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Occurrence of imazalil-resistant biotype of *Penicillium digitatum* in China and the resistant molecular mechanism*

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Abstract: Green mold caused by *Penicillium digitatum* (Pers.:Fr) Sacc. is one of the most important postharvest diseases of citrus fruits. Experiments were conducted to determine the occurrence of resistance of *P. digitatum* to imazalil in China. Four imazalil-Resistant (R) isolates were identified from 189 isolates of *P. digitatum*. The highest EC_{50} of an imazalil-R isolate was 0.578 mg/L, 29 times higher than that of the most imazalil-sensitive (S) isolate, suggesting that imazalil-R biotype of *P. digtatum* had occurred in China. In vitro assessment indicated that the imazalil-R isolates were not significantly different from imazalil-S isolates in their growth rate and sporulation, indicating that the imazalil-R biotype has competence similar to that of imazalil-S one and could co-exist with it in environment. To determine the mechanism of the resistance, *CYP51* gene was amplified from *P. digitatum* genome and sequenced. The results revealed that a tandem repeat of four extra copies of a unique 126-bp sequence in the upstream promoter region of *CYP51* gene present only in imazalil-R isolates, but not in imazalil-S isolates, implying this tandem repeat sequence may regulate the expression of *CYP51* positively, and lead to the sensitivity decrease.

Key words: *Penicillium digitatum*, Citrus, Imazalil, Resistance, *CYP51* gene
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INTRODUCTION

Green and blue mold caused by *Penicillium digitatum* (Pers.:Fr) Sacc. and *P. italicum* Wehmer are the most important postharvest diseases of citrus fruits, and usually cause losses of 20% to 30% during storage and marketing. Imazalil, one of the sterol demethylation-inhibitor (DMI) fungicides, has been used worldwide to control postharvest citrus fruits decay since the 1970s (Harding, 1976), and the natural imazalil-R biotype of *P. digitatum* was first detected in the 1980s (Eckert, 1987). Imazalil was introduced into China in 1993 as a replacement for the benzimidazoles because of benzimidazoles-resistance. Initially, dipping treatment of fruit with imazalil at concentrations 125 mg/L to 250 mg/L was highly effective. However, reduced performance or control failure has been reported frequently in Zhejiang since 1998 (Gan, Plant Protection Station of Kecheng District of Quzhou City, Zhejiang Province, China, personal communication). The objective of the present study was to determine if imazalil-R biotype has occurred and the probable molecular mechanism involving the resistance.

MATERIALS AND METHODS

Isolate collections

P. digitatum-infected citrus fruits were collected from storage houses located in Quzhou, Lishui, Jinhua, Ningbo and Taizhou, and from Hangzhou markets, between November and December of 1999 and 2000. *P. digitatum* conidia on the decayed fruits were

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scraped off with sterile scalpels and then suspended in 1 ml water containing 0.01% (v/v) Tween 20. Suspension of spores (50 μl) was diluted in water and spread evenly onto water agar medium in petri dishes containing 100 mg/L streptomycin sulfate. A single colony derived from single conidium was transferred to fresh PDA medium, with the resultant culture being used as an individual isolate.

Resistance determination

The *P. digitatum* isolates' sensitivity to imazalil was determined by their growth and sporulation on PDA medium (pH=6.0) containing 1.0 mg/L imazalil (Deccozil, 250EC, Elf Atochem North America, Inc.) as described previously (Holmes and Eckert, 1999). Four selected imazalil-R isolates were then further studied for EC_{50}. Four replicates were used for each treatment.

Efficacy of imazalil in controlling the infection of imazalil-R *P. digitatum*

Citrus fruits (*Citrus reticulata* Blanco, cultivars Pengan) were obtained from an orchard in Quzhou, Zhejiang, washed in a detergent solution and surface-sterilized by dipping in 70% ethanol for 1 min. After drying in air, the fruits were punctured with a 10-needles bundle to produce a wound on each fruit equator, and then were inoculated with conidial suspensions (1×10^6 ml^{-1}) of two imazalil-R isolates (PD01 and PD07) or two imazalil-S isolates (PD23 and PD27), respectively. An equal number of fruits (120 fruits/group) were inoculated with each of these 4 isolates and then stored at 20 °C (*RH*≈90%) for 18 h to allow the development of infection, followed by randomly dividing the fruits of each group into 4 subgroups of 30 fruits each. The subgroups were dipped for 1 min in a suspension of imazalil at concentrations of 0 (control), 250, 500 and 1000 mg/L and then incubated at 20 °C (*RH*≈90%) for 3 weeks before evaluation of disease incidence. Three replicates were used for each concentration of imazalil. The experiment was repeated twice.

Growth rate of imazalil-R/S *P. digitatum*

Four imazalil-R and four imazalil-S isolates of *P. digitatum* were chosen to compare mycelium growth rate in a liquid potato dextrose broth (PDB) medium. A plug (5 mm in diameter) was removed from a PDA plate and then inoculated into a flask with 100 ml PDB. After shaking the flask (120 r/min) at 25 °C for 5 d, the mycelia were filtered through two-layer cheesecloth. The dry weight of mycelia was determined after desiccation at 60 °C. The experiment was repeated twice.

Sporulation of imazalil-R/S *P. digitatum*

Conidial suspensions (10^6 ml^{-1}) from the 8 selected *P. digitatum* isolates were prepared as described above. Fifty microliters of this suspension were distributed evenly onto the PDA dish (90 mm in diameter). After incubation for 7 d at 25 °C, the spores were washed off by adding sterile water containing 0.01% (v/v) Tween 20 rubbing with a sterile glass rod. The mycelia were removed by two-layer cheesecloth filtration and the conidial concentration from each isolate was determined under microscope using a haemocytometer. Three replicates were prepared for each isolate, and the experiment was repeated twice.

Detection of the tandem repeated sequence in the promoter region of imazalil-R and imazalil-S *P. digitatum*

The mycelia were grown in potato dextrose broth (PD) at 25 °C and the total DNA of four imazalil-S isolates (PD23, PD26, PD27 and PD125) and four imazalil-R isolates (PD01, PD07, PD40 and PD110) were extracted by following the methods described by Nakaune *et al*.(1998). PCR was conducted following the parameters used in (Hamamoto *et al*., 2000). One fragment amplified from an imazalil-R isolates PD07 and another one from an imazalil-S isolate PD23 were cloned into PGEM-T easy Vector (Promega, Madison, Wis., USA) and sequenced on an ABI PRISM 377 HM (PE Applied Biosystems) DNA sequencer.

RESULTS

Occurrence of imazalil-R *P. digitatum*

A total of 189 monospore isolates were obtained from 256 *P. digitatum*-infected citrus fruits collected from Zhejiang Province. Four imazalil-R isolates were identified based on their ability to grow and sporulate on the PDA medium containing 1.0 mg/L

imazalil. The EC_{50} of imazalil to isolate PD01, PD07, PD110 and PD40 was 0.578, 0.224, 0.459 and 0.117 mg/L, respectively. While the EC_{50} for four randomly selected imazalil-S isolates (PD23, PD26, PD27 and PD125) was 0.020, 0.023, 0.025 and 0.030 mg/L, respectively. The EC_{50} of R isolate PD01 was 29 times higher than that of the S isolate PD23. This result clearly indicated the presence of imazalil-R biotype in China.

Table 1 List of *P. digitatum* isolates used in experiment

Regions	No. of isolates	No. of imazalil-R isolates*
Quzhou	21 (PD01~PD21)	2 (PD01, PD07)
Hangzhou	56 (PD22~PD77)	1 (PD40)
Ningbo	19 (PD78~PD96)	0
Jinhua	20 (PD97~PD116)	1 (PD110)
Lishui	38 (PD117~PD154)	0
Taizhou	35 (PD155~PD189)	0

*The isolates that could grow and sporulate on 1.0 mg/L imazalil-amended PDA medium were designated as imazalil resistant (imazalil-R), while the isolates could not grow or sporulate on this medium were designated as imazalil sensitive (imazalil-S)

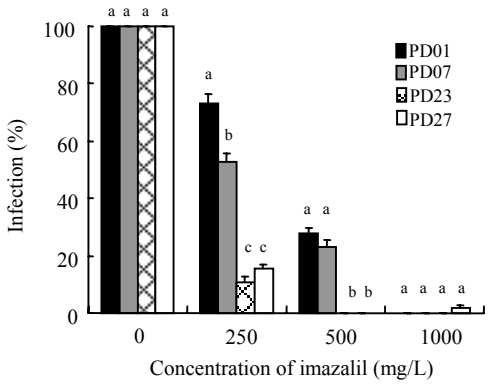

Fig.1 Efficacy of imazalil controls the infection caused by imazalil-R and imazalil-S *P. digitatum* isolates
The columns represented the means of percentage of infection of three replicates; the bars represented the standard errors, and bars attached with same letter in the same concentration of imazalil are not significantly different by the least square difference comparison (LSD, $P>0.05$); the isolates of PD01 and PD07 are imazalil-R, whereas the PD23 and PD27 are imazalil-S

Efficacy of imazalil against the infection of imazalil-R *P. digitatum*

The fruit decay caused by imazalil-S isolates PD23 and PD27 was controlled effectively by imazalil at concentration of 250 mg/L, and the decay was inhibited completely at a concentration of 500 mg/L. In contrast, disease development on fruits inoculated with imazalil-R isolates PD01 and PD07 was not effectively controlled by imazalil at concentration of 250 mg/L. Infection of the imazalil-R isolates was inhibited significantly at the concentration of 500 mg/L. Prevention of infection by the two imazalil-R isolates was achieved when the imazalil concentration was increased to 1000 mg/L (Fig.1).

Partial biological characteristics of imazalil-R *P. digitatum*

Four imazalil-S isolates (PD23, PD26, PD27 and PD125) and four imazalil-R isolates (PD01, PD07, PD110 and PD40) were tested to examine the difference of growth rate between the two biotypes. The mean dry-weight of mycelium from the 4 imazalil-R isolates was 0.519 g, just slightly less than that of the 4 imazalil-S isolates (0.557 g) and was not significantly different. The mean number of spores produced by the 4 imazalil-S strains was 0.678×10^7 ml^{-1}, similar to that of the 4 imazalil-R strains (0.612×10^7 ml^{-1}) when they were grown on imazalil-free media.

Tandem repeated sequence in the promoter region of *CYP51* gene

The PCR products amplified with primers of Pri 207 and Pri 1875c were about 2.6 kb and 2.1 kb for four imazalil-R isolates and four imazalil-S isolates, respectively. One fragment amplified from PD07 (resistant) with size of 2.6 kb and another fragment amplified from PD23 (sensitive) with size of 2.1 kb was cloned, respectively. Sequence analysis indicated that a tandem repeat of five copies of a unique 126-bp sequence in the upstream promoter region was presented in PD07, while this sequence was presented once in PD23.

DISCUSSION

This study indicated clearly that the imazalil-R biotype *P. digitatum* has occurred in Zhejiang, China. The controlling efficiency of imazalil against imazalil-R biotype of *P. digitatum* infection at concentration of 500 mg/L was significantly lower than that of imazalil-S one. However, the infection of imazalil-R biotype could be inhibited effectively by imazalil at 1000 mg/L. These results indicated the resistant levels of imazalil-R biotype are relatively

low in Zhejiang Province, compared to that reported in California (Eckert *et al*., 1994), and suggesting that increasing imazalil concentration to 1000 mg/L could be effective in green mold control of postharvest citrus. Continuously monitoring the changes of *P. digitatum* populations with different level of resistance to imazlil and rotation of fungicides with different mode of action is suggested.

The imazalil-R biotypes of *P. digitatum* and *P. italicum* induced in laboratory were less fit than wild biotypes (de Waard and van Nistelrooy, 1990). But Holmes and Eckert (1995) found out that some imazalil-R biotypes from storage house in California were more competitive than those of imazalil-S. The results of this experiment showed that the *in vitro* fitness (indicated by mycelium growth and spore formation) of imazalil-R *P. digitatum* from storage house of China were not significantly different from that of imazalil-S biotype and isolate-dependent, agreeing with that reported in California (Holmes and Eckert, 1995). The difference of laboratory-induced biotypes and nature-derived biotypes may be associated with the difference of selection pressure those biotypes endured.

Imazalil is a 14α-demethylation inhibitor of biosynthesis of ergosterol. Several molecular mechanisms have been revealed to be associated with the DMI fungicide resistance (reviewed in (Ma and Michailides, 2005)). The point mutation of 14α-demethylase (*CYP51*) gene, which encodes the target protein of DMIs, was demonstrated to be correlated to the emergence of resistance to DMIs in the grape powdery mildew fungus *Uncinula necator* and *Erysiphe graminis f.* sp. *hordei* (Délye *et al*., 1997). The overexpression of *CYP51* gene, resulting from the insertion located upstream of *CYP51* gene (the transcriptional enhancer region), was reported to confer the resistance of the partial MDI-resistant *Venturia inaequlis* strains (Schnabel and Jones, 2001). For *P. digitatum*, the ATP-binding cassette (ABC) transporters, involved multidrug resistance (MDR) in wide variety of organisms, were first reported to be associated with DMI-fungicide resistance (Nakaune *et al.*, 1998). However, Hamamoto *et al*.(2000) reported that the insertion of five-time tandem repeat with a unique 126-bp sequence in the promoter region of *CYP51* gene, leading to the overexpression of CYP51 is responsible for the imazalil-resistant formation. Our experiment confirmed that such insertions were presented in all four imazalil-R field isolates of *P. digitatum*, and that this is probably the main mechanism for resistance of *P. digitatum* to imazalil.

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