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ORIGINAL PAPER

An arbuscular mycorrhizal fungus and *Epichloë festucae* var. *lolii* reduce *Bipolaris sorokiniana* disease incidence and improve perennial ryegrass growth

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Abstract
Leaf spot of perennial ryegrass (*Lolium perenne*) caused by *Bipolaris sorokiniana* is an important disease in temperate regions of the world. We designed this experiment to test for the combined effects of the arbuscular mycorrhizal (AM) fungus *Claroideoglomus etunicatum* and the grass endophyte fungus *Epichloë festucae* var. *lolii* on growth and disease occurrence in perennial ryegrass. The results show that *C. etunicatum* increased plant P uptake and total dry weight and that this beneficial effect was slightly enhanced when in association with the grass endophyte. The presence in plants of both the endophyte and *B. sorokiniana* decreased AM fungal colonization. Plants inoculated with *B. sorokiniana* showed the typical leaf spot symptoms 2 weeks after inoculation and the lowest disease incidence was with plants that were host to both *C. etunicatum* and *E. festucae* var. *lolii.* Plants with these two fungi had much higher activity of peroxidases (POD), superoxide dismutase (SOD) and catalase (CAT) and lower values of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). The AM fungus *C. etunicatum* and the grass endophyte fungus *E. festucae* var. *lolii* have the potential to promote perennial ryegrass growth and resistance to *B. sorokiniana* leaf spot.

Keywords Arbuscular mycorrhizal fungi · Grass endophyte · Leaf spot disease · Anti-oxidative activity · Biocontrol

Introduction

Field-growing plants are exposed to a wide range of microorganisms both within and above the soil. The roots of many plants, including grasses, are extensively colonized by arbuscular mycorrhizal (AM) fungi of the sub-phylum Glomeromycotina (Spatafora et al. 2016). The presence of these fungi in roots typically enhances the growth of host plants, particularly in soils with low concentrations of available phosphorus. Other reported benefits to host plants include improved plant nutrient uptake (Marschner and Dell 1994) enhanced plant resistance to biotic stresses including insect pests (Pineda et al. 2010), disease (Whipps 2004; Saldajeno and Hyakumachi 2011; Elsharkawy et al. 2012; Lee et al. 2012; Maya and Matsubara 2013; Lenoir et al. 2017), and possibly abiotic environmental stresses (Zaefarian et al. 2013).

With many grasses of the sub-family Pooideae, a very specific type of mutualistic association is made with fungi of the genus *Epichloë*. These fungi are exclusively seed-borne, mostly by vertical transmission (Zhang et al. 2017), but some may infect developing seed following horizontal transmission (Chung and Schardl 1997). These fungi are in all tissues apart from the roots and are located in the intercellular spaces. The nature of the association of these systemic fungal endophytes can be thought of as if these fungi were a plant tissue as their growth in vegetative tissues is fully synchronized with that of the host grass (Christensen et al. 2008). A key function of the in situ hyphae is to produce secondary metabolites, particularly alkaloids, which protect the host from non-vertebrate pests. Some of the alkaloids also adversely affect the growth of grazing livestock (Gallagher et al.

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1984; Strickland et al. 1996; Popay and Hume 2011). These effects on non-vertebrate pests and grazing livestock can enhance the persistence of host plants.

In addition to the effects that the presence of an *Epichloë* sp. endophyte has on insects and grazing livestock, there are reports that there are effects against some pathogens (Ma et al. 2015), although as yet there are few reports of disease resistance associated with field-growing endophyte-infected grasses. There is strong field-based evidence that endophyte-infected tall fescue plants have enhanced tolerance to drought stress (West et al. 1993) and there are greenhouse-based studies of enhanced tolerance to harmful insects (Barker 1987; Vicari et al. 2002), toxicity from heavy metals, high salinity, and onset of cold conditions (Parsaeian et al. 2006; Baltruschat et al. 2008; Zhang et al. 2009). The best studied associations between *Epichloë* species and grasses are those with perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*), two species with widespread use in pasture-based agriculture (Johnson et al. 2013), and drunken horse grass (*Achnatherum inebrians*) (Li et al. 2004).

Perennial ryegrass is native to temperate regions of Europe but is now naturalized and widely used as pasture in temperate regions of North and South America, Japan, and China as well as in New Zealand where it is the most important grass for pastures. It is interesting that in New Zealand, there has been very strong selection pressure for endophyte-infected perennial ryegrass, especially for plants containing strains that produce three alkaloids that provide protection against herbivores: peramine, ergovaline, and lolitrem B. (Tapper and Latch 1999). Perennial ryegrass is increasing in use in parts of China (Wang and Zhao 2014). Fungal diseases are a problem with perennial ryegrass in China as in other parts of the world. Rust is one of the serious problems and another is a leaf spot disease caused by *Bipolaris sorokiniana*. Field trials with endophyte-infected (E+) and endophyte-free (E−) perennial ryegrass plants of the cultivar Fairway found that the presence of the *Epichloë festucae* var. *lolii* enhanced resistance against rust disease caused by *Puccinia coronata* (Ma and Nan 2011). There are no reports regarding the effects of the presence of this endophyte on the susceptibility of plants of this perennial ryegrass cultivar on leaf disease caused by *B. sorokiniana*. In another recent study involving this cultivar, the effects of the presence or absence of *E. festucae* var. *lolii* on four indicators of oxidative stress were measured in germinating seedlings exposed to five fungal pathogens, including *B. sorokiniana* (Ma et al. 2015). These indicators were the concentration of malondialdehyde (MDA) and proline and activity of peroxidases (POD) and superoxide dismutase (SOD). This study indicated that the presence of the fungal endophyte reduced oxidative stress of seedlings exposed to the fungal pathogens.

Roots of perennial ryegrass plants growing under field conditions are colonized by AM fungi (Zhu et al. 2000). Thus, plants which are hosts to mutualistic mycorrhiza in the roots also may be hosts to a mutualistic *Epichloë* sp. endophyte in the aboveground tissues. Several studies have looked at the interaction between endophytes and AM fungi in perennial ryegrass and the closely related grasses annual ryegrass (*Lolium multiflorum*) and tall fescue (*F. arundinacea*). Two studies using perennial ryegrass that looked at the effect of the presence of an epichloid endophyte on AM colonization reported that the foliage-confined endophyte reduced AM root colonization (Müller 2003; Liu et al. 2011). Similarly, the presence of *Epichloë coenophiala* (= *Neptyphodium coenophialum*) in tall fescue (Chu-Chao et al. 1992; Antunes et al. 2008) and of *Epichloë occultans* (= *Neptyphodium occultans*) in annual ryegrass (Omacini et al. 2006) reduced AM colonization. A further study showed that the presence of AM fungi in roots of perennial ryegrass with *E. festucae* var. *lolii* reduced the feeding deterrence effect against the insect pest Argentine stem weevil (*Listronotis bonariensis*) (Barker 1987). Another study, however, found that the presence of AM fungi in endophyte-infected perennial ryegrass reduced the survival of the angle shades moth (*Phlogophora meticulosa*) (Vicari et al. 2002). Based on these reports and studies in other endophyte-infected grasses (Novas et al. 2011; Larimer and Clay 2012), we undertook a study to understand the role and physiological mechanisms that each of the partners of the complex association with perennial ryegrass has on plant growth and infection by the fungal pathogen *B. sorokiniana*. The questions that we wanted to examine were would the presence of *E. festucae* var. *lolii*, an AM fungus species, *Claroideoglomus etunicatum*, and the combination of the two fungi, have beneficial effects on plant growth, and would the presence of *E. festucae* var. *lolii* and the AM fungus, either individually or in combination, provide resistance against *B. sorokiniana*? Our study utilized all combinations of the three types of fungi. We hypothesized that (1) the presence of *C. etunicatum* or *E. festucae* var. *lolii* would have beneficial effects on plant growth and increase resistance to the pathogen and (2) the presence of the AM fungus and the grass endophyte would reduce oxidative stress in perennial ryegrass such that the resistance of perennial ryegrass to *B. sorokiniana* will increase when plants are host to both *C. etunicatum* and *E. festucae* var. *lolii* versus host to each individually.

Materials and methods

Experimental design

Plants and fungi used in the study

Perennial ryegrass, with and without the systemic endophyte *E. festucae* var. *lolii*, provided by the Forage and Turfgrass Quality Supervision Testing Center, Ministry of Agriculture,

China, was the grass species used in this study. The cultivar used was 'Fairway,' the same perennial ryegrass cultivar that had been used in a study that looked at the effect of the presence of this seed-borne fungal endophyte on indicators of oxidative stress during invasion by fungal pathogens, including *B. sorokiniana* (Ma et al. 2015). The E+ and E− seeds used in this current study were obtained from plants that had been confirmed as being either E+ or E− by examination of leaf sheaths stained with aniline blue for the presence or absence of hyphae characteristic of *E. festucae* var. *lolii*. These hyphae were intercellular, seldom-branched, orientated parallel to the longitudinal leaf axis, 2–3 μm in diameter, and with non-staining septa.

The AM fungus used in the study was *C. etunicatum*. The AM fungus species was obtained from the Institute of Plant and Environmental Resource of Chinese Academy of Agriculture. Prior to 2010, the species was classified as *Glomus etunicatum*. The fungus had been maintained in dried-out pots of white clover (*Trifolium repens*) that were stored at room temperature. The pots had been allowed to dry when the number of spores of the AM fungus had reached the concentration of ~ 100 g^{-1} dry soil. We prepared inoculum of the AM fungus from the dry soil and from roots of the source white clover plants. The white clover plants were removed from the soil and the roots cut into pieces approximately 0.5 cm in length.

The isolate of *B. sorokiniana* used in the study was isolated from locally growing perennial ryegrass plants. Leaves with symptoms characteristic of *B. sorokiniana* were surface-sterilized by rinsing in 75% alcohol, then in 0.3% NaClO for 1–2 min and then washed several times with distilled water and dried on sterilized filter paper. The leaf tissue was then cut into small pieces (~ 0.2 cm in length), placed on potato dextrose agar (PDA) containing penicillin and streptomycin, each at a concentration of 0.5 mL L^{-1} and incubated at 25 °C. The isolates were observed every day for the presence of conidia, allowing identification of the pathogen. The *B. sorokiniana* isolated was sub-cultured on PDA and kept for 7–10 days at 25 °C (Nan 1995). Conidia were obtained by adding sterile water to Petri dishes containing conidia-producing *B. sorokiniana* colonies, scraping the surface with a sterilized spreader, filtering the suspensions through four layers of sterilized cheese cloth, and using a hemocytometer to obtain a concentration of approx. 1×10^6 conidia mL^{-1}.

Potting medium

We collected soil from the Xinglong Mountain, Lanzhou, China, to prepare the potting mix used for our experiment. The mix consisted of 50% of soil and 50% sand. Both components were dried at room temperature, and then sieved through a 2-mm sieve. The soil and sand mixture was twice sterilized via autoclaving at 121 °C for 1 h, over a period of 3 days, and then dried in an oven at 110 °C for 36 h. The mix had 19.17 mg P kg^{-1} plant-available P (resin extraction method) (McLaughlin et al. 1994) and a pH of 5.85.

Greenhouse study

The trial was conducted in a greenhouse and involved either E+ or E− perennial ryegrass plants growing in pots (20-cm high and 15-cm wide), containing 1500*g* of the potting mix. There were 16 pots of E+ and 16 pots of E− perennial ryegrass plants. Eight pots of E+ and eight pots of E− plants were inoculated with *C. etunicatum*, the same number of E+ and E− pots did not receive this AM fungus. Four of the eight pots of each treatment were inoculated with *B. sorokiniana.* There were four replicates per treatment, resulting in a total of 32 pots ryegrass.

We surface-sterilized the E+ and the E− perennial ryegrass seeds with 3% sodium hypochlorite (NaClO) for 30 s following which they were rinsed three times with sterile water. We placed the sterilized seeds on wet filter paper in the dark at 25 °C and following the onset of germination, six germinating E+ or E− seeds were transplanted into each pot containing the potting mix. Prior to the transplanting of seedlings, 20 g of the dry soil and white clover root fragments inoculum of AM fungus were added to 16 pots to provide the AM treatment. For the non-inoculated control plants (NM) treatments, 20 mL of the solutions that contains microbes excluding AM fungus were added to the soil with a previously prepared microbial filtrate (50 μm).

Each seedling was checked for the presence or absence of hyphae characteristic of *E. festucae* var. *lolii* after 2 weeks of growth. Small epidermal strips were peeled off the adaxial surface of the leaf sheaths and placed on a glass slide in 1 to 2 drops of aniline blue-lactic acid-glycerol solution, covered with a glass cover-slip, heated gently to boiling to drive out air bubbles and examined at ×100–400 magnification for the presence or absence of hyphae characteristic of epichloid endophytes (Florea et al. 2015). Each pot was thinned to three plants and maintained in a greenhouse for another 6 weeks. During those 6 weeks, the numbers of tillers and the height of each plant were recorded at weekly intervals.

Six weeks after seedling emergence, four of the pots of each of the AM fungus treatments involving either E+ or E− perennial ryegrass plants, a total of 16 pots were inoculated with *B. sorokiniana.* Ten milliliters of the suspension of *B. sorokiniana* conidia described above was sprayed onto each plant. The same amount of sterilized water was sprayed onto our control (non-inoculated) treatments. Immediately after being sprayed with either the suspension of conidia or sterilized water, each pot was covered with a black plastic bag for the following 2 days.

We conducted the experiment from April to July in a glasshouse with irradiance in the range of 180–850 mmol m^{-2} s^{-1}.

The average temperatures were 23–28 °C (day) and 20–25 °C (night). We applied a modified Long Ashton nutrient solution (−P) (Duan et al. 2011) to the pots every other day during the experiment.

Plant harvest and measurement

Two weeks after inoculation with *B. sorokiniana*, the plants were visually assessed for the presence of lesions. The incidence of lesions was noted on 25 leaves of each of four pots per treatment. Disease incidence was calculated by the number of leaves with lesions, divided 25 for each pot. The plants were then harvested by cutting the tillers at ground level. Tillers from the three plants of each pot were combined. Three subsamples of the combined tillers of each pot were taken and each was weighed. One of the samples was used to do all the analyses, another to do isolations from lesions, while the third was oven-dried to obtain the dry weight, and this was used to calculate the total aboveground dry weight of the plants of each pot. The pathogen was re-isolated with Nan's methods (1995) as previously described. The fungi were microscopically identified based on morphological characteristics of the asexual reproductive structures and colony characters. Phosphorous and soluble protein concentrations, the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and polyphenol oxidase (PPO), enzymes associated with the response of plants to pathogen invasion, along with malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), were determined. The oven-dried plant material was finely ground and weighed. Phosphate concentration of plant tissue was determined colorimetrically with a UV-Visible spectrophotometer (Shimadzu, Australia) at 390 nm, using the phosphovanado-molybdate method (Hanson 1950) after digestion of the dried, ground plant material (~ 0.5 g) in 5 mL of concentrated nitric acid. The activity of three of the enzymes, MDA, and hydrogen peroxide concentration were determined with an ultraviolet spectrophotometer (Pectrum, Shanghai 721 China). We determined the CAT activity with another UV spectrophotometer (PerkinElmer-Lambda 25-USA) at 240 nm by the published method of Beers and Sizer (1952). We determined the activity of SOD and POD and soluble protein content at 560, 470, and 595 nm, respectively (Li et al. 2000). We determined PPO activity at 495 nm via the method of Kumar and Khan (1982). Hydrogen peroxide content was determined at 390 nm (Velikova et al. 2000) and we determined MDA at 600, 450, and 532 nm using the method of Li et al. (2000).

We washed the roots in each pot and made three subsamples, two of approximately 0.1 g while the third was the remainder. One subsample was used to assess AM fungal colonization, the second to examine if *B. sorokiniana* had colonized some segments of roots using the method of Nan (1995) while the bulk of each root sample was used to determine total root dry weight. AM fungal colonization of roots was determined using the gridline intersect method of Giovannetti and Mosse (1980). After clearing in 10% KOH and staining in 1% trypan blue (Phillips and Hayman 1970), ~ 0.1 g 1-cm root segments were selected at random and observed using a compound microscope at ×400 magnification. The presence of vesicles, arbuscules, and intraradical hyphae was recorded in 25 fields of view. The possible presence of *B. sorokiniana* in roots was assessed by placing small pieces of surface-sterilized roots onto antibiotic PDA as done for pieces of leaves with symptoms.

Statistical analysis

All data are presented as means and standard errors of the mean of four replicates. Homogeneity of variances was tested with Levene's test and normality of distributions with the Kolmogorov-Smirnov test. The data for percent AM colonization were ARCSIN-transformed to achieve normality. We analyzed the data using the three-factor ANOVA analysis of variance, using the statistical analysis software SPSS 19.0 (SPSS Inc., Chicago, USA). Tukey's HSD all-pairwise comparisons were used to compare treatment means. Statistical significance was determined at $P \leq 0.05$.

Results

AM fungal colonization and disease incidence

All of the perennial ryegrass plants that were examined in the E+ treatments were found to be endophyte-infected whereas none of the perennial ryegrass plants examined in the E− treatments were infected. Examination of a representative sample of roots in the AM treatment revealed the presence of structures characteristic of this association, namely vesicles, arbuscules, and non-septate hyphae. In contrast, these structures were not observed in roots of plants in the non-inoculated treatments. E+ plants had 24% less *C. etunicatum* root colonization on average than E− plants, and *B. sorokiniana* decreased *C. etunicatum* colonization by 17%; plants with endophyte and the pathogen had the lowest *C. etunicatum* colonization (Fig. 1a).

Plants inoculated with *B. sorokiniana* showed typical leaf spot symptoms 2 weeks after inoculation. No leaf symptoms were present on non-inoculated plants. We re-isolated the pathogen *B. sorokiniana* from lesions of inoculated plants. Compared with the NM E− treatment, *C. etunicatum* alone or with the endophyte significantly decreased *B. sorokiniana* leaf spot incidence (Fig. 1b). Plants with *C. etunicatum* had 23% less disease incidence on average than NM plants (Fig. 1b).

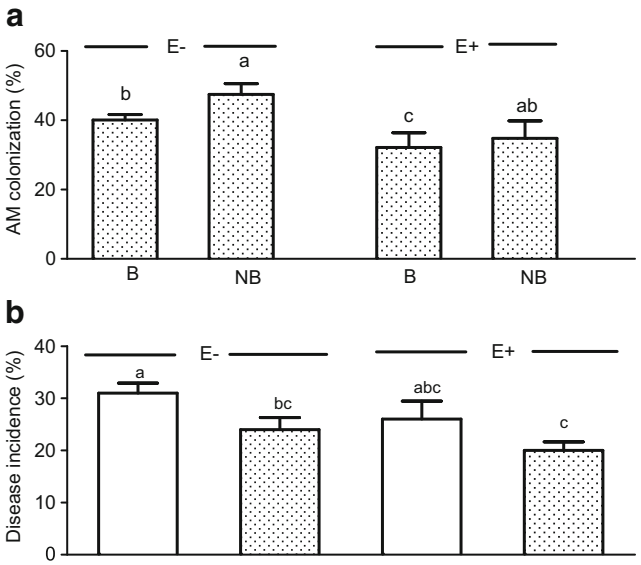

Fig. 1 AM colonization (**a**) and disease incidence (**b**) of perennial ryegrass with (E+) and without (E−) grass endophyte and colonized by *Claroideoglomus etunicatum* (stippled bars) or non-mycorrhizal (open bars) at harvest. Mean ± SEM of four replicates. B = *Bipolaris sorokiniana* applied, NB = no *B. sorokiniana*. Bars topped by the same lowercase letter do not differ significantly between treatments at $P \leq 0.05$ by Tukey's HSD. See Table 1 for ANOVA results

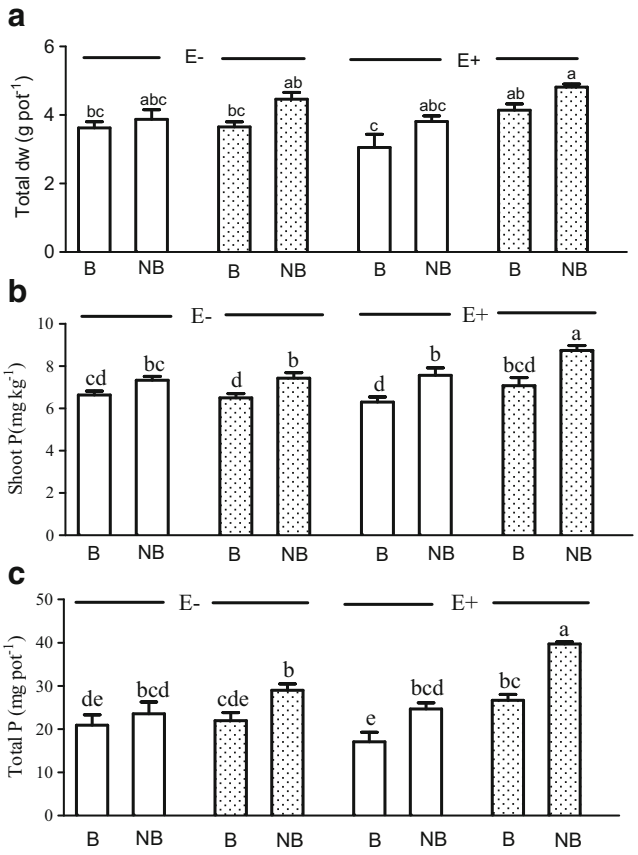

Fig. 2 Total dry weights (roots plus shoots) (**a**), shoot P concentration (**b**), and Total P content (roots plus shoots) (**c**) of perennial ryegrass with (E+) and without (E−) grass endophyte and colonized by *Claroideoglomus etunicatum* (stippled bars) or non-mycorrhizal (open bars) at harvest. Mean ± SEM of four replicates. B = *Bipolaris sorokiniana* applied, NB = no *B. sorokiniana*. Bars topped by the same lowercase letter do not differ significantly between treatments at $P \leq 0.05$ by Tukey's HSD. See Table 1 for ANOVA results

Plant growth

Perennial ryegrass responded positively to the combination of *E. festucae* var. *lolii* and *C. etunicatum* colonization. Effects first became apparent 14 days after being transplanted into the pots with the potting mix containing, or not containing, *C. etunicatum*, when all seedlings with both *E. festucae* var. *lolii* and AM fungus had visibly increased height compared to E− plants with and without *C. etunicatum* (Supplementary Fig. S1). The presence of *C. etunicatum* provided a significant increase in total dry weight (DW) only with E+ plants, whereas the total dry weight of E− plants was only slightly increased. The presence of *E. festucae* var. *lolii* and the pathogen had no significant effect on the total DW of plants (Fig. 2a). Root/shoot (R/S) ratios were not different between treatments (Table 1; Supplementary Fig. S2). The effects of *E. festucae* var. *lolii* and *C. etunicatum* colonization on shoot DW, root DW, and root length are very similar to total DW across all treatments (Supplementary Fig. S3). We found no interaction effects between *C. etunicatum*, *E. festucae* var. *lolii*, and the pathogen (see Table 1 for ANOVA results on these parameters).

Phosphorous nutrition

We found significant effects of *E. festucae* var. *lolii*, AM fungal colonization, and *B. sorokiniana* infection on phosphorous (P) concentration in shoots (Fig. 2b) and roots (Supplementary Fig. S4). Furthermore, the grass endophyte and the inoculation with *C. etunicatum* separately had interaction effects on foliage P concentration (Table 1). Values for pathogen-infected plants were significantly decreased compared with those of uninfected plants except for NM E−. The presence of both *C. etunicatum* and *E. festucae* var. *lolii* significantly increased foliage P concentration (Fig. 2b).

Total P content followed a trend similar to total DW, and was significantly affected by the presence of each of the three fungi (Table 1). E+ plants had the highest total P content in combination with *C. etunicatum* (Fig. 2c). The inoculation of *B. sorokiniana* decreased plant P uptake in all treatments except for NM E− plants (Fig. 2c).

Enzyme activity

The activity of enzymes was significantly affected by the presence of the grass endophyte and inoculation with *C. etunicatum* throughout all treatments except for CAT in plants with *C. etunicatum* (Fig. 3; Table 1). The co-colonization with *E. festucae* var. *lolii* and *C. etunicatum* gave

Table 1 ANOVA result for effects of grass endophyte (E), *Bipolaris sorokiniana* (B), mycorrhizal inoculation (M), and their interactions on the listed variables. Perennial ryegrass harvested at 2 weeks after pathogen inoculated

Source of variation	*Claroideoglomus etunicatum* (M)			Grass endophyte (E)			*Bipolaris sorokiniana* (B)			Interactions											
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	ExB			ExM			BxM			ExBxM		
										df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Shoot DW	1	13.442	0.0010	1	0.105	NS	1	7.895	0.0100	3	0.263	NS	3	2.254	NS	3	0.037	NS	7	0.303	NS
Root DW	1	5.699	0.0250	1	0.014	NS	1	9,627	0.0050	3	0.092	NS	3	4.441	0.0460	3	3.227	NS	7	2.202	NS
Total DW	1	18.992	< 0.0001	1	0.114	NS	1	15.974	0.0010	3	0.353	NS	3	5.605	0.0260	3	0.581	NS	7	1.072	NS
Root/shoot ratio	1	0.711	NS	1	0.082	NS	1	0.002	NS	3	0	NS	3	0.088	NS	3	2.291	NS	7	0.036	NS
Shoot P concentration	1	6.527	0.0210	1	5.616	0.0310	1	36.833	< 0.0001	3	2.967	NS	3	7.008	0.0180	3	0.712	NS	7	0.059	NS
Root P concentration	1	33.416	< 0.0001	1	18.488	0.0010	1	4.661	0.0460	3	14.038	0.0020	3	4.357	NS	3	7.648	00140	7	0.796	NS
Total P content	1	35.185	< 0.0001	1	5.856	0.0280	1	33.374	< 0.0001	3	4.361	NS	3	11.937	0.0030	3	3.512	NS	7	0.038	NS
AM colonization	1	332.55	< 0.0001	1	9.702	0.0070	1	4.637	0.0470	3	0.565	NS	3	5.604	0.0310	3	0.263	NS	7	0.257	NS
Disease incidence	1	7.141	0.0130	1	3.423	NS	1	431.028	< 0.0001	3	3.423	NS	3	0.042	NS	3	7.141	0.0130	7	0.042	NS
SOD	1	49.075	< 0.0001	1	30.928	< 0.0001	1	14.885	0.0010	3	0	NS	3	2.849	NS	3	0.048	NS	7	1.123	NS
POD	1	7.207	0.0160	1	10.894	0.0050	1	3.474	NS	3	0	NS	3	3.462	NS	3	0.492	NS	7	0.029	NS
PPO	1	10.035	0.0060	1	10.02	0.0060	1	9.285	0.0080	3	3.051	NS	3	6.656	0.020	3	0.995	NS	7	4.694	0.0460
CAT	1	3.583	NS	1	11.339	0.0040	1	0.556	NS	3	1.072	NS	3	7.566	0.0140	3	2.729	NS	7	0.739	NS
MDA	1	3.74	NS	1	10.446	0.0050	1	11.328	0.0040	3	0.124	NS	3	0.053	NS	3	0.023	NS	7	0.632	NS
H_2O_2	1	2.492	NS	1	11.009	0.0040	1	6.432	0.0220	3	0.34	NS	3	3.033	NS	3	0	NS	7	0.307	NS
Soluble protein	1	4.069	NS	1	8.048	0.0120	1	0.02	NS	3	0.619	NS	3	1.049	NS	3	0	NS	7	0.152	NS

Plant totally grown for 8 weeks

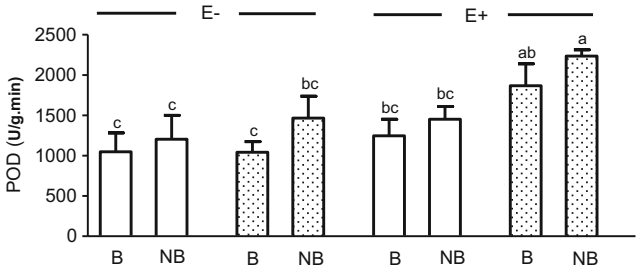

Fig. 3 Peroxidases (POD) enzyme activity of perennial ryegrass with (E+) and without (E−) grass endophyte and colonized by *Claroideoglomus etunicatum* (stippled bars) or non-mycorrhizal (open bars) at harvest. Mean ± SEM of four replicates. B = *Bipolaris sorokiniana* applied, NB = no *B. sorokiniana*. Bars topped by the same lowercase letter do not differ significantly between treatments at $P \leq 0.05$ by Tukey's HSD. See Table 1 for ANOVA results

much higher values of POD activity compared to NM E− and of each of them alone (Fig. 3). The SOD and CAT values largely followed the same pattern as for POD, with the values in AM E+ plants being higher than for other plants. Similarly, the values of SOD in plants inoculated with *B. sorokiniana* were lower than for paired un-inoculated plants in the NM E− and AM E+ treatments. In general, the levels of PPO were similar in all treatments with the exception being the extremely high relative level in the E+ plants that were host to *C. etunicatum* without inoculation of *B. sorokiniana* (Supplementary Fig. S5).

MDA, H_2O_2, and soluble protein concentrations

The presence of *E. festucae* var. *lolii* and infection with *B. sorokiniana*, had significant effects on MDA and H_2O_2 concentration. Colonization with *C. etunicatum*, however, had no significant effects on these parameters (Fig. 4a, b; Table 1). NM E− and AM E+ without infection of *B. sorokiniana* gave the highest and lowest value of MDA and H_2O_2 concentration, respectively (Fig. 4a, b). E+ plants had 18 and 33% less MDA and H_2O_2 concentration on average than E− plants, respectively, and *B. sorokiniana* increased the MDA and H_2O_2 concentration by 24 and 26%, respectively (Fig. 4a, b). Soluble protein concentration is similar across the treatments except co-colonization with *E. festucae* var. *lolii* and *C. etunicatum* that had the highest values although this was only affected significantly by *E. festucae* var. *lolii* (Fig. 4c; Table 1). .

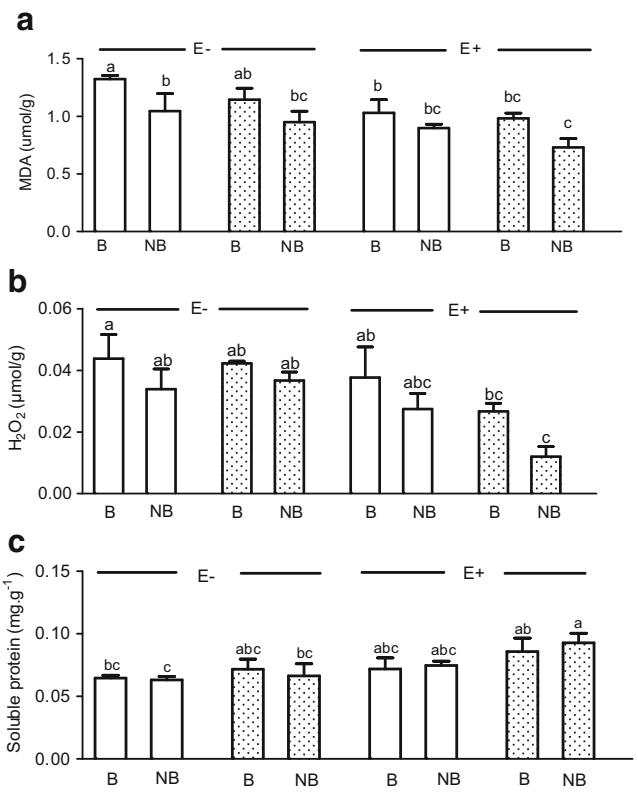

Fig. 4 Malondialdehyde (MDA) concentration (**a**), hydrogen peroxide (H_2O_2) concentration (**b**), and soluble protein concentration (**c**) of perennial ryegrass with (E+) and without (E−) grass endophyte and colonized by *Claroideoglomus etunicatum* (stippled bars) or non-mycorrhizal (open bars) at harvest. Mean ± SEM of four replicates. B = *Bipolaris sorokiniana* applied, NB = no *B. sorokiniana*. Bars topped by the same lowercase letter do not differ significantly between treatments at $P \leq 0.05$ by Tukey's HSD. See Table 1 for ANOVA results

Discussion

Using the AM fungus *C. etunicatum*, the pathogen *B. sorokiniana*, and perennial ryegrass with and without *E. festucae* var. *lolii* in a glasshouse experiment, we revealed that perennial ryegrass plants that are host to both the foliage-confined systemic endophyte and root-confined AM fungus had improved growth, even in the presence of the foliage pathogen *B. sorokiniana.* The improved growth resulting from the presence of the epichloid endophyte and *C. etunicatum* occurred even though perennial ryegrass plants that were host to *E. festucae* var. *lolii* had lower colonization of this AM fungus than plants that did not host the endophyte. Our hypothesis that the presence of *C. etunicatum* or *E. festucae* var. *lolii* will increase plant growth and resistance to the pathogen was supported. The presence of *C. etunicatum* in the roots of endophyte-infected plants gave the best growth and best reduction in the adverse effects of the foliage pathogen, which coincided with the best uptake of P. *E. festucae* var. *lolii* alone did not affect perennial ryegrass leaf spot incidence. Our hypothesis that the resistance of perennial ryegrass to *B. sorokiniana* will increase when plants are host to both *C. etunicatum* and *E. festucae* var. *lolii*, compared to each individually was upheld. Our results reveal that plants infected with *B. sorokiniana* had higher values of H_2O_2 for most treatments, and this was especially prominent in plants host to *E. festucae* var. *lolii*. The effects on levels of MDA are similar to those on H_2O_2. The activity of the disease-response related

enzymes, SOD, POD, CAT, and PPO, are mostly higher in treatments involving both *E. festucae* var. *lolii* and *C. etunicatum* than for other treatments.

As previously stated, a number of studies have examined the effects of the presence of an epichloid endophyte and AM fungi in host grasses. A key measureable outcome of several studies was that the presence of the foliage-confined epichloid endophyte resulted in a lower rate of root colonization by AM fungi (Chu-Chao et al. 1992; Müller 2003; Omacini et al. 2006; Attunes et al. 2008; Liu et al. 2011). Conversely, the finding of Novas et al. (2005), who studied two populations of *Bromus setifolius*, one with and one without endophyte association, that were grown in either the presence or the absence of AM fungi using two different sources of field soil, found that the roots of the E+ population were more extensively colonized than those of the E− populations. The differences that have been found in the different studies may be caused by differences in host genotype, *Epichloë* species and strain, and AM fungi species, as well as soil factors, in particular, available P content. Plants typically have higher AM fungal colonization under low P compared to high P conditions (Facelli et al. 2014). The high soil available P content of 19.18 mg kg^{-1} in our experiment might have affected the interactions between AM fungal colonization and grass endophyte. Moreover, our study revealed that AM fungal colonization in perennial ryegrass was decreased due to infection with *B. sorokiniana*, where there was the lowest AM fungal colonization when the E+ population was infected by *B. sorokiniana*. This finding agrees with Zambolim and Schenck (1983), who reported an average decrease of AM fungal colonization of 38% in soybean roots due to the infection of roots by *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Fusarium solani.* This may be because of competition for colonization sites and for photosynthates from the host plants between AM fungi and the pathogens. Furthermore, Maya and Matsubara (2013) reported the relationship between AM fungi and disease severity to show a negative correlation and that competition or antagonistic effects occurred between the AM fungus *Glomus fasciculatum* and root pathogens *Fusarium oxysporum* and *Colletotrichum gloeosporioides.* To our knowledge, however, our study is the first that has examined how the presence of an epichloid endophyte and AM fungus affects the incidence of a foliage fungal pathogen and the growth of the host plant.

The strength of our study on the effect of two mutualist fungal symbionts and the foliage pathogen on the host grass is that all combinations of partners, including the absence of all three fungal species, were assessed. Thus, our study provides an indication of what may be happening under field conditions where perennial ryegrass plants inevitably will have root systems colonized by AM fungi. Our study contrasts with many greenhouse studies that have examined the effects of the presence of *Epichloë* spp. endophytes of grasses on the susceptibility of host grasses to pathogenic fungi, where the grasses were growing in heat-treated soil or potting mix (Gu et al. 2007; Tian et al. 2008; Ma and Nan 2011; Sabzalian et al. 2012; Wang et al. 2014). Plants in those trials would have been unlikely to have a functional AM fungal root flora. Based on the finding of our study that the presence of both the systemic foliage endophyte and the root-confined *C. etunicatum* provided maximum enhancement of plant growth, even in the presence of a foliage pathogen, it is likely that previous studies have underestimated the role that both types of fungal mutualists are having on fungal diseases under field conditions.

Our results show that the AM fungus has overlying effects in improving plant disease resistance and which may be associated with biocontrol capacities (Lopez-Raez et al. 2010). This finding agrees with Martinez-Medina et al. (2009), who studied the effect of the interaction between different species belonging to the genus *Glomus* sp. and the saprotrophic fungus *Trichoderma harzianum* in growth promotion of melon plants and in a biocontrol effect against *Fusarium* wilt.

It is well established that AM fungal colonization is able to enhance plant resistance and growth by increasing P uptake (Marschner and Dell 1994; Smith and Read 1996). In our experiments, plants hosting *C. etunicatum* had increased P uptake. The positive growth responses and the reduced effects of *B. sorokiniana* on plants hosting *C. etunicatum* are consistent with increased P uptake. There are also research that has found that AM fungi do not have a positive effect or even have negative effect by plants (Klironomos 2003; Li et al. 2008) This difference between studies shows that the effects of AM fungi on plant P uptake depend on host genotype-AM fungi combinations and environmental conditions (Gavito et al. 2002; Schroeder and Janos 2005).

Another aspect of our study was its focus on the levels of H_2O_2 and MDA and of enzymes associated with reducing oxidative stress resulting from attack by pathogens. The expected results would have shown that plants infected with *B. sorokiniana* had elevated levels of H_2O_2 and MDA compared with non-inoculated plants. Increased levels would provide an indication of the level of oxidative stress experienced by plants infected with *B. sorokiniana.* Initially, the increase in levels of H_2O_2 in plants will inhibit the infection by plant pathogens, but as the concentrations increase further, the effects on plants are negative resulting from damage to proteins and lipids in plant cells due to peroxidation(Bolwell and Daudi 2009).

To alleviate the damaging effects of H_2O_2, plants will utilize antioxidant enzymes such as POD, SOD, CAT, and PPO to protect their cells from severe damage (Zheng et al. 2010). When plants are under stress such as from pathogens, the host will start removing active oxygen by producing antioxidant protection enzymes to prevent further damage of the pathogen. In our study, however, in all treatments, including the no-AM fungus controls, the activity of SOD, POD, and CAT was greater in the non-inoculated plants than in plants inoculated with

B. sorokiniana, which was contrary to our expectation. This is difficult to explain because the presence of the pathogen would be expected to increase stress and so the activities of these enzymes should increase to help reduce the levels of induced reactive oxygen species. The lower activity of SOD, POD, and CAT in plants inoculated with *B. sorokiniana*, however, is in agreement with a study of cyclamen plants, which reported that infection with *F. oxysporum* and *C. gloeosporioides* decreased antioxidative activity of the infected plants (Maya and Matsubara 2013). The findings are also in agreement with research that shows that SOD activity decreased when rice leaves were inoculation with *R. solani*, and the activity decrease was most obvious in the fourth day (Zhang et al. 2006). That consequence may be related to the increased content of cellulose and lignin when plants suffered stress (Wu et al. 2008). Not unexpectedly, in nearly all of our treatments, the levels of these enzymes were higher in the E+ than E− plants and this is in agreement with the finding of the study of exposure of seedlings of the same perennial ryegrass cultivar to *B. sorokiniana* and four other pathogens (Ma et al. 2015).

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