Impact of tricyclazole and azoxystrobin on growth, sporulation and secondary infection of the rice blast fungus, *Magnaporthe oryzae*

Running title: ***Magnaporthe oryzae*** **responses to tricyclazole and azoxystrobin**

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ps.3386.

ABSTRACT

BACKGROUND: Rice blast, caused by *Magnaporthe oryzae* B Couch sp. nov., is one of the most destructive rice diseases worldwide causing substantial yield losses every year. In Italy, its management is based mainly on the use of two fungicides, azoxystrobin and tricyclazole, that restrain the disease progress. The aim of this study was to investigate and compare the inhibitory effects of the two fungicides on the growth, sporulation and secondary infection of *M. oryzae*.

RESULTS: *Magnaporthe oryzae* mycelium growth was inhibited at low concentrations of azoxystrobin and relatively high concentrations of tricyclazole, while sporulation was more sensitive to both fungicides and was affected at similarly low doses. Furthermore, infection efficiency of conidia obtained from mycelia exposed to tricyclazole was affected to a higher extent than those produced on azoxystrobin-amended media, even though germination of such conidia were reduced after azoxystrobin treatment.

CONCLUSIONS: This study presents for the first time detailed azoxystrobin and tricyclazole growth-response curves of *M. oryzae* mycelium growth and sporulation. Furthermore, high efficacy of tricyclazole towards inhibition of sporulation and secondary infection indicate an additional possible mode of action of this fungicide that is different from inhibition of melanin biosynthesis.

KEYWORDS: Fungicide, Effective dose, Infection efficiency, *Oryza sativa*

1 INTRODUCTION

Rice (*Oryza sativa* L.) belongs to the most important food crops worldwide and although produced predominantly in Asia, it is cultivated since a long time in Europe and predominantly in Italy (1.6 million tons produced on 247 700 ha in 2010, http://faostat.fao.org). Rice pests and diseases can greatly reduce grain yield, and rice blast is one of the most widespread and destructive diseases. Estimates of annual losses caused by blast epidemics varied from 10-30% in different countries.[1]

The causal agent of rice blast is *Magnaporthe oryzae* B Couch sp. nov., a haploid filamentous fungus belonging to ascomycetes.[2,3] The blast infection initiates by the attachment and germination of conidia, the asexual spores of *M. oryzae,* on the rice-leaf surface. Germinating conidia develop at the end of the germination tube highly melanized, dome-shaped appressorium.[4,5] Here, the enormous turgor pressure (>8MPa), generated by accumulation of compatible solutes such as glycerol,[6,7] is used to penetrate the plant cuticle and cell wall by a penetration peg.[8] After the penetration, the peg differentiates into infectious hyphae that grow inter- and intracellularly and result in the development of blast lesions.[3,9] The appearance of necrotic lesions is accompanied by development of aerial conidiophores.[3] Under high relative humidity environment, numerous conidia are produced, which serve as a secondary inoculum and allow further spreading of the disease.[7,10,11]

In Europe, rice blast management is based upon the use of fungicides reducing disease progress. Chemical compounds with diverse modes of action have been used to manage rice blast, but two are the most used, melanin biosynthesis inhibitors (MBI) and mitochondrial respiration inhibitors. MBIs interfere with the melanin production in appressoria of *M. oryzae* that results in the lack of turgor pressure and consequent unsuccessful host infection.[12] The most significant representative of this class of fungicides is tricyclazole (5-methyl-1,2,4-triazolo(3,4-b)benzothiazole) that is extensively used for blast management worldwide. It was developed more than 35 years ago and it has been shown to specifically inhibit the two reduction steps in DHN-melanin biosynthesis pathway.[12-16] The direct effect of tricyclazole on the capability of germinating conidia to penetrate host epidermis is well documented,[14,17,18] whereas reduced secondary infection observed in the field

was inferred to be due to reduced conidia production or to lower virulence of conidia produced on tricyclazole-treated lesions.[12,19,20] Strobilurins, a class of mitochondrial respiration inhibitors, belong to the group of Quinone outside inhibitors (QoI) that inhibit the mitochondrial respiration by binding at the Qo-site of the cytochrome b. This blocks the electron transfer between the cytochrome b and c_1 in the inner mitochondrial membrane and the production of ATP, which in turn disrupts the energy cycle of the fungus.[21] The representative of this class of fungicides is azoxystobin (Methyl(2*E*)-2-(2-([6-(2-cyanophenoxy)pyrimidin-4-yl]oxy)phenyl)-3-methoxyacrylate). Its inhibitory effects on the mitochondrial respiration have been shown to negatively influence mycelium growth, conidia production and germination of diverse fungal pathogens.[22] However, because of its site-specific mode of action, strains resistant to azoxystobin have been detected for several pathogens including closely related *M. grisea*[23-26] whereas there is no evidence of *M. oryzae* strains resistant to tricyclazole.[27]

In Italy, fungicides containing tricyclazole and azoxystrobin have been authorized and extensively used to manage rice blast epidemics since the early 90's. Two years ago, the negative outcome of the European review of tricyclazole resulted in its withdrawal from use, and while waiting for the new European evaluation process, an emergency use of tricyclazole has been authorized. The new European legislation on plant protection products is imposing more stringent rules for their approval (EC 1107/2009) and sustainable use (Dir. 128/2009). Therefore the acquisition of new knowledge about tricyclazole mode of action is required to support its registration, thus avoiding a highly dangerous azoxystrobin selection pressure on *M. oryzae* populations that would enhance the risk of selecting azoxystrobin-resistant strains.[28,29] In addition, new insights concerning both fungicides are necessary for a more efficient use of the two molecules.

In this study we have investigated the inhibitory effects of tricyclazole and azoxystrobin on *M. oryzae* mycelium growth and conidia production and in order to evaluate the impact of fungicide treatment on rice blast progress we have studied the possible effects of both fungicides on conidia germination and infection efficiency.

2 MATERIALS AND METHODS

2.1 Materials

Strains of *Magnaporthe oryzae* used form a part of the laboratory collection of single-spore isolates obtained from Italian rice fields in Pavia and Novara province in years 1998 and 2010 (table1).

Rice plants of the variety Carnaroli were used for the *in vitro* infection trial. Seeds were imbibed in water for 30 minutes and then sawn in square plastic pots (10x10cm, WxH) containing a soil:bentonite mixture (3:1). Plants were grown in the growth chamber at 26°C with 18h light / 6h dark period.

The fungicides tricyclazole (Beam WP - wettable powder; 75% a.i.) and azoxystrobin (Amistar SC - suspension concentrate, 22,9% a.i.) were obtained from Dow Agrosciences Italia S.r.l. and Syngenta Crop Protection S.p.A., respectively.

2.2 Mycelium growth on media supplemented with fungicides

The activity of tricyclazole and azoxystrobin on mycelium growth was evaluated for 5 strains of *M. oryzae* on malt-extract agar medium (MEA) containing 20 g/l malt extract (Oxoid Ltd., United Kingdom) and 15 g/l bacteriological agar No. 1 (Oxoid Ltd., United Kingdom) dissolved in appropriate volume of sterile water, supplemented with increasing concentrations of the two fungicides: 0; 0.006; 0.01; 0.1; 1; 10; 33.3 or 100 mg/l for azoxystrobin and 0; 0.01; 0.1; 1; 10; 33.3; 66.65; 100; 216.65 or 333.3 mg/l for tricyclazole. A mycelium plug, 0.5 cm in diameter, obtained from 10-day-old culture, was transferred on MEA with or without fungicide. Five replicates for each strain were prepared and the plates were incubated at 26°C in the dark. The mycelium growth was measured after 7 days of incubation.

2.3 Mycelium sporulation

Mycelium plugs from 10-day-old cultures, 0.5 cm in diameter, of each strain were transferred on Rice-extract agar medium (RYA) containing 20 g/l ground rice (variety Gladio), boiled in

appropriate volume of water for 30 minutes, 15 g/l bacteriological agar No. 1 (Oxoid Ltd., United Kingdom) and 2.5 g/l yeast extract (Oxoid Ltd., United Kingdom) dissolved in appropriate volume of sterile water, amended with or without fungicide. Fungicide concentration was 0; 0.002; 0.01; 0.1 or 1 mg/l for azoxystrobin and 0; 0.01; 0.1; 1 or 5 mg/l for tricyclazole. Three replicates for each strain were prepared and plates were incubated for 12 days at 26°C under black fluorescent light (GBC, 23W, 365 nm, 1600 lm) to promote conidia formation. The area of the mycelium growth was calculated after 12 days of growth and conidia were scraped from the whole surface of the mycelium. They were collected in 2ml of sterile water into an Eppendorff tube. The conidial suspension was filtered through 2 layers of sterile gauze and centrifuged at 12.000 rpm for 10 min. Conidia were resuspended in 100 µl of sterile water and their density per cm^2 of mycelium was determined using a haemacytometer. The count was repeated for the three replicate plates of each strain, and the values averaged. Finally, sporulation (%) was calculated as conidia density on mycelium exposed to fungicide / conidia density of the control *100.

2.4 Conidia germination and appressoria diameter

Conidia were collected as described above and the concentration of conidial suspension was adjusted to $1x10^4$ conidia/ml. Fifty µl of conidial suspension placed on microscopic glass-slide were incubated in a wet-chamber at 26°C in the dark. The germination of 100 randomly chosen conidia for each strain exposed to different concentration of the fungicide was determined after 24h.

For the measurement of appressoria diameter, conidia were left to germinate as described previously in 5 replicates. For each strain and concentration of the fungicide, the image of 20 randomly chosen appressoria for each replica was taken using a microscope Olympus BX 51 equipped with a PD71 digital camera. Diameters of appressoria were measured using the Cell D software program (Olympus Soft Imaging Solutions GmbH, Germany).

2.5 *In vitro* conidia infection efficiency test

Conidia, collected as described previously, were resuspended in sterile water to a final concentration of $1x10^5$ conidia/ml. The top leaf, cut off at 5-leaf-stage of a Carnaroli plant, was placed on the surface of a Petri plate containing 1.5% water agar media supplemented with tetracycline (5mg/l). Sterile paper discs, 0.5 cm diameter were submerged in the conidia suspension and placed on the adaxial surface of the leaf. The plates containing leaf-segments were incubated at 26°C, 24 h in the dark and followed by 24 hours at 18 h light / 6 h dark conditions. Subsequently, paper-discs were removed from the leaf surface and the plates were incubated for additional 5 days at 26°C and the same photoperiod. The infection efficiency of conidia obtained from mycelia exposed to fungicide was evaluated 7 days after inoculation, and the disease incidence was calculated according to the formula I(%) = n/ N * 100, where n = number of infected points and N = total number of visible inoculated points. Each inoculated point was scored using four classes of lesion types: 0 – no infection; 1 – necrotic spot; 2 - necrotic spot with yellow halo; 3 – sporulating lesion. Frequency of type 3 sporulating lesions was calculated according to formula type 3(%) = n3/ N * 100; where n3= number of points with type 3 sporulating lesions and N = total number of inoculated points (Figure 1).

2.6 Statistical analysis

To calculate dose-response curves for mycelium growth and sporulation, non-linear regression analysis using the 4-parameter log-logistic model was employed. The concentration of fungicide causing 50% growth inhibition (ED_{50}) was estimated by interpolation from the fitted regression curve. Spore germination, appressoria diameter, disease incidence and frequency of type 3 lesions were submitted to liner and non-linear regression analyses. All the analyses were performed using R software.[30,31]

3 RESULTS

3.1 Inhibition of mycelium growth

The average diameter of *M. oryzae* mycelium after 7 days of growth on control medium was 4.29 cm (s.e. 0.03). Non-linear regression was employed to determine dose-response curves and ED_{50} and ED_{90} values (Figure 2a). ED_{50} for tricyclazole was 100.41 mg/l (s.e. 1.65, 95% conf. interval 97.17-103.66) whereas for azoxystrobin was 0.044 mg/l (s.e. 0.007, 95% conf. interval 0.030-0.057) with dose-responses different for the two fungicides. ED_{90} of the mycelium growth was obtained at concentrations 215.64 mg/l for tricyclazole and 69.58 mg/l for axozystrobin.

3.2 Inhibition of sporulation

The average density of conidia per cm^2 of mycelium of individual *M. oryzae* strains grown in the absence of fungicides varied from $1.6x10^3/cm^2$ up to $1.4x10^6/cm^2$ depending on the strain used. With increasing concentrations of both fungicides the sporulation was inhibited (Figure 2b). The 50% sporulation was achieved at 0.017 mg/l for azoxystrobin and at 0.072 mg/l for tricyclazole, respectively. Thus, azoxystrobin was approximately 4-times more effective than tricyclazole, based on ED_{50}. At concentrations of 1 mg/l for azoxystrobin and 5 mg/l for tricyclazole, above 95% inhibition of sporulation was obtained. Tricyclazole severely reduced conidia production even at concentrations at which the mycelium growth was not affected.

3.3 Germination of conidia and appressoria diameter

The average percentage of conidia germination of the control was 95.2% (s.e. 0.818). The germination of conidia from mycelia exposed to tricyclazole ranged in all tested concentrations between 89-92.4% and no correlation between the increasing tricyclazole concentration and germination was observed. Conidia from mycelium exposed to azoxystrobin behaved differently. Up to the concentration 0.1 mg/l, conidia germination was similar to control, however, at the

highest tested concentration 1mg/l, germination was only 67,1%. Conidia germination decreased with increasing azoxystrobin concentration (Figure 3a, R^2=0.819, *P=0.000*).

Conidia formed on mycelia in the absence of fungicides produced appressoria with average diameter of 8.67 µm (s.e. 0.033). The appressoria diameters of conidia produced on media supplemented with tricyclazole did not change with increasing fungicide concentration. Appressoria diameters of conidia obtained from mycelia grown in presence of azoxystrobin were slightly reduced, however this decrease was not statistically significant (Figure 3b).

3.4 Conidia infection efficiency

The disease incidence on detached leaves inoculated with conidia that had not been in contact with a fungicide (control), was 53.33% (s.e. 7.09), and the frequency of type 3 lesions was 19.56% (s.e. 4.24). When conidia harvested from mycelia exposed to different concentrations of fungicides were used, disease incidence and frequency of type 3 lesions decreased with increasing fungicide concentrations and this tendency was more notable for tricyclazole, although not statistically significant (Figure 4).

4 DISCUSSION

Rice blast is a widespread and highly destructive disease. In Italy, tricyclazole and azoxystrobin are predominantly used for blast disease management.[32] Tricyclazole is known to inhibit melanin synthesis in *M. oryzae* appressoria and thus affects the efficiency of pathogen penetration into the host,[12,14,18] while azoxystrobin disrupts the electron transfer between cytochromes in the mitochondrial membrane that decreases ATP production for energy-requiring processes.[21] In the presented study, capability of conidia developed on mycelia grown in the presence of increasing doses of the two fungicides to germinate and infect detached rice leaves even in the absence of the respective fungicide was assessed to simulate impact of these chemicals on spreading of secondary infection. For this purpose, response of several *M. oryzae* isolates to increasing concentrations of both fungicides in terms of their mycelium growth was determined as well.

Mycelia of *M. oryzae* are sensitive to relatively low concentrations of azoxystrobin (ED_{50}=0.044 mg/l), while they are more tolerant to tricyclazole (ED_{50}=100.41 mg/l). Our data present for the first time detailed growth-response curves of 5 strains of *M. oryzae* mycelia to both fungicides and confirm previous observations of low fungi-toxicity of tricyclazole.[27,33] Moreover, they contribute to better understanding of *M. oryzae* mycelium response to azoxystrobin, as until now, only scarce data have been available about its efficacy towards closely related *M. grisea* (ED_{50}=1.8 mg/l)[28] or *M. oryzae* from perennial ryegrass (ED_{50}=0.013 mg/l)[22].

Different situation was observed when sporulation of mycelia exposed to the two fungicides was evaluated. In the case of azoxystrobin, sporulation was approximately 3-times more affected than the mycelium growth based on ED_{50}. However, tricyclazole showed much higher efficacy towards the sporulation (ED_{50}=0.072 mg/l) compared to mycelium growth. This is very surprising as tricyclazole is known to act in demelanization of appressoria and interferes with penetration through the host epidermis.[14,18] A physical interaction of tricyclazole with tri- and tetra-hydroxynaphthalene reductases has been demonstrated, that are two enzymes involved in melanin biosynthesis pathway, while no other proteins have been reported to interact with tricyclazole.[34,35] It might be possible,

that also the sporulation in *M. oryzae* depends on melanin synthesis by an unknown mechanism, or the interference with melanin biosynthesis results in the production of toxic intermediate products, that have inhibitory effects on sporulation.[36,37] The association between conidiogenesis and mycelium melanization has been demonstrated in *Bipolaris sorokiniana*, where differentiation of secondary hyphae and conidiophores is dependent on melanin.[38] However, no connection between melanin and sporulation is known in *M. oryzae*, which could explain the high sensitivity of conidia formation process to tricyclazole. Also, analyses of melanin-deficient mutants of *M. oryzae* (such as *buf-*, *rsy-* or *alb-*) indicate that their sporulation is not compromised, which argues against direct influence of melanin biosynthesis inhibition on conidiogenesis.[39]

Another explanation of our results could be that, apart from hydroxynaphthalene reductases, tricyclazole targets additional enzyme or transcription factor involved in the regulation of conidiogenesis. Recently, mutants in two *M. oryzae* genes have been described, *mosom1* and *mocdtf1*, that affect diverse processes including melanin pigmentation and spore development.[40] At present, their mode of action has not been determined, neither has been evaluated their possible interaction with tricyclazole.

Conidia produced from mycelia exposed to the two fungicides were further examined for their capability to infect and develop typical disease symptoms on detached rice leaves, even though the fungicide was not present at the time of infection. Our results indicate that conidia produced on media supplemented with tricyclazole had decreased infection efficiency and produced less sporulating lesions, while lower reduction was observed for conidia developed in the presence of azoxystrobin. These results are very surprising as the conidia from tricyclazole-treated mycelia did not show significantly reduced germination, and the appressoria shape and length of such conidia did not differ from those of the control, as observed previously.[18] Instead, conidia that developed on mycelia grown in the presence of azoxystrobin showed significantly reduced germination and modest decrease in appressoria size, whereas spore infection efficiency and production of sporulating lesions was not reduced to such extent.

In field conditions, the initial deposition of tricyclazole on rice leaves is approximately 10 µg/g (corresponding cca. to 10 mg/l) at the applied dose 300g a.i./ ha, with the half-life 2.46-5.33 days.[41] Our results show significant inhibition of *M. oryzae* sporulation and subsequent infection efficiency of conidia at concentrations of tricyclazole 10- to 100-times lower, which indicate a possible effectiveness of tricyclazole in the field for 10-20 days. With a life cycle of *M. oryzae* of 5-12 days depending on environmental conditions, tricyclazole applications can substantially delay the disease progress.[32]

Okuno et al. (1983) observed that reduction in secondary infection after tricyclazole treatment in field conditions can be partially due to reduced conidia formation on mycelia exposed to tricyclazole.[19] They hypothesized that the effect of tricyclazole on secondary infection was caused by its deposition on *Magnaporthe* conidia, and at the onset of infection it inhibits appressoria melanization. However, in a similar study, where effects of pyroquilon (MBI fungicide) on secondary infection were observed, the authors demonstrated that the penetration of spores produced from mycelia exposed to pyroquilon was compromised only minimally, whereas the hyphal growth inside the host was significantly impaired.[36] This argues against the simple transfer of the fungicide within the conidia and its effect on appressoria melanization after spore germination. Similarly, our results show reduced formation of sporulating lesions from conidia developed on mycelia exposed to tricyclazole, while total disease incidence was impaired to lesser extent.

We conclude that an additional physiological process might be regulated by tricyclazole, resulting in decreased sporulation and reduced infection efficiency of *M. oryzae* conidia.

ACKNOWLEDGEMENTS

The authors would like to thank prof. Bruno Rossaro and Dr. Jorge Gomez Ariza for their valuable advice and assistance and the reviewer for his critical and constructive comments.

This research was supported in part by Dow Agrosciences Italia S.r.l.

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FIGURE LEGENDS

Figure 1. *Magnaporthe oryzae* infection types observed on Carnaroli rice leaves 7 days after inoculation.

Figure 2: Log-logistic models of dose-response curves of *Magnaporthe oryzae* mycelium growth (a) and mycelium sporulation (b) as a function of increasing concentration of azoxystrobin (A) and tricyclazole (T). Symbols represent mean values for 5 strains.

Figure 3: Germination (a) and appressoria diameter (b) of *Magnaporthe oryzae* conidia produced on mycelia exposed to azoxystrobin (A) or tricyclazole (T). The error bars represent standard error.

Figure 4: Total disease incidence (a) and frequency of type 3 lesions (b) caused by *Magnaporthe oryzae* conidia produced on mycelia exposed to increasing concentrations of azoxystrobin (A) or tricyclazole (T). Error bars represent standard error.

Table 1. *Magnaporthe oryzae* strains used in the study

Strain	Sampling site	Rice variety	Year	Fungicide exposure
TOA75	Ottobiano (Pavia province)	Thaibonnet	1998	N
TOA44	Ottobiano (Pavia province)	Thaibonnet	1998	N
TA61	Casalbeltrame (Novara province)	Thaibonnet	1998	N
TA102	Casalbeltrame (Novara province)	Thaibonnet	1998	N
BA43	Casalbeltrame (Novara province)	Balilla	1998	N
BA26	Casalbeltrame (Novara province)	Balilla	1998	N
BOA58	Ottobiano (Pavia province)	Balilla	1998	N
VNP46	Travacò Siccomario (Pavia province)	Vialone nano	2010	Y
VNP24	Travacò Siccomario (Pavia province)	Vialone nano	2010	Y

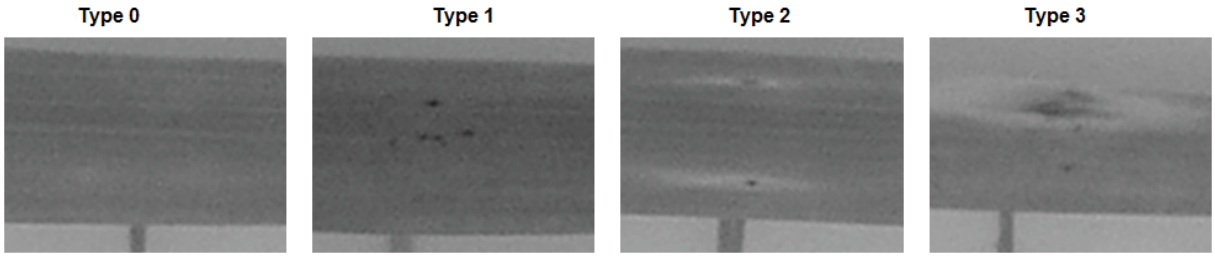
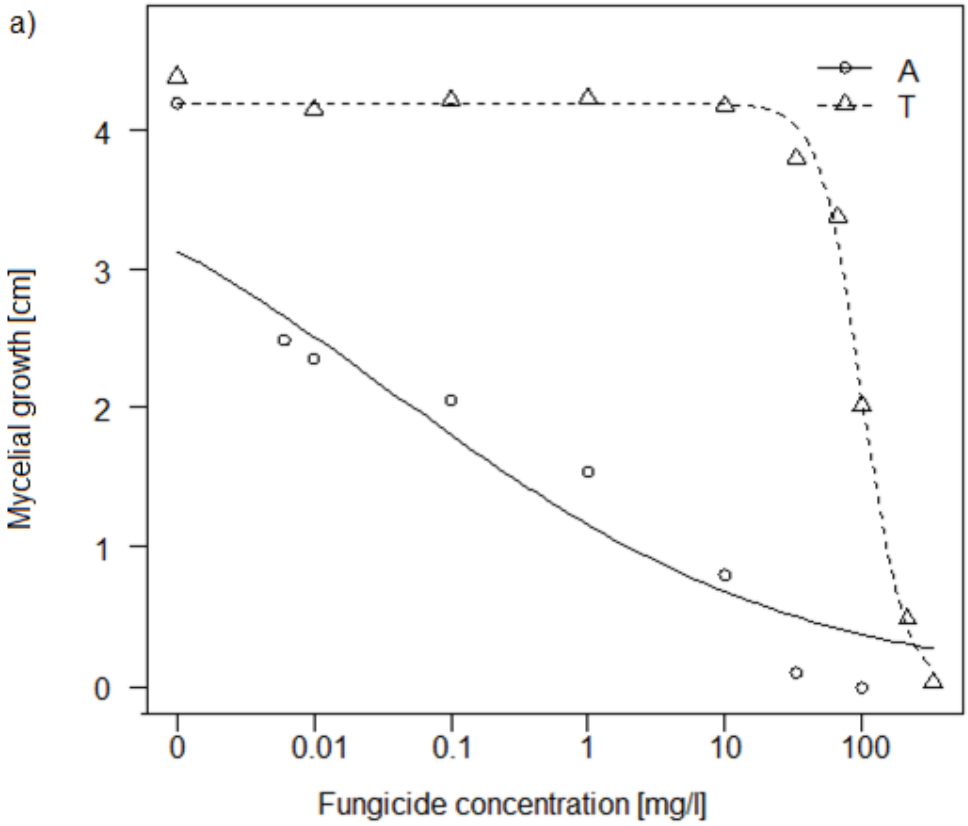


Figure 2: Log-logistic model of dose-response curves of *Magnaporthe oryzae* mycelium growth (a) and mycelium sporulation (b) as a function of increasing concentration of azoxystrobin (A) and tricyclazole (T). Symbols represent mean values for 5 strains.



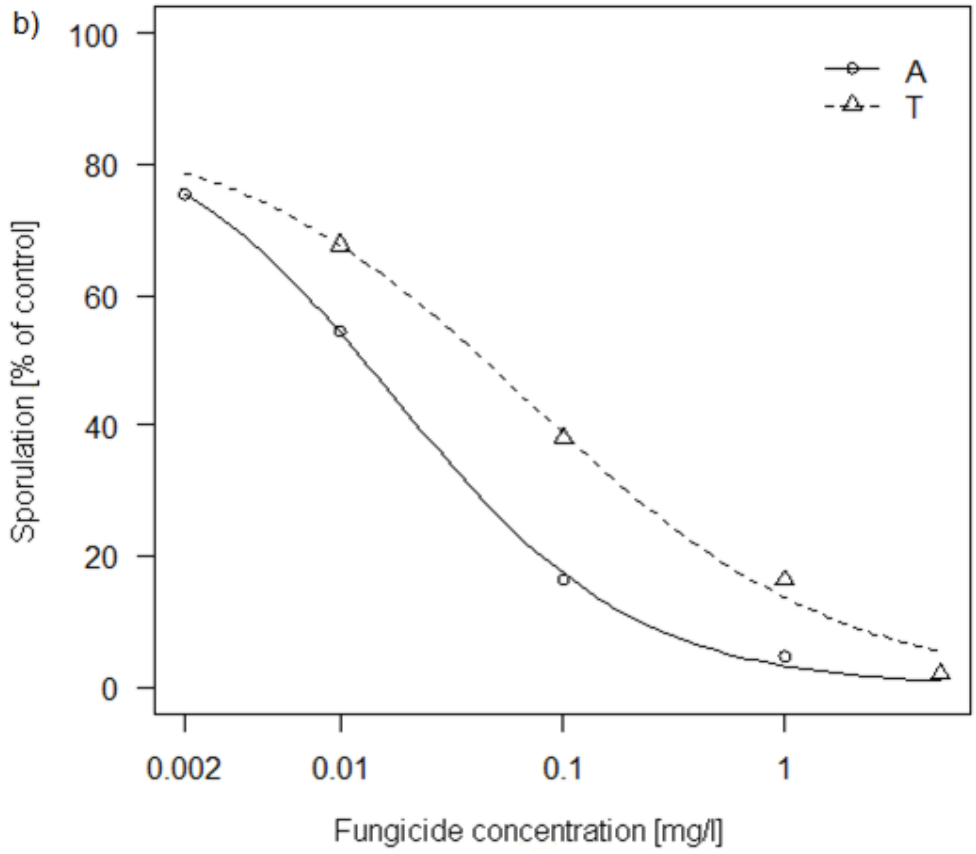
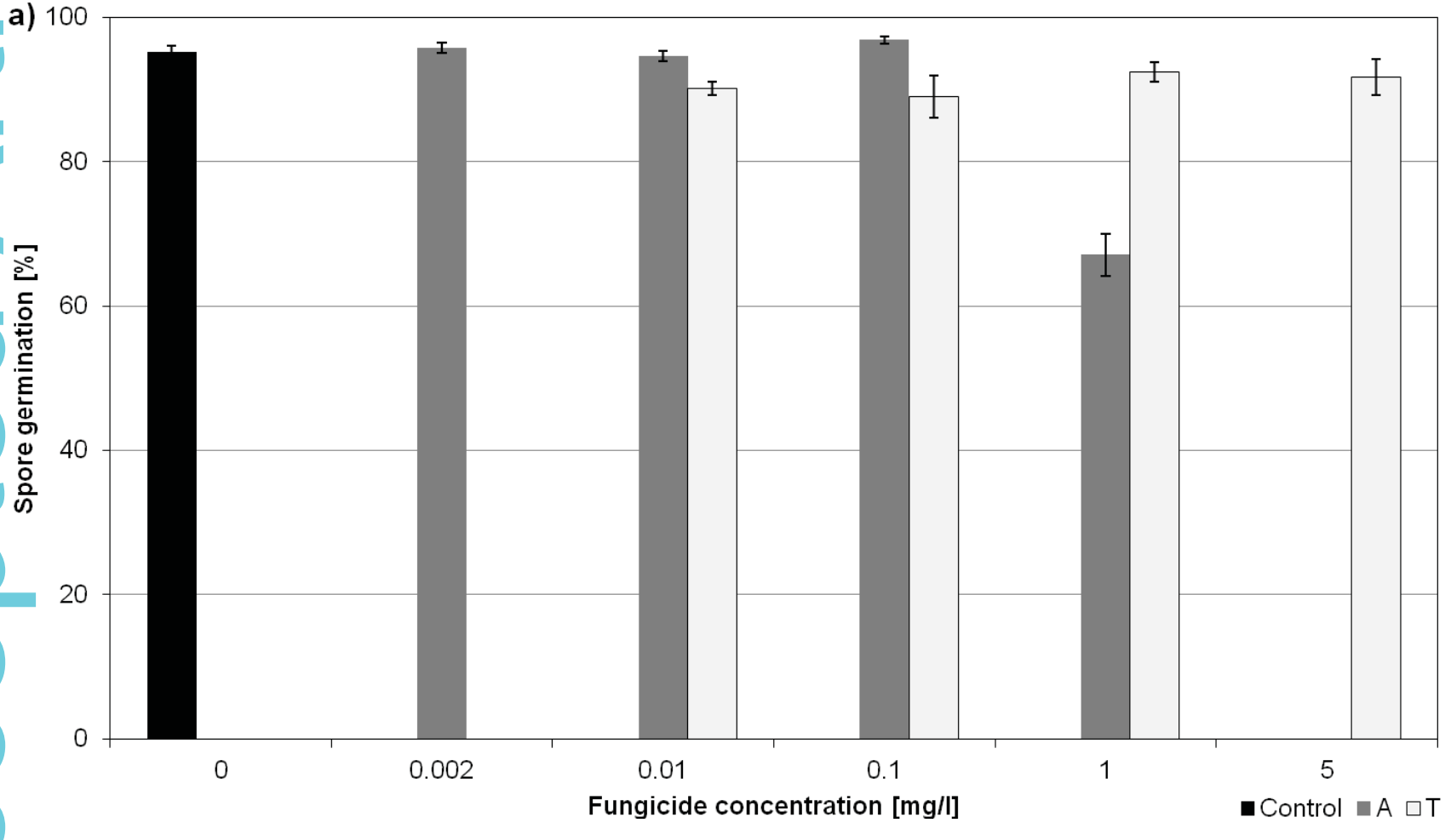


Figure 3: Germination (a) and appressoria diameter (b) of *Magnaporthe oryzae* conidia produced on mycelia exposed to azoxystrobin (A) or tricyclazole (T). The error bars represent standard error.



b)

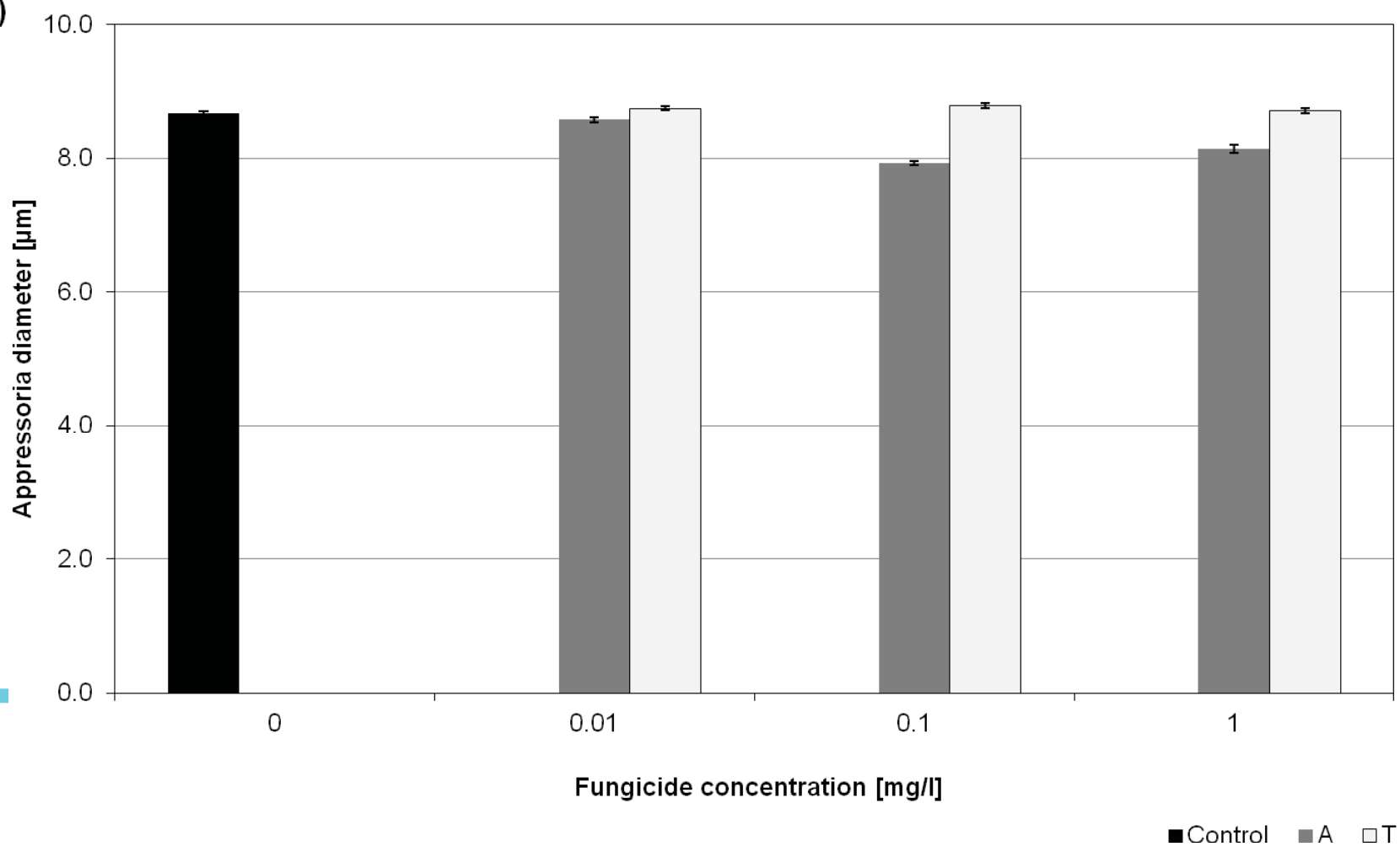
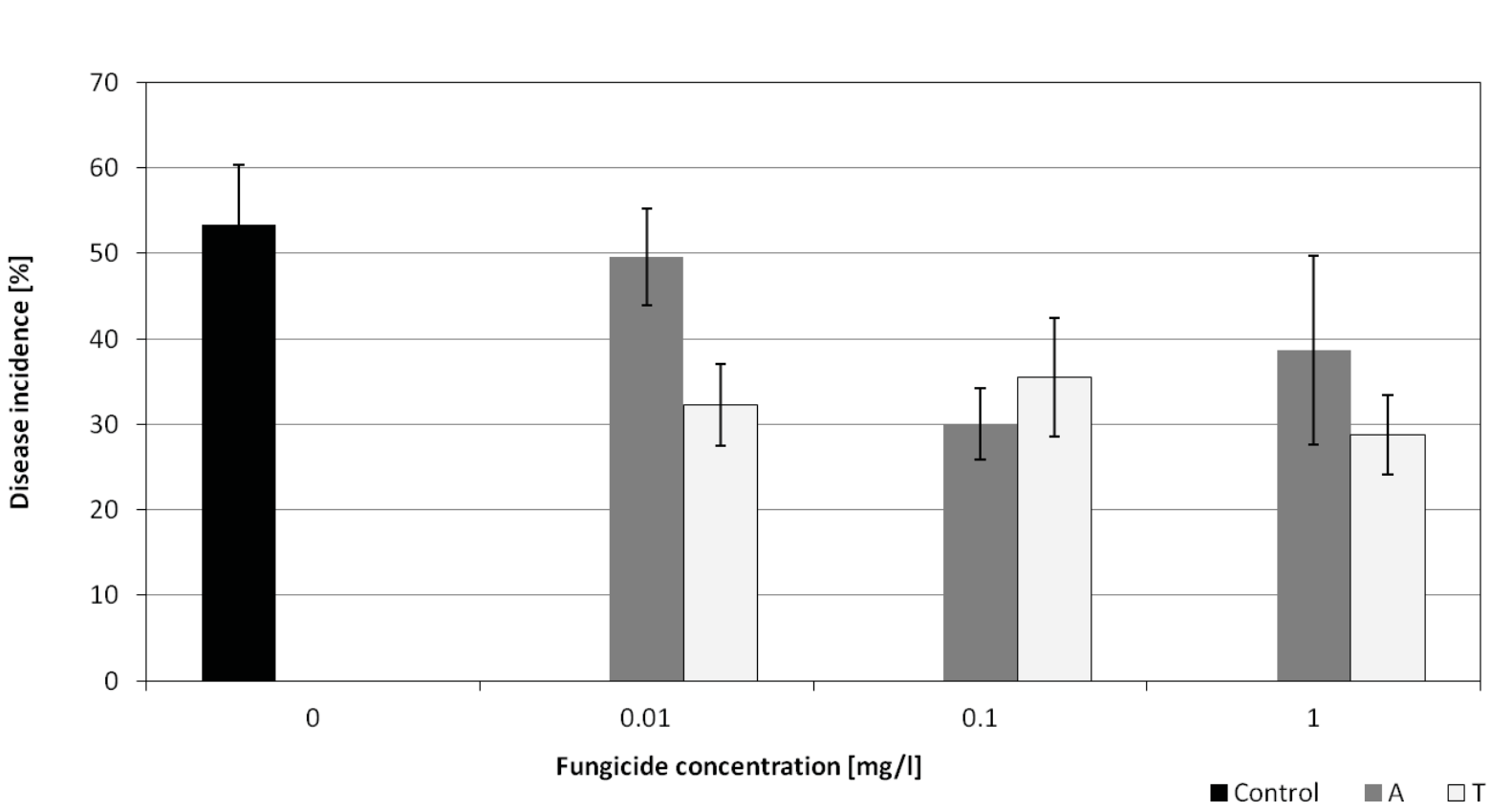


Figure 4: Total disease incidence (a) and frequency of type 3 lesions (b) caused by *Magnaporthe oryzae* conidia produced on mycelia exposed to increasing concentrations of azoxystrobin (A) or tricyclazole (T). Error bars represent standard error.



b)