	96.0 ab	1.9
EC-1	26.3 ab	53.8 ab	87.1 ab	84.2 abc	25.5
EC-2	22.8 ab	40.6 bcd	80.5 ab	74.2 c	40.7
EC-3	22.2 ab	42.4 abcd	79.3 b	80.8 bc	40.7
EC-4	19.5 ab	45.7 abcd	83.4 ab	84.3 abc	37.7
M-2	14.5 abc	46.3 abcd	100.0 a	100.0 a	28.1
EC-5	12.1 abc	45.3 abcd	82.7 ab	85.5 abc	41.7
EC-6	11.0 bcd	41.1 abcd	83.5 ab	86.6 abc	43.7
EC-7	10.9 bcd	50.7 abc	85.3 ab	83.4 abc	38.3
EC-8	10.9 bcd	45.8 abcd	80.2 ab	82.5 abc	43.2
EC-9	10.5 bcd	43.1 abcd	83.9 ab	83.9 abc	42.7
EC-10	6.3 bcd	41.9 abcd	82.9 ab	89.7 abc	38.9
M-3	5.1 cd	34.9 bcd	96.9 ab	85.3 abc	49.0
M-4	4.5 cd	31.3 bcd	92.2 ab	94.0 ab	45.7
M-5	−7.3 cd	22.3 d	90.2 ab	96.8 ab	53.0
M-6	−10.8 d	46.3 abcd	100.0 a	94.6 ab	41.3
M-7	−11.2 d	24.3 cd	89.5 ab	87.0 abc	53.3
M-8	−12.2 d	24.7 cd	79.4 b	92.2 abc	58.7
Mean	10.2	41.6	87.2	87.8	40.2
C.V. (%)	32.7	15.3	4.5	4.3	

Means followed by the same letter within columns are not significantly different (Tukey, $P<0.05$)

[a] *P. digitatum* isolates collected from Matao (M) and Engenheiro Coelho-SP (EC) packing houses

Table 8 Percent inhibition by thiabendazole of the mycelial growth of *Penicillium digitatum* strains isolated from PDA medium

Isolates[a]	Doses of thiabendazole (mg l^{-1}) in PDA				ED 50
	1	10	100	1000	
M-1	100.0 a	100.0 a	100.0 a	100.0 a	<1
M-2	100.0 a	100.0 a	100.0 a	100.0 a	<1
M-3	100.0 a	100.0 a	100.0 a	100.0 a	<1
M-4	100.0 a	100.0 a	100.0 a	100.0 a	<1
EC-1	83.3 ab	100.0 a	100.0 a	100.0 a	0.6
EC-2	71.5 ab	71.4 b	98.7 a	100.0 a	0.7
EC-3	41.4 bc	43.4 cd	84.2 b	81.8 b	22.7
EC-4	17.5 cd	48.3 c	86.0 b	87.7 b	36.1
EC-5	15.2 cde	44.4 cd	82.4 b	88.4 b	41.1
EC-6	13.6 cde	35.6 cd	81.1 b	83.5 b	47.2
M-5	10.1 cde	35.0 cd	99.2 a	100.0 a	39.2
M-6	3.4 de	32.9 cd	91.2 ab	97.5 a	45.9
M-7	−4.1 de	40.1 cd	88.0 ab	87.1 b	46.7
M-8	−5.9 e	28.6 d	82.0 b	84.8 b	54.7
Mean[b]	11.4	38.5	86.8	88.9	41.7
C.V. (%)	20.39	5.6	2.3	1.4	

Means followed by the same letter within columns are not significantly different (Tukey, $P<0.05$)

[a] *P. digitatum* isolates collected from Matao (M) and Engenheiro Coelho-SP (EC) packing houses

[b] Mean of EC-3 to M-8

presence of some fungal genera (Palou et al. 2001a). Although the quantity of fungal inoculum decisively influences the incidence of diseases, other factors, such as the intrinsic susceptibility of fruits to infections as well as the environmental conditions before and during the harvest season, also play an important role and prevent the establishment of a quantitative relation between the levels of fungal population in packing houses and the severity of rot (Palou et al. 2001a; Lennox et al. 2003; Fischer 2007). There is no general criterion that enables us to distinguish the critical limits of fungal contamination from which there is an inadmissible high risk of rot incidence. However, it is possible to define, based on fungal populational levels, clean and dirty zones, as well as to establish critical limits that enable the evaluation of the efficiency of sanitisation procedures in packing houses (Gardner et al. 1986; Sus and Viñas 1990; Orihuel et al. 1996, Palou et al. 2001a).

The fungal populational level in the two packing houses analysed was generally high in most of the zones sampled. The average fungal population frequency on the packing house surfaces was >50 cfu/plate, which corresponds to 2.1 cfu cm^{-2} when considering the use of 5.5 cm diam Rodac plates. This is higher than the critical upper limit of 0.7 cfu cm^{-2} proposed by Orihuel et al. (1996) as the maximum concentration following sanitisation. Figures >40 cfu/Rodac plate were found in the mycoflora sampling of Spanish packing houses (Palou et al. 2001a).

Sanitisation procedures were very different between the packing houses sampled. In the Matao packing house, washing of the processing line conveyors and the fruit reception zone was carried out daily, while in the Engenheiro Coelho packing house, where rotting fruits were frequently observed on the processing line, it was carried out only once a week.

Cladosporium was the most common genus in the Matao packing house and on the surfaces of the Engenheiro Coelho packing house, while *Penicillium* was the most prevailing genus in the environment of the Engenheiro Coelho packing house. Such results corroborate those found by Palou et al. (2001a, b), which showed that *Cladosporium* and *Penicillium* were the most frequent genera found in Spanish tangerine orchards and packing houses. Their study also showed that *Penicillium* increases during the crop season, suggesting, according to the authors, that the origin of fruit contamination in packing houses is the crop field. The *Penicillium* population, as well as the whole fungal population, varied in the packing houses throughout the sampled months (data not shown). The mycoflora composition in the packing houses depends on the local conditions of the producing region (Brown 1990). In Spain, the total mycoflora and the *Penicillium* population also show great variability, showing significant interactions among seasons, packing houses and orchards. There is also a positive correlation between the total mycoflora and local temperature (Palou et al. 2001a, b). It is difficult to analyse the influence of temperature due to the short sampling period in the packing houses studied. Comparative studies between summer and winter in Sao Paulo State, when temperature and RH figures show great discrepancy, may offer more conclusive results about the weather. In Turkey, a higher variability in the environmental mycoflora was observed in summer, followed by fall, winter and spring (Simsekli et al. 1999).

Contamination of clean zones (after-washing zone, packing table, boxes and containers) was not significantly lower than dirty zones (fruit arrival or reception zone and first manual selection zone). Such results, similar to those found by Palou et al. (2001a), are probably due to the outdoor environment of the processing line.

In order to minimise the rot incidence in citrus packing houses, especially if the intention is to work with an integrated production system, it is well to consider the effective physical separation of fruits arriving from the crop field from processed fruits. It is also necessary to optimise the selection process of injured fruits and the sanitisation of the processing line, introducing disinfestation procedures of harvest baskets and facilities (equipment, chambers, floor etc), using chlorine and quaternary ammonium products. Fruits also need to be protected by preventive fungicide applications. According to Gardner et al. (1986), the correct sanitisation procedures are effective in reducing rot incidence and avoiding the proliferation of fungal isolates resistant to fungicides. It is also more economical than increasing the use of fungicides.

The *Cladosporium herbarum* species is described as a post-harvest pathogen of minor importance in citrus (Tuset 1987). Its presence in degreening and cold chambers was verified in Spanish citrus packing houses by Díaz and Vila (1988). Due to the great colony variability found in the present work and the

low importance of this pathogen to citrus, it was decided not to characterise the *Cladosporium* species found in this study.

Another interesting result was the high populational levels of *Geotrichum*, which showed an average of 10.9 cfu/Rodac plate in the Engenheiro Coelho packing house, mainly on gloves and after-washing zone. Eckert and Brown (1986) and Eckert (1993) stated that sour rot, caused by *G. candidum*, was the second most important post-harvest disease in citrus, after molds, with infection through injuries, and is one of the most serious problems affecting lemons in California (Eckert and Brown 1986). An effective control requires intense prophylaxis involving hygiene and disinfestation of the whole processing line and workers' gloves.

Sampling showed a generalised presence of isolates of *Penicillium* spp. resistant to thiabendazole and imazalil, more commonly observed in the Engenheiro Coelho packing house, probably due to poorer sanitisation levels of the processing line. *Penicillium digitatum* isolates resistant to imazalil were generally less frequent than isolates resistant to thiabendazole, probably because benzimidazoles (thiophanate-methyl and carbendazime), which show a similar mode of action to this fungicide, have been commonly used in citrus orchards in Sao Paulo State for a long time to control blossom blight (*Colletotrichum acutatum*) and citrus black spot (*Guignardia citricarpa*). There is a low to moderate risk of pathogens becoming resistant to imazalil (Eckert 1987; Feichtenberger et al. 2005). Resistant *P. digitatum* isolates have become common in Japanese orchards, where benzimidazoles have been employed for many years for pre-harvest control (Kumaroto 1976). Most isolates of *Penicillium* resistant to imazalil were not *P. digitatum* or *P. italicum* species, which agrees with results from Palou et al. (2001a). Díaz and Vila (1989) described *P. variabile*, *P. steckii* and *P. velutinum* isolates, and less commonly *P. digitatum* and *P. italicum* isolates, showing cross-resistance to imazalil and prochloraz, both of which belong to the imidazole group. The low occurrence of *P. italicum* isolates resistant to the studied fungicides may be due to the low population of *P. italicum* in the packing houses, as well as the high sensitivities of this species to the fungicides and its low parasitic adaptability and reproductive capability when compared to *P. digitatum* (Holmes and Eckert 1999).

The frequency of *P. digitatum* isolates resistant to the fungicides, mainly to thiabendazole, is a reason for concern, as resistant populations may easily thrive and develop multiple resistance. As was observed, the effective control of green mold caused by isolates resistant to thiabendazole was lower when compared to non-resistant isolates inoculated in fruits previously treated with this fungicide. No adaptive differences were observed in the *in vitro* mycelial growth and in the pathogenicity of fruits between resistant and non-resistant isolates in the absence of thiabendazole, which agrees with data from California (Holmes and Eckert 1999). It is expected that the current selection pressure will remain over the next few years, since continuing use of these fungicides is anticipated. The combination of fungicides and different modes of action prevent or delay the development of resistant populations (Delp 1988). However, Holmes and Eckert (1999) observed that the proportion of *P. digitatum* and *P. italicum* isolates showing triple resistance to imazalil, thiabendazole and orthophenylphenol fungicides virtually doubled in 6 years in Californian packing houses due to the intensive use of these fungicides, not only in a sequence, but also as a combination. Heating the fungicide mixture reduces the possibility of the development of mold populations resistant to the currently used fungicides due to increasing the treatment effectiveness (Aquino et al. 2006; Smilanick et al. 2006). Alternative products showing different modes of action, such as pyrimethanil, will minimise the problems with populations of *Penicillium* spp. resistant to thiabendazole and imazalil (Aquino et al. 2006).

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