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Role of the Fungicide Seed Dressing in Controlling Seed-Borne *Fusarium* spp. Infection and in Enhancing the Early Development and Grain Yield of Maize

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Abstract: *Fusarium* spp. are key pathogens in maize seeds and seedlings. The aim of this study has been to evaluate the effects of applying fungicides to maize seeds to increase the survival of seedlings and to enhance the early vigor and grain yield of the plants. The protective effects of 2-way (fludioxonil, metalaxil-M) and four-way (fludioxonil, metalaxil-M, azoxystrobin, thiabendazole) fungicide seed treatments were compared with an *F. verticillioides* seed infected control in 11 field trials carried out in North Italy. A second study focused on the impact *F. verticillioides* and *F. graminearum* seed-borne infection on plant growth and on the possible advantages of applying the previously reported seed dressing. The seed dressing increased the plant density, vigor during the whole vegetative growth cycle for all the production situations, and grain yield. *F. verticillioides* led to a higher seedling mortality than *F. graminearum*, while both species reduced plant growth and delayed the flowering date. Seed-borne infection has an important impact on both the population and vigor of maize plants. The four-way fungicide enhanced both the defense of the seedlings and the vigor of *F. verticillioides* infected plants, which in turn resulted in a significant improvement in grain yield, compared to a conventional two-way fungicide.

Keywords: *Zea mays*; early vigor; *Fusarium verticillioides*; *Fusarium graminearum*; fludioxonil; metalaxil-M; azoxystrobin; thiabendazole

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1. Introduction

Soil contains a large and variable quantity of potentially pathogenic microorganisms, such as fungi, bacteria and viruses that interact with seeds and seedlings. Maize (*Zea mays* L.) seeds and seedlings are susceptible to infection from a number of fungal pathogens. This leads to the emergence of a small number of plants and, thus, heavy potential yield losses, for crops characterized by precision sowing and when there is not the possibility of self-regulating the plant population by means of tillering [1]. Moreover, the plant population may be reduced to such an extent that replanting is necessary. Numerous soil fungi are associated with maize seedling diseases, such as *Fusarium*, *Pythium*, *Rhizoctonia* and *Phytophthora* [2]. Among these, the *Fusarium* genus is the most widespread fungus that affects maize in temperate areas. Several *Fusarium* species can infect the seeds, seedlings and plants (root, stem) of maize and this can result in pre- or post-emergence damping-off [3,4]. The most common *Fusarium* species isolated from maize crops in temperate maize growing areas are *F. verticillioides* (Sacc.) Nirenberg, and *F. graminearum* Schwabe. These fungal pathogens can survive in the soil or on crop debris [5] and they affect the seeds and seedlings after planting germination (soil-borne infection). Moreover, seed inoculation with *Fusarium* spp. (seed-borne infection) may contribute to the stand losses caused by damping-off, particularly if seeds are produced in growing areas prone to *Fusarium* ear rot during ripening [6]. Previous studies that were conducted to evaluate the contribution of *F. verticillioides* seed-borne inoculum to maize seedling blight epidemics reported conflicting results [6–8]. However, there is a general agreement on the negative effect of seed-borne *Fusarium* spp. on germination and on a reduced seedling growth [3,9], while no field studies have highlighted the subsequent influence on plant growth or development considering the delay of anthesis and kernel maturity.

In North Italy, maize, with 800,000 ha located in the Po plain, is the most important crop, playing a key role in supporting agri-food supply chains. Prevention in the field is generally ineffective in reducing seed-borne infection, although any crop practice that favors a rapid germination and seedling growth can help to minimize its contribution to damping-off. However, only a few agronomic solutions are effective in reducing *Fusarium* soil-borne infection. Of these solutions, crop rotation or burying debris may be able to reduce the potential of soil inoculum [10]. Furthermore, in North Italy, as in several other temperate growing areas, the agronomic tendency is to anticipate the sowing time to such times when the soil temperature is above 10 °C in order to reduce water stress and injuries from insects and disease during ripening [11], which leads to a positive effect on grain yields. This practice, in addition to the application of strip tillage or other minimum tillage practices on cereal farms, has led to more critical conditions for seedling mortality and plant development due to the longer germination. A high plant density is required to fully benefit from the yield potential of modern hybrids [12], and the possible reduction in plant population after sowing led to clear yield gap. Thus, direct control solutions are necessary to minimize the risk associated with seedling mortality and the influence of fungal infection on plant growth and development. The use of chemicals is considered the best option for this purpose. As a result of the low cost and the specific action of fungicide seed treatments, they are considered an excellent solution to reduce early seed attacks from soil pathogens and to ensure emergence, even under critical environmental conditions [13]. Moreover, fungicide seed dressings may contribute to minimizing the damping-off and growth delay related to seed-borne infection [14]. Over the last two decades, fungicides from the phthalimide chemical family (e.g., captan) and dithiocarbamate (e.g., thiram) have progressively been substituted by phenylpyrroles (e.g., fludioxonil) phenylamides (e.g., metalaxyl, metalaxyl-M) and benzimidazoles (e.g., thiabendazole, carbendazim) [15]. A two-way fungicide combination (two active ingredients for a specific pathogen target) that is widely applied for maize seed dressing is fludioxonil and metalaxyl-M, the former of which shows good activity against *Fusarium* and *Rhizoctonia* spp. [16], while the latter is more effective on *Pythium* and *Phytophthora* [17,18]. Both of the previously reported compounds are non-systemic fungicides. Moreover, some of the fungicide formulations that have recently been proposed on the market are often mixtures of several active ingredients, which have different modes of action, in order to increase the control of a wide spectrum of pathogens. Strobilurins (e.g., azoxystrobin, pyraclostrobin and trifloxystrobin), triazoles (e.g., difeconazole, tebuconazole and prothioconazole) and pyrazole carboxamide (e.g., sedaxane) families, which are all characterized by a systemic activity, are some of the recent active ingredients that have been used for maize seed dressings.

Most of the studies conducted to evaluate the effect of fungicide seed treatments on *Fusarium* species have been performed in vitro, in growth chambers or in greenhouses to investigate the plant–fungus pathosystem in the first growth stages [3,19], while only a few experiments have taken into account the complete crop cycle under field conditions. An even smaller number have investigated the effects of fungicide seed treatments on plant growth and development until harvest [20], and in particular on grain yield [18,21]. Moreover, no information is available on the different effects of seed infection by *F. graminearum* and *F. verticillioides* from planting to harvest. The aim of the study has been to evaluate the role of fungicides applied to maize seeds in order to minimize the damping-off caused by seed-borne *Fusarium* infection and to enhance the early vigor of plants and grain yield under different production conditions.

2. Materials and Methods

2.1. Experimental Site and Treatments

Two different open field studies were carried out in North-West Italy to evaluate the ability of different fungicides to reduce the negative effect of seed infection from different *Fusarium* species on maize plants and their role in promoting a better plant development in the early stages. The first study was focused on *F. verticillioides* seed infection and considered a large number of production situations over a 5-year period (2015–2019). The second study was carried out in 2018 and 2019 and it was conducted to compare the efficacy of different fungicide applications in controlling *F. verticillioides* and *F. graminearum* seed infection.

2.1.1. Study I. Fungicide Seed Dressing Used to Control F. verticillioides Damage on Maize Seedlings and Plants under Different Environmental Conditions

Eleven field experiments were set up, from 2015 to 2019, in three locations: Chivasso (260 m above sea level, a.s.l), Poirino (249 m a.s.l.) and Carignano (240 m a.s.l.). At Chivasso and Poirino, the study was carried out on private farms with a long history of cereal cultivation, while the study in Carignano was conducted in the experimental fields of the University of Turin. The main physical and chemical characteristics of the soil in each site are reported in Table 1. The study in Carignano was carried out over all the growing seasons, while the study in Chivasso was performed in 2016 (with two different sowing times, first and second, considered as different experiments), and 2017 and 2018. The trial in Poirino was only conducted during the 2015 growing season. Daily temperatures and precipitations were measured at the meteorological stations of the Regione Piemonte located near (within 5 km) each experimental site.

Table 1. Main physical and chemical characteristics of the soils in the experimental sites.

Parameter		Chivasso	Carignano	Poirino
GPS Coordinate		N 45°12′42.4″ E 7°55′46.5″	N 44°53′10.6″ E 7°41′11.8″	N 44°54′55″ E 7°52′14″
USDA classification		Inceptic Hapludalf	Typic Ustifluvent	Aquic Haplustept
Soil texture		loam	silty loam	silty loam
Sand (2000–50 µm)	%	45.1	28.7	23.6
Silt (50–2 µm)	%	45.3	64.6	62.7
Clay (<2 µm)	%	9.6	6.7	13.7
Cation-exchange capacity	meq/100 g	12.5	12.2	15.8
Total limestone	%	-	1.8	1.2
pH		6.2	8.0	6.1
Organic matter	%	2.51	1.45	1.48
Total nitrogen	%	0.15	0.11	0.09
Assimilable phosphorus	mg kg^{-1}	47	7	17
Exchangeable potassium	mg kg^{-1}	118	49	135

The following fungicide seed treatments were compared in each trial under artificial infection conditions:

- Untreated control;
- Two-way fungicide, a mixture of fludioxonil (25 g L^{-1}) and metalaxil-M (10 g L^{-1}) applied at 6.25 g to 25,000 seeds (Celest® XL, Syngenta AG, Basel, Switzerland);
- Four-way fungicide, a mixture of fludioxonil (37.5 g L^{-1}), metalaxil-M (29 g L^{-1}), azoxystrobin (15 g L^{-1}) and thiabendazole (300 g L^{-1}), applied at 4.25 g to 25,000 seeds (Celest® Quattro, Syngenta AG).

Disinfected maize seeds, by soaking for 10 min in a 5% sodium hypochlorite solution, were artificially inoculated, before each fungicide seed dressing, by soaking them for 12 h in a conidic suspension of *F. verticillioides* (10^6 ufc mL^{-1}, using a strain isolated from grain of maize grown in

North Italy; the strain pathogenicity for maize seedlings has been previously verified in a preliminary greenhouse trial) and then drying them in air before the fungicide treatment. Fungicides were applied as water-based slurry using an automatic seed treater (Hege 11, Wintersteiger, AG, Ried im Innkreis, Austria). The treated seeds were air-dried and then stored at 4 °C for approximately 30–45 days prior to use. The fungicide treatments in each location were assigned to the experimental plots using a completely randomized block design, with four replicates. Each plot measured 30 m^2 (10 × 3 m) and consisted of four rows 0.75 m apart. All the measurements were conducted in the two middle rows.

In order to quantify and summarize the benefits of seed-dressing fungicide application in different scenario, according to the disease pressure, the results have been organized into three different groups. Data on seedling mortality recorded in each trial for the untreated control were used to cluster the experiments in the following classes: medium-high (mortality from 19% to 44%, five cases), high (mortality from 57% to 72%, three cases) and extremely-high (mortality from 90% to 94%, three cases). The subdivision of the experiments into seedling mortality classes is reported in Table 2. The previous crop in each experiment was maize. In all the locations, seeds of previous maize crops were always treated with the conventional two-way fungicide (mixture of fludioxonil and metalaxil-M). According to the conventional crop techniques of the growing area, planting was always carried out after autumn ploughing to a depth of 0.3 m, incorporating crop debris into the soil, followed by disk harrowing to prepare a proper seedbed. The maturity class (FAO 400 - 700) of the tested hybrids was selected according to the characteristics of the growing area and the adopted planting time. The maize hybrid, the sowing and harvest dates for each experiment are reported in Table 2. The maize seed were planted using a plot seeder, and the sowing density was eight plants per m^2 (six plants per linear meter of row). Phosphorus and potassium were applied before harrowing in each site according to the ordinary management practices of the farms. No starter fertilizers were distributed in the seed furrows at sowing to enhance the early vigor of the maize, but the micro-granulated soil insecticide tefluthrin was applied at 100 g AI ha^{-1} (Force®, Syngenta Crop Protection S.p.A, Basel Switzerland) close to the seed furrow, to protect seedlings and plants from injuries by ground insects. After sowing, a chemical weed control was carried out at pre-emergence on the soil surface with mesotrione (150 g AI ha^{-1}), S-metolachlor (1.25 kg AI ha^{-1}) and terbuthylazine (0.75 kg AI ha^{-1}) (Lumax®, Syngenta Crop Protection S.p.A.).

Table 2. Main agronomic information of the experimental sites clustered for seedling mortality recorded in each trial for the untreated control.

Seedling Mortality	Experiment	Year	Site	Sowing Time	Main Agronomic Information		
					Hybrid	**Sowing Date**	**Harvest Date**
Medium-high	A	2015	Carignano	First	NK Helico	2 April	10 September
	B	2015	Poirino	First	NK Helico	14 May	28 September
	C	2017	Chivasso	Second	NK Gigantic	10 May	14 September
	D	2018	Carignano	First	SY Zoan	20 April	18 September
	E	2018	Chivasso	First	SY Zoan	20 April	11 September
High	F	2017	Carignano	First	NK Gigantic	30 March	9 October
	G	2017	Chivasso	First	NK Gigantic	21 March	7 September
	H	2019	Carignano	First	SY Hydro	1 April	4 October
Extremely-high	I	2016	Carignano	First	NK Galactic	30 March	4 October
	L	2016	Chivasso	First	NK Galactic	23 March	14 September
	M	2016	Chivasso	Second	NK Galactic	15 April	14 September

The amount of nitrogen required to obtain the expected yield in each site was distributed in coverage in one solution at the 8th unfolded leaf growth stage (GS) using urea (46%). Different irrigation systems were adopted, according to the typical farm management practices used in the area, in order to avoid any drought stress for the crops: the furrow method was applied in Chivasso, while sprinkler was conducted in Carignano and Poirino.

2.1.2. Study II. Fungicide Seed Dressing to Control F. verticillioides and F. graminearum Damage on Maize Seedlings and Plants under Different Environmental Conditions

A field experiment was set up in 2018 (Chivasso) in 2019 (Carignano) in the previously described locations in order to further investigate the role of fungal infection and of the fungicide seed treatments on maize plant vigor. The compared treatments in each trial were factorial combinations of:

- Fungal seed infection (10^6 ufc mL^{-1}) before seed dressing, with the same previously reported procedure (study I):
    - ○ *F. verticillioides* artificial inoculation;
    - ○ *F. graminearum* artificial inoculation.
- Fungicide application as a seed dressing:
    - ○ Untreated control;
    - ○ Two-way fungicide, a mixture of fludioxonil (25 g L^{-1}) and metalaxil-M (10 g L^{-1}) applied at 6.25 g to 25,000 seeds (Celest® XL, Syngenta Crop Protection S.p.A, Basel, Switzerland);
    - ○ Four-way fungicide, a mixture of fludioxonil (37.5 g L^{-1}), metalaxil-M (29 g L^{-1}), azoxystrobin (15 g L^{-1}) and thiabendazole (300 g L^{-1}), applied at 4.25 g to 25,000 seeds (Celest® Quattro, Syngenta Crop Protection S.p.A, Basel, Switzerland).

An uninfected check, without fungal inoculation and fungicide application as seed dressing, was included in the experimental design, to quantify the influence of different fungal infections on plant development. This treatment was considered as a reference control to comprehend the role of fungicide in recovering an optimal early vigor. The seed inoculation and fungicide seed treatments were carried out as previously reported. The *Fusarium* strains have been isolated from grain of maize grown in North Italy and their pathogenicity on maize seedling has been previously verified in a greenhouse preliminary trial.

Treated and control seeds were assigned to the experimental plots each year using a completely randomized block design, with four replicates. Each plot measured 30 m^2 (10 × 3 m) and consisted of four rows 0.75 m apart. The crop management was carried out as previously described.

2.2. Crop Assessments

2.2.1. Emergence and Crop Density

Seedling emergence was calculated by counting the number of plants in the two middle rows of each plot for a length of 3 m, when approximately 100% of the seeds had emerged, and at least a few days after the beginning of the emergence stage. Data on seedling mortality recorded for the untreated control were used to cluster the experiments in study I into the three previously reported groups.

2.2.2. Crop Vigor

Different assessments were performed to establish vigor in the early vegetative stages. At the stem elongation stage (GS 32–35, BBCH Scale, [22]), the number of nodes that had completely developed was counted and the heights of the plants from the last node developed close to the ground were measured. This measurement was performed at the same time on 10 randomly selected plants from each plot.

The normalized difference vegetation index (NDVI) was measured using a hand-held optical sensing device, GreenSeekerTM® (Trimble, Sunnyvale, California, the USA). The NDVI measurement helped to quantify the development of the crop canopy throughout the season, since low values refer to naked soil, while high value is proportional to maize biomass. This device has its own consistent light emission source, photodiode detectors and interference filters for red [Red] and near infrared

[NIR] wavelengths in the 671 ± 6 nm and 780 ± 6 nm spectral bands, respectively; it provides the Normalized Difference Vegetation Index (NDVI), which is calculated as follows [23]

$$\text{NDVI} = \frac{\text{RNIR} - \text{RRed}}{\text{RNIR} + \text{RRed}} \tag{1}$$

where RNIR is NIR radiation reflectance and RRed is visible red radiation reflectance. The instrument was held approximately 60 cm above each single maize row and its effective spatial resolution was 0.75 m × the full length of the plot (10 m). This assessment was performed every 7 days, in the two middle rows of each plot, starting from the four-leaf stage (GS 14) until tassel emission (GS 55). The Area Under the Canopy Development Curve (AUCDC) was calculated, starting from the NDVI measurements, using the following formula

$$\text{AUCDC} = \sum_{i}^{n-1} \{[(R_i + R_{i+1})/2]\ (t_{i+1} - t_i)\} \tag{2}$$

where R is the NDVI value, t is the time of observation and n is the number of observations.

The plant growth rate was calculated as average daily NDVI increase during the vegetative period.

Date was registered when 50% of the plants in each plot reached the beginning of ear flowering (GS 62), albeit only for study II. This parameter was expressed as the day after flowering (DAS).

2.2.3. Grain Yield and Moisture

Ears were collected by hand at harvesting from 4.5 m^2 in each plot to quantify the grain yield. The ears were shelled using an electric sheller, and the kernels from each plot were mixed thoroughly to obtain a random distribution. A sample taken from the bulk production harvested in each plot was used to determine the grain moisture content, using a GAC® 2000 Grain Analyser (Dickey-John Auburn, IL, USA). The grain yield results were adjusted to a commercial moisture level of 14%.

2.2.4. Statistical Analysis

Normal distribution and homogeneity of variances were verified by performing the Kolmogorov–Smirnov normality test and the Levene test, respectively. In study I, an analysis of variance (ANOVA) was utilized for each seedling mortality group to compare all the detected parameters, using a randomized complete block in which the fungicide seed dressings and the experiment were the independent variables. In study II, an analysis of variance (ANOVA) was utilized to compare all the detected parameters, using a randomized complete block in which the seed treatment (combination of fungal infection and fungicide seed dressings) and the year were the independent variables.

Multiple comparison tests were performed in both studies, according to the Ryan–Einot–Gabriel–Welsh F (REGW-F) test [24], on the treatment means ($p < 0.05$). SPSS, version 25 (SPSS, IBM Corporation, Armonk, NY, USA, 2008), was used for the statistical analysis.

3. Results

3.1. Metereological Trends

The meteorological trend observed for each experimental field is reported in Table 3, considering both the data collected after the first 50 days after sowing and those pertaining to the whole crop cycle. The parameter that had an important impact on the clustering of the experiment, according to the seedling mortality, is the temperature in the period that followed planting: the growing degree days (GDDs) were higher (490 °C-day, with an average heat accumulation for maize of 9.8 °C) in experiments A, B, C, D, E, which were characterized by a higher seedling survival than those with high or extremely high mortality (282 °C-day, with an average heat accumulation for maize of 5.6 °C). Conversely, a distant relationship was observed between rainfall and seedling mortality: experiment I and L, both of which showed an extremely-high mortality of maize seedlings, reported the lowest and highest recorded rainfall both for the period after sowing and the whole cycle.

Table 3. Meteorological data [1] for the first 50 days after sowing and from sowing to harvest in the experimental sites clustered by seedling mortality.

Seedling Mortality	Experiment	First 50 DAS [2]		Whole Cycle [3]	
		Rainfall	GDDs [4]	Rainfall	GDDs
		(mm)	(°C-day)	(mm)	(°C-day)
Medium-high	A	132	351	289	1723
	B	147	568	354	1670
	C	324	594	565	1590
	D	323	442	439	1773
	E	448	496	650	1738
High	F	118	299	268	1980
	G	320	293	866	1814
	H	184	237	482	1848
Extremely-high	I	185	277	280	1871
	L	124	258	474	1726
	M	182	328	454	1616

[1] Data obtained from the agrometeorological service of the Regione Piemonte. [2] Days after sowing (DAS). [3] From sowing to harvest. [4] Growing degree days (GDDs): Accumulated growing degree days for each experiment for the first 50 days from sowing and for the whole cycle, using a 10 °C base.

3.2. Study I. Fungicide Seed Dressings to Control *F. verticillioides* Damage on Maize Seedlings and Plants under Different Environmental Conditions

The plant density at emergence and at harvesting was clearly affected by the artificial *F. verticillioides* inoculation, and showed a significant ($p < 0.001$) effect of fungicide seed dressing in all seedling mortality groups (Table 4). The two-way fungicide significantly increased the number of plants per square meter at emergence and at harvest, compared to the infected untreated control, in all the seedling mortality groups. On average, the recorded mortality was 30%, 67% and 91% in the untreated control for the medium-high, high and extremely-high mortality conditions, respectively, compared to the theoretical plant density (eight plants m^{-2}), while it was reduced to 19%, 30% and 58% as a result of the two-way fungicide seed application. A further significant increase in plant density at emergence was detected for the four-way fungicide seed dressing in the medium-high seedling mortality group (+8% compared to two-way fungicide) and in the extremely high (+56%) seedling mortality group, respectively. The interaction between the fungicide seed treatments and experiments was never significant in any of the seedling mortality groups.

In addition to the obvious effect on seedling survival during germination, the fungicide seed treatments also affected the early vigor and plant development during the vegetative stages. These differences were detected progressively, by means of the NDVI index, during the vegetative stages, from the four leaf stage (GS 14) to tassel emission (GS 55), and expressed by the AUCDC index (Table 4). The NDVI development, during the growing season, of the compared seed treatments in each seedling mortality group is represented in Figure 1, considering some of the representative experiments. Lower NDVI values are related to both a low plant density and a low plant development (vigor). It is possible to observe, from the reported curves, that maize growth was faster under medium-high seedling mortality conditions than under high or extremely high conditions, as confirmed by the higher daily NDVI increases (Table 5). Moreover, the fungicide seed dressing permitted a faster canopy development than for the untreated control in all seedling mortality clusters. Significant differences in NDVI growth rate during vegetative period were observed for two-way and four-way fungicides for the extremely high seedling mortality category.

Table 4. Effect of the fungicides applied to maize seeds artificially infected by *F. verticillioides* on the number of seedlings that emerged from the soil and the plant density at harvest and on the Area Under the Crop Development Curve (AUCDC) detected during the vegetative stages.

Seedling Mortality	**Factor**	**Source of Variation**	**Plants Density ($n°$ m^{-2})**		**AUCDC**
			Emergence	**Harvest**	**(NDVI-Day)**
Medium-high	Seed dressing	Untreated control	5.6 c	5.9 b	18.5 c
		2-way fungicide	6.5 b	6.6 a	20.4 b
		4-way fungicide	7.0 a	7.0 a	21.9 a
		P(F) [1]	<0.001	<0.001	<0.001
	Experiment	A	5.4 b	5.4 c	24.1 b
		B	5.2 b	5.2 c	11.5 e
		C	6.9 a	6.5 b	18.6 d
		D	7.1 a	7.8 a	21.3 c
		E	7.0 a	7.7 a	25.1 a
		P(F)	<0.001	<0.001	<0.001
	Seed dressing × Exp.	P(F)	0.698	0.414	0.016
High	Seed dressing	Untreated control	2.6 b	3.1 b	13.7 c
		2-way fungicide	5.6 a	5.8 a	20.6 b
		4-way fungicide	6.2 a	6.2 a	22.0 a
		P(F)	<0.001	<0.001	<0.001
	Experiment	F	5.2 a	4.7 a	17.6 b
		G	5.4 a	5.1 a	22.0 a
		H	3.9 b	5.2 a	16.6 c
		P(F)	<0.001	0.243	<0.001
	Seed dressing × Exp.	P(F)	0.063	0.652	0.006
Extremely high	Seed dressing	Untreated control	0.7 c	1.1 c	9.6 c
		2-way fungicide	3.4 b	3.1 b	14.5 b
		4-way fungicide	5.3 a	5.1 a	18.5 a
		P(F)	<0.001	<0.001	<0.001
	Experiment	I	2.9 a	2.3 b	7.9 c
		L	3.4 a	3.5 a	19.2 a
		M	3.2 a	3.5 a	15.6 b
		P(F)	0.090	<0.001	<0.001
	Seed dressing × Exp.	P(F)	0.722	0.075	0.956

[1] Means followed by different letters are significantly different (the level of significance P(F) is reported in the table), according to the REGW-F test.

Overall, seed dressing resulted in a significant ($p < 0.001$) increase in AUCDC in all the seedling mortality groups (Table 4): the seed dressing treatments were significantly different from each other for all the mortality groups considered for this vegetative index. Furthermore, a significant seed dressing × experiment interaction was reported for medium-high and high disease pressures. The C, D, E and G experiments did not show any statistically different results between the compared fungicide seed treatments, while experiment B did not show any difference between the two-way seed dressing and the untreated control (Figure 2). The environmental conditions (soil, meteorological trend) and agronomic ones (sowing time and hybrid) could be the main factors that interacted with the fungicide seed treatments. Confirmation of an effect of fungicide applied to seeds on plant vigor was observed in the growth stage and plant height measurements during stem elongation (Table 5).

The two-way fungicide seed treatment plants were significantly ($p< 0.001$) higher than the untreated control for the medium-high (+16%), high (+30%) and extremely high (+62%) seedling mortality groups, respectively. The plant height of the compared fungicide seed treatments was never different at flowering or at harvest (data not shown). A further significant increase as a result of the application of the four-way fungicide, compared to the two-way fungicide, was reported for all the conditions: increases in plant height of 8%, 20% and 35% were observed for the medium-high, high and extremely high seedling mortality groups. The interaction between seed dressing and experiment was never significant within each seed mortality group.

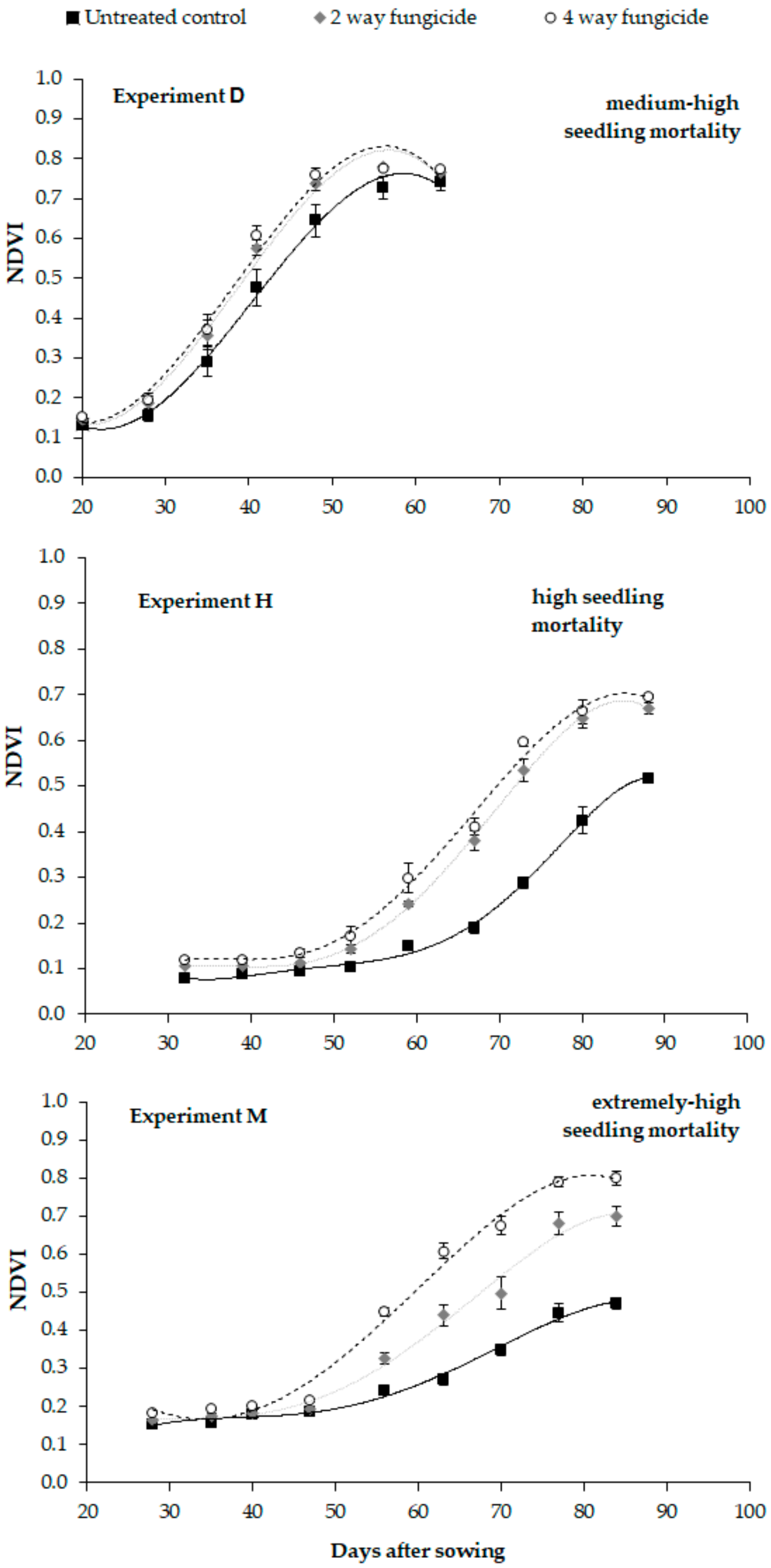

Figure 1. Effect of the application of fungicides to maize seeds artificially infected with *F. verticillioides* on the normalized difference vegetation index (NDVI) measured from the 4-leaf stage (GS14) until tassel emission (GS55). The reported data are an example for the experiments characterized by medium-low, high and extremely high seedling mortality. The reported values are based on 4 replications. The error bars represent the standard error of means (Sem).

Table 5. Effect of the fungicides applied to the maize seeds artificially infected by *F. verticillioides* on maize growth rate during the vegetative stage, expressed as daily NDVI increase, and on plant vigor measured at the stem elongation stage.

Seedling Mortality	Factor	Source of Variation	Growth Rate (NDVI Day^{-1})	Nodes [1] (n°)	Plant Height [2] (cm)
Medium-high	Seed dressing	Untreated control	0.016 b	3.8 c	64.9 c
		2-way fungicide	0.018 a	4.3 b	75.3 b
		4-way fungicide	0.018 a	4.6 a	81.3 a
		P(F) [3]	<0.001	<0.001	<0.001
	Experiment	A	0.015 d	4.0 c	77.7 b
		B	0.018 b	4.4 b	48.6 c
		C	0.021 a	4.8 a	96.5 a
		D	0.017 c	3.2 d	52.0 c
		E	0.017 c	4.7 ab	93.9 a
		P(F)	<0.001	<0.001	<0.001
	Seed dressing × Exp.	P(F)	0.025	0.624	0.967
High	Seed dressing	Untreated control	0.011 b	2.2 c	24.1 c
		2-way fungicide	0.016 a	2.7 b	31.3 b
		4-way fungicide	0.017 a	3.2 a	37.7 a
		P(F)	<0.001	<0.001	<0.001
	Experiment	F	0.013 b	2.4 b	25.6 b
		G	0.019 a	3.5 a	46.7 a
		H	0.012 c	2.2 b	19.4 c
		P(F)	<0.001	<0.001	<0.001
	Seed dressing × Exp.	P(F)	0.006	0.004	0.062
Extremely-high	Seed dressing	Untreated control	0.005 c	3.0 c	27.4 c
		2-way fungicide	0.010 b	4.0 b	44.3 b
		4-way fungicide	0.014 a	4.9 a	60.0 a
		P(F)	<0.001	<0.001	<0.001
	Experiment	I	0.006 c	3.1 b	28.1 c
		L	0.011 b	4.3 a	41.7 b
		M	0.013 a	4.6 a	61.8 a
		P(F)	<0.001	<0.001	<0.001
	Seed dressing × Exp.	P(F)	0.438	0.956	0.933

[1] Growth stage expressed as the average number of nodes detected at the stem elongation stage (GS 32–35). [2] Plant height expressed as the distance from the last detected node close to the ground. [3] Means followed by different letters are significantly different (the level of significance P(F) is reported in the table), according to the REGW-F test.

The maize yield was affected by the seed dressing treatment, and a significant ($p < 0.001$) effect of fungicide application was observed for all the seedling mortality groups (Table 6). The two-way fungicide seed dressing approximately increased maize production by 1.1% to 2.7% (compared to the control) in the medium-high and extremely high seedling mortality groups, respectively.

The yield results confirmed the superior capacity of the four-way fungicide seed dressing to minimize seedling mortality and enhance maize growth compared to the two-way fungicide. A significant difference between the two-way and four-way fungicides was observed for the medium-high (+13%) and extremely high (+45%) seedling mortality conditions. Only in trials carried out with a medium-high seedling mortality was the interaction between the seed dressing and experiment significant: no significant differences were detected between the two-way and four-way fungicides in the B, C and D experiments (data not shown). As far as the grain moisture at harvest is concerned, no significant differences between fungicide seed dressing were observed in any of the trials.

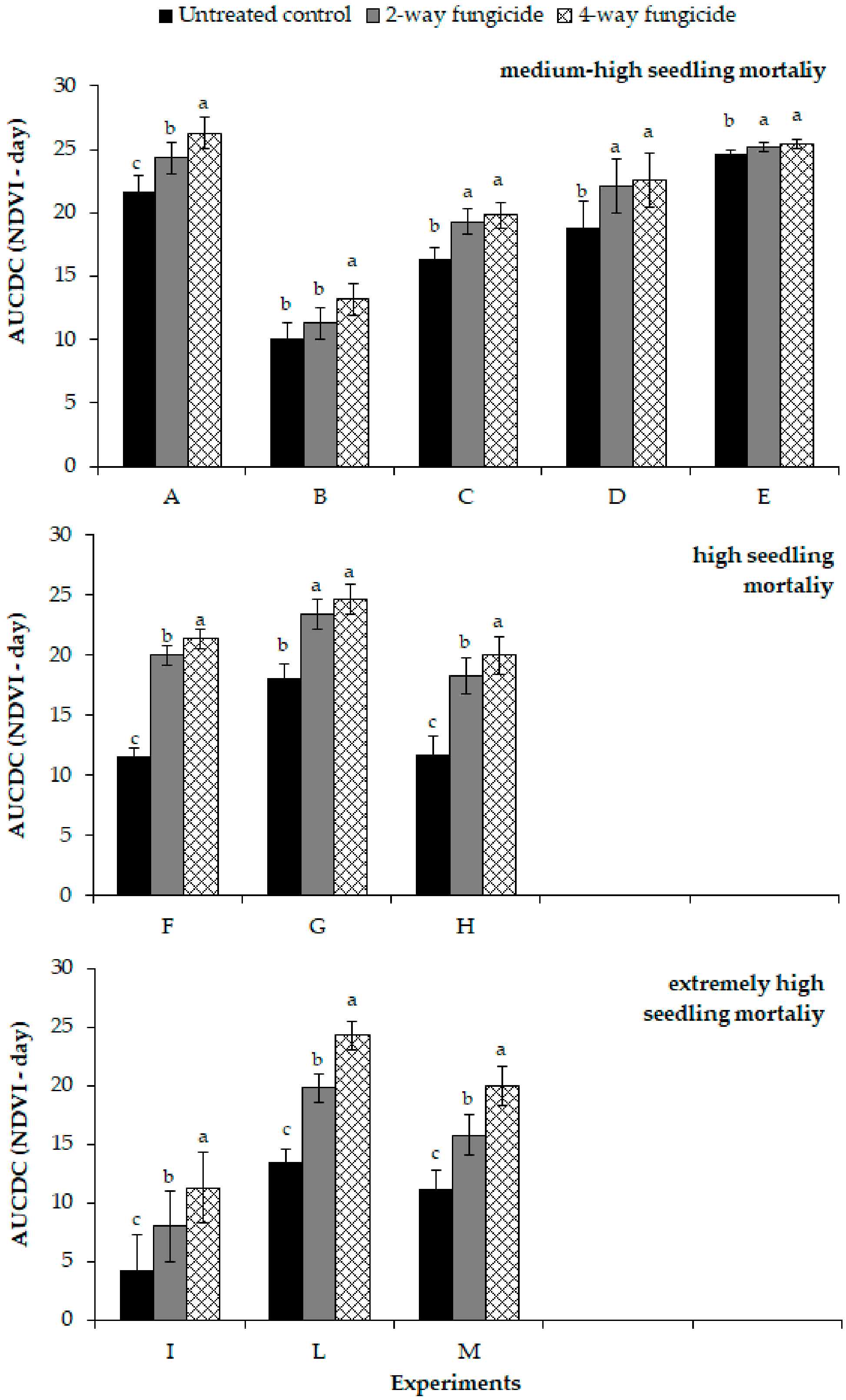

Figure 2. Effect of the fungicides applied to the maize seeds artificially infected with *F. verticillioides* on the Area Under the Crop Development Curve (AUCDC) detected during the vegetative stages in the different experimental sites clustered for seedling mortality. Bars in each experiment with different letters are significantly different (P(F) < 0.05), according to the REGW-F test. The reported values are based on 4 replications. The error bars represent the standard error of means (Sem).

Table 6. Effect of the fungicides applied to the maize seeds artificially infected with *F. verticillioides* on the grain yield and grain moisture content at harvest.

Seedling Mortality	Factor	Source of Variation	Grain Yield (t ha^{-1})	Moisture (%)
Medium-high	Seed dressing	Untreated control	9.6 c	25.8 a
		2-way fungicide	10.6 b	25.5 a
		4-way fungicide	12.0 a	24.8 a
		P(F) [1]	<0.001	0.168
	Experiment	A	12.0 b	19.4 d
		B	9.7 c	25.1 c
		C	4.5 d	31.1 a
		D	12.7 b	27.9 b
		E	15.7 a	23.9 c
		P(F)	<0.001	<0.001
	Seed dressing × Exp.	P(F)	0.001	0.846
High	Seed dressing	Untreated control	8.4 b	25.1 a
		2-way fungicide	11.8 a	25.5 a
		4-way fungicide	13.1 a	25.1 a
		P(F)	<0.001	0.605
	Experiment	F	11.1 b	22.3 b
		G	7.8 c	26.7 a
		H	14.2 a	26.7 a
		P(F)	<0.001	<0.001
	Seed dressing × Exp.	P(F)	0.679	0.870
Extremely high	Seed dressing	Untreated control	3.0 c	23.6 a
		2-way fungicide	8.0 b	23.0 a
		4-way fungicide	11.6 a	22.1 a
		P(F)	<0.001	0.120
	Experiment	I	6.9 a	18.0 c
		L	8.0 a	22.9 b
		M	7.6 a	27.7 a
		P(F)	0.243	<0.001
	Seed dressing × Exp.	P(F)	0.192	0.178

[1] Means followed by different letters are significantly different (the level of significance P(F) is reported in the table), according to the REGW-F test.

3.3. Study II. Fungicide Seed Dressings to Control F. verticillioides and F. Graminearum Damage on the Maize Seedlings and Plants

The effects of the fungicide seed dressings on maize emergence, development and yield, under *F. verticillioides* and *F. graminearum* artificial infection conditions, were compared in study II. Statistical differences ($p < 0.001$) were observed for *Fusarium* inoculation and the fungicide seed treatments on the parameters recorded during both the vegetative stages and at harvest (Tables 7 and 8). The artificial *F. verticillioides* infection was more harmful for maize seedlings (−45% of emerged plants, compared to the uninfected control) than the *F. graminearum* one (−33% of emerged plants). The interaction between seed dressing × year was significant for plant emergence. In 2018, *Fusarium* infection was less harmful (−14% plant emergence per square meter in the uninfected control) than the 2019 (−62%), and this resulted in a significant advantage for the seed dressing application, but without any significant differences between the two-way and four-way fungicides. Conversely, in 2019, the four-way fungicide significantly ($p < 0.001$) increased the plant density at emergence by 10%, compared to the two-way one.

Table 7. Effect of the fungicides applied to the maize seeds artificially infected with *F. verticillioides* or *F. graminearum* on plant emergence and plant vigor measured at the stem elongation stage.

Factor	Source of Variation		Plant Emergence (plant m^{-2})	Plant Height [1] (cm)
Seed treatment	Uninfected check	Untreated	7.7 a	65.9 a
	F. verticillioides infection	Untreated	4.3 e	43.7 c
		2-way fungicide	5.9 c	55.3 b
		4-way fungicide	6.5 b	67.2 a
	F. graminearum infection	Untreated	5.1 d	51.5 b
		2-way fungicide	7.5 a	64.2 a
		4-way fungicide	7.8 a	70.6 a
	P(F) [2]		<0.001	<0.001
Year	2018		7.2 a	92.5 a
	2019		5.6 b	21.5 b
	P(F)		<0.001	<0.001
Treatment × year	P(F)		<0.001	0.089

[1] Plant height expressed as the distance from the last detected node close to the ground detected at the stem elongation stage (GS 32–35). [2] Means followed by different letters are significantly different (the level of significance P(F) is reported in the table), according to the REGW-F test.

Table 8. Effect of the fungicides applied to the maize seeds artificially infected with *F. verticillioides* or *F. graminearum* on the Area Under the Crop Development Curve (AUCDC) detected during the vegetative stages, as well as on the flowering date and the grain yield.

Factor	Source of Variation		AUCDC (NDVI-Day)	Flowering Date (DAS) [1]	Grain Yield (t ha^{-1})
Seed treatment	Uninfected check	Untreated	24.2 a	82.4 b	16.8 a
	F. verticillioides infection	Untreated	17.2 e	84.8 a	12.3 c
		2-way fungicide	22.3 c	82.5 b	15.6 ab
		4-way fungicide	23.2 b	81.0 c	16.9 a
	F. graminearum infection	Untreated	19.4 d	84.3 a	14.6 b
		2-way fungicide	24.4 a	82.1 bc	17.3 a
		4-way fungicide	24.9 a	81.8 bc	17.4 a
	P(F) [2]		<0.001	<0.001	<0.001
Year	2018		25.0 a	67.6 b	16.4 a
	2019		19.2 b	97.8 a	15.3 b
	P(F)		<0.001	<0.001	0.013
Treat. × Year	P(F)		<0.001	0.001	0.145

[1] Flowering date expressed as days after flowering (DAS). [2] Means followed by different letters are significantly different (the level of significance P(F) is reported in the table), according to the REGW-F test.

Moreover, in both trials, the *F. verticillioides* infection led to a clear delay in plant development, as demonstrated by the reduced plant height at the stem elongation stage (Table 7) and by the AUCDC (Table 8), which overall resulted in a lower grain yield than for the *F. graminearum* infection. Compared to the inoculated untreated treatment, the two-way fungicide seed treatment significantly increased the number of emerged seedlings by 38%, when the pathogen was *F. verticillioides,* and by 46% for *F. graminearum*.

No significant further increase in plant density was detected for the four-way fungicide for seeds infected with *F. graminearum*, while this treatment led to a further rise in plant emergence of 9% for *F. verticillioides* infection.

As far as *F. graminearum* infection is concerned, the two-way fungicide was able to confer the same density, vigor and grain yield to the maize crops as the uninfected control, but no further benefits were observed for the application of the four-way fungicide. However, a significant improvement in plant vigor, which was also expressed as an acceleration of the flowering date, and in grain yield, was reported for the seeds infected with *F. verticillioides* treated with the four-way fungicide, compared to the two-way one. Although the four-way fungicide application to the *F. verticillioides* infected seeds resulted in a significantly lower plant density than the uninfected control, this systemic treatment could exert eradicant properties and was able to preserve the same plant vigor, measured as plant height at stem elongation, as the uninfected control, as well as a more anticipated flowering date and a similar grain yield.

4. Discussion

The data collected from a large number of field studies have clearly shown the advantages of fungicide seed treatments on controlling seed-borne *F. verticillioides* and *F. graminearum,* in terms of both maize emergency and vigor (speed of growth), as well as on reducing and, in some cases, totally eliminating, the productive losses caused by fungi. Eleven experiments were conducted from 2015 to 2019 in different production situations (soil, meteorological trend, agronomic techniques), which have clearly influenced the negative impact of fungal infection on the percentage of emerged plants. The experiments were grouped into three clusters on the basis of the seedling mortality at emergence (medium high, high and extremely high): as expected, the advantage of applying a fungicide as a seed dressing increased moving from a quick and prompt emergence, associated with high air and soil temperatures, to a slow process related to a low-growing degree accumulation [25]. Early sowings are often associated with low soil temperatures, but are also related to a higher water content in the soil, which in turn leads to slow and uneven emergence that promote seed-borne and soil-borne pathogens such as *Fusarium* [26].

As far as the comparison of seed-borne fungal species is concerned (study II), *F. verticillioides* led to a higher seedling mortality and grain yield loss than *F. graminearum* under the considered conditions. However, in experiments carried out under controlled conditions in Iowa [3,14] and in Brazil [9], *F. graminearum* was the most aggressive *Fusarium* species that affected maize emergence. The different susceptibility to the two *Fusarium* species could depend on the pathogenicity of the strains [27] used to infect the seeds and on their interaction with different environmental conditions during germination.

As observed in other research, most of the negative impacts of seed-borne fungal infection are due to the loss of plants that occurs during the emergence stages [21]. In the present study, the main cause of the yield gap, compared to the uninfected control, was clearly due to the decrease in the number of emerged seedlings, while no further loss of plants was observed in the successive growth stages until harvesting in any of the considered seedling mortality groups. Thus, the effect of *Fusarium* seed-borne infection on crop damping-off just seems to be concentrated in the germination phases. Furthermore, study II underlines that the loss of plant population, when it is lower than 15%, could be compensated by an increase in production of the single plant, resulting in a similar grain yield.

Nevertheless, the fungus activity, apart from influencing plant density, also has an effect on plant vigor and growth, and this was more evident in the experiments where fungal infection was more severe. Plants grown from artificially infected seeds clearly showed a slower growth than the uninfected control (study II); the height, measured at the elevation stage, the NDVI values, collected during the whole growing cycle, and thus AUCDC, were significantly lower. The infection of the *Fusarium*-inoculated seeds also slowed down plant development: in study II, the flowering date was postponed by about 2 days (approximately 30 GDDs) compared to the uninfected control. Pinto et al. [28] reported that systemic *F. verticillioides* infection in maize plants affected their photosynthetic performance, mainly as a consequence of a reduction in chlorophyll content, which in turn led to a decrease in the electron transport components and a consequent reduction in carbohydrate synthesis.

The fungicide seed treatments reduced the loss, and in some cases removed the gap in the expected plant density, and this led to no difference in the grain yield with the uninfected control (Study II). The seed dressing, apart from being effective in ensuring the desired plant density, also allowed a faster growth of the plants than those of the infected control, as it controlled the systemic infection of both *Fusarium* species. Previous studies, which were only carried out under controlled conditions (greenhouse), have reported a significant effect of the application of a fludioxonil and metalaxyl-M mixture on the plant vigor of infected maize [14,29] or soybean [17,30], as quantified by a higher dry mass of both the shoots and roots. Moreover, Rodriguez-Brljevich [20] reported that a fungicide seed dressing suppresses the soil-borne infection of *Fusarium* spp. in open fields, and results in enhanced photosynthesis and increased plant vigor. To the best of the authors' knowledge, our study is the first that has quantified the advantage in vigor associated with the control of *Fusarium* seed-born infection through a fungicide application in open fields, considering the complete growing cycle until harvest. In our medium-high seedling mortality experiments, the four-way fungicide did not increase the plant population at harvest, compared to the conventional two-way seed dressing, while the broad spectrum treatment increased plant vigor, resulting in a 16% grain yield increase. The seed dressing treatments resulted in a less detrimental vegetative growth, as a consequence of *Fusarium* infection, as highlighted by the NDVI measurement, which thwarted any possible delay in the flowering date. This effect could contribute to enhancing the competitiveness of maize, since a delay in flowering and in the consequent ripening is associated with a lower grain yield (e.g., lower solar radiation interception, [31]), a delay in the harvest date, or a higher grain moisture content at harvesting, and a higher risk of mycotoxin kernel contamination, because of late ripening, as well as a higher incidence of European corn borer injuries on the ears [11].

As far as the *F. verticillioides* infection is concerned, the broad-spectrum seed treatment (four-way) has proved to be more effective than the two-way fungicide one, and to result in a further significant advantage, even in the production situations with a lower disease pressure. The spectrum of the considered two-way mixture was probably not able to provide an analogous effective control of this pathogen in the considered growing areas, where *F. verticillioides* is the predominant and the more harmful species [5,32]. As noted in other works, the use of a greater number of active ingredients leads to a broad spectrum of action, which in turn leads to significant improvements in the control of fungal pathogens and, in particular, of *Fusarium* [21,33]. In addition, the use of active systemic fungicide ingredients with a greater ability to move in seedling tissue could significantly enhance the early season management of this disease. Benzimidazoles (thiabendazole), strobilurins (azoxystrobin), triazoles and pyrazole carboxamide are all able to penetrate the coating of maize seeds and translocate in the xylem to the endosperm, embryo, coleoptiles and radicle [15]. This could make these ingredients more active in controlling the detrimental effects of such systemic pathogens as *F. verticillioides*. Field experience with wheat [34] highlighted that systemic fungicides have eradicant properties and are able to slow down the progress of existing infections.

Conversely, the four-way fungicide did not induce any further advantage under the *F. graminearum* infection conditions. The benzimidazoles and strobilurins probably did not increase the control already provided by fludioxonil, which is highly effective in protecting seedlings from seed-borne *F. graminearum* infection [25]. Furthermore, the application of broad-spectrum fungicides may also determine an indirect advantage for diseases that are already well-controlled by simpler fungicide mixtures, in particular by reducing the risk of resistances [35]. In fact, although *F. graminearum* was included in group E (medium-low risk resistance) by the Fungicide Resistance Action Committee (FRAC), the resistance of its strains to fludioxonil has been reported [25]. In our conditions, the loss of vigor associated with infection from *F. graminearum* was significant, although less evident than that induced by *F. verticillioides*. The two-way fungicide seed dressing was able to prevent this negative effect on plant vigor [19], and an earlier flowering date than for the infected control was observed. As far as vegetative growth is concerned, the four-way fungicide did not lead to any further improvements in crop development or in the anticipation of flowering, compared to the two-way seed treatment.

A direct crop enhancement effect of fungicide seed dressing may be related to the physiological effect that certain fungicide compounds could exert on plants, even in the absence of a fungal infection. Strobilurins have been shown to induce physiological benefits for different crops, such as longer-lasting green leaf tissue and delayed plant senescence (stay green effect), through a reduction in oxidative stress [36], an increase in photosynthesis efficiency, for higher true photosynthesis, and a reduction in dark respiration [37]. Enhanced maize performance, even in the absence of disease, has also been reported for foliar applications of azoxystrobin [38] and pyraclostrobin [39] from the stem elongation stage to flowering. Conversely, no significant effects have been reported for earlier growing stage applications (five leaf-stage, [38]), and no data are available concerning the physiological effect of strobilurins applied to maize as seed dressings. The application of pyraclostrobin to soybean seeds under disease-free conditions improved the growth, vigor (plant height, root and shoot dry mass) and chlorophyll index after 14 days of emergency [40], while strobilurins enhanced rice seedling growth after root cutting injury by inducing reactive oxygen scavenging activity, thus inhibiting reactive oxygen species accumulation [41]. Under controlled sterilized conditions, pyrazole carboxamide sedaxane facilitates root establishment and intensifies nitrogen and the phenylpropanoid metabolism of maize seedlings [42].

In conclusion, the reported field experiments have confirmed the impact of the seed-borne infection of the two most common seed pathogens, *F. verticillioides* and *F. graminearum*, and quantified the negative effect of infection in different production situations from plant emergence to harvest. In addition, the collected data have highlighted the effectiveness of seed dressings with different fungicide treatments by detecting the advantages, in terms of plant population defense, stimulation of the plant development and final grain yield. The benefits of the broad-spectrum four-way formulation, compared to the conventional two-way fungicide seed dressing, clearly depend on the considered pathogens. As far as the *F. verticillioides* infection is concerned, the four-way fungicide enhanced both seedling defense and plant vigor, which resulted in a grain yield improvement under different disease infection conditions. In temperate maize-growing areas where the soil and seed occurrence of *F. verticillioides* inoculum is widespread, the application of four-way fungicide as seed dressing could allow to more successfully anticipate sowing time, even under conservative tillage conditions, often more prone to seedling disease and slow plant development.

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