

Effect of Septoria leaf blotch and its control with commercial fungicides, on arbuscular-mycorrhizal-fungal colonization, spore numbers, and morphotype diversity

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Abstract: Arbuscular-mycorrhizal internal structures (*i.e.* total root colonization, arbuscules, vesicles) and external structures (*i.e.* spore density), and Glomeromycota spore morphotypes, were evaluated in wheat severely infected with *Mycosphaerella graminicola* – the causal agent of Septoria leaf blotch. Plots in which the infection was controlled with a commercial fungicide at recommended field doses, were also examined. The commercial fungicide used was an admixture of trifloxistrobin and tebuconazole. No negative effects of the fungicide application on arbuscular-mycorrhizal fungi (AMF) were found. The *M. graminicola* fungicidal treatment actually favoured the formation of arbuscules and AMF spores, as there was a selective increase in the density of spores belonging to the glomoid morphotype. Arbuscular-mycorrhizal fungi have an absolute dependence on the carbon provided by the plant. A severe foliar disease leading to a diminished carbon supply to the roots would generate decreases in carbon availability. Such decreases would strongly affect mycorrhizal associations and development. Furthermore, the change in the green-leaf area produced by a severe foliar disease and/or a reversal of that condition through fungicide treatment could result in shifts in the composition of the AMF community so as to favour glomoid morphotypes. Glomoid species have been previously considered as *r*-strategists.

Key words: Glomeromycota, *Mycosphaerella graminicola*, strobilurin, triazole, wheat

Introduction

Arbuscular-mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota (Schüßler *et al.* 2001), are biotrophic fungi that form associations with most cultivated plant species. These fungi are, therefore, significant components of agroecosystems (Smith and Read 2008). In this symbiotic relationship, the fungi acquire their entire carbon supply from the plant. The colonization of roots by the AMF confers a wide range of benefits to the plant. Such benefits are an increase in nutrient uptake from the soil (Hodge *et al.* 2010) and an improvement in the plant's resistance to and/or tolerance to biotic stresses, particularly with respect to soil-borne diseases (Whipps 2004; Pozo *et al.* 2009). Arbuscular-mycorrhizal fungi can also provide long-term benefits to agroecosystems by increasing the degree of soil-carbon sequestration and the amount of organic matter (Verbruggen *et al.* 2013).

Foliar diseases produced by fungi cause major biotic limitations in the yield and quality of wheat. Leaf blotch, caused by *Septoria tritici* Rob. ex Desm. (teleomorph *Mycosphaerella graminicola* (Fuckel) Schroeter, in Cohn) is a serious disease in many wheat-producing areas of the

world causing major decreases in crop yields (Eyal *et al.* 1985, 1987; Simón *et al.* 2002; Mojerlou *et al.* 2009).

The control of fungal diseases in modern agriculture usually involves the use of fungicides. The effects of fungicides on non-target organisms, such as AMF, are of principal interest to agriculture. An inhibition of the effects of beneficial organisms would counteract the advantages derived from the disease control. The impact of fungicides on AMF associations depends on multiple conditions: the chemical's formulation, mode of action, and spectrum of activity along with the application methods used (Diedhiou *et al.* 2004; Zocco *et al.* 2008; Calonne *et al.* 2012). Consequently, no generalization can as yet be made as to the effects of fungicides on the status of the AMF.

The effects of fungicides on AMF have been evaluated mostly in the laboratory in the absence of pathogens. In the laboratory, *in vitro* studies were done where AMF structures were in direct contact with the compounds *e.g.* monitoring spore or sporocarp germination and mycelial growth in Petri dishes or in monoxenic cultivation with transformed roots (Giovannetti *et al.* 2006; Campagnac *et al.* 2008, 2009; Zocco *et al.* 2008; Calonne *et al.* 2012). The

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effects of fungicides on AMF were also studied in greenhouse experiments in pots by applying different fungicides to plants inoculated with AMF but these were plants not affected by fungal diseases (Kjoller and Rosendahl 2000; Diedhiou *et al.* 2004). Few studies have been carried out in field situations to examine the effect on AMF of foliar disease control by fungicides (Land *et al.* 1993).

Since the supply of carbon regulates arbuscular-mycorrhizal development, we speculated that maintenance of the green-leaf area through the application of a fungicide would promote AMF symbiosis, provided that the fungicide used did not affect the AMF *per se*. Conversely, an untreated severe foliar disease would inhibit that process.

A knowledge of AMF functional diversity is essential for the management of these fungi in agricultural systems (Verbruggen and Kiers 2010). A number of studies have shown that Glomeromycota taxa may have different life-history strategies (Sieverding 1991; Morton *et al.* 1993; Bever *et al.* 2001; Franke-Snyder *et al.* 2001; Lovelock *et al.* 2003; De Souza *et al.* 2005; Chagnon *et al.* 2013). Thus, fluctuations in carbon availability, caused by reductions in the green-leaf area through a severe foliar disease and/or the reversal of that pattern as a result of disease control, could affect the various Glomeromycota taxa in a differential fashion so as to lead to shifts in the AMF community.

In this study, our first aim was to compare the AMF internal structures (*i.e.* root colonization, entry points, internal mycelium, arbuscules, vesicles) and external structures (*i.e.* spore density) along with the Glomeromycota-spore morphotypes formed in wheat that had been severely affected by *M. graminicola*. Our second aim was to examine for comparison purposes, those plots where the disease had been controlled with a commercial fungicide at the recommended field doses.

Materials and Methods

A field trial conducted at the Experimental Station J. Hirschhorn, School of Agricultural and Forestry Sciences, National University of La Plata was planted on 11 June 2011. The experiment had a completely randomized factorial design with three replications. The wheat cultivar used was ACA 801. The factors investigated were the fungicide/inoculation treatments and the three growth stages sampled. The plots cultivated (1.40 m wide × 5.50 m long) were sown with a conventional experimental drill at a row spacing of 20 cm and a seeding rate of 300 seeds/m². The protocols of the application with the fungicide-inoculation were: (1) inoculation with an admixture of three virulent isolates of *M. graminicola*, and (2) no inoculation but a treatment with the fungicide Nativo® (trifloxistrobin 50 g and tebuconazole 25 g) at a rate of 600 cm³ of the product in 150 l water/ha applied from growth stage (GS) 22 every 15–20 days until physiologic maturity. The inoculated *M. graminicola* isolates were from the collection of the School of Agricultural and Forestry Sciences. The pathogens had been previously grown on malt extract agar at 20–22°C with 12-hour alternating light and dark cycles. The inoculum was prepared by scraping sporulating colonies aseptically with a scalpel

into deionized water and adjusting the conidial suspension to a concentration of 8.5×10^6 spores/ml. The plants were sprayed with the fungal suspension until runoff of the fluid. After inoculation, the plants were kept moist by sprinkling them with water several times a day for a period of 3 days.

The plants were fertilized with 50 kg of N as urea plus 50 kg of P₂O₅ as calcium triple superphosphate/ha at sowing, followed by 50 kg of N/ha as urea at tillering (GS 23, Zadoks *et al.* 1974). The herbicide Misil® (methylmetsulfuron: dry, flowable, 60% + Dicamba soluble liquid, 57.1% Dupont, Rosario, Argentina) at 100 cm³ in 120 l water/ha was applied at the three-leaf stage.

Samples of roots and rhizospheric soil were collected when the wheat plants reached GS 39, GS 60, and GS 82. Septoria leaf blotch severity was visually estimated as a necrotic lesion percentage (Simón *et al.* 2003) for the same GSs with all the green leaves of 10 plants in each plot. The area under the disease-progress curve was calculated with the data from the last three growth stages to summarize disease progression, according to Shaner and Finney's formula (1977). Composite random (Dick *et al.* 1996) root and soil samples were collected at a 15-cm depth at each GS evaluated. In each instance, 5 to 6 subsamples (250 g) were collected from a given plot and then pooled. In the laboratory, the roots and rhizospheric soil were separated. The roots were processed immediately and the soil was placed in storage at 4°C for subsequent determinations.

Colonization by AMF was determined by clearing and staining the roots (Phillips and Hayman 1970). The different intraradical fungal structures, such as entry points, internal mycelium, arbuscules, and vesicles were observed by microscopy. The percentages of each structure were estimated through the methodology according to McGonigle *et al.* (1990). The spores of AMF were isolated from 100-g soil samples by wet-sieving and decanting (Gerdemann and Nicolson 1963). The spores were then transferred onto a sucrose solution for centrifugation according to the method used by Walker *et al.* (1982). Only those spores that appeared healthy by directly observing them under a stereomicroscope, were counted in a 9-cm Petri dish. The AMF-spore counts are expressed as the number of spores per 100 g dry soil. The soil-moisture content was calculated for each soil sample as a per cent of the wet weight of soil, after drying at 80°C for 48 h in an oven and then reweighing.

For morphotype identification, spores were extracted and mounted on slides with polyvinyl alcohol/lactic acid/glycerol (Koske and Tessier 1983) or in a mixture of those three and Melzer's reagent (1 : 1, v/v; Blaszkowski *et al.* 2009). The spores obtained after sieving, decanting, and centrifuging were then classified according to the morphotypes glomoid, acaulosporoid, and gigasporoid on the basis of their morphologic features (Oehl *et al.* 2011).

Data were analyzed by the analysis of variance (ANOVA) for factorial designs using Genstat Release 12.1 (2009). The factors included were the inoculation and fungicide treatment, the growth stage, and the Glomeromycota morphotype.

Results and Discussion

The analysis of variance indicates that fungicide application significantly affected Septoria leaf blotch severity (Table 1). The *M. graminicola*-inoculated plots displayed high severities of infection (55.7% on the average over the three growth stages, Table 2). The Septoria leaf blotch increased significantly in severity during the crop's growth cycle, and was markedly and significantly reduced (by 39.2%, Table 2) with the application of the fungicide. Consequently, the area under the disease-progress curve was reduced by the fungicide treatment (to 1,513 vs. 2,839 units²; data not shown).

All the evaluated internal mycorrhizal structures (*i.e.* entry points, internal mycelium, arbuscules, and vesicles) along with the external structures (*i.e.* AMF spores) were affected by the crop-growth stages (Table 1). Significant interactions for fungicide-growth stage were found for arbuscule percentages and total AMF spore numbers. With the fungicide treatment, the former of those parameters was higher at GS 60 (flowering) and the latter at GS 82 (grain filling) than the respective values from the untreated plots (Table 3). That difference in the percentage of arbuscules found at flowering is reasonable since that parameter increases until anthesis but then declines at the end of the growing season (Schalamuk *et al.* 2004). The decrease in arbuscules in wheat at that time can be attributed to the reduction in the supply of carbon from photosynthesis to the roots that occurs during grain ripening (Mohammad *et al.* 1998).

No deleterious effects on the AMF were observed after fungicide application. In fact, the control of *M. graminicola*

favoured the formation of mycorrhizal structures. We propose that fungicide applications may affect AMF associations; directly by an action on the Glomeromycota fungi themselves or indirectly through changes in plant physiology derived from the reduction of the disease in the host plant. It should be taken into account that AMF are greatly affected by the physiological status of the plant since they are biotrophic microorganisms (Smith and Read 2008).

In the present study, a direct effect of fungicide application on the AMF could not be demonstrated. A comparison of mycorrhizal development in the plots with fungicide treatment with that from disease-free wheat without fungicide application, would have been necessary. In greenhouse conditions, a disease-free treatment without applying fungicides can be achieved. In the field, though, in the absence of a fungicide application, infection by spores within natural ambience, which is at least to some extent random, is inevitable. Previous studies in greenhouses, however, had already demonstrated that fungicides belonging to the strobilurins and triazoles at recommended doses, do not affect mycorrhization in crop plants (Schweiger and Jakobsen 1998; Kjoller and Rosendahl 2000; Diedhiou *et al.* 2004). Tebuconazole is a systemic fungicide (Homdork *et al.* 2000), while trifloxystrobin is mesostemic (Parvatha Reddy 2013). Nevertheless, even with fully systemic fungicides within the plant tissues, AMF do not come into direct contact with the pesticide. The reason is that all of those compounds are translocated acropetally into the leaves and the shoot tip. The low, or the entirely absent, basipetal transport of tebuconazole and trifloxystrobin, may help to explain

Table 1. Analysis of variance for Septoria leaf blotch severity and mycorrhizal structures (entry points, internal mycelium, arbuscules, vesicles and spores) on a wheat crop with and without fungicide at three growth stages

Source of variation	df	Severity	Entry point	Internal mycelium	Arbuscules	Vesicles	AMF spores
Fungicide	1	2156.5 (0.001)*	355.6 (0.235)	32.4 (0.602)	265.78 (0.014)	348.19 (0.068)	84.5 (0.510)
Growth stage	2	8630.7 (<0.001)	5881.6 (<0.001)	4437.7 (0.001)	2479.28 (<0.001)	1148.77 (<0.001)	928.2 (0.029)
Fungicide × growth stage	2	103.9 (0.451)	93.1 (0.673)	43.7 (0.687)	160.46 (0.026)	214.04 (0.127)	1066.2 (0.020)
Residual	12	122.0	227.2	112.8	32.02	86.84	180.9
Total	17	–	–	–	–	–	–

AMF – arbuscular-mycorrhizal fungi; *p > F, Fisher test

Table 2. Mean values for Septoria leaf blotch severity, entry points, internal mycelium, and vesicles on a wheat crop with and without fungicide at three growth stages

Treatments	Severity [%]	Entry point [%]	Internal mycelium [%]	Vesicles [%]
Growth stage				
GS 31	13.9 c	18.3 c	40.4 b	12.6 b
GS 60	33.3 b	59.7 b	82.1 a	14.9 b
GS 82	87.1 a	79.7 a	91.5 a	37.6 a
Fungicide				
With fungicide	33.9 b	57.0 a	72.7 a	26.1 a
Without fungicide	55.7 a	48.1 a	70.0 a	17.3 a

Mean values followed by the same letter in the same column among growth stages and between fungicidal treatments, are not statistically significant, LSD (p = 0.05)

Table 3. Mean values for arbuscules and AMF spores on a wheat crop with and without fungicide at three growth stages

Fungicides	Arbuscules			Total spores		
	GS 31	GS 60	GS 82	GS 31	GS 60	GS 82
With fungicide	6.9 a	50.0 a	5.0 a	43.3 a	27.3 a	76.3 a
Without fungicide	3.3 a	30.6 b	5.0 a	52.0 a	40.7 a	41.3 b

AMF – arbuscular-mycorrhizal fungi; GS – growth stage

Mean values followed by the same letter in the same column are not statistically significant, LSD ($p = 0.05$)

Table 4. Analysis of variance for the number of spores belonging to three Glomeromycota morphotypes (glomoid, acaulosporoid, and gigasporoid) on a wheat crop with and without fungicide at three growth stages

Source of variation	df	Mean squares	$p > F^*$
Morphotype	2	5622.72	< .001
Growth stage	2	309.39	0.003
Fungicide	1	28.17	0.433
Morphotype × growth stage	4	145.28	0.023
Morphotype × fungicide	2	13.39	0.743
Growth stage × fungicide	2	355.39	0.001
Morphotype × growth stage × fungicide	4	143.61	0.024
Residual	34	44.70	–
Total	53	–	–

*probability level, Fisher test

Table 5. Mean values for the number of spores belonging to three Glomeromycota morphotypes on a wheat crop with and without fungicide at three growth stages

Glomeromycota morphotypes	Fungicides	GS 31	GS 60	GS 82	Average morphotypes
Glomoid	with fungicide	33.33 a	22.00 a	55.00 a	35.17 a
	without fungicide	42.67 a	28.00 a	30.00 b	
Acaulosporoid	with fungicide	8.67 a	5.33 a	20.67 a	10.89 b
	without fungicide	8.00 a	12.00 a	10.67 a	
Gigasporoid	with fungicide	1.33 a	0.00 a	0.67 a	0.78 c
	without fungicide	1.33 a	0.67 a	0.67 a	

Mean values followed by the same letter in the same column within the same morphotype are not statistically significant, LSD ($p = 0.05$)

the lack of effect on microorganisms in the root zone by the foliar application of those compounds. Moreover, triazoles such as tebuconazole act as inhibitors of the biosynthesis of ergosterol, a major component of fungal membranes. The relative amounts of ergosterol in AMF, however, are low in comparison with the concentrations present in the tissues of other groups of true fungi (Schmitz *et al.* 1992; Frey *et al.* 1994). To achieve significant effects on AMF, high dosages or repeated applications of ergosterol inhibitors would be necessary (Kjoller and Rosendahl 2000).

Since AMF exhibit an absolute dependence on the carbon resources provided by the plant, a severe foliar disease as exemplified in this experiment, may generate fluctuations in carbon availability. Mycorrhizal development in turn, would be strongly affected. A reduction in photosynthesis under the influence of *M. graminicola* would be expected to lead to a decrease in the carbon supply to the roots and thus negatively affect the development of mycorrhizal associations.

Significant differences were found among the morphotypes and also for the two-way interactions morphotype × growth stage and growth stage × fungicide as well as for the three-way interaction morphotype × growth stage × fungicide (Table 4). The number of glomoid spores was higher than those belonging to the acaulosporoid and gigasporoid morphotypes (Table 5), whose distribution is consistent with other studies on agricultural systems in the same area (Schalamuk *et al.* 2006; Schalamuk and Cabello 2010). At GS 82, the spores belonging to the glomoid morphotype significantly increased with the application of fungicide. The acaulosporoid tended to increase as well, though not significantly so. In contrast, the number of gigasporoid spores was not affected by fungicide application.

Arbuscular mycorrhizal fungi have been classified within the classic life-history strategies 'r-K' on the basis of sporulation traits (De Souza *et al.* 2005; Ijdo *et al.* 2010; Verbruggen and Kiers 2010). While the AMF of the Gigasporaceae (with the gigasporoid morphotype) produce few and large spores within a long life cycle, and tend

to resemble *K*-strategists, the AMF of the Glomeraceae (with the glomoid morphotype) display opportunistic behaviour, such as a rapid colonization and production of many small spores, typical of *r*-strategists (Denison and Kiers 2011). Ijdo *et al.* (2010) found that, since AMF exhibit an obligate dependence on plant carbon, the fluctuations in the availability of that element produced by defoliation, can differentially affect AMF with contrasting life-history strategies. Those authors found that *Glomus intraradices*, of the glomoid morphotype renamed *Rhizophagus intraradices* by Schüßler and Walker (2010) modulated spore production directly in accordance with carbon availability. A direct investment in reproduction as would be expected for an *r*-strategist was shown. Whereas *Dentiscutata reticulata*, of the gigasporoid morphotype, was not affected after a single defoliation and thus showed a marked resistance to fluctuating carbon levels. In the present study, the higher glomoid spore number found after fungicide treatment involving a greater green leaf area and consequently more photosynthates reaching the roots is consistent with the findings by Ijdo *et al.* (2010). Thus, carbon fluctuations may affect glomoid and gigasporoid morphotypes in a different manner.

In conclusion, during AMF symbiosis, an alteration in green-leaf area, such as occurs during a severe foliar disease and/or its control by fungicides, may result in a shift in the AMF community to favour members belonging to the glomoid morphotype. Glomoid species have been previously considered as *r*-strategists. The present results demonstrated that the control of severe wheat infections by fungicides paradoxically enough can even positively affect the AMF present. This happens, for example, by selecting for glomeromycota morphotypes with the appropriate adaptive life-history strategies.

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