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RESEARCH

Fungicide seed treatments delay arbuscular mycorrhizal fungi colonization of winter wheat in the greenhouse, but the effect is attenuated in the field

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Abstract

Seed-applied fungicides support agricultural production by controlling seed- or soil-borne diseases. However, they can impact non-target soil organisms. In this study, we investigated the effect of eight seed treatments (including two authorized for organic farming) on root colonization of winter wheat (*Triticum aestivum* L.) by arbuscular mycorrhizal (AM) fungi. One experiment was conducted in greenhouse conditions, on a sterile substrate inoculated with the AM fungus *Rhizophagus irregularis* MUCL 41833 and one in field conditions, where winter wheat was colonized by native soil AM fungi. In greenhouse conditions, the six conventional seed treatments reduced root colonization five weeks after sowing. No difference with the control treatment was measured thereafter for a product containing triazole alone. In contrast, seed treatments containing fludioxonil (fungicide molecule alone or formulated with the triazole difenoconazole), and prochloraz formulated with the triazole triticonazole significantly reduced root colonization until 11 weeks after sowing. Notably, when formulated with sedaxane, the adverse effect of fludioxonil was reduced. The negative effect of seed treatments on AM fungal root colonization in field was smaller than in the greenhouse and generally not significant, with disparate results from one timestep to another. This may be related to the dilution or the degradation of the active ingredients in the soil during the winter period or AM fungal species/strain involved in symbiosis. Overall, our results outline that the direct effect of seed treatment is highly variable depending on the modes of action, half-lives and interactions between active ingredients. By contributing to highlight the undesired effects of pesticides on AM fungi (i.e., by delaying root colonization), this study pleads for a reduction of pesticide applications to encourage the rapid and efficient establishment of functional mycorrhizal symbioses.

Keywords Seed coating · Pesticides · Systemic and contact fungicides · Non-target soil organisms · *Rhizophagus irregularis* · Ecotoxicity

Introduction

Fungicides are essential agricultural inputs that support crop production in the face of fungal disease threats. Their application as seed treatments is almost systematic in conventional agriculture, to control seed- or soil-borne diseases (Munkvold 2009; Cameron et al. 2017; Ayesha et al. 2021). They generally contain one to multiple active ingredients (a.i.) that target different fungal metabolic processes (Cameron et al. 2017), thereby increasing the uniformity of plant populations and crop yields (Gaspar et al. 2014; de Souza Buzo et al. 2022). However, seed treatment with fungicides has raised some concerns regarding impacts on non-target microorganisms (Karlsson et al. 2014; Prior et al. 2017;

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Vasanthakumari et al. 2019; Ayesha et al. 2021), including plant symbionts such as arbuscular mycorrhizal (AM) fungi. These soil fungi are able to enhance their host nutrition and growth while improving resistance against pathogen infection, pest attack and environmental stresses (Smith and Read 2008). As AM fungi maintain not only plant health but also soil quality and resilience (Smith and Read 2008; Gianinazzi et al. 2010), fungicides could profoundly affect the ecosystem services provided by AM fungi. These microorganisms are present in most if not all agricultural soils and are obligatorily associated with the majority of economically important crops likely to be exposed to plant protection products (PPPs) (Sweeney et al. 2022). The effects of fungicides on this group of soil fungi either can be direct by contact between the pesticide and AM fungi or indirect by affecting the physiology of the host plant (Calonne et al. 2012; Edlinger et al. 2022). The European Food Safety Authority (EFSA) has recently included these root symbionts in a list of potential bioindicators for monitoring the environmental risks of pesticides on soil microbiota with a view to approving PPPs (Ockleford et al. 2017; Karpouzas et al. 2022; Sweeney et al. 2022).

The application of fungicides as seed-treatments can have a direct impact on the establishment of the symbiosis, as these molecules are active when AM fungi are still in the pre-symbiotic phase, as reported by Hage-Ahmed et al. (2019). Contact (or protective) fungicides target a wide range of fungi and protect the plant from infection on leaf surfaces and stems. Their efficacy often requires repeated applications because of continuous possible infection throughout the growing season. On the other hand, systemic fungicides can be absorbed and transported in the different tissues of the plant where they are toxic to fungi (Babaikova et al. 2019). Seed treatments with mixtures of systemic (i.e., trifloxystrobin, pyraclostrobin, azoxystrobin, penflufen, sedaxane, mefenoxam, prothioconazole, metalaxyl, tebuconazole, triticonazole, prothioconazole) and contact fungicides (i.e., fludioxonil) a.i. did not show any impact on root colonization of important crops such as corn (*Zea mays* L.), soybean (*Glycine max* Merr.) and oat (*Avena sativa* L.) (Cameron et al. 2017). Similar results were observed on AM fungi naturally present in soil colonizing soybean crops for which seeds were treated with mefenoxam (a xylem-mobile systemic fungicide), applied in association or not with fludioxonil (Murillo-Williams and Pedersen 2008). To the contrary, seed treatment with systemic fungicides [i.e., Allegiance™ FL (metalaxyl), Apron Maxx® RTA® (fludioxonil and metalaxyl), Vitaflo® 280 (carbathiin and thiram), Crown® (carbathiin and thiabendazole), and Trilex® AL (trifloxystrobin and metalaxyl)] was detrimental to the AM fungal root colonization of pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.), whereas contact fungicides such as Thiram 75WP (thiram) and Agrox® FL (captan) did not affect the root colonization (Jin et al. 2013). Finally, in field conditions, corn seeds treated with fludioxonil reduced root colonization by naturally present AM fungi by 46% (Castelli et al. 2014). In strategies using AM fungi-based bioinoculants to control pests and diseases and to stimulate plant growth, such reductions in symbiosis establishment could be particularly problematic. Therefore, the compatibility between conventional seed treatments containing fungicides and the use of AM fungi-based bioinoculants is questionable.

Worldwide, wheat (*Triticum spp*.) is one of the most widely cultivated cereals alongside maize (*Zea mays* L.) and rice (*Oriza sativa* L.). Winter wheat (*Triticum aestivum* L.) is by far the most extensively grown wheat species, accounting for around 95% of total wheat production (Shewry 2009). In Belgium, it accounts for around 63% of all grain cereals, with about 200 000 ha under cultivation in 2023 (Statbel 2024). Cereal seeds used in conventional agriculture are systematically treated with one or more fungicides to protect the crop from seed- and soil-borne diseases such as wheat bunt (*Tilletia caries*), loose wheat smut (*Ustilago nuda f. sp. Tritici*), Septoria (*Septoria tritici / Leptosphaeria nodorum / Septoria nodorum*) and Fusarium wilt (*Fusarium spp.*). Even in organic farming, seed lots usually are treated, with diluted vinegar being the main product used to sanitize seeds. To our knowledge, no study has been carried out on the effect of fungicide seed treatments on the development of AM fungi-winter wheat symbiosis under controlled greenhouse and field conditions.

Therefore in the present study, the objective was to investigate the effect of all treatments authorized in Belgium to sanitize cereal seeds on the AM fungal root colonization of winter wheat, with the following working questions: (1) do seed treatments decrease, delay or have no impact on AM fungal root colonization of winter wheat and (2) does the effect of the treatments differ depending on the a.i. involved. Two experiments were conducted: (i) in the greenhouse, under strict controlled conditions, to evaluate the effects of different seed treatments on the dynamics of root colonization of winter wheat by the AM fungus *Rhizophagus irregularis* MUCL 41833 and (ii) in the field, under non-controlled soil and climatic conditions, to evaluate the effects of seed treatments on root colonization of winter wheat by native populations of AM fungi.

Materials and methods

Seed treatments

Eight seed treatments were considered (Table 1). All formulations were liquid, in the form of a concentrated suspension and applied by seed coating with a liquid seed treater (Hege 11, Wintersteiger). The formulations comprised either a single a.i. (i.e., Redigo, Celest and Difend) or a combination of two a.i. (i.e., Difend Extra, Kinto Duo and Vibrance Duo). The two most frequently used seed treatments in organic farming (i.e., vinegar and Cerall) also were considered.

Several treatments have a.i. in common. Prothioconazole belongs to the triazole family, along with triticonazole and difenoconazole. Other families of a.i. include imidazole (prochloraz), pyrrole (fludioxonil) and pyrazole-4-carboxylic acid amide (sedaxane). The active ingredients tested cover a wide range of modes of action (Table 1; Lewis et al. 2016).

To carry out the greenhouse and field experiments, 1500 g of seeds of winter wheat (*Triticum aestivum L.* var. Chevignon) with a thousand grain weight of 49.4 g were coated. The application was made in agreement with the European Regulation (EC) No. 1107/2009 concerning the placing of plant protection products on the market and the Royal

Table 1 Main characteristics and mode of action of the eight seed treatments (Lewis et al. 2016). The products were all authorized in Belgium as seed treatments of winter wheat at the time of the experiment. Concentration refers to the respective concentration of active ingredients in the solution (g l^{-1}), with acetic acid expressed in mass percentage (%) and *Pseudomonas chlororaphis* MA 342, expressed in colony forming units per ml (CFU ml^{-1}). The application rate corresponds to the volume of solution applied for the treatment of 100 kg of seeds

Commercial name	Active ingredient(s)	Chemical group of active ingredient(s)	Concentration	Application rate (ml for 100 kg seeds)	Systemic/ contact fungicide	Mode of action	Target diseases*
Celest	Fludioxonil	Phenylpyrrole	25 g l^{-1}	200	contact	Inhibits transport-associated phosphorylation of glucose; Impacts osmotic signal transduction	*Septoria tritici*, *Leptosphaeria nodorum*, *Fusarium spp.*, *Tilletia caries*
Difend	Difenoconazole	Triazole	30 g l^{-1}	200	systemic	Disrupts membrane function - inhibits demethylation during ergosterol synthesis	*Tilletia caries*
Redigo	Prothioconazole	Triazolinthione	100 g l^{-1}	100	systemic	Inhibits sterol biosynthesis	*Fusarium spp.*, *Ustilago nuda f. sp. Tritici*, *Tilletia caries*
Difend Extra	Difenoconazole	Triazole	25 g l^{-1}	200	systemic	Disrupts membrane function - inhibits demethylation during ergosterol synthesis	*Fusarium spp.*, *Tilletia caries*
	Fludioxonil	Phenylpyrrole	25 g l^{-1}		contact	Inhibits transport-associated phosphorylation of glucose; Impacts osmotic signal transduction	
Kinto Duo**	Prochloraz	Imidazole	60 g l^{-1}	200	contact	Broad-spectrum; Disrupts membrane function;	*Fusarium spp.*, *Ustilago nuda f. sp. Tritici*, *Tilletia caries*
	Triticonazole	Triazole	20 g l^{-1}		systemic	Inhibits sterol biosynthesis	
Vibrance Duo	Sedaxane	Pyrazole	25 g l^{-1}	200	systemic	Broad spectrum; Inhibits succinate dehydrogenase activity	*Septoria nodorum*, *Fusarium spp.*, *Ustilago nuda f. sp. Tritici*, *Tilletia caries*
	Fludioxonil	Phenylpyrrole	25 g l^{-1}		contact	Inhibits transport-associated phosphorylation of glucose; Impacts osmotic signal transduction	
vinegar	Acetic acid	Organic acid	4%	1000		Inhibits spore germination by acidification	*Tilletia caries*
Cerall	*Pseudomonas chlororaphis* (MA 342)	Bacteria	10^9-10^{10} CFU ml^{-1}	1000		Produces anti-fungal metabolites	*Septoria nodorum*, *Ustilago nuda f. sp. Tritici*, *Tilletia caries*

* The product is efficient against the indicated diseases (Phytoweb.be, consulted on the 18th of September 2024)

** Product non authorized in Belgium since the 1st of July 2023

Decree of 28 February 1994 on the conservation, marketing and use of pesticides for agricultural use in Belgium. Concentration of a.i. in the formulated products and application rates are reported in Table 1.

Greenhouse experiment

One hundred eighty pots (1.05 l - Pöppelmann) were prepared, i.e., nine treatments (eight seed treatments + one untreated control) with five replicate pots and four sampling dates per treatment. The pots were filled with 400 g of a mixture of fine sand n°4 (Euroquartz) and 0–5 mm diam. vermiculite (Pull Rhenen) in equivalent proportions in volume, sterilized at 121 °C for 15 min. *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) (Schüßler and Walker 2010) [as 'irregulare'] MUCL 41833 was supplied by the Glomeromycota in vitro collection (GINCO, Belgium) and used as the inoculant. The fungus was maintained for 6 months in the greenhouse on maize plants for mass-production. One gram of tap water-washed AM fungal-colonized root fragments (0.5 cm in size) was homogeneously layered 5 cm below the surface of the substrate. A porous membrane was placed at the bottom of each pot before filling to prevent the roots from growing out of the pot. In each pot, 20 grains of winter wheat were sown at a depth of 3 cm. The pots were moistened with demineralized water. One week after germination, the plants were thinned to 10 individuals per pot, evenly distributed. Through harvest, no plants died. During the growing period, the plants were capillary fed with 200 ml per week of ¼ Hoagland low-P solution (90% phosphorus-reduced – Garcés-Ruiz et al. 2017). During the experiment, the average temperature, relative humidity and light intensity in the greenhouse were 20.7 ± 0.5 °C, 44.9 ± 7.7% and 31.0 ± 31.9 W/m, respectively. Root colonization by *Rhizophagus irregularis* was evaluated 3, 5, 7 weeks after sowing, at the first stages of the establishment of the symbiosis and 11 weeks after sowing, at the end of the tillering stage of the plants (see below). At each sampling time, five pots per treatment were harvested and the entire root system was cleaned with demineralized water prior to quantification of the mean percentage of colonized root length (%CRL).

Field experiment

The field trial was located in Thorembais-Saint-Trond (50.60°N, 4.75°E) in Belgium. The soil of the field trial is classified as hortic Luvisol according to the World Reference Base Classification (IUSS working group WRB 2014), developed on quaternary loess completely free of rock (Vanwindekens and Hardy 2023). It has a silt-dominated texture (81% silt, 13% clay and 6% sand) and a soil organic carbon concentration of 10.3 g kg^{-1}, which is a typical value for the soils of the loess belt of Belgium under arable cropping for centuries. The soil is frequently limed and has a pH measured in 1 M KCl of 7.2. The most probable number (MPN) of infectious propagules of AM fungi was 0.72 per gram of soil, determined according to the method of Jarvis et al. (2010). A detailed description of soil analyses is provided in Supplementary Information (SI 1).

The trial was sown at a density of 300 grains/m² on 16 October 2020, after the harvest of common flax (*Linum usitatissimum L.*). The trial includes the nine seed treatments (eight treatments + one untreated control) present in four replicates, i.e., 36 individual plots distributed according to a complete random block design (SI 2). Individual plots were 10 m in length and 1.5 m in width, 15 m² in total. The trial was conducted without foliar fungicide application and with three-fraction nitrogen fertilization (54 uN on the 25 of February, 51 uN on the 24 of March and 72 uN on the 06 of June, for a total of 177 kg N ha^{-1}). Root systems were sampled on 23 March, 13 April, 4 May and 27 May 2021 (i.e., 22, 25, 28 and 31 weeks after sowing), to evaluate the mean %CRL. These sampling dates corresponded to the plant shifts from vegetative to reproductive growth (GS30) until the flag leaf fully emerged (GS39). The mean temperature (°C) as well as the daily rainfall (mm) at each day of the field experiment are mentioned in SI 3. For each individual plot at each sampling date, 10 to 15 root systems were collected from four cores (volumes of soil of approximately 8000 cm³, to a depth of 20 cm) centered on a row of plants and randomly distributed throughout the plot. Root systems from the four samples were pooled to compose a composite sample. Individual plots were harvested on the 23rd of July 2021. Results and statistical analysis of grain yields are presented in SI 4.

Root colonization by arbuscular mycorrhizal fungi

The roots were stained according to the method of Vierheilig et al. (2005) with some adaptations. Namely, before staining, the air-dried roots were cut into 1 cm fragments and remoistened for 24 h in demineralized water. They were then bleached and softened in a 10% KOH bath for 60 min at 70 °C, rinsed in demineralized water, neutralized with 1% HCl and stained with 2% royal blue ink (Parker Quink Permanent Ink) in a 1% HCl solution. Stained roots were kept in a lactoglycerol solution (v:v:v, 1:1:1, lactic acid: glycerol: water) until observation. Root colonization was determined under a compound microscope (Olympus BH-2, Olympus Optical GmbH) at magnification of 25 to 400x. For the greenhouse experiment, 20 root fragments of 1 cm were used to estimate the mean %CRL, using the line-intercept method, noting mean %CRL as the presence of either

hyphae, arbuscules or vesicles. One hundred intercepts were considered for each individual sample, or 500 intercepts per sampling date and treatment. For the field experiment, the mean %CRL was determined using the same method on 196 to 224 intercepts of 28 to 32 fragments of 1-cm length, for each individual sample, for a total of ~800 intercepts per sampling date and treatment. The higher number of observed roots for the field experiment than the greenhouse experiment is because of higher expected heterogeneity in field conditions and the sampling strategy, with 10 to 15 plants randomly harvested from each plot and mixed before staining.

Data analysis

Data were analyzed with R 4.3.3 (R Core Team 2024). For the greenhouse experiment, the mean %CRL data were analyzed by analysis of variance (ANOVA) according to the variables "Seed Treatment", "Time" and the interaction "Seed Treatment x Time". Prior to analysis, data were transformed using the square root function (sqrt) to ensure that the fundamental conditions of normality of model residues and homoscedasticity were respected. Additional ANOVAs were performed for each individual time step to determine significant differences between seed treatments for a given time step, assessed using a post-hoc Tukey HSD (Honestly Significant Difference) test with the package Agricolae (Mendiburu 2023).

For the field experiment, the mean %CRL data were first analyzed with a linear mixed model (package lme4, Bates et al. 2015) according to the variables "Seed Treatment", "Time" and the interaction "Seed Treatment x Time" as fixed factors and "Block" and "Plot" variables as random factors. To meet the requirements of normality of model residues and homoscedasticity, the data were sqrt-transformed prior to the analysis. Fixed effects were tested by ANOVA and random effects by likelihood ratio tests. Fixed effects of seed treatments and time were both significant, therefore data were further analyzed with a post-hoc Tukey HSD test. Then, individual timesteps were analyzed for the variables "Seed Treatment", "Block" and the interaction "Seed Treatment*Block". Prior to analysis, data were sqrt-transformed to meet fundamental conditions of normality of model residues (tested with the Shapiro-wilk test) and homoscedasticity (tested with the Levene test). Linear models were then tested by ANOVA. Significant differences between seed treatments or blocks for a given time step were determined by a post-hoc Tukey HSD test.

Results

Greenhouse experiment

For the greenhouse experiment, the effect of seed treatments ($F_{8,144}$ = 7.15; $p<0.0001$), time ($F_{3,144}$ = 430; $p<0.0001$), and their interaction ($F_{24,144}$ = 2.68; $p=0.0002$) were highly significant. Overall, the mean %CRL increased from the first to the fourth sampling date, with seed-treated winter wheat generally less colonized than the control, and a specific effect of individual treatments that differed from one sampling date to another (Table 2). Looking individually at each sampling time (Table 2), no significant differences between treatments were recorded after three weeks of growth. At week 5, all seed treatments except Vibrance Duo (containing fluodioxonil and sedaxane), Cerall and vinegar (the two treatments authorized in organic farming) induced a significantly lower mean %CRL by the AM fungus compared to the control. After 11 weeks, several seed treatments (i.e., Difend, Redigo and Vibrance Duo) had a similar mean %CRL as the control, whereas other treatments (Celest, Difend Extra and Kinto Duo) still had a significantly lower mean %CRL than the control. Finally, seeds treated with vinegar induced a significantly lower mean %CRL as compared to the control after 11 weeks, whereas no differences

Table 2 Mean (±standard deviation) percentages of colonized root length of winter wheat *by Rhizophagus irregularis* MUCL 41833 for the eight seed treatments and the untreated control, 3, 5, 7 and 11 weeks after sowing, under greenhouse conditions

Seed treatment	Mean (±s.d.) percentages of colonized root length							
	Week 3		Week 5		Week 7		Week 11	
Control	3.0±2.8	**a**	35.2±19.1	**a**	59.0±13.7	**a**	73.8±11.3	**a**
Celest	1.4±2.0	**a**	7.0±7.1	**b**	30.0±6.5	**b**	47.6±12.7	**bc**
Difend	0.0±0.0	**a**	7.4±8.7	**b**	39.0±10.6	**ab**	66.2±8.0	**ab**
Redigo	1.0±1.0	**a**	7.2±6.8	**b**	37.0±14.0	**ab**	70.4±4.8	**ab**
Difend Extra	0.8±1.3	**a**	8.2±6.1	**b**	40.0±12.7	**ab**	38.8±12.7	**c**
Kinto Duo	1.0±1.4	**a**	7.2±5.4	**b**	40.2±6.1	**ab**	41.8±8.0	**c**
Vibrance Duo	0.6±1.3	**a**	11.2±6.8	**ab**	41.4±14.2	**ab**	66.2±12.2	**ab**
vinegar	0.6±1.3	**a**	16.8±5.4	**ab**	45.6±10.7	**ab**	47.6±14.1	**bc**
Cerall	0.0±0.0	**a**	26.0±13.4	**ab**	36.4±16.2	**ab**	53.6±11.2	**abc**

In a column, means followed by the same letter do not differ significantly by an ANOVA followed by a Tukey-HSD post-hoc test for a single time point ($n=5$)

with the control treatment had been observed for the previous sampling times.

To further support interpretation of the effect of seed treatments on AM fungal root colonization, the results were grouped by families of a.i. (Fig. 1). Among the products containing triazoles (Fig. 1a), two distinct behaviors were observed: (i) the simple products (Difend, Redigo) significantly inhibited the mean %CRL after 5 weeks but the gap with the control was closed by the end of the experiment, after 11 weeks; (ii) the products containing two a.i. (Difend Extra and Kinto Duo) also significantly delayed the mean %CRL compared to the control from week 5 but this delay remained until the end of the experiment.

Among the products containing fludioxonil (Fig. 1b), Celest (fludioxonil alone) and Difend Extra (fludioxonil and difenoconazole) had a similar behavior, with a significant delay in mean %CRL compared to the control that was not recovered at the end of the experiment. Conversely, Vibrance Duo (which contains sedaxane in addition to fludioxonil) was not significantly different from the control throughout the experiment.

Field experiment

The general model identified a significant effect of both seed treatment ($F_{8,24}$ = 2.88; p=0.02) and sampling time ($F_{3,225}$ = 119.7; p<0.0001), the latter attesting to the progressive significant increase in mean %CRL by AM fungi between the first and the third sampling dates. The interaction "Seed treatment*Time" was not significant ($F_{24,225}$ = 1.36; p=0.13). From the likelihood ratio tests, random factors did not significantly affect the mean %CRL, with p-values of 0.51 (Chi^2 = 0.37) and 0.54 (Chi^2 = 0.43) for the block and the plot effects, respectively. Post-hoc Tukey HSD tests identified five groups among seed treatments: Control (a)>Vinegar, Cerall (ab)>Difend Extra, Difend, Celest, Vibrance Duo (abc)>Redigo (bc)>Kinto Duo (c).

Statistical analyses of individual sampling dates are presented in Table 3, and boxplots of the mean %CRL of winter wheat for individual seed treatments at the four sampling dates are presented in SI 5. Plants from the control treatment presented the highest mean %CRL from the second sampling date. Whereas p-values indicated a significant difference between seed treatments for three sampling dates out of the four (i.e., 13th of April, 4th of May and 27th of May), the post-hoc test only identified significant differences between seed treatments for the last sampling date (27th of May), with only Redigo having a significantly lower mean %CRL than the control. A discrepancy between the results of the ANOVA and the post hoc test could be attributed to the conservative character of p-adjustment for multiple comparisons of the Tukey HSD test. For the last sampling date, a significant difference in the mean %CRL was measured within blocks ($p \leq 0.001$), with mean %CRL being significantly smaller in Block 1 than in Blocks 2, 3 and 4. A slight interaction between seed treatments and blocks was measured at the 2nd sampling date (13 of April, p=0.047), without any significant difference calculated by the post-hoc test for either of the two factors.

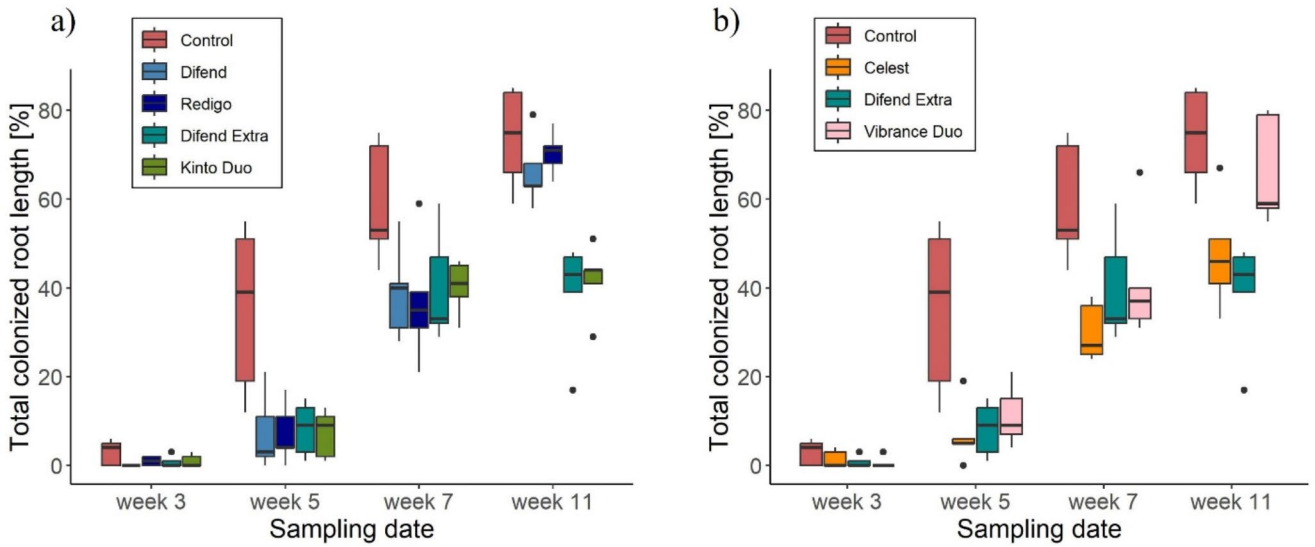

Fig. 1 Box-plots of mean percentages of colonized root length of winter wheat by *Rhizophagus irregularis* MUCL 41833 (**a**) for seed treatments containing triazoles and (**b**) for seed treatments containing fludioxonil 3, 5, 7 and 11 weeks after sowing under greenhouse conditions. Bold black line: median; box: interquartile range; whiskers highest and lowest data point within 1.5 interquartile range; dots: outliers outside the 1.5 times the interquartile range. Although outliers are illustrated, they were not excluded from analyses. Figures were drawn with the package ggplot2 (Wickham 2016)

Table 3 Significance levels (F-values and p-values) of the analysis of variance on the effects of 'seed treatment' and 'block' and their interaction for individual sampling dates of the field trial, and mean (± standard deviation (s.d.)) percentages of colonized root length of winter wheat by AM fungi naturally present in the soil for individual seed treatments and blocks at the four sampling dates (respectively 22, 25, 28 and 31 weeks after sowing)

	23 March		13 April		4 May		27 May	
	F-values (F) ; *p*-values (*p*)							
Seed treatment	$F_{8,36} = 1.31$; $p = 0.27$		$F_{8,36} = 3.24$; $p = 0.001$		$F_{8,36} = 2.30$; $p = 0.028$		$F_{8,36} = 2.90$; $p = 0.013$	
Block	$F_{3,36} = 1.26$; $p = 0.30$		$F_{3,36} = 1.04$; $p = 0.384$		$F_{3,36} = 1.64$; $p = 0.114$		$F_{3,36} = 9.97$; $p \leq 0.001$	
Seed Treatment * Block	$F_{24,36} = 0.59$; $p = 0.91$		$F_{24,36} = 1.85$; $p = 0.047$		$F_{24,36} = 1.47$; $p = 0.063$		$F_{24,36} = 1.43$; $p = 0.162$	
	Mean (± s. d.) percentages of colonized root length							
Seed treatments								
Control	0.7 ± 0.6	**a**	4.9 ± 2.9	**a**	10.4 ± 5.2	**a**	10.4 ± 5.0	**a**
Celest	0.7 ± 0.6	**a**	4.3 ± 2.9	**a**	7.8 ± 4.0	**a**	6.5 ± 3.9	**ab**
Difend	0.8 ± 1.3	**a**	3.9 ± 1.3	**a**	6.7 ± 1.8	**a**	7.5 ± 2.8	**ab**
Redigo	0.7 ± 1.2	**a**	2.1 ± 1.8	**a**	7.7 ± 3.1	**a**	5.0 ± 3.8	**b**
Difend Extra	0.9 ± 1.3	**a**	3.1 ± 2.7	**a**	9.5 ± 3.0	**a**	7.0 ± 3.5	**ab**
Kinto Duo	0.2 ± 0.5	**a**	1.9 ± 1.6	**a**	5.8 ± 2.3	**a**	5.6 ± 2.8	**ab**
Vibrance Duo	1.7 ± 2.1	**a**	2.9 ± 3.3	**a**	6.4 ± 2.1	**a**	8.7 ± 2.8	**ab**
vinegar	2.6 ± 2.1	**a**	4.9 ± 1.9	**a**	5.4 ± 4.1	**a**	8.6 ± 3.2	**ab**
Cerall	1.4 ± 2.3	**a**	4.6 ± 2.0	**a**	9.0 ± 4.9	**a**	8.7 ± 5.3	**ab**
	Mean (± s. d.) percentages of colonized root length							
Block								
Block 1	1.5 ± 2.1	**a**	3.4 ± 2.9	**a**	6.4 ± 2.9	**a**	4.5 ± 2.9	**b**
Block 2	1.2 ± 1.7	**a**	4.0 ± 3.0	**a**	7.1 ± 3.0	**a**	7.6 ± 3.3	**a**
Block 3	1.1 ± 1.3	**a**	3.3 ± 4.5	**a**	8.7 ± 4.5	**a**	9.2 ± 3.6	**a**
Block 4	0.5 ± 0.9	**a**	3.9 ± 4.1	**a**	8.4 ± 4.1	**a**	8.8 ± 4.2	**a**

In a column, means followed by the same letter do not differ significantly by an ANOVA followed by a Tukey-HSD post-hoc test for a single time ($n = 4$)

Discussion

The effect of single active ingredients on the AM fungal root colonization of winter wheat depends on their mode of action

In the greenhouse experiment, all tested seed treatments with one single a.i. diminished the mean %CRL of winter wheat, with differences being significant at one sampling date for all treatments. Difend and Redigo, containing difenoconazole and prothioconazole, respectively, significantly delayed the mean %CRL as compared to the control at week 5, an observation that disappeared after 7 and 11 weeks. Both a.i. are triazoles, known to affect ergosterol biosynthesis (Leroux 2003). Despite being heterotrophic organisms and obligate symbionts, AM fungi are able to synthetize their own sterols but do not produce ergosterol as do the majority of ascomycetes and basidiomycetes (Fontaine et al. 2001; Olsson et al. 2003). Nonetheless, just as for pathogenic fungi, triazoles were demonstrated to affect the growth of AM fungi, decreasing germination of spores, root colonization and extraradical growth and sporulation (Dodd and Jeffries 1989; Campagnac et al. 2008; Zocco et al. 2008; Calonne et al. 2010, 2012). This inhibitory effect was notably explained by a direct impact of propiconazole on the AM fungal extraradical mycelium by disturbing its sterol metabolism as in phytopathogenic fungi (Calonne et al. 2012). In the former study conducted under in vitro condition, the root colonization, although affected, was not totally inhibited, suggesting the degradation of the molecule in the culture medium. In the present study, a significant decrease of the mean %CRL was measured only 5 weeks after sowing, with a recovery measured at weeks 7 and 11. This recovery probably is explained by dilution of the a.i. in the substrate and the roots and/or the short half-life of the a.i., which was estimated from 10 to 21 days to more than 150 days for difenoconazole (a.i. of Difend) (Lewis et al. 2016; Man et al. 2021; Zhang et al. 2021) versus between 1 h and 14 days for prothioconazole (a.i. of Redigo) depending upon the growth conditions (Lewis et al. 2016; Wu et al. 2022).

Unlike the other seed treatments made of one single a.i., Celest (a.i.: fludioxonil) significantly decreased the mean %CRL compared to the control until the end of the experiment. Fludioxonil reduces mycelial growth by inhibition of transport-associated phosphorylation of glucose, preventing fungal respiration and osmoregulation (Lewis et. 2016, Wang et al. 2022). These results are concomitant

with the measured decrease in the mean %CRL after seed treatment with fludioxonil in association with metalaxyl of chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) in greenhouse conditions (Jin et al. 2013) and after sanitation of triticale (×*Triticosecale* Wittm.) seeds with a treatment containing fludioxonil, difenoconazole and tebuconazole (Caruso et al. 2018). The absence of root colonization recovery could be explained by the target metabolic site, the fact that fludioxonil is a contact fungicide directly affecting fungi, but also its half-life being much longer than that of triazoles in aerobic soil, from 164 to 219 days (Lewis et al. 2016) and from 119 to 559 days under typical environmental conditions (20 °C) in the dark in the laboratory (EFSA Scientific Report 2007).

Combinations of two active ingredients differentially affect AM fungal root colonization

In the greenhouse experiment, two contrasting behaviors in AM fungal root colonization were observed for seed treatments with a combination of two a.i. The two fungicides containing triazole [i.e. Difend Extra (fludioxonil and difenoconazole) and Kinto Duo (prochloraz and triticonazole)] significantly affected the mean %CRL through the end of the experiment (at week 11) while the formulations made of one single triazole (Difend and Redigo) were not significantly different from the controls at week 11. Inhibitory effects of seed-applied fungicides used in combination were observed in other studies (Jin et al. 2013; Caruso et al. 2018). The addition of fludioxonil (for Difend Extra) and prochloraz (for Kinto Duo) could explain the measured reduction in root colonization in linkage with the effect observed in presence of fludioxonil (Celest) applied alone. In the case of Difend Extra (fludioxonil + difenoconazole), both a.i. prevent the formation of fungal cell membranes by inhibiting sterol synthesis (difenoconazole) and inhibiting glucose phosphorylation (fludioxonil). Their addition did not induce an additive effect but a similar effect as measured for the fludioxonil applied alone.

Kinto Duo also affects sterol metabolism via the accumulation of non-functional sterols (triticonazole) and the inhibition of the enzyme lanosterol 14α-demethylase necessary for sterol synthesis (prochloraz). Prochloraz half-life was estimated between 8 and 18 days in soils (Fan et al. 2018), a relatively short half-life which suggests its rapid disappearance in soil, whereas a longer half-life (from 69 to 224 days) was reported by Lewis et al. (2016). The half-life of triticonazole was estimated from 84 to 112 days to more than 300 days in sandy loam soil (Ayliffe and Austin 1998; EFSA Scientific Reports 2005; Lewis et al. 2016). This persistent, systemic a.i. possibly affected the capacity of the AM fungi to colonize plants over an extended time period. These results suggest that the combination of a.i. prevented AM fungi from developing alternative metabolic pathways to circumvent toxicity.

In contrast, Vibrance Duo (fludioxonil + sedaxane) reduced the negative effect on the mean %CRL measured in presence of Celest (fludioxonil alone) for an equivalent amount of fludioxonil. This suggests a reduction of the toxic effect of fludioxonil on the AM fungal growth in the presence of sedaxane. Although we did not measure it, one possible explanation is improvement of photosynthetic activity and an increase CO_2 assimilation in wheat (potentially available for the AM fungi growth and metabolism), as suggested by Dal Cortivo et al. (2017) in maize cultivated in an unsterilized sand-soil mixture containing increasing concentrations of sedaxane. Overall, these results underline that the direct effects of fungicides on AM fungi may mostly differ depending on the combination of a.i. Nevertheless, the accumulation of pesticide residues and metabolites in soil over time appears to be overall adverse for AM fungal activity (Riedo et al. 2021).

For products containing fludioxonil (Celest, Difend Extra and Vibrance Duo), which have the same additive (1,2-benzisothiazol-3(2 H)-one), a potential effect of additives (indicated in SI 7) could be excluded. To date, the effect of additives on AM fungi is not yet known.

The effect of seed treatments on AM fungal root colonization is attenuated under field conditions in comparison with greenhouse conditions

In the field experiment and similarly to the greenhouse experiment, the control treatment had the highest mean %CRL by AM fungi overall, which supports the view that seed treatment with fungicidal formulations tends to decrease root colonization of winter wheat in field conditions.

As for the greenhouse experiment, the first sampling date showed similar (low) mean %CRL in all treatments, possibly because these samples represented the early colonization event (less than 3% of mean %CRL in both growth conditions). In contrast with the greenhouse experiment, the mean %CRL was much lower in field conditions, 10.4% for the control at the last sampling date (i.e., more than 7 months after sowing) in comparison with 73.8% for the control in the greenhouse at the last sampling date (i.e., 11 weeks or 2.5 months after sowing). These percentages accord with observations of control plants of winter wheat in the field which ranged from 9.9 to 18.2% depending upon the cultivar (Kirk et al. 2011) and from 6.5 to 16 depending upon the plant growth stage (Brito et al. 2012; Pellegrino et al. 2020).

In the field experiment, only Redigo (statistically significantly for the last sampling date and for the total period of sampling) and Kinto Duo (statistically significantly only

for the last sampling date) appeared to decrease the mean %CRL of winter wheat. This contrasts with the greenhouse experiment, for which all treatments but Cerall were significantly lower than the control for one timestep at least. One discrepancy between the field and greenhouse is the absence of effects of the treatments containing fludioxonil (Celest, Difend Extra, Vibrance Duo), an inhibitor of sterol metabolism, in field conditions. Fludioxonil being a contact fungicide, the long period of vernalization between sowing and the first sampling date could have contributed to dissipating the molecule by leaching or dilution in the rhizosphere. On the other hand, triazoles as prothioconazole (component of Redigo) and triticonazole (component of Kinto Duo) are systemic. Their activity in root tissues could have affected the root colonization of wheat by the AM fungi more persistently than the other products. The absence of an effect in the field of difenoconazole, a systemic a.i. component of Difend and Difend Extra could be explained by a different structural composition but also by its lower concentration in seed coating, 40 to 50% smaller than the a.i. of Redigo.

The grain yield, 8818 kg ha^{-1} on average, was not significantly affected by seed treatments ($F_{8,32} = 1.73$, $p = 0.14$; SI 4), regardless of their effects on root colonization. The lack of association between mean %CRL and grain yield does not negate similar mycorrhizal dependency or similar nutrient transfer from the fungi to the plant via the mycorrhizal pathway.

Several factors may have contributed to the less marked results in the field compared with the greenhouse: (i) the complexity of the soil matrix in comparison with the growth substrate used in the greenhouse, which could have led to a higher sorption of pesticides and/or a faster degradation through microbial metabolism; (ii) the volume of soil in the field experiment is much larger than in the pots of the greenhouse experiment. This could have resulted in a strong dilution effect of the molecules in the field experiment and therefore a lower concentration of the molecules in contact with spores or intraradical fungal structures. For instance, a diffusion effect of tebuconazole and prothioconazole coated around seeds to the surrounding soil clearly has been demonstrated (Albers et al. 2022). (iii) the diversity of AM fungi in the field does not rule out the possibility that some species/strains are more resistant to fungicides and therefore could colonize the plants. Furthermore, because the soil is cultivated in conventional farming and therefore regularly sprayed with pesticides, some species/strains may have acquired some resistance to fungicides which is absent from the standard strain (*R. irregularis* MUCL 41833) used in the greenhouse experiment. (iv) in the field, the spatial distribution of native AM fungal propagules in soil may be quite heterogeneous, which may explain the relatively high dispersion of the data, decreasing the power of the statistical analysis. Finally, (v) in the field, the delay between seed sowing and the mean %CRL of winter wheat was much longer than in the greenhouse. A possible degradation or dilution of the fungicides could have occurred between sowing and the first sampling date (i.e., after 5 months) during which the AM fungi were inactive because of the winter period. Indeed, soil temperatures below 10 °C prevented the AM fungal symbiosis between *Funneliformis mosseae* and winter wheat from establishing or developing (Daniels Hetrick and Bloom 1984). As a result, the root colonization of winter crops by AM fungi can be delayed to the spring, when soil temperature increases above ~10 °C (Daniels Hetrick et al. 1984). Consistently, under the oceanic temperate climatic conditions of Wallonia, Belgium, winter wheat generally is sown from mid-October to mid-November, when soil temperature already is about 10 °C or below. As a result, roots of winter wheat from the field trial started to be colonized by the AM fungi naturally present in the soil when the first sampling occurred in March (e.g., less than 2.6%CRL on March 26). Seed coating formulations seem therefore to have had limited effects on the mean %CRL of winter wheat by AM fungi in field conditions, but stronger effects might be expected on mean %CRL of a spring crop such as *Zea mays L*. or spring cereals.

Seed treatments authorized in organic farming have a smaller impact than others

The seed treatments approved for organic farming (vinegar, Cerall) affected AM fungi in different ways. Whereas Cerall (composed of *Pseudomonas chlororaphis* MA342) did not significantly affect the AM fungal root colonization either in the greenhouse or in field conditions, vinegar surprisingly decreased root colonization under greenhouse conditions at the last sampling date. A dilution effect (i.e., an increase in root growth induced by vinegar diluting AM fungal colonization across increased root volume) cannot be excluded as an explanation for this result. This discrepancy with the field experiment, for which no significant effect of vinegar was measured, could be explained by dilution, leaching or degradation of vinegar in field conditions. The same is true for chemical fungicides, but to a lesser extent, because the solubility of acetic acid is several orders of magnitude higher than that of other a.i. considered in this study (SI 6). An interesting perspective could be to test how seed treatments affect root colonization by AM fungi of spring cereals, for which the delay between seeding and root colonization by AM fungi is expected to be much shorter than for winter wheat. At this time, however a potential ecotoxicological effect of vinegar on AM fungi cannot be excluded. Another important point is that plant protection against seed diseases (e.g., wheat bunt) by vinegar is partial, and the efficacy of

Cerall in controlling wheat bunt has been shown to be even less (Fontaine et al. 2013; Bataille et al. 2021). Therefore, our results plead for the development of efficient alternative seed sanitization methods for organic farming to ensure the safety of the food chain and of soil beneficial microorganisms such as AM fungi. Several alternatives currently are under development or commercially available that can be applied as a seed coating. These include beneficial microorganisms such as AM fungi or plant growth promoting rhizobacteria as in Cerall, laminarin produced by the brown alga *Laminaria*, and chitooligosaccharides – oligogalacturonides (COS-OGA).

Conclusion

The results of this study support the view that seed treatments with fungicidal formulations may have undesired effects on root colonization of winter wheat by AM fungi. However, the direct effect of specific seed treatments on the AM fungal activity is hardly predictable because it may differ depending on several factors among which the modes of action, the half-lives and combinations of a.i., the AM fungal species/strains and the plant/fungi developmental stages. In field conditions, Redigo and Kinto Duo, two products containing systemic triazole fungicides, significantly decreased the mean %CRL by AM fungi, whereas no significant differences were measured for the other treatments. The attenuated effects of seed treatments in the field compared to greenhouse conditions may be attributed, at least partly, to the delay between sowing and the start of colonization (i.e., 5 months) of winter crops, leading to a likely dilution and/or degradation of the a.i. It might also relate to the larger heterogeneity of field conditions, responsible for a higher dispersion of data, decreasing the power of statistical analyses. Our results suggest that observations made in controlled conditions can be overinterpreted in comparison with the responses in real field conditions. Nevertheless, a stronger impact of fungicidal formulations applied as seed coatings cannot be excluded for spring crops such as maize or spring cereals that are expected to be colonized by AM fungi shortly after sowing. Overall, the present study highlights the potential unmeasured and undesired effects of PPPs on the colonization capacity of AM fungi. This pleads for a reduction in the use of pesticides and for improved assessment of the potential harmful effects of pesticides on non-target soil organisms.

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Author contributions All authors contributed to conception and design of the study. Material was acquired and prepared by BHa, EB and MCS. EB, BHa and MCS set up and conducted the experiments. EB quantified AMF colonization in winter wheat roots, under the guidance of MCS, SD and BHa. BHa analyzed the data with the help of EB and MCS. BHa drafted the manuscript, which was further commented, completed or corrected by all authors. All authors read and approved the final manuscript.

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Data availability Raw data from the greenhouse and field experiments are attached to the manuscript as a supplementary material.

Declarations

Competing interests The authors declare no competing interests.

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