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CICLO: XXXV

INNOVATION IN SEED DRESSING FOR THE CROP ENHANCEMENT OF ARABLE CROPS

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“Rimanete ambiziosi nei vostri obiettivi perché
rassegnarsi a una vita mediocre non vale mai la pena”

(Sergio Marchionne)

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PREFACE

This PhD dissertation is the result of the experimental work carried out at the Department of Agriculture, Forest, and Food Sciences (DISAFA) of the University of Torino under the supervision of Prof. Massimo Blandino.

The main theme of this work concerns the application of innovative seed treatment to cereal crops and the study of its potential benefit in terms of crop (plant vigor, grain yield and quality) and cropping system improvement (environmental and economical sustainability). In particular, two topics were taken into account: i) the application of fungicides to control fungal disease and their effect on plant development and the agronomic performance; ii) the use of a micronutrient and a biostimulant applied to seed, and their agronomic, yield and qualitative benefits. The thesis consists in seven chapters as described below:

- **Chapter I** introduces the role of seed treatment for arable crops, considering the technique, the different application methods and the benefits. This chapter also highlighted the aims of the research activity performed during the PhD period.

- **Chapters II** to **VI** present the five original papers published or submitted in International Peer-reviewed Journals:

    - ➢ **Chapter II** – "Minimizing yield losses and sanitary risks throught an appropriate combination of fungicide seed and foliar treatments on wheat in different production situations"

    - ➢ **Chapter III** – "Role of the fungicide seed dressing in controlling seed-borne *Fusarium* spp. infection and in enhancing the early development and grain yield of maize"

    - ➢ **Chapter IV** – "The role of zinc fertilization and its interaction with nitrogen and phosphorus starter fertilization on early maize development and grain yield"

    - ➢ **Chapter V** – "Agronomic strategies to enhance the early vigor and yield of maize. Part I: the role of seed applied biostimulant, hybrid, and starter fertilization on rhizosphere bacteria profile and diversity"

    - ➢ **Chapter VI** – "Agronomic strategies to enhance the early vigor and yield of maize. Part II: the role of seed applied biostimulant, hybrid, and starter fertilization on crop performance"

- **Chapter VII** summarizes and discusses the results achieved with the experimental research activities suggesting research perspectives.

1. CHAPTER I

The role of seed in agriculture sector is of prime importance because of it is a basic and vital input for sustained growth in agricultural productivity. Indeed, although the seed required for raising the crop yield is quite small and its cost is also lower if compared to other inputs, the farmer income and thus the fertilizers and plant protection products (PPP) efficacy depend mainly upon the quality of the seed he uses. Therefore, the seed is the most important input for crop production (Panda and Mondal, 2020) and its importance is growing: the global seed market accounted for 60 billion in 2022, and is expected to reach $80-90 billion by 2025 (Cardarelli et al., 2022; Lamichhane et al., 2022).

Faster and uniform germination of seeds and seedling emergence are the vital factors that determines better field stand of crops, with greater possibility to capture resources, tolerate stresses, compete with weeds and other biological adversities. Furthermore, limiting weather conditions and pathogens, such as fungi, bacteria and viruses able to attack seeds and seedlings can lead to heavy potential yield losses, if not managed timely. Seed quality plays an important role in crop production and lack of quality seed is one of the major hindrances in bridging the yield gap (Paravar et al., 2023). Sowing high-quality seeds is essential, but their use does not guarantee successful stand establishment (Afzal et al., 2020). To secure good seed quality and high yield, seeds are most often treated with plant protection products (PPP) before their planting because crop losses due to pests may achieve and exceed 80% (Lamichhane et al., 2022). Some of the biggest success stories in plant disease control involve the use of seed treatment fungicides; they generally have a low toxicity against plant and animal life, while the low application rate permit to reducte their pollution and increase the sustainable use.

Seed treatment is a reliable technique that refers to the exposure of the seeds to certain agents physical, chemical or biological which are not only employed to control pest or disease but also to improve seed quality (viability and vigor) and yield through enhancing the seed placement and performance. This practice is used to modify the physical properties of seed (improve seed handling and thus facilitate mechanized seed delivery through standardization of seed weight and size), and for the delivery of active ingredients (Afzal et al., 2020; Pedrini et al., 2017). Chemical seed treatment began with cereals with the use of brine in 1637, arsenic in 1755, and copper sulfate in 1760. This practice has been widespread in agriculture especially following the introduction of new chemistry classes (Lamichhane et al., 2020). Large-scale commercial utilization of seed treatment for field-scale precision agriculture began in the 1960s and constantly increases (Ma, 2019).

Seed enhancement technology, developed by the seed company is a standard procedure for the majority of arable crops (Pedrini et al., 2020) thanks to his numerous advantages over other crop management such as:

- lower amount of active ingredient (AI) per unit area used; the fungicide and insecticide seed treatments have a range of 10-100 times lower area of contact per hectare than the conventional in-furrow and foliar application. A precise and lower amount means less insecticide residues in the environment and a reduced risk to non target organism (Lamichhane et al., 2020; Vojvodić and Bažok, 2021);

- protection of seed during storage and after planting;

- increase flowability for precision planting (Pedrini et al., 2017);

- improve seed vigor and if necessary, break dormancy and thus improve uniform and rapid field emergence and establishment;

- uniform application of the treatment (Sohail et al., 2022);

- cost effectiveness, smaller amount of AI is distributed compared with broadcast applications. Moreover, the use of systemic PPP provides additional protection also in the early stages of plant development so foliar treatment is often not required. No additional equipment is required for sowing treated seeds and the number of runs of the agricultural machinery are limited as the fuel consumption and soil compaction (Sharma et al., 2015);

- reduced risk for farmers; seed treatment is carried out during seed processing by the seed industry and the farmers come into contact with treated seeds only when they fill the seed tanks;

- independent by weather conditions, in terms of field access, compared with foliar applications.

Seed treatment as a method of local application of pesticides in precision agriculture is considered the safest, cheapest and most ecologically acceptable method of protecting seeds and young plants from pests in the early stages of their development. Moreover, in addition to seed germination and protection this agronomic practice permits also to improve seed vigor and seedling establishment through various substances and functional activities.

1.1 Seed treatment technique

The materials used in seed treatment process include structural materials such as binder and filler and one or more active ingredients (Afzal et al., 2020; Paravar et al., 2023). Binder is a natural or synthetic polymer with adhesive properties used to ensure the adherence and cohesion of the active ingredient on the seed surface preventing the dusting-off (the release of dusty material from the surface of treated seeds as a result of frictions) during handling and sowing. Commonly used binders are polyvinyl alcohol, polyvinyl acetate, methylcellulose, carboxymethyl cellulose and gum arabic. Fillers are usually inert powders (such as bentonite, calcium carbonate, talc, diatomaceous earth, wood dust, sand, lime and clay) used to increase the volume and weight of the original seed (Pedrini et al., 2017). Color-coding (Figure 1) is the most widely used marker system in coating process for identification of a specific variety or seed treatment. Furthermore, the seed color can be applied to make treated seed less attractive to birds, differentiate between brands, alert farmers and others that seeds are treated and cannot be used for animal feed, and to facilitate cleaning operations in the case of an accidental spillage.

In addition to physical seed treatment, used to overcome different germination constraints, a wider range of active ingredients can be incorporated into seed treatment: the most common are protectants, including fungicides, insecticides, nematocides and herbicides. However, nutrient amendments, namely macronutrient (e.g., N, P, and K) and micronutrients (Bo, Cu, Mn, Mo, and Zn) and more recently biologicals can be also applied to seeds (Lamichhane et al., 2022).

Figure 1. Several wheat seed treatments compared to an untreated control.

1.1.1 Chemical seed treatment

The chemical seed treatment is the major group of active ingredients practiced worldwide for its wide spectrum ability; it was estimated between \$3 to \$5 billion in 2020, and accounts for at least 2/3 of the total seed treatment market (Afzal et al., 2020). Chemical seed treatments are generally aimed at controlling seed- and soil-borne pests affecting crop establishment and causing seed rot, pre-emergence damping-off, and seedling blight. In fact, soil contains a large and variable quantity of potentially pathogenic insects and microorganisms, such as fungi, bacteria and viruses that interact with seeds and seedlings. This could lead to the emergence of a small number of plants and, thus, heavy potential yield losses, especially for crops characterized by precision sowing such as maize and when there is not the possibility of self-regulating the plant population by means of tillering. For this reason, in the past, seed treatments were carried out mainly by applying fungicides, and even now this remains the most effective means (Mancini and Romanazzi, 2014). Nowadays various other active ingredients are used, in particular insecticides, nematicides and bactericides.

Among fungicides applied to seeds can be broad spectrum (toxic to all or many kinds of fungus) or narrow spectrum (effective only against a few species). Moreover, fungicides can be divided in three groups based on their mobility in the seed. The first group concerns contact fungicides; these are surface protectants that target seed surface-borne and soil-borne pathogens. Translaminar or cytotropic fungicides represents the second group of fungicides that are locally systemic and target both seed surface-borne and internally seed-borne pathogens. Finally, the third group of fungicides includes those that are characterized by systemic activity, and these are effective against fungal diseases deep within the seed, and can also give protection against early infection from airborne and soilborne diseases (Lamichhane et al., 2020; Mancini and Romanazzi, 2014).

1.1.2 Physical seed treatment

The physical methods for seed treatment are an alternative to chemical seed treatment for crop protection in order to obtain seed disinfection, protection in controlling seedborne pathogens, and invigoration. The most common physical treatments consist of heat treatments (hot water, hot air, solarization, and electron treatments), magnetic fields, electromagnetic waves, radiation (gamma ray, ultrasonic, microwave, UV, laser, ozone technology), ultrasounds, and non-thermal plasma (Attri et al., 2020; Sharma et al., 2015). Generally, thermotherapy inactivates or kills the pathogens leaving the seed viable (Mancini and Romanazzi, 2014). Although one of the most investigated physical seed treatment is based on the use of magnetic field, the gamma irradiation (at low dosage) is a valuable tool used both to abolish microbial contamination and insect pests and to increase at the same time seed vigor with a beneficial effect on germination percentage, and seedling establishment (Araújo et al., 2016).

1.1.3 Macro- and micronutrient seed treatment

Adequate nutrient availability is very important starting at the early stages of plant growth. Seed treatment with appropriate amounts of macro- (N, P, and K) and preferentially micro-nutrients (Zn, B, K, Mo, Fe, Mg, Mn) can reduce nutrient losses by placement on the seed, and also reduce competition from weeds. Both macronutrients and micronutrients are important but these ones, due to their reduced plants need are better suited to the application as seed treatment. Conventional broadcasting of fertilizers exhibited higher cost and losses, while coating with an equivalent rate of nutrients could produce a better cereal growth and potentially yield (Afzal et al., 2020). Furthermore, if any element is lacking in the soil or not adequately balanced with other nutrients, growth suppression or even complete inhibition may result. Micronutrient application through seed treatments improves the stand establishment, advances phenological events, and could increase grain yield and micronutrient grain contents in most cases (Farooq et al., 2012).

1.1.4 Biological seed treatment

The biological seed treatment market includes a wide range of biologicals including biofertlizers, biopesticides and biostimulants. If the biological seed treatment market was estimated between $1 to $1.5 billion in 2020 (Afzal et al., 2020), currently, it is projected to reach $1.7 billion by 2025 (Cardarelli et al., 2022). Therefore, there is increasing interest and demand for biological seed treatments as alternatives to chemical seed treatments as the latter have several negative human health and environmental impacts (Lamichhane et al., 2022).

Since the mobility of microorganisms in the soil is low, microbial inoculants should be placed in the vicinity of the rhizosphere. Thus, the seed treatment with beneficial microorganisms allows a precise application of minor amounts of inocula at the seedsoil interface (Rocha et al., 2019), ensuring that the biological agents are readily accessible at germination and early development plant stages, stimulating healthy and rapid establishment, and consequently maximizing crop production (Cardarelli et al., 2022; Paravar et al., 2023). Inoculation of seeds may represent an efficient and convenient way of introducing fungi and bacteria to soil and consequently the rhizosphere.

Selected fungal (arbuscular mychorrizal fungi, AMF) and bacterial microorganisms (Plant growth promoting bacterial, PGPB) are used commercially for plant protection (biopesticides) and/or plant growth (biofertilizers). Treatment of leguminous seeds with *Rhizobium* spp. is well known for many years for nitrogen fixation as essential contributor in agricultural productivity.

In addition to the biopesticides and biofertilizers, the biostimulants, which have been defined by the European Biostimulant Industry Council (EBIC) as “substances and/or micro-organisms whose function is to stimulate natural processes that enhance nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and crop quality” offer an eco-friendly option to reduce the use of external inputs, such as mineral fertilizers, and increase the sustainability of cropping system, without a reduction of crop productivity (du Jardin, 2015; Gupta et al., 2021). Biostimulants represent another promising developing strategy to enhance plant development and to reduce biotic and abiotic stresses, particularly during the most critical growth stages of the crop under suboptimal growing conditions (Li et al., 2022; Schütz et al., 2018). There is considerable research and development by industry in the broader field of biologicals but commercialization and implementation of bioinoculants still encounter limiting factors, particularly due to poor microbial survival, ineffective colonization of plant host and variable effectiveness due to plant species, growth stage, environmental conditions, and agronomy (Li et al., 2022; Ma, 2019). However, biological seed treatment is a promising alternative to traditional agricultural techniques as it can maintain the agroecosystem health and productivity.

1.2 Seed treatment type

In general, three major kinds of seed treatment tools containing a fluidized bed, rotary coater, and rotary pan or drum coater are used to procreate five kinds of seed treatments, namely dry powder coating, seed dressing, film coating, encrusting and pelleting (Figure 2). The classification of seed treatment types is usually based on the weight, size, and grouping properties of the coated seeds. In addition to the five reported seed treatment, another recent pre-sowing technique primarily used for high values crops such as vegetables is the seed priming. For each seed treatment type is possible to apply to the seeds chemical, micronutrient, and/or biologicals active ingredients (Afzal et al., 2020; Paravar et al., 2023; Rocha et al., 2019).

1.2.1 Dry powder coating

Seeds were mixed with a dry powder with fungal or bacterial activity followed by drying. This technology can be conducted on-farm for the application of labeled treatments for the control of a pest.

1.2.2 Seed dressing

Seed dressing is the most widely used method to apply low dosage of AI to create a thin layer around the seed. A wide range of active materials especially chemical plant protectants can be applied with this method.

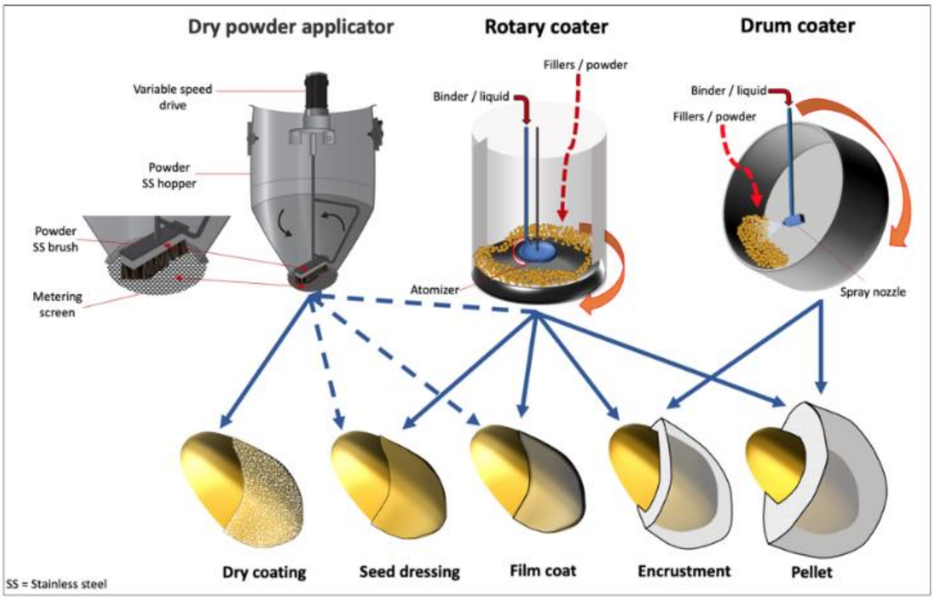

Figure 2. Seed treatments methods (Afzal et al., 2020).

1.2.3 Film coating

Seed coating consists in applying a thin layer of external material (less than 5–10% of the weight of the seed) onto the seed surface, altering little the seed shape, size, or weight and enhancing handling characteristics of seeds. This method allows better treatment precision and minimizes the production of dust compared to seed dressing. It is considered a well-established technique for treatment of several high-value horticultural species and other important agricultural arable crops, such as maize, sunflower, soybean, and canola.

1.2.4 Encrusting

Encrusting is a seed coating method where solid and liquids particulates are added to the seed without changing the original shape. The weight increase after encrusting can range from 8 to 500%. Encrusted seeds have been shown to improve seedling emergence.

1.2.5 Seed pelleting

Many crop seeds are small and irregular in shape that do not permit an accurate sowing by mechanical planting equipment. Seed pelleting, the most sophisticated seed treatment technology, leads to increase weight and volume of seeds and to uniform their shape and size throught adding inert materials (e.g. calcium peroxide, talc, perlite, bentonite, sand, and diatomaceous earth) to the seeds in order to enable precise metering and enhance handling and plantability, especially for irregularly shaped seeds. Seed pelleting is a continuation of the encrusting coating process resulting in changing physical shape of a seed (spherical or ovoid); the initial seed shape is indiscernible. The percent weight increase after pelleting and drying ranges from 500 to >5000 percent. Among seed treatments, seed pelleting is the most expensive application due to the requirement of specialized application machinery.

1.2.6 Seed priming

Seed priming is a promising strategy to provide a valuable solution to enhance the planting value of high-value crops. It refers to the controlled hydration of seeds before sowing, where seeds begin the germination process but are redried before overcoming the reversible phase, thus prior to the point of radicle/epicotyl extension. The physiological processes that occur during seed priming begins with water imbibition (Phase I), “activation” of metabolic activity (Phase II), and embryo and radicle/epicotyl growth (Phase III) (Figure 3). Seeds are tolerant to desiccation during the first two phases of water uptake, but become desiccation sensitive once embryo growth has been initiated (Pedrini et al., 2020; Thakur et al., 2022).

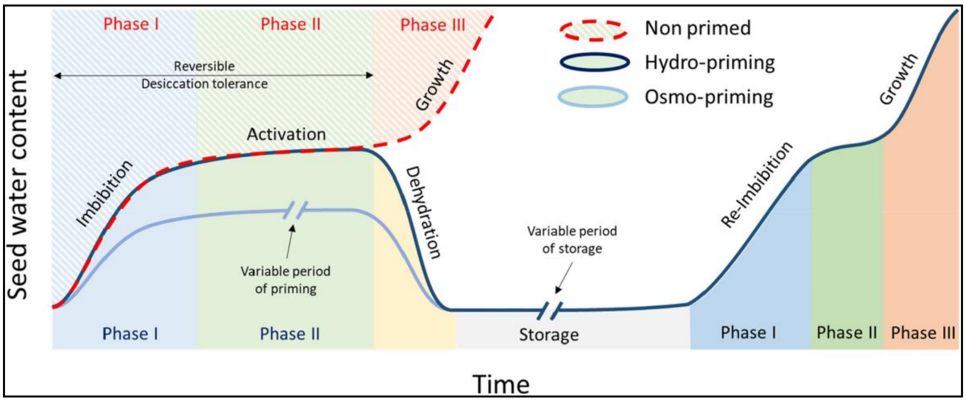

Figure 3. Seed priming process (Pedrini et al., 2020).

Depending on the priming material, various priming stategies can be classified into:

- Hydro-priming involves pre-soaking of seeds in pure water, typically in aerated conditions and at temperatures considered favorable for germination;
- Osmo-priming refers to soaking of seeds in aerated solutions of low osmotic potential like glycerol, polyethylene glycol (PEG), sorbitol, mannitol, and inorganic salts which regulate water imbibition;
- Hormo-priming is when seeds are soaked in phytohormone solutions (auxin, gibberellin, cytokinin, brassinosteroids and others);
- Matrix-priming is another approach in which seeds are primed in a solid insoluble substrate (e.g. compost, clay, peat, sand, or vermiculite) moistened with water to achieve desired water potentials;
- Nutripriming involves the synergistic benefit of biochemical effect of priming with water and the nutritional effect of the applied macro or micro-nutrient;
- Biopriming is realized by soaking seeds into a microbial suspension.

The optimal priming duration can depend on several factors, including priming method, species biology, seed size, dormancy status, and germination speed. Seed priming is an easy and effective technique to get speedy and uniform emergence (Figure 4), high seedling vigour and higher yields of crops. It can also confer greater resilience to thermal, moisture, and osmoticum (salt) stresses. The beneficial effects of seed priming techniques may be attributed to the physiological, biochemical and molecular alterations at the cellular level (Gour et al., 2019; Rhaman et al., 2020, 2021).

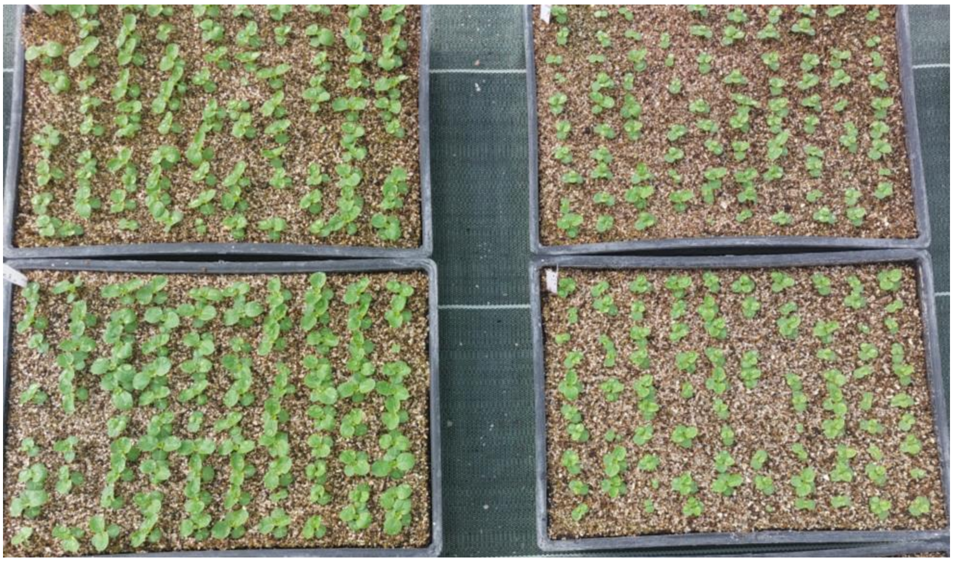

Figure 4. Seed priming effect (on the left) compared to untreated control (on the right) in corn salad (Picture: Luca Capo).

1.3 Aim and structure of the thesis

The research activity was carried out during the PhD period at the Department of Agriculture, Forest, and Food Sciences (DISAFA) of the University of Torino. An abroad period was also carried in Limagrain Europe and HM Clause seed indutries focusing to the optimization of the seed treatment technique in different crops. In particular, this thesis, consisting of five different studies, aimed to improve the knowledge about seed treatment in order to understand better its role on cereal crops (maize and wheat) in terms of effectiveness and sustainability in North-Italy through growth chamber and field experiments. The studies focused on two main topics: i) the fungal diseases control by means the use of innovative fungicide seed treatment application improving at the same time germination, plant development and therefore production (Chapter II and III); ii) and the early vigor and grain yield effect in bad weather conditions also by the application of a micronutrient (Chapter IV) or a biostimulant (Chapter V and VI) to maize seeds.

Chapter II and **III** evaluate the fungicide application on wheat and maize seeds, respectively. Chapter II reports the effects of a systemic fungicide in combination to different foliar treatments strategies on the control of *Septoria* leaf blotch and *Fusarium* head blight as well as on the deoxynivalenol contamination improving grain quality and yield, on wheat field experiment. Chapter III illustrates the protective effects of two different fungicides seed treatments in controlling *F. verticillioides* and *F. graminearum* seed-borne infection and in increasing the maize seedlings defense, vigor and yield.

Chapter IV refers to the application of zinc (Zn) micronutrient to maize seed in enhancing plant growth and yield in three year field experiment. In addition, the role of a Zn seed treatment has been investigated in three different soils characterized by different physical and chemical (mainly Zn availability and phosphorous content) properties, known for their antagonistic activity against the microelement.

Chapter V and VI studied, in both growth chamber and field experiments, the seed application of a biostimulant, based on a mixture of a bacterium and a plant extract, in combination to maize hybrids with different early vigor and the distribution of starter fertilizer with nitrogen and phosphorous in affecting the rhizosphere bacterial community composition and therefore the plant growth and grain yield.

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2. CHAPTER II - Minimizing yield losses and sanitary risks through an appropriate combination of fungicide seed and foliar treatments on wheat in different production situations

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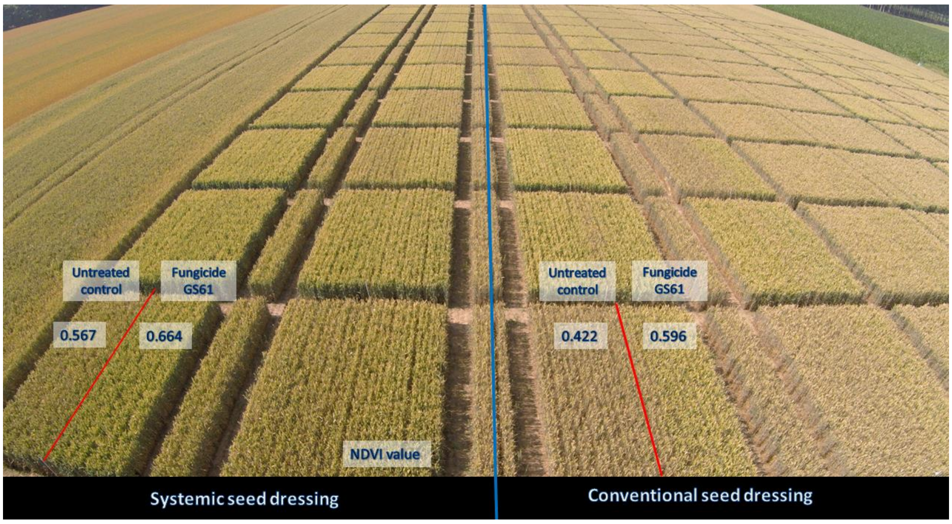
Graphical abstract



2.1 Abstract

Among the fungal diseases that affect wheat in temperate growing areas, *Septoria* Leaf Blotch (SLB) and *Fusarium* head blight (FHB) result in yield and sanitary risk losses that could be minimized through appropriate fungicide applications. Furthermore, the request from policy makers and the food market to reduce the use of chemical pesticides in agriculture has driven research in the direction of performant defense strategies with a reduced spraying of pesticides. The aim of this study was to evaluate the effects of different fungicide programs on the control of SLB and FHB, as well as on the grain yield and deoxynivalenol (DON) contamination of common wheat. Field experiments were carried out in 2016 and 2017 in North Italy. Two seed treatments (conventional vs. systemic) and four combinations of foliar fungicide applications (untreated control, application at the end of stem elongation, at flowering, and a double treatment at stem elongation and flowering) have been compared, according to a full factorial design, under two agronomic conditions: plowing vs. minimum tillage. Foliar sprayings at the end of stem elongation were found to be more effective in controlling SLB, while a triazole application at flowering was found to be an essential practice to reduce the FHB and DON contents. The double foliar treatment led to significant benefits, albeit only in the production situations with the highest SLB severity (e.g., in the 2017 experiment, after ploughing and the use of a conventional seed treatment). The systemic seed dressing led to a higher and prolonged SLB protection, with significant canopy greenness during ripening in all the production situations. In 2017, which suffered from high disease pressure, the seed treatment with systemic fungicide led to a significant increase in grain yield (+5%), compared to the conventional one. The combination of the systemic seed treatment and the triazole application at flowering guaranteed the highest control of both SLB and FHB, maximized grain yield, and minimized DON contamination. This study provides useful information that could be used to evaluate appropriate fungicide programs, based on a combination of seed and foliar treatments, for wheat yield and sanity in distinct SLB and FHB diseases pressure scenarios.

Keywords: *Triticum aestivum*, *Septoria* leaf blotch, *Fusarium* head blight, deoxynivalenol, seed treatment

2.2 Introduction

In 2018, a total of 734 million tons of wheat was harvested across the globe, making it the third-largest grain crop in the world (FAOSTAT). Of this, 33% was produced in Europe, where wheat, which is mainly cultivated as a winter crop, is the cereal that is grown the most, in terms of surface, and is a staple food for its citizens. Among the various factors that could contribute significantly to reducing wheat yield, several diseases, such as root and foot rot complex, powdery mildew, rusts, *Septoria* leaf blotch complex (SLB), and *Fusarium* head blight (FHB), could have a negative impact in temperate growing areas. It has been estimated that about 20% of the global wheat production is lost due to diseases every year (Fones et al., 2015; Serfling et al., 2017). Furthermore, the percentage of yield that could be lost, without plant protection, could exceed 70% in intensive temperate growing areas (Oerke 2006). The seedlings, crowns, roots, and feet of wheat may be attacked by fungi (*Fusarium* spp., *Microdochium nivale*, *Bipolaris sorokiniana* and others), even in the early phenological stages, causing tissue discoloration, slow growth, a low tillering capacity, and reduced grain filling (Scherm et al., 2013). Foliar diseases are able to colonize the leaves, stems, and internodes of wheat, and have been associated with yield losses, due to a reduction in the photosynthetic life of the canopy. SLB, which is caused by the ascomycete *Mycosphaerella graminicola* (asexual stage *Zymoseptoria tritici*), is the main foliar disease of wheat in Europe (Fones et al., 2015). Although leaves can be infected by SLB throughout the whole wheat life cycle, its effect on the loss of productivity and grain quality is more important when environmental conditions such as humidity and temperature are favorable for fungal growth during grain filling (Serrago et al., 2011). Crop protection strategies that are able to protect the flag leaf are required, since this leaf is responsible for 50% of grain filling assimilates (Sylvester-Bradley et al., 1990). The main agents of FHB in temperate areas, that is, *F. graminearum* and *F. culmorum*, are able to infect wheat spikelets at flowering, thereby causing total or partial premature senescence of the ears, in particular when rainy or wet periods occur between heading and the soft dough stage (Xu 2003). Both SLB and FHB are responsible for significant losses in yield and quality (low milling yield) whenever their attack strongly reduces grain test weight as a consequence of an early crop senescence (Figueroa et al., 2018).

In addition to grain yield loss, FHB is responsible for the accumulation of mycotoxins in the grains, and this remains a major hazard for human and animal health (Blandino et al., 2017). Deoxynivalenol (DON) is the most prevalent contaminant of wheat (Larsen et al., 2004). The European Commission (EC) has in fact set up regulatory limits to protect humans from exposure to this mycotoxin through cereal consumption (EC No. 1881/2006) (EUR-Lex). The agronomic practices adopted for the prevention of fungal diseases mainly focus on minimizing the pathogen inocula using crop rotation (Koch et al., 2006) or soil tillage to incorporate previous crop debris (Blandino et al., 2012), and the use of tolerant varieties (Blandino et al., 2017; Svarta et al., 2019). However, in climatic conditions that are conductive to fungal diseases, the previously mentioned preventive measures might not be sufficient, and direct control, through the use of a fungicide application, is often necessary (Ranson et al., 2008; Lori et al., 2009). Applying a fungicide to seeds minimizes the risks associated with seedling mortality and allows a further control of the root and foot rot complex. Phenylpyrroles (e.g., fludioxonil) and triazoles (14-demethylation inhibitors, e.g., difeconazole, tebuconazole and prothioconazole) are the most widespread wheat seed dressing for this purpose (Akgul and Erkilic 2016). On the other hand, spray applications to the canopy are necessary to control foliar disease and FHB. Fungicides containing triazoles, in particular metconazole and prothioconazole, applied at wheat flowering (growth stage, GS61, according to Zadoks et al., 1974) are the most active molecules for the control of FHB infection and the consequent DON contamination (Paul et al., 2008). This application timing also has a clear effect on delaying the decline of the green leaf area during grain filling and contributes to increasing grain yield (Blandino et al., 2011). Furthermore, in order to ensure a better control of SLB and other foliar diseases, fungicide spraying at a GS from the end of stem elongation (GS39) to booting (GS45) could guarantee a higher protection of the wheat canopy (Wiersma and Motterberg 2005). Such an application is in particular aimed at preserving the stay green of the flag leaf that has recently unrolled (Dimmock and Gooding 2002). Strobilurin (chemical quinone outside inhibitors, QoIs) and carboxamide (succinate dehydrogenase inhibitors, SDHI) fungicides are generally used to obtain a high efficacy against the main foliar diseases and a marked physiological activity on plants, as they are able to induce a longer duration of the green flag leaf area than triazoles (Fleitas 2018; Amaro et al., 2020). A double fungicide application of the fungicide at GS39 and GS61 is a crop protection strategy frequently adopted by farmers in temperate environments and where the agronomic conditions are more prone to fungal disease development, in order to maximize wheat yield (May et al., 2014). A recent innovation on the market is the availability of a fungicide seed treatment characterized by a marked systemic activity, which is able to prolong the control of foliar disease, even in later growth stages. Among the systemic active ingredients (AI) that may be applied as a seed dressing, fluxapyroxad, a carboxamide fungicide, has proved to provide an effective and long-term disease control, through a foliar application, but also physiological benefits connected to an increase in leaf greening, delayed senescence, reduced cell damage, reduced stomatal conductance, an improved photosynthetic rate, and water use efficiency with a positive effect on grain yield (Smith et al., 2013). The possibility of guaranteeing a profitable protection from the fungal diseases of winter wheat through the application of a systemic seed fungicide needs to be carefully evaluated, in order to check the role of these practices on the overall wheat protection programs and the interaction of such a fungicide with other fungicide treatments administered in spring. Considering the increasing request of lower pesticide applications in farming systems, as requested by politicians, supply chains and more in general by consumers, the possible substitution of a fungicide spray application with a seed dressing treatment would permit a clearly lower rate of active ingredients to be obtained per hectare.

The aim of the study was to evaluate the role of applying a systemic fungicide to wheat seeds in order to control fungal diseases and enhance grain yield and quality, considering the possibility of introducing this innovation into different crop protection programs for several agronomical and environmental conditions.

2.3 Materials and Methods

2.3.1 Experimental site and treatments

Field experiments were carried out in the 2015–2016 and 2016–2017 growing seasons in Buriasco (TO), in North-West Italy (44° 54’ N, 7° 24’ E; altitude 262 m.), in a sandy medium textured soil, classified as Typic Udifluvents (USDA classification), under naturally infected field conditions. Two adjacent experimental fields of winter wheat, one with a high agronomic risk of fungal diseases (related to the presence of previous crop residues on the soil) and the other with a low risk, were prepared each year. In both growing seasons, the previous crop was maize, grown according to a crop sequence normally applied in the growing area. The compared agronomic conditions were related to the tillage method, in order to favor diverse disease pressures:

- minimum tillage with double disk harrowing (15 cm depth), with previous maize crop residues left on the soil surface;
- fall ploughing (30 cm depth), which incorporated the maize debris into the soil, followed by disk harrowing to prepare a proper seedbed.

Different fungicides treatments were compared, under both agronomic conditions, according to a factorial combination of:

- a fungicide application as a seed dressing:
  - conventional: AI fludioxonil (Celest®, Syngenta Crop Protection S.p.A., Basel, Switzerland, fludioxonil 2.4%, 200 mL per 100 seed kg dose);
  - systemic: AI fluxapyroxad (Systiva®, BASF Agricultural Solutions S.p.A., Ludwigshafen, Germany, fluxapyroxad 28.7%, 150 mL per 100 seed kg dose);
- a foliar fungicide application:
  - an untreated control without any crop protection foliar treatment;
  - GS39, a single treatment at the end of stem elongation, in which a mixture of a strobilurin and a carboxamide (Priaxor®, BASF Agricultural Solutions, pyraclostrobin 150 g ha^{-1} and fluxapyroxad 75 g ha^{-1}) was applied;
  - GS61, a single treatment at the beginning of flowering in which a triazole AI mixture (Osiris®, BASF Agricultural Solutions, epoxiconazole 75 g ha^{-1} and metconazole 55 g ha^{-1}) was applied;
  - GS39 + GS61, a double treatment through the combination of the previously reported single foliar applications.

The fungicide treatments were assigned to experimental units using a completely randomized block design, with four replicates. The plot size was 12 m^2 (6 m X 2 m). The normal agronomic techniques adopted in the growing area were applied. Briefly, the wheat cultivar used in both growing seasons was Aubusson, which has a medium susceptibility to FHB and SLB diseases (Limagrain Italia S.p.A., Busseto, PR, Italy). Planting was conducted in 12 cm wide rows on October 23, 2015, and October 20, 2016, at a seeding rate of 450 seeds m^2. The experimental field received 140 kg N ha^{-1} as a granular ammonium nitrate fertilizer (26% N), split between wheat tillering, GS 31, (60 kg N ha^{-1}) and the end of stem elongation, GS 39, (80 kg N ha^{-1}). At the end of tillering, a chemical weed control was carried out with Pinoxaden 3.03% + Clodinafop-propargyl 3.03% + Florasulam 0.76% + Cloquintocet-mexyl 0.76% (Traxos One®, Syngenta Crop Protection S.p.A.). The fungicides were applied at the manufacturers' recommended field rates, by means of a four-nozzle precision sprayer (Honda Agricultural Sprayer T-Jeet A110/04; Honda Motor Europe, Ltd., London, UK), using a fine mist at a slow walk to ensure an effective coverage. The delivery pressure at the nozzle was 300 kPa. In 2016, the fungicide treatments were conducted on 29 April at GS 39 and on 17 May at GS 61, while in 2017, they were conducted on 27 April and 17 May at GS 39 and GS 61, respectively.

2.3.2 Crop assessments

a. Vegetation Index

A hand-held optical sensing device, GreenSeekerTM® (Trimble©, Sunnyvale, CA, USA), was used to measure the normalized difference vegetation index (NDVI) from the first leaf stage (GS11) to the end of the grain-filling stage (GS85), in all plots. The instrument was held approximately 60 cm above each single wheat plot, and its effective spatial resolution was 2 m x the full length of the plot (6 m). This assessment was performed every 2 weeks, until GS 39, and then every 7 days. The Area Under the Canopy Development Curve (AUCDC) was calculated, starting from the NDVI measurements, using the following formula:

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

where R is the NDVI value, t is the time of observation and n is the number of observations (12).

b. Septoria Leaf Blotch (SLB) symptoms

The SLB severity was evaluated on the leaves at the beginning of flowering (GS61) and at the early dough stage (GS83) in each plot. Leaf disease was classified into six classes (0 = 0%; 1 = 2%; 2 = 5%; 3 = 10%; 4 = 25%; 5 = 50%; 6 ≥ 50%), according to visible symptoms (Scaglioni et al., 2019). At GS

61, the measurement was carried out on 75 leaves per plot (the last 5 leaves for 15 randomly selected plants). Instead, 15 randomly selected flag leaves and 15 penultimate leaves were used at GS 83. In 2016, the assessments were performed on May 16 (GS 61) and on June 15 (GS 83); they were instead carried out on May 17 and on June 7 in 2017.

c. Fusarium Head Blight (FHB) symptoms

The incidence and severity of FHB was recorded in each plot by performing a visual evaluation of the disease on the grains at the early dough stage (GS83). The incidence was calculated as the percentage of ears with symptoms of the disease, using 200 randomly selected ears. The severity was calculated as the percentage of spikelets per ear with symptoms and was estimated on a scale from 0 to 7. Each numerical value corresponds to a percentage range of surfaces that exhibit visible symptoms of the disease (Parry et al., 1995), according to the scheme: 1 = 0–5%; 2 = 6–15%; 3 = 16–30%; 4 = 31–50%; 5 = 51–75%; 6 = 76–90%; 7 = 91–100%. The assessment was recorded on June 15, in 2016, and on June 7, in 2017.

d. Grain yield and production parameters

The plots were harvested, using a Walter Winterstaiger cereal plot combine harvester, on July 5, 2016, and July 13, 2017, and the grain yield results were adjusted to a 13% moisture content. Aliquots of 2 kgs of grain were taken from each plot to determine the test weight (TW), the thousands kernel weight (TKW), and the grain moisture content, using a GAC® 2000 Grain Analyzer (Dickens-John Auburn, IL, USA). TKW was determined on two 100-kernel sets for each sample (only whole seeds were considered) using an electronic balance. The harvested grains were mixed thoroughly, and an aliquot of 4 kg of grain was taken from each plot and ground completely using a Retsch ZM 200 (Retsch GmbH, Haan, Germany), fitted with a 1 mm aperture sieve. The resulting whole meal was analyzed for the DON content.

e. DON analysis

The DON concentration was determined using the ELISA method, by means of direct competitive immunoassays RIDASCREEN® DON (R-Biopharm, Darmstadt, Germany), according to the method reported by Nguyen et al., 2019.

2.3.3 Statistical analysis

The normal distribution and homogeneity of variances were verified by performing the Kolmogorov–Smirnov normality test and the Levene test, respectively. The effect of the fungicide seed and foliar treatments on the AUCDC vegetation index, SLB incidence and severity, FHB incidence and severity, grain yield, TW, TKW, and DON content was tested by means of an analysis of variance (ANOVA), using a randomized complete block. ANOVA was used separately for each year and tillage, to explore the specific effects of the fungicide treatments under different environmental conditions. Multiple comparison tests were performed, according to the Ryan–Einot–Gabriel–Welsh F (REGW-F) method, on the treatment means (*p*-value<0.05). Statistical analysis was performed with SPSS software, version 26 (IBM Corporation, Armonk, NY, USA, 2008).

2.4 Results

2.4.1 Meteorological trends

The two growing seasons showed different meteorological trends throughout the wheat crop cycle (Table 1). The precipitations in the 2016–2017 growing season were 200 mm higher than in the 2015–2016 season, with the difference in rainfall mainly being concentrated during the leaf emission stages (November and December). The growing degree days (GDDs) were higher (+86°C-day) from April to June in 2016–2017 than in 2015–2016.

Table 1. Monthly cumulative rainfall, rainy days, and growing degree days (GDDs)[1] *measured in the experimental areas from sowing (November) to harvesting (June) in the 2015–2017 period.*

Month	**Rainfall (mm)**		**Rainy days (n°)**		**GDDs (Σ °C-day)**[2]	
	2015-16	**2016-17**	**2015-16**	**2016-17**	**2015-16**	**2016-17**
November	2	257	4	7	293	250
December	1	77	0	5	188	159
January	5	12	4	3	151	111
February	164	62	12	13	188	175
March	90	69	7	8	295	356
April	96	51	9	6	430	412
May	117	77	11	11	516	558
June	34	103	14	7	636	698
November - March	261	477	27	36	1115	1050
April - June	246	231	34	24	1582	1668

[1] *Data obtained from the Regione Piemonte agrometeorological service.*

[2] *Accumulated growing degree days for each experiment using a 0°C base value.*

2.4.2 SLB symptoms and vegetative index

In both growing seasons, SLB affected the wheat canopy, although no symptoms of root rot or other foliar diseases were detected. The SLB incidence and severity in both GS61 and GS83 were higher in the 2016–2017 period than in the 2015–2016 growing season (Table 2). All the plant leaves showed SLB symptoms at GS83 (SLB incidence = 100%, data not shown). Furthermore, the SLB symptoms were clearly influenced by soil tillage, and in particular at this GS: the growth of the wheat under ploughing conditions resulted in a higher disease severity than under minimum tillage. At GS61, the systemic fungicide always significantly reduced SLB severity, by 47%, compared to the conventional seed treatment, except for the 2016 experiment under minimum tillage conditions. At GS83, the benefits, in terms of disease control of the systemic seed dressing, were significant for all the conditions and resulted in reductions of between 19% (2016, minimum tillage) and 27% (2017, ploughing). The fungicide application at GS39 significantly reduced SLB severity (-45%) for all the environmental conditions detected at flowering, compared to the untreated control. At the early dough stage, fungicide spraying at GS39 only resulted in a lower disease severity in the 2017 experiments. Compared to the untreated control, the disease symptoms during ripening were significantly lower than for the fungicide application at GS61 (-35%), while only under the ploughing conditions was a further reduction of SLB severity obtained with double spraying (GS39 + GS61).

The interaction between seed and foliar treatment was significant for SLB severity at GS83 in 2016 (ploughing) and in 2017 (under both ploughing and minimum tillage conditions). In all these production situations, the systemic seed dressing, without any further foliar applications, was able to significantly reduce SLB severity, reaching the same degree of protection obtained for the combination of conventional seed dressing and fungicide application at GS61 (Figure 1). When double foliar spraying was applied, no difference was recorded between the conventional and systemic seed treatments in any of the trials. In the production situation with the highest SLB pressure (2017, ploughing), the crop protection strategy with a single fungicide spraying was different according to the seed dressing. With the use of a systemic AI, the foliar applications at GS39 or GS61 resulted in a similar disease control, while with the conventional seed treatments, fungicide spraying at wheat flowering resulted in a significantly higher SLB severity. The positive effect of seed and foliar treatments on SLB control was confirmed by the NDVI values detected during the growing season (Figures 2 and 3). Low values are related to a lower plant biomass and/or greenness status of the wheat canopy, and NDVI therefore reached the highest values from GS37 to GS69. The crop development was slightly slower under the minimum tillage conditions than under the ploughing conditions in both years. Only in 2016 did the systemic seed treatment result in lower NDVI values than the conventional one until GS23, with a slower emergence and development in the early stages.

No difference was observed between the compared seed dressings from GS39 to GS69, while the systemic seed dressing resulted in a higher NDVI during grain filling than the conventional one, in all the production situations and considering the untreated control without foliar application, as a consequence of a delayed senescence (Figure 2). In both years, the seed treatment differences in NDVI were more visible under ploughing with higher SLB symptoms than under minimum tillage conditions. As far as the stay green evolution during grain filling is concerned (Figure 3), the application of the foliar fungicide led to higher NDVI values than the untreated control, with a more marked difference between the considered protection programs in the conventional seed treatment from the trials carried out in 2016 with minimum tillage than that in 2017 after ploughing. The systemic fungicide seed dressing alone (without any further fungicide application) was able to prolong the stay green, compared to the untreated conventional one. Moreover, when the systemic AI was applied to the seed, the differences between the foliar fungicide programs were smaller than those observed for the conventional seed dressing. Overall, the AUCDC vegetation index of the systemic seed dressing was significantly higher, that is, by 5%, than the conventional one (Table 2). A significant effect of the foliar treatments on AUCDC was observed for all the production situations (Table 2). Furthermore, only in 2017, under the ploughing conditions, were the differences between the single and double foliar fungicide treatments significant. In this experiment, the interaction between the seed and foliar treatments was significant: when a systemic fungicide was applied as a seed dressing, a single foliar application at GS39 was able to guarantee a higher stay green during wheat ripening, while a further benefit of the double foliar treatments was observed for the conventional seed treatment.

Table 2. Effect of the fungicide seed and foliar treatments on Septoria Leaf Blotch (SLB) incidence and severity at flowering (GS 61), at early dough (GS83) and on the Area Under the Canopy Development Curve (AUCDC) detected during the vegetative stages, from the beginning of flowering (GS61) to the soft dough stage (GS85) in the 2015–2016 and 2016–2017 growing seasons, North-Italy.

Year	**Soil tillage**	**Factor**	**Source of variation**	**SLB incidence (GS61) %**	**SLB severity (GS61) %**	**SLB severity (GS85) %**	**AUCDC (GS61-GS85) NDVI-day**
2016	Minimum tillage	Seed treatment[1]	Conventional	25.6	2.0	26.1 a	27.3 b
			Systemic	21.0	1.5	21.2 b	29.0 a
			p-value[3]	0.111	0.129	0.023	<0.001
		Foliar treatment[2]	Untreated	31.3 a	2.6 a	29.3 a	27.1 b
			GS39	16.3 b	0.7 b	24.5 ab	28.7 a
			GS61	28.1 a	2.6 a	20.6 b	28.5 a
			GS39 + GS61	18.8 b	0.9 b	20.3 b	28.4 a
			p-value	<0.001	<0.001	0.012	0.023
		Seed x Foliar	*p*-value	0.457	0.349	0.228	0.816
2016	Ploughing	Seed treatment	Conventional	40.1 a	3.2 a	38.1 a	26.7 b
			Systemic	31.5 b	1.0 b	30.8 b	28.6 a
			p-value	0.009	<0.001	0.006	0.001
		Foliar treatment	Untreated	37.7	2.6 ab	42.8 a	26.4 b
			GS39	32.7	1.5 b	35.7 ab	28.1 a
			GS61	36.4	3.0 a	32.6 bc	27.9 ab
			GS39 + GS61	35.3	1.3 b	26.8 c	28.3 a
			p-value	0.65	0.043	0.001	0.024
		Seed x Foliar	*p*-value	0.446	0.268	0.042	0.268
2017	Minimum tillage	Seed treatment	Conventional	39.3 a	14.2 a	17.6 a	30.6
			Systemic	17.8 b	3.2 b	12.8 b	31.1
			p-value	<0.001	<0.001	0.012	0.063
		Foliar treatment	Untreated	32.7 a	10.9 a	27.9 a	29.9 b
			GS39	24.8 b	6.3 b	11.9 b	31.2 a
			GS61	34.8 a	11.3 a	13.2 b	30.9 a
			GS39 + GS61	21.9 b	6.3 b	8.0 b	31.6 a
			p-value	0.001	<0.001	<0.001	0.002
		Seed x Foliar	*p*-value	0.081	0.002	0.046	0.14
2017	Ploughing	Seed treatment	Conventional	56.1 a	22.2 a	31.9 a	29.4 b
			Systemic	44.1 b	11.7 b	23.3 b	30.2 a
			p-value	<0.001	<0.001	<0.001	0.005
		Foliar treatment	Untreated	55.6 a	19.9 a	50.3 a	27.9 c
			GS39	46.5 b	15.1 b	18.0 c	30.7 a
			GS61	53.8 a	18.6 a	32.4 b	29.5 b
			GS39 + GS61	45.8 b	15.0 b	9.9 d	31.0 a
			p-value	0.005	<0.001	<0.001	<0.001
		Seed x Foliar	*p*-value	0.796	0.100	<0.001	0.007

[1] Fungicide seed treatments: conventional (fludioxonil AI) and systemic (fluxapyroxad AI); [2] Fungicide foliar treatment: untreated control; GS39, a single treatment at the end of stem elongation (pyraclostrobin + fluxapyroxad AI); GS61, a single treatment at the beginning of flowering (epoxiconazole + metconazole AI); GS39 + GS61, a double treatment through the application of a combination of GS39 and GS61; [3] Means followed by different letters are significantly different (the level of significance of the *p*-value is reported in the Table), according to the REGW-F test.

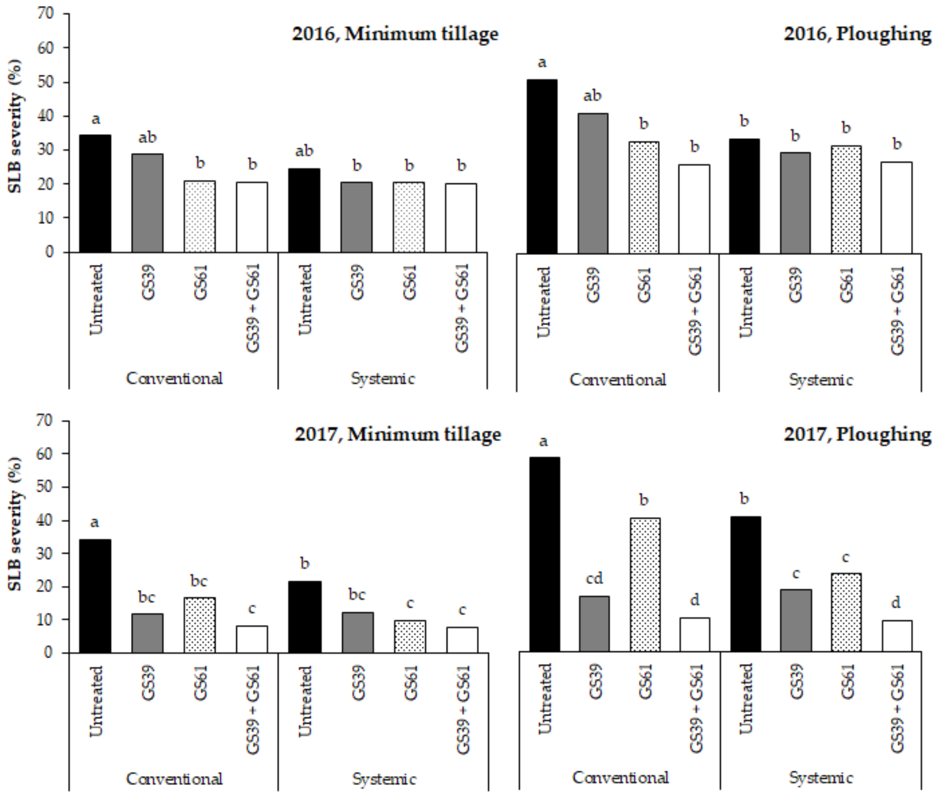


Figure 1. Effect of the fungicide seed ^{1} and foliar ^{2} treatments on Septoria Leaf Blotch (SLB) in different soil tillage and growing seasons (2015-16 and 2016-17) in North-Italy at the early dough stage (GS83). The bars in each experiment with different letters are significantly different (p-value < 0.05), according to the REGW-F test. The reported values are based on four replications. ^{1}Fungicide seed treatments: conventional (fludioxonil AI) and systemic (fluxapyroxad AI). ^{2} Fungicide foliar treatment: untreated control; GS39, a single treatment at the end of stem elongation (pyraclostrobin + fluxapyroxad AI); GS61, a single treatment at the beginning of flowering (epoxiconazole + metconazole AI); GS39 + GS61, a double treatment through a combination of the GS39 and GS61 applications.

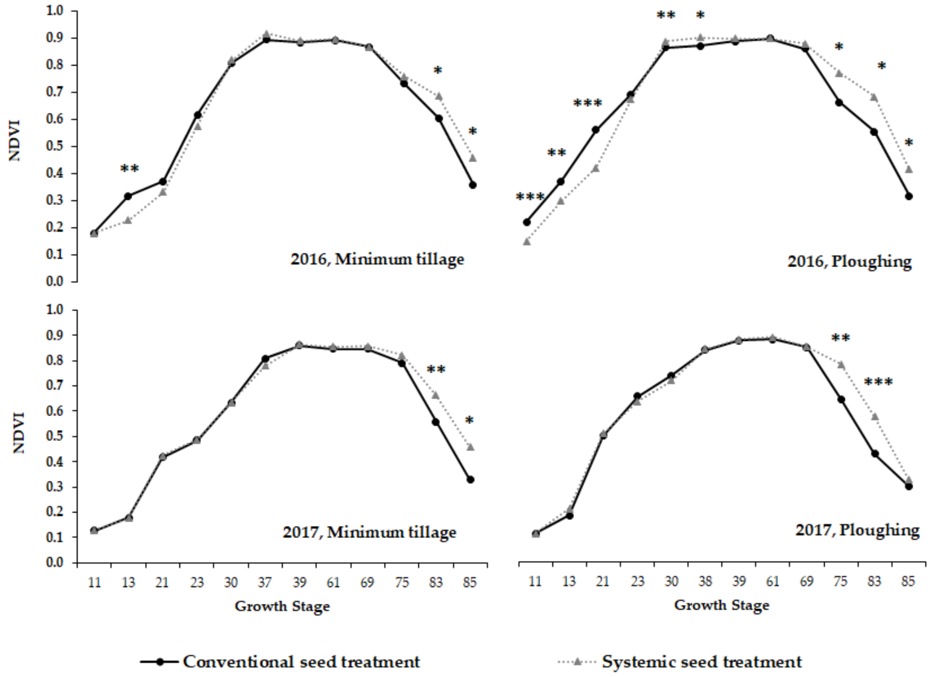


*Figure 2. Effect of the fungicide seed treatments [1] on the normalized difference vegetation index (NDVI) measured from the first unfolded wheat leaf (GS11) to the soft dough stage (GS85) in different soil tillage and growing seasons (2015-16 and 2016-17) in North-Italy. ANOVA was performed for each NDVI value: * significant difference at the <0.05 level; ** significant difference at the 0.01 level; *** significant difference at the <0.001 level. The reported data are based on four replications of the untreated control, without any foliar fungicide. [1]Fungicide seed treatments: conventional (fludioxonil AI) and systemic (fluxapyroxad AI).*

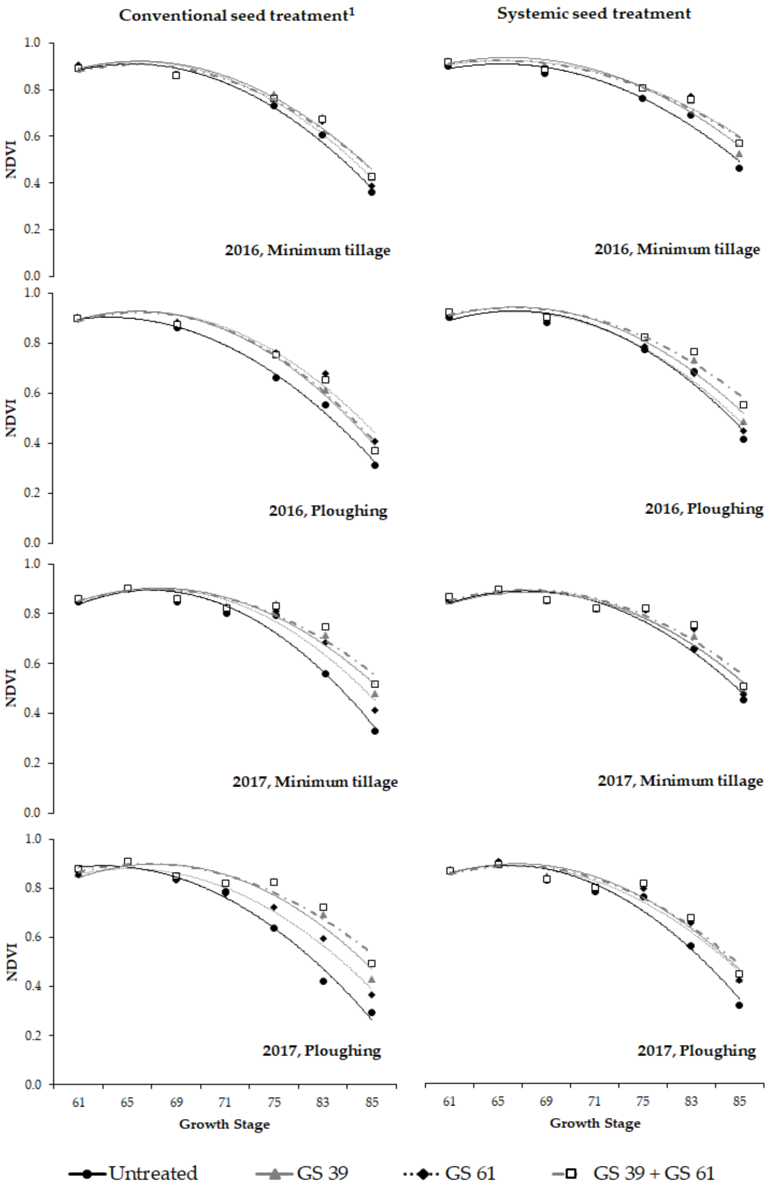


Figure 3. Effect of the fungicide foliar treatments [1] *on the normalized difference vegetation index (NDVI) measured from anthesis (GS61) until the soft dough stage (GS85), considering the seed treatments, soil tillage, and growing seasons (2015–2016 and 2016–2017). The reported values are based on four replications.* [1] *Fungicide foliar treatment: untreated control; GS39, a single treatment at the end of stem elongation (pyraclostrobin + fluxapyroxad AI); GS61, a single treatment at the beginning of flowering (epossiconazole + metconazole AI); GS39 + GS61, a double treatment through a combination of the GS39 and GS61 applications.*

2.4.3 FHB symptoms and DON content

The FHB incidence and severity and DON content are reported in Table 3. According to the SLB severity, the disease pressure was higher in 2017 than in 2016, as a consequence of the meteorological conditions, which were more prone to fungal development. As expected, FHB infection was higher under the minimum tillage conditions than in the ploughed soil, and the DON content in the kernels increased by 139% and 454% in 2016 and 2017, respectively. No significant difference was observed for FHB incidence and severity between the fungicide seed treatments. Furthermore, the DON content was significantly higher in 2016 (+33%) for the systemic seed dressing than for the conventional one. The FHB incidence and severity, and DON contamination were affected significantly by the fungicide foliar treatments (*p*-values < 0.001). The triazole application at GS61 significantly reduced the FHB symptoms and DON content (on average by 65%) in all the production situations, compared to the untreated control. The application of strobilurin and carboxamides (GS39 or GS39 + 61) could have resulted in a significantly higher DON content than the untreated control (2017, ploughing) or the single application at GS61 (2016, minimum tillage), respectively. The interaction between seed and foliar treatments was never significant as far as the DON content is concerned.

2.4.4 Grain yield and production parameters

The grain yield and production parameters were only affected significantly by the seed treatment in 2017 (Table 4). The systemic seed dressing increased the grain yield (+5%) and TKW (+5%) more than the conventional one under both soil tillage conditions. The effect of the foliar treatment on the grain yield was significant (*p*-value< 0.01) in 2016, under the ploughing conditions, and in 2017 in both trials. Furthermore, the interaction between the seed and foliar treatments was significant in this production situation. A significant increase in grain yield and TKW was recorded in 2016, albeit only for the conventional seed dressing, compared to the untreated control (Figures 4 and 5). A significant and similar increase in grain yield (+29%) and TKW (+10%) was recorded for both seed treatments in 2017, under minimum tillage conditions, as a result of the application of triazoles at flowering (GS61 or GS39 + GS61). In the same year, but in the ploughed plots, the highest TKW were obtained for the fungicide application at GS39 or at GS61, when the wheat seeds were treated with the conventional or the systemic AI (Figure 5).

Table 3. Effect of the fungicide seed and foliar treatments on Fusarium Head Blight (FHB) incidence and severity at the early dough stage (GS83) and on deoxynivalenol (DON) content in the 2015-16 and 2016-17 growing seasons in North-Italy.

Year	**Soil tillage**	**Factor**	**Source of variation**	**FHB incidence (GS83) %**	**FHB severity (GS85) %**	**DON µg kg^{-1}**
2016	Minimum tillage	Seed treatment[1]	Conventional	38.6	5.4	940 b
			Systemic	42.3	4.8	1126 a
			p-value[3]	0.245	0.427	0.025
		Foliar treatment[2]	Untreated	58.6 a	8.3 a	1245 a
			GS39	50.7 a	9.2 a	1457 a
			GS61	25.7 b	1.4 b	549 c
			GS39 + GS61	26.7 b	1.5 b	882 b
			p-value	<0.001	<0.001	<0.001
		Seed x Foliar	*p*-value	0.225	0.590	0.413
2016	Ploughing	Seed treatment	Conventional	26.7	1.6	342 b
			Systemic	32.3	1.8	501 a
			p-value	0.130	0.573	0.012
		Foliar treatment	Untreated	43.0 a	2.7 a	604 a
			GS39	39.2 a	2.5 a	645 a
			GS61	16.1 b	0.7 b	244 b
			GS39 + GS61	19.7 b	0.9 b	193 b
			p-value	<0.001	<0.001	<0.001
		Seed x Foliar	*p*-value	0.869	0.096	0.813
2017	Minimum tillage	Seed treatment	Conventional	51.2	19.3	3682
			Systemic	51.3	19.2	3966
			p-value	0.974	0.981	0.521
		Foliar treatment	Untreated	65.9 a	30.5 a	6001 a
			GS39	69.4 a	31.5 a	6593 a
			GS61	36.7 b	8.0 b	1457 b
			GS39 + GS61	33.1 b	6.9 b	1243 b
			p-value	<0.001	<0.001	<0.001
		Seed x Foliar	*p*-value	0.907	0.737	0.287
2017	Ploughing	Seed treatment	Conventional	30.1	5.9	530
			Systemic	30.0	5.8	839
			p-value	0.954	0.926	0.064
		Foliar treatment	Untreated	53.0 a	11.2 a	853 b
			GS39	41.2 b	10.1 a	1414 a
			GS61	13.0 c	1.3 b	276 c
			GS39 + GS61	13.1 c	0.8 b	275 c
			p-value	<0.001	<0.001	<0.001
		Seed x Foliar	*p*-value	0.008	0.058	0.209

[1] Fungicide seed treatments: conventional (fludioxonil AI) and systemic (fluxapyroxad AI); [2] Fungicide foliar treatment: untreated control; GS39, a single treatment at the end of stem elongation (pyraclostrobin + fluxapyroxad AI); GS61, a single treatment at the beginning of flowering (epoxiconazole + metconazole AI); GS39 + GS61, a double treatment through a combination of the GS39 and GS61 applications; [3] Means followed by different letters are significantly different (the level of significance of the *p*-value is reported in the Table), according to the REGW-F test.

Table 4. Effect of the fungicide seed and foliar treatments on the grain yield, test weight (TW) and thousand kernel weight (TKW) in the 2015-16 and 2016-17 growing seasons in North-Italy.

Year	**Soil tillage**	**Factor**	**Source of variation**	**Grain yield** ($t\ ha^{-1}$)	**TW** ($kg\ hl^{-1}$)	**TKW** (g)
2016	Minimum tillage	Seed treatment[1]	Conventional	7.4	81.8	46.4
			Systemic	7.5	81.4	44.9
			p-value[3]	0.572	0.085	0.057
		Foliar treatment[2]	Untreated	7.3	81.3	45.3
			GS39	7.5	81.5	45.1
			GS61	7.5	81.8	46.2
			GS39 + GS61	7.5	81.8	45.7
			p-value	0.468	0.303	0.817
		Seed x Foliar	*p*-value	0.465	0.032	0.485
2016	Ploughing	Seed treatment	Conventional	8.2	81.3	46.6
			Systemic	8.2	81.0	46.3
			p-value	0.527	0.598	0.362
		Foliar treatment	Untreated	7.8 b	80.5	45.0 b
			GS39	8.4 a	81.3	47.0 a
			GS61	8.4 a	81.4	46.8 a
			GS39 + GS61	8.3 a	81.7	47.0 a
			p-value	0.002	0.185	0.011
		Seed x Foliar	*p*-value	0.381	0.278	0.27
2017	Minimum tillage	Seed treatment	Conventional	7.1 b	72.2 b	42.1 b
			Systemic	7.4 a	73.2 a	42.9 a
			p-value	0.019	0.001	0.003
		Foliar treatment	Untreated	6.3 b	70.9 b	40.2 b
			GS39	6.6 b	71.1 b	41.0 b
			GS61	8.0 a	74.3 a	44.3 a
			GS39 + GS61	8.2 a	74.4 a	44.4 a
			p-value	<0.001	<0.001	<0.001
		Seed x Foliar	*p*-value	0.878	0.605	0.001
2017	Ploughing	Seed treatment	Conventional	7.6 b	72.9	41.0 b
			Systemic	8.1 a	72.7	43.9 a
			p-value	<0.001	0.622	<0.001
		Foliar treatment	Untreated	6.8 c	71.7 b	37.6 c
			GS39	7.9 b	72.6 ab	44.0 b
			GS61	8.0 b	72.9 ab	43.2 b
			GS39 + GS61	8.6 a	73.8 a	45.0 a
			p-value	<0.001	0.008	<0.001
		Seed x Foliar	*p*-value	<0.001	0.003	<0.001

[1] Fungicide seed treatments: conventional (fludioxonil AI) and systemic (fluxapyroxad AI); [2] Fungicide foliar treatment: untreated control; GS39, a single treatment at the end of stem elongation (pyraclostrobin + fluxapyroxad AI); GS61, a single treatment at the beginning of flowering (epoxiconazole + metconazole AI); GS39 + GS61, a double treatment through a combination of the GS39 and GS61 applications; [3] Means followed by different letters are significantly different (the level of significance of the *p*-value is reported in the Table), according to the REGW-F test.

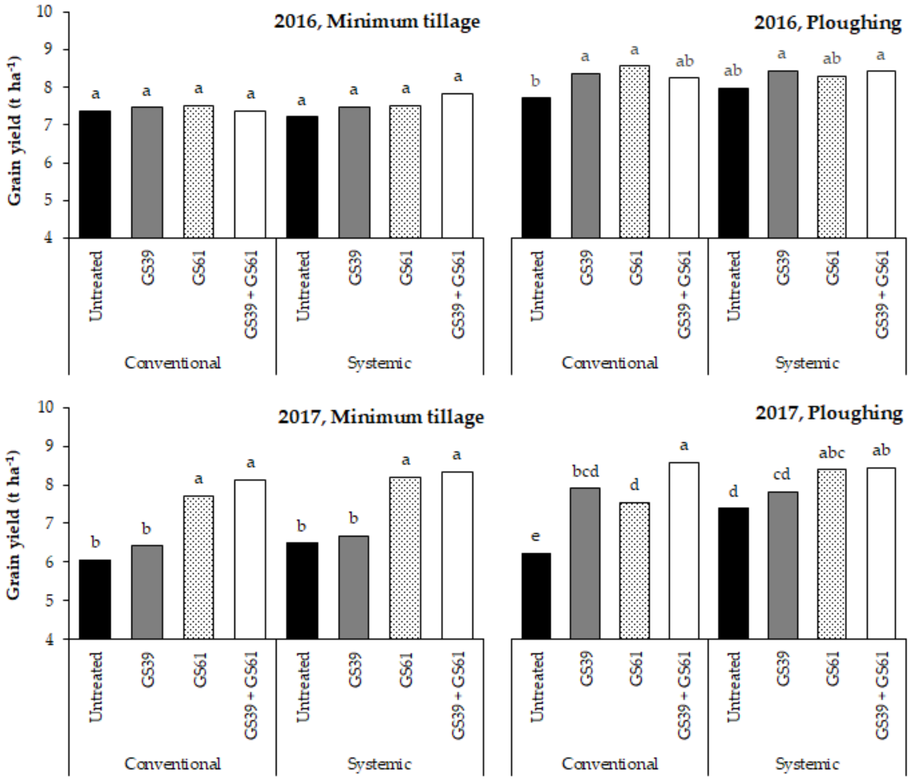


Figure 4. Effect of the fungicide seed [1] *and foliar* [2] *treatments on the grain yield under different soil tillage conditions and in different growing seasons (2015–2016 and 2016–2017) in North-Italy. The bars in each experiment with different letters are significantly different (p-value < 0.05), according to the REGW-F test. The reported values are based on four replications.* [1] *Fungicide seed treatments: conventional (fludioxonil AI) and systemic (fluxapyroxad AI);* [2] *Fungicide foliar treatment: untreated control; GS39, a single treatment at the end of stem elongation (pyraclostrobin + fluxapyroxad AI); GS61, a single treatment at the beginning of flowering (epossiconazole + metconazole AI); GS39 + GS61, a double treatment through a combination of the GS39 and GS61 applications.*

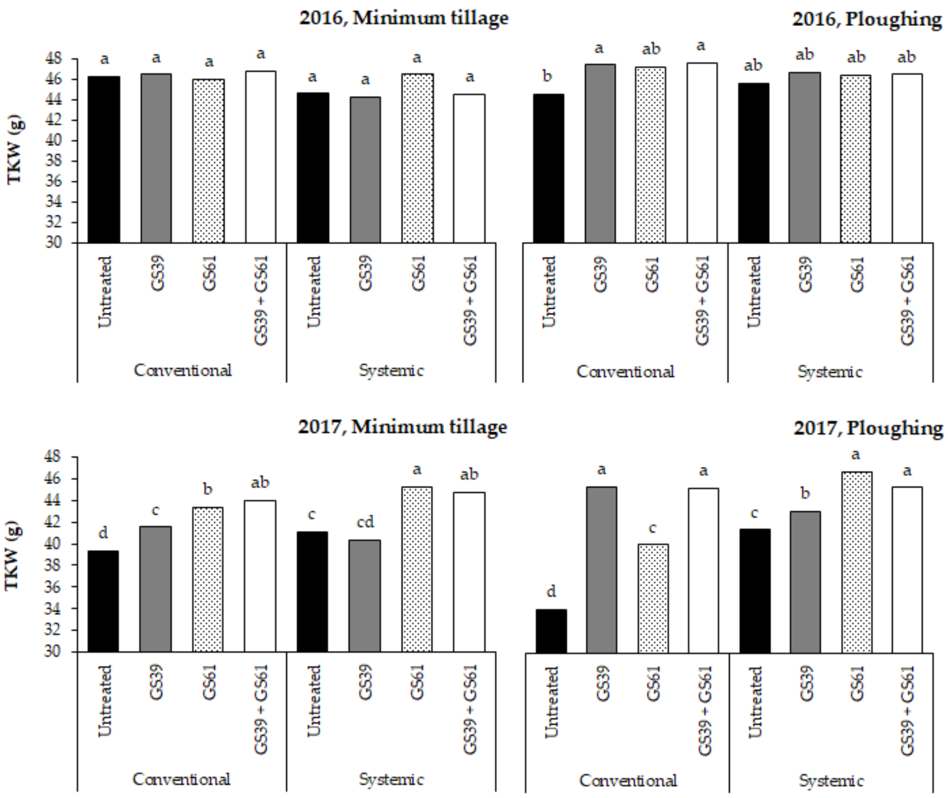


Figure 5. Effect of fungicide seed [1] *and foliar* [2] *treatments on the thousand kernel weight (TKW) under different soil tillage conditions and in different growing seasons (2015–2016 and 2016–2017) in North Italy. The bars in each experiment with different letters are significantly different (p-value < 0.05), according to the REGW-F test. The reported values are based on four replications.* [1] *Fungicide seed treatments: conventional (fludioxonil AI) and systemic (fluxapyroxad AI);* [2] *Fungicide foliar treatment: untreated control; GS39, a single treatment at the end of stem elongation (pyraclostrobin + fluxapyroxad AI); GS61, a single treatment at the beginning of flowering (epossiconazole + metconazole AI); GS39 + GS61, a double treatment through a combination of the GS39 and GS61 applications.*

2.5 Discussion

The obtained results confirm the significant link between environmental conditions, agronomic practices, and fungal protection programs. The wetter and hotter spring months in 2017 led to more severe SLB and FHB infections and development than in 2016, thus showing larger differences between the compared fungicide strategies and a more effective role of both the seed and foliar treatments in preserving grain yield. Furthermore, in both years, the presence of previous crop residues on the soil surface (minimum tillage) or their deep burial (ploughing) also clearly had an impact on the severity of the involved fungal species. It has been reported widely that the primary reservoir of FHB inoculum is debris from the previous crop, and DON contamination is more severe if the preceding crop is maize, since *Fusarium* survive longer on residues that do not degrade easily, and there is a direct relationship between debris biomass and fungal sporulation (Blandino et al., 2010). Thus, soil ploughing is the crop practice that is best able to reduce *Fusarium* infection on wheat (Baliukonienè et al., 2011). On the other hand, under the considered conditions, the SLB severity on the wheat canopy was lower for the minimum tillage than for ploughing. In experiments carried out in Canada (Gilbert and Woods 2001) and in Latvia (Bankina et al., 2018), SLB was found to be more frequent under conventional tillage, while tan spot (*Pyrenophora tritici-repentis*) was predominant under minimum tillage, thus suggesting a negative relationship between these pathogens. According to Bankina et al., 2014, *Z. tritici* can survive in living plants as pycnidia, and the presence of plant debris on the soil surface could therefore be less important for the development of this disease. The marked difference in SLB symptoms observed in our study for different soil tillage operations and maize as the previous crop, would seem to suggest that the high level of *Fusarium* inoculum produced under minimum tillage conditions may have had a biocontrol effect, thereby reducing the infection of *Z. tritici*. In all the production situations considered in the present study, the application of a foliar fungicide has led to a significant control of the fungal diseases, while the benefits, in term of grain yield have been observed more clearly for 2017, the year with the higher foliar and head disease pressure, than for 2016. Moreover, the collected data underline how the choice of the most appropriate fungal control strategies is closely related to the cropping systems. When the main target of a wheat crop protection program is FHB control, e.g., of the environments and cultivar, or crop practices, such as minimum tillage, which can lead to a higher risk of *Fusarium* infection and development, the application of a triazole fungicide at flowering should be mandatory to minimize the yield losses, to maintain acceptable TW values and to keep the contamination of DON below the regulatory limit thresholds. These results are in agreement with several research activities carried out in temperate growing areas, where applying triazoles at GS61 was found to be the best direct control solution against FHB infection and DON contamination (Haidukowski et al., 2005; Paul et al., 2008; Blandino et al., 2011). Moreover, in previous studies, carried out in North Italy (Scarpino et al., 2015; Blandino et al., 2017), this fungicide application led to a clear reduction, not only of DON, but also of several other mycotoxins and fungal metabolites produced by *F. graminearum* and *F. culmorum*, in addition to other emerging mycotoxins, such as enniatins and moniliformin, and metabolites produced by other fungal genus, such as *Alternaria* and *Claviceps*. As far as DON control efficacy is concerned, the double fungicide application (GS39 + GS61) did not result in any differences in most cases, compared to the single treatment (GS61), and the single application of the strobilurin and carboxamide mixture at the end of stem elongation did not lead to any advantages. Furthermore, the strobilurin and carboxamide mixture treatment carried out at GS39 could result in an increased risk of mycotoxin contamination, as a consequence of a slower dry down of the canopy during ripening, or a possible fungal competitive interaction phenomenon, with a shift of the fungal community. This change in the relative competition capacity among fungal species, as a result of the application of a control factor, which could result in an unexpected increase in the mycotoxin content, has been named the “flora inversion” phenomenon (Blandino et al., 2017).

It has been widely reported that the application of strobilurin AI at wheat flowering is less effective against *F. graminearum* and *F. culmorum*, but it is able to significantly reduce the non-toxigenic *M. nivale*, and could therefore increase DON contamination (Pirgozliev et al., 2003; Blandino et al., 2006). In the present experiments, this possible effect on the fungal microbial shift was also observed for earlier applications than those at flowering.

Although the fungicide application at GS61 led to a clear reduction in SLB severity at the dough stages, and significantly prolonged the canopy stay green, the fungicide application at the end of stem elongation (GS39) in the production situations that made wheat more prone to SLB attacks (ploughing and conventional seed dressing in the 2017 experiment) led to the highest level of protection of the canopy, and in particular of the flag leaf, in the early ripening stages, thereby resulting in overall greater yield benefits. Similarly, the double foliar fungicide application led to a significant control of SLB at the dough stage and to an increase in yield, compared to the single fungicide treatment for cropping systems and environmental conditions highly prone to SLB. In the environments and genotype (durum wheat) with a high SLB pressure, the double treatment, with a strobilurin application at the stem elongation stage and an azole application at flowering, showed clear advantages, in terms of the delay of flag leaf senescence and yield, compared to the treatment at flowering alone (Blandino et al., 2009). Several studies have reported a significantly higher apacity of strobilurin (Ruske et al., 2003; Blandino et al., 2011) and carboxamides (Castro et al., 2018; Fleitas 2018) to control foliar disease and to maintain the green leaf area longer than triazoles, as well as of reducing the decline in flag leaf physiological activity and ensuring higher grain yields. In addition, both strobilurin and carboxamide have demonstrated the capacity to provide physiological benefits that further improve the photosynthetic rate of wheat (Berdugo et al., 2012; Smith et al., 2013; Ajigboye et al., 2014; Carucci et al., 2020) and other arable crops (Kato et al., 2011; Testa et al., 2018).

Whenever a conventional fungicide seed dressing is applied, the profitability of the double foliar treatment could increase, with an anticipation of the stem elongation timing (from GS32 to GS35), extending the interval of canopy protection and reducing early disease development. Moreover, the collected data highlight how the application of a seed dressing with a systemic carboxamide fungicide to winter wheat could change the overall foliar fungicide programs applied at spring. Compared to a conventional seed treatment, the use of fluxapyroxad AI, which is able to translocate inside the plant and to be active for longer, guarantees a greater and longer lasting protection, and also leads to significantly lower SLB severity at the dough stage. The protection activity of this solution led to a clear delay in canopy senescence, in particular during the ripening stage, as observed from the NDVI trend for the whole crop cycle. The overall higher AUCDC vegetation index is the result of the expression of a higher photosynthetic activity, which resulted in a significant increase in TKW and TW in the 2017 trials, and thus in grain yield (Dimmock and Gooding, 2002). As expected, the benefits of a systemic seed treatment were more effective in production situations in which the development of SLB is the target disease. Under these agronomical conditions, the prolonged activity of a seed dressing in controlling fungal disease throughout the vegetative stages cancels out the advantage of administering a specific treatment at the emission of the flag leaf (GS39), thereby leading to more effective benefits for the combination with a late application at flowering, a timing in which it is crucial to control FHB and mycotoxin contamination. Moreover, no further yield benefits have been observed in any of the trials with the double fungicide foliar application. Thus, the systemic fungicide seed treatments, with a prolonged fungal control, permit the need for foliar treatments to be reduced, thereby allowing the number of pesticide treatments and the overall AI quantity per surface unit applied to be reduced. Moreover, compared to spray applications, the use of seed dressing is an easy strategy to apply and is safer for farmers and non-target organisms (Lamichane et al., 2019).

Since *Fusarium* infections at flowering occur from the inoculum produced on the soil surface and from previous crop residues, which reach the ears mainly through dispersal in rain splashes (Bateman 2005), the seed treatment did not influence the FHB symptoms. Furthermore, in the year 2016, which showed a moderate FHB infection, the conventional phenylpyrrole seed dressing resulted in a significantly lower DON content than the systemic carboxamide one, which is less effective against *Fusarium* spp. Although the systemic growth of a *Fusarium* fungus originating from seeds is not able to reach the wheat heads, Moretti et al., (2014) reported that a seed treatment prevented crown and root rot, and minimized the amount of DON that was able to translocate from the plant to the kernels because of its solubility in water. After comparing the role of seed treatments in different cropping systems, Blandino et al., (2011) stated that a fludioxonil seed application on average reduced DON by 10% at harvesting, compared to an untreated control. Although the effect was not significant in 2017, the year with the highest level of FHB symptoms, the DON contamination was lower after the conventional seed dressing than after the systemic one. It has been hypothesized that the higher relative contribution of aerial head infection in that year, compared to the quantity of DON originating from the systemic infection, led to a less quantifiable effect of the seed dressing on mycotoxin contamination. Since the considered carboxamide fungicide is not able to efficiently prevent several of the fungal species that affect seedlings, crown and root rot, its combination with other systemic AI, such as triazoles, which are able to contribute to the control of foliar diseases (Sundin et al., 1999), may represent a more efficient strategy for wheat seed dressing. Among the other benefits of a fungicide seed dressing, but which was not quantified in the present study, the key role such a dressing plays in controlling soilborne and seed borne pathogens that can attack seedlings and plants in the early growth stages should be mentioned, since no other effective direct control strategies can be applied (May et al., 2010). Moreover, as previously reported, in addition to the protection endowed in the first growing stages, seed treatments with systemic and prolonged activity could permit a late shift of foliar application, thereby reducing the lack of control of diseases whenever the environmental conditions prevent an operator from entering a field to carry out foliar spraying. Rios et al., (2017) highlighted that the early infection of leaves may have a negative impact on the physiology and photosynthesis of wheat.

In conclusion, our results, obtained under naturally infected field conditions, provide useful information to help evaluate the effects of different fungicide programs, based on the combination of seed and foliar treatments on wheat yield and sanity in distinct SLB and FHB disease pressure scenarios. The choice of the fungal control strategy is closely related to environmental (weather conditions, fungal population) and agronomic factors (mainly cultivar susceptibility, but also crop rotation and/or soil tillage as in the present study), thus it needs to be designed according to the overall fungal disease risk of the cropping system. In this context, the use of systemic seed treatments that are able to guarantee a prolonged protection from foliar diseases and to increase the duration of the green leaf area until the ripening stages, is a strategic practice that could be adopted to set up an effective crop protection program, in order to allow a greater sustainability of wheat cultivation to be obtained. Thus, because of the smaller amount of AI applied per hectare and the low risk for farmers and non-target organisms, seed application could represent a promising solution to reach the ambitious targets of a reduction in pesticide use and risks within the Farm to Fork Strategy proposed by the EU commission (Farm to Fork Strategy).

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3. CHAPTER III - 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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Graphical abstract

3.1 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

3.2 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 (Mueller et al., 2016). 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* (Agrios 2005). 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 (Munkvold and O'Mara 2002; Pascale et al., 2011). 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 (Venturini et al., 2020) 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 (Galperin et al., 2003). Previous studies that were conducted to evaluate the contribution of *F. verticillioides* seed-borne inoculum to maize seedling blight epidemics reported conflicting results (Galperin et al., 2003; Yates et al., 2005; Galli et al., 2005).

However, there is a general agreement on the negative effect of seed-borne *Fusarium* spp. on germination and on a reduced seedling growth (Munkvold and O'Mara 2002; Kuhnem et al., 2013), 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 (Marburger et al., 2015). 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 (Blandino et al., 2009), 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 (Testa et al., 2016), 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 (Rodriguez-Brljevich 2008). Moreover, fungicide seed dressings may contribute to minimizing the damping-off and growth delay related to seed-borne infection (Da Silva et al., 2015). 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) (Munkvold 2009). 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. (Bradley 2008), while the latter is more effective on *Pythium* and *Phytophthora* (Dorrance and McClure 2001; Acharya et al., 2018). 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 (Munkvold and O’Mara 2002; Aveling et al., 2013), 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 (Rodriguez-Brljevich et al., 2010), and in particular on grain yield (Solorzano and Malvick 2011; Acharya et al., 2018). 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.

3.3 Materials and methods

3.3.1 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 effcacy of different fungicide applications in controlling *F. verticillioides* and *F. graminearum* seed infection.

a. Study I. Fungicide seed dressing 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. 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* (106 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 X 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.

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	silt loam	silt 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

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 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.). 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.

b. *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.

3.3.2 Crop assessments

a. *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.

b. Crop vigor

Different assessments were performed to establish vigor in the early vegetative stages. At the stem elongation stage (GS 32–35, BBCH Scale, Lancashire et al. 1991), 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 Govaerts and Verhulst (2010):

$$\mathrm{NDVI} = \frac{\mathrm{RNIR} - \mathrm{RRed}}{\mathrm{RNIR} + \mathrm{RRed}}$$

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 (GS14) until tassel emission (GS55). The Area Under the Canopy Development Curve (AUCDC) was calculated, starting from the NDVI measurements, using the following formula:

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

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).

c. 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%.

3.3.3 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 (Tamhane 1996), on the treatment means (p-value < 0.05). SPSS, version 25 (SPSS, IBM Corporation, Armonk, NY, USA, 2008), was used for the statistical analysis.

3.4 Results

3.4.1 Meteorological 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 (mm)	**GDDs [4] (°C-day)**	**Rainfall (mm)**	**GDDs (°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.

3.4.2 Study I. Fungicide seed dressing 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 (GS14) to tassel emission (GS55), 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.

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.

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.

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**	**(Σ 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-value [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-value	<0.001	<0.001	<0.001
	Seed dressing × Exp.	*p*-value	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-value	<0.001	<0.001	<0.001
	Experiment	F	5.2 a	4.7	17.6 b
		G	5.4 a	5.1	22.0 a
		H	3.9 b	5.2	16.6 c
		p-value	<0.001	0.243	<0.001
	Seed dressing × Exp.	*p*-value	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-value	<0.001	<0.001	<0.001
	Experiment	I	2.9	2.3 b	7.9 c
		L	3.4	3.5 a	19.2 a
		M	3.2	3.5 a	15.6 b
		p-value	0.09	<0.001	<0.001
	Seed dressing × Exp.	*p*-value	0.722	0.075	0.956

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

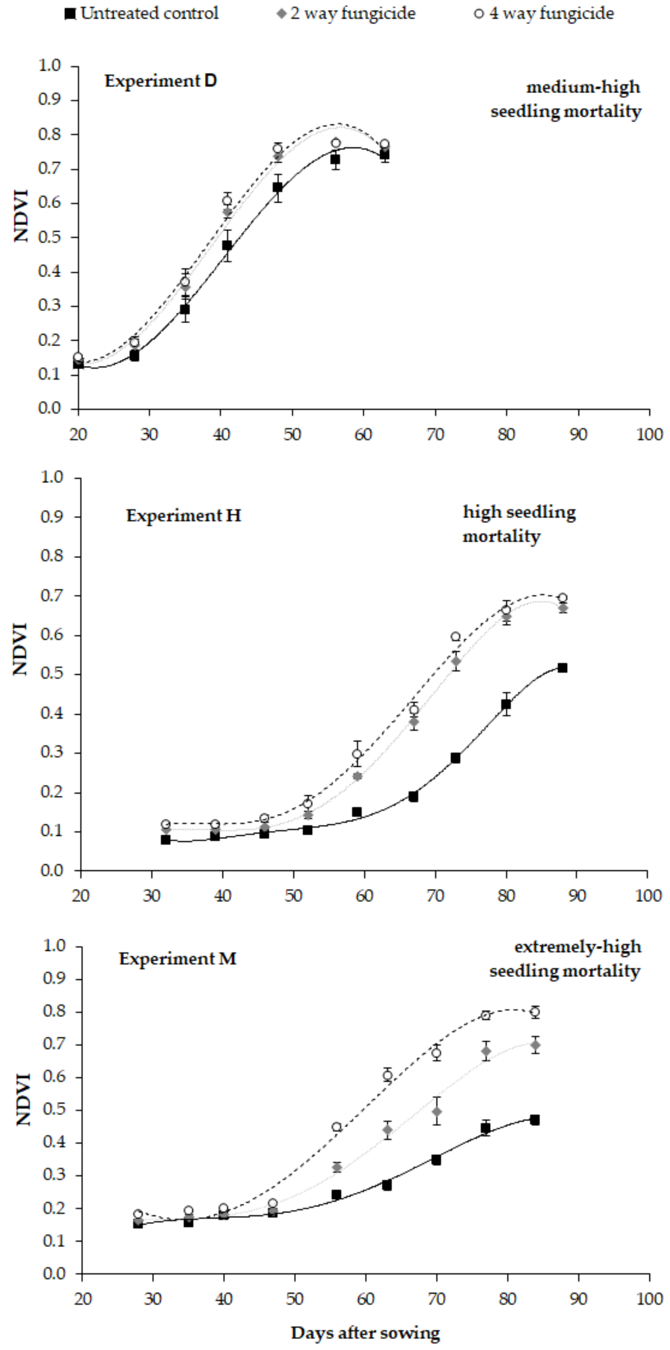

Figure 1. Effect of the application of fungicides to maize seeds artificially infected with F. verticillioides on the normalized difference vegetation index (NDVI) measured from the 4-leaf stage (GS14) until tassel emission (GS55). The reported data are an example for the experiments characterized by 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-value [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-value	<0.001	<0.001	<0.001
	Seed dressing × Exp.	*p*-value	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-value	<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-value	<0.001	<0.001	<0.001
	Seed dressing × Exp.	*p*-value	0.006	0.004	0.062
Extremely-high	Seed dressing	Untreated control	0.005 c	3.0 c	27.4 c
		2-way fungicide	0.01 b	4.0 b	44.3 b
		4-way fungicide	0.014 a	4.9 a	60.0 a
		p-value	<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-value	<0.001	<0.001	<0.001
	Seed dressing × Exp.	*p*-value	0.438	0.956	0.933

[1] Growth stag e 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*-value, is reported in the Table), according to the REGW-F test.

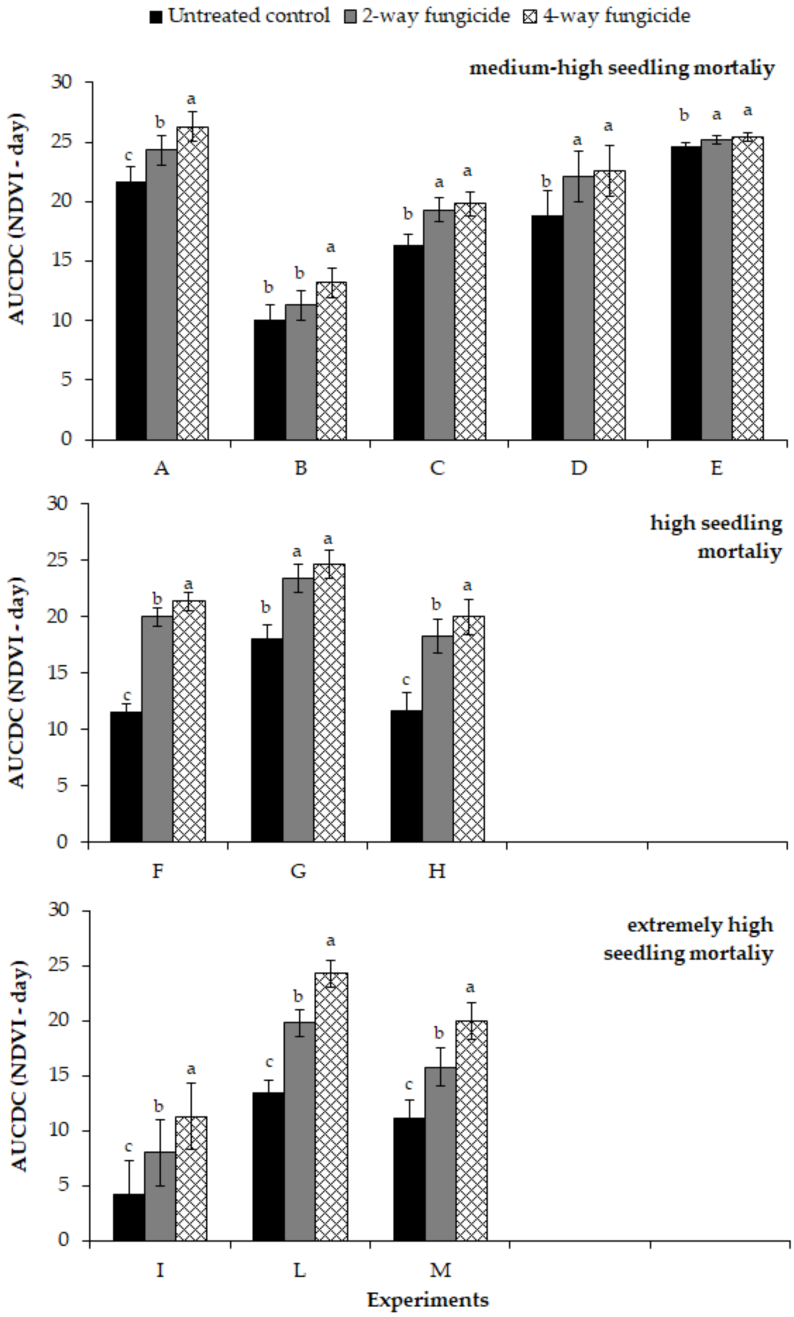

Figure 2. Effect of the fungicides applied to the maize seeds artificially infected with F. verticillioides on the Area Under the Crop Development Curve (AUCDC) detected during the vegetative stages in the different experimental sites clustered for seedling mortality. Bars in each experiment with different letters are significantly different ($p < 0.05$), 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
		2-way fungicide	10.6 b	25.5
		4-way fungicide	12.0 a	24.8
		p-value [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-value	<0.001	<0.001
	Seed dressing × Exp.	*p*-value	0.001	0.846
High	Seed dressing	Untreated control	8.4 b	25.1
		2-way fungicide	11.8 a	25.5
		4-way fungicide	13.1 a	25.1
		p-value	<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-value	<0.001	<0.001
	Seed dressing × Exp.	*p*-value	0.679	0.87
Extremely high	Seed dressing	Untreated control	3.0 c	23.6
		2-way fungicide	8.0 b	23.0
		4-way fungicide	11.6 a	22.1
		p-value	<0.001	0.120
	Experiment	I	6.9	18.0 c
		L	8.0	22.9 b
		M	7.6	27.7 a
		p-value	0.243	<0.001
	Seed dressing × Exp.	*p*-value	0.192	0.178

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

3.4.3 Sudy II. Fungicide seed dressing to control *F. verticillioides* and *F. graminearum* damage on maize seedlings and plants under different environmental conditions

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.

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.

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-value [2]		<0.001	<0.001
Year	2018		7.2 a	92.5 a
	2019		5.6 b	21.5 b
	p-value		<0.001	<0.001
Treatment × year	*p*-value		<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*-value, 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 (Σ 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-value [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-value		<0.001	<0.001	0.013
Treat. × Year	*p*-value		<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*-value, is reported in the Table), according to the REGW-F test.

3.5 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 (Pinto 2000). 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* (Broders et al., 2007).

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 (Munkvold and O'Mara 2002; Da Silva et al., 2015) and in Brazil (Kuhnem et al., 2013), *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 (Purahong et al., 2014) 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 (Solorzano and Malvick 2011). 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., (2000) 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 (Dragičević et al., 2011; Da Silva et al., 2015) or soybean (Dorrance and McClure 2001; Costa et al., 2019), as quantified by a higher dry mass of both the shoots and roots. Moreover, Rodriguez-Brljevich (2010) 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, Otegui et al., 1996), 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 (Blandino et al., 2009).

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 (Covarelli et al., 2012; Venturini et al., 2020). 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* (Solorzano and Malvick 2011; Da Silva et al., 2017). 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 (Munkvold 2009). This could make these ingredients more active in controlling the detrimental effects of such systemic pathogens as *F. verticillioides*. Field experience with wheat (Boyacioglu et al., 1992) 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 (Pinto 2000). 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 (Kitchen et al., 2016). 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 (Pinto 2000). 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 (Aveling et al., 2013), 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 (Testa et al., 2015), an increase in photosynthesis efficiency, for higher true photosynthesis, and a reduction in dark respiration (Amaro et al., 2020). Enhanced maize performance, even in the absence of disease, has also been reported for foliar applications of azoxystrobin (Blandino et al., 2012) and pyraclostrobin (Testa et al., 2018) from the stem elongation stage to flowering. Conversely, no significant effects have been reported for earlier growing stage applications (five leaf-stage, Blandino et al., 2012), 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 (Dalla Lana et al., 2009), while strobilurins enhanced rice seedling growth after root cutting injury by inducing reactive oxygen scavenging activity, thus inhibiting reactive oxygen species accumulation (Takahashi et al., 2017). Under controlled sterilized conditions, pyrazole carboxamide sedaxane facilitates root establishment and intensifies nitrogen and the phenylpropanoid metabolism of maize seedlings (Dal Cortivo et al., 2017).

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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4. CHAPTER IV - The role of zinc fertilization and its interaction with nitrogen and phosphorous starter fertilization on early maize development and grain yield.

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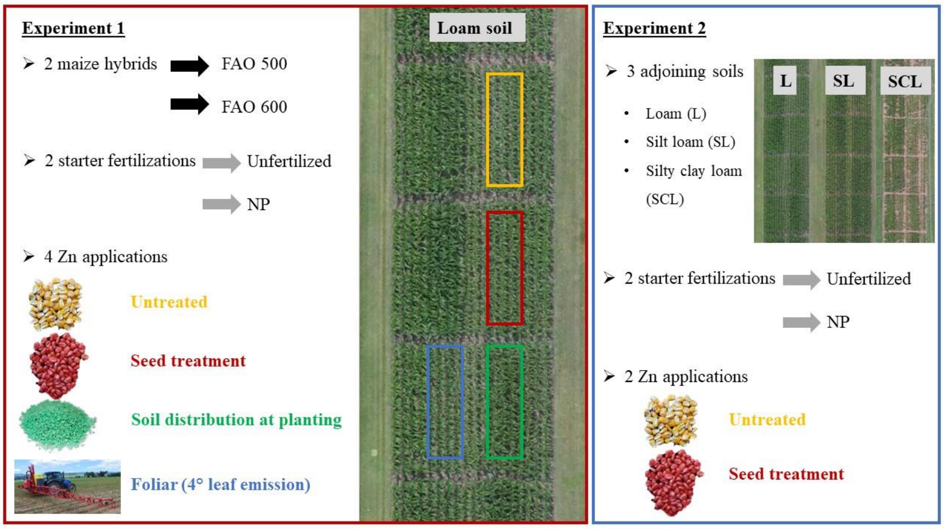
Graphical abstract



4.1 Abstract

The early planting of maize in temperate growing areas leads to yield and quality benefits, although a low soil temperature can affect the early nutrient uptake and delay plant development. Not only could the nitrogen (N) and phosphorus (P) uptake be limited, but also that of zinc (Zn) could be affected, while their application could result in an enhancement of maize growth. The aims of this study have been to evaluate the most effective Zn application strategy by comparing the seed treatment, soil distribution, and foliar application with an untreated control, and to investigate the role of a Zn seed treatment on the early development and yield of maize grown in three different types of soils characterized by a low, high, and medium P content. A subsurface NP fertilization in bands at planting was included in an experimental factorial design with a Zn application in two experiments, which were carried out in North Italy during the 2012-14 period. Zn fertilization significantly enhanced the early vigor and yield of maize, although the effects were lower than those of the NP fertilizer. Among the Zn fertilization strategies, maize growth was quicker for the seed and soil application, which both increased the plant height at stem elongation (+32%), shortened the planting-flowering period by 1 day, and increased the grain yield by 4%, than for the foliar application and the untreated control. The second experiment confirmed the positive impact of the Zn seed treatment in the different soils. The interaction between the NP fertilization and the Zn treatment was never significant, thus, the positive effect of Zn on the early vigor was an additional benefit to that caused by the starter NP fertilization. The study has demonstrated that a Zn application at planting in temperate growing areas significantly enhances the maize growth and yield with different hybrids, growing seasons and soils.

Keywords: corn, diammonium phosphate, early vigor, grain yield, seed treatment, foliar treatment, seed furrow

4.2 Introduction

Maize (*Zea mays* L.) is the first crop for worldwide production (1210 million tons, FAOstat, 2022), and it is increasingly being used in different kinds of food, as well as in the feed, starch, and biofuel sectors (Erenstein et al., 2022). Thus, the expected growth in the demand of this commodity will require a further increase in yield, which may be achieved by focusing on highly efficient crop practices. Breeding programs have increased the tolerance of modern maize hybrids to cold temperatures (Reis et al., 2022), thereby allowing a progressive anticipation of the planting date in temperate growing areas (Gillani et al., 2021). An early planting enhances the interception of solar radiation by the canopy during the crop cycle (Otegui et al., 1995; Zhang et al., 2019), reduces the risk of drought and heat stresses at flowering (Waqas et al., 2021), and leads to an earlier harvest in autumn with a lower drying cost (Xu et al., 2022) and a lower risk of mycotoxin contamination (Blandino et al., 2017). Therefore, an early planting date increases the efficiently of the grain yield, as well as the sustainability and economic profitability of the maize cropping system (Tsimba et al., 2013).

However, an early planting time also makes the crop more prone to cold and rainy weather conditions, which could lead to a slowdown in crop growth, thereby reducing the positive effects of this practice. Moreover, low soil temperatures constrain the uptake of phosphorous (P), nitrogen (N), and other macro and micronutrients by the root system, even in soils that have a high nutrient content, thus negatively affecting early crop development (Rehm and Lamb 2009; Imran et al., 2013; Battisti et al., 2022a). The application of NP fertilizers in bands close to maize seed furrows satisfies the early plant demand by providing nutrients in close proximity to the small and developing root system (Nkebiwe et al., 2016; Zhang et al., 2023), shortening the planting-flowering period, increasing the grain yield, and minimizing the mycotoxin content (Kaiser et al., 2016; Battisti et al., 2022b).

Low temperatures in the soil, apart from effecting the macronutrients, could also have an impact on the uptake of such micronutrients as zinc (Zn), and this could be a further growth-limiting factor (Cakmak and Kutman 2018). Furthermore, maize shows a higher impact of Zn deficiency than other crops (Fageria et al., 2002). Since Zn is a constituent of several enzymes, this micronutrient is involved in several plant functions and affects photosynthesis and the phytohormone activity, pollen formation, seed production, the carbohydrate metabolism, protein synthesis, membrane permeability, and signal transduction, and thus plays an important role in seedling physiology and plant establishment (Alloway 2008; Maqbool and Beshir 2019; Suganya et al., 2020). It has been estimated that half of the world’s cereal surface has low levels of available Zn for plants (Cakmak 2009), mainly in soils where the distribution of animal manure is infrequent (Ogiyama et al., 2005; Brock et al., 2006). Zn is taken up from soil primarily as divalent cations (Zn^{2+}) (Fageria et al., 2002), but the soil texture and the soil properties, such as pH, electrical conductivity, organic matter, calcium carbonate ($CaCO_3$) and nutrient interaction (mainly through phosphate fertilizers), can negatively affect the availability of Zn (Hafeez et al., 2013). Among the chemical properties of soil, the soil pH is the most important factor in controlling Zn mobility. Alkaline calcareous soils, which are characterized by low organic matter, high levels of oxides and high carbonate contents, favor Zn adsorption and decrease its concentration in the soil (Montalvo et al., 2016). Furthermore, lighter sandy textured soils with a low cation exchange capacity (CEC) have a lower retaining capacity (Alloway 2008). The Zn concentration in the soil is also influenced to a great extent by the P content (native P) and by fertilization: a high rate or prolonged use of P fertilizers can inhibit Zn mobility, thereby reducing its uptake by plants (Akhtar et al., 2019). Conversely, Karimian (1995) reported that N fertilization improved Zn crop nutrition by increasing the Zn concentration and its uptake.

Different Zn fertilization strategies can be applied: directly to soil (broadcast or banded), or as seed or foliar treatments (Cakmak and Kutman 2018; Martínez-Cuesta et al., 2021). Seed treatment applications could influence plant growth at the earliest growth stages after germination and also during establishment (Farooq et al., 2012; Maqbool and Beshir 2019; Tamindzic et al., 2021), while the distribution in seed furrows at planting or a foliar application in a mixture with a post-emergence herbicide could result in a delayed effect. Furthermore, these fertilization strategies permit higher nutrient rates than a seed treatment (Montalvo et al., 2016; Cakmak and Kutman 2018).

Since the effects of Zn fertilization on grain yield and grain Zn biofortification have generally been taken into account in other studies by considering just one of the possible application strategies, there is a lack of knowledge on the impact of different Zn fertilization strategies on the early development of maize under field conditions, as well as a direct comparison with a well-known strategy, such as NP starter fertilization banded at planting. The aims of this study, carried out as part of a large set of field experiments, have been: I) to study the effects of different Zn application strategies (soil, seed or foliar) on the early vigor and grain yield of maize in a subalkaline calcareous soil; II) to compare the effects of Zn application to the subsurface with an NP starter fertilizer on the early vigor and grain yield of the plant, considering the possible interaction between these practices; III) to verify the role of a Zn seed treatment, that is, a seed coating, on plant growth in soils with different textures, reactions, and P contents under the same environmental conditions.

4.3 Materials and methods

The study was carried out at the University of Turin research station in Carmagnola (44° 53'N; 7° 41'E; 245 m a.s.l.) in the North-West of Italy over three growing seasons, from 2012 to 2014. The climate in this area is temperate sub-continental, with a mean annual temperature of 12°C and an average annual rainfall of 760 mm. The daily temperatures and precipitation were measured over the three growing seasons by a meteorological station located in the experimental center. Two experiments were set up: the aim of experiment 1 was to study the most effective Zn application method, while experiment 2 was conducted to evaluate the effect of a Zn seed treatment on the early growth of maize in three soils with different textures and chemical properties. In addition, a banded subsurface NP starter fertilizer was placed at sowing in each experiment in order to compare this practice with the Zn fertilization application and evaluate their possible interaction.

4.3.1 Experiment 1 – Zn fertilizer application methods

The field experiment was carried out in a loam subalkaline soil, with a medium organic matter content and low cation-exchange capacity (C.E.C). The soil was sampled to a depth of 0-30 cm using Eijkelkamp cylindrical augers just before crop planting and analyzed for the total carbonate (Loeppert and Suarez 1996), exchangeable K (Summer and Miller 1996), total N (using a CHN elemental analyzer, Flash EA 1112, Thermoquest), available P (Olsen et al., 1954), and Zn-Di-Ethylene Triamine Penta Acetic acid (DTPA, Lindsay and Norvell 1978). Low total N, available P, and Zn contents were observed. The other main soil properties are reported in Table 1. Different maize hybrids, NP starter fertilization treatments, and Zn application methods were compared each year, according to a factorial experimental design:

- 2 maize hybrids,
    - PR33A46 (FAO maturity class 500; 128 days relative to maturity, Corteva Agriscience, Cremona, Italy),
    - P1547 (FAO maturity class 600; 130 days relative to maturity, Corteva Agriscience).
- 2 starter fertilization treatments,
    - unfertilized control (unfertilized), without any starter fertilization,
    - sub-surface NP starter fertilizer (NP) placed in bands close to the maize seed furrows, with 27 kg N ha^{-1} and 69 kg P_2O_5 ha^{-1} applied as diammonium phosphate (DAP, 18 and 46% for N and P_2O_5, respectively, w/w);

- 4 Zn application treatments,
    - untreated check (untreated); without any Zn application,
    - Zn seed treatment (seed), (Viener® Zn, 26% Zn w/w; BMS Micro-Nutrients NV, Bornem, Belgium) at a dose of 10 g kg^{-1} of maize seeds (0.07 kg Zn ha^{-1}),
    - Zn subsurface fertilization (soil), (Tradecorp® Zn, 14% Zn-chelate with ethylenediaminetetraacetic acid, EDTA, w/w; Trade Corporation International, Madrid, Spain) at a dose of 5 kg ha^{-1} in the seed furrows at maize planting (0.7 kg Zn ha^{-1}),
    - Zn foliar treatment (foliar), a single spray of 0.7 kg Zn ha^{-1} (Tradecorp® Zn, 14%, at 5 kg ha^{-1}, Zn-chelate with EDTA) in correspondence to the 4-leaf emission stage (growth stage, GS14, according to the BBCH-scale, Lancashire et al., 1991).

The treatments were assigned to experimental units using a completely randomized block design with four replicates. Each plot consisted of 4 rows 0.75 m apart, separated by two untreated buffer rows, one on either side; the plot length and the alleys between the plots were 10 and 1 m, respectively.

Table 1. Main physical and chemical characteristics of the first 0-30 cm layer of the compared soils for the field experiments carried out in the 2012-2014 period at Carmagnola (North Italy).

Soil Parameter	**Measurement unit**	**Experiment 1**	**Experiment 2**		
		L (loam)	**L (loam)**	**SL (silty loam)**	**SCL (silty clay loam)**
Sand (0.05 – 2 mm)	g kg^{-1}	323	393	203	84
Silt (0.002 – 0.05 mm)	g kg^{-1}	621	542	641	582
Clay (< 0.002 mm)	g kg^{-1}	56	65	156	334
pH (H_20) [1]		8.1	8.2	6.2	8.0
Total carbonate [2]	g kg^{-1}	16	23	-	12
Organic matter [3]	g kg^{-1}	20.2	13.0	12.4	18.2
C/N		11.5	8.6	9.0	7.6
Cation Exchange Capacity (C.E.C.)	cmol(+) kg^{-1}	8.7	8.0	11.6	24.1
Exchangeable K [4]	mg kg^{-1}	48	39	41	117
Total N [3]	g kg^{-1}	1.03	0.82	0.70	1.25
Available P [5]	mg kg^{-1}	9	8	37	24
Zn [6]	mg kg^{-1}	0.77	0.70	0.83	0.50

[1] pH 1:2.5 w/v H2O

[2] Volumetric calcimeter method (Loeppert and Suarez 1996)

[3] CHN elemental analyzer (Flash EA 1112, Thermoquest)

[4] BaCl2 extraction (Summer and Miller 1996)

[5] Bicarbonate-extractable-P (Olsen et al., 1954)

[6] Zn-DTPA (Lindsay and Norvell 1978)

4.3.2 Experiment 2 – Zn seed treatment in different soils

The study was carried out on 3 large adjoining plots, each measuring 15 × 150 m and consisting of different types of soil, as far as the texture, and the physical and chemical properties are concerned:

- a loam (L) soil (Typic Udifluvents, according to the USDA classification), which is the native soil of the experimental farm and has a medium-low C.E.C., and low N and plant-available P contents;
- a silt loam (SL) soil (Typic Dystrochrepts), which was transferred from a site in Riva presso Chieri (44° 59’ N, 7° 52’ E), and has a medium C.E.C., a low N content and a high plant-available P content;
- a silty clay loam (SCL) soil (Typic Hapludalfs), which was transferred from a site in Sale (44° 58’ N, 8° 48’ E), and has a high C.E.C, a medium N content and a medium P availability.

The SL and SCL soils were transferred in 1992 and were placed to a depth of 60 cm above the native soil (L). The Zn soil content in each soil was low, with the highest value in the SL soil and the lowest value in the SCL soil. The other physical and chemical properties are reported in Table 1.
Four fertilization strategies were compared in each year and for each soil, according to a factorial experimental design:

- 2 starter fertilization treatments:
  - unfertilized control (unfertilized), without any starter fertilization,
  - sub-surface NP starter fertilizer (NP) placed in bands close to the maize seed furrows with 27 kg N ha^{-1} and 69 kg P_2O_5 ha^{-1} applied as DAP.
- 2 Zn application treatments:
  - untreated check (untreated); without any Zn application,
  - Zn seed treatment (seed), (Viener® Zn, 26% Zn w/w) at a dose of 10 g kg^{-1} of maize seeds (0.07 kg Zn ha^{-1});

The treatments were assigned to experimental units in each soil using a split plot design, in which the Zn seed treatment was the main factor, and the NP starter fertilization was the subplot. The subplot size, replicated three times for each treatment, consisted of 4 rows 0.75 m apart and 15 m long. The study was performed using the P1543 commercial dent maize hybrid (FAO maturity class 600; 130 days relative to maturity, Corteva Agriscience).

4.3.3 Agronomic management of the experiments

The planting was carried out at the end of March – beginning of April (Table S1) in both experiments, according to the prompt planting time of the growing area, after a 0.3 m deep fall ploughing, followed by a suitable disk harrowing. When scheduled, the DAP was placed at a distance of 5 cm from the seed furrows, using a calibrated granular dispenser, at a depth of 10 cm from the soil surface, while the soil Zn application was performed through a calibrated micro-granulator; both dispensers were applied to the planter (Monosem NG, Largeasse, France). The Zn foliar treatment was performed over the entire plot surface using a four-nozzle precision sprayer (Honda Agricultural Sprayer T-Jeet A110/04; Honda Motor Europe, Ltd., London, the UK), held on the shoulders, at the 4 completely unfolded leaf growth stage (GS14).

The conventional crop practices of the growing areas were applied in both experiments. Briefly, the previous crop was maize each year. Before planting, 100 kg ha^{-1} of K_2O was applied (as potassium chloride, 60% K_2O w/w) each year, with no other N or P fertilizers applied, except for a starter in bands close to the seed furrows. The top-dressing N rates applied as urea (46% N w/w) were obtained as the difference between the crop needs (250 kg N ha^{-1}) and the amount of N supplied with the starter fertilization for the NP treatment.

The planting, harvesting, Zn foliar treatment as well as the N side-dressing fertilization dates are reported in Table S1 for each year and each experiment.

All the seeds were treated with a fludioxonil and metalaxil-m fungicide (Celest XL$^{®}$, Syngenta Crop Protection S.p.A., Milan, Italy). Systemic granular insecticide clothianidin was applied at planting in the seed furrows (Santana®, Sumitomo Chemical Agro Europe S.A.S, Lion, France) at the dose of 10 kg ha^{-1}, to limit and reduce insect damage. The weed control was conducted, at pre-emergence, with ciprosulfamide (66 g AI, Active Ingredient, ha^{-1}), isoxaflutole (100 g AI ha^{-1}), and tiencarbazone metile (40 g AI ha^{-1}) (Adengo®, Bayer Crop Science S.r.l, Milano, Italy) and at post-emergence with nicosulfuron (37 g AI ha^{-1}), rimsulfuron (9 g AI ha^{-1}), and dicamba (220 g AI ha^{-1}) (Principal® Mais, Corteva Agriscience). No foliar fungicide or insecticide was applied during the maize growing cycle. Irrigation was performed with a sprinkler, in both experiments, to avoid any drought stress until physiological maturity, according to the farm management system of the research center.

4.3.4 Crop development

A hand-held optical sensing device, GreenSeekerTM® (Trimble©, Sunnyvale, California, the USA), was used to measure the normalized difference vegetation index (NDVI) during the vegetative stages. 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 (10-15 m). This assessment was performed weekly in the two middle rows of each plot, starting from the three-leaf stage (GS13) until tassel emission (GS55). The NDVI measurement helped to quantify the development of the crop canopy throughout the season, since low values refer to bare soil, while high values are proportional to the maize biomass (Capo et al., 2020). The Area Under Canopy Development Curve (AUCDC) was calculated during the vegetative cycle, for each treatment, starting from the NDVI measurement and using the following formula:

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

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

The plant height was recorded at approximately the 3-leaf stage (GS13) and at stem elongation (GS34, approximately 4 detectable nodes) by measuring 10 consecutive plants randomly selected from the center two rows of each plot. The plant height was measured in centimeters from the ground level up to the collar of the uppermost fully developed leaf (GS13), or from the ground level up to the uppermost detectable node (GS34).

The flowering date of each plot was registered when > 50% of the plants in the two central rows of each plot had reached the beginning of ear flowering (GS61). This parameter was expressed as days after planting (DAS). All the observations described here were carried out in both experiments.

4.3.5 Grain yield and yield components

Ears were collected by hand at harvest maturity from a 4.5 m^2 area in the center of two rows in each plot to quantify the grain yield and to obtain a representative sample. The harvesting was performed on the same day in each experiment for all the compared treatments, when the grain moisture content was between 20% and 30%, according to the conventional harvesting practices in the growing areas (Table S1).

The collected ears from each plot were counted to record the density per square meter of the fully developed ears. The number of kernel rows and the number of kernels per row were also counted on 7 of these randomly selected and de-husked ears, and the theoretical amount of kernels per square meter (KSQ) was then calculated by multiplying the average number of kernels per ear by the number of ears per square meter (Battisti et al., 2022b). All the collected ears were shelled using an electric sheller. The kernels from each plot were mixed thoroughly to obtain a random distribution. Grain moisture was analyzed using a Dickey-John GAC2100 grain analyzer (Auburn, IL, the USA). The grain yield results were adjusted to a 14% moisture content. Two hundred dry kernels were randomly collected and weighed to assess the thousand kernel weight (TKW). The same measurements were performed in both experiments.

4.3.6 Statistical analysis

The statistical procedure was performed using the lmer function in the LME4 statistical package of the R software package (R Core Team, 2019). The data for Experiment 1 were analyzed using a mixed effects model, where the hybrid, starter fertilization and Zn treatment were considered as fixed effects, while the year and block were considered as random factors. The data for Experiment 2 were analyzed separately for each of the compared soils, using a mixed effect model in which the starter fertilization and the seed treatment were considered as fixed effects, while the year was considered as a random factor. A graphical method was used to verify the basic assumptions (Onofri et al., 2016).

When single factors or their interaction determined a significant effect, the means were compared using the Bonferroni post hoc test at $p \leq 0.05$.

4.4 Results and discussion

4.4.1 Meteorological trends

The three growing seasons showed slightly different meteorological trends for both rainfall and air temperature (expressed as growing degree days, GDDs) (Table S2). The 2012 and 2013 years showed similar and high amounts of rainfall during the spring months of April and May. Furthermore, the 2012 year had drier and warmer conditions from June to August and, as a result, the harvest was carried out at the beginning of September. Conversely, the 2014 year had less rainfall in the spring months (in particular in March and April), while it had the highest amount of rainfall in June and July, and lower GDDs in August during the ripening stage.

4.4.2 Experiment 1 – Zn fertilizer application methods

This research provides useful information on the role of Zn fertilization on the early vigor of maize, its potential benefits on grain yield, and the duration of the whole crop cycle, considering the impact of different application methods (soil, seed and foliar) and their interaction with other agronomic strategies, such as the maize hybrid and the subsurface NP starter fertilizer localized at planting.

A significant effect of the maize hybrid on plant vigor was only observed at GS13, with a better development of the later genotype (FAO 600) (Table 2) but, as expected, the flowering of the FAO 500 hybrid was 1 day earlier, and this led to a lower grain moisture at harvest. Although the flowering date and, consequently, the yield components of the compared genotypes differed, with a higher KSM for the FAO 500 hybrid, and a higher TKW for FAO 600 (Table 3), no significant effects on grain yield were recorded between the maize hybrids.

As far as the fertilization management is concerned, the application of an NP starter fertilizer at planting clearly affected the maize growing cycle each year, from the 3 leaf-stage to tassel emission, as confirmed by the canopy development, which was expressed by means of the NDVI measurements (Figure 1). Overall, the NP starter fertilization significantly influenced ($p < 0.001$) the AUCDC vegetative index (Table 2), which summarized the NDVI development during the vegetative stages, increasing this parameter by 39%, compared to the unfertilized control. The plant height measurements at GS13 and GS34 confirmed the AUCDC differences: the NP fertilized plants were significantly taller ($p < 0.001$), that is, by 1.3 and approximately 3 times, respectively, than the control with no starter fertilizer.

The enhancement of crop development in the early stages as a result of NP starter fertilization has been widely reported in literature (Jing et al., 2010; Ma et al., 2013; Zhang et al., 2023). The sub-surface placement of NP fertilizer close to the seed furrows at planting provides immediate nutrient access to emerging maize roots, which in turn leads to an increase in the concentration of immobile nutrients, such as P, even in soils with high N and P availability.

Moreover, the acidification of the rhizosphere in calcareous soils, which is related to the occurrence of N-NH_4^+, and the release of protons from the roots improve both the macro and micronutrient uptake and the nutrient use efficiency. In a previous study (Blandino et al., 2022), a synergistic effect on early plant growth was observed for the combined application of N and P. In fact, N and P supplied together enhance the root density and extensions, increase leaf expansion, and enhance the photosynthetic rate (Ma et al., 2014), thereby contributing to boosting the plant biomass in the early vegetative stages, as observed by the more rapid increase in NDVI, even before the stem elongation stages. Furthermore, differences in crop development were also detected in later growing stages, that is, at flowering and at harvest. On average, when the NP starter fertilization was performed at planting, flowering occurred -4.5 days before the unfertilized control, although the two maize genotypes led to different results: -5.1 days for the FAO 600 hybrid and -4.0 days for the earlier FAO 500. This advantage in the vegetative cycle was maintained and then transformed into a lower grain moisture (-2.1 percentage points) at harvest, and into an increase in the grain yield (+10%), which was influenced by an increased KSM (+8%) and TKW (+1%), compared to the unfertilized control (Table 3). Our data are in accordance with those of Kaiser et al., (2016), who found that the NP starter fertilization reduced the number of days between planting and silking, with a consequent reduced grain moisture content at harvest. In addition, the high radiation use efficiency and higher percent radiation interception led to a grain yield increase, with a positive effect that was related to the rise in KSM and TKW (Blandino et al., 2022).

Among the different Zn fertilization strategies, only the seed Zn treatment was able to affect plant vigor at GS13; the plants were +8% taller, while both the seed and soil Zn application methods significantly increased plant height at GS34 (+32%), compared to the untreated control. These differences, in terms of early development, were also observed by the increase in the NDVI values during the vegetative stages (Figure 1) and the AUCDC index (+6% and 8%, respectively), compared to the untreated control (Table 2). Zn covers an essential role for crop development, in particular in the early growth stages: this micronutrient is involved in the activity of such enzymes as alcohol dehydrogenase, which stimulates root development, mainly during seed germination (Cakmak 2008), and it is essential for the production of such auxin hormones as indoleacetic acid, which contributes to cell division (Suganya et al., 2020). Zn is also involved in the carbohydrate metabolism, as it increases the photosynthesis rate (Liu et al., 2016) and the chlorophyll content during the leaf development phase (Zhang et al., 2020). A positive effect on maize growth has been reported in semi-arid areas with a Zn deficiency of the soil for both seed (Harris et al., 2007) and soil treatments (Drissi et al., 2015a).

The enhancement in the maize development obtained with the Zn application (seed treatment and soil distribution) in the early growth stages reduced the number of days between planting and silking by 1 day, thus leading to an increase in grain yield (on average of 4%), compared to the untreated control (Table 3), while no differences were reported for grain moisture at harvest.

Stepic et al., (2022) reported a greater yield enhancement from a soil Zn application than from a seed treatment.

As far as the yield components are concerned, like the effect recorded for NP starter fertilizers, the Zn soil treatment only increased the number of KSM (+5%), due to the rise in the pollination rate resulting from an earlier flowering (Potarzycki and Grzebisz 2009; Liu et al., 2017, 2020).

The foliar Zn application did not differ significantly from the untreated control for any of the considered early vigor and grain yield indices. The interactions between the maize hybrid, starter fertilization, and Zn treatment factors were never significant in the early vigor or grain yield assessments, except for the previously mentioned flowering date.

This data highlighted that the Zn fertilization significantly enhanced the early vigor and, consequently, the ripening stages and the grain yield in the maize hybrids with different precocity, although the observed effect was less marked than the effect of the NP starter fertilization. The study has stressed the importance of the Zn application method on plant development in the early stages: the seed treatment or the banded distribution in the soil seed furrows were the most effective practices in promoting the early vigor of maize and, consequently, the overall agronomic benefits, with only a few significant differences between the two application strategies. Although the amount of the micronutrient applied in the seed coating treatment was 10 times lower than in the soil distribution treatment, it was readily available for the seedlings from the very first development stages. As it is easier for farmers to apply a seed treatment, results to be an efficient, feasible, and cost-effective practice to homogeneously distribute low rates of fertilizers, such as micronutrients, in order to support the early growth of maize, even under cool conditions (Imran et al., 2013; Gómez-Muñoz et al., 2018).

Nevertheless, the Zn foliar application at the 4-leaf stage did not affect the plant growth or grain yield of the maize, thus confirming the results reported by Golden et al., (2016). A possible explanation for this could be related to the later application timing than the early application provided by the seed coating or the distribution in seed furrows, which supports the idea that an early applications (soil and seed) improves plant early growth in the most critical stages, under low temperature conditions of the soil, while a late application, such as a foliar treatment, in Zn sufficient soil, it is not able to improve plant development, also because the nutrient limiting conditions reduce when the temperature increases. Furthermore, a 4-leaf treatment may not be as effective as a later one (6-8 leaf), when the foliar uptake of maize plant is expected to be higher. Only a few studies on Zn deficient soils (Martínez-Cuesta et al., 2021; Drissi et al., 2015b) have shown improvements in the plant growth and grain yield from a foliar distribution of Zn at the 6-7 leaf stage, when the leaf area index is higher and the Zn amount that reaches maize plants increases. Later applications (from tasseling) are only able to increase the Zn concentration in the grain (biofortification), without significant increase in grain yield (Wang et al., 2012).

Table 2. Effects of the maize hybrid, the NP starter fertilization and the Zn treatments on the plant height at the leaf emission (GS13) and stem elongation (GS34) stages, the area under canopy development curve (AUCDC), the flowering date, expressed as days after sowing (DAS), and the grain moisture content at harvest for field Experiment 1 carried out in the 2012-2014 period at Carmagnola (North Italy).

Factor	**Source of variation**	**Plant height GS13 (cm)**	**Plant height GS34 (cm)**	**AUCDC (Σ NDVI-day)**	**Flowering date (DAS)**	**Grain moisture (%)**
Hybrid (H)	FAO 500	7.8 b	43.3	21.7	88.3 a	26.2 b
	FAO 600	8.2 a	44.7	21.8	89.2 b	28.6 a
	p-value	0.012	n.s.	n.s.	<0.001	<0.001
Starter fertilization (SF)	Unfertilized	7.0 b	22.7 b	19.0 b	91.0 a	28.6 a
	NP	9.1 a	65.3 a	26.4 a	86.5 b	26.5 b
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001
Zn treatment (Zn)	Untreated	7.8 b	37.3 b	21.0 b	89.4 a	27.8
	Seed	8.4 a	49.0 a	22.3 a	88.4 bc	27.4
	Soil	8.1 ab	49.2 a	22.6 a	88.2 c	27.4
	Foliar	7.8 b	40.5 b	21.1 b	89.0 ab	27.7
	p-value	0.004	<0.001	<0.001	<0.001	n.s.
H × SF	*p*-value	n.s.	n.s.	n.s.	0.012	n.s.
H × Zn	*p*-value	n.s.	n.s.	n.s.	n.s.	n.s.
SF × Zn	*p*-value	n.s.	n.s.	n.s.	n.s.	n.s.
H × SF × Zn	*p*-value	n.s.	n.s.	n.s.	n.s.	n.s.

Means followed by different letters are significantly different for each factor. The level of significance (*p*-value) is shown in the Table. n.s, not significant

Table 3. Effects of the maize hybrid, the NP starter fertilization and the Zn treatments on the maize grain yield and the yield components: number of kernels per square meter (KSM) and thousand kernel weight (TKW) for field Experiment 1, carried out in the 2012-2014 period at Carmagnola (North Italy).

Factor	**Source of variation**	**Grain yield (t ha^{-1})**	**KSM (n°)**	**TKW (g)**
Hybrid (H)	FAO 500	15.6	4882 a	387 b
	FAO 600	15.7	4212 b	422 a
	p-value	n.s.	*<0.001*	*<0.001*
Starter fertilization (SF)	Unfertilized	14.9 b	4382 b	402 b
	NP	16.4 a	4711 a	406 a
	p-value	*<0.001*	*<0.001*	*0.033*
Zn treatment (Zn)	Untreated	15.4 b	4462 b	404
	Seed	15.9 a	4564 ab	404
	Soil	16.0 a	4669 a	406
	Foliar	15.3 b	4491 ab	402
	p-value	*<0.001*	*0.029*	n.s.
H × SF	*p*-value	n.s.	n.s.	n.s.
H × Zn	*p*-value	n.s.	n.s.	n.s.
SF × Zn	*p*-value	n.s.	n.s.	n.s.
H × SF × Zn	*p*-value	n.s.	n.s.	n.s.

Means followed by different letters are significantly different for each factor. The level of significance (*p*-value) is shown in the Table. n.s, not significant

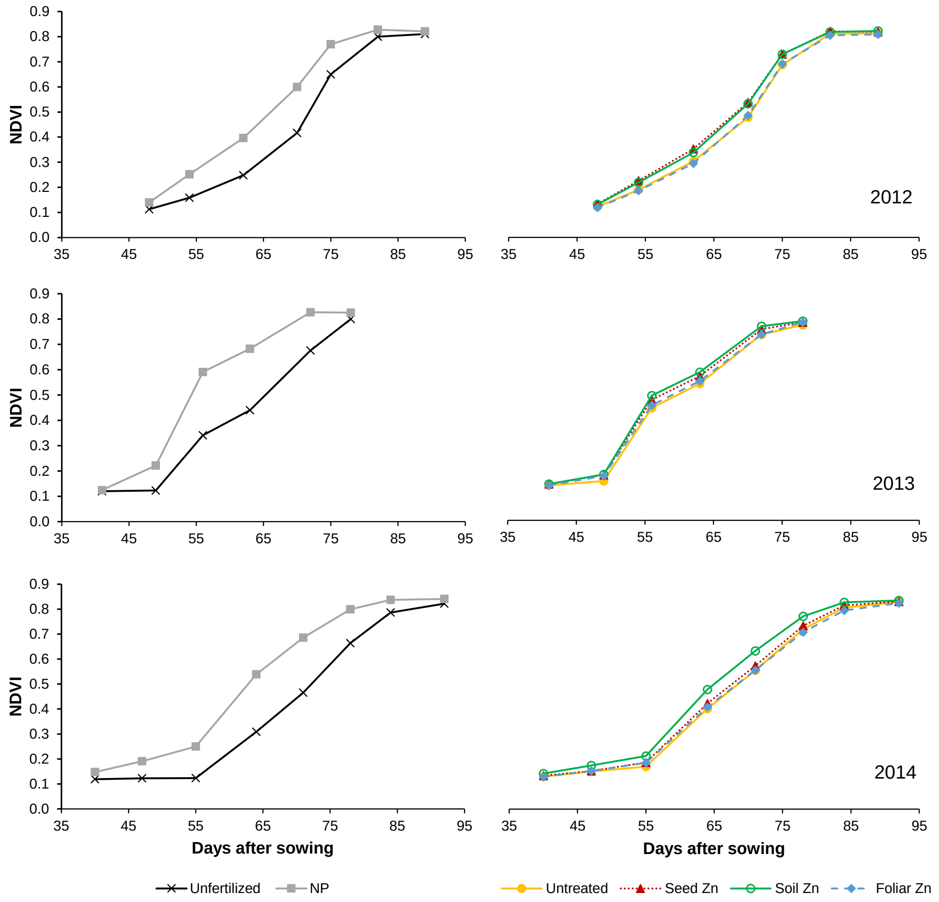


Figure 1. Effects of the NP starter fertilization (on the left) and the Zn treatments (on the right) on the canopy development of maize, expressed as the Normalized Difference Vegetation Index (NDVI), from the 3-leaf stage to tasseling during the 2012-2014 period for Experiment 1 at Carmagnola (North Italy). Unfertilized: no NP fertilization at planting; NP: 27 kg N ha^{-1} and 69 kg P2O5 ha^{-1} applied as diammonium phosphate at planting. Untreated: no Zn application; Seed Zn: Zn applied as a seed coating at a dose of 0.07 kg Zn ha^{-1}; Soil Zn: Zn applied in the maize seed furrows at planting at a dose of 0.7 kg Zn ha^{-1}; Foliar Zn: Zn foliar treatment at the 4-leaf stage at a dose of 0.7 kg Zn ha^{-1}.

4.4.3 Experiment 2 – Zn seed treatment in different soils

In this experiment, the early vigor of maize was also affected to a great extent by the NP starter fertilization banded at planting and by the Zn seed treatment. The NP fertilization recorded a significantly higher plant height (on average, +52% and +134% at GS13 and GS34, respectively) than the unfertilized control (Table 4) for each soil, whether subacid or subalkaline, with low and high P contents, and at both the leaf emission and stem elongation growth stages. Apart from the early vigor at GS13 in the SCL soil, the Zn seed treatment always showed a positive and significant effect on the early vigor of the plant, although this effect was lower than the NP starter fertilization: on average, compared to the untreated control, the plant height increased by 11% and 7% in the L and SL soils, respectively, at GS13, and by 7%, 7%, and 41% in the L, SL, and SCL soils, respectively, at GS34. A significant interaction between the starter fertilization × the Zn seed treatment occurred in the L soil for the plant height at GS13. However, the Zn seed treatment enhanced the maize growth in a different way when applied in combination with the NP fertilizer (+14%), compared to the Zn treatment without NP (+9%) (data not shown).

Higher AUCDC values were detected in all the soils for the NP starter fertilization (+22-27%) and, albeit to a lesser extent, for the Zn seed treatment (+3-9%), than the controls (Table 5).

In addition to the differences recorded for the plant vigor indices, the banded NP and seed Zn applications both significantly affected the flowering date to a great extent, while only the NP starter fertilization resulted in a significant effect on the grain moisture content. The NP fertilization at planting in the L, SL and SCL soils reduced the days to flowering by 3.8, 2.6, and 4.0 days, respectively, while the Zn seed treatment reduced them by 1.2, 0.6, and 1.2 days, respectively. At harvest, the shortening of the days to flowering caused by the NP starter fertilization on average reduced the grain moisture content at harvest by 2.3 percentage points, compared to the unfertilized control. A significant effect ($p < 0.001$) of the NP starter fertilization on grain yield and on its components was observed for all the compared soils: the yield rose as a result of the application of this fertilization practice by 2.1, 1.5, and 1.4 t ha^{-1} in the L, SL and SCL soils, while KSM increased by 8%, 8.6%, and 9%, respectively. In addition, the TKW increased significantly in the L (+2.3%) and SCL (+7.8%) soils (Table 6). Unlike Experiment 1, the grain yield increase and the KSM increase caused by the Zn seed treatment were not significant in any of the soils, while the TKW increased on average by 3% in the L and SL soils, compared to the control. The interactions between starter fertilization and seed treatment were never significant, except for the plant height at GS13 in the L soil.

Therefore, in addition to the early vigor benefits already reported in Experiment 1, this second experiment underlined the absence of antagonism between the Zn micronutrient applied as a seed application and such soil properties as texture, pH, organic matter, and available P content. Indeed, the here presented results, obtained by comparing the efficacy of a Zn seed treatment on maize development in soils with different chemical and physical traits, have shown a similar effect in term of early vigor.

Furthermore, our work provides new insights into the interaction between Zn and P. Some studies carried out in calcareous soils with high available P reported a lower Zn uptake by plants (Alloway 2008; Hafeez et al., 2013; Akhtar et al., 2019). Although, in the second experiment, the effect of the application of Zn as a seed treatment had a similar impact on promoting plant growth in soils with both low (e.g. the L soils) and high P (e.g. the SL soil) contents, a further quantifiable enhancement of the early vigor of maize was observed for the combination of the NP starter fertilizer and the Zn fertilization in both experiments (1 and 2), and for all the considered agronomic conditions. Drissi et al., (2015c) also reported an increase in the maize biomass at harvest in a sub-acidic soil after the supply of Zn to soil fertilized with a DAP fertilizer. Other studies carried out in calcareous subalkaline soils with low Zn and P availability have highlighted that the Zn supply (as zinc sulfate) combined with P (as DAP or calcium dihydrogenphosphate) increased maize growth and yield more than their separate application (Imran et al., 2016; Zhang et al., 2017). Moreover, in addition to a potential synergic effect of macro- and micronutrients on seedling physiology, which accelerate the vegetative growth of plants, particularly during cold periods, the application of an NP fertilizer could enhance the uptake of Zn and other micronutrients as a result of an acidification of the rhizosphere (Jing et al., 2012; Suganya et al., 2020).

Table 4. Effects of the NP starter fertilization and the Zn seed treatment on the plant height at the leaf emission (GS13) and stem elongation (GS34) stages for field Experiment 2 carried out on three natural soils with different textures placed side by side at Carmagnola (North Italy) in the 2012-2014 period.

Factor	**Source of variation**	**Plant height GS13 (cm)**			**Plant height GS34 (cm)**		
		L	**SL**	**SCL**	**L**	**SL**	**SCL**
Starter fertilization (SF)	Unfertilized	8.3 b	9.1 b	7.4 b	23.6 b	34.7 b	13.5 b
	NP	13.0 a	14.1 a	10.6 a	59.9 a	61.1 a	36.8 a
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Seed treatment (Zn)	Untreated	10.1 b	11.2 b	8.3	40.3 b	46.3 b	20.9 b
	Zn	11.2 a	12.0 a	9.2	43.1 a	49.5 a	29.4 a
	p-value	<0.001	0.022	n.s.	0.043	0.020	<0.001
SF x Zn	*p*-value	0.002	n.s.	n.s.	n.s.	n.s.	n.s.

Means followed by different letters are significantly different for each factor. The level of significance (*p*-value) is shown in the Table. n.s, not significant. L: loam, SL: silt loam, SCL: silty clay loam.

Table 5. Effects of the NP starter fertilization and the Zn seed treatment on the area under the canopy development curve (AUCDC), the date of flowering, expressed as days after sowing (DAS), and the grain moisture content at harvest for field Experiment 2, carried out on three natural soils with different textures placed side by side at Carmagnola (North Italy) in the 2012-2014 period.

Factor	**Source of variation**	**AUCDC (Σ NDVI-day)**			**Flowering date (DAS)**			**Grain moisture (%)**		
		L	**SL**	**SCL**	**L**	**SL**	**SCL**	**L**	**SL**	**SCL**
Starter fertilization (SF)	Unfertilized	22.3 b	22.3 b	17.9 b	98.7 a	97.0 a	98.8 a	30.1 a	27.9 a	30.3 a
	NP	28.1 a	27.1 a	22.8 a	94.9 b	94.4 b	94.8 b	27.7 b	25.7 b	27.9 b
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Seed treatment (Zn)	Untreated	24.9 b	24.3 b	19.5 b	97.4 a	96.0 a	97.4 a	28.8	26.6	29.2
	Zn	25.6 a	25.1 a	21.2 a	96.2 b	95.4 b	96.2 b	29.1	27.1	29.0
	p-value	0.012	<0.001	0.013	<0.001	<0.001	0.007	n.s.	n.s.	n.s.
SF x Zn	*p*-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Means followed by different letters are significantly different for each factor. The level of significance (*p*-value) is shown in the Table. n.s, not significant. L: loam, SL: silt loam, SCL: silty clay loam.

Table 6. Effects of the NP starter fertilization and the Zn seed treatment on the maize grain yield and the yield components: number of kernels per square meter (KSM), and thousand kernel weight (TKW) for field Experiment 2, carried out on three natural soils with different textures placed side by side at Carmagnola (North Italy) in the 2012-2014 period.

Factor	**Source of variation**	**Grain yield (t ha^{-1})**			**KSM (n)**			**TKW (g)**		
		L	**SL**	**SCL**	**L**	**SSL**	**SSL**	**L**	**SL**	**SCL**
Starter fertilization (SF)	Unfertilized	12.3 b	11.2 b	12.0 b	4001 b	3633 b	3861 b	349 b	347	344 b
	NP	14.4 a	12.7 a	13.4 a	4319 a	3946 a	4210 a	357 a	353	371 a
	p-value	<0.001	<0.001	<0.001	0.004	0.001	0.013	0.038	n.s.	<0.001
Seed treatment (Zn)	Untreated	13.1	11.9	12.5	4103	3718	3929	347 b	345 b	357
	Zn	13.6	12.0	12.9	4217	3861	4141	358 a	355 a	359
	p-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.008	0.032	n.s.
SF x Zn	*p*-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Means followed by different letters are significantly different for each factor. The level of significance (*p*-value) is shown in the Table. n.s, not significant. L: loam, SL: silt loam, SCL: silty clay loam.

4.5 Conclusions

The study has highlighted that the Zn fertilization of maize seedlings can lead to a significant improvement in the early vigor of maize plants in temperate growing areas. Although the gains in early vigor and, consequently, the reduction in the number of the days between planting and silking and the enhancement in the grain yield were less marked than those observed for the adoption of NP starter fertilization, the application of this micronutrient led to positive and consistent effects for different hybrids, different growing seasons and soils. Furthermore, the study underlined the possibility of also adopting the Zn fertilization practice at planting in combination with the sub-surface banded distribution of NP fertilizer, to support a greater enhancement of plant growth and the consequent agronomic benefits.

After comparing different Zn application methods, it was found that an early application involving a seed treatment or soil distribution in seed furrows at planting resulted to be the most effective practice, while a foliar application at the 4-leaf stage did not result in any significant plant development benefits. These findings also highlight the importance of introducing Zn and other micronutrient fertilizations for arable field crops whenever their contribution is able to translate into greater agronomic advantages, in order to make their application to cropping systems more effective. Moreover, the Zn seed treatment is a sustainable, low-cost, low-rate strategy, which is easy for farmers to apply in order to support seedling development, and it has a long-lasting effect, especially in those production situations in which the initial development of maize could slow down.

4.6 Supplementary material

Table S1. The main agronomic information: maize hybrids, sowing dates, top-dressing fertilization, and harvest dates for both Experiment 1 and Experiment 2 and the Zn foliar treatment date for Experiment 1 in the 2012-2014 period at Carmagnola (North Italy).

Experiment	**Year**	**Maize hybrid**	**Sowing date**	**Zn foliar treatment date**	**N fertilization date**	**Harvesting date**
Experiment 1	2012	PR33A46 (FAO 500) and P1547 (FAO 600)	22 March	9 May	17 May	13 September
	2013		15 April	21 May	31 May	8 October
	2014		20 March	14 May	23 May	29 September
Experiment 2	2012	P1543 (FAO 600)	27 March	-	17 May	31 August
	2013		19 April	-	31 May	24 September
	2014		21 March	-	26 May	16 September

Table S2. Cumulative monthly air growing degree days (GDDs; 10°C base) and rainfall during the 2012-2014 period at Carmagnola (North Italy).

Month	**GDDs (Σ °C-day)**			**Rainfall (mm)**		
	2012	**2013**	**2014**	**2012**	**2013**	**2014**
March	146	57	116	20	96	67
April	112	145	163	148	144	47
May	241	210	224	147	147	121
June	363	319	342	19	35	125
July	396	407	359	37	137	204
August	404	378	357	46	59	37
September	260	271	282	50	14	62
October	169	145	201	53	71	15
April-June	715	674	729	313	326	293
April-September	1775	1730	1727	447	537	597

4.7 References

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5. CHAPTER V - Agronomic strategies to enhance the early vigor and yield of maize. Part I: the role of seed applied biostimulant, hybrid, and starter fertilization on rhizosphere bacteria profile and diversity.

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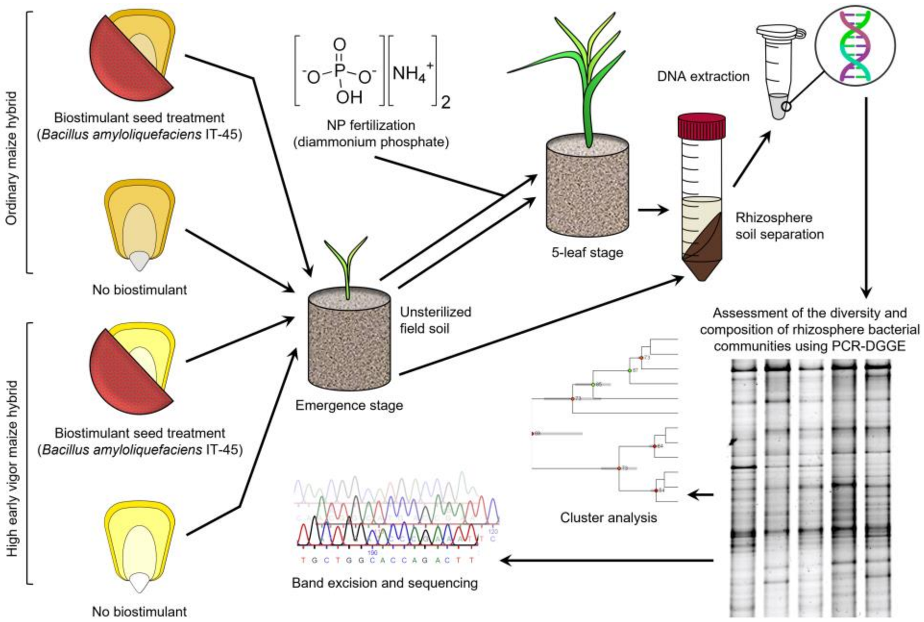
Graphical abstract



5.1 Abstract

The sustainable intensification of maize-based systems may reduce greenhouse-gas emissions and the excessive use of non-renewable inputs. Considering the key role that the microbiological fertility has on crop growth and resilience, it is worth of interest studying the role of cropping system on the rhizosphere bacterial communities, that affect soil health and biological soil fertility. In this work we monitored and characterized the diversity and composition of native rhizosphere bacterial communities during the early growth phases of two maize genotypes of different early vigor, using a NP starter fertilization and a biostimulant seed treatment, in a growth chamber experiment, by PCR denaturing gradient gel electrophoresis of partial 16S rRNA gene and amplicon sequencing. Our data showed that maize genotype was the major factor shaping rhizosphere bacterial community composition, as the root systems of the two hybrids, differing for their early vigor. Cluster analyses showed that the biostimulant treatment affected the rhizosphere bacterial microbiota of the ordinary hybrid more than that of the early vigor, both at plant emergence and at the 5-leaf stage. Moreover, the diversity indices calculated from the community profiles, revealed significant effects of NP fertilization on richness and the estimated effective number of species (H_2) in both maize genotypes, while the biostimulant had a positive effect on PGP community of the ordinary hybrid, both at the plant emergence and at the fifth leaf stage. Present findings support the interpretation of the effects of the biostimulant seed treatment, NP starter fertilizer, genotype early vigor and their combination on maize early development and grain yield, reported in the companion manuscript. Moreover, these results pave the way for further studies to be performed on the effects of cropping system and specific crop practices, considering also the application of biostimulants, on beneficial rhizosphere microorganisms.

Keywords: corn, seed treatment, rhizosphere, plant growth promoting bacteria, *Bacillus amyloliquefaciens,* diammonium phosphate

5.2 Introduction

Maize (*Zea mays* L.) is one of the most important crops worldwide, with an annual average production of 1115 million tonnes (FAOSTAT 2022), destined to several sectors, with a particular rising use in gluten free food and industrial (starch industry) or energy purpose (García-Lara and Serna-Saldivar 2019). The success of maize is related to the high productive efficiency in the use of agronomic inputs, with a marked response to the applied agronomic practices. Within the crop practices, the sowing time, and particular an early planting date, play a key role, in temperate growing areas, in achieving the highest profitability of maize, due to an increase in the length of the growing cycle (Long et al., 2017), a lower risk of environmental stresses, such as drought and heat (Waqas et al., 2021) and higher grain yield, quality and safety (Blandino et al., 2017). Furthermore, an early sowing date increased the risk to meet cold and rainy period during the maize emergence and the first vegetative stages, resulting in a slow plant development, with higher risk of damping-off and insect damages, thus reducing the beneficial effects of an early sowing. The cultivation of hybrids with a superior tolerance to low temperatures, therefore, characterized by a high early vigor, instead of ordinary one (Peter et al., 2009; Reis et al., 2022), and the application at sowing of starter fertilizers in bands close to seed furrows (Ma et al., 2015; Kaiser et al., 2016), are the main crop practices applied to limit the risk of maize slow development within the early planting times. In fact, although agricultural soils may contain large amounts of total nitrogen (N) and phosphorus (P), they are mainly in a form not available to the plant (Imran et al., 2013), while cool springs could further reduce plant uptake of these nutrients in the early vegetative stages, limiting crop growth rate and leaf chlorophyll content (Zhao et al., 2022). Blandino et al., (2022) reported a synergistic effect of N and P applied as diammonium phosphate (DAP) in sub-surface band at sowing in the increase of maize early vigor and grain yield, even in soils with high N and P concentrations. Furthermore, the excessive use of fertilizers was found to increase greenhouse-gas emissions (Robertson and Vitousek 2009) and to have potential negative impacts on soil health and long-term soil fertility, causing soil acidification (Juo et al., 1995; Matsuyama et al., 2005; Guo et al., 2010), reducing diversity in native microbial communities (Lazcano et al. 2013; Sun et al., 2015) and accelerating the eutrophication of water bodies (Carpenter et al., 1998; Withers and Haygarth 2007). Due to the potential environmental pollution and the low crop uptake at early growth stages, the need for additional N and P fertilizer applications to soils with a high availability in these nutrients is uncertain (Schröder et al., 2015). Moreover, the policy and consumer demand for a more sustainable food and feed production stimulate studies aimed at developing resilient environmental-friendly cropping systems with a reduced application of external inputs, such as non-renewable fertilizers. An expression of this request is the Farm to Fork program of the EU Commission, which will require a reduction of nutrient losses by 50% and a decrease of the use of synthetic fertilisers by 20% by 2030 (European Commission Communication COM/2020/381).

A sustainable tool for the management of cropping system could be the valorisation of soil microbial communities. Soil inhabiting bacterial populations are estimated to reach a density range of 10^8-10^{10} CFU g^{-1} dry soil, represented by a strikingly high taxonomic diversity (Roesch et al., 2007; Zhang et al., 2017). Compared to bulk soil, distinct bacterial communities live associated with plant roots, in the rhizosphere, affecting plant functions and productivity (van der Heijden et al., 2008; Mendes et al., 2013; Philippot et al., 2013). Many of the rhizosphere bacteria may enter in the functional category of plant growth promoting bacteria (PGPB), possessing specific metabolic traits enabling them to improve plant nutrient status and resistance to numerous biotic and abiotic stresses: via N fixation, phosphate and potassium solubilization, production of phytohormones, volatile organic carbon compounds (VOCs), siderophores, protective enzymes (e.g. chitinase, ACC-deaminase), induction of systemic resistance (ISR) and release of various antimicrobial substances (van der Heijden et al., 2008; Berg 2009; Hayat et al., 2010; Mendes et al., 2013; Gouda et al., 2018). Some studies have reported that the interaction between maize genotype and specific agronomic management could impact the microbiota composition, richness and functionality (Favela et al., 2021). The recruitment of soil bacteria in the rhizosphere and endosphere was affected by plant genotype in diverse crops, such as potato, bean, rice and durum wheat (Manter et al., 2010; Shenton et al., 2016; Pérez-Jaramillo et al., 2017; Agnolucci et al., 2019; Ujvári et al., 2021) which was ascribed to differences in root architecture and rhizodeposition patterns (Bais et al., 2006; Badri and Vivanco 2009). Large differences in rhizosphere microbial community composition were found among 27 maize hybrids and lineages (Peiffer et al., 2013; Walters et al., 2018), while qualitative differences in root colonization by bacterial endophytes were detected in different genotypes (Ikeda et al., 2013). However, the role played by hybrids with different agronomic attitude under stress conditions, such as the early vigor trait, in the regulation of plant-microorganism interactions has been less studied. P and N fertilization also proved to affect the composition and diversity of microbial communities occurring in maize rhizosphere and root endosphere (Zhu et al., 2016; Gomes et al., 2018; Miranda-Carrazco et al., 2022). In particular, root exudates, which are influenced by plant species, genotype and fertilization regime, are able to affect the rhizosphere microbial community composition and functionality (Sasse et al., 2018). Recently, the application of substances highly available and rapidly assimilated by soil microorganisms has been proposed as a way to enhance rhizosphere microbial community activity.

Moreover, several PGPB are today proposed to be applied as biostimulants and biofertilizers to the soil surface, in the seed furrow or to the seeds, in order to improve nutrient use efficiency or availability, while reducing agrochemical inputs, within a more sustainable crop management (Zaidi et al., 2015; Ruzzi and Aroca 2015; Rouphael and Colla 2020). Many bacterial taxa have been isolated and successfully used as PGPB and biostimulants, such as strains of *Azospirillum* sp. (Hungria et al., 2010), *Pantoea* sp. (Mishra et al., 2011), *Rhizobium* sp. (Chabot et al., 1996), *Serratia* sp. (Hameeda et al., 2006), *Pseudomonas* sp. (Kavino et al., 2010), *Paraburkholderia* sp. (Rahman et al., 2018), *Bacillus* sp. (Amaresan et al., 2019), *Lactobacillus* sp. (Shrestha et al., 2014), *Variovorax* sp. (Chandra et al., 2019) and *Ensifer meliloti* (Velásquez et al., 2020). PGP *Bacillus* species are considered optimal targets for bioinoculant development due to their distinctive trait of endospore formation, which results in longer product shelf-life, comparable with that of conventional agrochemicals (Qiao et al., 2014). *Bacillus amyloliquefaciens*, in particular, showed remarkable potentials for agricultural use (Qiao et al., 2014; Luo et al., 2022). Plant-associated strains of *B. amyloliquefaciens* demonstrated P solubilizing and N mineralizing abilities (Idriss et al., 2002; Hui et al., 2018), indole-3-acetic acid (IAA), cytokinin and ACC-deaminase production, siderophores, VOCs and several antifungal, antiviral and antibacterial secondary metabolites synthesis (Idris et al., 2007; Chen et al., 2009; Wang et al., 2016; Asari et al., 2017; Wu et al., 2019), as well as ameliorating capabilities through complex pathways in various stress conditions (Tiwari et al., 2017).

The effects of biostimulants inoculation on the complex habitat of the rhizosphere have not been adequately investigated in crop plants, also considering the interaction with other agronomic practices. The aim of this study was to monitor and characterize the native rhizosphere microbiota during the early growth phases of two maize genotypes, using a NP starter fertilization treatment and a biostimulant seed treatment, in a growth chamber experiment. To this aim, we assessed the diversity and composition of rhizospheric bacterial communities utilizing a culture-independent approach, such as PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis) analysis of the 16S ribosomal RNA (rRNA) gene and amplicon sequencing.

A companion manuscript (see Part II) will report the effect of biostimulant seed treatment, NP starter fertilizer, genotype early vigor and their factorial combination, on maize development in the early stages and the consequential effect on growth, grain yield and quality, in growth chamber and open field experiments.

5.3 Materials and methods

5.3.1 Microcosm experiment

A growth chamber experiment was set up in order to investigate the effect of a seed biostimulant, based on a PGPB strain and a plant extract, on the diversity and composition of the bacterial communities of maize rhizosphere, also considering the interaction with genotypes with different early vigor and the application of NP starter fertilization in seed furrow.

Sixteen kilograms of natural silt loam sub-alkaline soil (Typic Ustifluvents, USDA classification) were weighed and placed, after mixing it thoroughly, in each plastic pot (27 cm length x 24 cm width x 28 cm height). The soil was collected from the surface layer (0.2 m) in the field of the University of Turin experimental station, located in North-West Italy at Carmagnola (44° 53’ N, 7° 41’ E; elevation 245 m). The soil was characterized by a medium cation-exchange capacity (C.E.C.), low organic matter, potassium (K) and P content and medium nitrogen N availability. More information on soil physical and chemical parameters are reported in Table S1. Soil was not air dried, sieved, sterilized and mixed with quartz sand or other materials. The compared treatments were factorial combinations of:

- maize hybrids, considering genotypes with different early vigor after emergence but with similar growing cycle (FAO maturity class 600, 130 maturity days),
    - an ordinary hybrid (ordinary), with conventional early vigor (LG30600, Limagrain Europe, Saint-Beauzire, France),
    - a high early vigor hybrid (high early vigor), with a rapid growth in the first vegetative stages (LG31630, characterized by the Rapid’START trait, Limagrain Europe);
- NP starter fertilization,
    - unfertilized control (unfertilized),
    - sub-surface starter fertilization (NP), 27 kg N ha^{-1} and 69 kg P_2O_5 ha^{-1} were applied as DAP (18% and 46% for N and P_2O_5, respectively w/w) placed in bands close to the maize seed furrows;
- biostimulant seed treatment,
    - untreated control (no biostimulant),
    - biostimulant seed application (biostimulant), based on a mixture of a bacterium, *Bacillus amyloliquefaciens* strain IT-45 (Rise P®) and a leguminous plant extract *Cyamopsis psoraloides* (AgRho® GSB30) (Starcover, Limagrain Europe).

The experimental design was a completely randomized block design with three replications.

All maize seeds, independent from biostimulants treatment, were treated with a fungicide mixture of prothioconazole (100 g L^{-1}) and metalaxyl (20 g L^{-1}) applied at 15 mL to 50,000 seeds (Redigo® M, Bayer Crop Science S.r.l., Monheim am Rhein, Germany). Maize seeds shape, dimension and weight were carefully chosen in order to reduce seedling vigor variability. In each pot, 4 maize seeds were sown by hand at 2 cm of depth, equally distributed. After germination, only 2 plants per pot were maintained, to assure the conventional field density. NP fertilizer was placed manually in a hypothetical seed furrow band, 5 cm close to maize seed furrows, at a depth of 10 cm. No other fertilizers were applied before or after sowing.

Pots were placed in a controlled growth chamber with 50% relative humidity range, 12 h photoperiod, 700 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and 14/17 °C (night/day) air temperature range (Table S2). The air and soil temperatures have been controlled during the experiment by means of two data loggers: HOBO® Pro v2 (Onset Computer Corp., Bourne, MA, USA) and Tinytag Plus 2 GP-4020 with 10 cm thermistor probe (Gemini Data Loggers Ltd, Chichester, UK), respectively.

Soil moisture content was maintained at water holding capacity by adding weekly in each pot 0.57 L of water, corresponding to 10 mm of rain. The weed control was carried out manually to eliminate every undesired plant seedling just after germination. The experiment was terminated 49 days after sowing (DAS).

5.3.2 Sample collection and preparation

Maize plants were harvested at 13 (emergence) and 49 (5-leaf stage) DAS. The whole roots system of each plant was collected after cutting maize shoots at the collar and gently removing the soil by hand. Each treatment was represented by triplicate samples collected from separate pot cultures. Samples were stored on 4 °C until further analysis. Rhizosphere samples were separated from the roots in sterile Falcon tubes, adding 40 mL sterile physiologic solution (0.9% (w/v) NaCl; 0.005% (w/v) Tween80) to each sample and shaking them in a Lab-Line® Multi-Wrist™ shaker (Lab-Line Instruments, Melrose Park, IL, USA). After 10 minutes of shaking, clean roots were extracted from the solution. The remaining soil was centrifuged on 5500 rpm for 10 minutes, and the supernatant was eliminated.

5.3.3 DNA extraction

250 mg subsamples of rhizosphere soil were subjected to genomic DNA extraction using the DNEasy® Power Soil® Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. The extracted DNA was stored at −20 °C and subsequently used for the molecular analysis of soil bacterial communities.

5.3.4 Molecular analysis of bacterial community profiles with PCR - DGGE

For denaturing gradient gel electrophoresis (DGGE), the V3-V5 hypervariable region of the 16S rDNA was amplified. Polymerase chain reaction (PCR) was carried out using the primers 341F (5’-CCT ACG GGA GGC AGC AG-3’) and 907R (5’-CCG TCA ATT CCT TTR AG TTT-3’) (Eurofins, Ebersberg, Germany) (Yu and Morrison 2004). The primer 341F had an additional 40-nucleotide GC-rich tail (5’-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3’) on the 5’ end to prevent complete DNA denaturation during the DGGE process.

Reaction mixes were prepared in a final volume of 50 µL, containing 1 µL of 1:100 diluted DNA extract. Each reaction mixture contained 5 µL of ExTaq Buffer 10x (Takara Bio Inc., Kusatsu, Japan), 1.25 U of ExTaq (Takara Bio Inc.), 0.2 mM of each dNTP (Takara Bio Inc.) and 0.5 µM of both primers. The reaction was carried out in an iCycler-iQ™ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following thermal cycles: initial denaturation at 94 °C for 1’; 35 cycles of denaturation – annellation – elongation at 94 °C for 30”, at 60 °C for 30” and at 72 °C for 30”, respectively; and final elongation at 72 °C for 5’.

The expected product was about 560 bp long. The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) agarose gels in 1x TBE buffer (Tris-borate-EDTA, pH 8.3) (AppliChem GmbH, Darmstadt, Germany) stained with 20000x RealSafe Nucleic Acid Staining Solution (Durviz s.l., Valencia, Spain). DNA fragments were visualized over an UV transluminator (Uvitec Cambridge, Cambridge, UK), and pictures were captured with the UVI 1D v. 16.11 program (Uvitec Cambridge) in TIFF format.

For molecular analysis of the bacterial diversity, 20 µL of amplicon DNA was separated in 8% (w/v) polyacrylamide 4K (AppliChem GmbH) gels in the DCode™ Universal Mutation Detection System (Bio-Rad). The urea-formamide denaturing gradient was 36-52%. An unfertilized/no biostimulant sample of the ordinary hybrid at the 5-leaf stage was loaded on both sides and in the middle of the gels as marker. Gels were run at 80 V for 16 h in 1x TAE buffer (Tris-acetate-EDTA, pH 8.5) (AppliChem GmbH) at 60 °C. Subsequently, gels were stained in 1x TAE buffer with 10000x SYBR Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific, Waltham, MA, USA) and visualized over an UV transluminator as described above.

5.3.5 DGGE profile analysis

DGGE profiles were digitally processed with the BioNumerics software v. 8.1 (Applied Maths, St-Martens-Latem, Belgium) as reported in Turrini et al., (2017). Sample profiles were normalised to contain the same extent of total signal after background subtraction, and lanes were straightened and aligned following the manufacturer’s instructions. Markers were used for further normalisation between separate gels allowing their comparison. Bands were designated by manual supervision of the auto search bands function, and band positions were converted to Rf% values. Similarities between DGGE profiles were calculated with Pearson's similarity coefficients applied on the lane patterns using the band-matching tool with 0% of optimization. The similarity coefficients were then used for generating dendrograms with the Unweighted Pair-Group Method Using Arithmetic Average (UPGMA) cluster analysis tool.

Based on the banding data, and treating each band as an individual operational taxonomic unit (OTU), six different diversity indices were calculated. Richness (S) indicated the number of OTUs detected in the sample. Shannon-Weaver's diversity (H_s) and Simpson's dominance (D) indices were calculated as $H_s = \sum_{i=1}^{n} -\frac{h_i}{H} \cdot \ln\frac{h_i}{H}$ and $D = \sum_{i=1}^{n} \frac{h_i \cdot (h_i - 1)}{H \cdot (H-1)}$, where h_i was the peak intensity of a band and H was the sum of all peak intensities in a sample. Evenness (J_p) allowed to reveal the presence of dominant OTUs, calculated as $J_p = \frac{H_s}{\ln S}$. Hill 1 (H_1) and Hill 2 (H_2) numbers were computed as $H_1 = \frac{1}{D}$ and $H_2 = e^{H_s}$, respectively.

5.3.6 DGGE band sequencing

The main bands of the DGGE profiles were cut from the gel for further molecular analysis. Bands were eluted in 50 µL UltraPure™ DNase/RNase-free distilled water (Invitrogen, Waltham, MA, USA) for three days at 4 °C. Supernatants were diluted 1:100 and served as templates for PCR using the primers 341F and 907R without GC-clamp, following the protocol described earlier. PCR products were then purified with the QIAquick® PCR Purification kit (Qiagen GmbH) according to the manufacturer's instructions. Purified amplicons were eluted in 50 µL H_2O and controlled in a 2% agarose gel to confirm product quality, and their concentration for dsDNA was estimated with an Eppendorf Biophotometer (Eppendorf SE, Hamburg, Germany) measuring at λ= 260 nm. Partial 16S rDNA amplicons were 5'-end sequenced by Eurofins Genomics – Mix2Seq Custom DNA Sequencing Services (Ebersberg, Germany). Sequences were analysed as in Palla et al., (2022), using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in the NCBI-GenBank (https://www.ncbi.nlm.nih.gov/genbank) database, accessed in July, 2022. Related sequences were collected and aligned with the MUSCLE tool (Edgar 2004b, a) in the MEGA 11 software (Tamura et al., 2021). Phylogenetic trees were constructed using the Maximum Likelihood method based on Kimura's 2-parameter model (Kimura 1980) in MEGA 11 with 1000 bootstrap replicates. The DGGE band sequences were submitted to the European Nucleotide Archive under the accession numbers from OP964519 to OP964570; OP985320; OQ000256.

5.3.7 Statistical analysis

Two-ways analysis of variances (ANOVA) was conducted on the diversity indices of emergent rhizosphere samples considering the hybrid and biostimulant treatment as factors, each at two levels. Three-ways ANOVA was carried out on the diversity indices of 5-leaf stage rhizosphere samples considering the hybrid, NP starter fertilization and biostimulant seed treatment as factors, each at two levels. Homogeneity of variances was controlled with Levene’s test ($p < 0.05$). When needed, data were transformed to meet the variance homogeneity assumption. Statistical analyses were carried out with the SPSS v. 25 software (IBM Corp., Armonk, NY, USA).

5.4 Results

5.4.1 Analysis of PCR-DGGE profiles

Bacterial 16S rDNA fragments (ca. 560 bp) were successfully amplified in all samples. The DGGE separation of the PCR amplicons revealed rhizosphere bacterial community profiles, characterized by a high number of bands of variable intensities (Figures 1 and 2). DGGE profiles were compared by cluster analysis (UPGMA), and biodiversity indices (S, H_s, D, J_p, H_1, H_2) were estimated based on the banding patterns.

At the emergence stage, the rhizosphere bacterial communities of the two maize hybrids clustered separately in the UPGMA dendrogram (Figure 3), with a similarity of 78%. Interestingly, in the ordinary hybrid, samples treated with seed-applied biostimulant clustered separately from the control (91% similarity), while there was no such a separation in the high early vigor hybrid.

Two-ways ANOVA of the diversity indices revealed the early effect of the genotype on richness, which was higher in the ordinary hybrid, compared with the high early vigor hybrid, while biostimulant treatment did not significantly affect bacterial diversity at the time of emergence (Table 1). As a result of the UPGMA cluster analysis of the 5-leaf stage samples (Figure 4), the two hybrids grouped separately with a very low similarity value (20%). Within both maize genotypes, unfertilized/no biostimulant samples clustered separately from those treated with NP fertilizer, showing similarities lower than 74% and 88% for the ordinary hybrid and the high early vigor hybrid, respectively.

Concurrently, analysing the diversity indices calculated from the community profiles, three-ways ANOVA revealed significant effects of hybrid and NP fertilization on richness and H_2, with higher diversity values in the high early vigor hybrid and NP fertilized samples (Table 2). As variances were not homogeneous, H_1 and evenness indices were analysed by two-ways ANOVA, which showed significant increases induced by fertilization in the ordinary hybrid, but not in the high early vigor hybrid (Table 3). By contrast, seed-applied biostimulant did not influence any of the biodiversity indices.

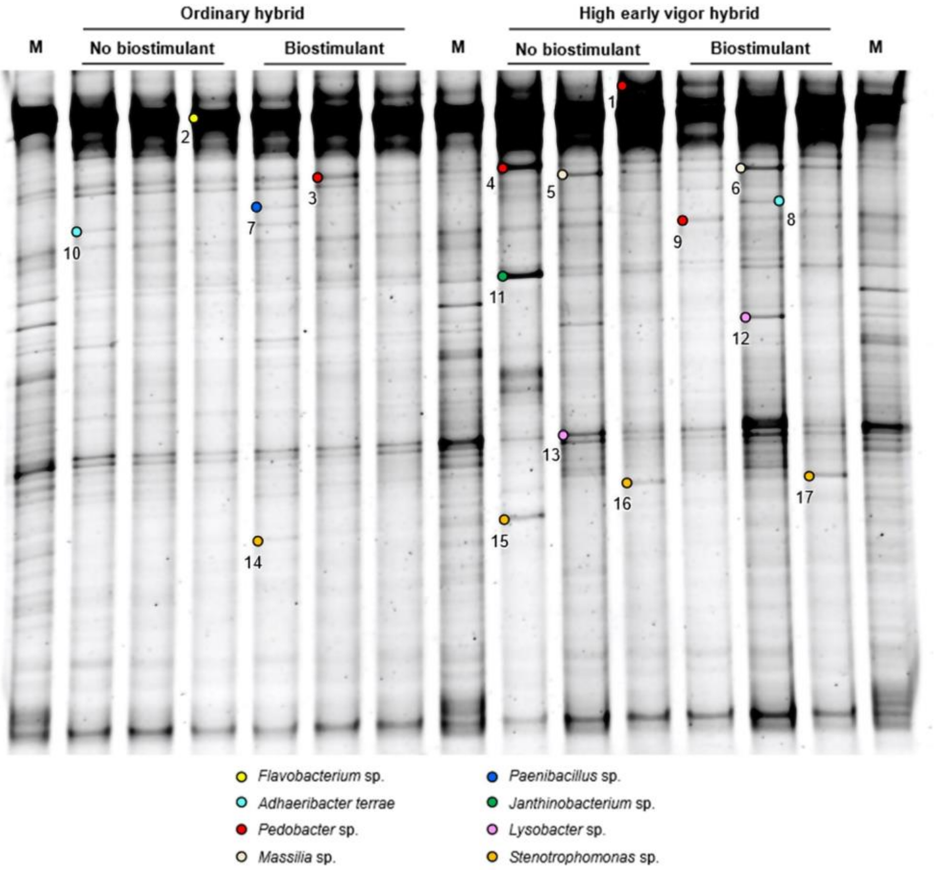


Figure 1. PCR-DGGE profiles of the rhizosphere bacterial communities of two maize hybrids at the emergence stage, treated or untreated with seed-applied biostimulant. Marker: M. The numbers indicate sequenced DNA fragments and the colored circles the relevant bacterial species affiliation.

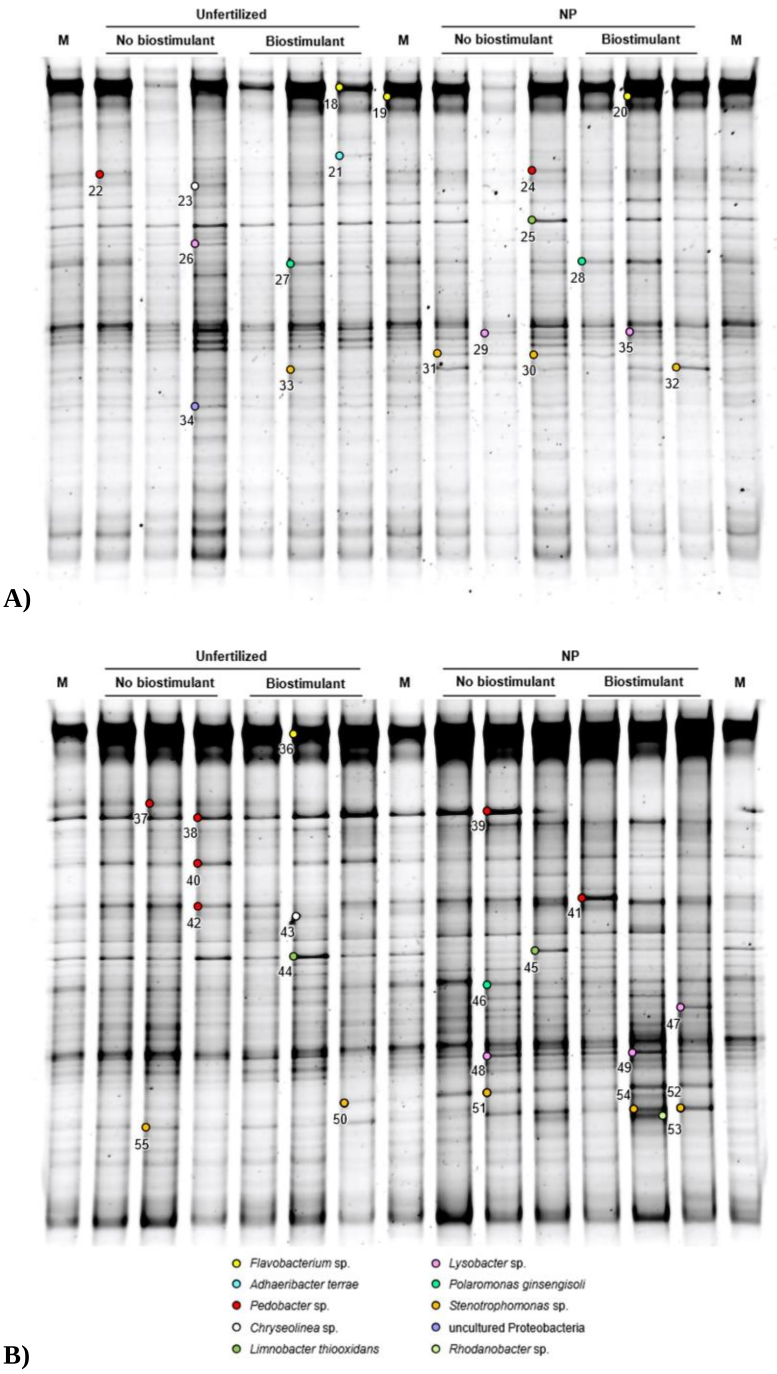


Figure 2. PCR-DGGE profiles of the rhizosphere bacterial communities of two maize hybrids at the 5-leaf stage, treated or untreated with NP starter fertilization and with seed applied biostimulant. ***A****) ordinary hybrid.* ***B****) high early vigor hybrid. M: Marker. The numbers indicate sequenced DNA fragments and the colored circles the relevant bacterial species affiliation.*

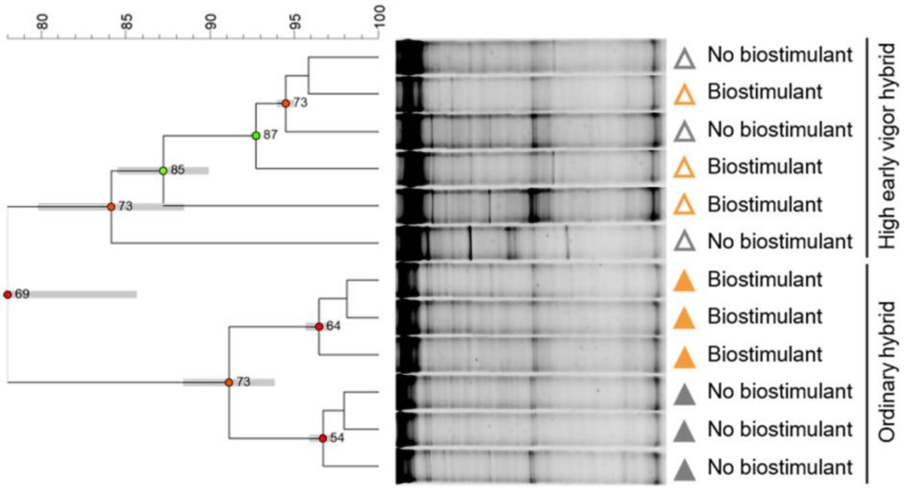


Figure 3. Cluster analysis of rhizosphere bacterial communities DGGE profiles indicating the relationships among samples, based on similarity, as shown by the numeric scale above each dendrogram, obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) analysis, using Dice's similarity coefficient. Dendrograms are based on DGGE profiles obtained from the rhizosphere of two maize hybrids at the emergence stage treated or untreated with seed applied biostimulant. Cophenetic correlation, expressing the consistency of clusters, is shown at each node by numbers and colored dots, ranging between green-yellow-orange-red, according to decreasing values. Standard deviation is shown at each node by a gray bar. Colors indicate the factorial treatments: unfertilized/no biostimulant (gray), biostimulant seed treatment (orange). Closed and open symbols refer to the ordinary and to the high early vigor maize hybrids, respectively.

Table 1. Effects of the hybrid and biostimulant seed treatment on diversity indices calculated from bacterial 16S rDNA DGGE profiles of the rhizosphere samples at the emergence stage.

Factor	**Source of variation**	**Richness (S) ± SD**	**Hill 2 (H_2) ± SD**	**Hill 1 (H_1) ± SD**	**Evenness (J_p) ± SD**
Hybrid (H)	Ordinary	12.67 ± 1.21 a	9.67 ± 1.25	8.09 ± 1.32	0.89 ± 0.02
	High early vigor	10.83 ± 1.47 b	8.37 ± 1.15	7.03 ± 1.21	0.89 ± 0.03
	p-value	0.039	0.106	0.198	0.937
Seed treatment (S)	No biostimulant	11.50 ± 1.87	8.93 ± 1.72	7.54 ± 1.80	0.89 ± 0.03
	Biostimulant	12.00 ± 1.41	9.10 ± 0.95	7.59 ± 0.78	0.89 ± 0.02
	p-value	0.521	0.811	0.943	0.804
H x S	*p*-value	0.156	0.275	0.289	0.960

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 6 observations ± standard deviation (SD).

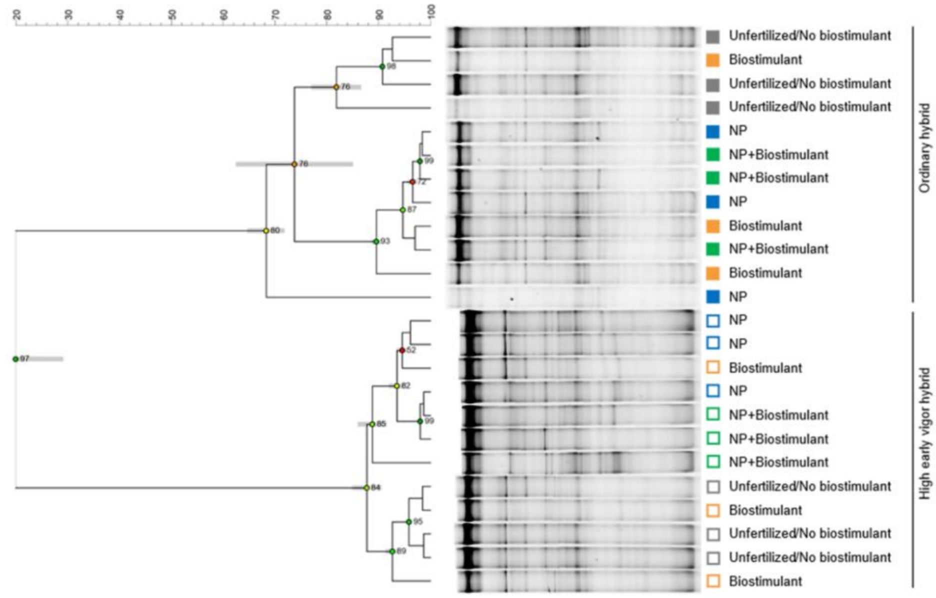


Figure 4. Cluster analysis of rhizosphere bacterial communities DGGE profiles indicating the relationships among samples, based on similarity, as shown by the numeric scale above each dendrogram, obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) analysis, using Dice's similarity coefficient. Dendrograms are based on DGGE profiles obtained from the rhizosphere of two maize hybrids at the 5-leaf stage treated or untreated with NP starter fertilization and with seed-applied biostimulant. Cophenetic correlation, expressing the consistency of clusters, is shown at each node by numbers and colored dots, ranging between green-yellow-orange-red, according to decreasing values. Standard deviation is shown at each node by a gray bar. Colors indicate the factorial treatments: unfertilized/no biostimulant (gray), biostimulant seed treatment (orange), NP starter fertilization (blue) and NP + biostimulant (green). Closed and open symbols refer to the ordinary and to the high early vigor maize hybrids, respectively.

Table 2. Effects of the hybrid, NP starter fertilization, and biostimulant seed treatment on diversity indices calculated from bacterial 16S rDNA DGGE profiles of the rhizosphere samples at 5-leaf stage.

Factor	**Source of variation**	**Richness (*S*) ± SD**	**Hill 2 (H_2) ± SD**
Hybrid (H)	Ordinary	11.83 ± 1.64 b	9.95 ± 1.53 b
	High early vigor	17.00 ± 1.91 a	13.32 ± 2.30 a
	p-value	< 0.001	< 0.001
Fertilization (F)	Unfertilized	13.08 ± 2.71 b	10.34 ± 1.99 b
	NP	15.75 ± 3.11 a	12.93 ± 2.50 a
	p-value	< 0.001	0.001
Seed treatment (S)	No biostimulant	14.25 ± 3.20	11.57 ± 2.53
	Biostimulant	14.58 ± 3.26	11.70 ± 2.73
	p-value	0.490	0.928
H x S	*p*-value	1.000	0.834
H x F	*p*-value	0.490	0.908
F x S	*p*-value	0.728	0.231
H x F x S	*p*-value	0.096	0.772

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 12 observations ± standard deviation (SD).

Table 3. Effects of NP starter fertilization and biostimulant seed treatment on diversity indices calculated from bacterial 16S rDNA DGGE profiles of the rhizosphere samples at 5-leaf stage for each hybrid.

Factor	**Source of variation**	**Hill 1 (H_1) ± SD**		**Evenness (J_p) ± SD**	
		Ordinary hybrid	**High early vigor hybrid**	**Ordinary hybrid**	**High early vigor hybrid**
Fertilization (F)	Unfertilized	7.82 ± 0.85 b	9.78 ± 2.03	0.92 ± 0.02 b	0.90 ± 0.04
	NP	10.12 ± 0.72 a	12.53 ± 3.16	0.94 ± 0.01 a	0.92 ± 0.04
	p-value	0.001	0.131	0.044	0.379
Seed treatment (S)	No biostimulant	8.80 ± 1.03	11.17 ± 2.86	0.93 ± 0.01	0.92 ± 0.04
	Biostimulant	9.14 ± 1.81	11.14 ± 3.24	0.93 ± 0.02	0.91 ± 0.04
	p-value	0.471	0.987	0.846	0.648
F x S	*p*-value	0.245	0.404	0.869	0.188

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 6 observations ± standard deviation (SD).

5.4.2 DGGE amplicon sequencing and identification of the main bacterial taxa

In order to identify major bacterial taxa characterizing the rhizosphere soils of different maize hybrids in the factorial combinations of fertilizer and biostimulant treatments, relevant bands were excised from DGGE gels (Figures 1 and 2), sequenced and affiliated to genera and species by using nBLAST and phylogenetic tree analyses. Partial 16S rDNA fragments belonged to three phyla, namely Proteobacteria (*Stenotrophomonas* sp., *Lysobacter* sp., *Polaromonas ginsengisoli*, *Limnobacter thiooxidans*, *Massilia* sp., *Rhodanobacter* sp., *Janthinobacterium* sp., uncultured Proteobacteria), Bacteroidetes (*Flavobacterium* sp., *Pedobacter* sp., *Chryseolinea* sp., *Adhaeribacter terrae*) and Firmicutes (*Paenibacillus* sp.) (Table S3; Figure 5). None of the sequenced bands affiliated with *B. amyloliquefaciens*.

16S rDNA fragments affiliating with *Massilia* sp. (5-6), *Paenibacillus* sp. (7) and *Janthinobacterium* sp. (11) could be recovered only from the emergence stage samples. At emergence stage, more variable and more intense banding patterns could be observed in the rhizosphere of the high early vigor hybrid, with occasional increases in the abundance of *Pedobacter* sp. (4), *Massilia* sp. (5-6), *Janthinobacterium* sp. (11), *Lysobacter* sp. (12-13) and *Stenotrophomonas* sp. (14-17), while bacterial communities showed much more uniform molecular profiles in the samples of the ordinary hybrid (Figure 1). Additionally, the higher abundance of the OTUs corresponding to *Paenibacillus* sp. (7) and *Stenotrophomonas* sp. (14) were associated with the biostimulant treatment in the ordinary hybrid.

Fragments corresponding to *Chryseolinea* sp. (23, 43), *Limnobacter thiooxidans* (25, 44-45), *Polaromonas ginsengisoli* (27-28, 46) and *Rhodanobacter* sp. (53) could be retrieved only from the 5-leaf stage rhizosphere samples. At the 5-leaf stage, despite most bacterial populations were represented uniformly in all samples of each hybrid, marginal fluctuations could be observed in the abundance of some taxa. Biostimulant application slightly increased the abundance of *Polaromonas ginsengisoli* (27-28) in the ordinary maize genotype, while other changes were detected mainly due to the NP fertilization treatment (Figure 2). In the ordinary hybrid, slightly higher abundance of *Limnobacter thiooxidans* (25), *Lysobacter* sp. (26) and an uncultured Proteobacteria (34) were associated to the unfertilized samples, while bands of *Stenotrophomonas* sp. (30-33) were more characteristic of the NP fertilization treatments. In the high early vigor hybrid, somewhat similar changes could be observed: bands affiliated with *Limnobacter thiooxidans* (44-45) appeared more intensely in the unfertilized samples, while *Stenotrophomonas* sp. (50-52, 54-55) remained more associated to the NP fertilizer treatments. Additionally, *Pedobacter steynii* (40) was more represented in the unfertilized maize rhizosphere, and *Polaromonas ginsengisoli* (46) and some *Lysobacter* sp. (48-49) in the NP fertilization treatments. Sequences affiliated to *Rhodanobacter* sp. (53) could be retrieved only from the NP fertilized samples of the high early vigor hybrid.

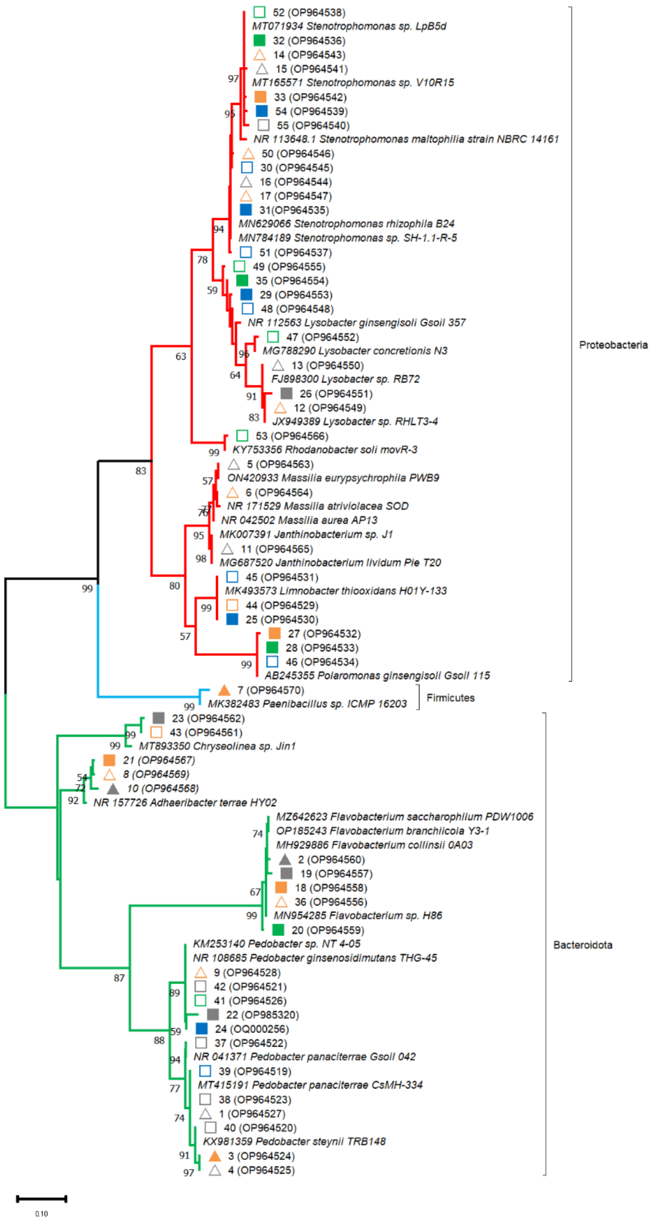


Figure 5. Affiliation of the sequences retrieved from DGGE gel fragments (marked in Figs. 2 and 4) with the sequences of the 16S rRNA gene retrieved in gene banks. Phylogenetic analysis was inferred by using the Maximum Likelihood method. The evolutionary distances were computed using the Kimura 2-parameter model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA11.The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are indicated by their corresponding band number and their accession number. Symbols indicate samples analysed at the emergence stage (triangles) and at the 5-leaf stage (squares). Closed and open symbols refer to the ordinary and to the high early vigor maize hybrids,

respectively. Colors indicate the factorial treatments: unfertilized/no biostimulant (gray), biostimulant (orange), NP starter fertilization (blue) and NP + biostimulant (green).

5.5 Discussion

Our data showed that maize genotype was the major factor shaping rhizosphere bacterial community composition, as assessed by cluster analyses of DGGE patterns, suggesting that the root system of the two maize hybrids recruited a different microbiota. Here, for the first time we identified at the species and genus level the predominant native bacteria associated with two maize hybrids differing for vigor. The biostimulant treatment affected the rhizosphere bacterial microbiota of the ordinary hybrid more than that of the early vigor, both at plant emergence and at the 5-leaf stage. Moreover, the 5-leaf stage rhizosphere bacterial community composition was differentially affected by starter NP fertilization, compared with that of the unfertilized/no biostimulant in both hybrids.

5.5.1 DGGE cluster analysis

Cluster analysis of the DGGE profiles detected significantly different rhizosphere bacterial community profiles among the two maize hybrids, which became more evident at the 5-leaf stage.

Differences in the rhizosphere microbiota of the two hybrids may be attributed to the influences of plant genotype on the assemblages of plant associated microbial communities (Manter et al., 2010; Shenton et al., 2016; Pérez-Jaramillo et al. 2017; Agnolucci et al., 2019). Genetical differences between cultivated crop varieties have been shown to affect root architecture, rhizodeposition patterns and plant-microbe signalling pathways (Hu et al., 2018; Kerstens et al., 2021; Semchenko et al., 2021). Rhizodeposition of sugars, organic acids, amino acids and secondary metabolites plays a crucial role in the recruitment and regulation of root associated microbiota, as some serve as signals, and some are easily available nutrients to heterotrophic bacteria (Philippot et al., 2013; Canarini et al., 2019).

Cluster analysis showed that seed-applied biostimulant preparations had contrasting effects on the two maize hybrids. Biostimulant treatment affected the rhizosphere bacterial microbiota of the ordinary hybrid since the emergence stage, which grouped separately from no biostimulant samples. Our data are consistent with those obtained in juvenile maize inoculated with *B. amyloliquefaciens* FZB42, revealing shifts in the PCR-DGGE rhizosphere bacterial community profiles, compared with uninoculated samples (Cozzolino et al., 2021). Furthermore, inoculation with *B. amyloliquefaciens* L-S60, B1408 and FZB42 caused changes in the rhizosphere bacterial communities of cucumber seedlings and tomato plants (Qin et al., 2017; Han et al., 2019; Eltlbany et al., 2019), while a biofertilizer preparation containing *B. amyloliquefaciens* NJN-6 had similar effects in field-grown banana plants (Shen et al., 2015). By contrast, our biostimulant treatment had marginal effects on the rhizosphere bacterial community of the high early vigor maize hybrid at both growth stages, consistently with previous findings obtained in field-grown wheat inoculated with a consortium of PGP *Azospirillum* spp., *Azoarcus* spp.

and *Azorhizobium* spp. (Dal Cortivo et al., 2020), and in lettuce and soybean treated with *B. amyloliquefaciens* (Correa et al., 2009; Chowdhury et al., 2013; Kröber et al., 2014). The different behaviour of the two maize genotypes may be ascribed to differential interactions between native rhizosphere bacteria and the biostimulant used in this study, which could affect the multipartite relationships in the rhizosphere microbiota. Accordingly, previous studies demonstrated differences in the compatibility of some crop genotypes with various microbial inocula in wheat (Akbari et al., 2020), tomato (Tucci et al., 2011), potato (Higdon et al., 2020) and sugarcane (de Oliveira et al., 2006). Although knowledge on this topic is scanty, our hypothesis is supported by the detailed analysis of the root-associated bacterial communities of two durum wheat varieties as affected by different microbial inocula (Agnolucci et al., 2019).

The UPGMA cluster analysis highlighted also differential effects of NP fertilization on the rhizosphere bacterial community composition at the 5-leaf stage, as fertilized samples clustered separately from unfertilized/no biostimulant samples in both hybrids. Mineral fertilization was previously found to impact root-associated microbial communities in several crop plants (Tang et al., 2016; Chen et al., 2019; Semenov et al., 2020), and to change the bacterial community composition in maize rhizosphere, including the abundance of important bacterial functional genes and PGPB groups (Zhu et al., 2016; Silva et al., 2017; Gomes et al., 2018; Wang et al., 2018). Besides the direct effects of increased mineral nutrient availability in fertilized soils, rhizosphere microbial communities may be affected by alterations of root morphology and root exudates quality and quantity, as the result of improved plant growth and nutrient status (Lu et al., 1999; Zhu et al., 2016; Chen et al., 2019).

At the emergence stage, bacterial OTU richness was significantly higher in the ordinary hybrid, while at the 5-leaf stage the high early vigor hybrid hosted a larger and more diverse rhizosphere bacterial community. N and P amendments had a positive effect on bacterial diversity indices, as OTU richness and the estimated “effective number of species” (H_2) increased in both maize genotypes, while evenness and another estimator of “effective number of species” (H_1) only in the ordinary hybrid, confirming previous data on the changes of biodiversity of the rhizosphere microbiota by mineral fertilization (Wang et al., 2018; Semenov et al., 2020).

5.5.2 Sequencing of predominant DGGE bands

The sequencing of the main DGGE bands allowed the detection of 13 taxa, all belonging to genera and species of widespread occurrence in soils. The high representation of Proteobacteria among the sequenced bands is not surprising, as this phylum was previously reported to be predominant in maize rhizosphere (Peiffer et al., 2013; Li et al., 2014; Silva et al., 2017; Gomes et al., 2018; Wang et al., 2018). It is interesting to note that in the ordinary hybrid treated with the biostimulant, some bacteria belonging to taxa reported as PGP, were more represented. In particular, *Paenibacillus* sp. and *Stenotrophomonas* sp. were more abundant in the rhizosphere of emergent plantlets and *Polaromonas ginsengisoli* in the 5-leaf stage samples. Indeed, *Paenibacillus* species are well known for their wide range of PGP properties, such as N_2-fixation, P solubilisation, plant hormones, siderophores and antibiotics production, and are used as biofertilizers and biocontrol agents (Campos et al., 2015; Goswami et al., 2016; Padda et al., 2017; Zhang et al., 2018). In agreement with our data, Eltlbany et al., (2019) reported increases in the population size of *Paenibacillus* sp. as a result of bacterial biostimulant treatments in the rhizosphere of tomato plants. The species *Polaromonas ginsengisoli*, isolated from a ginseng field in 2018 (Choi et al., 2018), has been functionally characterized as PGP in sugar beet plants (Okazaki et al., 2021). *Stenotrophomonas* spp. have been widely detected in plant-associated bacterial communities (Ryan et al., 2009; Hayward et al., 2010) and were reported to inhabit the maize rhizosphere (Medina-de la Rosa et al., 2016; Qaisrani et al., 2019; Ercole et al., 2021; Guo et al., 2022). Several isolates belonging to this genus were shown to promote plant growth via N_2-fixation, to solubilize P and to produce ACC-deaminase, plant hormones and siderophores, acting as stress protective agents (Yu et al., 2011; Alavi et al., 2013; Ghavami et al., 2017; Singh and Jha 2017; Youseif 2018; Ercole et al., 2021). Moreover, *Stenotrophomonas* sp. contributed to disease suppression in diverse crop plants, such as wheat, potato, cotton, cucumber and pepper (Messiha et al., 2007; Egamberdieva et al., 2011; Schmidt et al., 2012; Singh and Jha 2017). In the present study, this taxon reached higher abundance also in the NP fertilized rhizosphere of both hybrids, suggesting a possible responsiveness to P fertilization levels, as reported by Guo et al., (2022) in maize rhizosphere.

In this work, certain taxa were more represented in the early vigor hybrid, particularly in NP treated samples, such as *Lysobacter* sp., which occurred in diverse habitats including soils (Hayward et al., 2010) and was previously reported to colonize or even dominate maize rhizosphere (García-Salamanca et al., 2013; Li et al., 2014; Maarastawi et al., 2018). Interestingly, strains of *Lysobacter* sp. have shown multiple PGP activities *in vitro*, such as P solubilisation, siderophores and antibiotics production with promising biocontrol potentials (Hayward et al., 2010; Gómez Expósito et al., 2015; Puopolo et al., 2018; Sharma et al., 2021), successively demonstrated in pepper (Liu et al., 2019), tomato (Puopolo et al., 2010), Chinese cabbage (Zhou et al., 2014) and rice (Tu et al., 2022). *Rhodanobacter* sp. was detected only in the rhizosphere of the NP fertilized high early vigor hybrid, in agreement with previous findings showing that maize genotype and inorganic fertilizers may strongly affect its abundance in the rhizosphere soil (Wen et al., 2017; Semenov et al., 2020). However, although its occurrence was reported in the rhizosphere of maize plants by other authors (Shen et al., 2021; Chen et al., 2021), little is known of *Rhodanobacter* metabolic traits (Van Den Heuvel et al., 2010; Kostka et al., 2012; Damo et al., 2022).

In this work, certain bacterial taxa occurred only either at the emergence stage, such as *Janthinobacterium* sp., *Massilia* sp. (family Oxalobacteriaceae), consistently with previous findings describing such genera as dominant in maize rhizosphere at early growth stages, with a sharp decline during the vegetative growth (Li et al., 2014). Interestingly, an isolate of *Janthinobacterium* sp. was found to express antagonism against a wide range of plant pathogens (Yin et al., 2021), while strains of *Massilia* sp. revealed important PGP characteristics, such as the production of phosphatases, siderophores and IAA and antagonism against pathogens (Hrynkiewicz et al., 2010; Turnbull et al., 2012; Raths et al., 2020; Li et al., 2021).

The phylum Bacteroidetes was represented mostly by *Flavobacterium* and *Pedobacter* species. The genus *Flavobacterium* (family Flavobacteriaceae) was uniformly distributed in the different treatments, regardless sampling time and maize genotype, consistently with previous data reporting high abundance of this genus in maize rhizosphere (Li et al., 2014; Correa-Galeote et al., 2016; Yang et al., 2017). Isolates of *Flavobacterium* sp. showed PGP traits, such as P-solubilization, ACC-deaminase and IAA production, while they provided significant plant growth promotion in maize, disease suppression in pepper, onion and cucumber and improved drought stress tolerance in wheat (Sang and Kim 2012; Gontia-Mishra et al., 2016, 2017; Youseif 2018; Nishioka et al., 2019). Also, the genus *Pedobacter* (family Sphingobacteriaceae) was found in all our samples, consistently with their described global occurrence (Steyn et al., 1998; Yoon et al., 2007; Gordon et al., 2009). It was previously described as associated with the roots of potato, canola and wheat (Manter et al., 2010; Lay et al., 2018; Dai et al., 2020), and showed prolonged fruiting season and improved plant growth, fruit quality and leaf gas exchange parameters in strawberry plants (Morais et al., 2019).

Fragments of *B. amyloliquefaciens* 16S rDNA was not retrieved from the DGGE gels, suggesting that the biostimulant strain was not a dominant member of the maize rhizosphere microbiota. Unfortunately, it is not possible to compare our data with previous ones obtained with the same molecular method, as similar works utilizing *B. amyloliquefaciens* BNM122 and FZB42 for soybean and maize inoculation, respectively, and PCR-DGGE, did not perform the identification of the main DGGE bands (Correa et al., 2009; Cozzolino et al., 2021). Moreover, other studies, utilizing different methods to monitor the persistence of *B. amyloliquefaciens* strains, such as serial dilutions and plate counting, were carried out in the absence of native bacterial communities (Correa et al., 2009; Ben Abdallah et al., 2018). Overall, the studies aimed at verifying the root persistence of *B. amyloliquefaciens* reported significant decreases over the course of time (Chowdhury et al., 2013; Kröber et al., 2014).

5.6 Conclusions

This work showed that rhizosphere bacterial community composition of maize was mainly affected by the genotype, as the root systems of the two maize hybrids, differing for their early vigor, were characterized by different rhizosphere microbiota. The predominant native bacteria associated with the two maize hybrids, identified at the species and genus level, belonged to well-known PGPB taxa, such as *Stenotrophomonas* sp., *Lysobacter sp.*, *Massilia* sp., *Paenibacillus* sp. and *Flavobacterium* sp., which were reported to be able to solubilize P and to produce IAA, siderophores and antibiotics, providing significant plant growth promotion and disease suppression. The starter NP fertilization strongly affected PGP rhizosphere bacterial community composition of both maize hybrids at the 5-leaf stage compared with that of the unfertilized treatments, while the biostimulant treatment had a positive effect on PGP community of the ordinary hybrid more than that of the early vigor maize both at the plant emergence and at the fifth leaf stage.

Present findings could support the interpretation of the effects of the biostimulant seed treatment, NP starter fertilizer, genotype early vigor and their combination on crop early development and grain yield, reported in the companion manuscript. Moreover, these results pave the way for further studies to be performed on the effects of cropping system and specific crop practices, considering also the application of biostimulants, on beneficial rhizosphere microorganisms.

5.7 Supplementary material

Table S1. The main physical and chemical characteristics of the natural topsoil (0-30 cm) used in the growth chamber experiment.

Parameters	
Soil type	Typic Ustifluvent
Soil texture	Silt loam
Sand (0.05 - 2 mm)	272 g kg^{-1}
Silt (0.002 - 0.05 mm)	680 g kg^{-1}
Clay (< 0.002 mm)	48 g kg^{-1}
pH (H_20)	7.9
Total carbonate ($CaCO_3$)	12 g kg^{-1}
Organic matter	18.2 g kg^{-1}
C/N	8.6
Cation Exchange Capacity (C.E.C.)	11.0 cmol(+) kg^{-1}
Total Nitrogen	1.23 g kg^{-1}
Exchangeable Potassium	52 mg kg^{-1}
Olsen Phosphorus	6 mg kg^{-1}

Table S2. The main agronomic information pertaining to the maize growing cycle in the growth chamber experiment.

Medium temperature of the air	Day	16.7	C°
	Night	14.1	C°
Air GDDs [1]	Sowing - 6 leaves	273	C°-day
Medium temperature of the soil	Day	15.6	C°
	Night	14.2	C°
Soil GDDs	Sowing - 6 leaves	237	C°-day
Air Humidity		50	%
Day/night		12	h
Light intensity		700-1000	PAR [2]
Water irrigation		10 mm every 7 day	

[1] GDDs: accumulated growing degree days on a 10°C basis.
[2] PAR: photosynthetically active radiation: µmol m^{-2} s^{-1}

*Table S3. Best match identification of bacterial sequences retrieved from PCR-DGGE analysis of the rhizosphere samples at emergence (**A**) and at 5 leaf-stage (**B**).*

A)

Fragment	***Taxon name***	***Isolation source***	***Closest match (% similarity)***	***GenBank accession number***
1	*Pedobacter panaciterrae CsMH-334*	*karst caves*	*100.00*	*MT415191.1*
2	*Flavobacterium sp. H86*	*Astragalus mongholicus root endosphere*	*99.24*	*MN954285.1*
3	*Pedobacter steynii TRB148*	*soil*	*99.43*	*KX981359.1*
4	*Pedobacter sp. CCBAU 10902*	*maize rhizosphere*	*99.42*	*JF772566.1*
5	*Massilia eurypsychrophila PWB9*	*meltwater*	*99.81*	*ON420933.1*
6	*Massilia aurea AP13/Massilia atriviolacea SOD*	*drinking water/soil*	*98.12*	*NR_042502.1/NR_171529.1*
7	*Paenibacillus sp. ICMP 16203*	*Clianthus puniceus root nodules*	*98.25*	*MK382483.1*
8	*Adhaeribacter terrae HY02*	*mountain soil*	*98.68*	*NR_157726.1*
9	*Pedobacter sp. NT 4-05*	*root endosphere*	*99.25*	*KM253140.1*
10	*Adhaeribacter terrae HY02*	*mountain soil*	*97.30*	*NR_157726.1*
11	*Janthinobacterium sp. J1/ Janthinobacterium lividum Pie_T20*	*Medicago polymorpha root endosphere/ Typha latifolia rhizoplane*	*100.00*	*MK007391.1/MG687520.1*
12	*Lysobacter sp. RHLT3-4*	*glaciers*	*99.81*	*JX949389.1*
13	*Lysobacter sp. RB72*	*spring water*	*99.06*	*FJ898300.1*
14	*Stenotrophomonas sp. So3Pt_86*	*forest soil*	*99.44*	*AB836481.1*
15	*Stenotrophomonas sp. LpB5d*	*Lotus parviflorus root nodules*	*99.41*	*MT071934.1*
16	*Stenotrophomonas sp. PN3-B04P1-9*	*wheat rhizosphere*	*99.81*	*MK638446.1*
17	*Stenotrophomonas rhizophila B1*	*Brassica spp. seed endosphere*	*100.00*	*MN629046.1*

B)

Fragment	Taxon name	Isolation source	Closest match (% similarity)	GenBank accession number
18	*Flavobacterium* sp. H86	*Astragalus mongholicus* root endosphere	99.42	MN954285.1
19	*Flavobacterium saccharophilum* PDW1006/*Flavobacterium collinsii* 0A03	poultry drinking water/tundra soil	98.46	MZ642623.1/MH929886.1
20	*Flavobacterium* sp. H86	*Astragalus mongholicus* root endosphere	99.04	MN954285.1
21	*Adhaeribacter terrae* HY02	mountain soil	97.88	NR_157726.1
22	*Pedobacter* sp. NT 4-05	root endosphere	97.40	KM253140.1
23	*Chryseolinea* sp. Jin1	unknown	95.72	MT893350.1
24	*Pedobacter* sp. NT 4-05	root endosphere	99.07	KM253140.1
25	*Limnobacter thiooxidans* H01Y-133	*Prymnesium saltans* algal culture	99.81	MK493573.1
26	*Lysobacter* sp. RHLT3-4	glaciers	98.17	JX949389.1
27	*Polaromonas ginsengisoli* Gsoil 115	ginseng field soil	99.24	AB245355.1
28	*Polaromonas ginsengisoli* Gsoil 115	ginseng field soil	99.43	AB245355.1
29	*Lysobacter* sp. YC6725	rice field soil	97.73	EU707563.1
30	*Stenotrophomonas rhizophila* B24	*Brassica* spp. seed endosphere	99.26	MN629066.1
31	*Stenotrophomonas* sp. SH-1.1-R-5	*Dendrobium officinale* endosphere	100.00	MN784189.1
32	*Stenotrophomonas* sp. LpB5d	*Lotus parviflorus* root nodules	99.81	MT071934.1
33	*Stenotrophomonas* sp. T2BM2-2	agave plant microbiota	99.81	OP210260.1
34	Uncultured proteobacterium clone 39	freshwater	89.85	MN844051.1
35	*Lysobacter* sp. YC6725	rice field soil	97.93	EU707563.1
36	*Flavobacterium* sp. H86	*Astragalus mongholicus* root endosphere	99.42	MN954285.1
37	*Pedobacter panaciterrae* WR144	ginseng rhizosphere soil	100.00	AB365796.1
38	*Pedobacter panaciterrae* CsMH-334	karst caves	100.00	MT415191.1
39	*Pedobacter panaciterrae* CsMH-334	karst caves	99.81	MT415191.1
40	*Pedobacter steynii* TRB148	soil	100.00	KX981359.1
41	*Pedobacter* sp. NT 4-05	root endosphere	100.00	KM253140.1
42	*Pedobacter* sp. NT 4-05	root endosphere	99.81	KM253140.1
43	*Chryseolinea* sp. Jin1	unknown	96.33	MT893350.1
44	*Limnobacter thiooxidans* H01Y-133	*Prymnesium saltans* algal culture	99.81	MK493573.1
45	*Limnobacter thiooxidans* H01Y-133	*Prymnesium saltans* algal culture	99.62	MK493573.1
46	*Polaromonas ginsengisoli* Gsoil 115	ginseng field soil	99.63	AB245355.1
47	*Lysobacter concretionis* N3	nickel sludge waste	98.85	MG788290.1
48	*Lysobacter ginsengisoli* Gsoil 357	ginseng field soil	98.86	NR_112563.1
49	*Lysobacter* sp. YC6725	rice field soil	97.56	EU707563.1
50	*Stenotrophomonas* sp. 12C_21	lake water	100.00	AY689084.1
51	*Stenotrophomonas* sp. BIS1040	soil	99.43	MN810222.1
52	*Stenotrophomonas* sp. LpB5d	*Lotus parviflorus* root nodules	99.81	MT071934.1
53	*Rhodanobacter* sp. movR-3	rhizosphere soil	99.81	KY753356.1
54	*Stenotrophomonas* sp. V10R15	*Phragmites australis* roots	99.25	MT165571.1
55	*Stenotrophomonas* sp. V10R15	*Phragmites australis* roots	98.66	MT165571.1

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6. CHAPTER V - Agronomic strategies to enhance the early vigor and yield of maize. Part II: the role of seed applied biostimulant, hybrid and starter fertilization on crop performance.

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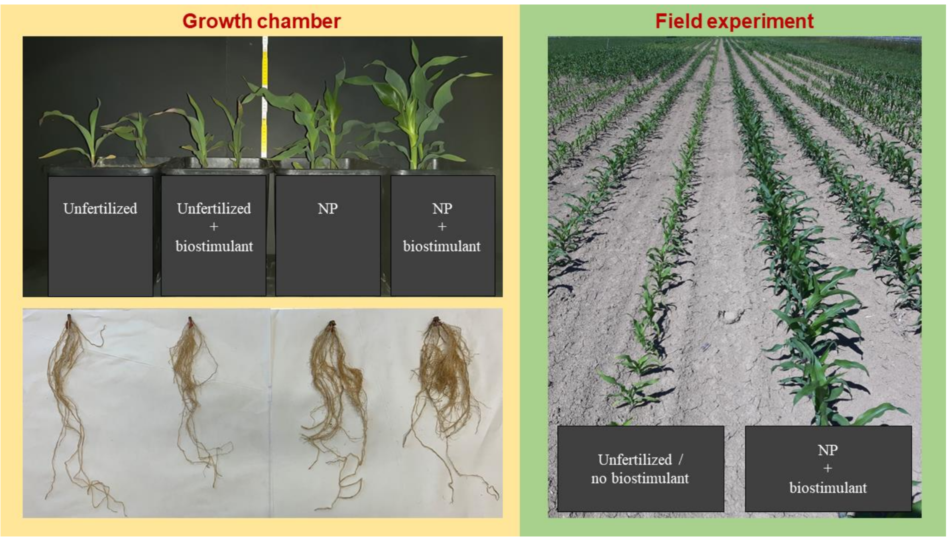
Graphical abstract



6.1 Abstract

Maize cropping systems need to be re-designed, within a sustainable intensification context, by focusing on the application of high-use efficiency crop practices, such as those that are able to enhance an early plant vigor in the first critical growth stages; such practices could lead to significant agronomic and yield benefits. The aim of this study has been to evaluate the effects of the cultivation of hybrids with superior early vigor, of the distribution of starter fertilizers at sowing, and of the seed application of biostimulants on promoting plant growth and grain yield in full factorial experiments carried out in both a growth chamber and in open fields. The greatest benefits, in terms of plant growth enhancement (plant height, biomass, leaf area, NDVI) and cold stress mitigation (CRI and NPCI indices), were detected for the starter fertilization, followed by the use of a high early vigor hybrid and a biostimulant seed treatment. The starter fertilization and the high early vigor hybrid led to earlier flowering dates, that is, of 2.1 and 2.8 days, respectively, and significantly reduced grain moisture at harvest. Moreover, compared to the control treatment, the high early vigor hybrid, the starter NP fertilization, and the biostimulant treatment increased grain yield by 8.5%, 6.0%, and 5.1%, respectively, compared to the untreated controls. The combination of all the considered factors resulted in the maximum benefits, compared to the control cropping system, with an increase in the plant growth of 124%, a reduction of the sowing-flowering period of 5 days, and a gain in grain yield of 14%. When choosing the most suitable crop practice, the diversity of each cropping system should be considered, according to the pedo-climatic conditions, the agronomic background, the yield potential, and the supply chain requirements.

Keywords: corn; early vigor; grain yield; seed treatment; diammonium phosphate; *Bacillus amyloliquefaciens*.

6.2 Introduction

Maize (*Zea mays* L.) is the first commodity, in terms of the worldwide annual production of dry grain, and it plays a key role in global agri-food systems, since it is used for food and feed consumption and several non-food purposes, such as the production of starch, chemical compounds, and energy. The global maize production has surged in recent decades, driven by the increasing demand, and by a combination of yield increases and area expansions (Erenstein et al., 2022). Furthermore, the global use of maize is destinated to increase, although the prospects for further area expansions are limited, thus highlighting the need to innovate cropping systems to further increase their production capacities and enhance a sustainable intensification of maize-based systems.

One of the agronomic strategies that has allowed maize yields to be increased in temperate growing areas, but also the qualitative and sanitary traits, is the progressive ability to anticipate the sowing period in spring, which leads to an increase in the length of the growing cycle and avoids possible heat and drought stress during the flowering or the ripening (Van Roekel and Coulter 2011; Waqas et al., 2021). Moreover, in order to prevent the negative impact of cold and rainy periods during the first vegetative stages, which can limit the advantages of early sowing, this practice is often associated with the use of good early vigor hybrids and/or the application of starter fertilizers, mainly those containing nitrogen (N) and phosphorus (P), applied in bands close to the seed furrows (Kaiser et al., 2016; Reis et al., 2022). Since a fast and uniform plant growth plays a key role in achieving the full potential yield of maize and in promoting higher resource use efficiencies, these crop practices are adopted to efficiently limit a delay in maize development, which could reduce the benefits of early sowing, thereby significantly contributing to the profitability of the crop.

Biostimulants represent another promising developing strategy to enhance plant development and to reduce biotic and abiotic stresses, particularly during the most critical growth stages of the crop (du Jardin 2015; Cardarelli et al., 2022). Biostimulants, which have been defined by the European Biostimulant Industry Council as “substances and/or micro-organisms whose function is to stimulate natural processes that enhance nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and crop quality” can offer an eco-friendly option to reduce the use of external inputs, such as mineral fertilizers, and increase the sustainability of cropping system, without a reduction of crop productivity (Gupta et al., 2021; Li et al., 2022).

The application of specific species of plant growth promoting bacteria (PGPB) and/or substances that are readily available and rapidly assimilated by the rhizosphere microbial community has been proposed, as biostimulants and biofertilizers, as they could lead to beneficial effects on the crop, as a consequence of the production of plant growth regulators (phytohormones), antifungal secondary metabolites, and an increase in plant nutrient uptake (Tahir et al., 2018; Aloo et al., 2022). In addition to a direct effect on plant growth, such crop practices as the choice of hybrids with different early vigor, or the application of starter fertilizers or biostimulants at sowing, could also influence the rhizosphere bacterial richness and functionality (Favela et al., 2021). Since plants rely on their microbiota for several functions, including nutrient acquisition and protection against stress (Compant et al., 2019), the effect of enhancing rhizosphere microbiota through the application of crop practices could lead to further indirect benefits for the early development of plants, particularly in the most critical early vegetative stages. In part I of the present study (Ujvári et al., 2023), the role of maize genotype, NP starter fertilization, a biostimulant seed treatment, and their interaction, was studied on native rhizosphere microbiota during the early growth phases of maize in a growth chamber experiment. The diversity and composition of the rhizosphere bacterial communities was mainly affected by the genotype, when hybrids with different early vigor were considered, and by the starter fertilization. Moreover, the collected data highlighted that the use of a high early-vigor hybrid, and NP fertilization, also increased the abundance of specific bacterial taxa (*Stenotrophomonas*, *Lysobacter, Massilia*, *Paenibacillus* and *Flavobacterium*), which have been reported to be able to provide a significant plant growth promotion (Hayward et al., 2010; Kour et al., 2019; Li et al., 2021). Moreover, the collected data also showed that a biostimulant seed application resulted in a significant enhancement of the rhizosphere bacterial community, but, interestingly, the microbiota of the ordinary hybrid was affected more than the early vigor one by the biostimulant treatment, thus showing an interaction among the compared crop practices.

To the best of our knowledge, the effects of a biostimulant application on crop development, and the related benefits, in terms of both grain yield and quality, have not been adequately investigated for crop plants, by considering a comparison and interaction with other agronomic practices that are able to enhance plant vigor. Moreover, the benefits associated with the application of PGPB have generally been reported for marginal environments (Li et al., 2022), while their contribution to intensive systems has yet not been clarified.

Considering the current need to re-design maize cropping systems in order to enhance their inputs and use efficiency, and to lead to a more sustainable production, the aim of the present study, part II of the work, has been to evaluate and compare the agronomic and yield effects of different crop practices that are able to enhance the early plant vigor. The effect of genotype early vigor, NP starter fertilizer, and a biostimulant seed treatment on maize development in the early stages, and the consequent effect on the crop cycle length, and on the grain yield and quality, has been studied in depth in both a growth chamber experiment and open field experiments, considering factorial combinations of the compared agronomic factors in different locations (i.e. in different soils) and in different growing seasons.

6.3 Materials and methods

A growth chamber experiment was set up to investigate the effects of i) various maize hybrids, considering genotypes with different early vigor, ii) NP starter fertilization in seed furrows and iii) a biostimulant, based on a PGPB and plant extract, applied to seeds, in promoting maize plant development in the early stage under cold conditions. The same experimental design was replicated under open field conditions in the 2018 and 2019 growing seasons, to demonstrate the role of different agronomic factors in favoring the early growth of maize in a real environment, and to quantify the benefits in terms of grain yield and quality.

6.3.1 Growth chamber experiment

a. Experimental design

A detailed description of the management of the growth chamber experiment is reported in part I of the present work (Ujvári et al., 2023). Briefly, an amount of sixteen kilograms of natural silt loam sub-alkaline soil (Typic Ustifluvents, USDA classification), was weighed and placed, after mixing it thoroughly, into each plastic pot (27 cm long x 24 cm wide x 28 cm high). The soil was collected from the surface layer (0.2 m) of a field in the University of Turin experimental station, located in Carmagnola, North-West Italy (44° 53’ N, 7° 41’ E; elevation 245 m). The soil was characterized by a medium cation-exchange capacity (C.E.C.), low organic matter, K, and P contents, and medium nitrogen availability. More information on the physical and chemical parameters of the soil are reported in Table 1.

Table 1. The main physical and chemical characteristics of the topsoil (0-30cm) in the Carmagnola and Poirino experimental sites.

Parameters	**Measurement units**	**Carmagnola** [1]	**Poirino**
Geographical coordinates		44°53' N, 7°41' E	44°56' N, 7°52' E
Soil type		Typic Ustifluvent	Fluventic Haplustepts
Soil texture		Silt loam	Silt loam
Sand (0.05 - 2 mm)	g kg^{-1}	272	233
Silt (0.002 - 0.05 mm)	g kg^{-1}	680	696
Clay (< 0.002 mm)	g kg^{-1}	48	70
pH (H_20)		7.9	6.5
Total carbonate ($CaCO_3$)	g kg^{-1}	12	4
Organic matter	g kg^{-1}	18.2	19.8
C/N		8.6	9.2
Cation Exchange Capacity (C.E.C.)	cmol(+) kg^{-1}	11.0	13.1
Total Nitrogen	g kg^{-1}	1.23	1.26
Exchangeable Potassium	mg kg^{-1}	52	70
Olsen Phosphorus	mg kg^{-1}	6	73

[1] the Carmagnola soil was used in the growth chamber experiment.

The soil was not air dried, sieved, sterilized or mixed with quartz sand or any other materials. The compared treatments were factorial combinations of:

- maize hybrids; considering genotypes with different early vigor after emergence, but with a similar growing cycle (FAO maturity class 600, 130 maturity days),
    - an ordinary hybrid (ordinary), with conventional early vigor (LG30600, Limagrain Europe, Saint-Beauzire, France),
    - a high early-vigor hybrid (high early vigor), with a rapid growth in the first vegetative stages (LG31630, characterized by the Rapid'START trait, Limagrain Europe);
- NP starter fertilization,
    - unfertilized control (unfertilized),
    - sub-surface starter fertilization (NP), whereby 27 kg N ha^{-1} and 69 kg P_2O_5 ha^{-1} were applied as diammonium phosphate (DAP, 18-46%, for N and P_2O_5, respectively w/w) and placed in bands close to the maize seed furrows;
- biostimulant seed treatment,
    - untreated control (no biostimulant),
    - biostimulant seed application (biostimulant), based on a mixture of a bacterium, *Bacillus amyloliquefaciens*, strain IT45 (Rise P®), and a leguminous plant extract *Cyamopsis psoraloides* (AgRho® GSB30) (Starcover, Limagrain Europe).

The adopted experimental pot design was a completely randomized block design with three replications for each treatment.

All the maize seeds were treated with a fungicide mixture of prothioconazole (100 g L^{-1}) and metalaxyl (20 g L^{-1}), which was applied, at 15 mL, to 50,000 seeds (Redigo® M, Bayer Crop Science S.r.l., Monheim am Rhein, Germany).

The shape, dimensions and weight of the maize seeds were chosen carefully to reduce the variability of the seedling vigor. Four maize seeds were sown by hand and equally distributed in each pot at a depth of 2 cm. After germination, only 2 plants per pot were maintained, while the other 2 plants were manually removed at the first leaf stage to ensure the conventional field density.

The starter NP fertilizer was manually placed in band at a distance of 5 cm from the maize seed furrows, and at a depth of 10 cm. No other fertilizers were applied before or after sowing. The pots were placed in a controlled growth chamber, with 50% relative humidity, a 12 h photoperiod, 700 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and a 14/17 °C (night/day) air temperature range (Table S1).

b. *Crop measurements*

The plant height was measured in centimeters, from the ground level up to the collar of the tallest fully developed leaf, at the 2 (growth stage, GS, 12, 21 days after sowing, DAS), 3 (GS13, 35 DAS), and 5 (GS15, 47 DAS) leaf stages (BBCH scale, Lancashire et al., 1991). At the same time, the Leaf Area (LA) was estimated according to Ruget et al., (1996).

The chlorophyll leaf content was measured at the 4-leaf stage (GS14), using a SPAD 502Plus-chlorophyll meter® (Konica-Minolta, Osaka, Japan), and expressed as SPAD units. Two SPAD readings were taken around the midpoint of each unrolled leaf for both maize plants in each pot and averaged.

The spectral signature (from 350 to 2500 nm) of each unfolded leaf was acquired at GS14 using a NaturaSpec™ RS-5400® Portable spectroradiometer (Spectral Evolution, Haverhill, USA). We used a leaf clip bundle (Spectral Evolution, Haverhill, USA), which was specifically designed for leaf reflectance measurements, and which has an integrated light source and white reference, to collect sample spectra. One observation was considered for each maize leaf for both pot plants. The collected data allowed the vegetative indices related to the P (normalized phosphorus content index, NPCI, according to Mahajan et al., (2014)) and carotenoid (Carotenoid Reflectance Index, CRI_{700}, according to Gitelson et al., (2001)) leaf content to be collected, according to the following formulas:

$$NPCI = \frac{(R_{1080} - R_{1460})}{(R_{1080} + R_{1460})}$$

$$CRI_{700} = (R_{510})^{-1} - (R_{700})^{-1}$$

where R is the reflectance at the corresponding subscripted wavelength (nm).

The normalized difference vegetation index (NDVI) of each pot was measured, at the 5-leaf emission stage (GS15, 47 DAS), by means of a pistol grip mounted onto the RS-5400 spectroradiometer, and elaborated with DARWin SP Data Acquisition® software (Spectral Evolution, Haverhill, USA). The instrument was held at a height of 1.36 m above each single pot, using a 25-degree exercise fiber cone, to detect the spectral signature (in the 350-2500 nm range) of both plants sown in each pot. This assessment was carried out under standard conditions, placing the pot in a room with only artificial light (ILM-550 Tungsten-Halogen Light Sources) and using an opaque plastic black cloth placed on the floor and on the surface of the pot to only detect the plant reflectance. An NDVI measurement permits the development of the crop canopy to be quantified, since the obtained values are proportional to the maize biomass (Capo et al., 2020).

The shoots and root system of each plant were collected at 49 DAS, after cutting the maize shoots at the collar and gently removing the soil by hand. The shoot and root dry biomasses were determined after their biomass had been oven dried at 105°C for 24h. The obtained data were expressed in grams per plant of dry weight (d.w.).

6.3.2 Field experiments

a. Site and treatments

Two field experiments were set up in the 2018 and 2019 growing seasons in two locations in North-West Italy: Carmagnola, in the same soil used in the previous growth chamber experiment, and Poirino. The main physical and chemical characteristics of these soils are reported in Table 1. The greatest differences between the soil in the considered locations concerned the pH and the available P content (Olsen et al., 1954): Carmagnola is characterized by a sub-alkaline soil with a low P content, while the Poirino soil is sub-acidic and has a high P availability. The 2019 experiment was performed in a new area, adjacent to the one used in 2018, in both sites to avoid carry-over effects resulting from the treatments.

The daily air temperatures and precipitations were measured at Regione Piemonte meteorological stations located near (within 5 km) each site.

The soil temperature was measured, during the crop emergence and seedling stages, using a GP-4020 Tinytag Plus 2 devices, with a 10 cm thermistor probe (Gemini Data Loggers Ltd, Chichester, UK), which was placed along the seed rows.

The same experimental design as the one adopted in the growth chamber trial was adopted at each location and in each year, that is, a factorial combination of 2 hybrids (ordinary vs high early vigor), 2 fertilizations close to the sowing furrows (unfertilized vs NP), and 2 seed treatments (no biostimulant vs biostimulant).

The treatments were assigned to experimental units in each site using a completely randomized block design, with four replicates. Each plot consisted of 4 rows 0.75 m apart; the plot length and the alleys between the plots were 12 and 1 m, respectively. All the measurements were conducted in the two middle rows.

The starter fertilizers were placed at a distance of 5 cm from the side of the seed furrows, using a calibrated granular dispenser attached to the planter (Monosem NG, Largeasse, France), at a depth of 10 cm from the soil surface.

The conventional crop technique of the growing areas was adopted. Briefly, in Carmagnola, sowing was carried out after autumn ploughing (at a 0.3 m depth), followed by disk harrowing, while, in Poirino, a seedbed was prepared by means of a sub-soiler, followed by power harrowing. The previous crop was maize in each field. The sowing density was 8.0 plants per m^2. The sowing and harvest dates are reported for each year and site in Table 2. A granular soil insecticide (tefluthrin 0.183 kg AI ha^{-1}) was applied at sowing to the seed furrows to protect the seedlings from injury by ground insects (Force® Ultra 1.5%, Syngenta Crop Protection S.p.A.). The weed control was conducted, at pre-emergence, with mesotrione (0.15 kg 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., Basel, Switzerland), and at post-emergence with nicosulfuron (36.8 kg AI ha^{-1}), rimsulfuron (9.2 kg AI ha^{-1}), and dicamba (220 kg AI ha^{-1}) (Principal® Mais, Corteva Agriscience, Cremona, Italy).

Table 2. The main agronomic information, cumulative monthly rainfall, air growing degree days on a 10°C basis (GDDs), medium temperature of the soil, and GDDs during the maize crop cycle in the 2018-2019 period at Carmagnola and Poirino (North-West Italy).

Agronomic information		**2018**		**2019**	
		Carmagnola	**Poirino**	**Carmagnola**	**Poirino**
Sowing date		April 20	April 26	March 22	March 21
Harvesting date		September 25	October 1	September 16	September 24
Rainfall (mm)	March	103	105	25	6
	April	116	82	184	85
	May	310	183	272	103
	June	14	52	54	25
	July	74	85	177	120
	August	6	21	119	86
	September	35	51	82	63
	Sowing - 6 leaves	244	184	402	139
	Sowing - Harvest	439	397	884	482
Air GDDs^{1} (C°-day)	March	5	9	58	48
	April	151	155	129	80
	May	255	238	195	165
	June	372	360	417	395
	July	462	463	496	466
	August	457	467	457	436
	September	327	345	318	286
	Sowing - 6 leaves	290	275	265	211
	Sowing - Harvest	1942	1912	1901	1798
Medium temperature of the soil (C°)	Sowing - 6 leaves	20.1	18.8	14.8	15.2
Soil GDDs (C°-day)	Sowing - 6 leaves	353	307	288	327

Before sowing, 157 kg ha^{-1} of K_2O was applied (as potassium chloride, 60% K_2O w/w), but no other N or P fertilizations were distributed, except for the starter in bands close to the seed furrows. A total of 230 kg ha^{-1} of N was applied at approximately the 8th unfolded leaf growth stage (GS18), using urea (46% N w/w), for all the treatments at each site and in each year.

Irrigation was carried out, at both sites, by means of the sprinkler method, according to the conventional farm management system in force in the experimental area, to avoid any drought stress until physiological maturity (GS87).

b. *Crop vigor*

Different assessments were performed to establish plant vigor in the early growth stages.

The NDVI was measured from the 3-leaf stage (GS13) until tassel emission (GS55) for each plot using a hand-held optical sensing device, GreenSeekerTM® (Trimble©, Sunnyvale, California, USA). 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 (12 m). This assessment was performed approximately every 7 days, on the two middle rows of each plot.

The NDVI measurement helped to quantify the development of the crop canopy throughout the season, since low values refer to naked soil, while high values are proportional to the maize biomass. The Area Under Canopy Development Curve (AUCDC) (Capo et al., 2020) was calculated during the vegetative stage for each treatment, starting from the NDVI measurement for each observation date and using the following formula:

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

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

Plant height was recorded at approximately the 4-leaf stage (GS14) and at stem elongation (GS33, approximately 3 detectable nodes) by measuring 20 consecutive randomly selected plants within the central two rows of each plot. Plant height was measured, in centimeters, from the ground level up to the collar of the tallest fully developed leaf (GS14), or from the ground level up to the tallest detectable node (GS33).

The number of days from sowing until the day when > 50% of the plants in the two central rows of each plot had reached the beginning of ear flowering (GS62) was recorded. This parameter was expressed as DAS.

c. *Grain yield and yield parameters*

Ears were collected by hand at harvest maturity from 4.5 m^2 in the two central rows of each plot to quantify the grain yield and to obtain a representative sample. The harvesting was performed when the grain moisture content was between 23 and 30%, according to the conventional harvesting practice in the growing areas. The collected ears from each plot were counted to record the density per square meter of the fully developed ears. The number of kernel rows and the number of kernels per row were also counted on 7 of these randomly selected and de-husked ears, and the theoretical amount of kernels per square meter (KSM) was then calculated by multiplying the average number of kernels per ear by the number of ears m^{-2} (Blandino et al., 2022).

All the collected ears were shelled using an electric sheller. The kernels from each plot were mixed thoroughly to obtain a random distribution. Grain moisture was analyzed using a GAC2100 Dickey-John grain analyzer (Auburn, IL, USA). The grain yield results were adjusted to a commercial moisture level of 14%. Aliquots of 5 kg were taken and dried at 60°C for 72 hours to reduce the kernel moisture content to 10%. Two hundred dry kernels were randomly collected, considering only whole seeds, and weighed using an electronic balance to assess the thousand kernel weight (TKW).

6.3.3 Statistical analysis

The Kolmogorov–Smirnov normality test and the Levene test were carried out to verify the normal distribution and homogeneity of variances. An analysis of variance (ANOVA) was performed for the growth chamber data (plant height, LA, vegetative indices, shoot and root biomass) and field experiment data (plant height, AUCDC vegetative index, date of flowering, grain moisture, grain yield, and yield parameters), with the maize hybrid, starter fertilization, and biostimulant seed treatment being considered as independent factors. ANOVA was carried out separately in the field experiment for each compared soil and for each year.

Multiple comparison tests were performed, according to the Ryan-Einot-Gabriel-Welsh F (REGW-F) test, on the treatment means. Statistical data analysis was carried out with the SPSS software package, version 27.0.

6.4 Results

6.4.1 Growth chamber experiment

The plant height and LA of the plants, as measured during the first vegetative stages, that is, from GS12 to GS15, are reported in Table 3. The high early vigor hybrid showed a significantly higher plant height than the ordinary one at GS15 (+10%); although a significantly higher LA was already observed at the 2-leaf stage for this genotype and it was maintained in the following growth stages. The starter NP fertilization led to the first significant increase in plant height at GS13 (+30%, compared to unfertilized control), which resulted in an increased advantage at GS15 (+53%), while a significant positive effect of starter fertilization, in terms of LA, was already detected at GS12. The biostimulant seed treatment led to a significantly higher plant height and LA than the untreated control (no biostimulant), from GS13. As far as the NDVI value at GS15 is concerned, the greatest benefits, in terms of plant growth and development, were on average observed for the starter NP fertilization (+60%), followed by the biostimulant seed treatment (+11%), and the use of a high early vigor hybrid (+8%). The interaction between starter fertilization and seed treatment was significant: the biostimulant seed treatment resulted in a significant increase in LA, albeit only when the NP starter fertilizer was applied at both GS13 and GS15 (Figure 1).

All the compared agronomic factors resulted in a significant effect on the vegetative indices related to leaf greenness or to the manifestation of plant stress, which was associated with red-yellow colors at GS14 (Table 4). The maize hybrid and fertilization significantly affected CRI_{700} (red-yellow color). The starter NP fertilization resulted in the strongest effect, as it increased the chlorophyll content (green color) by 42% and reduced CRI_{700} (red-yellow color) by 44%. The high early vigor hybrid showed a lower CRI_{700} value than the control one (-67%), thus highlighting a lower level of stress, although the chlorophyll content was higher in the ordinary hybrid (+5%). A significant interaction was reported between hybrid and fertilization on CRI_{700}: the NP starter fertilization significantly decreased this vegetative index in the ordinary hybrid control, while no significant differences were detected for the high early vigor one (Figure 2). Conversely, the biostimulant seed treatment did not result in a significant effect on the chlorophyll content or on $CRI_{700.}$

All the compared factors influenced NPCI to a great extent: on average, the starter NP fertilization resulted in the highest increase (+77%) of this index, which is related to the capacity of a crop to reduce the stress symptoms associated with P deficiency, and this was followed by the hybrid (+23%), and by the biostimulant seed treatment (+22%).

The NP starter fertiliser and biostimulant seed treatment significantly affected both the shoot and root biomass measured at GS15 (Table 4). The application of the NP starter fertilizer at sowing showed the greatest effect: on average, the dry biomass increased by 4.6 times and 2.2 times for the shoots and roots, respectively. The biostimulant seed treatment significantly enhanced maize biomass production, that is, by 21% (shoots) and by 20% (roots), compared to the untreated control (no biostimulant). The interaction between the starter fertilization and the seed treatment was significant for the shoot biomass: the application of a seed biostimulant significantly improved the shoot biomass (+24%), albeit only under the NP fertilized conditions (data not shown).

Table 3. Effects of the hybrid, starter fertilization, and biostimulant seed treatment on the early vigor of maize, expressed as plant height and leaf area (LA) at the two- (GS12), three- (GS13) and five-leaf (GS15) emission stages, and the Normalized Difference Vegetation Index (NDVI) value, in the growth chamber experiment.

Factor	**Source of variation**	**Plant height (cm)**			**LA (cm^2)**			**NDVI**
		GS12	**GS13**	**GS15**	**GS12**	**GS13**	**GS15**	**GS15**
Hybrid (H)	Ordinary	5.1 a	7.9	11.8 b	26.3 b	68.9 b	164.8	0.250 b
	High early vigor	4.4 b	8.3	13.0 a	29.2 a	76.4 a	181.1	0.271 a
	p-value	0.024	0.530	0.006	0.006	0.029	0.053	0.013
Fertilization (F)	Unfertilized	4.5	7.1 b	9.8 b	27.0 b	57.5 b	93.3 b	0.200 b
	NP	5.0	9.2 a	15.0 a	28.7 a	89.6 a	254.1 a	0.320 a
	p-value	0.078	<0.001	<0.001	0.031	<0.001	<0.001	<0.001
Seed treatment (S)	No biostimulant	4.7	7.9 b	12.1 b	27.5	68.0 b	160.9 b	0.247 b
	Biostimulant	4.8	8.4 a	12.8 a	28.2	78.2 a	186.5 a	0.274 a
	p-value	0.586	0.004	0.002	0.174	<0.001	<0.001	0.006
H x F	*p*-value	0.951	0.073	0.085	0.815	0.612	0.524	0.053
H x S	*p*-value	0.166	0.218	0.574	0.869	0.673	0.406	0.910
F x S	*p*-value	0.416	0.009	0.106	0.112	<0.001	<0.001	0.460
H x F x S	*p*-value	1.000	0.405	0.661	0.398	0.688	0.672	0.392

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 12 observations.

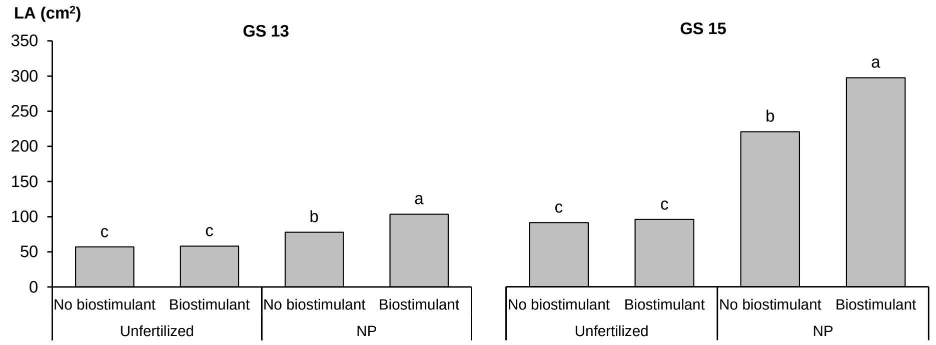


Figure 1. Effects of the starter fertilization and the biostimulant seed treatment on the maize leaf area (LA) at the three- (GS13) and five-leaf (GS15) emission stages in the growth chamber experiment. Bars with different letters are significantly different (p-value < 0.05), according to the REGW-F test. The reported values are based on 6 observations.

Table 4. Effects of the hybrid, starter fertilization, and biostimulant seed treatment on the carotenoid reflectance index (CRI_{700}), on the chlorophyll content measured by means of the SPAD device, and on the Normalized Phosphorous Content Index (NPCI) of the leaves at the 4-leaf emission stage (GS14), and on the shoot and root maize biomass at the 5-leaf stage (GS15) in the growth chamber experiment.

Factor	**Source of variation**	**CRI_{700}**	**Chlorophyll (SPAD unit)**	**NPCI**	**Biomass (g plant^{-1} d.w.)**	
					Shoot	**Root**
Hybrid (H)	Ordinary	0.265 a	40.1 a	0.144 b	1.1	1.0
	High early vigor	0.087 b	38.1 b	0.177 a	1.2	1.1
	p-value	<0.001	<0.001	0.049	0.126	0.117
Fertilization (F)	Unfertilized	0.225 a	32.3 b	0.118 b	0.4 b	0.6 b
	NP	0.126 b	45.9 a	0.209 a	1.8 a	1.4 a
	p-value	0.001	<0.001	<0.001	<0.001	<0.001
Seed treatment (S)	No biostimulant	0.195	39.2	0.146 b	1.0 b	0.9 b
	Biostimulant	0.156	39.0	0.178 a	1.2 a	1.1 a
	p-value	0.127	0.632	0.019	<0.001	0.014
H x F	*p*-value	0.008	0.736	0.083	0.592	0.842
H x S	*p*-value	0.257	0.326	0.486	0.186	0.260
F x S	*p*-value	0.440	0.234	0.420	0.002	0.056
H x F x S	*p*-value	0.404	0.988	0.686	0.218	0.231

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 12 observations.

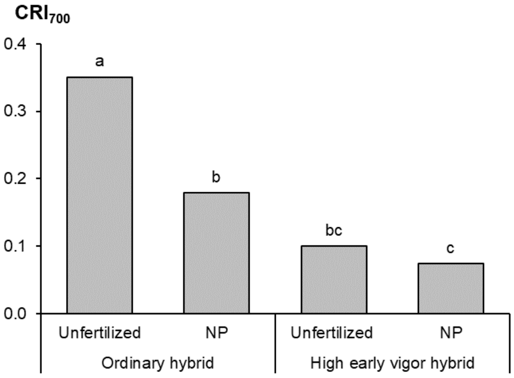


Figure 2. Effects of the maize hybrid and the starter fertilization on the carotenoid reflectance index (CRI_{700}) at the four-leaf emission stage (GS14) in the growth chamber experiment. Bars with different letters are significantly different (p-value < 0.05), according to the REGW-F test. The reported values are based on 6 observations.

6.4.2 Field experiments

a. Meteorological trends

The meteorological trends observed in each site and in each year are shown in Table 2. The two growing seasons showed different weather conditions throughout the maize crop cycle, in particular as far as the rainfall is concerned: the precipitations were more frequent in 2019 than in 2018, above all during the ripening stages (July and August). Carmagnola had a larger amount of rainfall and more air GDDs than Poirino in both years. The soil temperatures, measured from sowing to the 6-leaf stage (GS16), were higher in 2018 than 2019, as a consequence of the later sowing time in both sites.

b. Early vigor

The NDVI development of the compared treatments, from GS13 to GS55, is reported in Figure 3; low values are related to a lower plant biomass and/or greenness status of the maize canopy. Maize growth was faster for the NP starter fertilization than for the unfertilized control in each site and for each year, but a positive effect of the biostimulant seed treatment on plant growth was observed in both sites in 2018 as well as in Carmagnola in 2019. Moreover, these differences were influenced by the maize hybrid: the development of the high early vigor maize was faster than that of the ordinary maize, and the effect of the seed biostimulant and NP fertilization treatments on NDVI development was therefore generally more noticeable for the ordinary hybrid. A positive synergistic effect on plant development between the starter fertilization at sowing and the biostimulant seed treatment was reported for the control hybrid maize cultivated in Poirino in 2018 and in Carmagnola in 2019.

All the NDVI measurements registered during the vegetative growth have been summarized in the AUCDC index (Table 5). The NP starter fertilization at sowing resulted in a significant (+13%) increase in AUCDC in both sites and in both years. Both the hybrid and seed biostimulant showed a significant effect on this vegetative index in Poirino in 2018, and at both sites in 2019. The average increase in AUCDC of the high early hybrid, compared to the ordinary one, was 9% in these environments, while the biostimulant application had a 6% greater value than the control (no biostimulant). The interaction between the maize hybrid and seed biostimulant was significant in Carmagnola in 2019: the biostimulant seed application led to a significantly higher development of the plants, albeit only in the ordinary control hybrid (data not shown).

The plant development results, which are summarized by the AUCDC index, were confirmed by considering the height of the plants measured at GS14 and GS33. The high early vigor hybrid significantly increased (+30%) the plant height at the leaf emission stage in all the environments, compared to the ordinary maize, with the exception of the experiment carried out in 2018 in Carmagnola. No significant differences between hybrids were observed at the stem elongation stage. The NP starter fertilization had a significant effect on plant vigor in all the experiments and at both growth stages. On average, the plant height increased by 43% and 90%, compared to the unfertilized control, at GS14 and GS33, respectively. The biostimulant seed treatment only increased the plant height significantly (+22%) at GS33, although no effect was reported in the experiment carried out in 2019 in Poirino. The interactions between factors were never significant for the plant height parameter at GS14. Conversely, a significant starter fertilization × seed biostimulant interaction was reported in Carmagnola in 2018 at GS33, where the biostimulant seed treatment led to a significantly higher development of the plants, albeit only in the unfertilized control (data not shown).

Crop development differences were also detected between the compared factors in the later growth stages, at flowering and at harvest (Table 6). The hybrid and the NP starter fertilization significantly affected the flowering date in all the experiments. The use of the NP fertilization at sowing and the high early vigor hybrid, instead of the ordinary one, led to an earlier flowering date, that is, of 2.1 and 2.8 days, respectively. Furthermore, the NP fertilization showed a more consistent effect on the grain moisture content at harvest: the NP starter fertilizer significantly reduced the moisture content, compared to the unfertilized control, on average by 1.1 percentage points, in all the experiments. The hybrid with a high early vigor was instead harvested with a significantly lower moisture content than the ordinary one in the experiment carried out in Poirino in both years. A significant anticipation of the flowering date (-0.8 days) was observed for the biostimulant seed treatment, albeit only in the experiment carried out in Poirino in 2018, while the seed treatment did not affect the grain moisture at harvest in any experiment.

A significant interaction between hybrid and seed treatment was reported for the flowering date in 2018 in Carmagnola: the biostimulant only led to an anticipated flowering in the high early vigor hybrid (data not shown). The interaction between the fertilization and the seed treatment was significant for the grain moisture at harvest in both sites in 2018. The application of a seed biostimulant decreased the grain moisture by 1 percentage point in the unfertilized control in Carmagnola and by 2 percentage points in Poirino, where the NP starter fertilizer was applied, respectively (data not shown).

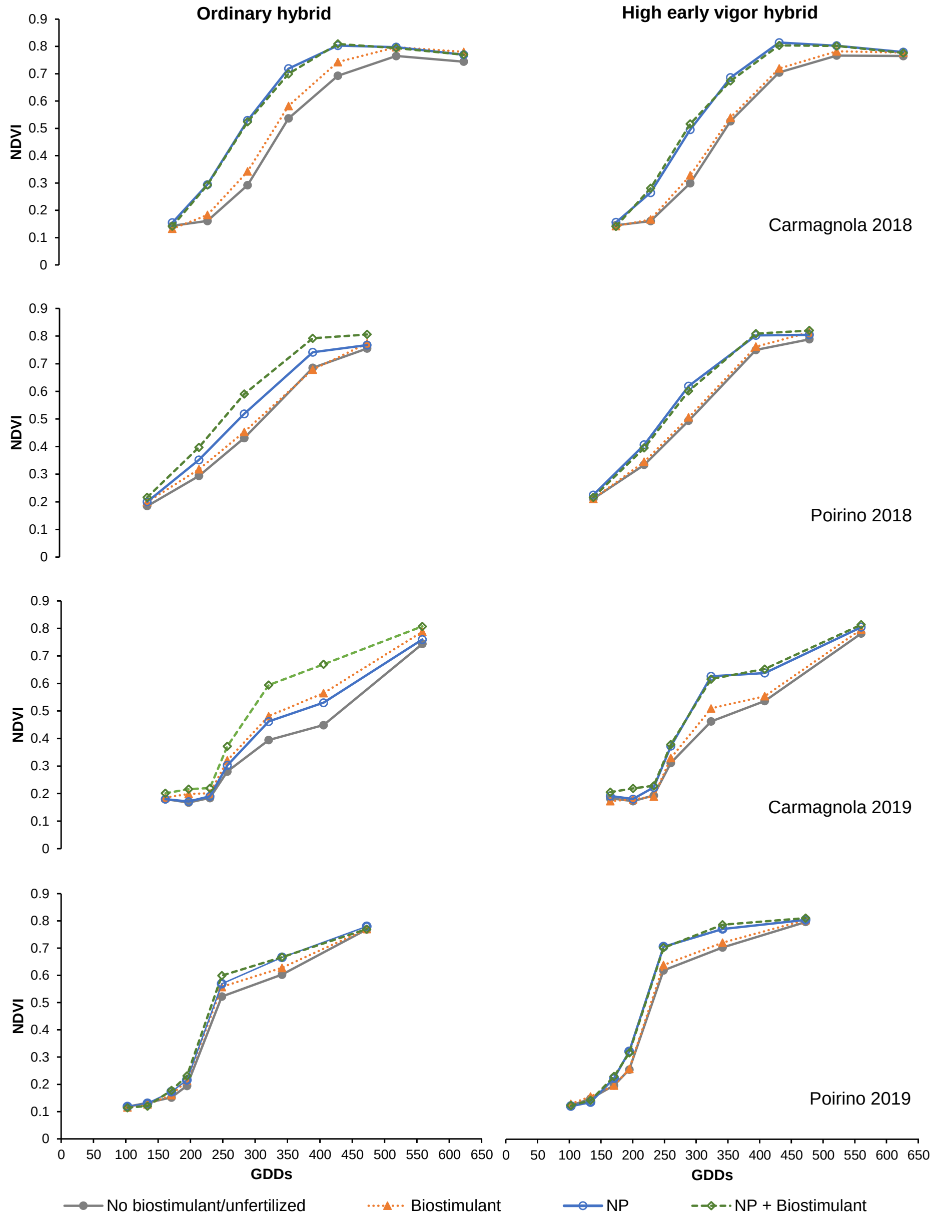


Figure 3. Effects of the hybrid, starter fertilization, and biostimulant seed treatment on the normalized difference vegetation index (NDVI) measured from the 3-leaf stage (GS13) until tassel emission (GS55) in each site (Carmagnola and Poirino) and each year (2018 and 2019). Colors indicate the factorial treatments: no biostimulant/unfertilized (gray), biostimulant (orange), NP starter fertilization (blue) and NP + biostimulant (green). The reported data are based on 4 observations. Growing degree days (GDDs): accumulated air growing degree days measured on a 10°C basis.

Table 5. Effects of the hybrid, starter fertilization, and biostimulant seed treatment on the early vigor of maize, expressed as the area under the canopy development curve (AUCDC) and the plant height at the leaf emission (GS14) and stem elongation (GS33) stages for the Carmagnola and Poirino field experiments in the 2018 and 2019 growing seasons.

Factor	**Source of variation**	**AUCDC (Σ NDVI-day)**				**Plant height GS14 (cm)**				**Plant height GS33 (cm)**			
		2018		**2019**		**2018**		**2019**		**2018**		**2019**	
		Carmagnola	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**
Hybrid (H)	Ordinary	23.6	20.3 b	19.9 b	19.9 b	15.9	17.5 b	6.9 b	11.3 b	70.9	55.6	23.4	49.1
	High early vigor	23.0	21.6 a	21.2 a	23.0 a	16.0	20.3 a	8.8 a	16.5 a	62.7	63.5	25.4	53.5
	p-value	0.212	<0.001	0.001	<0.001	0.417	<0.001	<0.001	<0.001	0.223	0.106	0.096	0.126
Fertilization (F)	Unfertilized	21.0 b	19.9 b	19.1 b	20.6 b	12.3 b	16.0 b	6.4 b	12.2 b	45.6 b	42.6 b	14.0 b	41.1 b
	NP	25.4 a	21.8 a	21.7 a	22.1 a	19.9 a	21.3 a	9.3 a	15.9 a	85.5 a	76.0 a	34.0 a	61.5 a
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Seed treatment (S)	No biostimulant	22.9	20.5 b	19.4 b	21.1 b	15.7	18.5	7.4	13.7	62.0 b	54.1 b	21.1 b	50.3
	Biostimulant	23.7	21.3 a	21.6 a	21.6 a	16.3	19.0	8.2	14.4	72.2 a	63.7 a	28.1 a	52.4
	p-value	0.084	0.034	<0.001	0.034	0.494	0.195	0.076	0.215	0.018	0.032	0.015	0.618
H x F	*p*-value	0.688	0.473	0.241	0.065	0.489	0.102	0.091	0.215	0.639	0.152	0.482	0.006
H x S	*p*-value	0.933	0.128	0.001	0.754	0.763	0.775	0.186	0.863	0.395	0.670	0.292	0.805
F x S	*p*-value	0.053	0.632	0.549	0.277	0.080	0.660	0.972	0.326	0.033	0.563	0.164	0.923
H x F X S	*p*-value	0.843	0.142	0.369	0.777	0.302	0.444	0.983	0.977	0.349	0.993	0.181	0.657

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 16 observations.

Table 6. Effects of the hybrid, starter fertilization, and biostimulant seed treatment on the early vigor of maize, expressed as the date of flowering (Days after sowing, DAS) and grain moisture at harvest for the Carmagnola and Poirino field experiments in the 2018 and 2019 growing seasons.

Factor	**Source of variation**	**Date of flowering (DAS)**				**Grain moisture (%)**			
		2018		**2019**		**2018**		**2019**	
		Carmagnola	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**
Hybrid (H)	Ordinary	75.9 a	75.1 a	105.7 a	108.2 a	22.7	24.3 a	25.2	28.7 a
	High early vigor	74.3 b	72.7 b	101.8 b	104.9 b	23.2	23.1 b	25.2	26.3 b
	p-value	<0.001	<0.001	<0.001	<0.001	0.334	0.008	0.934	<0.001
Fertilization (F)	Unfertilized	76.4 a	74.8 a	104.7 a	107.6 a	23.5 a	24.3 a	25.9 a	28.0 a
	NP	73.8 b	72.9 b	102.9 b	105.4 b	22.4 b	23.2 b	24.6 b	26.9 b
	p-value	<0.001	<0.001	<0.001	<0.001	0.029	0.017	0.023	0.004
Seed treatment (S)	No biostimulant	75.3	74.3 a	103.9	106.4	23.1	24.1	25.4	27.4
	Biostimulant	74.9	73.4 b	103.7	106.6	22.8	23.4	25.0	27.5
	p-value	0.237	0.003	0.586	0.948	0.479	0.103	0.500	0.883
H x F	*p*-value	0.039	0.028	0.586	0.473	0.485	0.783	0.898	0.394
H x S	*p*-value	0.039	1.000	0.459	0.948	0.654	0.454	0.744	0.336
F x S	*p*-value	0.237	0.643	0.303	0.743	0.011	0.045	0.631	0.458
H x F X S	*p*-value	0.810	1.000	0.520	0.647	0.840	0.472	0.485	0.542

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 16 observations.

c. *Grain yield and yield components*

The NP fertilization at planting significantly increased the grain yield in all the experiments, although a significant effect of hybrid and the seed treatment was only reported for Poirino in 2018 and Carmagnola in 2019 (Table 7). On average, the starter NP fertilization increased the grain yield, compared to the unfertilized control, by 6%, while the average yield enhancement values obtained for adopting a high early vigor hybrid, instead of the ordinary one, and for the application of seed biostimulant, instead of no biostimulant control, were 8.5% and 5.1%, respectively. The grain yield increase was mainly related to an enhancement of KSM for all the factors, while the hybrid and NP fertilization significantly affected the TKW, albeit only in Poirino in 2018. In this experiment, the higher KSM that was observed for the high early vigor hybrid and NP starter fertilization led to a significantly, but less proportional, lower TKW, although a significant yield increase was maintained. The interaction between the involved agronomic factors for grain yield and yield components was only significant under a few of the considered conditions. The NP starter fertilization led to a higher grain yield in the high early vigor hybrid in Poirino in 2018 and in Carmagnola in 2019.

Table 7. Effects of the hybrid, starter fertilization, and biostimulant seed treatment on the maize grain yield and the yield component, kernels per square meter (KSM), and thousand kernel weight (TKW) for the Carmagnola and Poirino field experiments in the 2018 and 2019 growing seasons.

Factor	**Source of variation**	**Grain yield (t ha^{-1})**				**KSM (n° m^2)**				**TKW (g)**			
		2018		**2019**		**2018**		**2019**		**2018**		**2019**	
		Carmagnola	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**	**Carmagnola**	**Poirino**
Hybrid (H)	Ordinary	17.1	16.1 b	14.9 b	15.0	4850	4902 b	4190 b	4301	401	418 a	369	388
	High early vigor	17.0	17.2 a	16.4 a	15.6	4764	5205 a	4796 a	4321	393	400 b	367	384
	p-value	0.941	0.001	<0.001	0.101	0.361	0.036	<0.001	0.798	0.111	<0.001	0.674	0.514
Fertilization (F)	Unfertilized	16.6 b	16.2 b	15.2 b	14.9 b	4425 b	4858 b	4163 b	4164 b	396	412 a	367	386
	NP	17.7 a	17.1 a	16.1 a	15.7 a	5287 a	5248 a	4823 a	4471 a	398	407 b	370	385
	p-value	<0.006	0.004	0.021	0.032	<0.001	0.011	<0.001	0.028	0.637	0.019	0.530	0.865
Seed treatment (S)	No biostimulant	16.8	16.3 b	15.2 b	15.1	4847	4902 b	4392 b	4281	401	410	372	386
	Biostimulant	17.4	17.0 a	16.1 a	15.5	4766	5205 a	4627 a	4341	393	409	365	386
	p-value	0.312	0.004	0.005	0.344	0.221	0.036	0.031	0.543	0.142	0.628	0.220	0.952
H x F	*p*-value	0.080	0.003	0.018	0.211	0.852	0.409	0.052	0.078	0.328	0.123	0.573	0.419
H x S	*p*-value	0.752	0.124	0.124	0.829	0.023	0.572	0.224	0.402	0.466	0.021	0.155	0.358
F x S	*p*-value	0.067	0.239	0.631	0.972	0.025	0.476	0.475	0.507	0.988	0.004	0.835	0.784
H x F X S	*p*-value	0.357	0.906	0.026	0.664	0.901	0.748	0.139	0.942	0.970	0.195	0.058	0.771

Means followed by different letters are significantly different. The level of significance (*p*-value) is shown in the Table. The data reported for each factor are based on 16 observations.

6.5 Discussion

This study, which pertains to part II of the work, provides useful data that can be used to compare the agronomic and grain yield benefits of different crop practices that are able to enhance the early plant vigor of maize, which is considered, in temperate growing areas, a key stage to maximize the production and thus increase the overall sustainability of maize cropping systems. The effects of the maize genotype, the NP starter fertilizer, and the biostimulant seed treatment on the early stages of maize development, and the consequent effect on the length of the crop cycle, as well as on the grain yield and quality, were studied in depth in both a growth chamber experiment and open field experiments, considering the factorial combination of the compared agronomic factors in different locations and different growing seasons.

First, this experiment has allowed a direct comparison to be made of the efficacy of the tested crop practices in enhancing plant vigor. The starter fertilization, which involved the distribution of N and P in the furrows at sowing, led to the greatest and steadiest effect, in terms of plant development and final grain yield, in both the growth chamber experiment and the open field experiment. The effect on plant vigor of the starter fertilization, or the use of a vegetative index, such as NDVI, from the 3-leaf growth stage, was evident across the growth stage assessments in both the field experiments. In agreement with Ma et al., (2013), the positioning of N and P close to plants led to the early root growth, and hence, the nutrient uptake being increased, which in turn influenced the plant morphology and physiology by creating a larger leaf area. This resulted in a greater radiation interception throughout the entire maize growing cycle. Such an increased leaf area was closely correlated with a high rate of photosynthesis and a high chlorophyll content (Rostami et al., 2008), as suggested by the SPAD and NPCI values. A starter fertilization is primarily aimed at satisfying the crop P uptake. Moreover, this experiment confirms that the effect of P distribution at sowing also enhances the early vigor of maize in soils (see, for example, the Poirino site), where this macronutrient is present in large quantities, thus confirming that the cold meteorological conditions of an early sowing could limit its absorbance, even in agronomic conditions where this element is abundant (Roth et al., 2006). Furthermore, N and P subsurface banded together showed a positive and synergistic interaction on nutrient uptake and plant growth, in comparison with N (Blandino et al., 2022) or P (Jing et al., 2010) on their own. A better early growth and early establishment allowed the flowering of maize to be anticipated, thereby reducing the interval between sowing and silking, making the interception of the solar radiation within the growing season more efficient and increasing the final grain yield (Tsimba et al., 2013). The starter fertilization always significantly impacted the duration of the crop cycle in both experimental fields, since it shortened the time from sowing to flowering, and it decreased the grain moisture of the maize at harvest (Kaiser et al., 2016). Thus, the possible advantages for the maize cropping system are not only linked to a lower drying cost, but also to the possibility of harvesting in advance, thereby reducing the mycotoxin contamination risk

(Blandino et al., 2009), or of using a later hybrid with the same growth stage duration, in order to enhance the grain yield potential (Tsimba et al., 2013).

Overall, a starter fertilization is expected to stimulate a faster root system establishment, which could lead to a more effective uptake of all the nutrients by the crop. In addition to the direct effect on plant growth, the first part of the present study (Ujvári et al., 2023) highlighted that the application of an NP fertilizer could modify the rhizosphere bacterial community to a great extent, and thus positively increase several richness and diversity indices. Moreover, the rise in the availability of mineral nutrients and the higher production of root exudates, as a consequence of a higher plant development (Zhu et al., 2016), showed an increase in the occurrence of specific genera (e.g., *Stenotrophomonas* and *Lysobacter*) which are P solubilizers (Ghosh et al., 2020; Dai et al., 2023), thus leading to a further improvement in the nutrient use efficiency.

The high early vigor hybrid showed a more rapid crop establishment in the earliest vegetative growth stages, and this resulted in a significant anticipation of the crop cycle and an increase in the grain yield, compared to the ordinary one, in half of the considered production situations. Maize breeding has produced an increase in tolerance to low temperatures in modern hybrids, in order to reduce the physiological impacts of cold temperatures, which could limit the uptake of nutrients by the root system and plant photosynthesis (Haldimann 1999; Gillani et al., 2021). The data collected in the growth chamber experiment, carried out at a low temperature, which is typical of an early sowing time, highlighted that the ordinary hybrid showed more nutritional stress symptoms, with more yellow/orange leaves, than the early vigor one. These symptoms are related to a higher occurrence of carotenoid (Li et al., 2008) and anthocyanin (Pietrini et al., 2002) compounds in the leaves, which play a precursor signalling role of plant stress defense. The high early vigor hybrid showed significantly higher LA values than the ordinary one, already from the 2-leaf stage. An early larger leaf area allows plants to capture sunlight more effectively during early canopy development (Wijewardana et al., 2016), thereby increasing the photosynthesis rate, which could help support a further, more rapid plant growth. In addition to the effect of epigeal development, the root system might also be affected by a more rapid growth, with a more extended root volume and a higher root exudation of organic acids being able to increase the nutrient availability and uptake (Hund et al., 2008). Furthermore, the root traits, in particular the production of root exudates, could influence the microbial rhizosphere community, as reported in part I of the present study (Ujvári et al., 2023), in which a clear difference in the composition of the microbiota was reported between the two maize hybrids. Although the richness of the bacterial species was slightly higher in the ordinary hybrid at plant emergence, both the richness and the Hill2 biodiversity indices were much higher in the high vigor hybrid at the 5-leaf stage.

The biostimulant seed treatment showed a significant effect on several plant growth indices in both the growth chamber and the field experiments. Furthermore, this treatment was less effective in reducing the interval between sowing to flowering and in lowering the moisture content of the grain at harvest, although a significant increase in grain yield was detected in 2 of the 4 compared production situations. As already reported in the literature (Mickan et al., 2021), the biostimulant effect of adding microbial inoculants may vary, according to the environmental and agronomic conditions. Several studies have underlined that biostimulants can play a role under conditions of great crop stress (Schütz et al., 2018; Li et al., 2022). In our field trials, the biostimulant seed treatment was more effective in enhancing plant growth and grain yield in the experiments carried out with the coldest soil temperatures from sowing to the 6-leaf stage (e.g. Poirino 2018 and Carmagnola 2019). Although beneficial microorganisms have been used largely as inoculants for crops, their agronomical benefits have generally been reported in marginal environments, with a low yield potential (Li et al., 2022), while their contribution to intensive cropping systems has not yet been clarified. Instead, in this study, the agronomic advantages of a biostimulant, based on a PGPB strain, has been reported in production situations with high soil fertility and a high yield potential, but focusing on a specific critical factor that could limit plant productivity.

A *B. amyloliquefaciens* seed application response in wheat (Donn et al., 2015; Wolińska et al., 2020) has also been reported to be influenced to a great extent by its interaction with other crop practices, such as the genotype and fertilization. In the present study, the best advantage of the seed biostimulant, in terms of NDVI value, was reported for the ordinary hybrid, which is more prone to environmental and nutritional stress. Although the DGGE analysis did not detect an increase in the occurrence of this species in the maize rhizosphere (see Part I), a contribution of this PGPB, especially in the first growth stage, should not be excluded. *B. amyloliquefaciens* is one of the most efficient bacteria, and it is able to solubilize organic and inorganic P (Nkebiwe et al., 2017; Mpanga et al., 2020), and to release auxin and ACC deaminase (Borriss 2015). Moreover, in part I of the work, the biostimulant seed treatment affected the composition of the rhizosphere bacterial community of the two hybrids in different ways, and showed a greater effect on the ordinary one. The application of the biostimulant led to a more abundant occurrence of the *Paenibacillus* and *Stenotrophomonas* species, which are well known for their wide range of plant growth promoting properties (N_2 fixation, P solubilization, as well as plant hormone and siderophore production). Hui et al., (2018) reported that the application of *B. amyloliquefaciens* to soil led to an initial increase in the concentration of nitrate, which affected the soil microbial community composition. *B. amyloliquefaciens* seed inoculation has in particular been shown to promote the abundance of the rhizosphere microorganisms involved in the soil nutrient cycles (Luo et al., 2022). *B. amyloliquefaciens* has also been shown to be able to directly influence the plant roots of *Arabidopsis thaliana*, changing their structure by increasing lateral outgrowth and elongation, and root-hair formation, thus promoting plant growth (Asari et al., 2017).

In addition to the close relationship between the maize hybrids and the biostimulant seed treatment, a synergistic effect between the biostimulant and the NP starter fertilizer was observed in both experiments. The biostimulant, in combination with the NP fertilizer, determined a faster growth rate for each maize hybrid, especially the ordinary one. Since it was applied to the seed, the biostimulant may have produced an early growth-promoting effect on the seedling during germination, thus allowing the roots to reach the fertilizer localized in the seed furrow more quickly and to anticipate its benefits. Moreover, the effect on the microbiota composition, that is, an increase in PGPB that was able to solubilize nutrients, may have improved the maize plants' use of the starter fertilizer, which resulted in greater availability of such macronutrients (Xue et al., 2021). Thus, the role of biostimulants in cropping systems should not only be considered as an eco-friendly alternative to applying fertilizers, but also as a solution to improve the effectiveness of fertilizing practices, especially when using a high-efficiency strategy, such as its application to seed furrows. However, this synergistic effect requires further studies to evaluate how to maintain the same effectiveness on the early vigor of maize but limiting the quantity of applied N and P, especially to soils with high P availability, where the need of an additional application is questionable (Schröder et al., 2015).

In addition to the single effect of each treatment, the full factorial combination of the considered agronomic factors has allowed a direct comparison to be made of their importance and of the benefits of their combination on enhancing the initial plant growth across the whole cropping system. The influence of the three agronomic factors (hybrid, starter fertilization, and seed treatment) and their interaction on the NDVI value detected at the 5-leaf stage in the growth chamber and on AUCDC (sum of the NDVI value detected for the 3-leaf stage at tassel emission) in the field experiments were evaluated by means of three-way ANOVA (Figure 4). In both experiments, the starter fertilization accounted for the highest percentage of variation, in particular in the growth chamber experiment where maize growth was limited to the 5-leaf stage. The use of hybrids with different early vigor explained the lower amount of variation in the growth chamber experiment, while it accounted for 29% of the total variation in the field trials. The biostimulant seed treatment accounted for 8-9% of the total variation in both experiments. Overall, the interaction between the involved agronomic factors explained less than 6% of the total variation in the early growth of the plants.

Furthermore, the collected data highlighted some significant interactions among these practices, and it was considered interesting to address the overall benefits of their different combined application within the maize cropping system. With this aim, the advantages, in terms of plant vigor, the shortening of the sowing – flowering interval, and grain yield, are summarized in Figure 5, taking into account some of the possible cropping systems and comparing them with a control situation (an ordinary maize hybrid with no seed biostimulant or starter NP fertilizer): I) an early vigor hybrid; II) an early vigor hybrid plus the biostimulant seed treatment; and III) the combination of the above mentioned two strategies together with NP localized fertilization at sowing. Compared to the use of a high early vigor hybrid, its combination with the biostimulant seed treatment led to a significant further enhancement of plant growth and a reduction of the sowing-flowering period. The combination of all 3 factors resulted in the maximum benefits, compared to the control cropping system, with a 124% increase in the plant growth, a 5-day reduction of the sowing-flowering period and a 14% gain in grain yield. The complexity of the interaction among the crop practices, including the genotype, and with the pedo-climatic conditions, but also the close relationship of the crop management practices with the rhizosphere microbiota, as highlighted in the present study, underline the need for further research to define the most suitable cropping systems to maximize the profitability and sustainability of maize cultivation in different production situations (Busby et al., 2017).

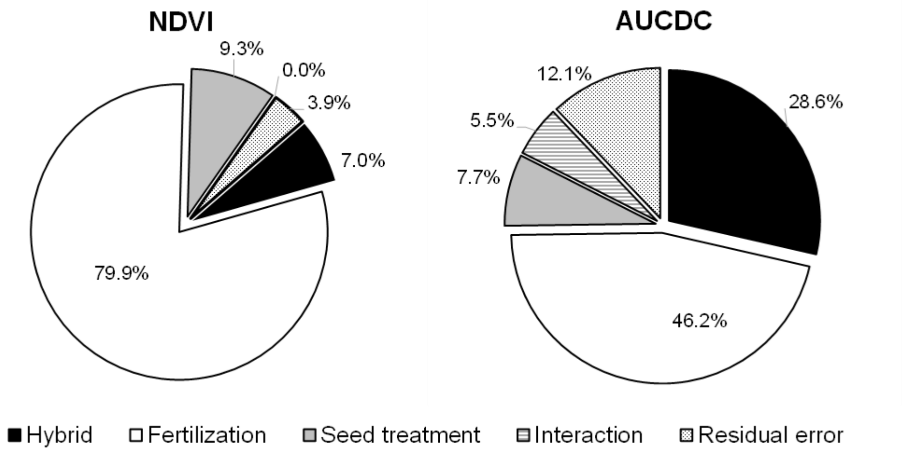


Figure 4. Variance components of the NDVI index at the 5-leaf stage (growth chamber experiment) and of the AUCDC index (field experiments). The variance components were calculated as the ratio of the variance of each agronomic factor to their interaction on the total variance of ANOVA. The data on AUCDC refer to ANOVA, as applied to all 4 field experiments.

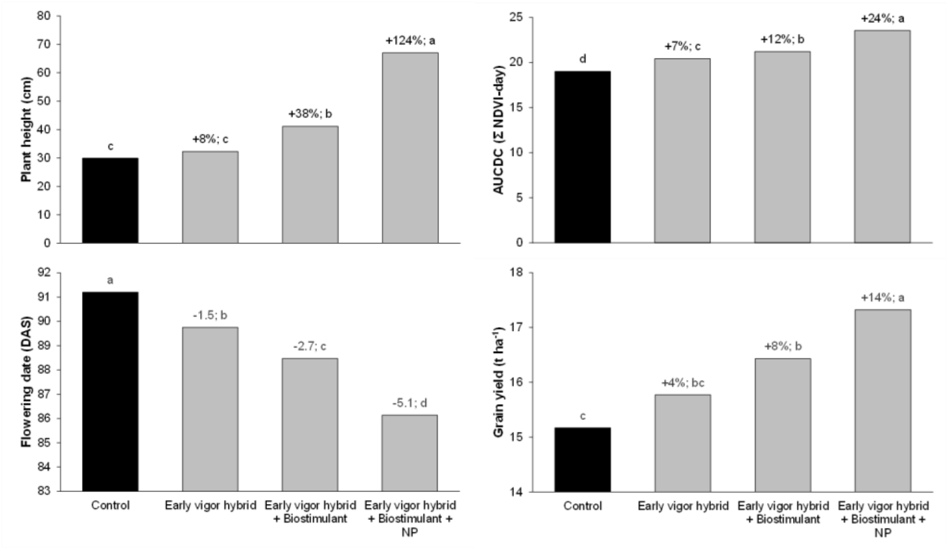


Figure 5. Effect of the hybrid, starter fertilization, and biostimulant seed treatment on the high early vigor of maize, expressed as plant height at the stem elongation stage (GS33), the area under the canopy development curve (AUCDC), the flowering date, and the grain yield. Bars with different letters are significantly different (p-value < 0.05), according to the REGW-F test. The reported values are based on 16 observations.

Control: ordinary hybrid (LG30600) with no biostimulant or NP starter fertilizer.
Early vigor hybrid: LG31630 with no biostimulant or NP starter fertilizer.
Early vigor hybrid + biostimulant: LG31630 with a biostimulant seed treatment (mixture of a bacterium, Bacillus amyloliquefaciens IT-45 strain (Rise P®), and a leguminous plant extract Cyamopsis psoraloides (AgRho® GSB30).
Early vigor hybrid + biostimulant + NP: LG31630 with a biostimulant seed treatment and the distribution of 27 kg N ha^{-1} and 69 kg P_2O_5 ha^{-1} as diammonium phosphate at sowing.

6.6 Conclusions

In the context of the development of future cereal cropping system, considering the introduction of environmentally-friendly innovations in a sustainable intensification approach, this study has highlighted the importance of innovating the crop practices characterized by a high input use efficiency. As far as the maize cropping system is concerned, the application of agronomic techniques that are able to promote plant vigor in the early vegetative stages is a key factor that leads to significant and sustainable yield advantages. Here, the starter fertilization, with the application of N and P close to seed furrows, had the greatest effect, thus suggesting that simply reducing fertilizer inputs may represent a significant drawback for high N- and P-requiring crops, such as cereals, and that it is necessary to re-design the fertilization strategies by above all enhancing the input use efficiency, focusing on the most critical growth stages for nutrient uptake.

As far as alternative practices are concerned, the use of innovative genotypes, with high early vigor, or biostimulant seed applications, are crop techniques that can attenuate the abiotic stress factor of an early sowing, thus leading to a prompter crop development. These innovations can therefore represent a solution that can be used to reduce or, in certain cases, replace a starter fertilization. Furthermore, the study has highlighted a positive additive effect of these practices, that is, of further increasing the initial plant vigor and the associated agronomic advantages. The choice of the most suitable crop practices should consider the diversity of each cropping system, according to the pedo-climatic conditions, the agronomic background, the yield potential, and the requirements of the supply chain. Moreover, in a more holistic view, the present study (see part I) has highlighted that the crop practices evaluated in our work positively influenced the rhizosphere microbiota composition, thereby playing a clear role in the management of microbial soil fertility and leading to a possible further contribution, that is, favoring the early development of maize. These findings indicate the need to study the interactions between crop practices and plant microbiota in more depth, in order to better understand their potential microbial role in extending specific plant functions, in sustainably enhancing the associated agronomic benefits, and in increasing the plasticity and resilience of plants to fluctuating environmental conditions.

6.7 Supplementary material

Table S1. The main agronomic information pertaining to the maize growing cycle in the growth chamber experiment.

Medium temperature of the air	Day	16.7	C°
	Night	14.1	C°
Air GDDs [1]	Sowing - 6 leaves	273	C°-day
Medium temperature of the soil	Day	15.6	C°
	Night	14.2	C°
Soil GDDs	Sowing - 6 leaves	237	C°-day
Air Humidity		50	%
Day/night		12	h
Light intensity		700-1000	PAR [2]
Water irrigation		10 mm every 7 day	

[1] GDDs: accumulated growing degree days on a 10°C basis.

[2] PAR: photosynthetically active radiation: µmol m^{-2} s^{-1}

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7. CHAPTER VI

7.1 Conclusions and perspectives

Among the biggest challenges that humanity is now facing there is the stabilization and the improvement of production despite numerous difficulties related to the climate change, overbuilding, environment pollution and wars; at the same time, the food demand of the growing global population requires increases in production in a more sustainable way. Therefore, it is necessary to produce more and better (high quality) but with less land, water, manpower and inputs, such as pesticides and synthetic fertilizers (Umesha et al., 2018). Field crops, especially wheat and maize that are the world's leading staple cereals, are generally low value crops, compared with industrial and vegetable crops, and consequently input costs often play a decisive role in farmers' cost of production (Lamichhane et al., 2020). One possible strategy is represented by the seed treatment technology where agriculturists, for centuries, used this simple technology to treat seeds for their protection from pathogens and pests. Today, the seed treatment is a highly sophisticated strategy that has evolved into a very valuable, effective, and environmentally friendly component of agricultural production practices (Munkvold et al., 2014).

The research activity carried out in this PhD thesis was aimed to improve the knowledge about the benefits of innovative seed treatments in cereal crops cultivated in North-West Italy area under high yield production situation. To this purpose, five different studies were set up to analyze deeply the role and the benefits in terms of fungal disease protection, early development and grain yield that can be achieved with this widespread agronomic practice taking into account fungicide, micronutrient and biostimulant active ingredients.

The chemical control via soil/foliar application has its limitation such as high cost, selectivity, affect non target organisms, development of pest resistance, resurgence of pests, pollution of food and feed, health hazards, toxicity towards plants and animals, environmental pollution, etc (Sharma et al., 2015). Fungicide seed treatments played and are still playing a pivotal role in sustainable crop production as it was observed on wheat and maize in **Chapter II** and **III** respectively, especially thanks to the availability on the market of fungicide seed treatments characterized by a marked systemic activity.

Among the most dangerous fungal diseases of wheat, in temperate growing area, the *Septoria* leaf blotch complex (SLB) and the *Fusarium* head blight (FHB) contribute significantly to reduce wheat yield and quality (mycotoxins accumulation) by means the attack and infection of leaves and spikelets, respectively. The agronomic practice adopted for the prevention of fungal diseases focuses on minimizing the pathogen inocula using crop rotation, soil tillage to incorporate previous crop debris and the choice of tolerant varieties. Although leaves can be infected by SLB throughout the whole wheat cycle and the FHB is able to colonize and infect wheat spikelets at flowering, a double fungicide application at the flag leaf emission and at the beginning of flowering is a crop protection strategy frequently adopted by farmers in North-Italy temperate environments leading to the highest level of protection of the canopy and the ears. The data collected in the study reported in Chapter II highlighted how the application of a seed treatment with a systemic fungicide to winter wheat could change the overall foliar fungicide programs. Compared to a conventional seed treatment, the use of fluxapyroxad AI, which is able to translocate inside the plant and to be active for longer, guarantees a greater and longer lasting protection against SLB disease and leads to a clear delay in canopy senescence resulting in higher photosynthetic activity, and therefore in a significant increase in TKW and TW, and thus in grain yield. The prolonged activity of a seed treatment in controlling fungal disease throughout the vegetative stages cancels out the advantage of administering a specific treatment at the emission of the flag leaf, thereby leading to more effective benefits for the combination with a late application at flowering, a timing in which it is crucial to control FHB and mycotoxin contamination. The systemic fungicide seed treatment, with a prolonged fungal control, is a strategic practice that permits the need for foliar treatments to be limited, thereby allowing the number of pesticide treatments and the overall AI quantity per surface unit applied to be reduced (-51% AI/ha) in order to obtain a greater sustainability of wheat cultivation. In fact, in addition to the reduction of active ingredient rate applied to seed, the fungicide seed treatment eliminates the need for foliar application later in the season reducing also the quantity of pesticides required to manage diseases.

Among the other benefits of a fungicide seed treatment, a key role of seed treatment is related in controlling soil-borne and seed-borne pathogens that can attack seedlings and plants in the early stages resulting in damping-off and thus yield losses. Therefore, in **Chapter III**, the role of innovative fungicide seed treatment in limiting maize seed-borne *Fusarium* infection was investigated. The fungicide seed treatment, apart from being effective in ensuring the desired plant density, also allowed a faster growth of the maize plants than those of the infected control, as it controlled the systemic infection of *Fusarium* species. Furthermore, a broad-spectrum seed treatment (four-way), with a mixture of four AI, compared to the conventional one (two-way) was able to further increase plant vigor, resulting in grain yield increase (+16%) without affecting plant population at harvest. A direct crop enhancement effect of the fungicide seed treatment may be related to the physiological effect of the two systemic fungicides added to the conventional one, even in the absence of a fungal infection. Therefore, in addition to enhance both seedling defense and plant vigor which resulted in a grain yield improvement under different disease infection conditions, the use of a greater number of AI with a systemic fungicide activity as in the four-way seed treatment could also reduce the risks of resistance due to the broad spectrum of action.

The benefits reported in Chapter II and III all contribute to maximizing crop yield while minimizing negative impacts through efficient use of crop protection chemicals. Seed treatment increases precision and effectiveness of crop protection product by reducing the applications rate of pesticides applied to the land area and thereby, it is a leading technology in precision agriculture in present days. However, if fungicide seed treatments are considered an excellent solution to reduce seed attacks and ensure good and uniform emergence, micronutrient and biostimulant seed application play a primary role in increasing the performance of seeds and seedlings in terms of germination and growth, even under critical environmental conditions.

Another key function that could be pursued throught innovative seed treatment refers to the enhancement of spring crops. In North Italy farmers tendency is to anticipate the maize sowing date in order to increase the length of the growing cycle, avoid heat and drought stress during the flowering stage, harvest earlier in autumn with lower drying cost and mycotoxin contamination. By contrast, an early planting make the crop more prone to cold and rainy weather conditions and to a low nutrient uptake. If the application of NP starter fertilizer in band close to maize seed furrows, practice commonly used in temperate growing areas, satisfy efficiently the early plant demand, micronutrients such as Zn could be a further growth-limiting factors. **Chapter IV** investigated through three years field experiments, the possibility to apply Zn to the maize seed and its effects on the whole crop cycle. Among different application strategies (seed, soil, foliar), the Zn seed treatment as seed coating, was as effective in promoting early vigor as the soil banded distribution although the amount of micronutrient applied was 10 times lower than the second one. Although the effect observed was lower compared to the NP starter fertilization, Zn seed treatment led to enhance maize early vigor, anticipate the flowering period and thus increase the grain yield. Furthermore, the micronutrient supply through seed application did not seem to be affected by soil chemical and physical properties and by NP fertilization but rather further advantages in terms of early vigor and yield can be achieved by the combination of both types of fertilization, NP starter fertilizer + the Zn seed treatment. Seed treatment is a low cost, easy and sustainable practice for homogeneously distributing low rates of fertilizers such as micronutrients, and at the same time increase Zn uptake for the seedlings since the first development stages, demonstrating long-lasting effects, even in different soils and weather conditions.

In addition to the micronutrient seed application, another promising strategy to increase the nutrient efficiency and thus environmental sustainability of the agricultural cropping system is represented by the biostimulants: substances and microorganisms that are able to enhance plant development and tolerate biotic and abiotic stresses. This topic covered **Chapter V** and **VI** where in addition to the role of maize hybrids with a superior early vigor and the distribution of a starter NP fertilization, the effects of a biostimulant seed treatment, based on a mixture of a bacterium and a plant extract, on promoting early plant growth were also evaluated in both a growth chamber and open field experiments.

Since the maize genotype and the NP fertilization at planting detected the greatest effects in terms of rhizosphere bacterial community modification with an increase of P solubilizers genera and thus on plant growth, the biostimulant also, in the ordinary hybrid more than that of the early vigor maize, affected PGP community leading to increase maize development from three leaf stage and grain yield in 2 of 4 production situations. In addition to the agronomic and yield benefits, a close relationship was observed between the seed biostimulant and the crop practices such as the maize hybrid and the NP fertilizer. In particular, the study reported the best advantages of the biostimulant in combination to the ordinary hybrid, which was more prone than the high early vigor hybrid to environmental and nutritional stresses affecting also the rhizosphere bacterial composition. Furthermore, a synergistic effect was detected between the biostimulant seed treatment and the NP starter fertilizer determining a faster growth rate and at thus more efficient fertilizer exploitation. For these innovative results Chapter VI highlighted the complexity of the interactions among the crop practices underlining the need to re-design the fertilization strategies and the new role of the research that has to take into account both agronomy and plant microbiota to define the most resilient and sustainable cropping system without changing productivity. Although the effectiveness of a given biological may significantly vary across contrasted systems or environmental conditions biological seed treatments can offer an eco-friendly option to reduce the use of external inputs, such as mineral fertilizers and a valid solution to feed the increasing global populations while avoiding negative effects on human health and thus ensure environmental sustainability. At the same time, among the microorganisms that can be distributed into the soil or directly on the crop, microbial formulations that include the inoculation of non-native strains should be further explored under local conditions as the awareness of potential risks on the native microbioma (Rocha et al., 2019). However, the future of biostimulant seed treatment is related to formulations that best adjust to local growing conditions and to agricultural practices (e.g., use of pesticides/fertilizers, irrigation management) maybe combining this AI with the seed priming technique, not yet applied for cereal crops despite the large number of scientific researches available reporting interesting results. In this way, it is possible to further increase plant establishment even in bad weather conditions where fungal and insect attacks and the reduced macro- and micronutrient availability could be deleterious to plant growth. All these numerous seed treatment virtues that reduce the use of agrochemicals and simultaneously ensure agricultural sustainability, cost-effectiveness, and food security are completely in accordance to the consumer demand for a more sustainable food and feed production, the limitations with the principles of integrated pest management and the recent EU “Farm to fork” strategy where it is required a reduction of overall use of chemical pesticides by 50% and of synthetic fertilizers by 20% by 2023 (European Commission Communication COM/2020/381). Furthermore, the objective of at least 25% of the EU’s agricultural land under organic farming by 2030 can have a positive impact on industries to study new biologicals to apply to the seeds (biopesticides, biofertilizers and biostimulants).

Therefore, the seed treatment is an easy, attractive and effective alternative compared to other application methods (soil and foliar) because of the reduced application rate of external inputs such as non-renewable fertilizers and pesticides, more than 10 times lower, can develop of resilient and more efficient environmental-friendly cropping system. Moreover, the proximity to the seedling ensures a constant and faster release of the active ingredient (fungicide or micronutrient or biostimulant) especially when climatic conditions (low soil temperature) can inhibit its assimilation. In conclusion, the seed treatment is a low cost, eco-friendly technology, and viable option for farmers to ensure the crop productivity by means the plant defense and insurance of a uniform stand and early invigoration across a wide variety of soil types, cultural practices and environmental conditions, playing a pivotal role in increasing the productivity as well as the sustainability of the agricultural sector.

7.2 References

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