Significance of 195bp-enhancer of *PdCYP51B* in the acquisition of *Penicillium digitatum* DMI resistance and increase of fungal virulence

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ABSTRACT

Two sterol 14α-demethylase genes from *Penicillium digitatum*, *PdCYP51A* and *PdCYP51B,* were evaluated and revealed that 95% of Imazalil (IMZ)-resistant isolates carried a 195-bp insertion in the *PdCYP51B* promoter. We functionally characterized both sterol 14α-demethylases by overexpression. Molecular analysis of overexpression mutants showed that the introduction of *PdCYP51B* insertion is more stable than the five-tandem repeat *PdCYP51A* sequence previously described that confers DMI fungicide resistance. The both enhancers can coexist in *P. digitatum* isolates that initially contained the 195-bp *PdCYP51B* insertion but the introduction of 195-bp *PdCYP51B* enhancer promoted the loss of the five-tandem repeat of *PdCYP51A*. The incorporation of 195-bp *PdCYP51B* resulted in an increase of DMI fungicide resistance in mutants from already resistant isolates and confers resistance to DMIs in mutants from sensitive isolates. Transcription evaluation of the both genes showed noticeable induction in all overexpression mutants, except for those coming from the five-tandem repeat *PdCYP51A* sequence, whereas *PdCYP51A* expression dropped dramatically. Only *PdCYP51B* exhibited up-regulation during citrus infection compared to axenic growth, and the role of *PdCYP51B* in fungal virulence was further reinforced since strains with low virulence showed increased infectivity in overexpression mutants. This study suggested the predominant role of the *PdCYP51B* enhancer in the acquisition of DMI resistance and fungal virulence, by replacing homologues genes with same putative function.

Keywords: *CYP51*; DeMethylation Inhibitors; Fungicide resistance; *Penicillium digitatum*; Virulence

1. Introduction

Imazalil is a broad-spectrum systemic fungicide used to control post-harvest decay of citrus fruits worldwide because of its curative and anti-sporulant action against green mold, caused *Penicillium digitatum* (Pers.: Fr.) Sacc (Holmes and Eckert, 1995). Imazalil is a demethylation inhibitor (DMI) that inhibits the biosynthesis of ergosterol (Hamamoto et al., 2000; Nakaune et al., 2002). DMIs comprise one of the most important groups of fungicides, controlling numerous plant diseases by inhibiting cytochrome P450-dependent sterol 14α-demethylase (P45014DM) activity (Yoshida and Aoyama, 1991). *CYP51* encodes sterol 14α-demethylase, an enzyme involved in ergosterol biosynthesis (Parks et al., 1995) and is targeted by DMI fungicides.

The mechanisms that confer DMI resistance are (i) mutations in *CYP51* or (ii) overexpression of *CYP51*. (i) While one amino acid substitution of phenylalanine to tyrosine at position 136 (Y136F) of *CYP51* has been found to confer resistance to DMIs in *Uncinula necator* (Délye *et al*., 1997), *Erysiphe graminis* f.sp. *hordei* (Délye *et al*., 1998), *Erysiphe necator* (Ralloe and Badiun, 2015), and *Penicillium expansum* (Ali and Amiri, 2018), two single nucleotide mutations resulting in amino acid substitutions Y136F and K147Q have been found in *CYP51 of Blumeria graminis* (Wyand and Brown, 2005). Furthermore, different mutations have been reported in *Tapesia* sp. (Albertini et al., 2003), *Penicillium italicum* (Joseph-Horne and Hollomon 1997), *Ustilago maydis* (Butters et al., 2000) *Blumeriella jaapii* (Ma et al., 2006), *Mycosphaerella graminicola* (Leroux and Walker, 2011), and *P. digitatum* (Wang et al., 2014). (ii) The other mechanism responsible for DMI resistance was change in *CYP51* expression level (Cools et al., 2012). Insertions in the promoter region were found in the phytopathogenic fungus *B. jaapii* (Ma et al., 2006), *Venturia inaequalis* (Schnabel and Jones 2001), *Monilinia fructicola* (Luo et al., 2008), and *M. graminicola* (Leroux and

Walker, 2011). This mechanism has been also associated with imazalil resistance of *P. digitatum* that causes green mold, the major postharvest fungal disease of citrus. The presence of five tandem repeats of a 126-bp transcriptional enhancer, in the *PdCYP51A* promoter region, has been reported, which results in the overexpression of *PdCYP51A* (CA-R1; Hamamoto et al., 2000). Moreover, the insertion of a 199-bp sequence within the 126-bp transcriptional enhancer, has been seen to result in the elevated expression of *PdCYP51A* (CA-R2; Ghosoph et al., 2007). Furthermore, a unique 199-bp insertion observed in the promoter region of *PdCYP51B* has been associated with its overexpression and DMI fungicide resistance (CB-R3; Sun et al., 2011).

In a previous work done on 75 Spanish *P. digitatum* strains, the majority of *P. digitatum* DMI-resistant isolates could not be explained by the presence of 126-bp tandem repeats of *PdCYP51A* that confers fungicide resistance (Sánchez-Torres and Tuset, 2011). Thus, the aim of this study was to investigate the contribution of both sterol 14α-demethylase genes, *PdCYP51A* and *PdCYP51B*, in the acquisition of DMI fungicide resistance and their putative role in fungal virulence during citrus fruit infection. We found that *PdCYP51B* enhancer played a predominant role in the acquisition of DMI resistance and fungal virulence.

2. Materials and Methods

2.1. Biological material and culture conditions

The *P. digitatum* isolates used in this study have previously been described (Sánchez-Torres and Tuset, 2011; de Ramón-Carbonell and Sánchez-Torres, 2017a). *P. digitatum* Pd1, Pd2, Pd27, and Pd149 isolates were used for gene isolation and fungal transformation. *Penicillium* spp. used in this work were obtained from the fungi collection of our lab. All strains were grown in potato dextrose broth (PDB; Liofilchem

Laboratories, Teramo, Italy) or potato dextrose agar (PDA; Liofilchem Laboratories, Teramo, Italy). Liquid cultures were grown at 25ºC with continuous light for 1, 2, or 3 d depending on requirement or up to 1 week in agar media. Fungal spores were obtained from 1-week-old PDA plates by scraping the plates with a sterile spatula and transferring the spores to sterile water. Spores were filtered, counted using a hemocytometer, and suspended at desired concentrations.

Escherichia coli DH5α was used for cloning and maintaining plasmids. *E. coli* cultures were grown in LB plates or LB liquid media, supplemented with 100 μg/mL of kanamycin, at 37ºC.

Fungal transformations were carried out using *Agrobacterium tumefaciens* $C_{58}C_1$. Bacteria containing plasmid constructs were cultured in LB plates or LB liquid medium with 50 μg/mL rifampicin and 100 μg/mL kanamycin, at 28ºC, as described by de Ramón-Carbonell and Sánchez-Torres (2017a).

Mature oranges (*Citrus sinensis* L. Osbeck) from the cultivars 'Navelina' or 'Navelate' not treated with fungicides, were harvested from different orchards at IVIA in Moncada (Valencia, Spain) and used for this study.

2.2. Molecular manipulations

P. digitatum genomic DNA was isolated from mycelium growing in liquid culture, as previously reported by Sánchez-Torres and Tuset, 2011. PCR amplicons obtained in this work were purified using Ultra Clean TM PCR Clean-up (MoBio, Solana Beach, CA, USA).

Trizol method (Ambion Inc., Austin, USA) was used for RNA extraction from *P. digitatum* frozen mycelium. Extraction of total RNA from infected samples was processed as described previously (López-Pérez et al., 2015).

2.3.Isolation of PdCYP51B

Primers CB1 and CB2 were designed, based on the sequence of a 248-bp cDNA fragment isolated from *P. digitatum* suppression subtractive hybridization (SSH) cDNA library (López-Pérez et al., 2015) and were used to amplify a PCR product using *P. digitatum*-Pd1 genomic DNA as template. The PCR reaction consisted of an initial denaturation at 94ºC for 4 min, followed by 30 cycles at 94ºC for 30 s, 55ºC for 45 s, and 72ºC for 60 s. A final elongation step was carried out at 72ºC for 10 min. The 500-bp PCR amplicon was sequenced using the same primers used for PCR amplification. CB1 and CB2 oligos were used for the screening of the *P. digitatum* Pd1 genomic DNA library (López-Pérez et al., 2015), as described previously (de Ramón Carbonell and Sánchez-Torres (2017a).

2.4. Construction of vectors and fungal transformation

Overexpression constructs OCYP51A and OCYP51B, were constructed using the binary plasmid pRFHU (Frandsen et al., 2008). *PdCYP51A* was isolated from Pd2 strain that exhibits CA-R1 genotype (five tandem repeat enhancer-*CYP51*) and *PdCYP51B* was isolated from Pd1 that exhibits CB-R3 genotype (195-bp enhancer-*PdCYP51B*). Both plasmids were constructed using their own promotors and coding regions fused to the hygromycin cassette. OCYP51B-*A. tumefaciens* (Fig. S1A) was used to transform *P. digitatum* strains: DMI^R strain Pd2 (CA-R1 and CB-S) and DMI^S strains Pd27 and Pd149 (CA-S and CB-S) (Fig. S2).

OCYP51A-*A. tumefaciens* (Fig. S1B) was used to transform Pd1 *P. digitatum* strain DMI^R (CA-S and CB-R3) (Fig. S2).

Overexpression mutants were selected on PDA plates containing 100 μg/mL of hygromycin B. Overexpression mutants were checked by PCR, using genomic DNA from monosporic isolates and copy number quantification was carried out using real time quantitative PCR (qRT-PCR), with beta-tubulin as the reference gene (de Ramón-Carbonell and Sánchez-Torres, 2017b).

2.5. Assay of fungicide sensitivity

Four different chemical fungicides were selected for sensitivity evaluation: imazalil (Textar I; Tecnidex), prochloraz (Ascurit; Tecnidex), and philabuster (Decco Ibérica) at increasing concentrations of 0, 0.5, 1, 2, 4, 8, and 10 μg/mL and thiabendazol (Textar 60 T; Tecnidex) at 0, 5, 10, 20, 40, 80, and 100 μg/mL. Each treatment was done in triplicates in two independent experiments. The measurements, comparing growth at different fungicide concentrations, were performed in 96-well microtiter plates and the respective untransformed wild-type strains were assayed at the same time. The activity and efficacy of different fungicides were evaluated following the protocol described by Sánchez-Torres and Tuset (2011). Sensitivity to chemical compounds was measured as relative growth, expressed as percentage, by comparing growth in absence and presence of the chemical.

2.6. Virulence evaluation

Pathogenesis experiments were performed using freshly harvested oranges (*C. sinensis*) that were wounded at four places, along the equatorial axis, infected with 10 μL of a 10^5 conidia/mL spore suspension, and kept at 20°C and 90% RH. Three replicates of five fruits each were used, and the infection experiments were done twice. As control, mock-inoculated fruits were used. Infection progression was measured as

the percentage of infected fruits (disease intensity) and diameter of macerated tissue (disease severity).

2.7. Gene expression

PrimeScript™ RT reagent Kit (Takara Bio Inc. CA, USA) was used for synthesis of the first strand of cDNA in a 20 μL reaction, following the instructions of the manufacturer. Quantitative PCR was done as reported by de Ramón-Carbonell and Sánchez-Torres, (2017b).

Experimental values obtained were an average of two repetitions of three biological replicates. Oligos qCAF-qCAR and qCBF-qCBR were used for *PdCYP51A* and *PdCYP51B*, respectively, and genes coding for fungal β-tubulin (qTubF-qTubR), ribosomal protein 28S (q28SF-q28SR) and histone H3 (qH3F-qH3R) were independently used as reference genes (Table S1).

LightCycler 480 Software, version 1.5 (Roche Diagnostics) was used for cycle quantification. Primer melting temperature allowed the selection of each primer set for specific amplification. Relative gene expression was carried out as previously described by Ballester et al., 2015.

2.8. Statistical Analysis

Significant differences in pathogenicity were evaluated using analysis of variance (ANOVA) with SAS software (SAS Institute Inc., Cary, USA). Statistical significance was defined as $P<0.05$; when the analysis was statistically significant, Tukey's test for separation of means was performed.

3. Results

3.1. Isolation of P. digitatum PdCY51B

Previously, a cDNA fragment was identified from an SSH cDNA library-enriched in *P. digitatum* genes that were induced during citrus fruits infection (López-Pérez et al., 2015). SSH-42 deduced amino acid sequence showed high homology to sterol 14α-demethylases. Comparison of genomic and cDNA sequence confirmed an open reading frame of 1750-bp and three introns-73, 51, and 52-bp-in positions 611..856, 930..1127, 1179..2243, and 2296..2361, respectively, encoding a 524 amino acid protein. Gene sequences confirmed the identity of *PdCYP51B* = PDIP_01820 gene. *PdCYP51B* sequence of different *P. digitatum* isolates were deposited with accession numbers GU124181-89.

3.2. Analysis of PdCYP51B in different P. digitatum isolates

Characterization of *PdCYP51B* was carried out in 75 *P. digitatum* isolates, of which 58 were DMI-resistant (DMI^R) and 17 DMI-sensitive (DMI^S) (Sánchez-Torres and Tuset, 2011). No differences were observed in the nucleotide sequences of the coding regions. Analysis of the promoter region using CB8-CB9 primers (Table S1) revealed the presence of a 195-bp insertion in 55 of 75 strains. These 55 isolates corresponded to *P. digitatum* IMZ-resistant strains that did not present the 126bp five-tandem repeat of *PdCYP51A* in the promoter region (CA-R1) (Fig. S2) and represented the 73% of the *P. digitatum* isolates evaluated and the 95% of IMZ-resistant ones (Fig. 1A).

The same primers were used to amplify *PdCYP51B* promoter region from different *Penicillium* strains, like *P. brevicompactum, P. chrysogenum, P. digitatum, P. expansum, P. italicum,* and *P. simplicissimum.* Both strands of the PCR amplicons were sequenced revealing differences for each *Penicillium* spp. Of all isolates studied, only

DMI-resistant strains of *P. digitatum* (Pd1 and Pd12) and *P. italicum* (Pi1), both citrus pathogens, exhibited the 195-bp insertion. Phylogenetic tree analysis using maximum likelihood of promoter sequences showed that both *P. digitatum* and *P. italicum* DMI^R cluster in the same group (Fig. 1B).

3.3.Characterization of PdCYP51B overexpression mutants

In order to characterize *PdCYP51B* (Pd2, P27, and Pd149) overexpression mutants, PCR evaluation using primers HygB-F and HygB-R (Table S1) was carried out.

All overexpression mutants that showed a 488 bp fragment of the hygromycin gene (*hph*), were selected and further analyzed by PCR using CB8-CB9 oligos, to test for the presence of the 195-bp insertion in the *PdCYP51B* promoter. Overexpression mutants were compared to their respective wild type strains and Pd1 strain was used as the positive control. All mutants, regardless of which progenitor they came from, exhibited two patterns. Pattern A: two bands corresponding to the *PdCYP51B* promoter region with and without 195-bp. Pattern B: a unique band of 695-bp, corresponding to the *PdCY51B* promoter, carrying the 195-bp insertion, indicating that homologous recombination had occurred (Fig. 2A–B).

Moreover, *PdCYP51A* promoter analysis was performed using primers CA1 and CA2. Surprisingly, Pd2-overexpression overexpression mutants showed the loss of 126-bp five-tandem repeat insertion (CA-S pattern), in contrast to CA-R1 exhibited for the progenitor strain Pd2 (Fig. 2C), while all mutants derived from Pd27 or Pd149 strains maintained the CA-S pattern, similar to their respective parental strains (Fig. 2D).

3.4. Analysis of PdCYP51A overexpression mutants

All Pd1-overexpression mutants were checked by PCR using primers HygB-F and HygB-R (Table S1) confirming the presence of *hph*, as described above.

Selected overexpression mutants were further characterized for the two sterol demethylase promoters. Evaluations carried out, as described above, showed that Pd1 mutants had both forms of *PdCYP51A* promoter, with and without five 126-bp repeat insertions (Fig. 3A). Analysis of the *PdCY51B* promoter confirmed the presence of the 195-bp insertion found in wild-type Pd1 (Fig. 3B).

3.5. Fungicide evaluation of P. digitatum mutants

Evaluation of fungicide resistance of *PdCYP51A* overexpression mutants T1 and T2 showed the same level of azole resistance. No differences on percentage of growth were observed for all fungicides assayed (IMZ, PHI, PCL and TBZ). Both overexpression mutants behaved like Pd1 parental strain (Fig. 4).

Fungicide sensitivity analysis for *PdCYP51B*-overexpression mutants was carried out on two mutants of each parental strain. The results showed that Pd2 (T1 and T4) overexpression mutants exhibited increased DMI fungicide resistance, especially for PCL. No change in resistance to TBZ was observed in T1 and T4 mutants which behaved like the parent strain Pd2 (Fig. 5). All Pd27 (T1 and T2) and Pd149 (T4 and T5) overexpression mutants showed resistance to IMZ, PHI, and PCL fungicides at different concentrations. All four overexpression mutants exhibited DMI-resistant unlike their respective progenitors. None of them showed resistance to TBZ as in the case of their respective progenitors (Fig. 6).

3.6. Infection assays

Evaluation of infection was done for all overexpression mutants compared to each parental strain. Pd1 *PdCYP51A*-mutants did not exhibit variation regarding disease incidence (Fig. 7A) or disease severity (Fig. 7B) compared to wild-type Pd1.

In contrast, *PdCYP51B*-mutants showed a significant increase of disease incidence for Pd149-mutants, where aggressiveness increased by 40%, as compared to Pd149 wild-type strain (Fig. 8A). Moreover, disease severity was also seen to increase (Fig. 8B). Notably, the increase observed during infection of citrus fruits, for Pd2- and Pd27-mutants, was less noticeable and not statistically significant (Fig. 8).

3.7. Expression analysis of P. digitatum sterol 14α-demethylases

qRT-PCR was performed to assess gene expression. *In vitro* transcriptional profiling of *PdCYP51A* showed highest expression in Pd2 wild type (Fig. 9A); however, in all Pd2 mutants of *PdCY51B*, the expression fell to levels of azole-sensitive strains (Fig. 9B) and gene expression was almost undetectable. Pd27- and Pd149-overexpression mutants showed a four-fold increase in *PdCYP51A* expression, compared to their respective wild-type strains at 2 dpi (days post-infection) (Fig. 9C–D).

Evaluation of *PdCYP51B* transcription in axenic growth showed clear induction of Pd1 wild-type, compared to Pd2, Pd27, and Pd149 wild-type strains, which scarcely showed gene expression (Fig. 10A). Analysis of *PdCYP51B* expression resulted in a significant induction in all *PdCYP51B*-overexpression mutants, regardless of the origin of each mutant. Pd2- and Pd27 induction was four-folds that of Pd1 wild type, while Pd149-overexpression mutants showed three-folds induction (Fig. 10B–D)

Evaluation of *PdCYP51A* transcription was also carried out in Pd1, Pd2, and P149, during citrus fruit infection. *PdCYP51A* expression was very little in all *P.*

digitatum strains during infection, particularly in Pd2, in which *PdCYP51A* exhibited the highest level of transcription *in vitro*. Only Pd1 showed similar levels of expression, both *in vitro* and *in vivo* (Fig. 11A–C). In contrast, *PdCYP51B* expression was highly induced during citrus fruit-pathogen interaction although the magnitude of up-regulation was different in each strain. Pd1 exhibited six-fold induction at 2 dpi, compared to axenic growth, and the rate of transcription was higher; while Pd2 and Pd149 reached two-fold induction at 1-2 dpi, but the rate of transcription was reduced (Fig. 11D- F).

4. Discussion

To date, several mechanisms involving DMIs resistance have been described; mediated either by specific mutations in the coding region (Ali and Amiri, 2018; Snelders et al., 2015; Wang et al., 2014) or by increasing gene expression due to an insertion in the promoter (Fan et al., 2013).

In this study, we identify a sterol 14α-demethylase gene from an SSH cDNA library enriched in genes involved in *P. digitatum* virulence (López-Pérez et al., 2015). The resultant gene was identical to *PdCYP51B*, reported by Sun et al., 2011. The characterization of *PdCYP51B*, in the same 75 *P. digitatum* isolates previously studied for *PdCYP51A* (Sánchez-Torres and Tuset, 2011), revealed that 95% of isolates resistant to IMZ (IMZ^R) exhibited the presence of a 195-bp insertion in the *PdCYP51B* promoter. This sequence was identical, with the exception of four nucleotides, to the 199-bp promoter insertion described by Sun et al., (2011) which was later confirmed to be a MITE element, PdMLE (Sun et al., 2013). Contrary to what other authors reported (Ghosoph et al., 2007; Sun et al., 2011), we never found this insertion in the *PdCYP51A* promoter. Phylogenetic analysis of *PdCY51B* promoter showed that this sequence was only found in *Penicillium* spp. infecting citrus (*P. digitatum* and *P. italicum*), which

were resistant to IMZ. This suggests that this enhancer might be involved in disease progression and host specificity apart of conferring fungicide resistance.

Overexpression studies with the two sterol 14α-demethylase genes, *PdCYP51A* and *PdCYP51B*, were conducted in different genetic backgrounds to understand their role in the acquisition of resistance to DMIs. The molecular analysis of both sterol 14α-demethylase promoters in all different overexpression mutants, IMZ^R strain (Pd2) and in two IMZ^S strains (Pd27 and Pd149), revealed that the 195-bp insertion could be present as an ectopic element or could be introduced as a result of homologous recombination, thereby eliminating the original promoter. Although, all mutants showed both patterns, the most commonly occurring one was in which both forms coexisted. The most striking result was that the respective insertions present in both *PdCYP51A* and *PdCYP51B*, the five-tandem repeats and the 195-bp insertion, could not coexist when the progenitor previously carried the 126-bp five-tandem repeats (IMZ^R CA-R1). Thus, when the *PdCYP51A* promoter was characterized in Pd2-mutants, the five-tandem repeats were seen to be lost and DMI resistance was exclusively conferred by the 195-bp insertion. In contrast, the introduction of five-tandem repeats in Pd1 (IMZ^R CB-R3) allowed the coexistence of both enhancers. These results seem to indicate that *PdCYP51B* enhancer behaves as a transposon and acts as a MITE element (Sun et al., 2013) and is more stable and predominant than the *PdCYP51A* enhancer, consisting of 126-bp repeats. In fact, this sequence has been previously detected in *PdCYP51A*, as a 199-bp insertion (Ghosoph et al., 2007), suggesting high ability to transpose. This could explain why most *P. digitatum* isolates found with IMZ^R phenotype, acquired DMI resistance due to the presence of the 195-bp insertion. Transposition of these elements usually resulted after fungicide pressure (Chen et al., 2015). However, in this study the presence of the enhancer was not related to fungicide pressure and it was found in

isolates coming from the fungicide-untreated fields or from packing houses with fungicide treatment.

Moreover, the predominant role of *PdCY51B* in the acquisition of DMI resistance was confirmed through fungicide sensitivity studies. Results obtained with all *PdCYP51B* mutants proved the predominance of *PdCYP51B* in DMI resistance mechanism, since there was an increase in DMI resistance in previously resistant isolates and the reversion to a resistance phenotype to DMIs in the sensitive isolates. In contrast, the introduction of *PdCYP51A* in already resistant isolates (provided by the presence of 195-bp of *PdCYP51B*) did not show a summing effect on the resistance to DMIs, as seen in Pd1-mutants.

Further, the effect of *PdCYP51B* on pathogen virulence was confirmed, demonstrating that disease incidence increased in all mutants carrying the *PdCYP51B* enhancer. This result was more evident in Pd149-mutants, since they were of a less virulent origin, and the increase in their aggressiveness was greater and more visible. The contribution of *PdCYP51A* to virulence has been previously described for the rice blast fungus, *Magnaporthe oryzae*, in which sterol 14α-demethylase was crucial for conidiation and virulence (Yan et al., 2011), and in *Fusarium graminearum* (Fan et al., 2013) and *Verticillium dahliae* (Zhang et al., 2016).

The preponderance of *PdCYP51B* was further confirmed by the analysis of the gene expression. The high transcription level of *PdCYP51B* was confirmed in the Pd1 isolate compared to the parental strains that lacked the 195-bp insertion. Similarly, all *PdCYP51*B-overexpression mutants showed a significant increase in the rate of gene expression, independent of the progenitor.

Although Pd2 had the highest level of *PdCYP51A* transcription, Pd2-overexpressing *PdCYP51B* mutants showed reduction in *PdCYP51A* expression rate to

almost zero, by eliminating the *PdCYP51A* five-tandem repeat enhancer. Surprisingly, the transcription levels of *PdCYP51A* in isolates Pd27 and Pd149, increased slightly in the presence of 195-bp *PdCYP51B*, possibly because the enhancer could modulate the transcription of other genes. Previous work reported that this insertion (*PdMLE1*) is could act as a putative promoter and recruit transcription factor(s) (Sun et al., 2013). The effect of transposons in fungicide resistance has been elucidated in several fungal pathogens, such as *M. fructicola* (Luo and Schnabel, 2008), *V. inaequalis* (Schnabel and Jones, 2001), *Botrytis cinerea* (Kretschmer *et al*., 2009), *P. digitatum* (Hamamoto *et al*., 2000; Ghosoph *et al*., 2007; Sun *et al*., 2011), and *Aspergillus fumigatus* (Albarrag et al., 2011).

The role of *PdCYP51B* in the acquisition of resistance to DMIs and virulence was confirmed by the increase of transcription level seen, during the infection process of citrus fruits, particularly in the Pd1 strain, which is much more virulent. Recently, a review on fungal CYPs revealed the crucial role of *CYP51* in invasive fungal growth and virulence (Zhang et al., 2019). However, not all CYP51 genes are involved in virulence; in this work *P. digitatum PdCYP51A* did not showed any increase in transcription during fruit-pathogen interaction.

Very little is known about the regulatory mechanisms of both sterol 14α-demethylases genes. Recently, sterol regulatory element binding proteins (SREBPs) have been described as modulators of *CYP51*. For instance, factor SrbA, an SREBP in *A. fumigatus*, is induced by azole stress and is related to fluconazole resistance of *A. fumigatus* (Gsaller et al., 2016, Song et al., 2017). *sreA* has been reported to be required for resistance to prochloraz and the regulation of expression of *CYP51* in *P. digitatum* (Liu et al., 2015). Functional analysis revealed that both *PdsreA* and *PdsreB* play global regulatory roles in ergosterol biosynthesis and fungicide resistance (Ruan et al., 2017).

However, when both SREBPs were blocked, other transcription factors mediating ergosterol biosynthesis might be activated. In that respect, previous works have shown that both *PdCYP51A* and *PdCYP51B* could be modulated by *PdSte12* transcription factor (Vilanova et al., 2016) and *PdSlt2* MAP kinase (De Ramón-Carbonell and Sánchez-Torres, 2017c). Another fungal-specific Zn2-Cys6 transcription factor (AtrR) in *Aspergillus* spp. also modulates the expression of *CYP51A* by directly binding to the promoter region of this gene (Hagiwara et al., 2017). Hence, several mechanisms and signaling pathways could be involved in the regulation of sterol demethylases and further studies will be required to understand these mechanisms.

In summary, we functionally characterized two sterol-14α-demethylases of *P. digitatum*, demonstrating that the 195-bp *PdCYP51B* enhancer drives the acquisition of DMI fungicide resistance and contributes to increased virulence. With this work, we provide information on the main mechanisms responsible for azole resistance that could offer a solution for sustainable and long-term disease management.

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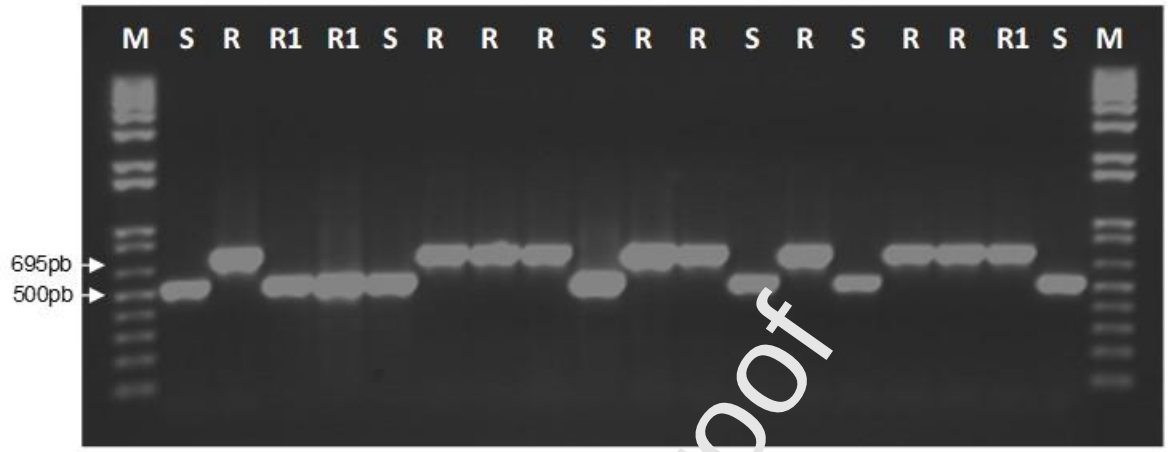

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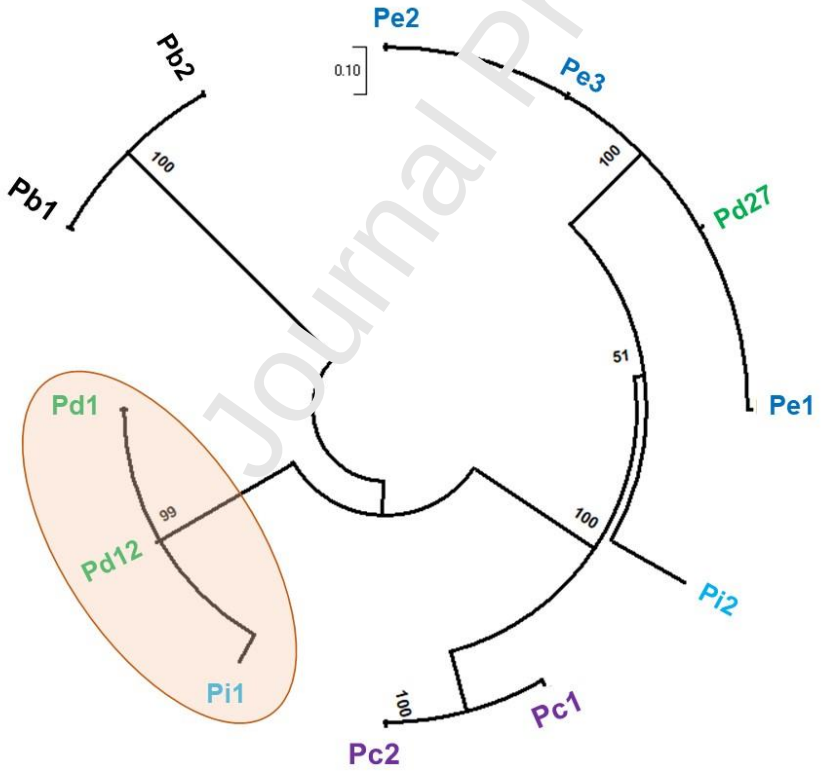

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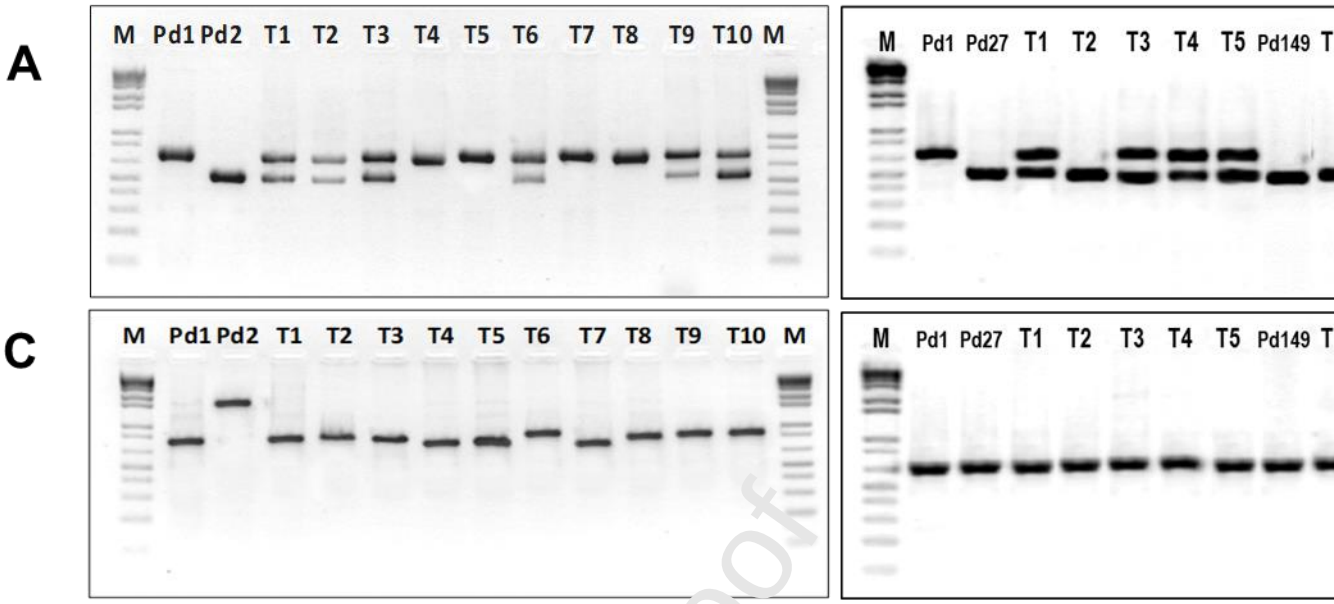

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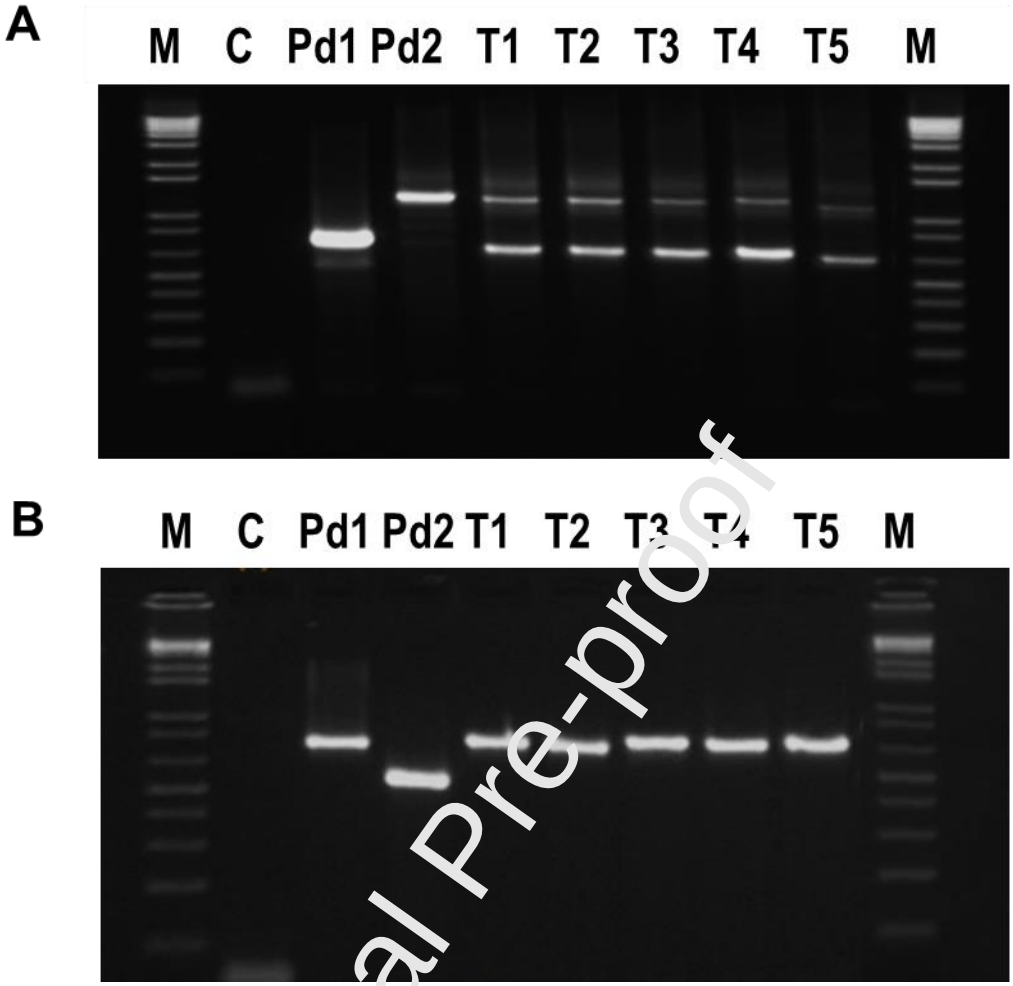

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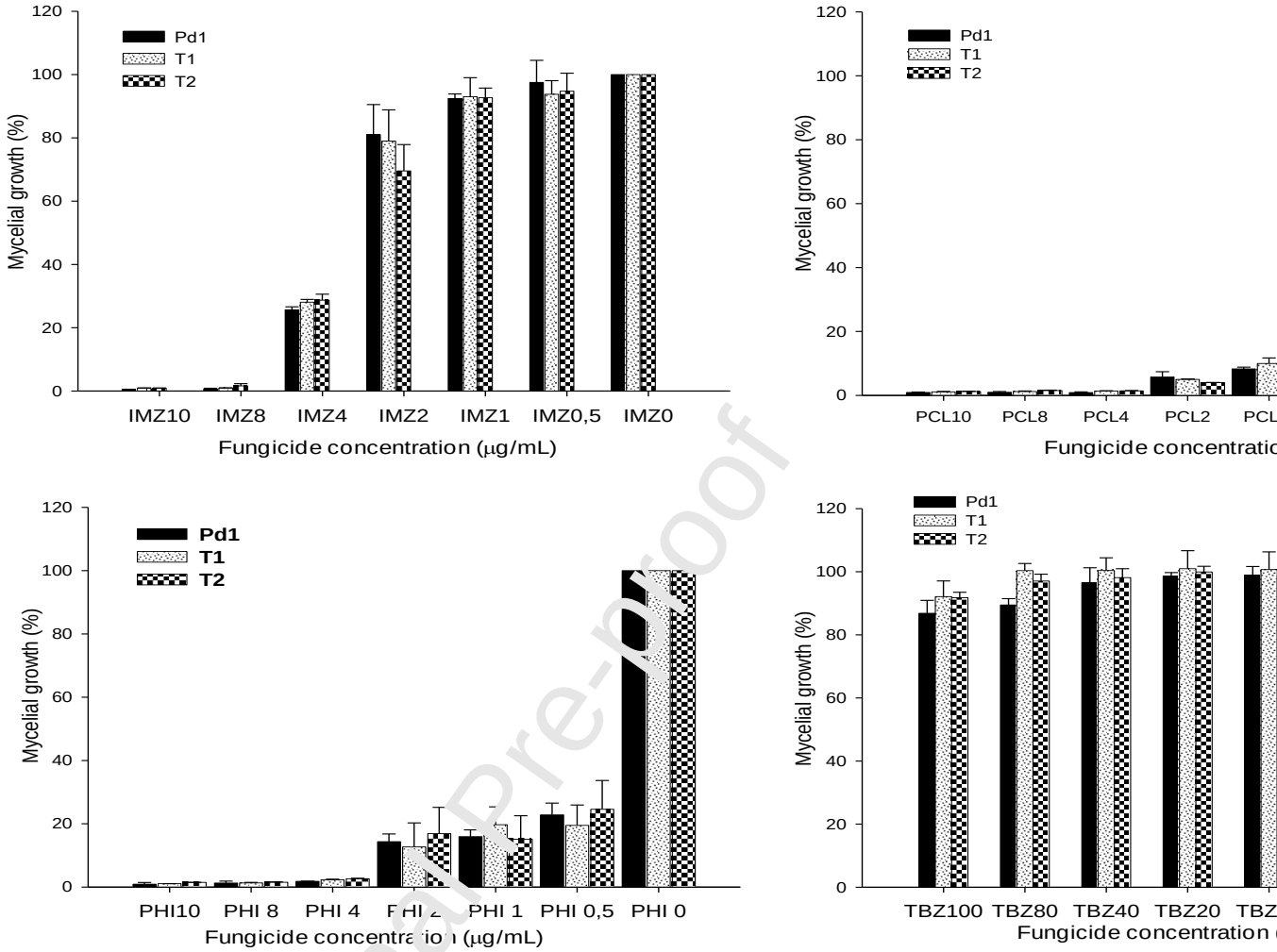

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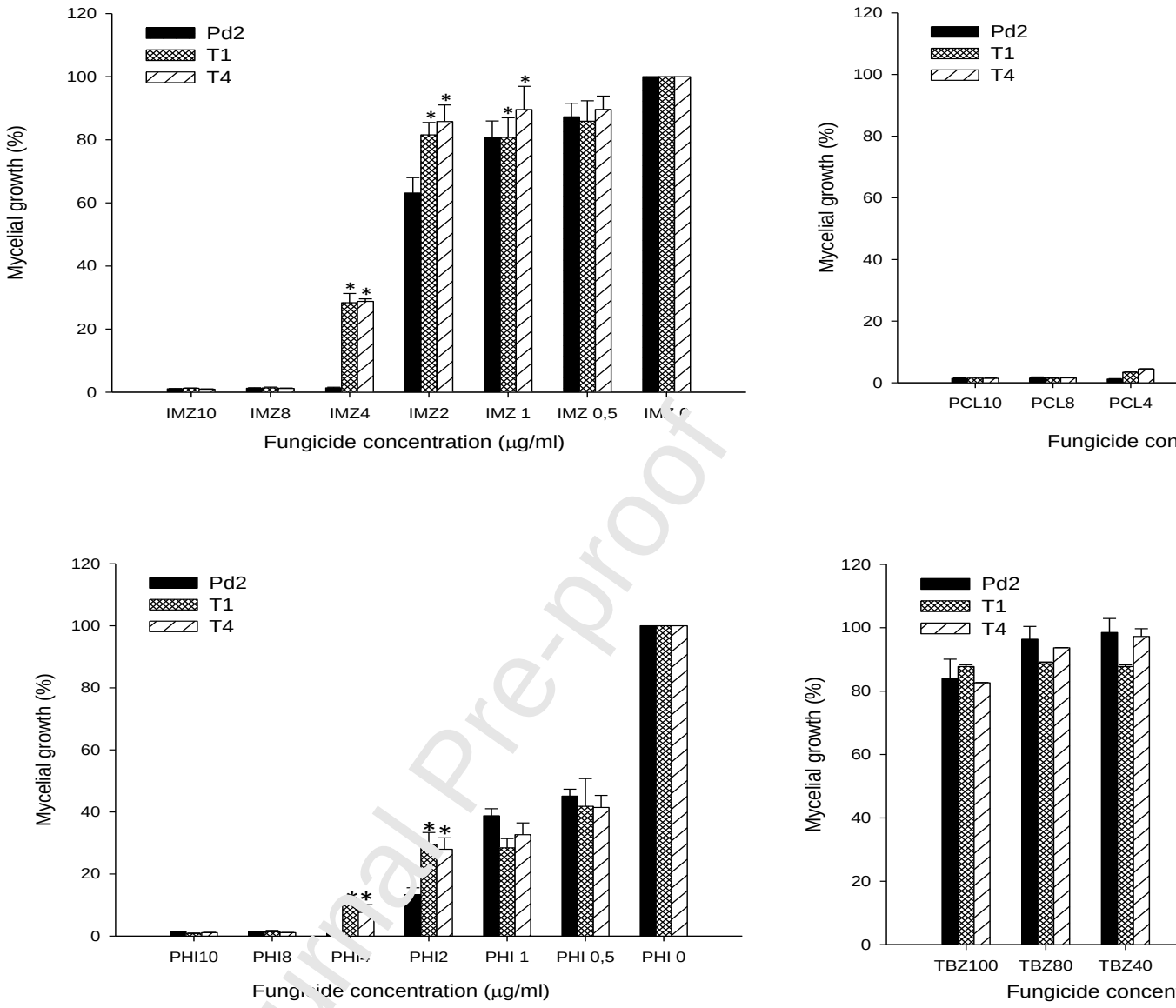

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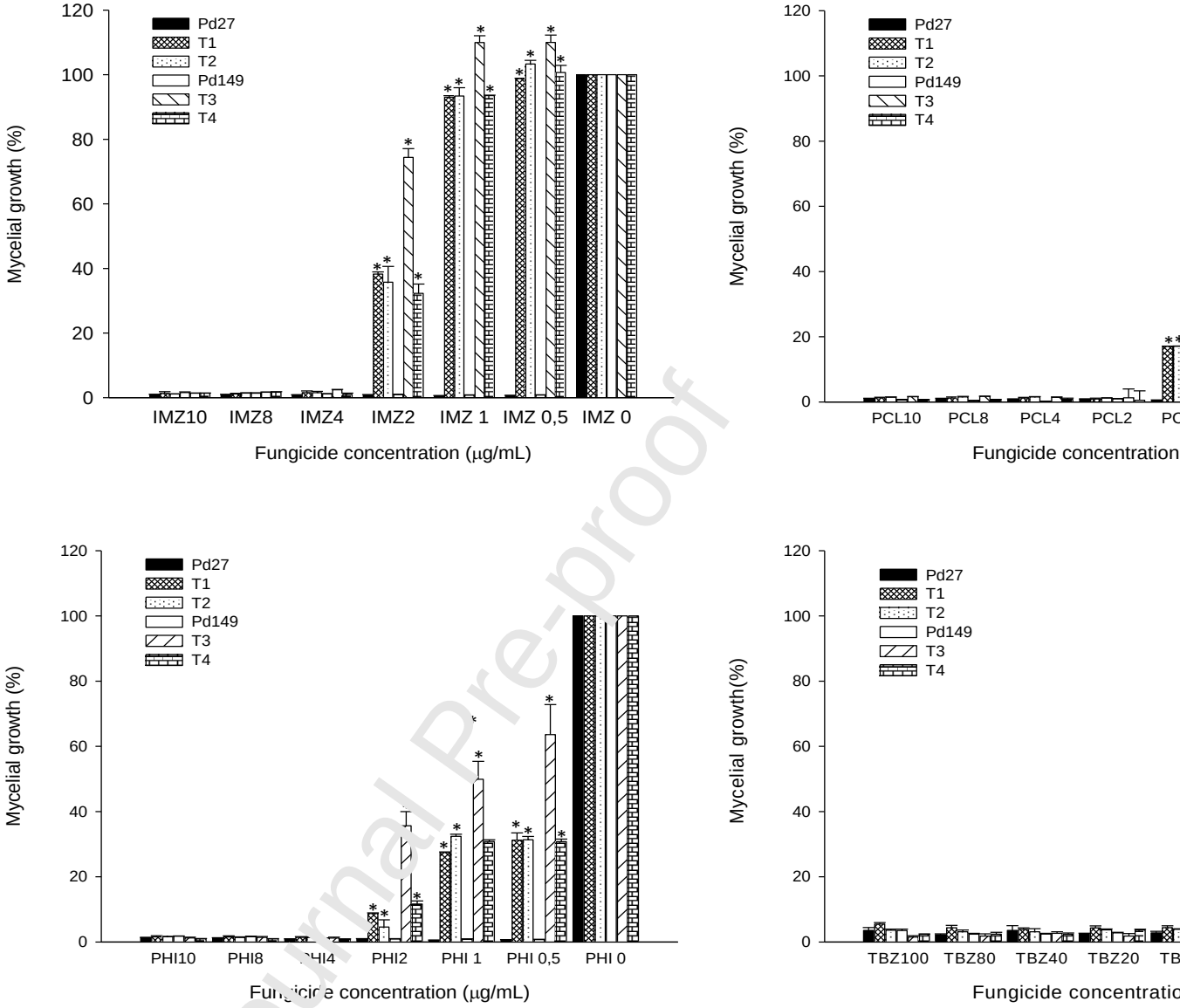

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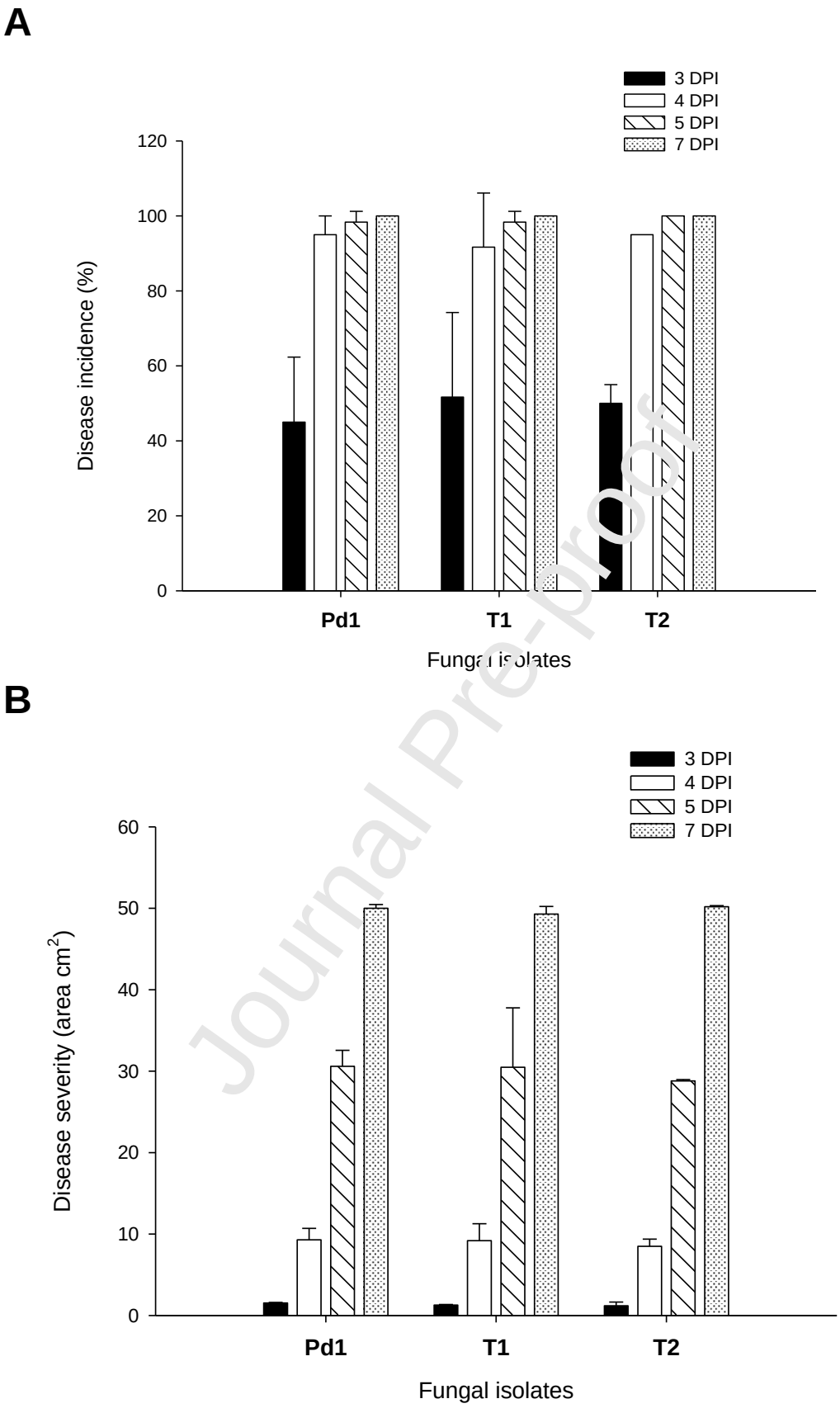

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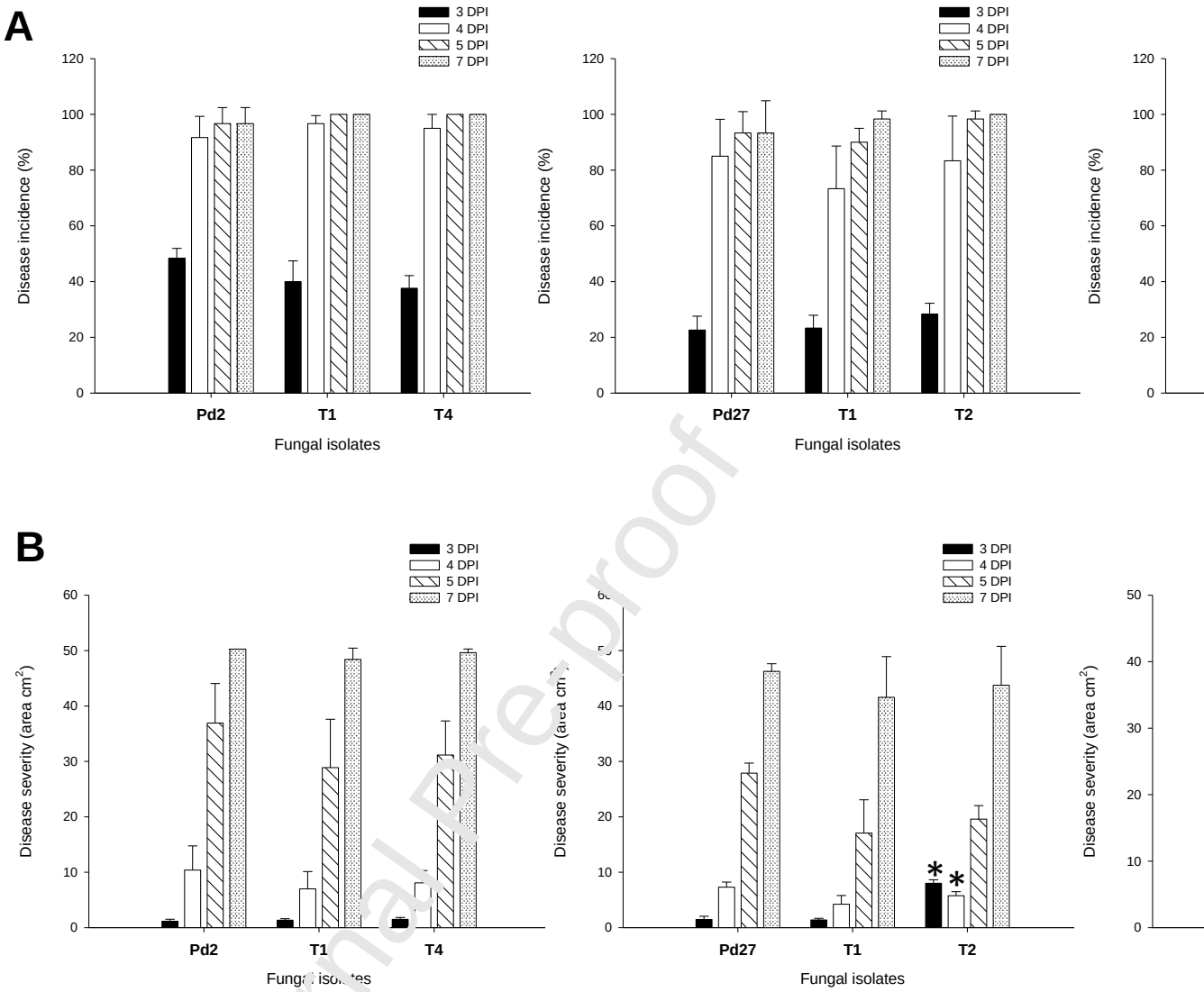

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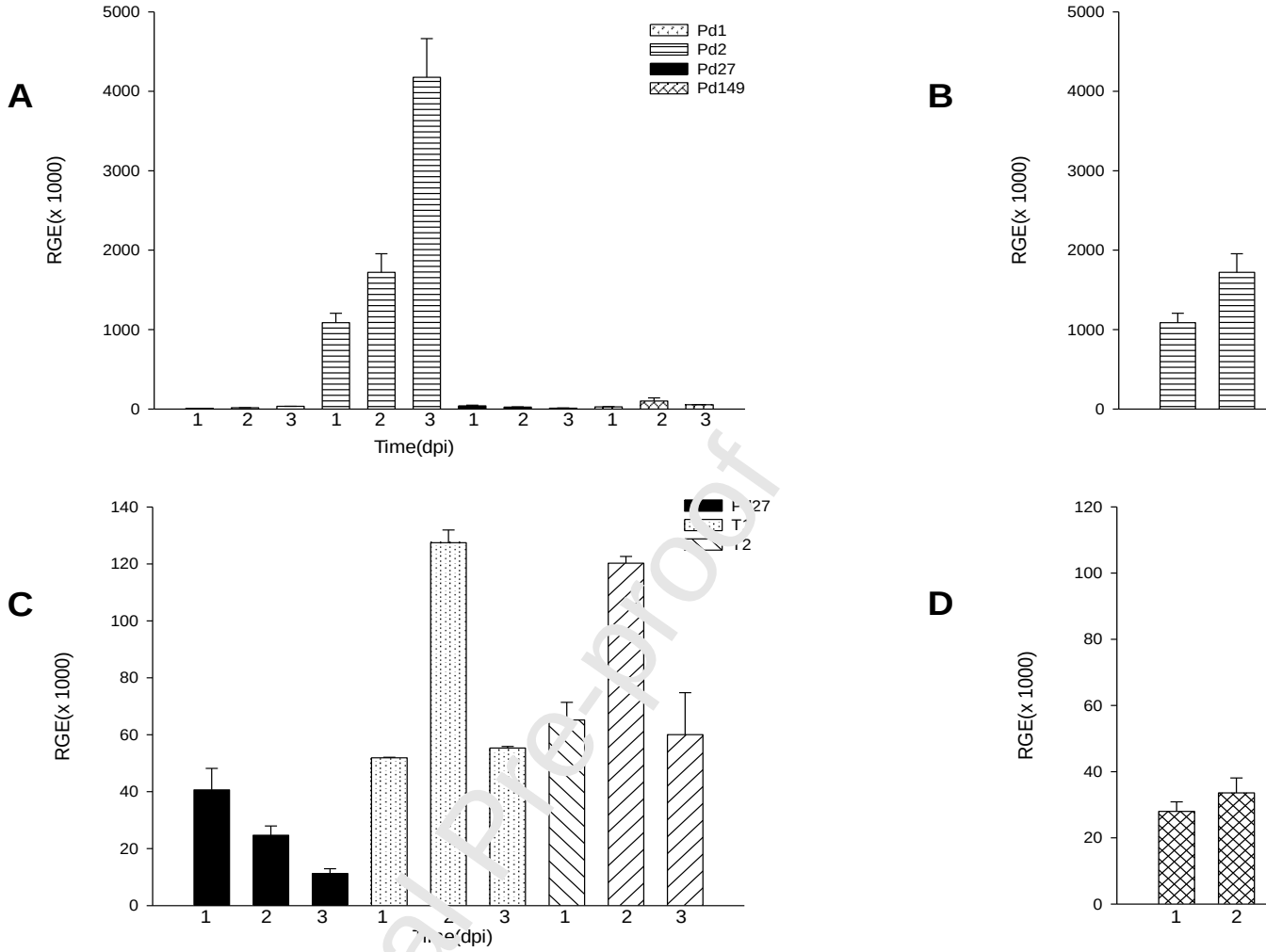

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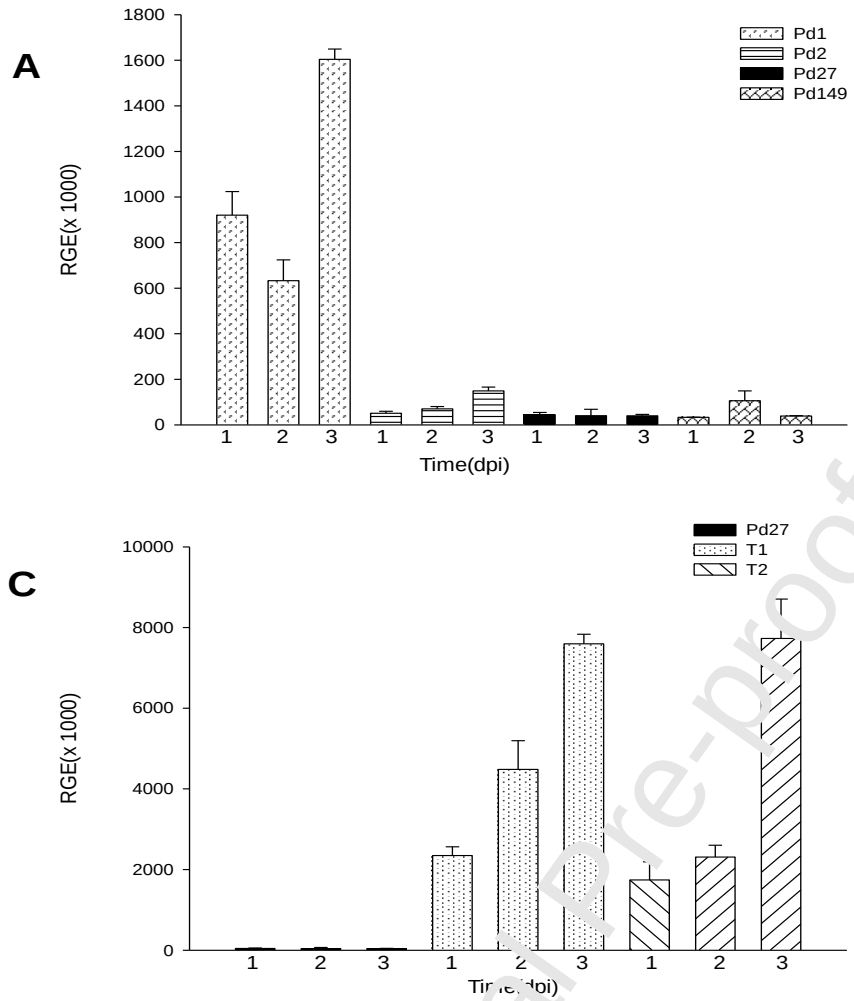

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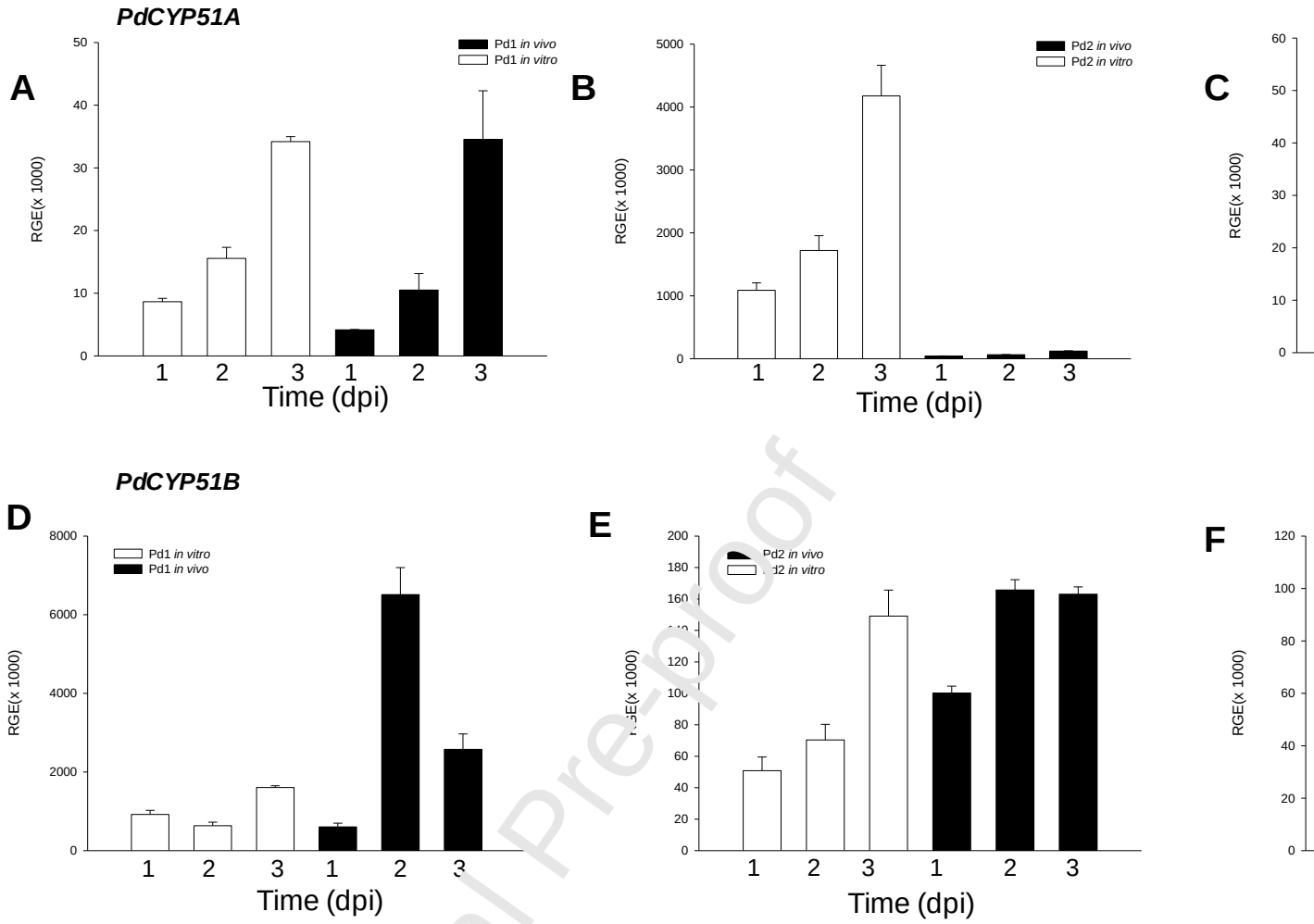


Fig. 11

Figures Legends

Figure 1. A: Evaluation of *PdCYP51B* promoter by PCR. S: sensitive isolates with absence of 195bp insertion. R: DMI resistant strains with 195bp insertion R1: DMI resistant strains without 195bp insertion. **B:** Unrooted Maximum Likelihood phylogenetic tree of different CYP51B promoter. Pe: *Penicillum expansum* strains (Pe1, Pe2, Pe3). Pi: *Penicillum italicum* strains (Pi1, Pi2). Pc: correspond to *Penicillum chrysogenum* strains (Pc1, Pc2). Pb: *Penicillum brevicompactum* (Pb1, Pb2). Pd: *Penicillum digitatum* strains (Pd1, Pd12, Pd27). Strains within blue circle are related to strains with presence of 195-bp insertion. Values indicate the number of times (in percent) that each branch topology was found during bootstrap analysis.

Figure 2. Evaluation of sterol 14α-demethylases promoters by PCR. Analysis of *PdCY51B* promoter in Pd2-overexpression mutants (lanes T1-10 after Pd1 and Pd2 wild-types) (A) and Pd27- (lanes T1-T5 after Pd27 wild-type) and Pd149-overexpression mutants (lanes T1-T5 after Pd149 wild-type) (B). Analysis of *PdCYP51A* promoter in Pd2-overexpression mutants (lanes T1-10 after Pd1 and Pd2 wild-types) (C) and Pd27- (lanes T1-T5 after Pd27 wild-type) and Pd149-overexpression mutants (lanes T1-T5 after Pd149 wild-type) (D). M: 1kb plus ladder marker.

Figure 3. Evaluation of CYP51s promoters' by PCR in Pd1-overexpression mutants. (A): Evaluation of *PdCYP51A* promoter. C corresponded to negative PCR control. Lanes Pd1 and Pd2 DNA from wild-type isolates and lanes T1-T5 Pd1-overexpression mutants (B): Evaluation of *PdCYP51B* promoter. C corresponded to negative PCR control. Lanes Pd1 and Pd2 DNA from wild-type isolates and lanes T1-T5 Pd1-overexpression mutants.

Figure 4. Fungicide sensitivity analysis in Pd1 mutants compared to wild type. IMZ=imazalil, TBZ=Thiabendazol, PCL=Prochloraz, PHI=Philabuster. Percentage of relative growth was calculated respect to the each strain grown without fungicide. Error bars represent standard deviation among three replicates. *Significant differences between treatments using Tukey's test ($P < 0.05$).

Figure 5. Evaluation of fungicide sensitivity in Pd2-overexpression mutants (T1 and T2) compared to wild type Pd2. IMZ=imazalil, PCL=Prochloraz, PHI=Philabuster, TBZ=Thiabendazol. Percentage of relative growth was calculated respect to the each strain grown without fungicide. Error bars represent standard deviation among three replicates. *Significant differences between treatments using Tukey's test ($P < 0.05$).

Figure 6. Evaluation of fungicide sensitivity in Pd27-(T1 and T2) and Pd149-(T3 and T4) overexpression mutants compared to their respective wild types. IMZ=imazalil, PCL=Prochloraz, PHI=Philabuster, TBZ=Thiabendazol. Percentage of relative growth was calculated respect to the each strain grown without fungicide. Error bars represent standard deviation among three replicates. *Significant differences between treatments using Tukey's test ($P < 0.05$).

Figure 7. Evaluation of virulence as (A) disease incidence (%) and (B) disease severity (cm^2). Virulence evaluation of Pd1 (wild-type) and the overexpression transformants (T1 and T2). DPI: days post inoculation. All are mean of three infection experiments. Control corresponded to oranges mock inoculated. Error bars represented standard deviation among replicates. *Significant differences between treatments using Tukey's test ($P < 0.05$) at each dpi.

Figure 8. Evaluation of virulence as (A) disease incidence (%) and (B) disease severity (cm^2). Virulence evaluation of Pd2 (wild-type) and Pd2 overexpression mutants (T1 and T4), Pd27 (wild-type) and Pd27 overexpression mutants (T1 and T2) and Pd149 (wild-

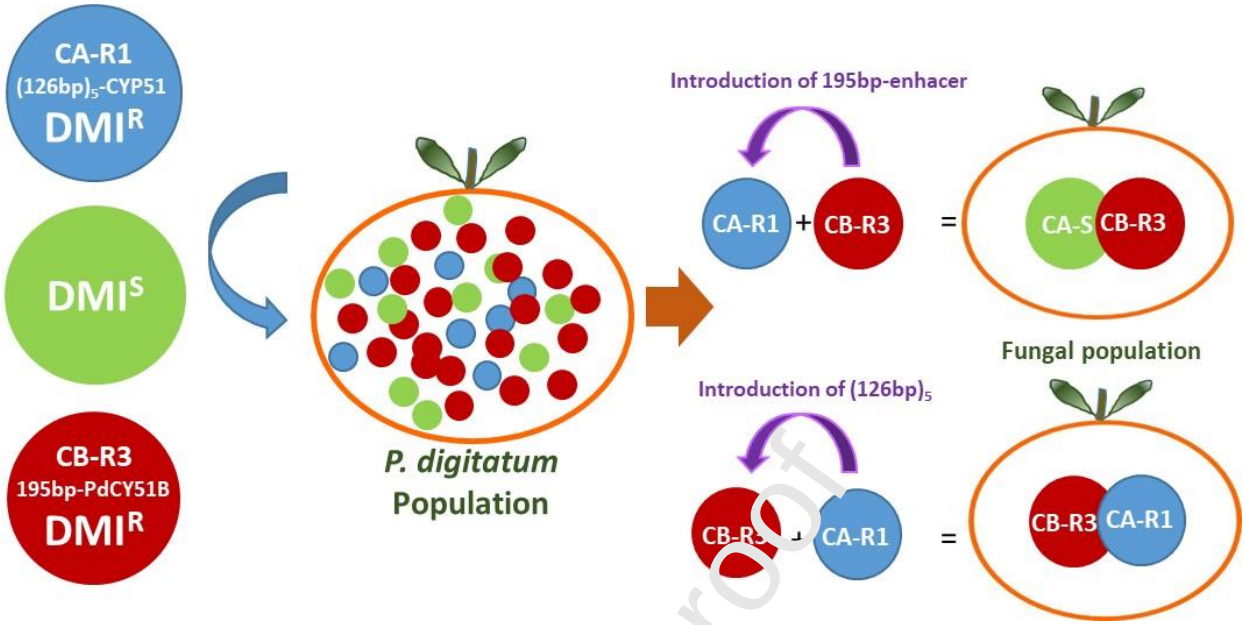
type) and Pd149 overexpression mutants (T4 and T5). DPI: days post inoculation. All are mean of three infection experiments. Control corresponded to oranges mock inoculated. Error bars represent standard deviation among replicates. *Significant differences between treatments using Tukey's test ($P < 0.05$) at each dpi.

Figure 9. Time course evaluation of *PdCYP51A* relative gene expression (RGE) of *P. digitatum* strains grown in PDB liquid culture at 25 ºC. (A): Analysis of all *P. digitatum* wild-type strains (B): Gene expression of Pd2-overexpression mutants T1 and T4 (C): Gene expression of Pd27-overexpression mutants T1 and T2. (D): Gene expression of Pd149-overexpression mutants T4 and T5. The expression levels are relative to three reference genes: ribosomal 28S, β-tubulin and histone H3. Error bars indicate standard deviations of three biological replicates.

Figure 10. Time course evaluation of *PdCYP51B* relative gene expression (RGE) of *P. digitatum* strains grown in PDB liquid culture at 25 ºC (A-D) and *P. digitatum* strains infecting citrus fruit. (A): Analysis of all *P. digitatum* wild type strains (B): Gene expression of Pd2-overexpression mutants (C): Gene expression of Pd27-overexpression mutants. (D): Gene expression of Pd149-overexpression mutants. (E): Gene expression of Pd1 wild type during orange infection (F): Gene expression of Pd149 wild type during orange infection.. The expression levels are relative to three reference genes: ribosomal 28S, β-tubulin and histone H3. Error bars indicate standard deviations of three biological replicates.

Figure 11. Time course evaluation of relative gene expression (RGE) of wild type *P. digitatum* isolates grown in PDB liquid culture at 25 ºC (*in vitro*) and wild type *P. digitatum* isolates infecting citrus fruit (*in vivo*). Evaluation of *PdCYP51A* gene A=Pd1, B=Pd2 and C=Pd149. Gene expression of *PdCYP51B* gene D=Pd1, E=Pd2 and

F=Pd149. The expression levels are relative to three reference genes: ribosomal 28S, β-tubulin and histone H3. Error bars indicate standard deviations of three biological replicates.



Highlights

- Reduced sensitivity to DMIs was not attributable to *PdCYP51B* coding region mutations.
- *Penicillium digitatum* DMI resistance is influenced by 195bp-enhacer of the sterol 14α-demethylase *PdCYP51B*
- 195bp-enhacer of *PdCY51B* stays more stable than 126bp five tandem repeats of *PdCYP51A.*
- Increase in *P. digitatum* virulence was associated to *PdCYP51B* sterol 14a-demethylase gene.