An active role of systemic fungicides to curb wheat powdery mildew caused by *Blumeria graminis* f. sp. *tritici*

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Abstract: Efficacy of five systemic fungicides (flusilazole, propiconazole, diniconazole, cyproconazole and tetraconazol) in curbing powdery mildew disease of wheat (Sakha93) and its impact on yield components were evaluated under field condition during two successive season. Both fungicides, propiconazole and diniconazole exhibited the highest value of efficacy (88.80% each) recording the lowest rate of disease infection (1 each). The contrast was noticed with tetraconazol (55.50% efficacy, four disease infection) with significant differences to untreated control during both seasons. The results of fungicide application reflected on yield components where 1000-kernel and 1000-mL weights recorded the highest values with propiconazole (49.03 g and 742.65 g) followed by diniconazole (47.69 g and 737.62 g), respectively. Tetraconazol come in the last order in this respect (44.39 g and 672.07 g). An active role of fungicide (propiconazole) on *Blumeria graminis* f. sp. *tritici* was examined on infected wheat leaves (eight-day old seedlings) by scanning electron microscopy (SEM). To investigate the effect on early develop of conidia, fungicide was applied at 24 h post inoculation (hpi), and collapsed conidia along with blocked development beyond the primary appressoria were observed. Where conidia germinated, appressoria formed two or three lobes. To investigate the effect on fungal morphogenesis, applying of fungicide at 2 day post inoculation (dpi) greatly reduced the mycelium formation associated with a rapid collapse of hyphae compared with untreated control. Fungicide treatment at 10 dpi inhibited and delayed sporulation alongside formation of aberrant and collapsed conidiophores. The conidiophores formed elongated tubes with no regular or sepatated chain of conidiospores. Results concluded that fungicide is an efficient curative fungicide at very low concentrations if it is applied early enough after infection. Also, fungicide has a significant impact on fungal survival.

Keywords: wheat, powdery mildew, fungicide activity, *Blumeria graminis* f. sp. *tritici*

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1 Introduction

The ascomycete powdery mildew fungi, *Blumeria graminis* (DC) E.O. Speer f. sp. *tritici* Marchal (*Erysiphe graminis* f. sp. *tritici*) continue to rank among the most harmful pathogens on wheat (*Triticum aestivum* L.) in the world (Opalski et al., 2006). This is reflected by the size of the cereal powdery mildew fungicide market, which is estimated to exceed $300 million per year (Hewitt, 1999). In temperate regions, powdery mildew causes significant economically decreases on wheat yield approximately 10% losses and occasionally as much as 40% when chemical control is neglected (Jørgensen, 1988). In Egypt, yield losses caused by powdery mildew on wheat ranging up to 22.52% have been reported on susceptible cultivars on high severity levels of the disease (Ashmawy et al., 2014). Along with cultural measures, the main measures of powdery mildew control in integrated crop protection systems are the use of less susceptible cultivars and the use of effective fungicides.

Applying chemical fungicides are commonly used to control powdery mildew. Systemic fungicides play an important role in controlling wheat powdery mildew in the recent years. However, the development of pathogen strains resistant to the mostly used fungicide has been reported and the resistant strain is now widespread (Opalski et al., 2006). A great challenge is the remarkable

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capacity of mildew populations to evolve virulent and fungicide-resistant genotypes that overcome control measures (Limpert et al., 1990; De Waard et al., 1993). Indeed, powdery mildew has a number of characteristics, which support rapid adaptation, such as its relatively short generation time, with potential sexual recombination throughout the year, and the nature of its airborne spread. Consequently, finding novel and efficient fungicides against powdery mildew fungi represents an important challenge to crop protection research. Novel systemic fungicides represent an active ingredient that had commonly been used in chemical plant protection. They were developed after earlier work had shown that exhibited promising effects. Systemic fungicide differs from other commercial fungicides in that its action differs from currently known mechanisms (Opalski et al., 2006).

Following contact the ectoparasitic obligate biotrophic fungus *Bgt* with the host surfaces, the conidia form a primary germ tube and an appressorial germ tube approximately 0.5-2 and 4-8 h after inoculation respectively. The appressorial germ tube begins to elongate and after 9-12 h differentiates a lobed appressorium. A peg forms under this appressorium, which penetrates the host cell wall and establishes a digitate haustorium within an epidermal cell. Papilla formation occurs in the leaf epidermal cell subjacent to the germ tubes. This local response in the outer epidermal cell wall excludes or delays a significant proportion of the attempted penetrations by the fungus (Carver, 1986). Successful establishment of a haustorium, the only fungal organ that invades the host, is followed by the formation of secondary hyphae from the appressorium. An elongating secondary hypha is the starting point for the development of a fungal colony. From the epicuticular hyphae, secondary appressoria are formed and, from these, secondary haustoria are established in epidermal cells. About 3-4 days after the primary infection, conidiophores are formed on the hyphae, sporulation starts and spores can be wind spread to initiate new infection cycles (Thordal-Christensen et al., 1999).

This work mainly aimed to estimate the efficacy of systemic fungicides in controlling the powdery mildew of wheat and to describe the potential active role of the best systemic fungicide on *B. graminis* f. sp. *tritici* observed by using scanning electronic microscope.

2 Materials and methods

2.1 Applying of systemic fungicides

The present investigations were carried out at the Experimental Farm of Sakha Agriculture Research Station, Agricultural Research Center of Egypt. The efficacy of five fungicides against powdery mildew disease of wheat at adult plant stage under field condition was studied. The main objective of this experiment is to determine the best fungicide assigned for controlling powdery mildew and its reflection on yield components.

A completely randomized design with three replicates was proposed for this experiment. Five treatments represented by the tested systemic fungicides used in controlling wheat powdery mildew and their specifications are listed in Table 1. The experimental unit was represented by a plot consisted of five rows with 2.5 m long, 25 cm apart and 5 g seed rate. The experiment was surrounded by 1 m ally and 1.5 m belt of spreader. The tested fungicides were applied twice (14 days interval) onto plants in the proper concentration until run-off using a portable sprayer at the flag leaf stage. However, the control treatment was left to natural infection.

Efficacy of fungicide was determined according to the method adopted by Rewal and Jhooty (1985). On the other hand, yield components were estimated as 1000-kernel weight and 1000-mL weight to the methods of Griffey et al. (1993).

$$\text{Efficacy of fungicide (\%)} = (C - T) / C \times 100$$

where, C = Infection in the control; T = Infection in the treatment

Data were calculated from the repeated tests during two successive seasons before subjection to analysis of variance (ANOVA) using International Rice Research Institute (IRRI) stat computer program. Means were compared using least significant difference (LSD) method (Steel and Torrie, 1980) and multiple range test (Duncan, 1954).

Table 1 Systemic fungicides used in controlling wheat powdery mildew and their specifications

Fungicide		Chemical composition	Rate of use, $cm^3\ L^{-1}$	Registrant
Active ingredient	Concentrate & Formula			
Flusilazole	75 EC	1- [bis (fluorophenyl) methysily methyl] -1 H -1, 2, 4 triazol	0.06	Syngenta
Diniconazole	5 EC	(E) – (R) - 1(2,4-dichlorophenyl) -4, 4- dimothey -2 – (1, 2, 4-triazol – yl)- pent -1-en-3-ol (IUPAC)	0.35	Sumitomo
Propiconazole	25 EC	(±) -1- [2- (2, 4- dichlorophenyl) – 4 -propyl -1, 3- dioxolan - 2 -ylmethyl] – H - 1, 2, 4 –triazole	0.25	Syngenta
Cyproconazole	50 SL	(2 RS, 3 RS; 2 RS, 3RS) -2- (4- chorophenyl) – 3cyclopropyl 1- (1 H- 1, 2, 4 – triazol -1-YL) butan -2 – 01	0.40	Syngenta
Tetraconazol	12.5 EW	(±) -2- ((2, 4- dichlorophenyl)-3- (1 H – 1, 2, 4- triazol -1 - yl) pro-pyl 1. 1, 2, 2- tetra & luoro – ethylether (IUPAC)	1.00	Syngenta

Note: EC = Emulsifiable Concentrate, SE = Suspension Emulsion, SL = Soluble Concentrate, EW = Emulsion

2.2 Examination *Bgt* by scanning electron microscope (SEM)

To investigate an active role of systemic fungicide propiconazole for curbing wheat powdery mildew, fungicide was directly applied to wheat leaves inoculated with *Blumeria graminis* f. sp. *tritici*.

2.2.1 Inoculation process and treatments

The inoculation protocol as described by El-Salamony (2002) and Opalski et al. (2006) was used in this investigation**.** Eight-day old seedlings of wheat (Sakha-93) were inoculated with pure isolates of *Blumeria graminis* f. sp. *tritici* pathogen. The inoculated seedlings were incubated in a moist chamber for 24 hrs at 20˚C±2˚C, 70%-90% relative humidity under 16 hrs photoperiod with light intensity of approximately 14000 luxmeter. Ten-day old cultures of the pure isolates were used for re-inoculation the tested seedling leaves concerned for SEM examination. Three leaves bearing raised powdery mildew colonies were gently removed from the target culture and then shaken over the tested seedlings leaves.

Samples of infected leaves were treated with fungicide propiconazole 24 h post-inoculation (hpi). Untreated samples of infected leaves sprayed with sterilized-distilled water 24 hpi were used for comparison. At 24 hpi, samples were prepared and examined to investigate the effect of fungicide on early develop of fungal conidia. To investigate the effect of fungicide on the development of fungal morphogenesis, samples were treated with fungicide 2 and 10 days post-inoculation (dpi), then were prepared and examined by SEM.

2.2.2 Sample preparation for SEM examination

Cuts (4 mm^2) from infected leaf of wheat were immediately fixed in glutraldhyde (2.5%) for 24 hrs at 4˚C, then post-fixed in osmium tetraoxide (1% OsO_4) for one hour at room temperature (Harley and Fergusen, 1990). Sample were dehydrated with passing through ascending concentration of acetone (30%-100%). Sample were dried till the critical point finally, leaf was sputter coated with gold. The examination and photographing were done through a Jeol Scanning Electron Microscopy (T.330 A) in the Central Laboratory of agriculture faculty, Ain shams university.

3 Results

3.1 Efficacy of systemic fungicides in controlling wheat powdery mildew

Efficacy of five systemic fungicides in controlling wheat powdery mildew was estimated during two successive seasons. Data presented in Table 2 indicated that the efficacy percentage of the tested fungicides on disease reaction ranged from 55.5% (tetraconazol) and 88.8% (propiconazole and diniconazole). Both of fungicides, propiconazole and diniconazole exhibited the lowest value of disease reaction (1 each) consequently they have the highest value of efficacy during both seasons (88.80% each). The contrast was noticed with tetraconazol that exhibited the rate of 4 of disease reaction and the least value of efficacy (55.50%).

As regard to the reflection of fungicide application on yield components, the obtained data about 1000-kernel weight (Table 2) indicated that a significance between propiconazole and each of flusilazole, cyproconazole and tetraconazol. No significance was observed between propiconazole and diniconazole. It was also observed that propiconazole exhibited the highest values in 1000-kernel weight during both seasons (49.03 g) followed by

diniconazole (47.69 g), however these fungicides were distinctive in this regard. Concerning the effect of treatments on 1000-mL weight, the similar trend was detected with the fungicides during both seasons, since propiconazole (742.65 g) and diniconazole (737.62 g) showed significance and distinction in their performance as compared with the rest of fungicides.

Table 2 Efficacy of systemic fungicides in controlling powdery mildew disease of wheat (Sakha-93) naturally infected under field condition

Fungicide	Disease reaction	Efficacy, %	1000-kernel weight, g	1000-mL weight, g
Flusilazole	2	77.70 b	46.75 b	704.96 b
Diniconazole	1	88.80 a	47.69 ab	737.62 a
Propiconazole	1	88.80 a	49.03 a	742.65 a
Cyproconazole	3	66.60 c	45.87 bc	672.56 c
Tetraconazol	4	55.50 d	44.39 c	672.07 c
Control	9	0.00 e	40.64 d	641.17 d
LSD		3.71	1.99	6.09

Note: Figures with the same letter are not significantly ($P \leq 0.05$) different.

3.2 An active role of fungicide observed by SEM examination

Fungicide (propiconazole) was directly applied to wheat leaves inoculated with *Blumeria graminis* f. sp. *tritici* at 24 hpi to investigate the early development of conidia and at 2 and 10 dpi to investigate fungal morphogenesis development.

3.2.1 Effect on germling morphogenesis

To examine whether fungicide affected the early development of conidia of Bgt, fungal germlings were observed by SEM. On untreated leaves, typical germinated conidia and primary germ tube was observed and the appressoria formed a typical apical hook-shaped lobe (Figure 1). On the fungicide treated leaves, collapsed conidia, and, where conidia germinated, subsequent appressoria frequently presented two or three lobes, and mostly lobes were malformed (Figure 2). The appressoria were functional in the sense that they could form infection pegs, but penetration was mostly blocked in host cell wall papillae. Hence the fungus rarely developed a haustorium and elongated secondary hyphae. If the germling established a haustorium, it was frequently malformed and encapsulated by host cell wall-like material, and consequently the pathogen stopped growing.

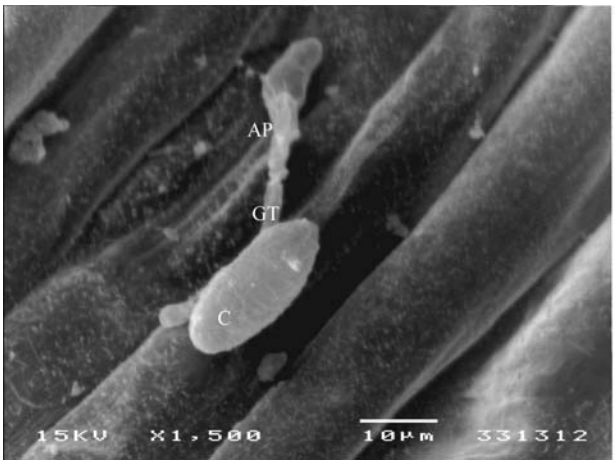

Figure 1 Scanning electronic microscope examination of *Blumeria graminis* f sp. *tritici* on untreated wheat leaves. Typical germinated conidia (C), germ tube (GT) and appressorium (AP) were observed 24 h post- inoculation

Figure 2 Scanning electronic microscope examination of *Blumeria graminis* f sp. *tritici* on wheat leaves treated with fungicide (propiconazole). Collapsed Conidia (C) and numerous primary germ tubes were observed 24 h post-inoculation

3.2.2 Effect on fungal morphogenesis

Fungal morphogenesis development was observed by SEM. In untreated controls, at 2 dpi, the colonies consisted of a conidium with the primary and the secondary germ tube, primary appressorium and numerous hyphae. The tip-growing cells comprised a cylindrical shank of constant diameter and an apical dome. From the superficial hyphae, secondary appressoria were formed with small globular structures disposed alone or in pairs on opposite sides of the hyphae. At 10 dpi, leaves showed spreading colonies with a dense surface of mycelium and numerous conidiophores consisting of a mother cell producing chains of conidia separated by regularly spaced septa (Figure 3).

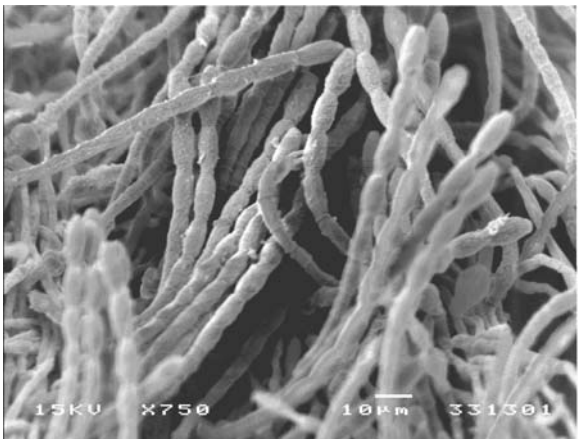

Figure 3 Scanning electron microscopy examination of wheat leaves infected with *Blumeria graminis* f sp. *tritici* observed 10 days post-inoculation

In treated leaves, fungicide caused a fungal growth delay, a number of morphological anomalies and a rapid collapse of mycelium. The diameter of treated hyphae was larger than that of untreated hyphae. The form of the apices changed from cylindrical to spherical and the tip of the apical cells became swollen, often followed by a burst and release of globular structures. Hyphae with ruptured hyphal tips were often collapsed. Swelling and bursting were occasionally seen in subapical regions or near secondary appressoria. Furthermore, several hyphal tips were bifurcated. Secondary appressoria were more abundant than normal and grouped closely together. They were also more round or bifurcated. Additionally, sporulation was delayed or inhibited by fungicide. Concurrently, fungicide caused formation of aberrant and collapsed conidiophores. The conidiophores formed elongated tubes with no regular or sepatated chain of conidiospores. Irregular sepatation was also observed in surface hyphae (Figure 4).

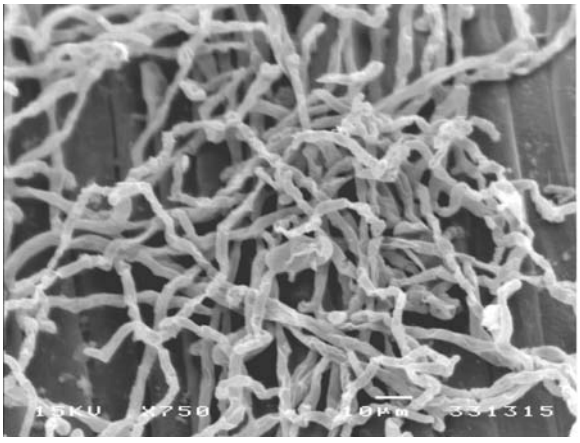

Figure 4 Scanning electron microscopy examination of wheat leaves infected with *Blumeria graminis* f sp. *tritici* treated with fungicide (propiconazole) observed 10 days post-inoculation

4 Discussion

The efficacy of systemic fungicides in controlling wheat powdery mildew indicated that fungicide application resulted in highest efficacy for disease reduction which recorded with propiconazole and diniconazole exhibiting the lowest value of disease reaction. The application of fungicides reflected on yield components where the highest 1000-kernel and 1000-mL weights were recorded with propiconazole and diniconazole. The contrast was noticed with rest fungicides, tetraconazol, cyproconazole and flusilazole. Such results are in agreement with Kingsland (1982), Leath and Bowen (1989), Meeus et al. (1989), Peltonen and Karjalainen (1992), Iliev (1994), Lebedev et al. (2000), Singh and Ramesh (2000), Oroian et al. (2004), Bensoltane et al. (2006), Opalski et al. (2006), Felsenstein et al. (2010), Reis, Moreira, et al. (2008), Reis, Basso, et al. (2013) who indicated the efficacy of the same fungicides or other groups as: cyproconazole, flusilazole, triadimenol, triadimefon, metrafenone, tobaconazole, propiconazole, bayfidan, and rubegan, fluriafol, fempropimorf. Most of these groups were tested for their efficacy against powdery mildew and its reflex on the yield components.

An active role of systemic fungicide propiconazole on *Blumeria graminis* f. sp. *tritici* was investigated by SEM examination. Fungicide was directly applied to wheat leaves inoculated with *Bgt* at 24 hpi to investigate the early development of conidia and at 2 and 10 dpi to investigate fungal morphogenesis development. Treatment of powdery mildew-infected wheat with fungicide resulted in effective curbing against the fungus. When fungicide was applied to wheat leaves (eight-day old seeding) at 24 hpi, collapsed conidia, and blocked development beyond the primary appressoria were observed. Where conidia germinated, appressoria typically formed two or three lobes. Similar multilobed appressoria appear on resistant host cultivars in the case of unsuccessful penetration by the apical lobe (Thordal-Christensen et al., 1999; Opalski et al., 2006). Fungicide enhanced the number of appressoria giving rise to papillae whilst reducing the proportion that succeeded

in forming a haustorium. Gene expression and mutant analysis revealed that a Ras-type guanosine triphosphatase (GTPase) activating protein is most likely involved in quinoxyfen resistance of *B. graminis* f. sp. *hordei* Marchal, and hence this fungicide might disrupt Ras signalling in appressorium formation (Wheeler et al., 2003). Since, treatment increased multilobed appressoria and reduced germination, we conclude that propiconazole exerts a direct effect on the fungus and likely does not induce host resistance. The fungicide might rather delay fungal development, giving the host more time to prevent penetration.

As regard applying of fungicide at 2 dpi, the mycelium proportion was greatly reduced compared with control in the treatment. Fungicide is an efficient curative fungicide at very low concentrations if it is applied early enough after infection. Also, fungicide has a significant impact on fungal survival. The reduction in mycelium formation was associated with a rapid collapse of hyphae. Hyphal collapse was associated with swelling and bursting of hyphal tips. Similar observations were detected by Opalski et al. (2006). Cytological analysis by Bachewich and Heath (1998), Torralba et al. (1998) and Heath et al. (2003) indicated that swelling of hyphal tips might be caused by weakening of the cell wall at the apex, disturbance of apical vesicle delivery and disruption of the F-actin cap at the apex. It has been reported that cytochalasin A or latrunculin B treatment results in swelling of hyphal tips, with disruption of actin, disturbed vesicle delivery and irregular wall deposition at the hyphal tips. However, although the effect of cytochalasin D on *B. graminis* was very similar to that of fungicide (data not shown), an *in vitro* actin polymerisation assay by Tanaka and Takai (1998), Momany (2002) and Harris and Momany (2004) indicated that fungicide did not directly inhibit actin polymerisation, suggesting that the chemical rather induced changes in the actin pattern.

Proteins that play an important role in actin organization include the Ras and Rho GTPases Rho, Cdc42 and Rac. In *Ashbya gossypii* Guill the Agrho3 mutant showed swelling with delocalised actin at the hyphal tips Wendland and Philippsen (2001). In *Colletotricum trifolii,* Bain & Ess the mutational activation of Ras gene exhibited hyphal tips tending to burst (Truesdell et al., 1999). Moreover, fungicide caused bifurcation of hyphal tips and secondary appressoria, likely indicating the loss of cell polarity. A mutation in the *Neurospora crassa* Shear & Dodge actin gene results in branching of hyphal tips and alteration of actin at the tip (Virad and Griffiths, 2004). In a temperature-sensitive mutant of *Aspergillus niger* van Teighem, apical branching involves dislocation and disappearance of the Spitzenk orper (Reynaga-Peňa and Bartnicki-Garcia, 1997). The process of apical branching was suggested to be caused by a shift of vesicle deposition from the tip to the side, consequently initialising the formation of new hyphal outgrowths (Raudaskoski et al., 1994). Similarly, fungicide might cause bifurcation by delocalisation of actin, vesicles and the Spitzenk orper at hyphal tips. Thus in fungicide treated hyphae, the defects in cell polarity could be a consequence of the failure in maintaining polarity of the actin cytoskeleton. Additionally, fungicide treatment resulted in abundant secondary appressoria grouped together at abnormally close distance. A high number of lateral branches were also observed in hyphae of *Saprolegnia ferax* (Gruithuisen) Thuret after application of latrunculin B. The phenomenon was preceded by formation of radial arrays of actin in regions without detectable surface protrusion. These sites were consistent with future branches (Bachewich and Heath, 1998). Accordingly, in the absence of focused actin at the apex, hyphal growth might be not directed and thus cells grew in multiple directions. Hyper branching possibly revealed multiple randomly distributed initiation sites of polar growth after fungicide treatment.

Fungicide treatment at 10 dpi inhibited and delayed sporulation. Hence fungicide has curative activity. The inhibition of sporulation was associated with the malformation of conidiophores that found to be collapsed and showed multinucleate cells and irregular sepatation. These observations were in agreement with that of Opalski et al. (2006). This suggests that mitosis proceeded without septum formation, indicating that cytokinesis was impaired. Actin is known to play a central role in septum formation and to be involved in cytokinesis (Harris and Hamer, 1995). In turn, actin was

delocalised in the tip of the young conidiophores, and actin associated with septa was hardly detectable after fungicide treatment. The effect of fungicide on *B. graminis* f. sp. *tritici* shows interesting similarities to the deletion of cflB, the *Penicillium marneffei* Rac homologue. ΔcflB results in cell division and growth defects in conidiophores so that cells become multinucleate and exhibit inappropriate septation (Boyce et al., 2003).

These results suggest that the potential active role of systemic fungicide propiconazole is involved in the effect on early develop of conidia, appressorium and hyphal morphogenesis development, rather polarised hyphal growth and the establishment and maintenance of cell polarity in *B. graminis* f. sp. *tritici*. Fungicide might interfere with processes that are essential to establish and maintain polar actin organization. Our findings, combined together, concluded that the finding of novel and efficient systemic fungicides against wheat powdery mildew in the conjugation with the determination of the potential active role on the causal fungus *Bgt* represents an important challenge to crop protection research that have been successfully achieved in the presented study.

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