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Efflux Pumps and Multidrug-Resistance in *Pyricularia oryzae Triticum* Lineage

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Abstract: Widespread resistance to QoIs, DMI and SDHIs fungicides has been reported for Brazilian populations of the wheat blast pathogen *Pyricularia oryzae Triticum* lineage (*PoT*l). A pre-existing resistance mechanism not associated with target site mutations has been indicated for resistance to DMIs and SDHIs, with strong indication that *PoT*l has multidrugresistance (MDR). Therefore, the main objective of this study was to test the hypothesis that resistance to DMI and SDHI fungicides detected in *PoT*l was due to efflux pump mediated MDR mechanism(s) by characterizing the sensitivity to antifungal efflux pump substrates. Four antifungal substrates were tested: tolnaftate (TOL), cycloheximide (CHX), rhodamine 6G (RH6G) and triphenyltin chloride (TPCL). TPCL and RH6G were considered the most relevant indicators for enhanced MDR activity. Among the 16 *PoT*l isolates tested, 9 were insensitive to TPCL, 1 to TOL, 16 to RH6G and 1 to CHX. The *PoT*l isolates were grouped into four distinct multidrug resistance phenotypes (MDRPs) based on resistance to combinations of fungicides and antifungal efflux pump substrates. Insensitivity to TPCL, RH6G and or TOL correlated well with DMI insensitivity, but MDR was not associated with SDHI resistance. The identification of multiple MDRP phenotypes associated with DMI resistance in our study warrants further research aimed at revealing the exact mechanisms of multidrug resistance in the wheat blast pathogen, including efflux pumps overexpression via transcriptomic analyses of differentially expressed genes; identification and discovery of mutations associated with changes in promoter regions or transcription factors of efflux transporters associated with multidrug resistance.

Keywords: wheat blast; MDR; QoI; DMI; SDHI

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1. Introduction

Wheat blast is caused by the ascomycete fungus *Pyricularia oryzae Triticum* lineage (*PoT*l) [1,2]. This is a very aggressive disease, and its management is hampered by the absence of durable host plant resistance sources due to the extremely variable pathogen populations, both genetically and phenotypically [3,4]. In addition, the frequent and extensive use of site-specific fungicides without adopting anti-resistance strategies, including other integrated disease management strategies, has resulted in a general loss of fungicide efficacy for management of the disease [2,5].

Resistance to the site-specific fungicides QoI (quinone-outside inhibitors) and DMI (sterol 14α-demethylation inhibitors) has become pervasive and persistent in *PoT*l populations from all wheat-cropping regions throughout Brazil over a time span of seven years, from 2012 to 2019 [6–10]. Resistance to QoI was mainly associated with cyt*B* target site

alteration G143A (substitution of glycine (G) by alanine (A) at codon 143), which has been reported for many plant pathogens [6,11]. Due to the absence of *CYP51* mutations and promoter inserts linked with azole resistance, the mechanism(s) conferring azole resistance remains unclear for *PoT*l populations in Brazil.

Moreover, resistance to the new generation carboxamide fluxapyroxad (a succinate dehydrogenase (Sdh) inhibitor (SDHI) fungicide) was detected in *PoT*l populations sampled in 2012 and 2019 [10]. Since SDHI resistance was detected in Brazil before the introduction of this fungicide MOA in the cereals market and no changes in Sdh subunits B, C and D, forming the SDHI binding site, were detected, it was suggested that resistance is mediated by a pre-existing non-target site resistance mechanism.

Reduced sensitivity phenotype to multiple chemically unrelated compounds is defined as multidrug resistance (MDR), which is often caused by overexpression of efflux transporters with broad substrate specificity, as has been reported for a range of plant pathogens [12–15]. In fungi, the major drug efflux pumps are members of the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter proteins [16]. Together, these two superfamilies of integral membrane transporters represent roughly half of all the genes coding for transporters in fungal genomes [17]. As an example, *Zymoseptoria tritici* genome analysis indicated the presence of 47 ABC transporters and 288 MFS transporters, of which at least 87 of the latter are putative multidrug transporters [18].

ABC transporters consists of transmembrane domains (TMD) and structurally conserved nucleotide-binding domains (NBDs) and function as molecular machines by coupling ATP binding, hydrolysis, and phosphate release to the translocation of diverse substrates across membranes [19]. In comparison, MFS transporters use proton motive forces to drive substances across membranes. These proteins play fundamental roles, such as the transport of small molecules, metabolites, and ions, as well as fungicides, toxins, and heavy metals removal as protection against biotic and abiotic stresses [19–21]. ABC or MFS transporters are located in different types of membranes, from the external plasma membrane to intracellular compartments membranes such as vacuole, endoplasmic reticulum, peroxisomes and mitochondria [16,19,22,23].

Overexpression of efflux pumps can be caused by modifications in their promoter regions or due to gain-of-function mutations in transcription factors that control expression. Omrane et al. [24] identified for the Septoria leaf blotch pathogen *Z. tritici* a 519-bp long terminal repeat (LTR) insert (relic of transposon activity) in the *MFS1* promoter cosegregating with the MDR phenotype using a bulk progeny sequencing approach. Through gene replacement, they showed that the promoter insert was responsible for *MFS1* overexpression and the MDR phenotype. In addition, different types of shorter promoter inserts were found in more recent MDR strains. In MDR strains of human pathogen *Aspergillus fumigatus*, the C_2H_2 zinc finger transcription factor *SltA* was pointed as relevant for azole resistance by coregulating the expression of the fungicide target gene *CYP51A* and the efflux pump *Mdr1* [25]. In MDR isolates of the rice blast pathogen *PoO*l, single point mutations in the *MoIRR* gene encoding a Zn_2Cys_6 zinc finger transcription factor-like protein confers resistance to isoprothiolane and to iprobenfos plus tricyclazole [15]. This Zn_2Cys_6 domain is also present in the *Mrr1* transcription factor found in the opportunistic human fungal pathogen *Candida albicans* and in grape's gray mold pathogen *Botrytis cinerea* [13,26]. Specifically, mutations in the *Mrr1* regulates gene expression of *CDR1* (ABC) transporter in *C. albicans* and the *BcAtrK* (ABC) transporter in *B. cinerea* with MDR phenotype MDR1. Another mechanism reported for MDR2 in *B. cinerea* is the *BcmfsM2* (MFS) transporter gene promoter region rearrangement [13,27]. MDR3 in *B. cinerea*, generated through sexual crossing, combines both the MDR1 and MDR2 mechanisms, resulting in increased substrate range, and resistance to fludioxonil, cyprodinil and tolnaftate.

In general, multidrug transporters have a wide range of substrates with differences in specificity roles according to transporter types, their abundance and recognition of a domain's structure. For example, the *MgMFS1* transporter system in *Z. tritici* has redundancy roles in the transport of rhodamine 6G, once its gene deletion is compensated by expression

changes in other transporters with an affinity for this drug [28]. However, the knockout of *MgMFS1* caused an increase in sensitivity to QoI fungicides, with no redundancy in this case. In fact, there is no obvious correlation between homology in the transporter amino acid sequence and specificity to substrates. For instance, the *PoO*l ABC2 transporter has high amino acid homology with BMR1 (BcatrK) in *B. cinerea*, although they have different ranges of substrates [29]. The *PoO*l ABC2 transports the DMI tebuconazole, cycloheximide, bitertanol, myclobutanil and camptothecin, while BMR1 extrudes ibrobenfos and polyoxin.

Chemically unrelated antifungal compounds have been used as efflux pump substrates in order to test hypotheses about the role of MDR mechanisms and to distinguish different MDR phenotypes caused by enhanced activity of MFS and/or ABC transporters. Triphenyltin chloride, tolnaftate, rhodamine 6G and cycloheximide are amongst the most common efflux pump substrates used as indicators of MDR phenotypes [14,30–32]. In our study, we tested the hypothesis that resistance to DMI and SDHI fungicides detected in *PoT*l was due to efflux pump mediated MDR mechanism(s) by characterizing the sensitivity to these four antifungal efflux pump substrates. Based on the sensitivity levels to the efflux pump substrates, we can also identify if there are different MDR phenotypes amongst the *PoT*l strains previously sampled in 2012 and 2018.

2. Materials and Methods

2.1. Fungal Strains

To carry out this study, twenty-one isolates of *Pyricularia* spp. previously classified according to their insensitivity levels to QoI, DMI and SDHI fungicides were randomly selected from our collections spanning 2007, 2017, 2018 and 2019 sampling years [6,9,10]. The strains selected harbor distinct combinations of resistance phenotypes from fully sensitive isolates to fully resistant isolates to DMI and SDHI fungicides.

2.2. Sensitivity to Efflux Pump Substrates

The antifungal efflux pump substrates tested were tolnaftate (TOL), cycloheximide (CHX), rhodamine 6G (RH6G) and triphenyltin chloride (TPCL) (all from Sigma-Aldrich, St. Louis, MO, USA). These drugs were dissolved to 1 mg·mL^{-1} in acetone followed by dilution in deionized water to obtain a 100 µg·mL^{-1} stock solution.

Sensitivity tests with efflux pump substrates were conducted with flat-bottomed 96-well microtiter plates (Kasvi, India) using mycelial fragments. Mycelium fragments were obtained from fungal colonies that were grown for 10 days in PDA at 25 °C under 12 h photoperiod using a protocol adapted from Brito et al. [33] and Arango Isaza et al. [34]. Pathogen mycelial samples from 10-day old colony from a single agar plate were transferred to a mortar and macerated using a pestle in 3 mL sterilized water and Tween 20 (one drop L^{-1}). Each 1 mL of the macerate was transferred to tubes containing a volume of 1 mL of sterile 2 mm glass beads and vortexed for two minutes. The mycelial fragments suspension recovered from tubes were mixed and pre-diluted to a final volume of 15 mL, counted under a microscope using a Neubauer chamber and adjusted to 10^5 fragments mL^{-1}. Each microplate well was filled with 50 µL of inoculum suspension and 100 µL of PD broth (20.7 g·L^{-1} of potato dextrose (Kasvi, India), 1 L of distilled water)) amended with different concentrations of the efflux pump substrates tested and in the presence of chloramphenicol and streptomycin (50 µL·mL^{-1} each).

Stock solutions from each of the substrates were added to the wells to obtain the following final concentrations: tolnaftate: 0.0, 0.032, 0.16, 0.8, 4 and 20 µg·mL^{-1}; cycloheximide, rhodamine 6G and triphenyltin chloride: 0.0, 0.016, 0.08, 0.4, 2 and 10 µg·mL^{-1}. For strains extremely sensitive to triphenyltin chloride, the following dilution series was used: 0.0, 0.000128, 0.00064, 0.0032, 0.016 and 0.08 µg·mL^{-1}. The microplates were incubated at 25 °C for 5 days under a 12 h photoperiod. After incubation, mycelial growth (*mg*) was measured based on absorbance values at 620 nm ($mg = t_5 - t_0$; where t_0 = reading (absorbance at 620 nm) immediately after plate assembly; t_5 = reading after five days

incubation) using a microplate reader (Multiskan^{TM} FC Microplate Photometer, Thermo Scientific TM, Waltham, MA, USA).

Sensitivity to efflux-pump substrates was determined as 50% effective concentration to inhibit fungal growth (EC_{50} in $\mu g \cdot mL^{-1}$), estimated using the software *ED50 plus* v1.0 [35]. Resistance factors (RF) were determined to estimate the relative fold-inhibition of all the strains tested based on their EC_{50} values and normalized by the lowest EC_{50} for each substrate. For EC_{50} means comparison within each substrate (TPCL, TOL, RH6G or CHX), the experimental design consisted of complete randomized blocks, with four replicates per treatment and experiments in duplicate.

2.3. Statistical Analysis

Analysis of variance (ANOVA) by the *F* test and means comparison by the Scott-Knott test (at 5% probability) were performed using the R environment with the statistical libraries *agricolae* and *laercio* [36].

Pearson's correlation analysis was performed using EC_{50} data from all fungal strains to explore the relationships among sensitivity to efflux pump substrates and the fungicides tebuconazole (DMI) and fluxapyroxad (SDHI). The DMI and SDHI EC_{50} data were obtained from previous studies [9,10]. To verify the correlation significance, *t* tests were applied using the R environment with the statistical library *performance analytics* [36].

3. Results

3.1. Sensitivity to Efflux Pumps Substrates

The statistical analysis indicated no significant difference between replicates (R) of the experiments, as well no significant interaction between replicates and the strain (S) factor (R*S). This indicated the reproducibility of the observations allowing for the joint analysis of the replicates. The strain effect was highly significant ($p < 0.01$) for all four efflux pump substrates checked (Table 1).

Table 1. Analysis of variance of the effect of strains on the EC_{50} values for efflux pump substrates.

Source of Variation	*df*	Triphenyltin Chloride		Tolnaftate		Rhodamine 6G		Cycloheximide	
		Mean Square	*F* Values	Mean Square	*F* Values	Mean Square	*F* Values	Mean Square	*F* Values
Strain (S)	20	0.0802	53.923 **	5,043	43.241 **	2.946	6.325 **	0.5575	20.851 **
Block	3	0.0021	1.431 ^{NS}	0.0965	0.828 ^{NS}	0.1237	0.265 ^{NS}	0.0881	3.295 ^{NS}
Residuals	122	0.0015		0,1166		0.4659		0.0267	

** Significance by the *F* test at $p < 0.01$; ^{NS} non-significant. The experiment was repeated once and a joint analysis of the data was performed, since no interaction strain vs. experiment was detected in the individual analyses.

Nine out of the 16 *PoT*l strains tested were insensitive to the efflux pumps substrate triphenyltin chloride (TPCL), as shown by a greater than 5-fold elevated EC_{50} level in comparison with the average EC_{50} value of 0.0085 $\mu g \cdot mL^{-1}$, recorded for the two fungicide sensitive *PoO*l strains 421 and 656 (Table 2). The strains *PoT*l 18SPK6 (Figure 1A), and 18SPC10 showed the highest significant EC_{50} values for TPCL, 0.31 $\mu g \cdot mL^{-1}$, resulting in a resistance factor (RF) of ~36-fold in comparison with the average EC_{50} value of the two fungicides sensitive PoOl strains (Table 2). The RFs for the other seven TPCL insensitive *PoT*l strains ranged from 7.1 to 31-fold, while the two other *PoT*l strains were sensitive, showing RFs < 5.

Only one *PoT*l strain, 12.1.130 (Figure 1D), was insensitive to tolnaftate (TOL), with an EC_{50} of 4.40 $\mu g \cdot mL^{-1}$, showing a RF of 13 in comparison with the average EC_{50} value of 0.34 $\mu g \cdot mL^{-1}$ measured for the two fungicide sensitive *PoO*l strains 421 and 656 (Figure 1B and Table 2). All other 15 *PoT*l strains and 2 *PoO*l strains tested were sensitive, showing RFs between 0.7 and 3.9.

Table 2. Field isolates of *Pyricularia* spp. sampled in Brazil in 2012 and 2018 and respective resistance phenotype classes to three major fungicide classes and sensitivity to efflux pump substrates and respective resistance factors.

[a] Species and Isolates	[b] Previously Determined Fungicide Resistance Phenotype			[f,g,h,i] Mean EC_{50} (μg·mL^{-1}), Standard Deviation and RF												[j] MDR Phenotype
	[c] QoI Azoxystrobin	[d] DMI Tebuconazole	[e] SDHI Fluxapyroxad	Triphenyltin Chloride (TPCL)		RF TPCL	Tolnaftate (TOL)		RF TOL	Rhodamine 6G (RH6G)		RF RH6G	Cycloheximide (CHX)		RF CHX	
Pyricularia oryzae Triticum lineage—PoTl																
12.1.015	S	R	RS	0.030 (0.01)	d	3.5	0.25 (0.1)	d	0.73	1.01 (0.9)	b	29	0.10 (0.08)	d	0.83	MDRP1
12.1.045i	R	R	RS	0.036 (0.01)	d	4.2	0.36 (0.1)	d	1.1	1.26 (0.41)	b	37	0.13 (0.09)	d	1.1	MDRP1
12.1.037	R	R	RS	0.04 (0.002)	d	4.7	0.31 (0.3)	d	0.91	1.22 (0.8)	b	35	0.09 (0.07)	d	0.75	MDRP1
18MGH25	R	MR	RS	0.06 (0.03)	d	7.1	0.62 (0.33)	d	1.8	0.80 (0.54)	c	23	0.18 (0.11)	d	1.5	MDRP2
18MGH11	R	R	HR	0.04 (0.005)	d	4.7	0.25 (0.07)	d	0.74	0.47 (0.27)	c	13	0.17 (0.14)	d	1.4	MDRP1
12.1.005	R	R	S	0.04 (0.03)	d	4.7	0.55 (0.29)	d	1.6	1.76 (1.1)	a	50	0.18 (01.5)	d	1.5	MDRP1
12.1.183	R	R	MR	0.16 (0.06)	b	19	0.87 (0.49)	c	2.6	1.07 (0.83)	b	31	0.42 (0.15)	c	3.5	MDRP2
18MGH19	R	R	RS	0.12 (0.02)	c	14	1.02 (0.30)	c	3.0	0.73 (1.08)	c	21	0.27 (0.45)	c	2.3	MDRP2
18SPK6	R	R	RS	0.31 (0.09)	a	36	1.33 (0.68)	b	3.9	1.77 (0.80)	a	51	0.15 (0.05)	d	1.3	MDRP2
12.1.299	R	R	MR	0.17 (0.04)	b	20	0.79 (0.57)	c	2.3	2.13 (0.65)	a	61	0.11 (0.09)	d	1.1	MDRP2
12.1.312	R	R	MR	0.19 (0.08)	b	22	0.89 (0.64)	c	2.6	1.03 (0.34)	b	29	0.11 (0.04)	d	0.92	MDRP 2
18MGF23	R	R	MR	0.15 (0.03)	b	18	0.31 (0.12)	d	0.91	1.35 (0.29)	b	39	1.24 (0.53)	a	10	MDRP4
18SPC10 b	R	R	HR	0.31 (0.04)	a	36	0.50 (0.31)	d	1.5	1.25 (1.28)	b	36	0.13 (0.03)	d	1.1	MDRP2
12.1.165	S	R	S	0.27 (0.05)	a	32	0.36 (0.09)	d	1.1	1.47 (1.00)	b	42	0.16 (0.06)	d	1.3	MDRP2
12.1.130	S	HR	RS	0.04 (0.002)	d	4.7	4.40 (2.2)	a	13	1.87 (0.6)	a	53	0.11 (0.03)	d	0.92	MDRP3
18PRH9	R	R	S	0.03 (0.004)	d	3.5	0.87 (0.45)	c	2.6	0.50 (0.6)	c	14	0.12 (0.03)	d	1.0	MDRP1
Pyricularia oryzae Oryza lineage—PoO																
656	S	S	S	0.013 (0.007)	d	1.53	0.45 (0.28)	d	1.32	0.06 (0.05)	d	1.71	0.12 (0.1)	d	1.0	-
421	S	S	S	0.004 (0.001)	d	0.47	0.23 (0.08)	d	0.68	0.01 (0.01)	d	0.29	0.80 (0.34)	b	6.7	-
674	S	S	RS	0.033 (0.03)	d	3.88	0.42 (0.33)	d	1.24	0.25 (0.17)	c	7.14	0.09 (0.09)	d	0.75	-
704	S	S	MR	0.004 (0.001)	d	0.47	0.28 (0.09)	d	0.82	0.05 (0.03)	d	1.43	0.09 (0.06)	d	0.75	-
Pyricularia grisea—Pg																
363	R	S	S	0.04 (0.004)	d	4.71	0.26 (0.08)	d	0.76	1.67 (1.4)	a	48	0.11 (0.05)	d	0.92	-

[a] The *PoT*l strain coding indicates the respective sampling year: '12' for 2012 populations and '18' for 2018 populations; *PoO*l and *Pg* isolates were from 2012 populations. [b] Sensitivity phenotype classes: S = sensitive, RS = reduced sensitivity, MR = moderately resistant, R = resistant, HR = highly resistant. [c] According to Castroagudín et al. [6]: R isolates grew at the discriminatory dose of 10 μg·mL^{-1}. [d] According to Poloni et al. [9]: S, EC_{50} = 0.007–0.07 μg·mL^{-1}; MR, EC_{50} = 0.62–0.98 μg·mL^{-1}; R, EC_{50} = 1.03–1.44 μg·mL^{-1}; HR ≥ 1.62–1.70 μg·mL^{-1}. [e] According to Vicentini et al. [10]: S, EC_{50} = 1.15–2.94 μg·mL^{-1}; RS, EC_{50} = 3.30–19.31 μg·mL^{-1}; MR, EC_{50} = 21.83–43.67 μg·mL^{-1}; HR ≥ 50–100 μg·mL^{-1}. [f] Effective concentration to inhibit fungal growth in 50% (EC_{50}) using mycelial fragment method evaluation by absorbance at 620 nm in microplates. [g] Means followed by the same letters in the column did not differ by the Scott-Knott test (at $p \leq 0.05$). Standard deviation in parenthesis. [h] Resistance factor (RF) indicates the ratio of the strain's EC_{50} tested to the mean average EC_{50} value for strains PoOl 421 and 656 for each antifungal- isolate combination, with exception for cycloheximide where only the EC_{50} value of PoOl 656 was used due to insensitivity of strain PoOl 421. [i] RF within each substrate: dark purple = very high, orange = high, light purple = medium. [j] Multidrug-resistance phenotype of *PoT*l strains (MDRP) classification according to the combination of resistance to different efflux pump substrates and resistance to tebuconazole (DMI) and/or fluxapyroxad (SDHI).

All 16 *PoT*l strains and 1 *PoO*l strain (674) showed raised EC_{50} values to rhodamine 6G (RH6G) (Table 2). The RFs ranged from 13- to 61-fold for the *PoT*l strains with the highest EC_{50} value, 2.13 μg·mL^{-1}, measured for strain 12.1.299 (Figure 1B). A lower RF value of 7.1 was measured for the RH6G sensitive *PoO*l strain 674.

The cycloheximide (CHX) EC_{50} values for the 20 *Po* strains tested ranged from 0.09 to 1.24 μg·mL^{-1} (Table 2). The fungicide sensitive *PoT*l reference strain 421 was insensitive and showed a RF of 6.7 in comparison with *PoT*l strain 656. Only one *PoT*l strain, 18MGF23, was also insensitive with an EC_{50} value of 1.24 μg·mL^{-1} and a RF of 10 (Figure 1C).

The strain *P. grisea* 363 was resistant only to RH6G, while *PoO*l 421 was resistant only to CHX (Table 2).

*PoT*l strains were subsequently classified into multidrug-resistance phenotype patterns (MDRP) based on the resistance levels to the four different efflux pump substrates tested (Table 2). Isolates resistant to either one of the target-site fungicides, DMI or SDHI, or both (Table 2), and also to at least one unrelated efflux pump substrate, were considered as expressing MDR (Table 2). The resistance to QoIs was not used as a parameter, because high levels of resistance in these strains was linked to the target-site alteration G143A in cytochrome *b* gene.

Four distinct MDR phenotypes (MDRP1-4) were observed amongst the 16 *PoT*l strains (Table 2): (i) strains with high levels of RH6G and DMI resistance, and with varying levels of SDHI insensitivity (MDRP1, six strains); (ii) strains resistant to RH6G, TPCL and DMI with different levels of SDHI insensitivity (MDRP2, eight strains); (iii) strains resistant to TOL, RH6G, DMI and SDHI (MDRP3, strain 12.1.130) and (iv) strains resistant to TPCL, RH6G, CHX, DMI and SDHI (MDRP4, strain 18MGF23).

*PoO*l strains 656 and 704 were sensitive to all four efflux pump substrates, but only *PoO*l 704 was also resistant to the SDHI tested. *PoO*l strain 421 showed insensitivity to CHX but was sensitive to both DMI and SDHI, while *PoO*l strain 674 was insensitive to both RH6G and SDHI but sensitive to DMI.

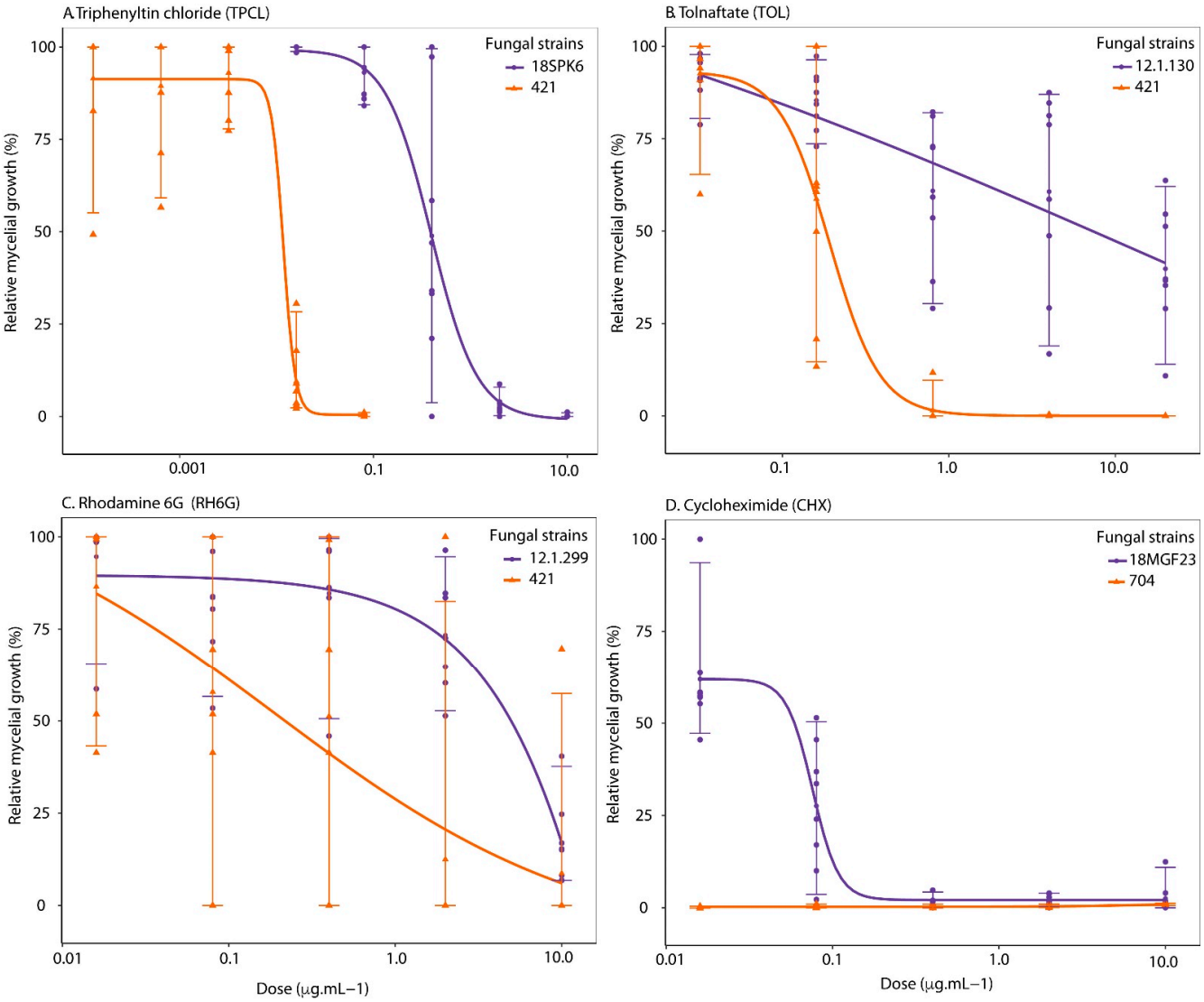

Figure 1. Contrasting in vitro relative growth of *Pyricularia oryzae Triticum* and *Oryza* lineages in different efflux pump substrates in PD broth on a microplate assay. (**A**). Triphenyltin chloride (TPCL)-resistant strain 18SPK6 from *P. oryzae Triticum* lineage (*PoT*l), and the sensitive strain 421 from *P. oryzae Oryza* lineage (*PoO*l). (**B**). Tolnaftate (TOL)-resistant *PoTl* 12.1.130 and the sensitive strain *PoO*l 421. (**C**). Rhodamine 6G (RH6G)-resistant strain *PoT*l 12.1.299 and the sensitive strain *PoO*l 421. (**D**). Cycloheximide (CHX)-resistant strain *PoT*l 18MGF23 and the sensitive strain *PoO*l 704. Bars contain the sample median and outer quantiles at 95% confidence interval (the 2.5th and the 97.5th percentiles as the lower and the upper confidence limits). Data points are distributed along the bar.

3.2. Correlation Analysis

Positive significant correlation ($p \leq 0.01$) was observed among the EC_{50} values of the fungicide tebuconazole (DMI) and of the substrates TPCL (55%) and RH6G (58%), besides lower significance ($p \leq 0.10$) between DMI and TOL (42%) (Figure 2). However, the EC_{50} values for fluxapyroxad (SDHI) did not correlate with any chemical tested (Figure 2). The sensitivity levels between the substrates TPCL and TOL were significantly correlated ($p \leq 0.001$) with high positive linear relationship (close to 80%) (Figure 2). The TPCL also

correlated with RH6G ($p \leq 0.01$), in which the linear relationship was close to 60%. For TOL and TPCL the correlation was lower than 40% (significance at $p \leq 0.1$) (Figure 2). The EC_{50} values for the efflux pump substrate CHX did not correlate with any chemical tested (Figure 2).

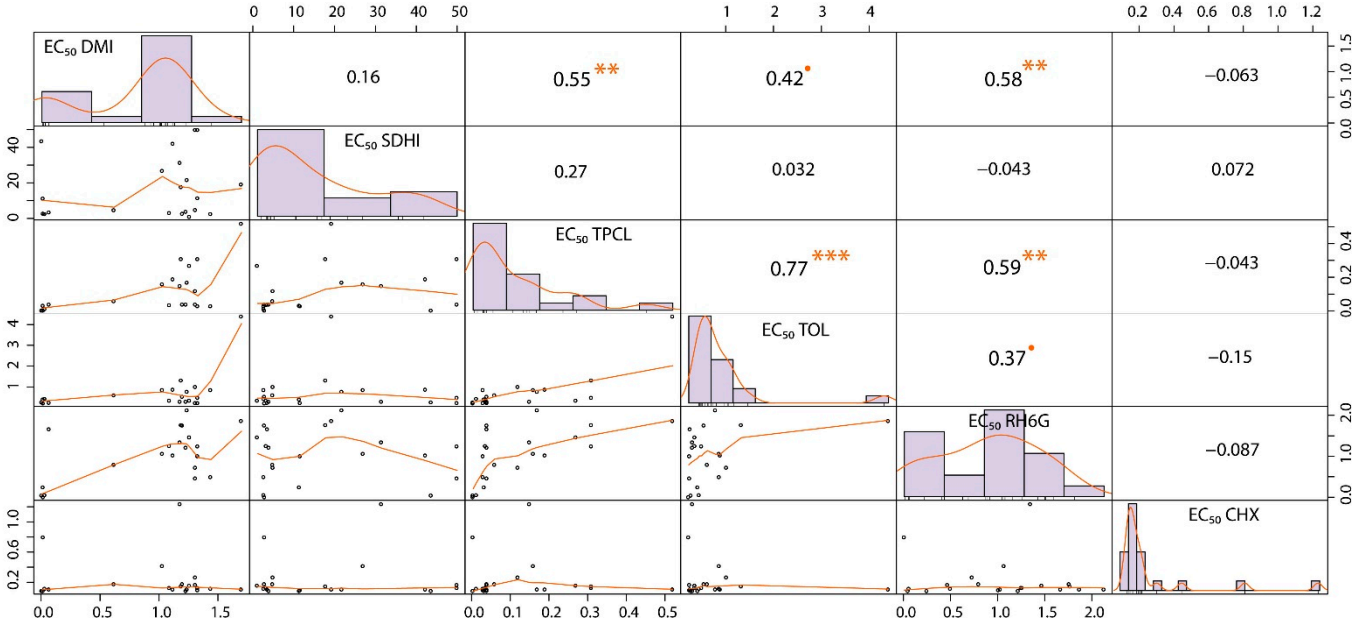

Figure 2. Pearson correlation analysis among the EC_{50} values for the efflux pump substrates tolnaftate (TOL), cycloheximide (CHX), rhodamine 6G (RH6G) and triphenyltin chloride (TPCL) and the site-specific fungicides tebuconazole (DMI) and fluxapyroxad (SDHI) determined for *Pyricularia oryzae Triticum* and *Oryza* lineages and *P. grisea*. Diagonal frames represent the distribution of the EC_{50} values for each chemical; frames below, the bivariate dispersion and adjusted lines; frames above, the correlations values and the significance levels. Significance of the correlation was determined by *t* tests. *p* values were represented by: *** = 0.001; ** = 0.01; • = 0.1.

4. Discussion

Resistance to site-specific fungicides in fungi can vary for different unrelated chemicals according to the recognition domain's structures and the expression patterns of different transporter's genes, and this variation is population- and strain-associated. Therefore, it is plausible that resistance to the site-specific DMI and SDHI fungicides could occur both due to target-site mechanisms in association with the efflux pump's overexpression in the MDR isolates [16]. A pre-existing mechanism not associated with target site mutations has been associated with the resistance to DMI and SDHI fungicides, with a strong indication that *PoT*1 has evolved efflux-based multidrug-resistance (MDR).

We tested this hypothesis by testing a selection of 20 *Po* strains (16 *PoT*1 and 4 *PoO*1) with different resistance levels to DMI and SDHI fungicides for sensitivity to four antifungal efflux pump substrates, cycloheximide (CHX), triphenyltin chloride (TPCL), rhodamine 6G (RH6G) and tolnaftate (TOL).

CHX is an antifungal and antibiotic isolated from *Streptomyces griseus* and inhibits protein synthesis. CHX exerts its effect by interfering with the movement of tRNA molecules on the mRNA-ribosomal complex, paralyzing the translocation step of protein synthesis [37]. CHX has been frequently used in efflux pumps studies with transporters such as *PoO*1 ABC2 [29], *AtrD* in *B. cinerea* [13] and *PdPMR1* in *P. digitatum* [38]. Yeast heterologous gene expression studies with ABC and MFS transporters from both *Z. tritici* [18] and *C. Albicans* [39] show CHX can act as a substrate for both efflux pump families. In comparison with the fungicide sensitive strain *PoO*1 656, only two strains, *PoT*1 18MGF23 and *PoO*1 674, showed raised levels of CHX insensitivity, with RFs of 10 and 6.7, respectively. However, *PoO*1 674 did not have a MDR phenotype, being sensitive to QoI, DMI and SDHI

fungicides, and MDR in the remaining *PoT*l strains (*PoTl* strain 18MGF23) is therefore also not likely associated with CHX insensitivity.

Triphenyltin chloride (TPCL) and/or rhodamine 6G (RH6G) can be considered as the most useful indicators of efflux pump mediated MDR activity, since seven strains were insensitive to RH6G alone (MDRP1 and MDRP3) and nine out of 16 fungicides-resistant *PoT*l strains were insensitive to both RH6G and TPCL (MDRP2 and MDRP4), using a RF of 5 as a cut-off, with a high correlation between their EC_{50}s and the EC_{50}s recorded for the DMI tebuconazole. Fentin compounds such as TPCL inhibit mitochondrial ATPase activity, affecting membrane permeability and calcium influx regulation [40]. *Saccharomyces cerevisiae* and *Z. tritici* are examples of fungi screened for MDR using this substrate [32,41]. Rhodamine 6G (RH6G), known to be an inhibitor of mitochondrial oxidative phosphorylation [42], has been used as a transporter substrate to test hypotheses on MDR in many other fungal species, in particular ABC or MFS transporter systems such as *C. albicans* (*Cdr1*) [43], *Cryptococcus neoformans* and *C. gattii* (*Afr1*, *Afr2*, *Mdr1*) [30]. RH6G is a fluorescent dye with chemical properties appropriate for accumulation or export assays based on fluorescence detection of its activity, which has been applied, for instance, to determine the activity of the ABC transporters *CgCDR1* and *CgCDR2* in *C. glabrata* [44].

In our study, only one *PoT*l strain, 12.1.130, was insensitive to tolnaftate (TOL) (Table 2). This strain, representing MDRP3, was also insensitive to RH6G and showed the highest level of DMI resistance (Table 2). Furthermore, a high level of cross-resistance was found between TOL and RH6G (Figure 2). TOL is a synthetic thiocarbamate used as an antifungal agent, acting as an inhibitor of squalene epoxidase and, consequently, inhibiting the sterol biosynthesis [45]. Tolnaftate has been used for MDR phenotyping in *Z. tritici* (*MgMFS1*), *B. cinerea* (*AtrB*, *mfsM2*) and *C. albicans* (*Cdr1* and *2*) [13,14,32,45].

In summary, four different MDR phenotypes (MDRP1-4) were observed amongst the 16 *PoT*l strain tested based on insensitivity levels to multiple fungicides and four antifungal efflux pump substrates (TPCL, TOL, RH6G and CHX). Insensitivity to DMI correlated well with insensitivity levels to TPCL and/or RH6G, and the Pearson's correlation between the DMI's EC_{50} with both TPCL and RH6G EC_{50} values corroborated this observation.

Regarding DMI fungicides, the widespread reduced triazole sensitivity of the wheat blast pathogen in Brazil could have evolved to its current high levels of resistance in response to intensive exposure of *PoT*l populations to triazoles over 30 years of selection pressure due to repeated spray applications of DMI fungicides in the local wheat agroecosystem [9]. Although the authors detected five different *PoT*l *CYP51A* haplotypes (H1 to H5), four of them all carrying non-synonymous substitutions in *CYP51A* at high frequencies within field populations, none of these substitutions correlated with elevated EC_{50} values. For a selection of strains, including *PoT*l 12.1.299, sequencing of regulatory and coding sequences of both *CYP51A* and *CYP51B* revealed no alterations and the operation of a non-target site resistance mechanism was suggested [9]. In our present study this strain was categorized as MDRP2, with resistance to the transporter substrates TPCL and RH6G (Table 2).

In the sister species *PoO*l, the rice blast pathogen, an ABC2 transporter system was identified as a relevant mechanism for azole resistance. Disruption of genes associated with the ABC transport resulted in increased sensitivity to tebuconazole in *PoO*l, and also to CHX and other efflux pump drugs [29]. Many other examples of ABC transporters linked to azoles resistance have been reported for different fungi species such as *A. nidulans* (*AtrG*), *A. fumigatus* (*Mdr1, abcE*), *B. cinerea* (*BcatrB*), *C. albicans* (*Cdr1p* and *2p*), *C. neoformans* (*Afr1p*) and *Penicillium digitatum* (*PdPMR1*). Moreover, MFS efflux pumps have been associated with DMI resistance in *A. fumigatus* (*mfsC*), *Z. tritici* (*MgMFS1*), *B. cinerea* (*Bcmfs1*) and *C. albicans* (*CaMdr1p*) [14,25,46–49].

Regarding the mechanisms of the resistance to the SDHI fungicide fluxapyroxad, none of the SDHI-resistant *PoT*l strains harbored any amino acid substitutions in SdhB, C or D [10], also pointing to the existence of a non-target site associated resistance mechanism such as enhanced efflux pump activity. For instance, *MgMFS1* promoter insertions con-

ferring resistance to many SDHI molecules, including fluxapyroxad, have been reported for *Z. tritici* [24], and *Mrr1* transcription factor changes altering the ABC-*AtrB* and the MFS-*mfsM2* expression levels have been reported to confer resistance to the SDHI fungicide boscalid [13]. However, one DMI-sensitive *PoO*l strain without a MDR phenotype, isolate 704, was found moderately resistant to fluxapyroxad (Table 2), whereas three *PoT*l strains, 12.1.005, 12.1.165 and 18PRH9, showing high levels of resistance to the DMI tebuconazole and classified as MDRP1 or MDRP2 (12.1.005, 12.1.165 and 18PRH9), were sensitive to the SDHI fluxapyroxad (Table 2), indicating another resistance mechanism might be responsible. Interestingly, another non-target site resistance mechanism for SDHI resistance was recently suggested to operate in some *Z. tritici* strains [32], but further research revealed that duplication of a SdhC paralog was responsible for this phenotype [50]. The presence of an additional SdhC paralog was found in several other fungi, including *Fusarium graminearum* and *Alternaria alternata*, but multiple copies have not been predicted for *Magnaporthe oryzae*.

The identification of multiple MDRP phenotypes associated with DMI resistance in our study warrants further research aimed at revealing the mechanisms of multidrug resistance in the wheat blast pathogen, including efflux pumps overexpression via transcriptomic analyses of differentially expressed genes; identification and discovery of mutations associated with changes in promoter regions or transcription factors of efflux transporters associated with multidrug resistance.

If allelic variation in the promoter regions, in the transcription factors or even in the coding region of the transporter genes could be unequivocally associated with MDR phenotypes, these alleles could be converted into molecular markers for monitoring, in real time, the spread of different MDR lineages of the pathogen in airborne inoculum [51–53]. This would foster the adoption of rational and sustainable anti-resistance strategies to manage wheat blast, using real time spatial and temporal disease surveillance tools based on the molecular detection of resistant inoculum of the pathogen in air samples from wheat fields. The adoption of such a smart, real-time anti-resistance strategy would minimize unneeded sprays and guide the choice of fungicides that are still effective when MDR is detected.

Future studies could be conducted by testing the role of specific transporter inhibitors such as *BLT-4* [N-(2-methoxyphenyl)-N′-2-naphthalenyl-urea, ≈ *INF271*, a MFS transporter type inhibitor] and verapamil hydrochloride (for ABC transporter type inhibition) [16,54–57]. The main objective would be to assess if these two superfamily-distinct transporter inhibitors could interfere with the ability of *PoT*l strains for MDR resistance. One would look at any phenotypic changes induced by *BLT-4* and verapamil hydrochloride in the fungal strain's resistance responses to DMI and SDHI fungicides (i.e., altering the *PoT*l strain's distinct MDRPs phenotypic patterns for MDR and restoring fungicide sensitivity).

Over the past few years, strategies have been prospected to combat MDR yeasts and bacterial human pathogens, as well as multi-resistant cancer cells, based on the concomitant administration of efflux pump exporters inhibitors using chemicals in synergism with azoles and other chemo drugs to restore their efficacy [58–60]. Perhaps this strategy could also be applied for managing MDR plant pathogens in the agroecosystem. Further studies could also be carried out in planta to assess whether the use of efflux pump inhibitors in association with DMI or SDHI fungicides would have an additive effect, restoring the efficacy of the fungicides for wheat blast control.

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Data Availability Statement: The phenotypic data on EC_{50} values for sensitivity to the antifungal efflux pump substrates tolnaftate (TOL), cycloheximide (CHX), rhodamine 6G (RH6G) and triphenyltin chloride (TPCL) presented in this study are available upon request to the corresponding author. The data are not publicly available due to the authors' option.

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