



Assessment of sensitivity and virulence fitness costs of the *AvrPik* alleles from *Magnaporthe oryzae* to isoprothiolane

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ABSTRACT. The *in vitro* sensitivity of *AvrPik* allele isolates of *Magnaporthe oryzae* to isoprothiolane was examined and the virulence fitness costs of *AvrPik* allele isolates to isoprothiolane were assessed. Isoprothiolane was found to suppress the radial growth of *AvrPik* allele isolates at all concentrations (1, 5, 10, 15, and 20 $\mu\text{g/mL}$). Generally, a higher isoprothiolane concentration has a stronger inhibitory effect on mycelial growth in *AvrPik* allele isolates at 6 and 10 days after inoculation. The inhibitory effect of isoprothiolane also increased with treatment time. To determine whether a correlation existed between the *in vitro* sensitivity of *AvrPik* allele isolates and virulence, the half-maximal inhibitor concentration and 75% of the maximum inhibitor concentration were calculated for each mutation isolate and wild-type isolate. Based on these values and virulence, no significant correlation between the susceptibility of *AvrPik* allele isolates and virulence was

detected. In summary, no fitness costs were associated with sensitivity of blast isolates carrying specific *AvrPik* alleles to different virulence.

Key words: *AvrPik* alleles; Fitness costs; Isoprothiolane; *Magnaporthe oryzae*

INTRODUCTION

Rice blast disease caused by the filamentous ascomycete fungus *Magnaporthe oryzae* is one of the most devastating diseases of rice worldwide (Talbot, 2003; Liu et al., 2010). The disease causes heavy yield losses, ranging from 35-50% during the epidemic years (Padmavathi et al., 2005). The use of resistant varieties is the most economical and effective method of controlling rice blast mainly in resource-poor fields (Séré et al., 2007). To date, more than 80 resistance genes related to rice blast disease have been reported, and some have been used to control the disease (Ballini et al., 2008). For example, in the southern United States, *Pita*-based resistant cultivars including Katy, Drew, Kaybonnet, Madison, Cybonnet, Ahrent, and Banks have been widely utilized to control blast disease since the release of the first *Pita*-containing cultivar, Katy, in 1990 (Moldenhauer et al., 1990; Jia et al., 2004). Although *R* gene-mediated resistance is highly effective once it is triggered, breeding of resistant cultivars often shows only short-term success, which is mainly because of the frequent appearance of new races (or pathotypes) of the fungus that are virulent to previously resistant cultivars (Lee et al., 2005). New virulent races of the pathogen may have emerged by genetic modification of avirulence (*Avr*) genes through several mechanisms, including deletion of the entire gene (Dai et al., 2010; Takahashi et al., 2010), nonsynonymous point mutations (Yoshida et al., 2009), frameshift mutations (Dai et al., 2010), or transposon insertions (Fudal et al., 2005; Zhou et al., 2007; Li et al., 2009). Generally, *Avr* genes in *M. oryzae* are highly diverse and are predicted to be capable of rapid changes in nature (Jia et al., 2000). Thus, the pathogenic *Avr* gene product is no longer 'recognized' by the host *R* gene or can no longer modify the plant guard protein, rendering host resistance ineffective (Jones and Dangl, 2006). To date, more than 40 *M. oryzae* *Avr* genes have been genetically analyzed (Ma et al., 2006), 9 of which have been cloned: *PWL1* (Kang et al., 1995), *PWL2* (Sweigard, 1995), *Avr1-CO39* (Farman and Leong, 1998), *Avr-Pita* (Orbach et al., 2000), *ACE1* (Fudal et al., 2005), *Avr-Pizt* (Li et al., 2009), *Avr-Pia*, *Avr-Pii*, and *Avr-Pik/km/kp* (Yoshida et al., 2009).

The durability of an *R* gene may be predicted from the information regarding the fitness cost of the pathogen's virulence at the corresponding locus (Leach et al., 2001). The fitness cost associated with pathogen evolution from avirulence to virulence to overcome host resistance can also affect the durability of resistance (Vera Cruz et al., 2000). A study by Huang et al. (2010) suggested that there are fitness costs of virulence at both the *AvrLm1* and *AvrLm4* loci in *Leptosphaeria maculans*, but there are also differences in fitness costs of virulence between the 2 loci. Recently, the rice blast *Pik* alleles, which confer high levels of resistance to blast, have been used in many rice breeding programs in China (Wang et al., 2009; Zhai et al., 2011). The resistance gene *Pik* corresponds to the avirulent gene *AvrPik*. The *AvrPik* coding region harbors 4 ubiquitous, non-synonymous single-nucleotide polymorphisms (C136A, C139G, G143A, and G234A) resulting in four major variations (H46N, P47A, G48D, and M78I) (Yoshida et al., 2009). The four major single-nucleotide polymorphisms at *AvrPik* can give rise to 16 genotypic combinations. All possible alleles were created by site-directed muta-

genesis and then subjected to pathotype testing and identification of 5 pathotypes (Wu, 2012). However, the fitness cost of virulence at *AvrPik* locus has not been examined.

Chemical control is an effective method of controlling rice blast. Isoprothiolane (diisopropyl 1,3-dithiolan-2-ylidene malonate), a dithiolane-related fungicide belonging to the organo sulfur group of compounds, can be used as an insecticide. It is used in the cultivation of rice plants because of its insecticidal effect on brown planthoppers (Uesugi, 2001). Isoprothiolane has been the primary chemical used for blast disease control since the early 1980s in China (Yuan et al., 2005; Qi et al., 2013). However, the sensitivity of *AvrPik* allele isolates of *M. oryzae* to isoprothiolane is unknown. To obtain a comprehensive understanding of the sensitivity of *AvrPik* allele isolates of *M. oryzae* to isoprothiolane, 8 of the 16 *AvrPik* allele isolates with different genotypes and pathotypes were subjected to susceptibility testing. We determined that there is fitness costs associated with the evolution to virulence at the *AvrPik* locus in *M. oryzae*.

MATERIAL AND METHODS

Fungal isolates and fungicides

Two wild-type isolates of *M. oryzae* CHL724 and CHL346 and 8 of the 16 *AvrPik* allele isolates with different genotypes and pathotypes were used throughout these experiments (Table 1). The field blast isolate CHL724 carrying $N_{46}A_{47}D_{48}I_{78}$ was virulent to all 4 *Pik* alleles (IRBLkp-K60, *Pik-p*; IRBLk-Ka, *Pik*; IRBLkm-Ts, *Pik-m*; and IRBLkh-K3, *Pik-h*; V/V/V/V) and the wild-type isolate CHL346 carrying $H_{46}P_{47}G_{48}M_{78}$ was avirulent to all 4 *Pik* alleles; the former was used as a recipient and the latter as a donor (wild) isolate. Among the 8 mutation isolates, KM1 was derived from the *Pik* allele-avirulent isolate CHL346. Additionally, the other 7 mutants derived from the 4 key single-nucleotide polymorphism site-directed mutations of the wild type were introduced into the *Pik* allele-virulent isolate CHL724 via *Agrobacterium*-mediated transformation (Table 1). Isoprothiolane 40 WP was supplied by Zhongnong Zhushang (Tianjin) Agricultural Chemicals Co., Ltd. (Tianjin, China) and dissolved in H₂O to 2 x 10³ µg/mL.

Fungal growth inhibition assay

The inoculums in all experiments were obtained from colonies grown in complete medium (50 mL 20X nitrate salts, 1 mL trace elements, 10 g D-glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acid, 1 mL vitamin solution, 20 g agar, and added H₂O to a total volume of 1L; the pH of the complete medium was adjusted to pH 6.5 using 1N NaOH). A range of concentrations (0, 1, 5, 10, 15, and 20 µg/mL) of a current anti-rice blast fungicide (isoprothiolane 40 WP) was used to detect the mycelial growth of blast isolates carrying specific *AvrPik* alleles. A control (without fungicide) was seeded with 5 mm agar plug containing blast mycelium, which was initially grown for 10 days and incubated at 28°C. The spread of mycelium was recorded at 6 and 10 day intervals after inoculation (DAI) by measuring the diameter along the 2 perpendicular lines on the underside of the Petri dishes. The percent inhibition of blast isolates carrying specific *AvrPik* alleles in each treatment was recorded over the control and calculated using the following formula: Inhibition of blast isolates (%) = [(colony growth in control - colony growth in treatment) / (colony growth in control)] x 100%.

The growth rate as a function of isoprothiolane concentration was estimated by polynomial regression, from which the half-maximal inhibitor concentration (EC_{50}) and 75% of the maximum inhibitor concentration (EC_{75}) values were estimated. Three independent biological replicates with 3 technical replicates per blast isolated were conducted. Data collected were subjected to statistical analysis using simple analysis of variance (SAS, 2002).

RESULTS

AvrPik allele sensitivity evaluation

To detect the sensitivity of *AvrPik* allele isolates, we evaluated the percent inhibition at different concentrations of isoprothiolane. The results showed that the inhibitory effect of isoprothiolane generally increased with increasing concentration with respect to mycelial growth at 6 and 10 DAI (Table 2). At 6 DAI, the inhibitory effect of isoprothiolane at 1, 5, 10, and 15 $\mu\text{g/mL}$ did not significantly differ among the 10 isolates, while a significant effect was observed among the 10 isolates when 20 $\mu\text{g/mL}$ isoprothiolane was applied. Minimum inhibition was observed in KM12 (14.76%), KM12 (33.51%), KM4 (62.78%), and KM16 (74.29) at 1, 5, 10, and 15 $\mu\text{g/mL}$, respectively, and the maximum was observed in KM1 (22.32%), CHL346 (41.81%), CHL346 (66.63%), and CHL346 (80.68%; Table 2). For the 20 $\mu\text{g/mL}$ concentration, minimum inhibition (79.1%) was observed in KM16 and maximum (88.19 and 84.74%) in CHL346 and KM1 (Table 2). The remaining mutants showed a similar percent inhibition, ranging from 81.34-83.18%, which was similar to the wild-type CHL724 (81.03%). These results suggest that isoprothiolane effectively suppressed the radial growth of the *AvrPik* allele isolates at all concentrations, including 1, 5, 10, 15, and 20 $\mu\text{g/mL}$ (Figure 1).

Table 1. Different *AvrPik* alleles with different genotypes and pathotypes tested.

Isolate/mutant ^a	Four major minor amino acids mutated ^b				Pathotypes ^c <i>Pikp/k/km/kh</i>
	46	47	48	78	
CHL724	N	A	D	I	V/V/V/V
CHL346	H	P	G	M	A/A/A/A
KM1	H	P	G	M	A/A/A/A
KM2	N	P	G	M	V/A/A/A
KM4	H	P	D	M	A/A/A/A
KM6	N	A	G	M	V/V/A/A
KM8	N	P	G	I	V/V/A/V
KM12	N	A	D	M	V/V/A/A
KM14	N	P	D	I	V/V/V/V
KM16	N	A	D	I	V/V/V/V

^aThe *Magnaporthe oryzae* isolates CHL724 and CHL346 were used as recipient and donor (wild-type) isolates, respectively, and mutants were prefixed by KM. KM1-16 is a transformant derived from the target gene isolated from the donor isolate (CHL346) and introduced into the recipient isolate (CHL724), respectively. ^bMutated positions are underlined. ^cMonogenic lines carrying the cognate allelic *R* genes (*Pik-p*, *Pik*, *Pik-m*, and *Pik-h*) were used for the pathotype test: A = avirulent; V = virulent.

At 10 DAI, however, the inhibitory effect of isoprothiolane at 1, 5, and 10 $\mu\text{g/mL}$ did not differ significantly among the 10 isolates. The inhibitory effect differed significantly at 15 and 20 $\mu\text{g/mL}$. The minimum inhibition was observed in KM16 (11.24%), KM12 (27.51%), and KM8 (56.05%) at 1, 5, and 10 $\mu\text{g/mL}$, respectively, and maximum in KM6 (16.15%), CHL346 (35.07%), and CHL346 (61.78%), respectively (Table 3). At 15 $\mu\text{g/mL}$, minimum inhibition (68.91%) was observed in CHL724 and the maximum (77.77%) in CHL346 (Table 3).

At 20 µg/mL, minimum inhibition (77.34%) was observed in KM14 and maximum (85.63%) in CHL346 (Table 3). This suggests that the inhibitory effect of isoprothiolane at all 5 concentrations at 10 DAI were better than those at 6 DAI, so treatment time affects the inhibitory effect of isoprothiolane. These results indicate that isoprothiolane inhibits mycelial growth of blast isolates carrying specific *AvrPik* alleles *in vitro* at all 5 concentrations used, particularly at high concentration. *AvrPik* alleles isolates were sensitive to isoprothiolane.

Table 2. Effect of varying concentrations of isoprothiolane on mycelial growth of blast isolates carrying specific *AvrPik* alleles *in vitro* at 6 days after inoculation.

Isolate/mutant	Percent inhibition at different concentrations of isoprothiolane				
	1 µg/mL	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL
CHL724	17.47 ^A	37.44 ^A	64.98 ^A	76.67 ^A	81.03 ^{BC}
CHL346	16.38 ^A	41.81 ^A	66.63 ^A	80.68 ^A	88.19 ^A
KM1	22.32 ^A	39.69 ^A	65.07 ^A	75.74 ^A	84.74 ^A
KM2	19.35 ^A	37.65 ^A	63.56 ^A	76.66 ^A	81.42 ^{BC}
KM4	15.84 ^A	34.19 ^A	62.78 ^A	76.00 ^A	81.34 ^{BC}
KM6	18.50 ^A	40.73 ^A	64.70 ^A	75.80 ^A	83.18 ^{BC}
KM8	20.72 ^A	35.54 ^A	65.53 ^A	76.24 ^A	81.75 ^{BC}
KM12	14.76 ^A	33.51 ^A	65.15 ^A	74.37 ^A	81.85 ^{BC}
KM14	18.00 ^A	38.54 ^A	63.69 ^A	74.93 ^A	79.96 ^{BC}
KM16	16.85 ^A	41.11 ^A	64.30 ^A	74.29 ^A	79.10 ^C

Means with the same letter in each column were not significantly different ($P = 0.01$).

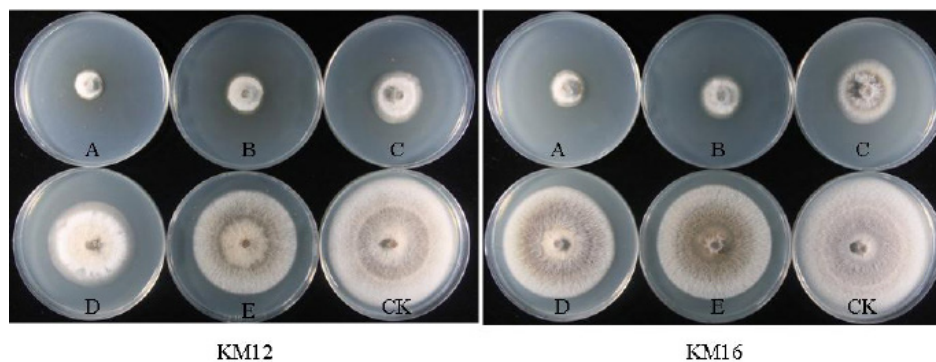


Figure 1. *In vitro* effect of isoprothiolane at 6 concentrations on the mycelial growth of blast, 10 DAI. A, B, C, D, E, and CK are 20, 15, 10, 5, 1, and 0 µg/mL, respectively.

Table 3. Effect of varying concentrations of isoprothiolane on mycelial growth of blast isolates carrying specific *AvrPik* alleles *in vitro* at 10 days after inoculation.

Isolate/mutant	Percent inhibition at different concentrations of isoprothiolane				
	1 µg/mL	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL
CHL724	11.80 ^A	29.89 ^A	59.38 ^A	68.91 ^{AB}	77.97 ^B
CHL346	11.92 ^A	35.07 ^A	61.78 ^A	77.77 ^A	85.63 ^A
KM1	14.94 ^A	30.27 ^A	58.57 ^A	70.42 ^B	80.44 ^{AB}
KM2	15.27 ^A	31.46 ^A	60.62 ^A	72.95 ^{AB}	79.36 ^{AB}
KM4	11.79 ^A	27.87 ^A	57.26 ^A	72.64 ^{AB}	79.46 ^{AB}
KM6	16.15 ^A	33.74 ^A	59.99 ^A	73.32 ^{AB}	78.04 ^B
KM8	14.48 ^A	29.64 ^A	56.05 ^A	72.02 ^{AB}	80.12 ^{AB}
KM12	11.49 ^A	27.51 ^A	58.74 ^A	70.50 ^B	81.37 ^{AB}
KM14	11.96 ^A	32.22 ^A	57.49 ^A	71.29 ^{AB}	77.34 ^B
KM16	11.24 ^A	33.37 ^A	59.74 ^A	71.15 ^{AB}	78.35 ^B

Means with the same letter in each column were not significantly different ($P = 0.01$).

Comparison of virulence fitness costs of *AvrPik* alleles isolates to isoprothiolane

To determine whether there was a correlation between the *in vitro* susceptibility of *AvrPik* allele isolates and virulence, EC_{50} and EC_{75} were calculated for each mutation isolate and each wild-type isolate. EC_{50} and EC_{75} values for inhibition of the isolates are shown in Table 4. The differing virulence isolates KM1 (A/A/A/A) and KM6 (V/V/A/A) exhibited relatively lower EC_{50} values than the wild-type isolate CHL724, whereas KM4 (A/A/A/A) and KM12 (V/V/A/A) showed higher values than the wild-type isolate CHL724 at 6 DAI, respectively. These results suggest that different virulence isolates exhibited nearly the same EC_{50} . The remaining isolates carrying different pathotypes, V/A/A/A, V/V/V/V, and V/V/A/V, showed a similar EC_{50} value to the wild-type isolate CHL724. Moreover, the EC_{50} value increased with longer treatment times and showed slight to moderate changes in 10 DAI. Specifically, the KM4 (A/A/A/A), KM8 (V/V/A/V), KM12 (V/V/A/A), KM14 (V/V/V/V), and KM16 (V/V/V/V) showed similar EC_{50} values to the wild-type isolate CHL724. The remaining isolates KM1, KM2, and KM6 showed significantly lower values compared with the wild-type isolate CHL724.

Table 4. Effect of isoprothiolane on mycelial growth of the *AvrPik* alleles isolates of *Magnaporthe oryzae*.

Isolate/mutant	Pathotype <i>Pikp/k/km/kh</i>	6 days		10 days	
		EC_{50} (μ g/mL)	EC_{75} (μ g/mL)	EC_{50} (μ g/mL)	EC_{75} (μ g/mL)
CHL724	V/V/V/V	5.61 ^{AB}	16.13 ^{AB}	7.40 ^{AB}	20.02 ^A
CHL346	A/A/A/A	4.89 ^B	12.19 ^B	6.04 ^B	14.27 ^B
KM1	A/A/A/A	4.89 ^B	15.40 ^{AB}	6.89 ^{AB}	19.66 ^A
KM2	V/A/A/A	5.46 ^{AB}	16.49 ^{AB}	6.59 ^{AB}	18.86 ^A
KM4	A/A/A/A	5.98 ^A	16.47 ^{AB}	7.36 ^{AB}	19.29 ^A
KM6	V/V/A/A	5.26 ^{AB}	15.10 ^{AB}	6.44 ^{AB}	18.94 ^A
KM8	V/V/A/V	5.31 ^{AB}	16.07 ^{AB}	7.02 ^{AB}	19.80 ^A
KM12	V/V/A/A	6.06 ^A	16.26 ^{AB}	7.43 ^A	19.40 ^A
KM14	V/V/V/V	5.63 ^{AB}	16.92 ^A	7.25 ^{AB}	19.93 ^A
KM16	V/V/V/V	5.65 ^{AB}	16.73 ^A	7.07 ^{AB}	18.61 ^A

Means with the same letter in each column were not significantly different ($P = 0.01$).

For the EC_{75} value the most virulent pathotype V/V/V/V isolates KM14 and KM16 exhibited higher EC_{75} values compared to the wild-type isolate CHL724 at 6 DAI. The remaining mutation isolates, including the KM1 (A/A/A/A), showed similar EC_{75} values compared to the wild-type isolate CHL724. However, in all mutation isolates, the EC_{75} value was similar to CHL724 at 10 DAI. Overall, these results suggest that there are no fitness costs associated with the sensitivity of blast isolates carrying specific *AvrPik* alleles to different pathotypes with respect to the EC_{50} and EC_{75} values at 6 and 10 DAI.

DISCUSSION

The inhibitory effect of isoprothiolane against many plant and animal pathogenic fungi has been reported in several studies. Isoprothiolane is effective for treating fat necrosis in Japanese Black cattle (Oka et al., 1988) and experimentally induced fatty liver in Holstein cattle (Nagasawa et al., 1989) and rats (Imaizumi et al., 1981). Isoprothiolane increased proliferation of mammary epithelial cells in a dose-dependent manner at concentrations of 0.05-5 μ M when cultured either with or without serum-supplemented medium. In contrast, isoprothiolane

(0.0005-5 μ M) significantly inhibited the production of IL-1 and IL-6 by mammary epithelial cells (Okada et al., 1999). Although isoprothiolane has been widely used to control rice blast in China since the 1980s (Yuan et al., 2005; Qi et al., 2013), this is the first study examining blast isolates carrying specific *AvrPik* alleles. The results showed that isoprothiolane inhibits mycelial growth of blast isolates carrying specific *AvrPik* alleles *in vitro* at all 5 concentrations used, particularly at high concentrations.

The fitness of an organism is defined as its combined ability to survive and reproduce (Pringle and Taylor, 2002). For example, in fungi, smaller spores can be dispersed more effectively and therefore be more fit than larger spores, even if larger spores have greater germination rates; moreover, stabilizing selection can optimize spore size (Meerts, 1999). The fitness of an individual genotype can also be measured by choosing a single spore and using the asexually derived progeny of that spore to measure a specific aspect of fitness, such as mycelial growth rate (Antonovics and Alexander, 1989). A number of recent studies have investigated the fitness costs of virulence at the *Avr* locus. The *L. maculans*-*Brassica napus* pathosystem confirmed a fitness cost of virulence at the *AvrLm4* locus in *L. maculans* on hosts without the corresponding *R* gene *Rlm4* (Huang et al., 2006). Studies suggest that there is no fitness cost for virulence at the *AvrLm2* and *AvrLm3* loci because the avirulent alleles *AvrLm2* or *AvrLm3* are very rare or completely absent in European populations (Rouxel et al., 2003; Balesdent et al., 2006; Stachowiak et al., 2006). In contrast, there may be a fitness cost at the *AvrLm1* and *AvrLm4* loci because *AvrLm1* and *AvrLm4* are still present in *L. maculans* populations, although cultivars containing *R* genes *Rlm1* and *Rlm4* have been used commercially for over 10 years. However, in this study, no relationship between *AvrPik* allele virulence and EC_{50} or EC_{75} values was observed for all *AvrPik* alleles isolates. No fitness costs were associated with the virulence trait in the *AvrPik* loci. Similar results have been reported by Ziogas and Kalamarakis (2001), who found that a fungicide-resistant *Botrytis cinerea* pathogen was as virulent as the wild-type strain, demonstrating that chemical resistance may not impose a fitness cost. Although no fitness costs were observed in the *AvrPik* loci in this present study, fitness costs cannot be ruled out in other aspects, as various aspects of individual fitness during the immature and adult stages may be affected by biotic and abiotic factors. Therefore, the fitness of *AvrPik* alleles can be accurately compared by testing for a variety of fitness parameters, including the radial growth of mycelia on petri dishes, spore germination, mean length of germ tubes, and pathogenicity.

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