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Article

Marine Natural Product Antimycin A Suppresses Wheat Blast Disease Caused by *Magnaporthe oryzae Triticum*

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Abstract: Application of chemical pesticides to protect agricultural crops from pests and diseases is discouraged due to their harmful effects on human and environment. Therefore, alternative approaches for crop protection through microbial or microbe originated pesticides have been gaining momentum. Wheat blast is a destructive fungal disease caused by Magnaporthe oryzae Triticum (MoT) pathotype, which poses a serious threat to global food security. Screening of secondary metabolites against MoT revealed that antimycin A isolated from a marine Streptomyces sp. had significant inhibitory effect on mycelial growth in vitro. This study aimed to investigate the inhibitory effects of antimycin A on some critical life stages of MoT and evaluate the efficacy of wheat blast disease control by this natural product. Bioassay indicated that antimycin A suppressed mycelial growth, conidiogenesis, germination of conidia and formation of appressoria in germinated conidia of MoT in a dose-dependent manner with minimum inhibitory concentration 0.005 µg/disk. If germinated, antimycin A induced abnormal germ tubes (4.8%) and suppressed the formation of appressoria. Interestingly, application of antimycin A significantly suppressed wheat blast disease in both seedling and heading stages of wheat supporting the results from invitro study. This is the first report on inhibition of mycelial growth, conidiogenesis, conidia germination, detrimental morphological alterations in germinated conidia, and suppression of wheat blast disease caused by a Triticum pathotype of M. Oryzae. Further study is required to unravel the precise mode of action of this promising natural compound for considering it as a biopesticide to combat wheat blast.

Keywords: Antimycin A; wheat blast; inhibition; biopesticide; biological control

1. Introduction

The wheat blast fungus *Magnaporthe oryzae Triticum* (MoT) pathotype is considered one of the utmost destructive pathogens of the major food crop, wheat [1-4]. This fungal pathogen has been a threat to 3 million hectares of wheat crop in some South American countries viz. Brazil, Argentina, Bolivia and Paraguay since its first outbreak in Brazil in 1985. Recently, this fearsome wheat killer was introduced into Bangladesh and Zambia posing a serious threat to global food and nutritional security [3, 5-7]. The MoT fungus can infect wheat plants at any growth stages but infection at heading stage is considered the most destructive [4,8,9]. Infection on the spike at heading stage blocks the vascular system resulting in white head symptoms with shriveled or n grains in the spike [1,3,4].

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Under a favorable environment, yield loss due to wheat blast can be up to 100% [3]. Infected seeds are thought to be the source of inoculum for long distance dispersion of this pathogen while both seeds and airborne asexual spores serve as inoculum for short distance dispersion of the pathogen [10,11]. The asexual spore of the fungus is called conidium, a three-celled, hyaline, and pyriform structure, which attaches to the surface of the host by secreting adhesive biochemicals [3,8,12]. After attachment to the host surface, the conidium germinates to form a germ tube. Later on, an appressorium and infection peg is formed to rupture the host epidermis to proceed to the infection process [12,13]. The process of plant tissue invasion is accomplished by the penetrating fungal hypha through the host epidermis and invading the plasma membrane of the host [8,12,13]. Therefore, disruption of any of these asexual life stages eliminates the possibility of pathogenesis by this fungus [14].

The use of synthetic fungicides is one of the approaches to control plant disease. Strobilurin (QoI) fungicides either alone or in combination with other fungicides have long been used to manage wheat blast disease. However, indiscriminate and frequent use of strobilurin (QoI) fungicides has led to the emergence of pathogenic strains resistant to this group of fungicides [15-17]. In addition, extensive use of these fungicides can cause serious damage to human health, animals, and disturbance in the natural ecosystem [18,19]. Moreover, traditional breeding strategies take longer time to develop resistant variety, which often breaks down at field conditions after a few years due to the development of new pathogen races [15,20]. Due to hexaploidy, genetic modification of wheat is considered extremely complicated [21]. Therefore, an eco-friendly alternative approach is needed to manage this fearsome disease of wheat.

Biological control of plant disease may offer a better alternative to the management of plant diseases. Several bacterial genera including *Bacillus*, *Pseudomonas*, *Streptomyces* are well known for their biocontrol activity [22]. They produce a wide array of antifungal compounds like lytic enzymes, antibiotics to suppress growth of pathogens or induce systemic resistance in plant. Among bacterial genera, the genus *Streptomyces* has received special attention to researchers due to their potential to produce diverse class of antibiotics [23]. Approximately two third of economically important antibiotics have been isolated from *Streptomyces* spp. that were used for disease management in agriculture [24,25]. The biocontrol activity of those isolated compound against phytopathogens have been reported by various workers [25-30]. Moreover, they also produce lytic enzymes such as chitinases and glucanases which are also used to control phytopathogens [31-33]. Either whole cells or metabolites have been used to formulate *Streptomyces*-based fungicides. For example, mycelia and spore of *Streptomyces* have been used for formulation of Mycostop (containing *S. griseoviridis* K61), Actinovate and actinoiron (containing *Streptomyces lydicus* WYEC 108) and RhizovitR (*S. rimosus*) for the control of foliar and root diseases of various crops [34-37]. In addition, three secondary metabolites; polyoxin D, streptomycin, and kasugamycin produced by *Streptomyces* spp. have currently been distributed as foliar fungicides and bactericides [38].

Antimycin A, a member of antimycins antibiotic isolated from marine *Streptomyces* sp., composed of acyl and alkyl side chains and a nine-member dilactone ring [39]. Antimycins are known as specific inhibitors of respiratory chain of mitochondria at the level of complex III [40]. The antimycin A has drawn considerable attention due to their toxicity toward both human and phytopathogenic fungi [40-42]. They inhibited the growth and development of fungi, *Rhizoctonia solani* and *Magnaporhe grisea* [43,44]. In a screening of

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secondary metabolites against a newly introduced destructive wheat blast disease caused by MoT, we found that antimycin A isolated from a marine *Streptomyces* sp. significantly inhibit growth of MoT *in vitro*. This study aimed to investigate in detail the effects of antimycin A on asexual development of wheat blast fungus in vitro and evaluate the control of disease *in vivo*. Therefore, the specific objectives of this study were to (i) investigate the effect of antimycin A on the suppression of mycelial growth of *MoT*; (ii) test its effect on conidiogenesis, conidial germination, and subsequent steps of the development; and (iii) evaluate the suppression of blast disease at the seedling and heading stage of wheat.

2. Materials and Methods

2.1 Chemicals

Antimycin A (AMA) (Fig 1) is a chemical compound produced by a marine *Streptomyces* sp. [39]. This pure compound was generously provided by Dr. Hartmut Laatsch of Georg-August University Goettingen, Germany. The fungicide Nativo® 75WG (a combination of 50% tebuconazole and 25% trifloxystrobin) was purchased from Bayer Crop Science Ltd. Dhaka, Bangladesh.

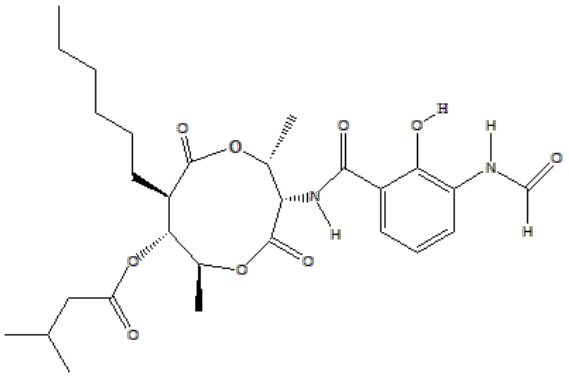

Figure 1. Structure of Antimycin A

2.2 Culture of wheat blast isolate

A fungal isolate BTJP 4-5 was obtained in pure culture from the field infected wheat blast samples by picking up a single conidium and then preserved it at 4°C on dry filter paper following the method described by [45]. For this study, this virulent isolate BTJP 4-5 of MoT was retrieved from the storage in potato dextrose agar (PDA) medium and incubated at 25°C for conidia production [45].

2.3 Preparation of chemical solution and conidial suspension

Stock solution of antimycin A was prepared using a small quantity of dimethyl sulfoxide (DMSO). Preparation of 1, 5, and 10 µg/disk concentrations of this compound was then carried out in distilled water where the final concentration of DMSO never exceeded 1% (v/v) in the final solution, which is proven not to affect the hyphal growth or sporulation of MoT. Nativo® 75WG concentrations were 1, 5, and 10 µg/disk were prepared in distilled water. Sterilized water with 1% DMSO served as a negative control. The conidial suspension was prepared from ten-day old culture plates and spore concentration was adjusted to ca. 5×10^4 conidia mL^{-1} as described by [45].

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2.4 Fungal growth inhibition and morphological changes of hyphae

Hyphal growth inhibition of MoT isolate BTJP 4-5 by antimycin A and Nativo® 75WG was calculated by using the modified disk diffusion method [14,46]. A series of concentrations ranging from 0.005 to 2 µg/disk of antimycin A and Nativo® 75WG were prepared by dissolving required amounts in DMSO and water. Nine-millimeter diameter filter-paper disks (Sigma-Aldrich Co., St. Louis, MO, USA) were soaked with the test compounds. The treated disks were placed at 2 cm distance from one side of 9 cm dia Petri dishes containing 20 mL fungal growth media. Five-millimeter diameter mycelial blocks were placed on the opposite side of the filter paper disk. Filter paper disks treated with DMSO followed by evaporation in room temperate functioned as a negative control. Petri dishes were incubated at 25˚C until the fungal colony fully covered the media surface of the control plates and the experiment was repeated five times. Radial growth of the fungal colony was measured in centimeters with a ruler along with two perpendicular lines drawn on the lower side of each plate. After ten days of incubation, data were recorded by measuring the inhibition zone formed by the test compounds and corresponding mycelial growth. Radial growth inhibition percentage (RGIP) (± standard error) [47] was determined from mean values as:

$$\text{RGIP \%} = \frac{\text{Radial growth in control plate - Radial growth in treated plate}}{\text{Radial growth of control}} \times 100$$

Zeiss Primo Star microscope at 40X and 100X (100x was an oil immersion lens) was used to observe hyphal morphology at the leading edge of the colonies facing the treated and control disks. Canon DOS 700D digital camera was used to capture images of the disk diffusion experiment. Photographs of the hyphae were captured with a Zeiss Axiocam ERc 5s microscope.

2.5 Inhibition of conidiogenesis

To induce conidiogenesis, the mycelia of a ten-day old MoT culture plate was washed to reduce nutrients [3,14,48]. MoT mycelial agar blocks measuring 10 mm were treated with 50 µL of antimycin A and Nativo® 75WG at 1, 5, and 10 µg/mL and put into Nunc multiwell plates. On the other hand, same amount of sterilized water was used on the MoT mycelial block with 1% DMSO served as a negative control. Then, treated MoT mycelial agar blocks were incubated at 28˚C with >90% RH. Additionally, light and dark periods were adjusted at 14 h and 10 h, respectively. After 24 hours, conidiogenesis was examined using Zeiss Primo Star microscope at 40x magnification. All of the images were captured with a Zeiss Axiocam ERc 5s microscope and the experiment was repeated five times.

2.6 Germination inhibition and morphological modifications of germinated conidia

The conidial germination assay was conducted according to the previously described protocol [14,49]. For each treatment, a 100 µl solution of respective concentration was added directly to 100 µl of 5×10^4 conidia mL^{-1} of MoT to make a final volume of 200 µL into a well of a 96-multiwell plate. Glass rod was used to mix the solution immediately, and incubated at 25˚C. In this experiment, 1% DMSO with sterile water served as a control. A moisture

 doi:10.20944/preprints202205.0156.v1

chamber was used to incubate the multiwell plate at 25 ^{0}C for 6 h, 12 h, and 24 h in the dark. For each replication, a total of 100 conidia were observed under a Zeiss Primo Star at 100x magnification. Calculation of germination percentage of conidia and its developmental process were examined and photographs were captured with a Zeiss Axiocam ERc 5s. The time course was repeated five times. Conidial germination percentage (± standard error) was calculated from mean values as: CG % = (C–T)/C × 100; Where, CG = conidial germination, C = percentage of germinated conidia in control samples, and T = percentage of germinated conidia in treated samples.

2.7 Growing of seedlings

Wheat seeds of Bangladeshi cultivar BARI Gom-26 were surface-sterilized with 70% ethanol for 10 minutes, soaked in 1.5% active chlorine for 1 hour, and rinsed five times in sterile distilled water (SDW) [50]. Twenty-five seeds were planted in each of 20-cm-diameter plastic pots filled with NPK fertilizer amended soil. Finally, twenty healthy seedlings per pot were allowed to grow under natural conditions until seedling bioassay following previously described protocol by Gupta et al. [45] was performed. Watering was done as a regular management practice.

2.8 Field Evaluation of Antimycin A against wheat blast

2.8.1 Preparation of land and fertilization

The experiment was set at confined land in the research field of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, Bangladesh. The experimental site was located at 24.09° north latitude and 90.26° east longitude with an elevation of 8.4 m from the mean sea level. The land was well ploughed and cleaned properly by uprooting weeds and stubbles. Well decomposed cowdung was applied in adequate amount during land preparation. Chemical fertilizers like nitrogen, triple super phosphate, muriate of potash and gypsum were applied at the rate of 70-28-50-11 kg ha^{-1} N-P-K-S, respectively [51]. Two-third of urea and all other fertilizers were applied at the final land preparation as basal dose 3-4 days before seed sowing. Immediately after sowing the plots were lightly irrigated to ensure uniform germination. Irrigation and other intercultural operations were done whenever necessary. The rest one-third urea was top dressed at 1st irrigation at 20 days after sowing (DAS). Randomized complete block design (RCBD) was followed for conducting the experiment.

2.8.2 Seed sowing and management of the plot

The seeds of wheat variety BARI Gom-26 were sown in the first week of December. S**eed** treatment was done with fungicide Vitavex 200 (3 g/kg seed) before sowing. All the plots were properly labeled. Irrigation and other intercultural operations were done as necessary.

2.9 Plant infection assay at the seedling stage

After fourteen days of emergence, the pots were covered with sterilized transparent polyethylene bags to maintain humidity and laid in a completely randomized design. Seedlings were sprayed with freshly prepared test compounds at respective concentrations

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mentioned above and left overnight to dry up. Pots were then inoculated by spraying conidial suspension containing 5×10^4 MoT conidia mL^{-1}. Inoculated seedlings were incubated inside sterilized transparent polyethylene bags (> 95% relative humidity) at 25°C and kept in dark for 24 h after inoculation. The seedlings were then transferred into a growth room operating at 28 ± 1°C and a minimum of 90% relative humidity with 12 h light per day [52]. In addition, sterilized water was sprayed on the seedlings 5 to 7 times a day to provide a conducive environment for disease development in the growth room conditions. Disease development data were recorded after five days of inoculation. Each treatment was replicated for five times.

2.10 Infection assay in the wheat field at the reproductive phase

Freshly prepared 1, 5, and 10 µg/disk concentrations of the test compound were sprayed in the respective plot and left overnight to dry, sterilized water with 1% DMSO served as a negative control. Spore suspension was applied in wheat fields just after the flowering stage of wheat plant. Fungicide Nativo® 75WG was applied as positive control and deionized distilled water was applied as a negative control. Before inoculation, plots were covered with transparent polyethylene sheets to ensure humid condition congenial for spore germination.

2.11 Recording of data, measurement of disease intensity and severity

At the reproductive phase, data were collected on total tiller, effective tiller and infected tiller $hill^{-1}$, full length and infected part of spike, seeds $spike^{-1}$, 1000-grain weight and grain yield $hill^{-1}$. At the vegetative phase, the data were collected on total seedlings, infected seedlings pot^{-1}, full length, and infected part of the leaves. The disease intensity was calculated using the formula:

$$DI = \frac{\text{Total no. of infected plants}}{\text{Total no. of plant observed}} \times 100$$

Likewise, blast disease severity assessment was done using a 5 scale basis where % infection means the length of the spike infected by blast. The scales were 0 = no lesions; 1 = 1-25% infection; 2 = 26-50% infection; 3 = 51-75% infection and 4 = 76-100% length of the leaves infected by blast. The severity of blast was calculated using the formula:

$$DI = \frac{n \times v}{N \times V} \times 100\ \%$$

DS = disease severity
n = number of leaves infected by blast
v = value score of each category attack
N = number of leaves observed
V = value of highest score

2.12 Design of experiment and statistical analysis

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Experiments in the laboratory and field condition were performed using a completely randomized design (CRD) and Randomized complete block design (RCBD), respectively to determine the fungicidal activities of the pure antimycin A compound compared to a standard fungicide. All statistical analyses were conducted using the statistical software package, IBM SPSS Statistics 25, and Microsoft Office Excel 2015 program package. Analysis of means comparison of the treatments was accomplished by Tukey's HSD (Honest Significance Difference) test ($p \leq 0.05$). Each treatment was replicated five times and mean value ± standard error was used in Tables and Figures.

3. Results

3.1 In vitro assays of fungal growth inhibition

The antifungal activity was tested by measuring the inhibition of fungal growth by antimycin A using a plate assay against wheat blast fungus MoT (Fig. 2). The compound antimycin A showed a strong inhibition of hyphal growth of the fungus MoT. Mycelial growth inhibition by antimycin A was 68.6±0.64% at 2 µg/disk (Fig. 3). Comparative pictures of the suppression of fungal growth by test compounds are shown in Fig. 2.

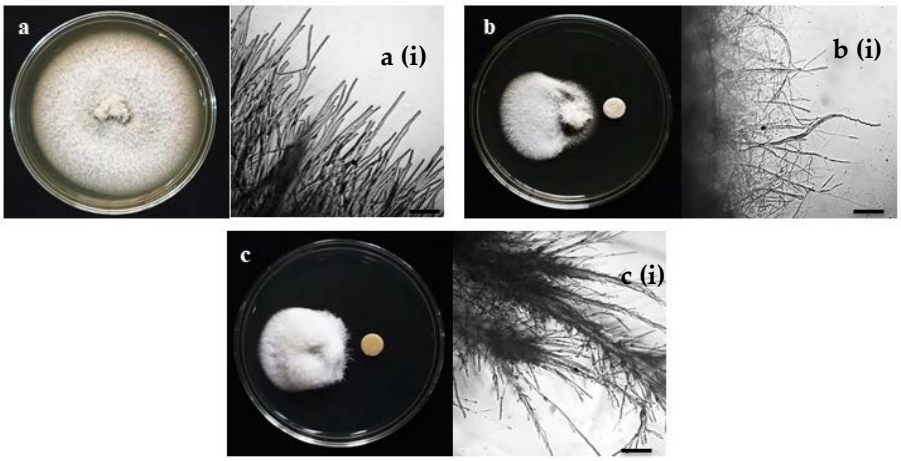

Figure 2. Macroscopic and microscopic view of *in vitro* antifungal activity of antimycin A and the commercial fungicide Nativo® 75WG against *M. oryzae Triticum* (MoT) at 20 µg/disk. The macroscopic images were (a) Control, (b) Antimycin A, and (c) Nativo® 75WG, whereas, a (i), b (i), and c (i) were microscope images of control, antimycin A, and Nativo® 75WG, respectively. Bar = 50 µm.

Bioassay revealed that antimycin A inhibited the growth of mycelia in a concentration-dependent manner. Inhibitory effects of antimycin A increased with the increasing doses ranging from 0.005 to 2 µg/disk. Based on the lower and higher concentration, inhibition percentage of mycelial growth by antimycin A were 9.6±0.38% and 62.9±0.42%, respectively (Fig. 3). Antimycin A showed a slightly lower inhibition rate of fungal mycelia than Nativo® 75WG (Fig. 3).

Antimycin A showed extensive inhibition of hyphal growth at 2 µg/disk (62.9 ± 0.42%) followed by 1.5 µg/disk (52.3 ± 1.29%) and 1 µg/disk (50.6 ± 1.19%), which was indicative of a positive correlation of suppression with an increase in concentration. However,

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this natural product didn't show any activity against MoT lower than the dose of 0.005 µg/disk.

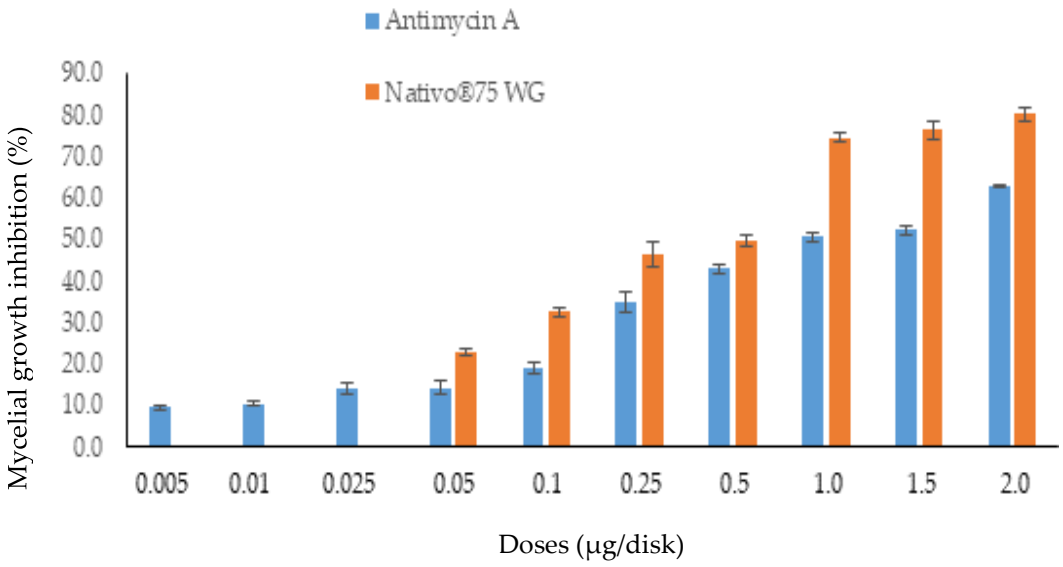

Figure 3. Inhibitory effects of Antimycin A and the commercial fungicide Nativo® 75WG on mycelial growth of MoT in PDA media. The data are the mean ± standard errors of three replications for each dose of the tested compound.

Minimum inhibitory concentration (MIC) required for growth inhibition (MIC's) for each inhibitor were obtained. The MIC of Nativo® 75WG was 0.05 µg/disk, which was ten times higher than the MIC of antimycin A (0.005 µg/disk). The percentage of fungal growth inhibition at MIC of antimycin A and Nativo® 75WG was 9.6±0.38 and 22.9±0.64, respectively.

The observation of hyphal growth under a microscope revealed that the hyphae of untreated MoT had polar and tubular growth with smooth, hyaline, regularly branched, septate, plump, and intact structures (Fig 2ai). MoT containing Petri dish treated with antimycin A showed irregular growth and frequently increased branch per unit length of fungal hyphae. Antimycin A treated Petri dish also showed rough hyphal cell walls but displayed ridges with a corrugated existence and irregular swelling of cells (Fig 2bi). Likewise, morphological abnormality also occurred in the case of MoT where the hyphae were close to the disk containing fungicide Nativo® 75WG (Fig 2ci). Nevertheless, the altered morphological appearance of MoT by antimycin A was slightly different than those observed with the Nativo®75WG signifying a possible dissimilar mode of action. Overall, antimycin A is stronger inhibitor than the commercial fungicide, Nativo® 75WG.

3.2 Antimycin A block conidiogenesis in MoT

Generation of conidia asexually from conidiophres is critical for infecting wheat plant by MoT. Both antimycin A and fungicide Nativo® 75WG significantly decreased conidia formation in MoT at 5 and 10 µg/mL when compared to the control. Inhibition increased with an increase in concentration from 1, 5, and 10 µg/mL (Fig 4). MoT failed to

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develop any conidia at the concentration of 10 µg/mL of both natural antimycin A and synthetic fungicide Nativo® 75WG. Microscopic investigation showed broken mycelial tips and a complete lack of conidiophores when treated the mycelia at 10 µg/mL of both tested compounds. On the other hand, control dish treated with sterilized water containing 1% DMSO produced 5–6 × 10^5 conidia/mL.

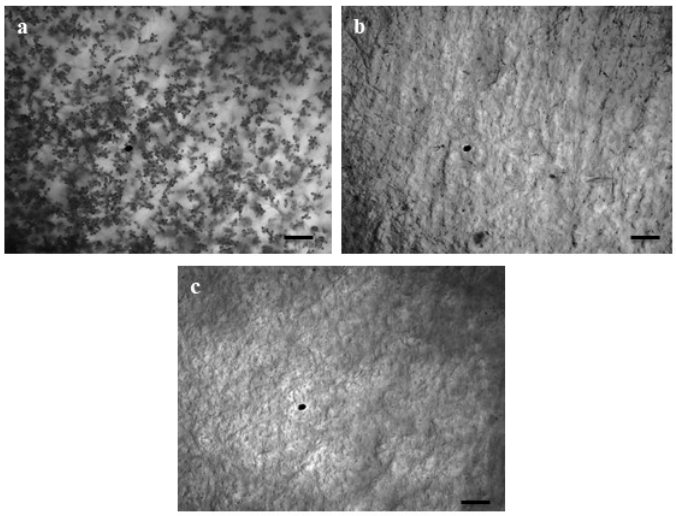

Figure 4. Effects of antimycin A and a commercial fungicide Nativo® 75WG on the suppression of conidiogenesis of MoT in Nunc multidisc at 10 µg/mL. (a) Control, (b) Antimycin A, (c) Nativo® 75WG. Bar = 50 µm.

3.3 Antimycin A alters conidia germination and developmental transitions of germinated conidia

To determine the germination of conidia and formation of appressoria of MoT, we used antimycin A and Nativo® 75WG at 10 µg/mL in multi-well plates. The percentage of germinated conidia and their altered morphology were recorded after 6, 12, and 24 h of incubation (Table 1). Both treatments remarkably reduced conidial germination after six hours of incubation compared to the control. At the same time, 100% conidial germination was observed in water, whereas, 44.3±2.33% in plates treated with fungicide Nativo® 75WG. In antimycin A solution, fungal spore germination was 42.1±0.35%, whereas commercial fungicide showed 44.3±2.33% after 6 h of incubation. At all the incubation time (6 h, 12 h and 24 h), control treatment supported 100% germination of conidia, developed normal germ tubes and showed standard mycelial growth at 25°C in dark (Table 1 and Fig 5a). At 10 µg/mL concentration, antimycin A displayed significant adverse effect on both germinations of conidia and impaired post germination developmental process of MoT. Overall, antimycin A showed abnormal transitional advancement from one step to the next during developmental processes of germinated conidia.

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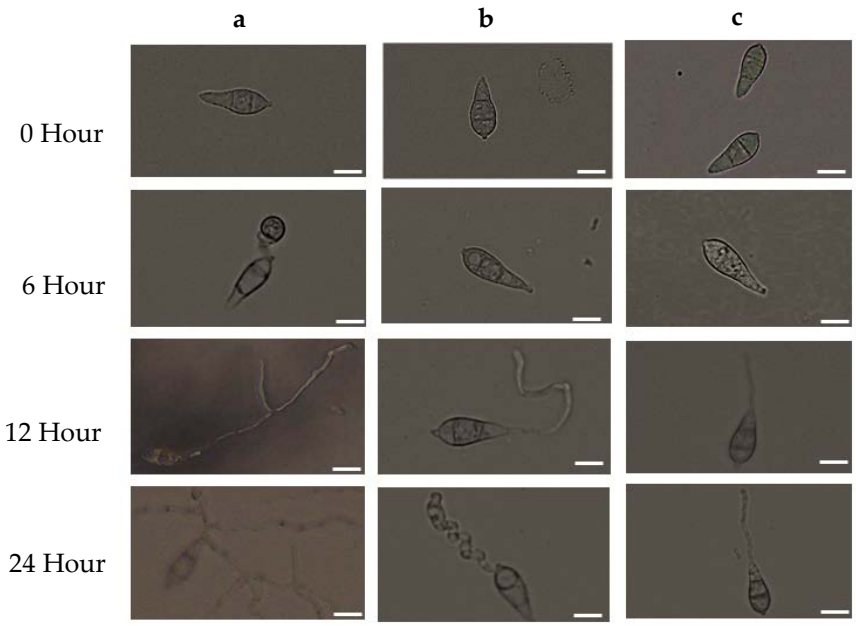

Figure 5. Time-dependent alterations in MoT germination of conidia and subsequent morphological changes in the presence of Antimycin A and the commercial fungicide Nativo® 75WG. Dose of Antimycin A was 10 µg/mL. (a) Control, (b) Antimycin A, and (c) Nativo® 75WG. Germinated conidia; Short germ tube; Elongated germ tube. Bar = 10 µm.

Afterward 6 h, in presence of antimycin A, 26.7±0.41% of conidia germinated with shorter germ tubes compared to the control and 15.4±0.44% conidia lysed. Similar developmental abnormalities were also observed among the germinated spores after 12 h of incubation, which showed 12.6±0.40% normal and 4.8± 0.29% with the formation of abnormally elongated germ tubes, while no additional germination was found after 24 h of incubation (Table 1, Fig 5b).

Table 1. Effects of Antimycin A and the fungicide Nativo® 75WG on germination of conidia and morphology of germ tubes and appressoria of *Magnaporthe oryzae Triticum* at 10 µg/mL *in vitro*

Treatment	Time (h)	Germination of conidia, morphology of germ tubes, and appressorial formation	
		Germinated conidia (% ± SE[a])	Morphological change/developmental transitions in the treated conidia
Water	0	0±0b	No germination
	6	100±0a	Germination with normal germ tube and normal appressoria
	12	100±0a	Normal mycelial growth
	24	100±0a	Normal mycelial growth
Antimycin A	0	0±0c	No germination
	6	42.1±0.35a	26.7 ± 0.41% Short germ tube and 15.4 ±0.44% conidia lysed
	12	26.7±0.41b	12.6 ± 0.40% Normal germ tube, 9.3 ± 0.68% short and 4.8 ± 0.29% Abnormally elongated germ tube
	24	0±0c	No appressoria, no mycelial growth
Nativo® 75 WG	0	0±0b	No germination
	6	44.3±2.33a	Germinated with a short germ tube
	12	44.3±2.33a	Normal germ tube
	24	0±0b	No appressoria; no mycelial growth

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[a] The data presented here are the mean value ± SE of three replicates in each compound. Means within the column followed by the same letter(s) are not significantly different from those assessed by Tukey's HSD (Honest Significance Difference) post-hoc ($p \leq 0.05$). Conidia germination percent at different incubation times is not cumulative, rather at different time intervals in separate experimental unit.

In the presence of Nativo® 75WG, the germination of conidia was the same at 6 and 12 h of incubation (44.3±2.33), although after 6 h conidia showed shorter germ tubes and after 12 h all the conidia exhibited normal germ tubes. However, they didn't form any appressoria. Similar to antimycin A, commercial fungicide Nativo® 75WG also completely suppressed germination of spores after 24 h (Table 1, Fig 5c). Interestingly, antimycin A yielded abnormally short and long germ tubes and lysed conidia, while the fungicide did not exhibit such type of changes. Both natural compound antimycin A and synthetic fungicide blocked formation of appressoria that are essential for pathogenesis suggesting their potentials for control of wheat blast.

3.4 Antimycin A suppresses wheat blast disease at seedling stage

Application of antimycin A significantly inhibited the development of blast symptoms in the leaves of artificially inoculated wheat seedlings. In this study, blast lesion on wheat seedlings treated with Antimycin A and Nativo® 75WG fungicide compared with water treatment control. In case of antimycin A, percentage of disease incidence and severity were 16.33±2.19% and 10.67±2.96%, respectively at 1 µg/mL preventive dose, whereas, 5 µg/mL produced 6.67±0.88 and 3.33±0.88 % disease incidence and severity, respectively [Fig 6(i) and Table 2]. Wheat blast caused the highest level (100±0%) of disease intensity and severity (82±4.73%) in case of untreated control, whereas healthy control did not show any blast symptoms [Fig 6(i) and Table 2].

Table 2. Effect of antimycin A in suppression wheat blast disease development in artificially inoculated wheat seedlings.

				Preventive (µg/mL)			Curative (µg/mL)		
Parameter	Untreated control	Healthy control	Commercial fungicide Nativo® 75WG	1	5	10	1	5	10
% disease incidence	100±0.00a	0±0.00d	0±0.00d	16.33±2.19b	6.67±0.88c	0±0.00d	19±1.15b	8.33±0.67c	0±0.00d
% disease severity	82±4.73a	0±0.00c	0±0.00c	10.67±2.96b	3.33±0.88b	0±0.00c	12.33±2.40b	5.33±1.20bc	0±0.00c

Additionally, both of the curative doses, 1 and 5 µg/mL developed 19±1.15 and 8.33±0.67% plant infection as well as 12.33±2.40 and 5.33±1.20% leaf infection, respectively. However, 10 µg/mL preventive and curative doses of antimycin A didn't show any blast symptoms on wheat seedlings (Table 2). However, in case of both preventive and curative control measures, 100% suppression of wheat blast were achieved at 10 µg/mL concentration of antimycin A as well as commercial fungicide (Fig 6 (d & e) and Table 2).

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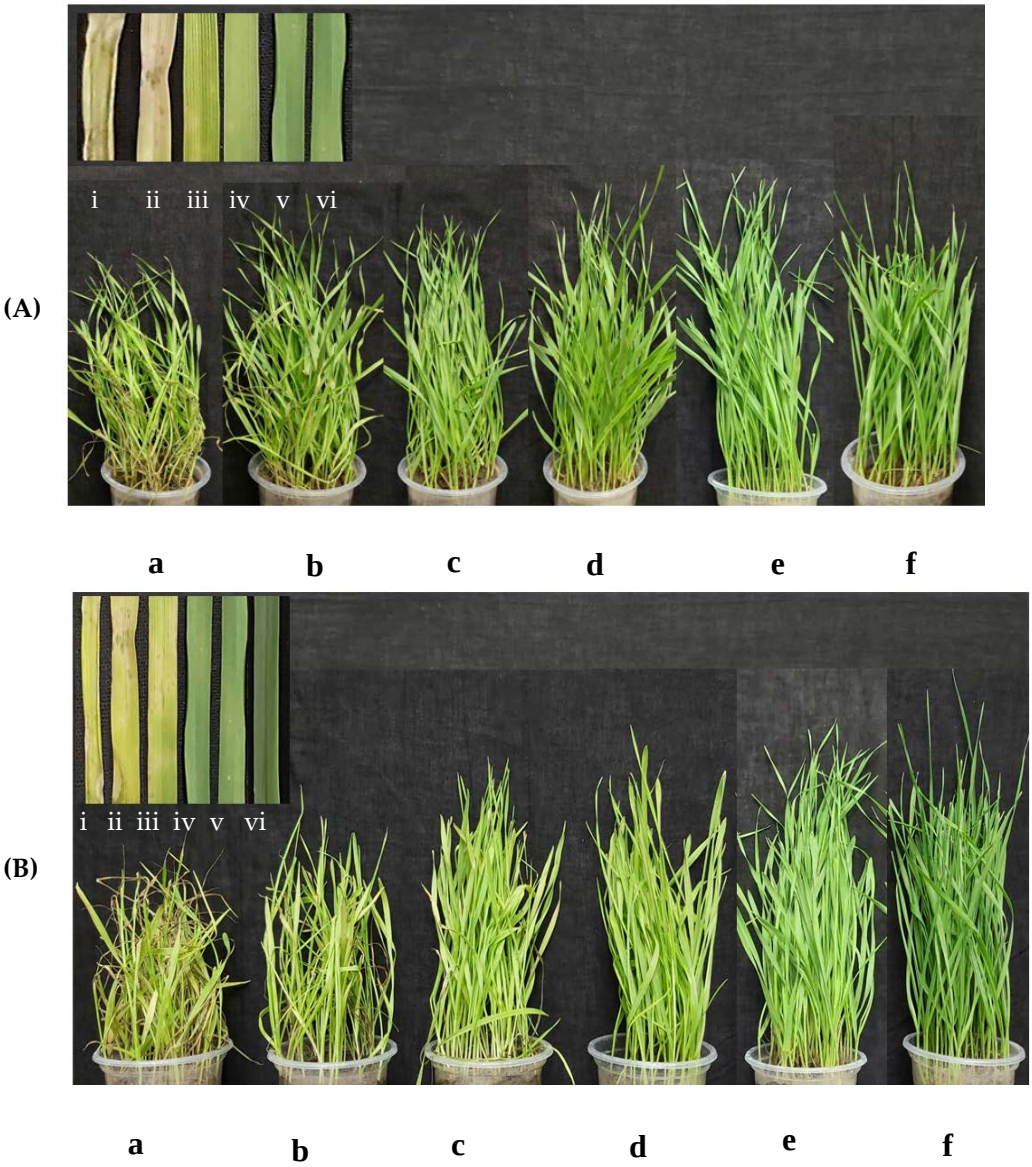

Figure 6. Suppression of wheat blast disease by antimycin A and Nativo® 75WG. Herein, blast lesions suppressed by different doses of antimycin A, commercial dose of Nativo® 75WG as well as untreated and healthy control of wheat seedlings were presented as (A) preventive, and (B) curative. In both cases of preventive and curative assay, (a) Water control+MoT, (b-d) Antimycin A+MoT inoculation, (e) Commercial dose of Nativo®75WG+MoT inoculation, and (f) Non-inoculated, non-treated seedlings. Whereas, 1 µg/mL, 5 µg/ml, and 10 µg/mL dose of antimycin A represented as (b), (c), and (d), respectively. In inset, representative leaf sample was presented as i) Water control+MoT, (ii-iv) Antimycin A at 1 µg/mL, 5 µg/mL, and 10 µg/mL, respectively +MoT inoculation, (v) Commercial dose of Nativo® 75WG+MoT inoculation, and (vi) Non-inoculated, non-treated seedlings as healthy control. Photos were taken 21 days after inoculation.

3.5 Suppression of wheat blast disease by antimycin A at heading stage of wheat under field conditions

Wheat blast is predominantly a head disease. To assess the efficacy whether the natural product antimycin A suppress wheat blast disease in artificially inoculated wheat spikes, we conducted an experiment in the field conditions together with a commercial fungicide, Nativo®75WG at 10 µg/mL. In field condition, application of antimycin A remarkably reduced the incidence of wheat blast (33%) (Fig. 7(c), Table 3), whereas the disease incidence in untreated control plot was 87% (Fig. 7(b), Table 3).

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(a) (b) (c) (d)

Figure 7. Suppression of wheat blast symptoms with Antimycin A and Nativo® 75WG. Herein, a) Non-inoculated, non-treated seedlings as healthy control, b) Water control+MoT, c) Antimycin A at 10 µg/mL+MoT inoculation and d) blast lesions suppressed by commercial dose of Nativo® 75WG. In inset, clear images of representative spike samples were presented.

Besides, antimycin A treated wheat plants had blast severity of 23.67% compared to 73.67% in the untreated control. Application of both antimycin A (1.95±0.06 gm) and Nativo® 75WG (2.05±0.13 gm) had statistically similar but significantly increased grain yield compared to the untreated control (0.86±0.04 gm). Grain yield in both antimycin A and Nativo treatments were comparable to the negative (non-inoculated, non-treated) check (2.05±0.05 gm) (no artificial inoculation) (Table 3).

Table 3 Effect of Antimycin A on yield or yield components of the wheat variety BARI Gom-26 under field condition after artificial inoculation with wheat blast fungus

Treatment	Grain yield per spike (gm) *	1000-grain weight (gm) *	Disease incidence (%)*	Disease severity (%)*
Healthy control	2.05±0.05a	53.57±1.37a	0.00±0.00c	0.00±0.00c
Untreated control	0.86±0.04b	34.43±0.27c	87.00±2.91a	73.67±2.65a
Antimycin A	1.95±0.06a	42.73±0.71b	33.00±1.45b	23.67±2.31b
Nativo® 75WG	2.05±0.13a	44.94±1.55b	31.71±2.96b	23.33±2.95b

*Any two means having a common letter are not significantly different at the 5% level of significance

We also recorded 1000-grain weight data for the treatments and found 44.94, 42.73 and 53.57 gm weight for Nativo® 75WG, antimycin A and negative control plot, respectively. These grain yields were significantly higher than those of untreated control plot (34.43 gm) (Table 3).

4. Discussion

 doi:10.20944/preprints202205.0156.v1

Microorganisms are a vital source of novel antimicrobials as they usually yield toxins to combat different microbes. Biological activity of microbe derived secondary metabolite can successfully prevent the growth and developmental morphological features of wheat blast spores. In the current study, a natural product, antimycin A, isolated from a marine *Stretomyces* sp. significantly inhibited mycelial growth and pre-infectional development of wheat blast fungus MoT *in vitro*. Interestingly, bioactivity of antimycin A was stronger to the widely used commercial fungicide Nativo®75WG. Furthermore, this natural compound also suppressed wheat blast disease in artificially inoculated wheat seedlings and spikes, which is comparable to the commercial fungicide Nativo®75WG. Bioassay revealed that inhibition of conidial germination, suppression of appressoria formation, and induction of abnormal mycelial growth by antimycin A are likely to be correlated with blast disease suppression in wheat. To the best of our knowledge, this is the first report of suppression of the devastating wheat blast fungus by antimycin A extracted from the *Streptomyces* sp., which has the potential to become a fungicidal product or used as a lead compound for controlling the MoT, a killer of wheat.

Biological activities of antimycins are well documented [53-59]. In an earlier study, a crystalline antibiotic isolated from *Streptomyces kitazawaensis* nov. sp. has significantly inhibited the growth of rice blast fungus, *Pyricularia oryzae Oryzae* pathotype [60]. The antibiotic was identified as antimycin A based on its physical, chemical and biological properties. Due to its high cost, antimycin A was first considered as a promising agent to control of blast of rice in the green house rather in the field [60]. Later, a series of experiments in mammalian cells revealed the mode of action of this bioactive secondary metabolite. Antimycin A inhibits mitochondrial electron transport, which breaks down membrane potentials of mitochondria through the proton gradient across the mitochondrial inner membrane [61,62]. Additionally, antimycin A significantly increases the production of reactive oxygen species (ROS) and ATP inhibition as well as glutathione depletion [53-59]. The reactive oxygen species (ROS) production and membrane depolarization by antimycin A lead to apoptosis through opening the mitochondrial permeability transition pore. Thus, it releases pro-apoptotic molecules like cytochrome C into the cytoplasm [62-64]. In some instances, antimycin A-induced cell death is associated with increased activity of caspase and damage of DNA [57, 64, 65]. Although several researchers reported the activity of antimycin A in mammalian cells, however, a very few reports describe the antifungal activity of antimycin A to control human and plant pathogenic fungi [40, 42]. Considering to the inhibition of the electron transport chain in the mitochondria by antimycin A, a fungicide can be designed against wheat blast using antimycin A as a lead compound.

In this study, we found that hyphal growth of MoT was inhibited at lower concentration of antimycin A compared to a commercial fungicide Nativo®75WG. The microscopic observation suggested that antimycin A leads to irregular hyphal growth with frequent branches per unit length of fungal hyphae (Figure 2bi). These observations are in consistent with a recent observation made by [14], where the authors demonstrated that two secondary metabolites of *Streptomuyces* sp. (oligomycin B and F) altered morphological features of MoT hyphae [14]. Some other *Streptomyces*-derived secondary metabolites also cause hyphal growth inhibition and deformed hyphal growth [42].

One of the notable findings of this study is the induction of swelling on the MoT hyphae by the antimycin A (Fig 2bi and 2ci), which is generally reflected as a mode of inhibitory action of a compound against the normal growth and development of pathogenic fungi [66, 67]. We

 doi:10.20944/preprints202205.0156.v1

examined a series of concentrations of antimycin A ranging from 0.005 to 2 µg/disk. With increase in concentrations of the antimycin A, swelling in hyphae also increased (data not shown). This type of swelling has also been reported earlier in various fungal hyphae by the treatment of polyoxin B [68], fengycin [69], tensin [70], linear lipopeptides, oligomycin B and F [14,22]. Fungal morphological changes such as profuse branching and swelling of hyphae of an oomycete pathogen *viz. Aphanomyces cochlioides* by phloroglucinols isolated from *Pseudomonas fluorescence* or xanthobaccin A extracted from *Lysobacter* sp. SB-K88 has already been documented [71-74]. Another earlier study showed that antimycin A exhibits remarkable inhibitory effect against various fungi including *Pyricularia oryzae, Alternaria kikuchiana, Gloeosporium laeticolor, Torula utilis, Candida alicans, C. krusei, C. parakrusei* and *Penicillium chrysogenum* [60]. However, this is the first report of the development of swelling-like structures in the hyphae of MoT by antimycin A.

Most of the pathogenic fungi enter into the host plants via infecting propagule-like spores or conidia and the process by which conidia are produced is known as conidiogenesis [75,76]. Suppression of conidiogenesis and germination of conidia reduces the chance of infection by the pathogen. Antimycin A inhibited both conidiogenesis and conidial germination of MoT in dose-dependent manners in this study. In addition, other unique behaviors discovered in this study include conidia lysis and abnormally extended hypha-like germ tubes (Fig 5b and 5c). Chakraborty et al. [14] reported that secondary metabolites from *Streptomyces* sp. suppressed conidiogenesis and germination of conidia of MoT. In other example, reveromycins A and B from *Streptomyces* sp. inhibited spore germination of *B. cinerea* and *R. stolonifer* [77]. It has also been reported that during the conidiogenesis and conidial germination process, fungal cells need high energy consumption in the form of ATP [78]. Antimycin A have been reported to interfere with mitochondrial electron transport by targeting ubiquinol-cytochrome C oxidoreductase, which breaks down membrane potentials and inhibit ATP synthesis. Inhibition of conidiogenesis and conidial germination by antimycin A is likely to be linked with the inhibition of ATP synthesis in MoT cells. Recent experiment with *Fusarium oxysporum* cell provide evidence that an elevated level of ATP content is positively associated with conidial germination. Higher ATP production supports breaking of the dormancy and formation of a germ tube [78]. Therefore, a reasonable justification for the inhibition of spore germination and hyphal growth of MoT described in this study might be associated with inhibition of ATP synthesis in mitochondria by antimycin A. Another explanation of the present results could be that, apart from ubiquinol-cytochrome C oxidoreductase, antimycin A targets an additional protein Bcl-2 [79]. The Bcl-2 protein is found in the mitochondria of *Colletotricum gloeosporioides*, as well as other cellular compartments including the ER, and is involved in many stages of the fungal life cycle, including growth, morphogenesis, morpho-pathogenesis, and reproduction. In comparison to wild-type, Bcl-2 isolates produced 8–10 times more conidia. Furthermore, Bcl-2 isolates are more virulent than wild type and cause infection faster [80]. It has been reported that Bcl-2 family regulate apoptosis in mammals by controlling mitochondria efflux of cytochrome C and other apoptosis-related proteins [80]. Whether antimycin A inhibit conidiogenesis in MoT fungus by interfering with the function of Bcl-2 is needed to be confirmed by a further investigation.

The hallmark of the findings of this study is that antimycin A remarkably suppressed wheat blast disease in both green house and in the field conditions, which is comparable to the commercial fungicide Nativo® 75WG (Fig 6). Herein, wheat seedlings treated with antimycin A had a lower infection rate than the untreated control (Fig 6). In contrast, water-treated healthy control seedlings had a higher infection percentage indicating that conducive environment for higher infection and disease was present during the experiment. Products or active compounds

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that are capable of reducing plant disease under high disease pressure in an experiment should be considered viable options for the future commercial use. However, blast lesions didn't form on the seedlings treated with the antimycin A compound and Nativo® 75WG at the highest concentration (Fig 6). At the heading stage of wheat, we found similar results. Antimycin A significantly inhibited blast disease development on the artificially inoculated wheat spikes (Fig 7). Excitingly, the antifungal effect of antimycin A on the inhibition of wheat blast fungus was found equivalent or stronger than that of the commercial fungicide *in vitro*. Herein, the commercial fungicide, Nativo® 75WG has two main active ingredients *viz.,* tebuconazole and trifloxystrobin. Tebuconazole acts as a demethylase inhibitor (DMI), which is known as a systemic triazole fungicide. Demethylase inhibitors suppress the biosynthesis of ergosterol, which is a key component of the plasma membrane of certain fungi crucial for fungal growth and development [81]. On the other hand, trifloxystrobin is a strobilurin fungicide that interferes with the respiration of plant pathogenic fungi by inhibiting the production of energy in mitochondria, thereby halting the germination of fungal conidia [82]. The adverse effects on conidial germination caused by antimycin A (µg/mL), reduced the infection of wheat seedlings by MoT conidia, suggesting a potential function of antimycin A in blast disease suppression. Taken together, both *in vitro* and *in vivo* field tests revealed that antimycin A inhibited the growth of mycelia, conidiogenesis and conidial germination thus reduced the disease incidence in wheat plants. Our results suggest that antimycin A has a high potential for formulating an effective biopesticide against wheat blast either using it directly or as lead compound. Further study is required to elucidate the underlying mechanism of wheat blast disease control by the marine natural product, antimycin A.

5. Conclusion

Our experimental findings revealed that a marine natural product, antimycin A significantly inhibited mycelial growth, asexual sporulation and the developmental transitions of the conidia of wheat blast fungus *MoT in vitro*. *In vivo* seedling assay and field evaluation confirmed that antimycin A effectively suppressed wheat blast disease in artificially inoculated wheat at both seedling and heading stage. These assessments proved that this natural compound could be considered as biofungicide or a lead compound to design a new fungicide to control the destructive wheat blast disease. Further extensive research is also needed to understand the precise mode of action of antimycin A against the devastating fungus, *M. oryzae Triticum*.

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and will hold themselves jointly and individually responsible for its content. All co-authors agreed to this submission.

Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. You might choose to exclude this statement if the study did not report any data.

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