Isolation, identification and antifungal susceptibility of lemon pathogenic and non pathogenic fungi

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Summary

Numerous species of filamentous fungi were isolated from lemon on different plantations in the province of Tucuman, Argentina. The techniques suggested by the Subcommittee of Antifungal Susceptibility of the National Committee for Clinical Laboratory Standards, (USA) were adapted. The effect of three different concentrations of the fungicides imazalil, guazatine, SOPP and thiabendazole on the fungi *Fusarium oxysporum, Fusarium moniliforme, Aspergillus niger, Aspergillus flavus, Aspergillus clavatus, Geotrichum candidum, Rhizopus* sp*, Penicillium* sp*, Penicillium digitatum* and *Mucor* sp were studied.
All the tested strains were resistant to thiabendazole. We assayed a mixture of SOPP (5%), guazatine (350 ppm) and imazalil (100 ppm), which showed a synergic effect on *Rhizopus* sp*. Mucor* sp was the only fungus resistant to the four fungicides tested as well as to the above mentioned mixture.

Key words Phytopathogenic fungi, Saprophytic fungi, Lemon, Susceptibility, Fungicides

Aislamiento, identificación y sensibilidad a antifúngicos de hongos patógenos y no patógenos del limón

Resumen

Numerosas especies de hongos filamentosos se aislaron de limones provenientes de diferentes plantaciones en la provincia de Tucumán, Argentina. Se adaptaron las técnicas recomendadas por el National Committe for Clinical Laboratory Standards, Subcommitte of Antifungal Susceptibility (EE.UU.), para estudiar el efecto de tres concentraciones de los antifúngicos imazalil, guazatina, SOPP y tiabendazol sobre los hongos: *Fusarium oxysporum, Fusarium moniliforme, Aspergillus niger, Aspergillus clavatus, Aspergillus flavus, Geotrichum candidum, Rhizopus* sp*, Penicillum* sp*, Penicillum digitatum y Mucor* sp*.* Todas las cepas fueron resistentes al tiabendazol. Los otros fungicidas tuvieron una acción diferente para cada cepa. Se ensayó una mezcla de SOPP (5%), imazalil (100 ppm) y guazatina (350ppm) y se demostró un efecto sinérgico en *Rhizopus* sp que es resistente a cada antifúngico en forma individual. *Mucor* sp fue el único hongo resistente a los cuatro antifúngicos ensayados y a la mezcla mencionada.

Palabras clave Fitopatógenos, Saprofitos, Limón, Sensibilidad, Antifúngicos

The citrus industry in Tucuman has placed Argentina among the main lemon producers in the world. Large quantities of lemons are being exported at present and the microbiological quality control requirements are becoming increasingly more strict.

Lemons are liable to infections caused by filamentous fungi and, to a lesser degree, by yeasts. This is a matter of concern if we consider, in the case of lemons for export, an average of 30 days elapses from the moment they are harvested till they reach consumers. This period is long enough for pathogenic fungi to develop and cause diseases such as sour rot, brown rot, green mold, penicillium rot, etc. Numerous fungicides are commercially available. In view of the constant apparition of new pathogens and the increased resistance to fungicides, the National Committee for Clinical Laboratoty Standards (USA) Subcommitte on Antifungal Susceptibility Testing has developed procedures to test the antifungal susceptibility of filamentous fungi [5,7]. Reliable standard conidial suspensions can be prepared using spectrophotometric methods [8]. The main parameters to carry out the test are the preparation of the inoculum, the composition of the culture medium, the pH, the incubation time and temperature and the scoring of MIC results or end point [9].

The purpose of this work was to isolate and identify the pathogenic and non pathogenic fungi for lemon and to adapt the methodology developed to determine the susceptibility of the fungicides used in postharvest treatments to control lemon diseases as well as to determine the optimum concentrations for fungicide application.

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Samples were taken from lemon trees of three different plantations located in several areas of the province of Tucuman. Ten lemons were collected from each plantation every three weeks during two years. The surface of injured lemons was scraped with a scraping blade and the samples were seeded in Sabouraud-glucose medium with 50 µg/ml of chloramphenicol or 40 µg/ml of streptomycin plus 40 UI/ml of penicillin. They were incubated at 28 °C for 15 days. The isolated fungi were identified through a macromorphological, micromorphological and physiological studies as stated in the litterature [1,11,13].

The isolated fungi were cultured in potato dextrose agar tubes and incubated at 35 °C for 7 days to induce sporulation, with the exception of *Fusarium*, which was incubated at 35 °C for 48-72 h and then at 28 °C for 4 days. When fungal growth was observed, 2 ml of sterile distilled water was added to the tube and then the surface of the culture was scraped off with the tip of a sterile Pasteur pipette. Thus we obtained a suspension made up of spores and mycelium pieces that was transferred to a sterile tube. After standing for 5 min for the thicker particles to settle, the supernatant was placed in another tube, shaken in a vortex for 15 s, and absorption (O.D) was adjusted at 530 nm as follows:

Aspergillus flavus: 0.09-0.11; *Aspergillus niger*: 0.09-0.11; *Aspergillus clavatus*: 0.09-0.11; *Rhizopus* sp: 0.15-0.17; *Penicillium digitatum*: 0.09-0.11; *Penicillium* sp: 0.09-0.11; *Fusarium moniliforme*: 0.15-0.17*; Fusarium oxysporum*: 0.15-0.17*; Mucor* sp: 0.15-0.17.

The conidial size of these fungi ranged between 2 and 7 µm, which resulted in an inoculum size of 0.5 - 4 x 10^6 CFU/ml. In the case of *Geotrichum*, it was cultured in yeast nitrogen base (YNB) for 24 h, time after which a few colonies were picked up and resuspended in physiological solution. Finally, they were homogenized in a vortex and turbidity was adjusted to 0.5 in the Mc Farland's scale (equivalent to 1 - 5 x 10^6 CFU/ml) [10].

The following fungicides were assayed at different concentrations obtained by dilution in sterile distilled water: thiabendazole: 500, 1000 and 2000 ppm; imazalil: 500, 1000 and 2000 ppm; guazatine: 175, 350 and 700 ppm; SOPP (Sodium O-phenilphenate): 2.5, 5 and 10 %. Each solution was filter sterilized using cellulose membranes with a pore size of 0.4 µm.

Disks were prepared according to the specifications in the United States Food and Drug Administration Standards. Paper disks of 6.35 mm of diameter were cut with a hollow punch. For the disks we used filter paper weighing 30 ± 4 mg/cm^2 with a water adsorption capacity of 2-3 times their own weight. Twenty microliters of each fungicide solution at the above mentioned concentrations were placed on the center of each disk. Then the disks were dried in clean bench and kept in a dessicator at 4 °C. Before use, they were allowed to return to room temperature for 15 min to prevent condensation [12].

For the study of antifungal activity in vitro, we used the agar Neo Sensitab diffusion technique in a modified Shadomy medium and RPMI 1640 with L-glutamine buffered with 0.165 mol/l morpholine propane sulphonic acid (MOPS) plus 2% glucose, pH 7.0 ± 0.1. A sterile cotton swab was immersed in the inoculum and the excess liquid was removed by pressing the swab several times against the tube wall. Then the inoculum was uniformly spread over the whole surface of the plate. The disks were placed on the center of the agar surface and incubated at 35 °C for 72 h [10]. A fungi culture without any fungicide was used as control. Results were expressed as follows: Susceptible (with an inhibition halo of 30 mm or more), moderately susceptible (23-29 mm) and resistant (22 mm or less). All determinations were carried out in both media by triplicate.

The strains isolated from lemon were *F. oxysporum, F. moniliforme, A. niger, A. flavus, A. clavatus, Geotrichum candidum, Rhizopus* sp*, Rhodotorula* sp*, Scopulariopsis* sp*, P. digitatum, Penicillium* sp*, Diaporthe citrus* and *Mucor* sp. During the two years of sampling the same microorganisms were founded.

When studying the effect of fungicides on the different fungi, we noticed that they had a fairly good diffusion on the media. For any antimicrobial agent, the rate of diffusion in a given medium under controlled conditions is called diffusion coefficient. Under standardized conditions, the diffusion of an antimicrobial agent is in proportion to its concentration in the container.

The effect of the fungicides assayed against the various strains is summarized in table 1. All the strains were resistant to thiabendazole. *Penicillum* sp, which causes penicillum rot, is sensitive to imazalil and moderately sensitive to SOPP, while *P. digitatum* is also sensitive to guazatine, an encouraging finding since the postharvest disease that causes green mold is much more common than penicillum rot. *P. digitatum* appearing in oranges has been controlled by imazalil decreasing the disease incidence from 98.8 to 17.4% [14]. This fungus is a prolific spore producer, and airborne spores easily contaminate soak and drench tanks, degreening and storage rooms, packing houses, equipment and transit containers. Due to its ability to produce large masses of spores, the fungus has the potential to develop resistance to postharvest fungicides [2,4,6].

Table 1. Effect of fungicides againt various strains.

Strains	Thiabendazole (ppm)			SOPP (%)			Imazalil (ppm)			Guazatine (ppm)		
	500	1000	2000	2,5	5	10	500	1000	2000	175	350	700
Fusarium oxysporum	R	R	R	S	S	S	MS	S	S	S	S	S
Fusarium moniliforme	R	R	R	MS	S	S	MS	S	S	S	S	S
Aspergillus niger	R	R	R	S	S	S	R	R	MS	R	R	R
Aspergillus flavus	R	R	R	R	R	MS	MS	MS	MS	R	R	R
Aspergillus clavatus	R	R	R	R	R	MS	R	MS	MS	MS	MS	S
Geotrichum candidum	R	R	R	R	MS	S	R	MS	MS	MS	MS	S
Rhizopus sp	R	R	R	R	R	R	R	R	R	R	R	R
Penicillium sp	R	R	R	MS	MS	MS	S	S	S	R	R	R
Penicillium digitatum	R	R	R	MS	MS	MS	S	S	S	MS	S	S
Mucor sp	R	R	R	R	R	R	R	R	R	R	R	R

R: Resistant
MS: Moderately susceptible
S: Susceptible

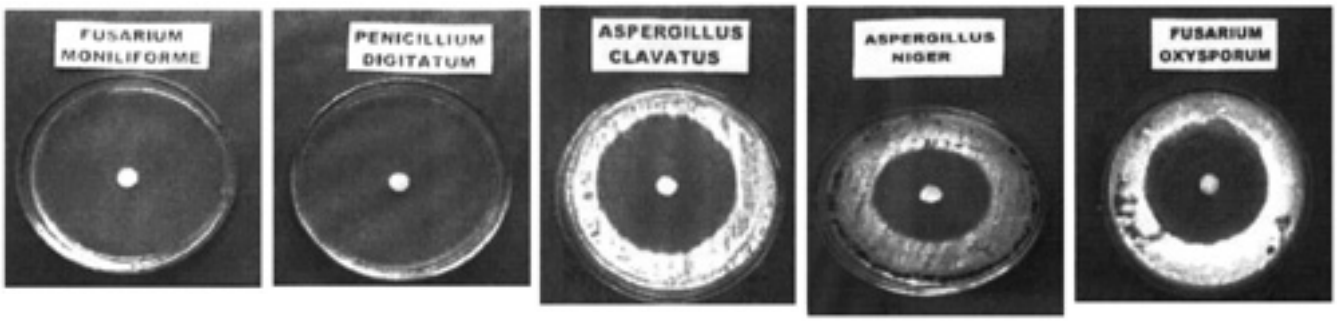

Figure 1. Susceptibility to the antifungal mixture in several fungi.

G. candidum proved to be sensitive to SOPP and guazatine and moderately sensitive to imazalil. This fungus causes a postharvest disease in injuries to mature and overly mature citrus fruits. When infected fruits are packed together with healthy ones, the characteristic sour odor of the former attracts fruit flies, which can spread the fungi to other injured fruit [3,5].

The remaining isolated fungi belong to a group of saprophytic (non-pathogenic) fungi. Both *F. oxysporum* and *F. moniliforme* proved to be sensitive to the three SOPP concentrations assayed, while *Rhyzopus* sp and *Mucor* sp were resistant to all the fungicides assayed. It should be noted that although postharvest treatments often effectively control pathogens, they do not necessarily affect the growth of the remaining saprophytic fungi. Out of the three isolated *Aspergillus* species, only *A. niger* was sensitive to SOPP and *A. clavatus* to guazatine. These results agree with the literature since, on the whole, saprophytic fungi are more resistant to postharvest treatment [15].

Finally, we assayed a mixture of SOPP (5 %), imazalil (1000 ppm) and guazatine (350 ppm). As shown in figure 1, *A. clavatus, A. niger, P. digitatum, F. moniliforme* and *F. oxysporum* exhibited a marked susceptibility to the antifungal mixture, the strongest effect being observed in *A. clavatus, P. digitatum* and *F. moniliforme*.

Mucor was the only fungus resistant to the four fungicides assayed as well as to the mixture of SOPP, imazalil and guazatine. The mixture acted in a synergic manner on *Rhizopus* sp, but this fungus was resistant to the above fungicides when they were used separately.

The methodology used in this paper would allow lemon packinghouses to determine the best concentrations and the most effective antifungal mixtures to be used in lemon and as to extend storage time in the very best sanitary conditions.

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