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Exploiting arbuscular mycorrhizal fungi and soil microbiota diversity to increase the sustainability of seed production in corn (Zea mays L.)

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Abstract

Conventional agriculture is facing increasing public criticism for both the environmental impact of high-input production systems and concerns about food safety. The seed industry is in turn affected by this trend as it faces a decrease in the tools for controlling the most important adversities in the field and a constantly evolving environmental and regulatory context.

Studies on the root microbiota as a factor influencing plant growth and health are constantly revealing new aspects of the role that beneficial microorganisms play in current and future agriculture.

In this PhD thesis, soil microorganisms were investigated in relation to their biodiversity and possible applications, to improve the sustainability, yield and quality of seed maize production.

This was pursued through two complementary approaches:

- The greenhouse and field experimentation of two species of arbuscular mycorrhizal fungi (AMF) on maize inbred lines, in the years 2019 and 2020 (described in Chapter 3);
- Complete characterization of the soil microbiota and rhizosphere (bacteria and fungi) of 20 different seed production fields in Northern Italy, in 2021 (described in Chapter 4).

Both approaches highlighted the role of the soil microbiota in the successful production of maize seed and allowed to define strategies to improve the reliability of seed production through a more accurate field selection and the application of adequate microbial inocula.

Riassunto

L'agricoltura convenzionale sta affrontando crescenti critiche da parte dell'opinione pubblica sia per l'impatto ambientale dei sistemi di produzione ad alto input sia per le preoccupazioni sulla sicurezza alimentare. L'industria delle sementi è a sua volta interessata da questa tendenza poiché deve far fronte a una diminuzione degli strumenti per il controllo delle più importanti avversità di campo e ad un contesto ambientale e regolatorio in continua evoluzione.

Gli studi sul microbiota radicale come fattore che influenza la crescita e la salute delle piante rivelano costantemente nuovi aspetti sul ruolo svolto dai microrganismi ad effetto benefico per l'agricoltura presente e in prospettiva per quella futura.

In questo lavoro di tesi di dottorato, i microrganismi del suolo sono stati studiati in relazione alla loro biodiversità e alle possibili applicazioni, per migliorare la sostenibilità, la resa e la qualità della produzione di mais da seme.

Ciò è stato perseguito attraverso due approcci complementari:

- La sperimentazione in serra e in campo di due specie di funghi micorrizici arbuscolari (AMF) su linee parentali di mais, negli anni 2019 e 2020 (descritta nel Capitolo 3);

- La caratterizzazione completa del microbiota del suolo e della rizosfera (batteri e funghi) di 20 diversi campi di produzione di seme nel Nord Italia, nel 2021 (descritta nel Capitolo 4).

Entrambi gli approcci hanno messo in evidenza il ruolo del microbiota del suolo nel successo della produzione di mais da seme, e hanno permesso di definire strategie per migliorare l'affidabilità della produzione di semente attraverso una migliore selezione degli appezzamenti e l'applicazione di adeguati inoculi microbici.

Chapter 1

General introduction

Conventional agriculture is facing increasing criticism from the public opinion both due to the environmental impact of high input production systems and to food safety concerns related to the use of synthetic agrochemicals. Minimizing both issues, while maintaining a high level of crops productivity, is a primary objective for the agribusiness companies and for the whole agricultural context.

The seed industry is also touched by this tendency as it faces a decrease in the tools available for controlling the most important field adversities and new challenges generated by a constantly evolving scenario (global warming, resistant pests, environmental policies, market demand, etc…).

The studies about root microbiota as a factor affecting plants growth and health are constantly revealing new aspects about the role that microorganisms play, as potentially useful microorganisms are being tested and their effect on crops is being evaluated.

1.1 The importance of maize worldwide and in Italy

Maize (also known as corn) is a cereal crop originated from the Americas, belonging to the Poaceae family and is one of the most widely distributed food and feed crops in the world (Shiferaw et al., 2011; Garcia-Lara and Serna-Saldivar, 2019). It is being used for several different purposes:

- Livestock feed: corn is a very common grain in the main livestock's diet, thanks to its relatively higher energy level if compared to other cereals, due to its high concentration of starch and oil and to its low content of fibers. For this purpose, it can be used as grain, forage, silage or industrial byproducts.
- Human food: maize is used for several traditional foods like polenta, masa, tortillas etc., which have been the nutritional base for people in many regions. Today corn has a plethora of additional utilizations in the food industry, making it a paramount source of ingredients for thousands of food products. Some of these are corn starch, mainly used

as a thickening agent, corn oil, used for frying, for condiments, or to make margarine and sauces, dextrose or high-fructose corn syrup, used as a sweetener and preservative, etc…

- Biofuels: corn grain is used to produce bioethanol, which is the global main biofuel. This production mainly occurs in North America. On the other hand, Europe is the world leading Biogas producer, mainly using silage maize.
- Industrial materials: as well as for food products, corn derivatives are key components for several industrial products like drugs, adhesives, textiles, inks, lubricants, polishes, coatings, explosives, paints, tires, etc…

As shown in Figure 1, maize is the world's most important grain, based on production volume (Garcia-Lara and Serna-Saldivar, 2019), with an estimated total production of 1126 million metric tons for the 2019/20 season (IGC – International Grain Council), followed by wheat and rice:

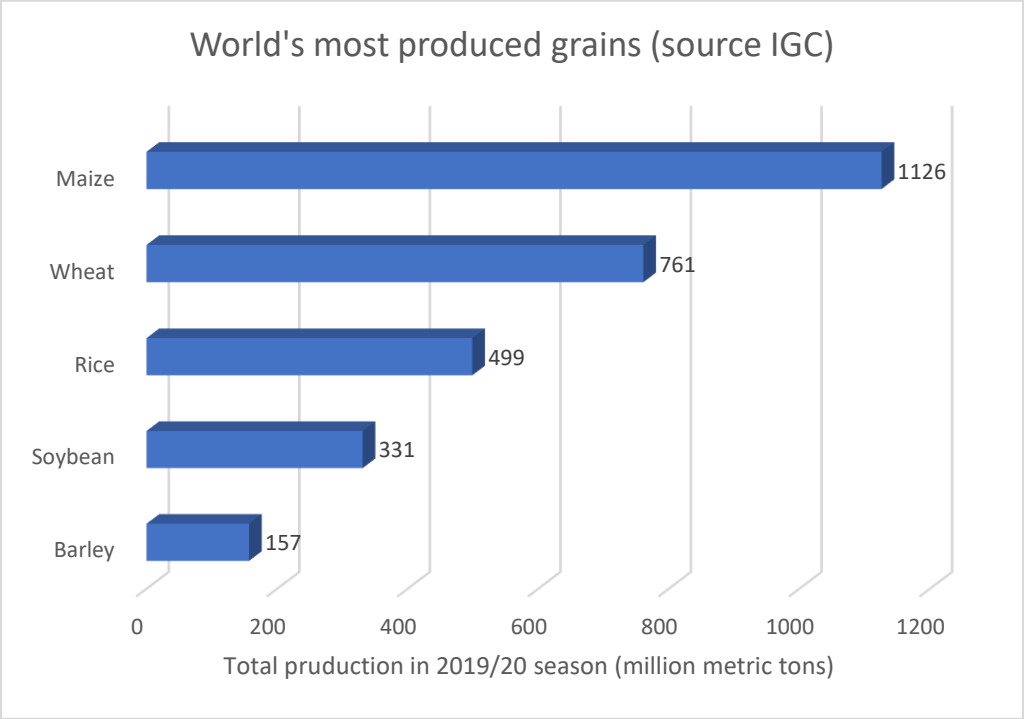


Figure 1: production volume for the world's most important grains (Source: IGC)

The US are by far the most important producer (Figure 2), with about 360 million metric tons, followed by China (261 million mt), Brazil (109 million mt) and the European Union (64 million mmt) (USDA, 2021).

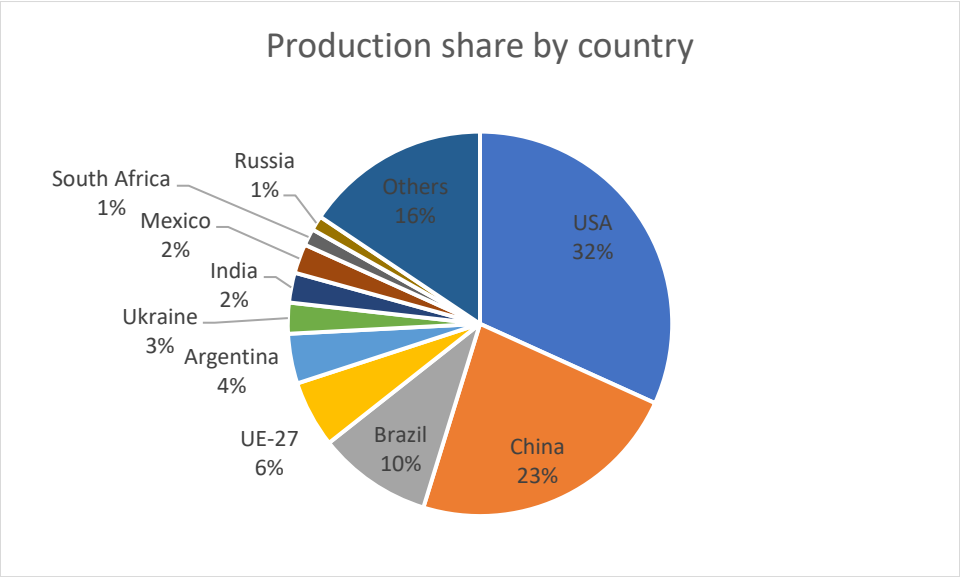


Figure 2: maize production share by country/region (Source: USDA, FAS Grain: World Markets and trade, Jan. 2021)

Considering the European Union (Figure 3), maize is the second most important crop after wheat. Romania is the first maize producer (2019 data), followed by France, Hungary and Italy (European Commission, 2019).

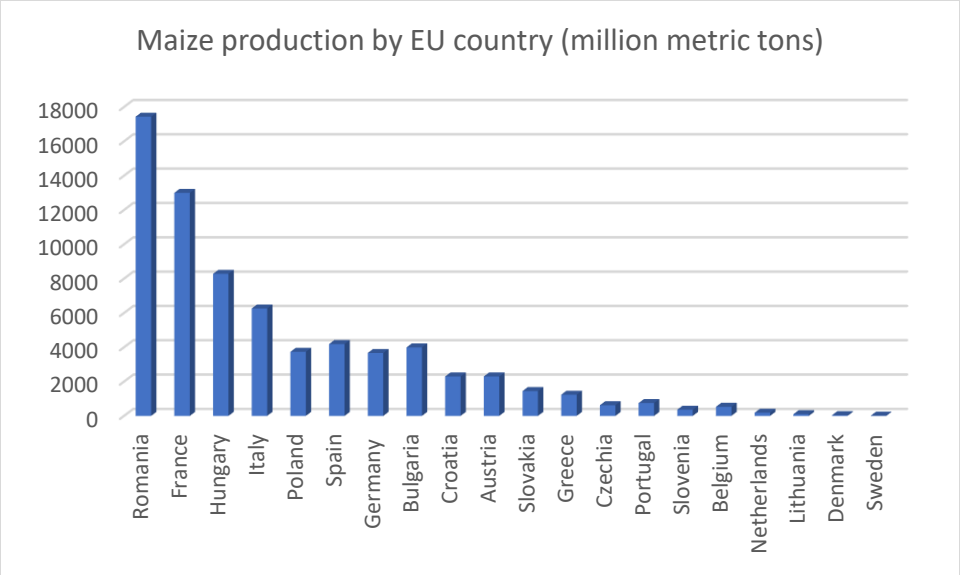


Figure 3: maize production by EU country (Source: European Commission, cereal statistics, 2019)

The Italian maize sector has recorded negative economic and structural performances in the last decade, despite the significant importance of this cereal within crop rotations in extensive herbaceous cultivation systems, especially in Northern Italy. The difficult competitiveness with other European and non-EU countries and, at the same time, increasing sanitary issues (mycotoxins) also linked to fluctuating performances due to climate change, have led to a gradual decline in the main production areas. In addition, the sector complains of unsatisfactory average market prices until 2020, which put a strain on Italian farmers because they do not guarantee the economic sustainability of the crop with often unsatisfactory financials margins (ISMEA, 2020).

As reported in Figure 4, in 2011, Italy counted about 1 million hectares cultivated with corn, while 10 years later, the areas contracted to about 600000 ha (ISTAT, 2021). The area reduction and the consequent decrease in domestic corn production led to an increase in imported raw material and to a decrease in the self-sufficiency rate to around 50%. This means an import/export balance of about -1000 M€, compared to about -567 M€ in 2011 (Ismea Mercati, 2021).

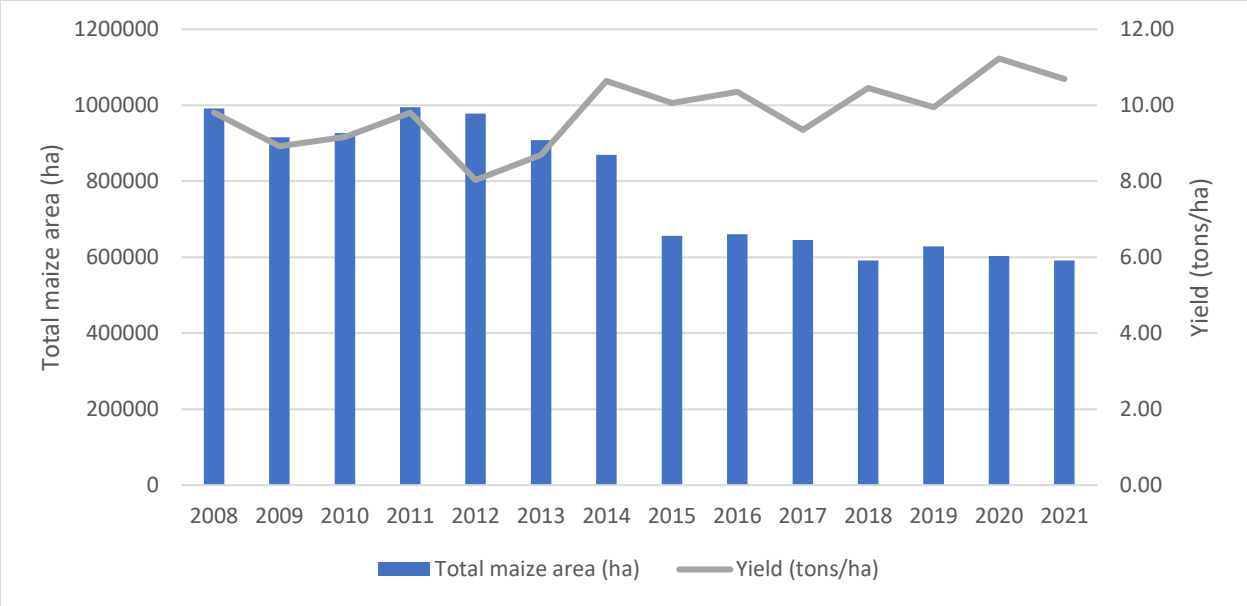


Figure 4: total maize area and yield in Italy 2008-2021 (Source: ISTAT, 2021)

1.2 The biology of maize

Maize (*Zea mays* L.) belongs to the tribe Maydeae, family Poaceae. Among the few species belonging to the *Zea* genus, all native of Mexico and Central America, maize is the only one of economic importance, while the other ones are annual and perennial wild grasses, collectively called *Teosinte* and are the ancestors of modern maize (Doebley, 1990; Sanchez Gonzalez et al., 2018). Maize as we know it is then the result of a transformation, mediated by human breeding activities, of *Teosinte*.

Maize is a diploid plant with 2n = 20 chromosomes (Gaut et al, 2000) and is characterized by a relatively high level of polymorphism (Tenaillon et al., 2001), especially for the shape and composition of its kernels. On the base of these characteristics, the cultivated corn can be divided into seven main groups, not relevant from a taxonomic point of view:

- *Zea mays* indentata (dent corn)
- *Zea mays* indurata (flint corn)
- *Zea mays* amilacea (soft or flour corn)

- *Zea mays* saccharata (sweet corn)
- *Zea mays* everta (pop corn)
- *Zea mays* ceratina (waxy corn)
- *Zea mays* tunicata (pod corn)

The most important groups in terms of produced volume are dent corn, mainly used as an animal feed, followed by flint corn, mainly important as a human food or for poultry farming. The remaining groups are secondary, but have a certain importance as a human food (sweet and pop corn), for the chemical industry (waxy corn) and as ornamental plants.

Corn growth stages (Figures 5 and 6) are typically divided into vegetative (V) and reproductive (R) stages (Ciampitti et al.,2011). Vegetative stages start from seedling emergence (VE) and are then numbered on the base of the number of visible leaf collars (V1, V2, V3, etc.) until the last leaf collar before the tassel become visible. The tasseling stage, when the male inflorescence is emitted is called VT and at this point the vegetative growth ends.

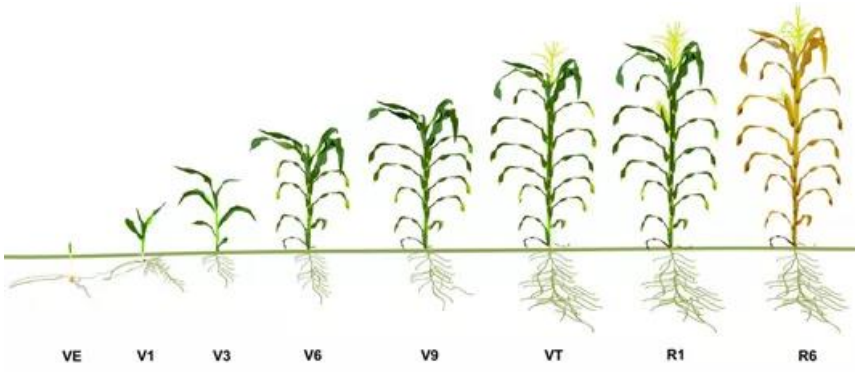


Figure 5: Corn growth stages (Source: Corteva)

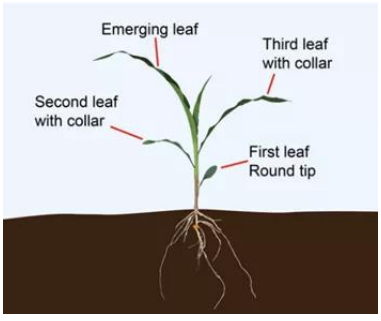


Figure 6: a V3 stage corn plant (Source: Corteva)

The reproductive stages start with silking (R1), the emission of female inflorescence, and then move forward according to kernels development, as shown in Figure 7: blister stage (R2) when kernels are white and liquid inside, milk stage (R3) when kernels turn yellow and have a milky white liquid inside, dough stage (R4) when the inner liquid become pasty, dent stage (R5) when most kernels start to show a dent in their upper face, physiological maturity stage (R6) when the black layer is formed at kernel attachment and the milk line is no longer visible.

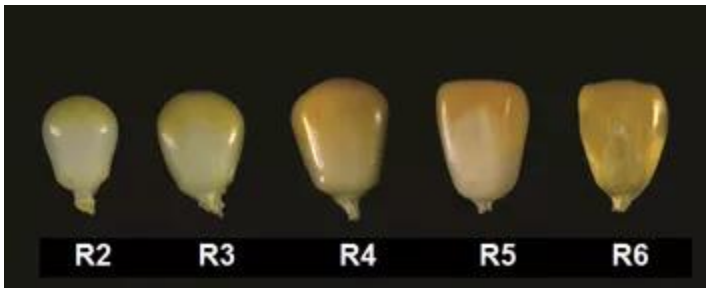


Figure 7: Kernel development stages (Source: Corteva)

Maize is an annual monoecious C4 plant with a single tall stem, rarely tillering, between 0.50 m and 5-6 m high, composed by nodes and internodes that give plant a telescopic-like growth that give corn plants the capacity to grow very quickly during the so-called “rapid growth” stages, between V5-V6 and VT.

Maize has a fibrous root system, typical of Poaceae family. According to Hochholdinger et al. (2018) maize’s root system comprises embryonic roots, generated during the embryogenesis and postembryonic roots, developed after germination:

- the embryonic root system is composed by a single primary root and by a variable number of seminal roots, that are important for determining the seedling vigor during the first stages of plant development;
- the postembryonic roots are shoot-borne and are by far the most relevant root system in adult plants.

All root types can generate lateral postembryonic roots, that have high importance on increasing the absorbing surface and soil exploration capability of corn plants.

Leaves are alternate, one per each node, on the two sides of the stem and can be in variable number, from 8-10 on early hybrids to 22-24 on the latest ones. Each leaf is composed by 3 parts:

- the leaf sheath, embracing the internode, right above the node where the leaf originates;
- the leaf blade, typically lanceolate, with longitudinal, parallel ribs, representing the real leaf and separated from the leaf sheath by the leaf collar;
- the ligule, a membrane originated by the leaf collar, firmly surrounding the stem to avoid the access of water and parasites.

The male inflorescence, emitted at the top of the stem, is a loose panicle, composed by couples of spikelets, each of them carrying 2 flowers with 3 stamens. A tassel is able to produce millions of pollen grains, that are released in the air to be carried by wind toward the silks (anemophilous pollination). The female inflorescence is a spadix, formed by a thick central axis, the cob, carrying a variable number of spikelet rows. The female spikelets are normally grouped into couples and therefore corn spikes (also known as ears) normally carry a pair number of kernel rows. A well-developed hybrid corn spike can carry up to 18-20 kernel rows and a total number of 800-1000 kernels (Garcia-Lara et al., 2019). An inbred corn plant is normally much weaker and less productive than a hybrid plant and normally produces a maximum of 4-500 kernels per ear. Each female spikelet contains two flowers of which only one is fertile. This fertile flower is composed by an ovary and a threadlike stigma lengthening until it reaches the top of the bracts: at full flowering all the stigmas are visible at the top of the ears, forming the so-called silks. When a pollen grain reaches a silk, it germinates and emit a pollen tube that is able to grow along the silk until it reaches the ovule. The pollen tube carries two nuclei: one of them fecundate the egg cell and generates the embryo, while the other one merge with the pole nuclei to form the first endosperm cell. When the pollination is over, the silks completely dry.

The maize grain is a kernel and is an indehiscent fruit: it is composed by the embryo, the aleurone and the endosperm and all these structures are enclosed into the pericarp. The embryo is formed by a plumule, a radicle and the scutellum which is a modified first leaf. The second modified leaf is the coleoptile, that already contains the first 4-6 embryonic leaves into it. The endosperm is mainly composed by starch, that start developing about two weeks after the flower fertilization.

The starch start accumulating from the upper part of the kernel (the one opposed to the seed attachment) and gradually progresses toward the tip of the seed, defining the so-called “milk line”. The starch quality and quantity are important parameters for corn: in fact, they define the grain characteristics (dent, flint corn, etc..) and its final use (food, feed, industrial, etc…).

1.3 Seed corn production overview and technique

Most of the world’s corn is produced from hybrid genotypes, taking advantage of the heterosis effect that allow to grow stronger, more productive and more resilient plants (Lippman and Zamir, 2007; Birchler et al., 2010). Since male and female reproductive structures are brought into separate inflorescences, the technique for producing hybrid corn seed was developed relatively early, compared to other seeds. Starting from the 30s the commercial availability of hybrid corn seed led to a quick replace of traditional varieties (Figure 8), first in the United States, then in Europe. This process triggered a massive increase in corn yield over the following decades.

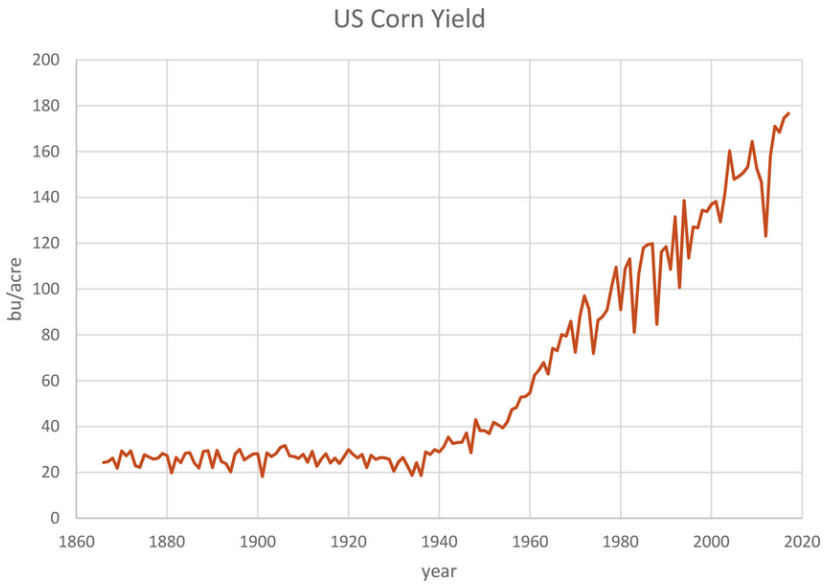


Figure 8: historical US corn yield 1860-2020 (Source: Chavas, Jean-Paul & Mitchell, Paul. (2018), USDA data)

As illustrated in Figure 9, seed corn production has seen a constant increase in the last few years, thanks to a sustained growth in the most important production countries. In France, the leading producer and first world exporting country, the area reached a new record of 82760 ha in 2020,

representing 47% of the total EU area. Romania occupies the second position, with 31145 ha, then followed by Hungary (27685 ha), Italy (7750 ha) and Slovakia (5290 ha) (ESCAA).

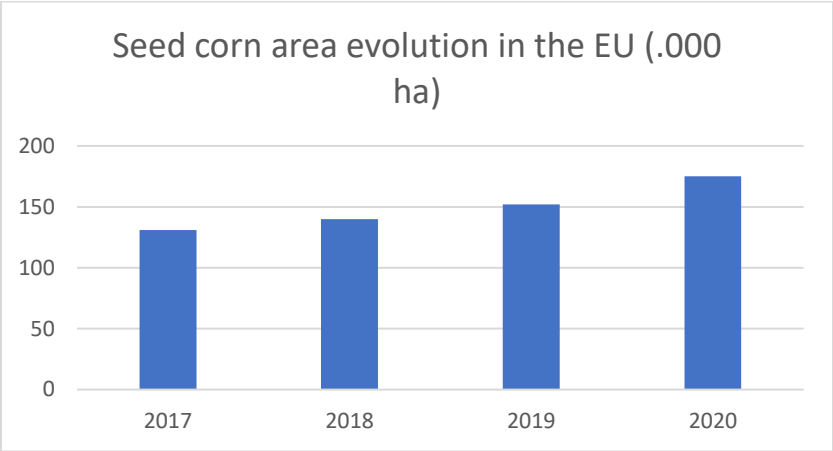


Figure 9: seed corn area evolution in Europe 2017-2020 (Source: ESCAA, European seed certification agencies association)

In terms of cultivated area, corn appears to be the eighth most widespread seed crop in Italy with an incidence of about 4.5% of the total area invested in these crops (CREA, 2020 basis).

The cultivation of seed corn in Italy has assumed an oscillating trend over the years (Figure 10), partly connected to the commodity price and to industrial dynamics, seeing a peak in 2012 and 2013, followed by a decline in the following years and then a constant recovery after 2017. As for the surfaces, the quantities of certified corn seeds have undergone an oscillatory trend.

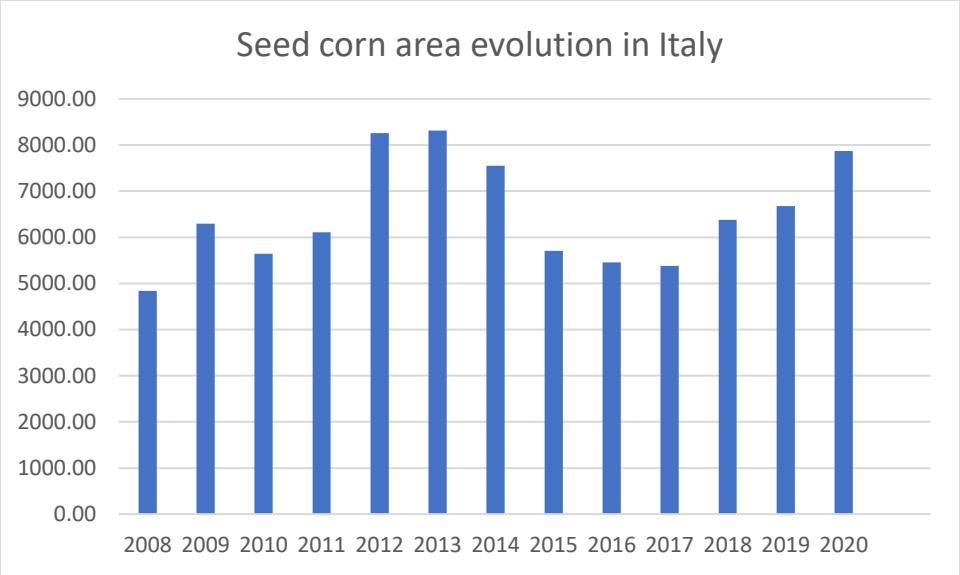


Figure 10: seed corn area evolution in Italy 2008-2020 (Source: CREA, statistiche di certificazione)

To introduce the seed corn production technique, it is important to clarify the definition of variety, inbred line and hybrid:

- Variety: within a species there can be a wide range of different types of plant, but farmers need plants with particular characteristics, which are well adapted to their environment and their agricultural practices. According to UPOV (Union for the Protection of New Varieties of Plants), a variety is then “a more precisely defined group of plants, selected from within a species, with a common set of characteristics”. It is a population of individuals that are still genetically heterogeneous, but show a uniformity of some phenotypical characteristics (e.g. grain color, etc…). They normally have limited productivity, compared to hybrid plants.
- Inbred line: first defined by W. L. Johannsen in 1909, an inbred line is “a relatively true-breeding strain resulting from at least five successive generations of controlled self-fertilization or of backcrossing to a recurrent parent with selection, or its equivalent, for specific characteristics” (AOSCA). An inbred line shows a high level of homozygosis (>99%) which increases at each progressive self-fertilization and is characterized by plants that

are all identical to each other. This high level of homozygosis generates the so-called inbreeding depression that leads to weak, short and less productive plants. An inbred plant, when self-fertilized, generates a progeny that is identical to the mother plant.

- Hybrid: a hybrid derives from the crossing of two parents, genetically distant and with a high level of homozygosis (inbred lines). A hybrid is characterized by plants that are all identical to each other and that are much more vigorous, productive and stable (Figure 11), thanks to the so-called heterosis effect.

Figure 11: hybrid plant and ear (center) compared to their parent inbred lines (sides). Source: Iowa State University.

Different types of hybrids can exist, depending on the parents they originated from:

  - Single cross hybrids: they originate from two inbred lines and are then the hybrids in the strict sense. They are characterized by a high level of uniformity. Their high level of heterosis confers them the best agronomic fitness, but their cost of production is high, due to the low productivity of the mother parent.
  - Triple cross hybrids: they are the result of a cross between an inbred line and a single cross hybrid, normally used as a seed carrying parent. They show a lower level of heterosis and uniformity, but their cost of production is lower, thanks to the high productivity of the mother parent.
  - Double cross hybrids: obtained by crossing two single cross hybrids, they have the lowest cost of production and the lowest level of heterosis and uniformity. Their large genetic base makes them more adaptable to various environment.

1.3.1 Field production technique

As anticipated above, the field production technique of seed corn presents some differences, compared to commercial hybrid corn. Despite they share many of the field operations, the simultaneous cultivation of two inbred lines, namely the female “seed” line and the male “pollen” line, requires spatial arrangement in order to grant timely and abundant amount of pollen from the male and, at the same time, maximize the area occupied by the female, from which the product is obtained.

The ordinary planting pattern normally consists of female blocks, made of 4-6 consecutive female rows (70-75 cm row spacing), spaced out by 2 male rows, as visible in Figure 12.

Planting is done between the end of march and the beginning of June and normally consists of 2-3 different stages, since males and female inbreds are planted at different times in order to ensure a perfect flowering synchrony and a certain delay between the males. This delay ensures a longer pollination time and a better fertilization success.

The sowing planning involves a careful selection of the plots in which the seed crop can actually be sown. Being the inbred lines much weaker than hybrid corn, they require special care, especially for the following reasons:

- Inbred lines are less efficient in using resources (Troyer and Wellin, 2009; Araus et al., 2010) and then more sensitive to abiotic stresses. For this reason, this genetic material needs additional irrigation and requires a higher amount of nitrogen per kg of produced grain.
- Inbred lines are weaker and shorter than hybrids, they need more time to close the interrow space and then they suffer more the competition with weeds. For this reason, it is important to dedicate special care to weed-control strategies and it is normally mandatory to have a pre-emergence herbicide intervention to control the weed emergence from the beginning of the cropping cycle.

- Inbred lines are more sensitive to biotic stresses, namely insects and need an accurate IPM strategy and additional interventions. The main biotic adversities are corn cutworm (*Agrotis* sp.), European corn borer (*Ostrinia nubilalis*), corn rootworm (*Diabrotica virgifera*) and spider mites (*Tetranychus urticae*).

Corn is an anemophilous pollinated and mostly allogamous plant that disperses its male propagules thanks to the action of wind. This exposes seed crops to a high risk of genetic contamination due to the potential presence of unwanted corn pollen in the air, in case of other corn crops in the neighborhoods. It is therefore necessary to ensure that the neighboring plots are correctly isolated from the seed corn fields. There are mainly two ways to do this: through physical distance and by offsetting the planting time of the seed crop and the commercial crop in order to avoid a contemporary pollination.

The minimum distance between seed corn and commercial corn fields is defined by law and can be slightly different, according to different countries' regulations. The minimum distance for Italy is 200m, but it can be reduced up to 70 m by interposing a male barrier on the border of the seed corn field, composed by one male row every 10 meters of distance reduction (plus the two male rows typical of the planting pattern). Studies confirm the efficacy of such prescriptions (Ireland et al., 2006).

The seed-carrying lines can be male-fertile, and then able to produce pollen and self-fertilize, or male sterile (cytoplasmatic male sterility), so that the plant is unable to shed pollen, making self-fertilization impossible. These variants lead to a substantial difference in the cultivation process: for male-fertile plants it is necessary to recur to plants detasseling to remove the male inflorescence (tassel) while for male-sterile ones it's normally not necessary to recur to this important and expensive operation. The result, in this last case, is a reduced cost for mechanical operation and manual labor and an untouched photosynthetic area that leads to a higher seed yield.

On fertile female lines, a few days before tasseling, emasculation is performed by cutting or pulling the male inflorescence through a dedicated high-clearance machine, capable of entering between corn rows. The operation is repeated 2-3 times in order to eliminate most of the tassels

and is then followed by a manual finishing, in order to eliminate all the remaining inflorescences (late and shorter plants).

The cutting action must be less invasive as possible to safeguard the photosynthesizing leaf surface: the plant must therefore be prepared to overcome this type of stress with preventive irrigation if necessary.

Figure 12: A typical seed corn production field, with emasculated female rows, spaced by a couple of male rows

A continuous field monitoring is essential for this crop in order to reach the high-quality standards required for marketing. This is particularly important for guiding production planning, detasseling operations, removal of off-type and segregant plants and pest management. There are in fact strict regulations defining the minimum requirements in terms of genetic purity, and even stricter limits imposed by Corteva's internal standards of production.

Before flowering, it's especially important to detect and remove any off-type plants, both from female and male lines, by carefully checking several phenotypic characters, in order to identify even the most subtle off-types.

Flowering is especially monitored when the ears begin to emit their first silks, then becoming receptive to both wanted and unwanted pollen. This is also being done to understand if the female and male lines are temporally coordinated. At this stage, it is critical to ensure the absence of any pollen from female inbred plants (including sterility breakings in sterile lines).

At the end of flowering, the corn begins the filling and ripening phase. In this phase the male plants are destroyed, through a mechanical operation, only leaving the female lines in the field and waiting for their complete maturation.

The cycle ends with harvest. This operation is carried out with corn pickers collecting the entire ear. The ears are then transported to the factory where they are carefully received, husked sorted, dried and shelled.

1.3.2 Cytoplasmatic male sterility (CMS) in hybrid seed corn production

Maize inbred lines derive from a careful breeding process and are characterized by a high level of homozygosity. These lines carry a high genetic load (pool of rare alleles) and therefore, following the increase in the level of homozygosity, they are subject to inbreeding depression. This phenomenon leads to a gradual decrease in fitness and therefore, potential performance in the field.

Cytoplasmic male sterility (CMS) is a phenomenon through which a male-sterile plant is obtained through the contemporary action of nuclear and cytoplasmic genes (Weider et al., 2009). This can be a naturally occurring trait, and is being exploited for seed production, since it leads to a plant that is unable to produce pollen or produces non-viable pollen (Laser and Lersten, 1972; Schnable and Wise, 1998). The CMS inbred lines do not need to be emasculated in the pre-flowering period: this favors a significant decrease in the operations in the field which, in addition to the economic benefit, involves the absence of leaf tissues removal, and allows the plant to avoid a state of stress and keep the photosynthetically active leaf surface unaltered with evident repercussions on production.

CMS mainly happens due to mutations in mitochondrial DNA that can have negative implication in the plant respiratory metabolism and generates abnormal phenotypes, with an abnormal gametes production (Budar et al., 2003). This mechanism does not anyway affect female fertility and corn plants can then develop and carry seeds, if viable pollen is available.

Three main types of male-sterile cytoplasm are known: T-cytoplasm of maize (Texas) (Rogers and Edwardson, 1952), S-cytoplasm (USDA) (Jones, 1957) and C-cytoplasm (Charrua) (Beckett, 1971). Those three CMS types differ in the nuclear restorer genes (rf genes) that are able to interrupt CMS and restore fertility in the progeny (Schnable and Wise, 1998).

At first, the most used CMS type was the T-cytoplasm, which was reliable and easy to adopt and became the main type between 1950s and 1970s, but it unfortunately conferred susceptibility to *Cochliobolus heterostrophus* (Duvick, 1959; Levings, 1993) the fungal agent of the Southern leaf spot disease (also known as disease T). This led to a huge phytosanitary issue in the American corn belt in 1970 and caused an immediate interruption in the use of T-cytoplasm in favor of fertile line or other CMS types.

The S and C cytoplasms are actually less reliable than the T type and they can spontaneously revert to fertility and generate erratic amounts of viable pollen (Weider et al., 2009). Despite this, in the following decades, the use of CMS lines has constantly increased and today is widely popular.

1.4 Plant-microbiota interactions in the rhizosphere

Plants in nature and in agro-ecosystems are constantly surrounded by microbial communities that interact with their root system as well as with their aerial structures, with the potential to influence plants physiology, genetic expressions and ultimately growth and production performances (Lemanceau et al., 2017). This indefinite set of microorganisms is called the plant microbiota, while the overall repertoire of microbial genomes is defined as microbiome (Compant et al., 2019).

The history of such relationships likely started with the first colonization of emerged land by the first ancestors of plants. Genetic evidence suggested that the first plants ancestors developed from a symbiosis between an alga and a primitive fungus (Strullu-Derien et al, 2018). Such archaic organisms did not likely have a root system and took advantage of mycorrhizal relationships to get water and nutrients (Chalva et al, 2021;).

It is today estimated that 80% of land plants species, referring to 92% of land plant families are establishing symbiosis with mycorrhizal fungi (Wang and Qiu, 2006) and likely all plants in nature have to interact with bacteria and other microorganisms at different levels and through different organs, both on the root system or on the aerial system.

The soil layer in close contact with the roots of plants is called rhizosphere and represents a microbial habitat clearly distinct from the bare soil in terms of physical, chemical and biological characteristics. The microbial community that populates this soil-root interface, referred to as the rhizosphere microbiome, establishes a range of interactions with plants that includes examples of mutualism, commensalism and parasitism (Schlaeppi and Bulgarelli, 2015).

In turn, plants are not passively exposed to this microbial environment but play an active role on shaping mutualistic relationships with useful species and reacting to pathogens.

Through photosynthesis, the plants convert light energy and carbon dioxide into carbohydrates and a remarkable part of the photosynthates are released by the root in the form of exudates in the rhizosphere, which is the interface layer between the plant roots and the soil. Root exudates are carbon-rich, and contain several organic compounds like carbohydrates, amino acids, organic acids, flavonoids, glucosinolates, auxins, etc. (Badri and Vivanco 2009). The aim of these exudates is to act as a signal and to create small ecological niches in order to attract microbes from the soil surrounding the roots and thus shaping the so-called rhizosphere microbiota.

Plants and their associated microbial communities can be intended as superorganisms, in which the plant genome interact with the plant microbiome (Figure 13), considered as the plants' other genome, similarly to the terminology and models used for human microbiome. In fact, plants rely in part on their microbiome for specific functions and traits (Mendes et al., 2013). In exchange, they provide nutrition and ensure favorable conditions to their microbial guests.

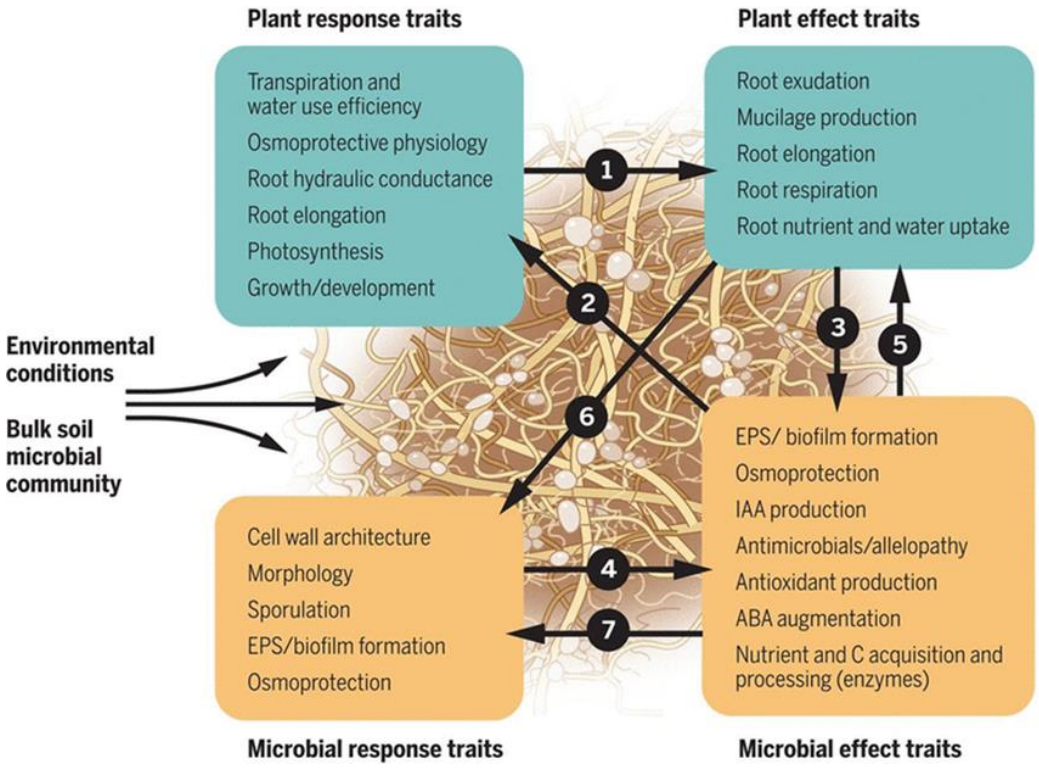


Figure 13: the plant/microbiota interactions in the soil (Source: de Vries et al., 2020)

1.4.1 The role of root microbiota on plant growth and health

The agricultural soil is one of the most complex microbial ecosystems on Earth: it is estimated that in one gram of soil can live billion of bacteria belonging to thousands of different species and more than 200 meters of fungal hyphae (Sellitto, 2020). Despite this, microbial communities that are populating the rhizosphere, tend to show a limited and defined number of taxonomic groups. Plant roots are clearly exposed to this microbial crowd.

As well as some of these microbes might express pathogenic effects or simply depressing plant growth and development, several studies conducted on microorganisms isolated from different organs of the plants demonstrated that they can have beneficial effects on different plant biologic activities like enhancing nutrients and water intake, protecting the plant from abiotic stresses and from other pathogenic microbes (Schlaeppi and Bulgarelli, 2015).

Root-associated microbes can enhance plant nutrition by providing the plants with nutrients that are normally inaccessible to them. A well-known example is the symbiosis between leguminous plants and nitrogen-fixing bacteria, capable of capturing atmospheric nitrogen and making it available to plants. This process can also happen for other plant families, involving different bacteria (Franche et al.,2009). Another example is the decomposition of soil minerals that are not accessible to plants: for instance, some bacteria and fungi are able to produce phytase that mobilize inorganic phosphate (Richardson and Simpson 2011), while some others are capable of mobilizing iron by producing siderophores (Bulgarelli et al., 2015).

Root microbiota can also provide several other benefits to plants, like protecting plants from infection by soil-borne pathogens (Bakker et al.,2013, Berendsen et al., 2012), producing or inducing plants to produce phytohormones (Gaiero et al., 2013) and helping plant to tolerate abiotic stresses such as drought (de Vries et al., 2020), cold or heat stress (Caradonia et al.,2019; El-Daim et al., 2013), high salinity (Kumar et al., 2020), and heavy metals contamination (Caracciolo and Terenzi, 2021). All these beneficial roles played by rhizosphere microbiota, summarized in figure 14, are critical for an optimal plant growth and development and, in turn, for getting the most from crops.

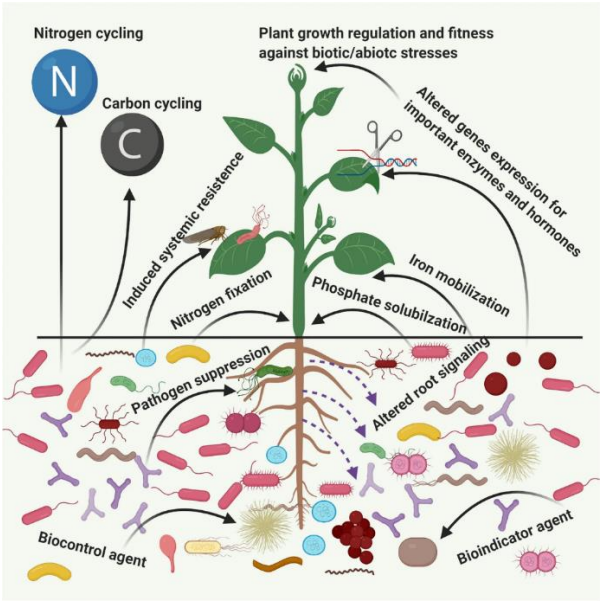


Figure 14: soil microbiome mediated benefits to plants and nutrient cycling (Source: Bano et al., 2021)

The way how these microorganisms interact with each other and with superior organisms and their cells is mainly through secretion systems, which are the key way how prokaryotes transfer protein-based materials from the cytoplasm to the external environment and into other prokaryotic and eukaryotic cells, in order to send chemical signals and to explicate different effects on the receiving cells. This is for example the mechanism used by pathogenic microorganisms to deactivate the plant immune system, but also the mechanism used by microbes to interact with each other, thus shaping such a complex environment. At the same way, beneficial microorganisms can use this system to stimulate useful reactions or pathways to plants. Studies conducted on barley, revealed that rhizosphere microbiota triggered the expression of some biological functions that are at the base of plants adaptation and survival into root-associated microhabitats like adhesion, stress response, secretion, regulation of host-pathogen and microbe-microbe interactions and improvement of sugar and iron mobilization (Bulgarelli at al. 2015).

Therefore, it is clear that the rhizosphere microbiota is not a random assortment of microbial species, but it derives from a precise set of microbe-microbe and plant-microbe interactions, mediated by soil. Eventually, a real selection process takes place at the root-soil interface.

1.4.2 The role of soil as a driver for plants' root microbiota

The soil has a primary role on shaping the microbial communities associated to plants rhizosphere: according to several studies, it actually serves as the main reservoir for most of the microorganisms that can approach the plants or be recruited by them (Bulgarelli et al., 2012; Lareen et al., 2016). Physical and chemical characteristics of soils like pH (Lauber et al., 2009), content of organic matter or nutrients availability (Lauber et al., 2008), can influence the population of different bacterial and fungal species and the overall microbial biodiversity. As a consequence of this, it's clear that human impact on soils can have profound consequences on edaphic microbial communities: differences in land use can shape them due to variations in both soil conditions and plant diversity and coverage. As an example, shifting from natural ecosystems

to arable land can cause a drop in microbial diversity as a consequence of the mutated and more artificial conditions (Muñoz-Arenas et al., 2020). Narrowing focus to agricultural land, it has been demonstrated that organic management can increase the taxonomic and phylogenetic richness of soils (Lupatini et al., 2017) as well as some sustainable agricultural practices, that can be applied in conventional agriculture too, like reduced tillage and the use of cover crops (Vukicevich et al., 2016; Schmidt et al., 2018).

These findings also suggest that many other agricultural practices, like the amount and typology of mineral fertilization (Caradonia et al., 2019), likely have an impact on the edaphic communities and it is therefore important to understand how to optimize them. This in order to create the best pedological and microbiological conditions for crops and for preserving soil health and improve the overall sustainability of agriculture.

1.4.3 How plants influence and recruit root microbiota

Despite the soil plays a primary role on selecting the microorganisms to which plants are exposed, they are not completely passive to it and can react and adapt to different soil conditions. As anticipated at paragraph 1.3, plants can actively select what species to promote and recruit, mainly through the production of root exudates that influence the chemical conditions of the rhizosphere and create niches where useful microbes can thrive and cooperate with plants. This process has been called rhizodeposition and can be influenced by the plant genetic and by its developmental stage (Jones et al., 2009).

Plant genetic is a key factor on determining the root-associated microbiota (Miethling et al., 2000), since the composition of root exudates can vary between species and cultivars. Studies done on the model plant *Arabidopsis thaliana* demonstrated the host genotype effect on shaping the root-associated microbial population, despite this influence was secondary to that of soil (Bulgarelli et al., 2012; Lundberg et al., 2012). This sheds light on the effect of human domestication and breeding activities on influencing the way how plants interact with their surrounding microbial communities (Bulgarelli et al., 2015). This also highlights the opportunity

to shape root-associated microbial population at our advantage, through modern breeding activities (Clouse and Wagner, 2021).

Another important factor affecting this mechanism is the root system architecture (RSA): this characteristic can be shaped by genetic drivers but is partly influenced by soil characteristics and by the availability and distribution of nutrients. A different shape and different physical characteristics of RSA can play a role in defining the rhizosphere microbiome (Pérez-Jaramillo et al., 2017).

The recruiting and composition of rhizosphere microbiota can vary according to plant developmental stage: despite there is evidence of a core microbiota that does not significantly change throughout plant development, studies demonstrated that a subset of it significantly varies, according to plant developmental stages. This is likely affected by different plant needs during crop development, in addition to different seasonal conditions. It was in fact demonstrated by several studies that plants can shape their root microbiome through the release of different exudates at different growth stages, and this activity is clearly connected to the expression of an evolving gene activity and its related transcripts (Chaparro et al., 2014). Maize microbiome development as related to plant growth, is in line with the above reported findings (Xiong et al.,2021).

Another important mechanism used by plants to shape their own microbiome is by recurring to keystone microbial species (Jones et al., 2019), which are those species capable of modifying the composition and physical appearance of the surrounding community (Paine, 1966). Both arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF) are recognized as being part of these keystone species (Gamper et al., 2010; Soka and Ritchie, 2015). This is a top-down mechanism of ecosystem control that take place when plants start recruiting keystone microbes in order to manipulate their own rhizosphere microbiome. The study of keystone microbe is a relatively recent research area, but looks promising for improving our understanding of the mechanisms used by plants to shape their surrounding microbiome and for taking advantage of it. Keystone microorganisms can in fact be used as a way for improving agricultural performance

and sustainability through their direct manipulation of rhizosphere microbiome (Busby et al., 2017; Trivedi et al., 2017; Hamonts et al., 2018).

1.5 The use of beneficial soil microorganisms in sustainable agriculture

The rapid growth of world population and the increasing needs following the improved living conditions of people in many regions are progressively driving an excessive exploitation of agricultural soils, accompanied by the necessity to expand the agricultural land and conjugate food production with energy crops. This leads to increasingly intensive practices, with the risk to progressively degrade soil fertility and biological diversity. A potential solution for maintaining high yield and minimizing the negative effect on soil of intensive agriculture is offered by microorganisms that are able to proliferate in any conditions. Soil is normally characterized by a high biodiversity, hosting a huge number of organisms, classified on the base of their dimensions and ecosystemic functions. Soil microorganisms, and particularly microflora (bacteria, archaea, fungi and algae), are a key component of the pedological trophic chain: they have a primary role in the degradation of the organic materials derived from plants, in the generation of humus and in the mineralization of the organic matter. Soil microorganisms have close relationships with plant: soil rhizosphere, the thin interface layer between soil and root cells, is normally the area where the highest number of microbes is concentrated, thanks to the release of root exudates, that provide a precious food source for the soil microbial world. Despite many species probably have a neutral effect on plants, many others can positively or negatively influence plant growth with remarkable effects on agriculture. This research report will mainly focus on beneficial microorganisms, mainly bacteria and fungi, on their influence on seed corn productivity and on how their biodiversity can be influenced by agricultural practices and drive crop performances.

1.5.1 Plant growth promoting rhizobacteria (PGPR)

Plant growth-promoting rhizobacteria (PGPR) are bacteria that live in the rhizosphere, where root exudates are released and carry out their activity. Kloepper and Schroth first defined this category of useful microorganisms in 1978 and then many studies reported them in the following

years. Several bacterial species have been reported as being PGPR thanks to their ability to stimulate plant growth, while helping plants to control pathogens. To cite some examples, some of the most common genera are *Azospirillum, Azotobacter, Bacillus, Burkholderia, Herbaspirillum, Pseudomonas, Rhizobium* and others. A more detailed list of PGPR species is reported by Kumari et al. (2019). They are considered an environmentally friendly alternative to chemical fertilizers, thanks to their positive activity on plant growth, allowing farmers to reduce inputs on crops. Despite legumes are a well-known example of plants taking advantage of PGPR, several other species are able to exploit different bacterial species to improve their own growth (de Souza et al., 2015; Backer et al., 2018).

PGPRs can have both direct and indirect beneficial effects on plants. Direct growth promotion implies that root-colonizing bacteria synthesize compounds providing/facilitating the plant with the absorption of nutrients from the environment or with various mechanisms; e.g.:, fixing atmospheric nitrogen or solubilizing inorganic compounds containing phosphorus, potassium or zinc thus making all these nutrients more bioavailable for plants (Vejan et al., 2016; Kamran et al., 2017; Wang et al., 2020). The indirect promotion occurs Instead when the PGPRs reduce or prevent the deleterious effects of one or more phytopathogenic microorganisms. They can in fact act as biocontrol agents through different modes of action: they can compete with pathogens for vital elements or for the binding sites on the root surface, synthesize antagonistic substances such as antibiotics or lithic enzymes or act as elicitors for the induced systemic resistance (ISR) of plants (Annapurna et al. 2012; Beneduzi et al., 2012). Some PGPR can carry out both direct and indirect beneficial effects at the same time, like increasing nutrient availability and acting as biocontrol agents (Glick, 2012).

Despite this, PGPRs not only play a beneficial role in improving crop productivity but also have a fundamental role in the regular functioning of agroecosystems, in restoring degraded soils and in reducing pollutants in the environment (Khatoon et al., 2020; Chitara et al., 2021). For all the above-mentioned reasons, they represent a great tool for sustainable agriculture.

Many beneficial effects of different PGPR on maize have been demonstrated by several studies. Breedt et al. (2017) highlighted the potential of five different rhizobacterial strains and their

consortia to increase yield, when seed-applied. Similarly, Di Salvo et al. (2018) demonstrated the positive effect of two strains of *Azospirillum brasilense* and of a consortium of *Azospirillum brasiliense* and *Pseudomonas fluorescens* on corn yield. Cassán et al. (2009) improved seed germination rate and seedling development, thanks to strains of *Azospirillum brasiliense* and *Bradyrhizobium japonicum*, alone or combined. Regarding mineral nutrition, Kuan et al. (2016) improved dry biomass of maize plant's top, root and ear thanks to an improved nitrogen fixation activity mediated by a strain of *Bacillus pumilus*. Also, thanks to a better mobilization of nitrogen and phosphorous, promoted by seed-applied strains of *Bacillus subtilis*, Lobo et al. (2019) were able to increase maize plant's growth and grain yield.

Additionally, PGPRs can have a bioprotective role on maize. Pereira et al. (2011) demonstrated the protective effect of *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* against *Fusarium verticillioides* when applied as seed coating. Bevivino et al. (1998) found that some species like *B. cepacia* could act as both biocontrol agents against *Fusarium* spp. and biofertilizers by improving iron nutrition and then growth of corn through siderophore production.

Like for other crops, maize's response to PGPR is complex as it is the result of multiple interactions between plants and microbes and between different microbial species. For this reason, it is critical to better understand the ecological relationships in the rhizosphere under different agricultural practices and regimes, to be able to exploit the useful activity of these bacterial species and strains (Mendes dos Santos et al., 2020).

1.5.2 Plant growth promoting fungi (PGPF) and arbuscular mycorrhizal fungi (AMF)

Soil contains a huge variety of both beneficial and pathogenic fungi, colonizing plants' roots in any stages of their development. Soil fungi normally represent the major rate of biomass among microbial communities and are considered among the most important actors in all the soil processes connected to the cycles of different nutrients, especially nitrogen and phosphorous (Paul, 2014; Sellitto, 2020).

Plant growth-promoting fungi (PGPF) are non-pathogenic fungi belonging to several diverse genera that are able to provide benefit to plants, by stimulating various aspects of plant growth.

As for the PGPR, the use of PGPF is considered as an important opportunity to improve the agricultural sustainability, by reducing the inputs needed for an optimal productivity. These fungi have in fact demonstrated to improve crop yields thanks to several positive effects, depending on fungal and plant species, like increased plant growth, better seed germination and seedling vigor, enhanced root development and photosynthesis, improved tolerance to biotic and abiotic stresses. Similarly to PGPR, they can do that through direct and indirect mechanisms, like making nutrients more available to plants through solubilization or mineralization, influencing the phytohormonal balance, producing organic compounds and enzymes and compete with pathogenic microorganisms (Hossain and Sultana, 2020).

PGPF can be divided into three categories: endophytic, when they are able to colonize the inner parts of the root and exchange metabolites directly with plants, without causing diseases (Wilson, 1995); epiphytic, when they live on the root surface (Kharwar et al., 2009); free-living PGPF, when they live outside the plant cells, like in the rhizosphere.

Most of PGPF are naturally present in the soil; many of them belong to the genus *Trichoderma, Alternaria, Aspergillus, Aureobasidium, Candida, Cladosporium, Metarhizium, Paecilomyces, Phoma, Penicillium* and others (Sellitto, 2020). They can put in place non-obligate mutualistic relationships with plants and can provide benefit to them, not only when they are associated to roots. Therefore, they tend to be distinguished by AMF, that belong to different taxonomic categories and need to create obligate symbiotic relationships (Hossain and Sultana, 2020).

Focusing on corn, studies reported beneficial effects of PGPF like augmented shoot growth and improved resistance to several diseases and abiotic stresses promoted by *Trichoderma* spp. (Harman et al., 2007; Saravanakumar et al., 2017; Estévez-Geffriaud et al., 2020; Degani and Dor, 2021), better tolerance to cold stress after seed treatment with *Penicillium* sp. (Gómez-Muñoz et al., 2018), improved growth, related to a better phosphorous mobilization mediated by *Mortierella elongata* (Li et al., 2018).

Contrarily to ordinary PGPF, AMF are obligate biotrophic, belonging to the subphylum *Glomeromycotina*. They can put in place the most commonly established symbiosis in plants: the arbuscular mycorrhizal symbiosis. It's in fact estimated that 80-90% of all land-plant species are

able to interact with AMF through this symbiosis (Luginbuehl and Oldroyd, 2017), where fungi receive fixed carbon from plants and in exchange provide them better access to water and nutrients, especially phosphates (Smith and Smith, 2011). AMF are in fact able to get better access to plant nutrients, thanks for example to their ability to solubilize normally insoluble compounds, to get better access to soil colloidal structures and to guarantee a better soil exploration and exploitation thanks to their thin hyphae and dense hyphal network (Gianinazzi et al., 2010). In addition to that, they can provide better tolerance to abiotic stresses like cold, drought and salinity (Volpe et al., 2018; Caradonia et al., 2019). As mentioned at paragraph 1.3.2, AMF are keystone species, then able to influence the recruiting of other microorganisms by plants. They can then have additional indirect effect, by mediating the attraction of other beneficial PGPR and PGPF.

AMF are endomycorrhizal fungi, meaning that they are able to establish endosymbiosis, where the fungus lives within the plant cells, contrarily to ectomycorrhizal fungi (EMF), mainly present in forest environments, setting up symbiosis on the surface of plant roots. AMF penetrate the root tissues and cells and can occupy the intercellular and intracellular spaces, creating ovoidal structures called vesicles and branched structures called arbuscles. While vesicles are intended to be reserve bodies, arbuscles are likely the structures where most of the exchanges between plant and fungus take place (Balestrini and Bonfante, 2014). AMF development is mediated by chemical signals and exchange of molecules between the symbionts: this triggers the hyphal movement toward the root, where a hyphopodium is generated on the surface, through which the fungus can penetrate the epidermal cell layer. Here the hyphae can spread inside the root intercellular spaces, until they reach the inner cortex where the arbuscles are developed, inside the cortical cells. The fungus can in fact penetrate the cell wall, but it does not break the host plasma membrane, that expands and surrounds the hyphal branches, forming the so-called periarbuscular membrane, separating the fungal structures from the host cytoplasm. Being the site of nutrient exchange between pant and fungus, the arbuscules are critical for the AM symbiosis (Luginbuehl and Oldroyd, 2017).

Maize is a host for mycorrhizal fungi (Kahn, 1972; Karasawa, 2000). Several positive effects of AMF on this crop have been reported and include, for instance, improved growth and

phosphorous uptake, especially under drought conditions (Karasawa, 2000), better tolerance to zinc deficiency (Saboor et al., 2021), alleviation of drought stress (Sylvia et al., 1993; Polcyn et al., 2019), yield and phosphorous uptake increase, especially in soils with limited phosphorous availability (Camargo Gomes Stoffel et al., 2020), yield increase and improved resistance to insects (Wang et al., 2021), increased biomass production and roots development (Bender et al., 2019), improved growth and influence on grain quality (Berta et al, 2014), increased nutrient uptake with seed-applied AMF (Rocha et al., 2017)

On the other hand, some null or adverse effects have also been found, like reduction of nitrogen uptake by corn plants treated with AMF, in a pot experiment, under limited nitrogen availability (Wang et al., 2018), AMF efficacy dependent on field nutrient availability and abundance of native AMF species (Bender et al., 2019), null or very limited root colonization in field conditions (Berruti et al., 2017)

As a summary of the previously mentioned studies, it appears that the positive effects of AMF tend to be more constant when tested in controlled environmental and soil conditions, while they become much more variable in the case of field experiments where soil conditions, especially in terms of nutrient (mainly P) availability and presence of native AMF tend to return erratic responses by AMF inoculants.

In both cases, the positive responses tend to be more pronounced under stressing crop conditions (drought, limited phosphorous availability, and other abiotic and biotic stresses), while in optimal agronomic conditions the advantage is more limited or null.

Considering these results, in the case of a high-input agricultural system, like that of seed production, AMF appear as an opportunity to consolidate field production reliability by minimizing the negative effects of adverse seasonal conditions and to improve corn production sustainability by better balancing the agricultural inputs.

1.6 The importance of soil microbial biodiversity

Biodiversity expresses a measure of richness and variability of living communities, species and genes, in space and time (Wilson, 1988; Sepkoski, 2015). A high level of biodiversity is normally desirable and considered the expression of a healthy ecosystem. In particular, microbial diversity, which is still largely uncharted, is fundamental for preserving the global genetic resources (Gill and Dhanda, 2018).

Soil ecosystems harbor a huge microbial diversity, much higher than that of eukaryotic organisms. Despite some activities of soil microorganisms are being known for decades, like their role in the degradation of organic matter or the cycle of nitrogen, until recent years the study of microorganisms have been limited due to the lack of technologies able of deeply investigating the genetic complexity of the microbial world. The introduction of molecular technologies, capable of analyzing the nucleic acid composition of microorganisms, then allowed to boost our knowledge on microbial diversity and especially on those species that cannot be grown in-vitro, that account for at least 99% of the total species present in the soil (Vartoukian et al., 2010; Chaudhary et al., 2019).

Despite the well-known role played by many microorganisms in several processes and biogeochemical cycles in the soil, the mechanisms at the base of their activity and those that regulate their efficacy are still partly unclear. For instance, soil organic matter is estimated to be by far the largest carbon reservoir in nature, containing more than three times as much carbon as that stored either in water or in the atmosphere. Beside this, it remains unclear why the soil organic matter, which is by nature a thermodynamically unstable compound, can persist for millennia under certain conditions, while it can be quickly degraded in others. This phenomenon led to the view that the destiny of soil organic matter is not mainly defined by its intrinsic properties, but it is mainly influenced by the physicochemical and biological properties of the environment that surrounds it. In other words, the stability of soil organic matter depends on ecosystemic properties, more than molecular properties (Schmidt et al., 2011).

Given the importance of soil as a carbon reservoir, it becomes clear how these mechanisms can also be important for the release of greenhouse gases (GHG), as a result of soil organic matter degradation. This is mediated by soil microorganisms through processes that are influenced by

physicochemical soil properties. A better understanding of these processes and of the interactions between soil microbiota, soil organic matter, soil properties and pedoclimatic conditions at the base of GHG emission, can be of great help on facing the global warming issue (Sellitto, 2020).

As mentioned in previous paragraphs, soil microbes play an important role in the cycle of nutrients and in making them more available to plants: this can happen through the mineralization of organic matter, but also through the deterioration of primary minerals into secondary minerals. Moreover, the microbic biomass turnover and the release of microbic substances, like enzymes are supposed to play a role in the development and stabilization of soil aggregates (Sellitto, 2020). Microbes are also supposed to play a role on determining soil magnetic properties (Chiellini, 2019).

On the base of the above-mentioned examples and of the other known and suspected roles that microorganisms play in the soil, it appears clear how soil health and properties are strongly influenced by them. This is not just the role of a single or few species but it's a complex ecosystemic interaction that might be understood as much as possible in its entirety and complexity.

Studying the soil microbial biodiversity is then paramount for better understanding some of the most important problems and challenges of our time, like global warming and soil health depletion, but also for putting in place the adoption of sustainable agricultural practices that can conjugate the need to produce more food with that of protecting the health of our soils and of our planet.

Chapter 2

Aim of the project

Increasing the sustainability of human activities is one of the biggest challenges of our time and it's an unavoidable way to mitigate the global warming effect that is rapidly affecting our world and that threatens the environment, our safety, and our future. Doing this in a context of growing world population and while safeguarding our security and the world's economic growth is surpassingly hard: as we often move on an unexplored land, the role of research is critical as well as it's paramount the involvement of all the different subjects that can play a role in this process. The collaboration between UniMoRe and Corteva Agriscience on this project goes in this direction, joining the effort of public research to a private company with a strong commitment on sustainability and a large influence on world's agriculture.

Agriculture is in a pivotal position in this context. Farming needs in fact to reduce its environmental impact and its greenhouse gas emissions, but at the same time, can provide resources for minimizing the impact of other activities like providing renewable energy sources or increasing carbon storage in the soils via atmospheric sequestration. On the other hand, agriculture is also primarily threatened by global warming that affect its productivity and reliability.

In line with the strategic objective reported above, this research project aims to improve the sustainability of Corteva's seed corn production activities, in line with Corteva's sustainability commitment, while maintaining or improving the current levels of yield, quality and reliability.

To reach this objective, the study and exploitation of soil microorganisms was identified as the most promising and feasible strategy. In this sense, two main approaches have been identified and pursued:

1. The study and development of novel sustainable practices based on soil microorganisms to be applied in the current seed corn production system
2. The study of soil microbiota to understand how it can impact seed production results and how we can take advantage of it

The first approach is described in chapter 3. In this experimental activity, the use of arbuscular mycorrhizal fungi (AMF) in seed corn production was tested to improve seed corn production

yield and reliability by taking advantage of biological inoculants as a way to minimize stresses on plants in an eco-friendly way.

The second approach is related to the soil microbiota characterization, reported in chapter 4, through which the soil microbial population from 20 different seed corn fields across Northern Italy have been characterized and studied, with the objective to understand the main microbial drivers for yield and acquire information about their exploitation for a more sustainable production.

Chapter 3

Application of AMF on seed corn in greenhouse and field conditions

3.1 Introduction

Maize is among the thousands of plant species that act as mycorrhizal host. The symbiosis between a plant and an AMF establishes a mutual trade between the two actors: while the fungus can benefit of an increased carbon flow coming from the host, the plant can obtain a number of advantages tied to an increased water and nutrient uptake and to a better tolerance to biotic and abiotic stresses (Smith et al., 2009; Rasmann et al., 2017).

Despite some literature is available about the effects of AMF on maize inbred line, they tend to mainly be focused on root colonization by naturally available AMF and on their ability to reduce the negative effects of phosphorous deficiency (Almagrabi and Abdelmoneim, 2012; Hao et al., 2008; Wang et al., 2020) or drought (Boomsma and Vyn, 2008). Moreover, most of them are aimed at studying the effects of naturally available AMF, rather than that of additional inputs of selected AMF species, under ordinary growth conditions.

To better evaluate the effects of mycorrhizal inoculants on maize inbreds, a series of dedicated greenhouse and field trials have been planned during 2019 and 2020, in Northern Italy, thanks to the collaboration between UniMoRe and Corteva Agriscience. The main aim of these trials was to explore the potential benefits of AMF on seed corn production performances and seed quality traits, with special regard on their ability to colonize maize roots, on putting in place symbiosis and on improving seed production reliability.

3.2 Materials and methods

Both greenhouse and field experiments have been conducted on the same maize inbred line, which won't be furtherly identified, as well as the reasons why it was selected for these trials, due to confidentiality clauses.

Two different species of AMF have been selected for these trials, after having been identified as the most promising ones, both for their intrinsic characteristics and for the purposes of the study.

The names of these species are also covered by confidentiality clauses and will henceforth be identified as AMF1 and AMF2.

A preliminary greenhouse trial was designed with the main aim to better understand the level of root colonization associated to the application of AMF inocula and to identify the most effective fungal species. Parallel to this, a field experiment was planned in order to test the same AMF on the same genetic material in realistic agronomic conditions.

3.2.1 Greenhouse trials

Two greenhouse experiments were carried out. The first experiment was planned at the beginning of 2019 in order to collect some data about the behavior of the 2 selected AMF species with the target maize inbred line, in a controlled environment.

The trial was conducted taking advance of the greenhouse available at the Corteva seed production plant in Sissa Trecasali (PR), Italy. No heating system was used: the temperature control was only operated through an automated windows control, set to an optimal level of 25 °C. This however did not avoid to reach some cold temperature peaks (< 5°C) at the beginning of the trial, as well as some hot peaks (> 32°C) at the end, but allowed to collect some preliminary observations prior to the field trials, by anticipating maize growth cycle of about 1 month, compared to the production fields.

Two separated sectors were planned, one per each tested AMF. Each sector was organized as a randomized complete block design (RCBD), with four blocks.

Per each sector/AMF, the following treatments have been considered:

- 2 levels of AMF treatment:
    - Granular AMF applied at planting (AMF1 and AMF2)
    - Control check (CTRL)
- 2 levels of seed treatment:
    - Seed treated with fungicide (t)
    - Untreated seed (nt)

The seed applied fungicide is a standard commercially available seed treatment, that cannot be more precisely identified, due to confidentiality reasons.

Each experimental unit corresponds to a single corn plant, grown into a 15 L plastic pot (Figure 15). The growing medium was a universal gardening medium, which characteristics are reported in Table 1.

pH	7	**Exchangeable Na**	127.1 ppm
Limestone	0%	**Ca/Mg**	5.15
Organic C	28.20%	**Mg/K**	1.9
Total N	1.06%	**Soluble B**	2.091 ppm
Organic matter	48.70%	**Exchangeable Fe**	195.52 ppm
C/N	26.6	**Exchangeable Cu**	54.57 ppm
Assimilable P2O5	291.2 ppm	**Exchangeable Zn**	12.48 ppm
Exchangeable K2O	3029.8 ppm	**Exchangeable Mn**	58.15 ppm
Exchangeable Ca	12564.9 ppm		

Table 1: growing media composition

The medium was not sterilized and was mixed with 3% of perlite, prior to planting, in order to increase a bit its porosity and water retention characteristics, and with the following fertilizers, in order to provide a base fertilization level:

- NPK 18-5-10: 5 g pot^{-1}
- Single superphosphate 19% P_2O_5: 4 g pot^{-1}

Each pot was planted with 3 seeds to make sure to have one healthy plant per plot. In each planting hole an amount of granular AMF inoculum, equivalent to 20 propagules, was deposited right beneath the seed. Immediately after emergence, the redundant seedlings were thinned and only the best plants were kept for the subsequent data collection.

During plants growth, the following amount of ammonium sulphate (20% N_2O) was applied, to avoid nitrogen deficiencies, especially at later stages:

- 3 g pot^{-1} at V5 stage
- 3.3 g pot^{-1} at V6 stage

- 1.5 g pot^{-1} at V12 stage
- 1.5 g pot^{-1} at VT stage

All along the plant growth, the irrigation was provided through an automatic dripping system, able to distribute the same amount of water per plant, at exactly the same time.

Figure 15: the greenhouse trial at V5, V8 and VT growth stages

The data collection plan was defined, including the following traits and evaluations:

- Reaching of the main phenological growth stages in terms of days after planting (DAP) and observation of any remarkable phenological characteristic, potentially related to the different treatments, like plant height and stalk diameter.
- Evaluation of physiological traits, by using the SPAD-502 (Minolta, Japan) and Dualex (FORCE-A, Orsay, France) instruments at V8 and VT stages. While the first instrument measures the leaf content of chlorophyll, the second one measures the leaf chlorophyll content, the flavonoids (as sum of adaxial and abaxial side of the leaf) and anthocyanins. In addition, Nitrogen balance index (NBI) was calculated as the ratio between Chlorophyll and Flavonoid contents (Cerovic et al. 2005, 2012). Each measurement was conducted on the top leaf of each plant, at both growth stages, by replicating the measurements three times.
- Destructive root sampling to evaluate the level of root colonization by AMF, done at V8 stage on replication n. 1 and at full flowering on replications 2, 3, 4. During this sampling, fragments of thin roots were washed and sent to the lab for a dedicated staining

procedure, preliminary to the microscope evaluation of root colonization. The frequency of root colonization (F%) and average intensity of root colonization (M%) were evaluated.

- Destructive plants sampling at flowering: the whole aerial part of the plant was sampled and then completely dried in an oven at 65 °C for determining the total content of dry matter. The dried material was then milled and analyzed through an X-ray analyzer.
- Destructive root sampling to evaluate the level of root colonization by AMF, done at V8 stage and at full flowering. During this sampling, fragments of thin roots were washed in distilled water. Then, root samples were submerged in a staining solution of lactic acid 90% and cotton blue at 0,2 % concentration for 12 hours. Then the samples have been washed two times by transferring them into lactic acid for 2 hours per each washing stage (Berruti et al., 2013). Roots have then been stored into lactic acid, waiting for microscope analysis. Mycorrhizal frequency (F%), root system AMF colonization intensity (M%), and the occurrence of arbuscules in the root system (A%) were determined and calculated according to Trouvelot et al. (1986).

In 2020, the greenhouse experiment was carried out in the same way of 2019. After the positive results obtained in the first year, only the treated seeds were used.

3.2.2 Field trials

In order to test AMF1 and AMF2 in field conditions, during spring-summer 2020 a field trial was conducted into two seed corn production fields, representative of the main seed corn production areas in Northern Italy. In both locations, the same inbred line used for the greenhouse trials was being grown as a female inbred for hybrid seed production. In addition, the soil type was very similar except for the content of available phosphorous, which is supposed to play an important role in the activity and efficacy of mycorrhizae.

The first location was in Gossolengo (Piacenza province) (Figure 16), it had a silty clay loam soil, non-calcareous, sub-alkaline pH, high CEC and medium content of organic matter and a very high level of exchangeable phosphorous. The second location was in Castel Guelfo (Bologna province), it had a silty clay loam soil, slightly calcareous, sub-alkaline pH, high CEC and medium content of organic matter and low level of exchangeable phosphorous.

The experimental design was a randomized complete block design (RCBD), with three blocks and three replications. The following thesis were tested, both for AMF1 and AMF2, on 3 m^2 plots, cautiously separated by 5 m interspaces in any directions:

- AMF applied at planting time (Plant)
- AMF applied at V4-V6 stage as sidedress (Side)
- Untreated check (CTRL)

At planting time, the AMF have been applied directly into the planting furrow, while at V4-V6, they have been applied at 5-10 cm from the plants and then immediately buried. This was in order to simulate the most likely application strategies in view of a future machine distribution. In fact, granular mycorrhizae can likely be applied by the planter's microgranulator or through a dedicated distributor mounted on an inter-row cultivator.

An average AMF application rate of 25 propagules $plant^{-1}$ was considered for each AMF thesis.

Figure 16: the field trial in Gossolengo (Piacenza)

The data collection plan was defined, including the following traits and evaluations:

- Root sampling to evaluate the level of root colonization by AMF, done at flowering stage, in the first 10 cm of soil, close to the plant stem. During this sampling, fragments of thin roots were collected, washed. Then, in the laboratory, the staining procedure and microscope evaluation of root colonization were carried out, similarly to the greenhouse trials.
- Maize ears sampling: ears from each plot were harvested by hand when the grain moisture was at around 35%, similarly to the industrial process. Ears were collected into

separate mash bags and dried until the grain moisture decreased to around 12%. Ears were then shelled and grain yield data were collected, by simply weighing the grain and adjusting the grain moisture differences.

- Seed quality analysis: each grain sample was divided into 4 sizes on the base of seed dimension and shape (large round, large flat, medium round, medium flat) and the following analysis have been performed for each of them: count of the number of kernels per Kg, warm germination test, cold germination test. Both warm germination and cold germination tests were performed on two replications of 100 seeds each, on moistened filter paper rolls. For the warm germination test, they were put at 25 °C and 100% moisture for 7 days, while for the cold germination test a significant cold stress was generated in order to simulate the most difficult conditions that seeds can face in field conditions. Details about this last test cannot be disclosed, since it's a proprietary procedure.

3.3 Results

3.3.1 Greenhouse trials

In the first trial, the evolution and reaching of growth stages was uniform among the different thesis: no remarkable differences were observed during the trial execution about the average results in terms of days after planting (DAP) to reach each growth stages (Figure 17).

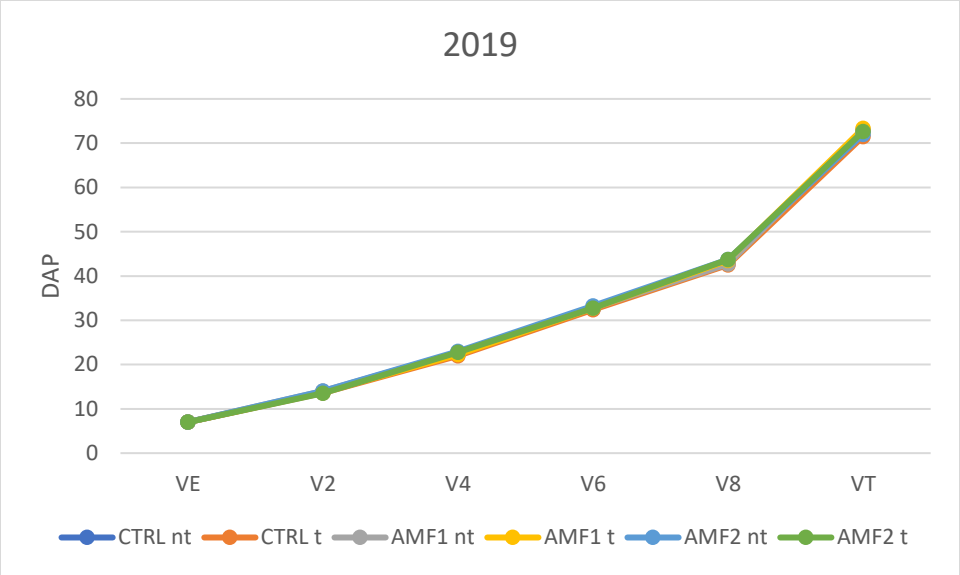


Figure 17: evolution of growth stages in 2019

In 2020, the GDU (Growing Degree Unit) accumulation was more intense, so that the plants reached the different growth stages quicker than in 2019 (Figure 18).

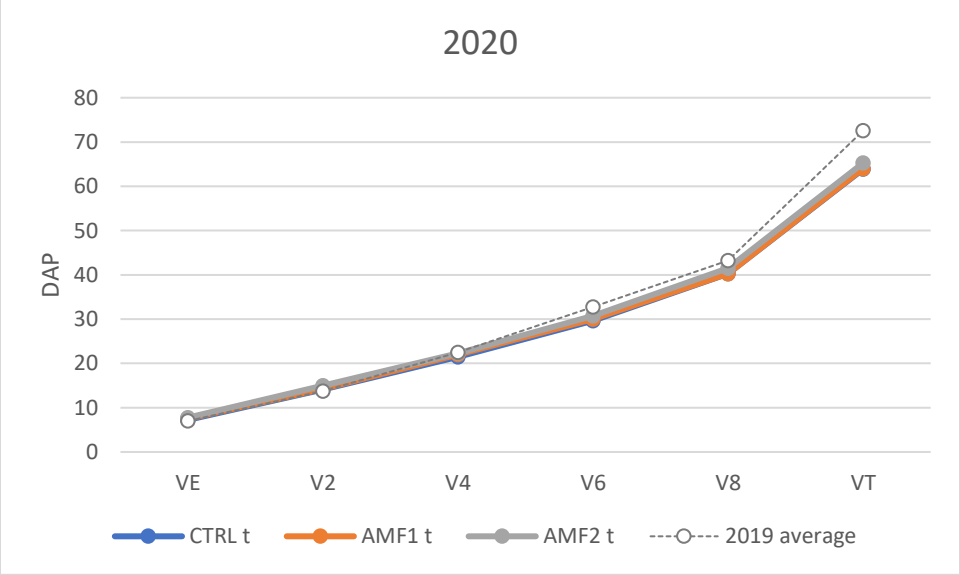


Figure 18: evolution of growth stages in 2020

Measurements about plants physiological activity, as related to leaf content of chlorophyll and polyphenols, done with SPAD and Dualex instruments, are reported in Figure 19 and didn't return

statistically significant differences between the thesis, across the two years of trials. Despite this, an overall increase in the concentration of polyphenols (Flavonoids, Anthocyanins) was observed, along with a decrease in the Nitrogen Balance Index (NBI) and chlorophyll concentration (measured both with the SPAD and Dualex instruments), when AMF were applied on treated seeds. This general (but not significant) trend was observed in 2019 for both the AMF and in 2020, when it was mostly detectable for AMF2. Overall, in 2019, the seed treatment was the main driver for all these parameters, while the AMF treatment was secondary.

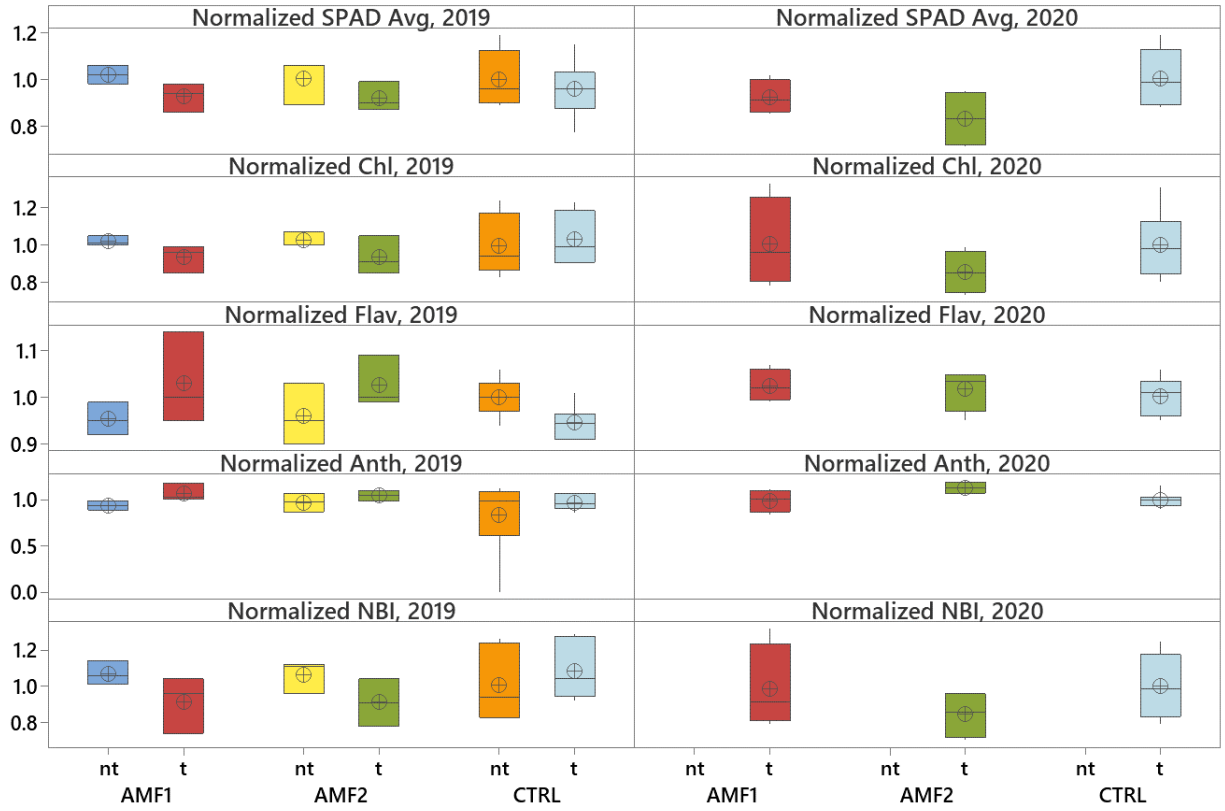


Figure 19: Results of SPAD and Dualex measurements on leaves at VT stage.

AMF1 and AMF2: mycorrhizal treatments; CTRL: Control treatment; Chl: leaf chlorophyl content; Flav: Leaf flavonoid content; Anth: leaf anthocyanin content; NBI: nitrogen balance index; t: Seed treated with fungicide; nt: Untreated seed

Regarding the phenological traits, AMF2 had a significant effect at 95% C.I. (Confidence Interval) in 2019, on reducing the maximum stalk diameter, compared to the CTRL check. No other significant effects were found. However, an overall reduction of the plants stalk diameter (Figure

20) was observed for the thesis treated with both AMF1 and AMF2 in 2019 and with AMF2 in 2020.

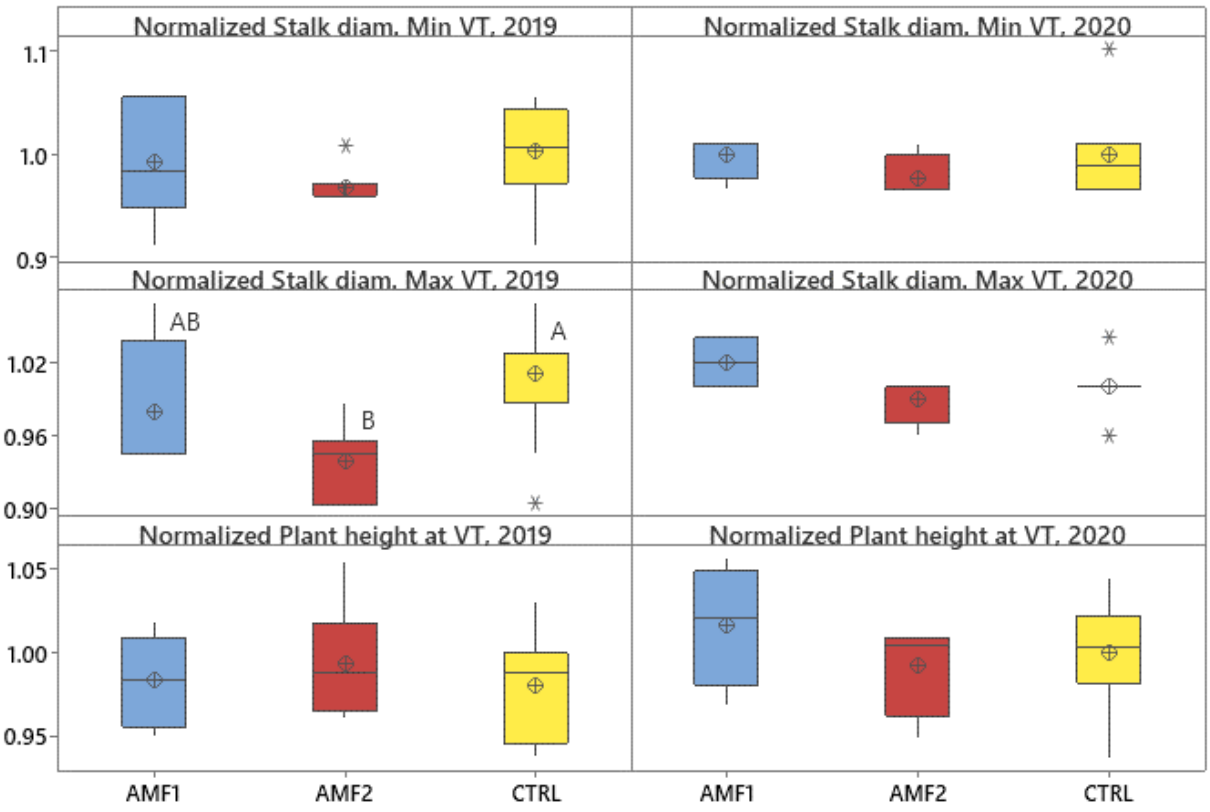


Figure 20: results of phenological measurements at VT stage. 2019 boxplots combine both treated and untreated seed. Different letters indicate statistically significant differences among treatments. * = outliers

The aerial dry matter (DM) production and repartition (Figure 21) was perfectly aligned with the above results and showed significant effects. In 2019 the total aerial dry matter was significantly lower for both AMF and this was mainly due to a significant reduction in the stalk dry matter accumulation, in line with the diameter decrease previously mentioned. In 2020, only AMF2

showed a significant negative effect on the total dry matter, again related to a significant stalk dry matter decrease. An average (but not significant) reduction was also observed for leaves dry matter.

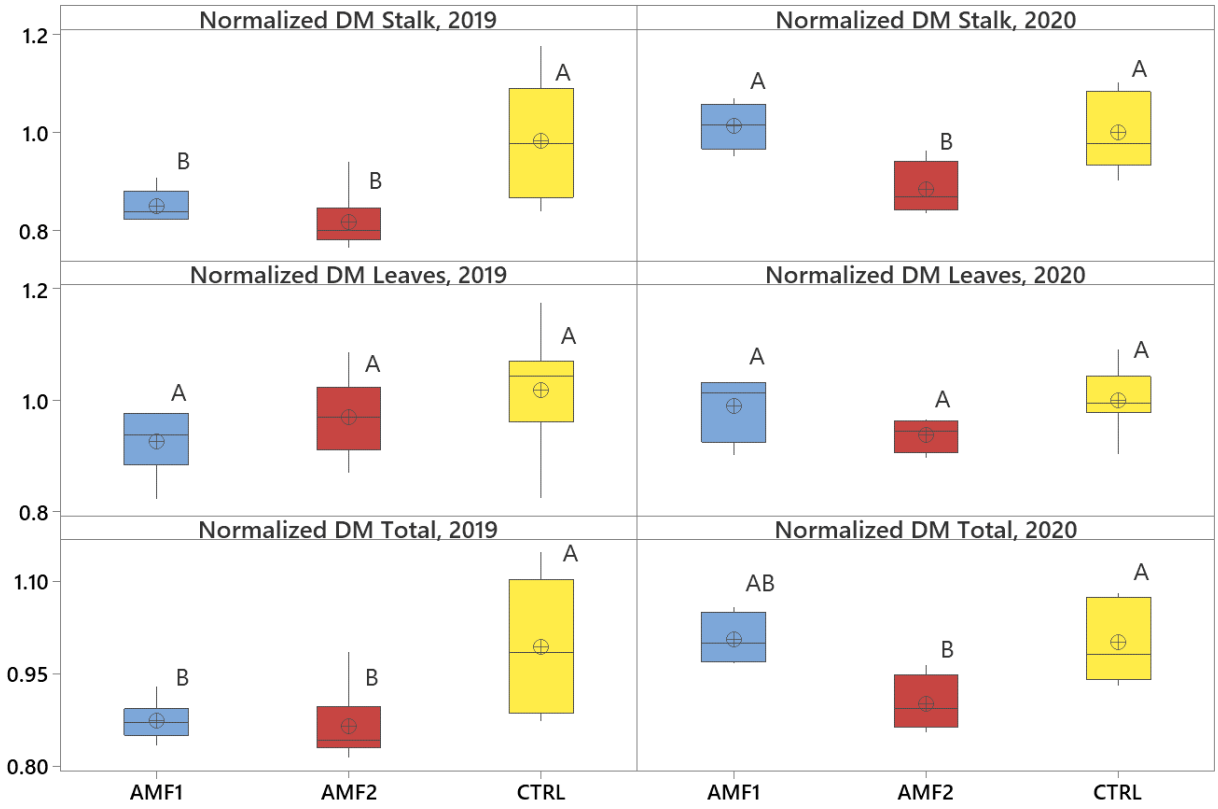


Figure 21: aerial dry matter accumulation and repartition at VT stage. 2019 boxplots combine both treated and untreated seed. Different letters indicate statistically significant differences among treatments. DM = Dry matter

The analysis of the concentration of the main chemical elements into stalks and leaves, summarized in Figures 22 and 23, only highlighted few significant differences between the tested thesis. The main differences found are:

- A significantly increased Zinc (Zn) accumulation in stalks in 2019 for both the AMF thesis compared to the CTRL check and a significant decrease for the seed treated thesis (t) compared to the untreated ones (nt). In 2020, only AMF2 led to a significant zinc increase in the stalks, while AMF1 led to a non-significant average decrease.
- A significant increase of magnesium (Mg) in leaves in 2019, associated to both AMF, not confirmed in 2020.
- A decrease in leaves nitrogen (N) concentration in 2019 for both AMF, but only significant for AMF1. This effect was not found in 2020, even though AMF1 led to a non-significant nitrogen decrease.
- An increase in leaves silicon (Si) concentration in 2019 for both AMF, but only significant for AMF2. No significant differences were measured in 2020, but an average silicon increase was observed for both the AMF thesis.
- A significant decrease in leaves calcium (Ca) concentration in 2020, associated to AMF2. This effect was not observed in 2019 where a non-significant calcium increase was observed for both AMF.
- A significant increase in leaves phosphorous (P) concentration in 2020, associated to AMF2, not observed in 2019.

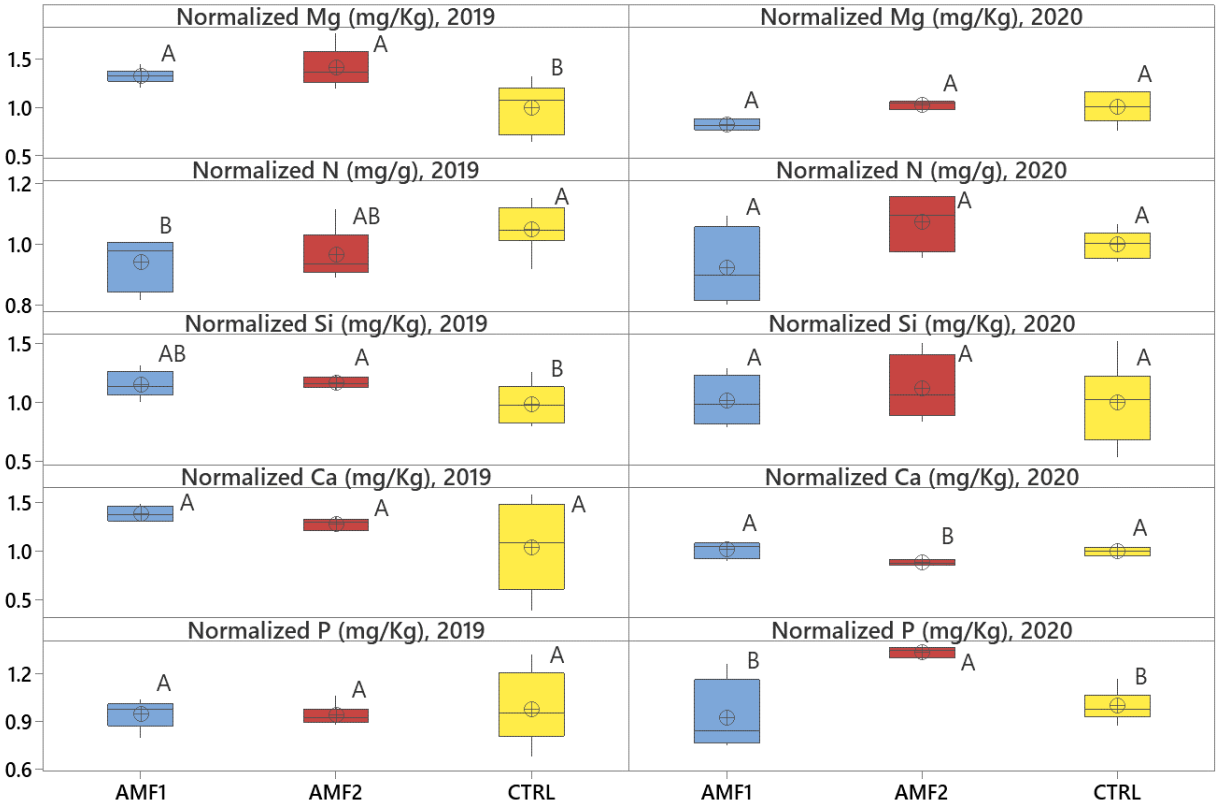


Figure 22: most significant comparisons of mineral elements content in leaves. 2019 boxplots combine both treated and untreated seed. Different letters indicate statistically significant differences among treatments.

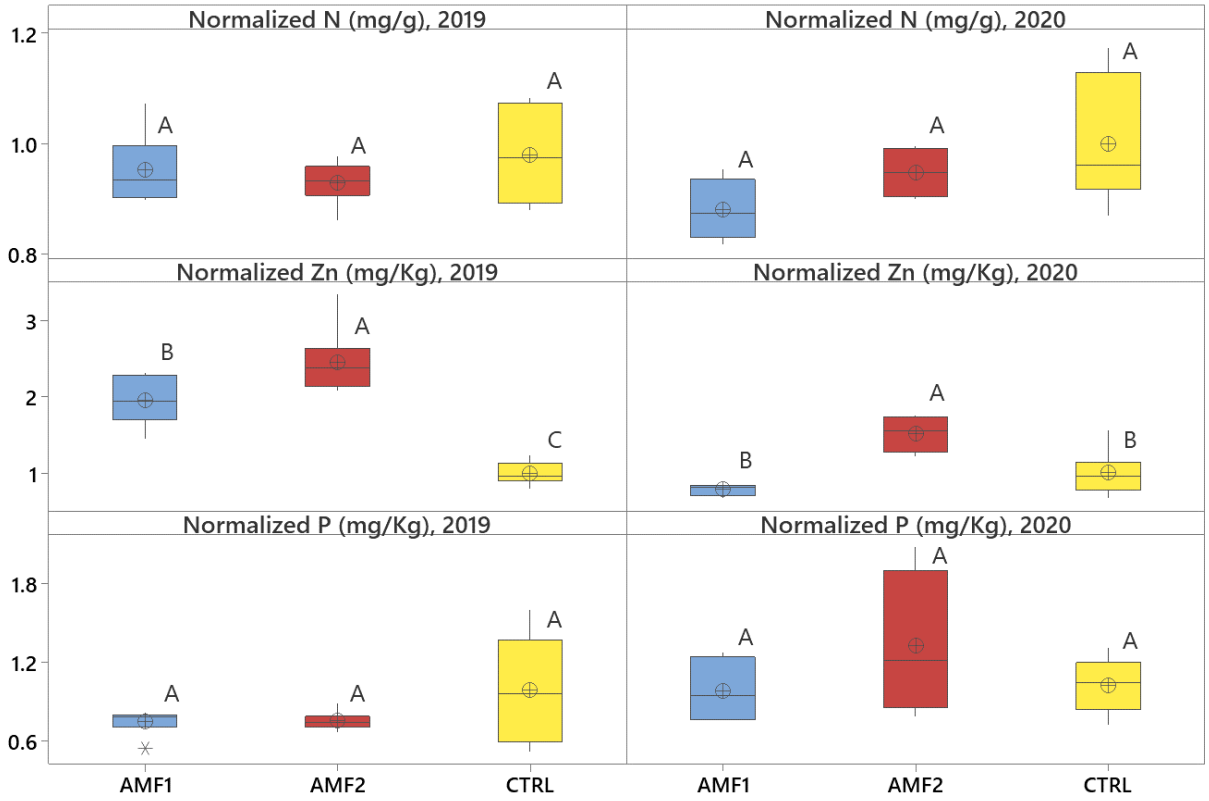


Figure 23: most significant comparisons of mineral elements content in stalks. 2019 boxplots combine both treated and untreated seed. Different letters indicate statistically significant differences among treatments.

The microscope evaluation of actual root mycorrhization highlighted no root mycorrhization at v8 stage in 2019. Despite this, a clear success of both the AMF inocula on effectively colonizing the roots was evaluated at flowering stage in both years. The frequency of mycorrhization (F%), indicating the percentage of roots where traces of AMF were observed, was clearly and significantly higher for both AMF1 and AMF2, compared to CTRL (Figure 24). In 2020, AMF2 returned a significantly higher level of F%, compared to AMF1, with around 95% of root fragments showing the presence of mycorrhizal structures. In 2019, plants from fungicide treated seeds showed a slightly lower level of F%, compared to those from untreated seeds. The level of F% remained anyway consistently and significantly higher than that of the CTRL thesis. In both years, the CTRL thesis showed a very low level of mycorrhization, likely due to few AMF, natuarally available in the soil media used for this experiment.

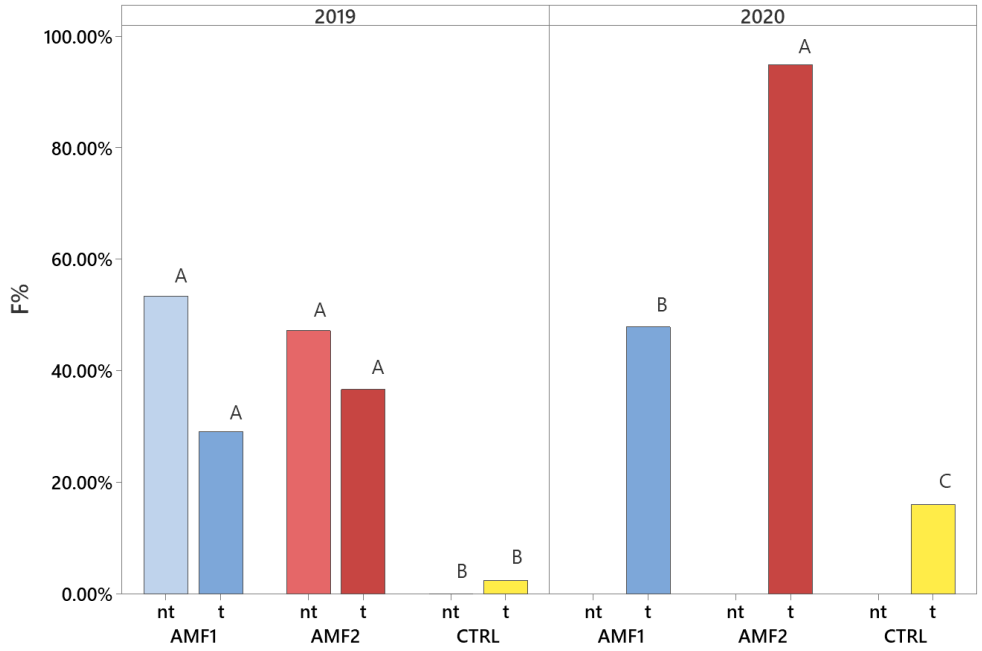


Figure 24: frequency (F%) of root mycorrhization in 2019 and 2020 plants. Different letters indicate statistically significant differences among treatments.

The mycorrhization intensity (M%), expressing average mycorrhizal coverage of the analyzed root fragments, follows exactly the same trend. The only difference with F% is that in 2019, AMF1 and AMF2 samples from fungicide treated seeds, are not significantly different from the CTRL thesis, even though consistently more intensely colonized (Figure 25).

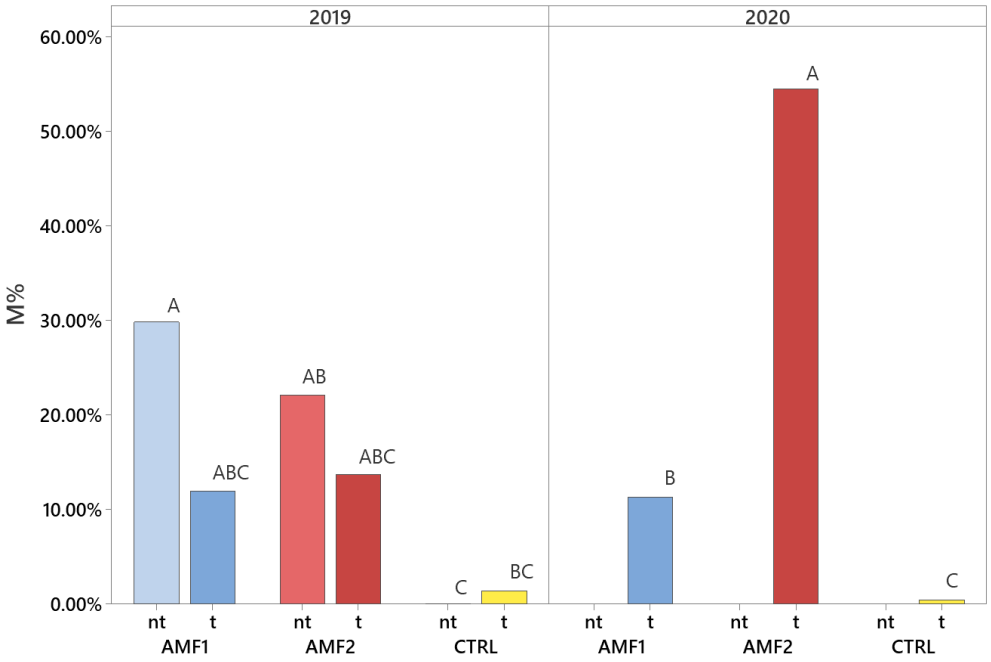


Figure 25: intensity (M%) of root mycorrhization in 2019 and 2020 plants. Different letters indicate statistically significant differences among treatments.

3.3.2 Field trials results

The microscope analysis of root fragments highlighted a certain efficacy of both the AMF inocula. In terms of mycorrhization frequency (F%), the field in Bologna area showed an important and statistically significant increase for all the inoculated thesis, except for AMF1 applied at sidedress. A higher mycorrhization frequency was observed in case of application at planting in comparison with sidedress.

The field in Piacenza area showed a not significant increase of F% for both the AMF1 thesis and an average slight decrease for AMF2, compared to the CTRL check. Both the AMF1 thesis were significantly better than the AMF2 applied at planting (Figure 26).

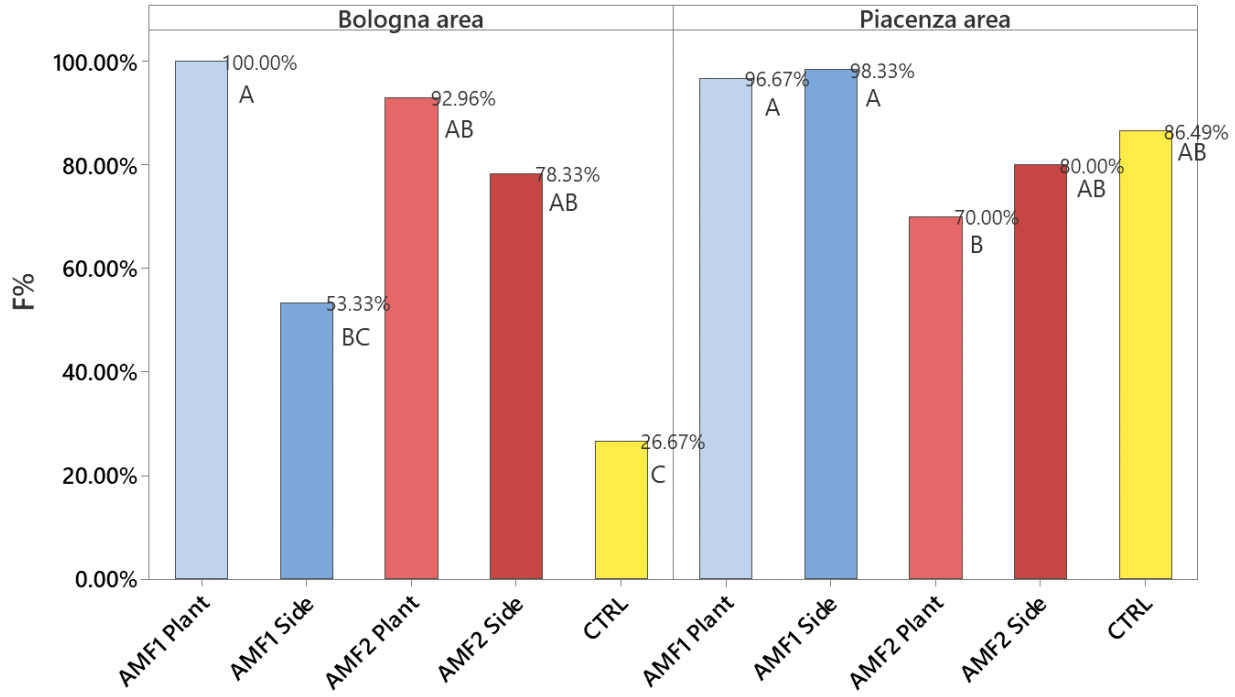


Figure 26: frequency (F%) of root mycorrhization in 2020 field experiments. Different letters indicate statistically significant differences among treatments.

In terms of mycorrhization intensity (M%), all the inoculated samples, from both locations, showed an increase of M% values (Figure 27). In Bologna area only the AMF1 applied at planting showed a significantly increase in M% values, compared to the CTRL check, while in Piacenza location, there were no statistically significant differences.

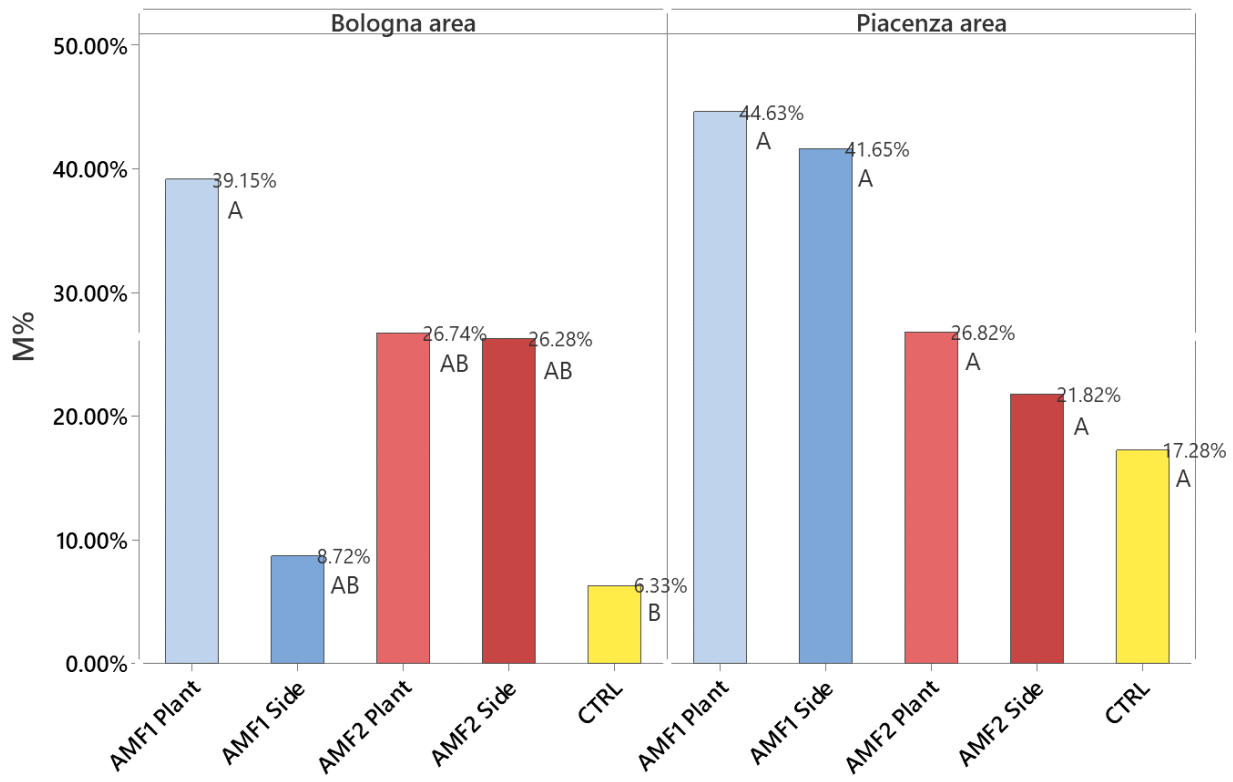


Figure 27: intensity (M%) of root mycorrhization in 2020 field experiments. Different letters indicate statistically significant differences among treatments.

Regarding yield results, thesis where AMF was applied showed an overall 3-5% increase in grain yield compared to control untreated. However, this increase was not statistically significant. Combined normalized yield results from both locations are showed in figure 28.

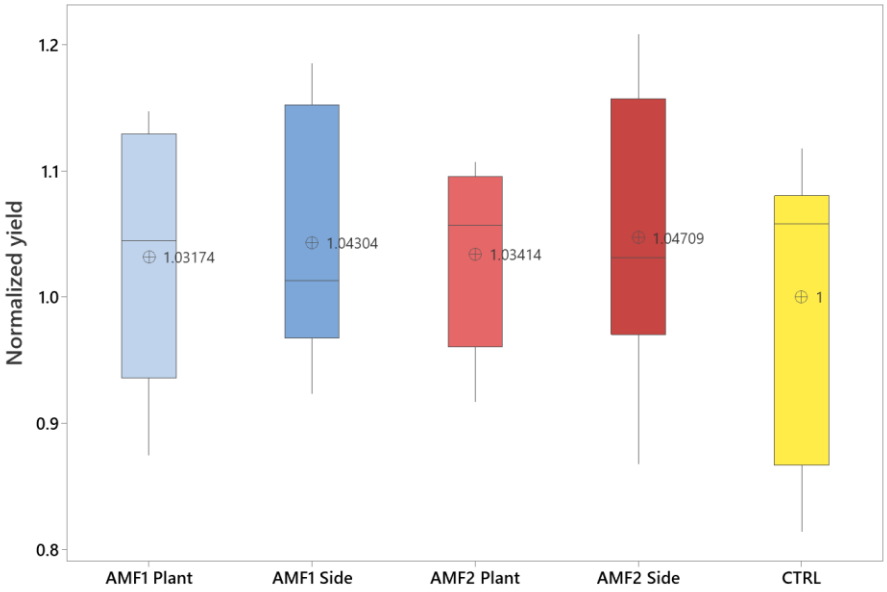


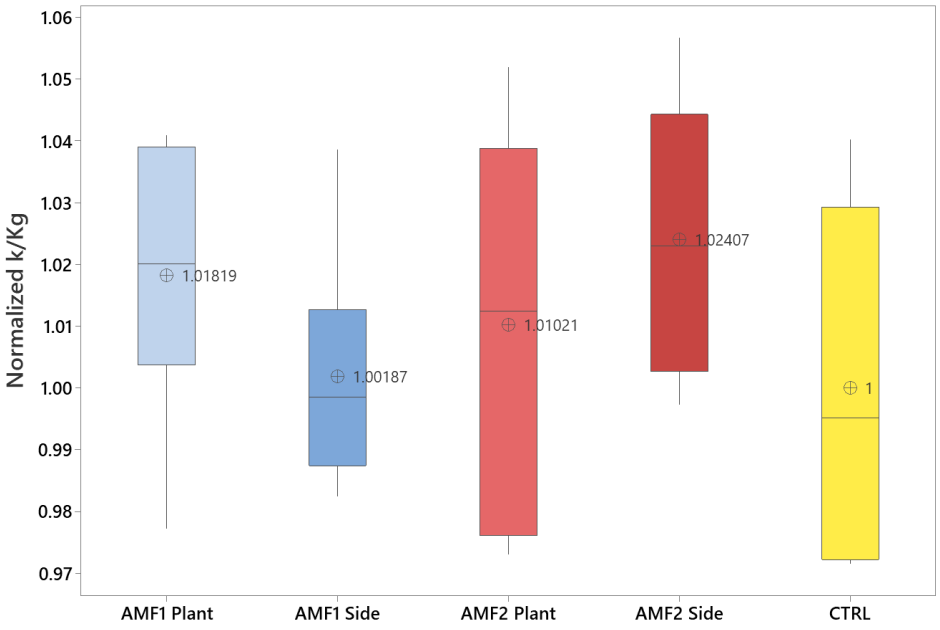
Figure 28: normalized yield results, combining both locations.

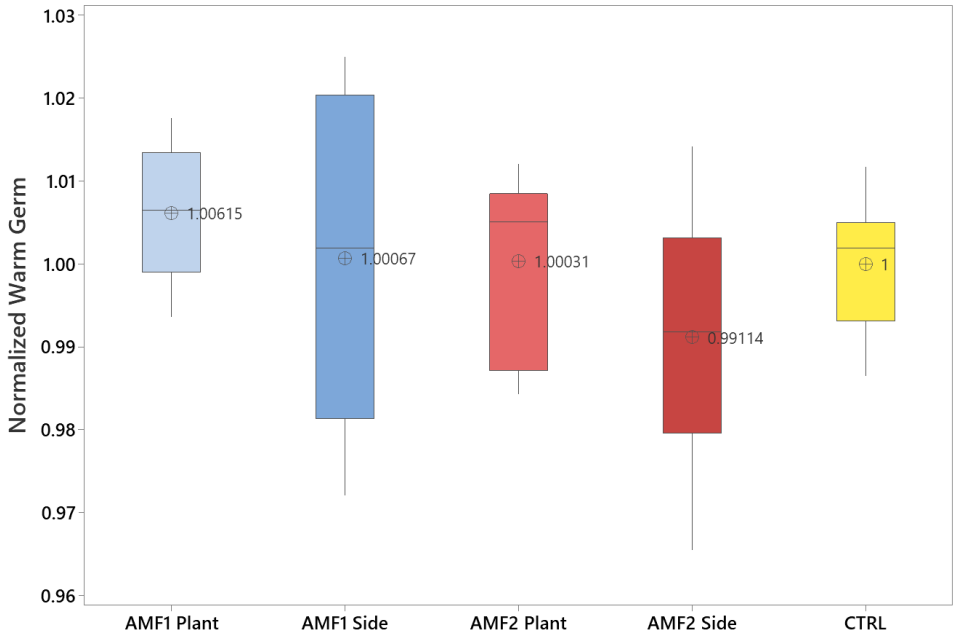
This was mainly due to a consistent average yield increase measured in Bologna area, while in Piacenza, the AMF effect wasn't clear, as summarized in table 3:

Location	Treatment	Average grain yield (kg/ha)	%Diff vs CTRL
Piacenza area	CTRL	3269	
	AMF1 Plant	3277	0.26%
	AMF1 Side	3223	-1.39%
	AMF2 Plant	3338	2.11%
	AMF2 Side	3243	-0.79%
Bologna area	CTRL	3260	
	AMF1 Plant	3327	2.07%
	AMF1 Side	3564	9.34%
	AMF2 Plant	3348	2.72%
	AMF2 Side	3420	4.90%

Table 3: main yield results by location and treatment

In terms of seed quality, no statistically significant differences were found for any of the parameters evaluated in this study (Figure 29). The number of kernels per Kg was practically unaffected by the different treatments, as well as the warm germination. The cold germination, despite the lack of significant differences, showed a certain average decrease for the AMF2 thesis.


Normalized k/Kg
1.06
1.05
1.04
1.03
1.02
1.01
1.00
0.99
0.98
0.97
1.01819
1.00187
1.01021
1.02407
1
AMF1 Plant
AMF1 Side
AMF2 Plant
AMF2 Side
CTRL


Normalized Warm Germ
1.03
1.02
1.01
1.00
0.99
0.98
0.97
0.96
1.00615
1.00067
1.00031
0.99114
1
AMF1 Plant
AMF1 Side
AMF2 Plant
AMF2 Side
CTRL

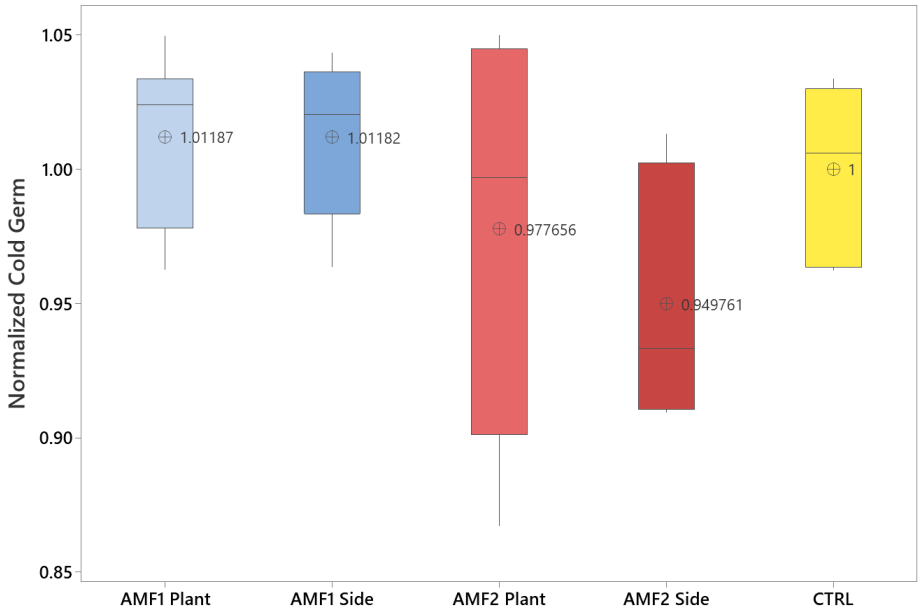


Figure 29: normalized seed quality results, combining both locations

3.4 Discussion

During the execution of the greenhouse trials, the AMF did not significantly influence the plant growth cycle and did not generate any remarkable effect on seedlings and plant vigor. In fact, all thesis reached the key growth stages contemporarily.

The overall trend observed for the SPAD and Dualex measurements, despite not significant, could likely be associated to an increased nitrogen stress for both the AMF thesis. In fact, it was already observed that in case of nitrogen deficiency, the plant produces less chlorophyll (Ercoli et al., 1983; Varvel et al., 1987) and directs its metabolism toward an increased production of flavonoids (Davies et al., 1964; Stewart et al., 2002). The Nitrogen Balance Index parameter summarizes those two effects and demonstrates this link. This effect seems in line with what was observed by Wang et al. (2018), when a reduced uptake of nitrogen was measured in plants treated with AMF, grown in limited pot conditions. This might explain the phenomenon observed especially in 2019, when plants treated with AMF started to show symptoms of nitrogen

deficiency on basal leaves before the CTRL checks. Despite working with wild perennial plants, Reynolds et al. (2005) also observed that AMF could compete for nitrogen and depress plants growth in a constrained rooting volume at low nitrogen supply. It is not actually clear why this phenomenon was mainly detectable on plants grown from treated seeds. No conclusions can anyway be drawn on this, since no statistically significant differences were found: further tests would be needed to better investigate the role of AMF on this phenomenon.

The stalk diameter reduction tied to AMF1 and AMF2 in 2019 and to AMF2 in 2020 appears to be the result of a stress associated to AMF and it's perfectly in line with the physiological measurements. The same is true for the dry matter (DM) accumulation where, again, a consistent stalk and total dry matter decrease, were tied to both AMF in 2019 and to AMF2 in 2020.

Regarding the analysis of mineral concentration in plant tissues, zinc concentration in stalks looks connected with the AMF activity and somehow correlated with the above reported effects. In addition, zinc accumulation was reported as typically related to AMF (Subramanian et al., 2011; Coccina et al., 2019). Also, some other differences (P increase, N decrease) look related to the activity of AMF and in-line with some previous literature (Reynolds et al., 2005; Wang et al., 2018; Karasawa, 2000; Camargo Gomes Stoffel et al., 2020), even though the results are sometimes inconsistent when comparing 2019 with 2020.

The microscope analysis done on root samples in both years confirm the activity of both AMF on successfully colonizing corn plants. This activity was not measurable at V8 stage and was very clear at flowering, suggesting that it takes time for the AMF and the plant to establish the symbiosis. Having it in place at flowering stage looks important, since this is the most sensitive stage for maize plants as related to water and environmental stresses and the one when they might receive the biggest benefit from an improved water and nutrients uptake, thanks to AMF. The level of both mycorrhization frequency and intensity at flowering stage was in fact consistently and significantly higher than the CTRL checks in both years. The fungicide seed treatment reduced only slightly those parameters, but still maintaining a remarkable level of mycorrhization: this can suggest both a potential negative interaction of the fungicide with the

AMF, and at the same time, it confirms the possibility to get a satisfactory level of root inoculation and symbiosis also in case of treated seeds.

Root fragment analysis from the field trials confirmed these results, even though the differences are less clear, likely due to natural presence of AMF in the trial locations and especially in Piacenza area. Despite this, a clear overall mycorrhization increase was observed in Bologna, both in terms of frequency and intensity, and an increase in terms of mycorrhization intensity was measured in Piacenza, even though not significant. Results from both locations suggest that sidedress application of AMF inocula can be an effective possibility, though planting application appear to be the most effective option.

The effect of both AMF treatments on yield, despite not being statistically significant, was clearly positive in Bologna location, but substantially null in Piacenza. As previously reported, Bologna location had a much lower content of exchangeable phosphorus in the soil, the lowest level of root mycorrhization on the CTRL thesis and showed the most important differences in terms of mycorrhization frequency and intensity, when AMF were applied. This could explain the different results in the 2 locations. Despite further tests are needed to better confirm this hipothesi, the application of AMF looks promising as a way to increase productivity and minimize the negative effects of stresses on plants.

The quality results showed almost no differences, apart from the negative, but not significant effect of AMF2 treatments on cold germination. This last observation is an important point to consider in view of further stress, since it will be critical to measure and minimize similar negative effects and better understand what is causing them, in case they didn't just happen by chance.

3.5 Conclusions

Both the greenhouse and field experiments showed a clear activity of either AMF on effectively establishing symbiosis with corn plant and influencing the plant physiological activity and some phenotypic parameters. The field experiments also highlighted a potential application of AMF for seed corn production, paving the way for further studies and more detailed experiments, to be

able to run stronger and more targeted statistics and better investigate any negative consequences on seed quality. Since both AMF provided positive and promising results, it's not easy to say which one worked better, but combining results from both experiments, AMF2 provided the most stable results in terms of root inoculation, independently of the trial (greenhouse, field), field location and application time. It also ensured good seed production results, even though similar to AMF1.

Chapter 4

Characterization of soil and rhizosphere microbiome to improve seed corn yield and quality

4.1 Introduction

Most of the Italian maize production is concentrated into the Po valley, where maize growers apply very intensive agricultural techniques with a massive use of chemical fertilizers. Po valley is characterized by very different soil types and a fragile territory, already subject to high rates of nitrate pollution of fresh water, also due to a high concentration of livestock farming activities (European Environment Agency, 2012). Seed corn is in turn located into the Po Valley and mainly concentrated into few production areas in Emilia-Romagna, Lombardy and Veneto regions.

Maize seed production faces a series of biotic and abiotic constraints, and these issues are related to specific crop genetic and management factors. Since these factors alone cannot fully explain differences related to the problems detected, we hypothesize that a significant part of the remaining variability could be ascribed to soil microbiota x inbred line combination. Moreover, maize inbred lines are much less resilient than hybrid corn to stresses (Troyer and Wellin, 2009; Araus et al., 2010): in fact, yield results could decrease significantly in case of sensitive genetics and seed quality could be even more erratic. For this reason, tailoring maize root-microbiota combination can be crucial for the improvement of both yield and quality of hybrid seed production. The study of microbiome would allow a comprehensive analysis of the interactions between corn plants and the soil microbial communities leading to a better understanding of those taxa which can play a key role (positive or negative) on determining the crop success.

Harnessing the soil microbiota interacting with plants is a pivotal way to sustainably enhance maize production, however studies and knowledge are still limited by several constraints and namely the complexity of the edaphic microbial communities (Köberl et al., 2020), their interactions with corn plants, and the lack of biomarkers linking microbial with soil and plant traits. Exploiting new insights into the soil microbiota can be crucial to reduce the use of external inputs and provide farmers with more sustainable approaches, even in conventional systems, while maintaining or improving the current level of productivity and quality.

To overcome bottlenecks and better understand how soil microbes and their diversity influence crops biosustainability, health, nutrition and then performances, a project was defined as a

collaboration between the University of Modena and Reggio Emilia, Corteva Agriscience and BiomeMakers, an American provider of high-throughput microbiome characterization.

This project was possible thanks to BiomeMakers' Fields4ever initiative, a global initiative dedicated to conserving and monitoring soil health, with the aim to support the ongoing development of sustainable and respectful agriculture, ensuring quality soil and quality food for generations to come. This initiative was completely funded by European Union's "Horizon 2020" research and innovation program.

As a long-term objective, gaining a better awareness on how the soil microbiota can influence crop performances will allow us to advise breeders and farmers on the most effective plant-microbiota composition. Such microbiome management could sustainably enhance maize production in a given soil, and will be based on agricultural practices that can lead to an increased microbial biodiversity and to a more sustainable exploitation of soil potential and fertility. Furthermore, this study has the potential to become a reference framework to predict seed yield and quality for additional crops. We believe this will be particularly relevant for crops which genomic information are not available (e.g., minor crops) and/or accessible (e.g., developing countries). In the frame of a long-term goal of tailoring the type of soil and suitable crops, we aim to associate different soil microbial populations to specific seed corn yield and quality traits towards selection of suitable maize inbred line–microbiota combination for the best hybrid seed production in terms of quality and quantity. The results of this research can also be a reference baseline for developing microbial inocula tailored to seed corn or other crops in order to ensure the best growing conditions and prevent biotic and abiotic stresses.

4.2 Materials and methods

Experimental set up was based on consolidate experience of the team on microbiome analysis (Caradonia et al., 2019) and according to what reported recently on maize literature (Walters et al., 2018).

Four different female inbred lines, used as female parents in the production of F1 hybrids, were identified, as representative of Corteva's Italian production portfolio, including genotypes with stable genetics, as well as with more erratic ones, in terms of yield or quality performance. For each genotype, five different locations (fields) were chosen across Norther Italy and namely in the Po Valley, in order to explore the main seed corn production environments. Seed corn is in fact mainly being produced into three main areas (Figure 30):

- Western Po Valley (Piacenza, Parma, Cremona, Lodi provinces) with mainly silty-clay soils in Piacenza and Parma and loam soils in Cremona and Lodi
- Eastern Po Valley (Bologna, Ferrara and Ravenna provinces) with clay-loam to silty-clay soils in Bologna and Ravenna and silty-clay to loam in Ferrara
- North-eastern Po Valley (Venice province) with mainly silty-loam soils

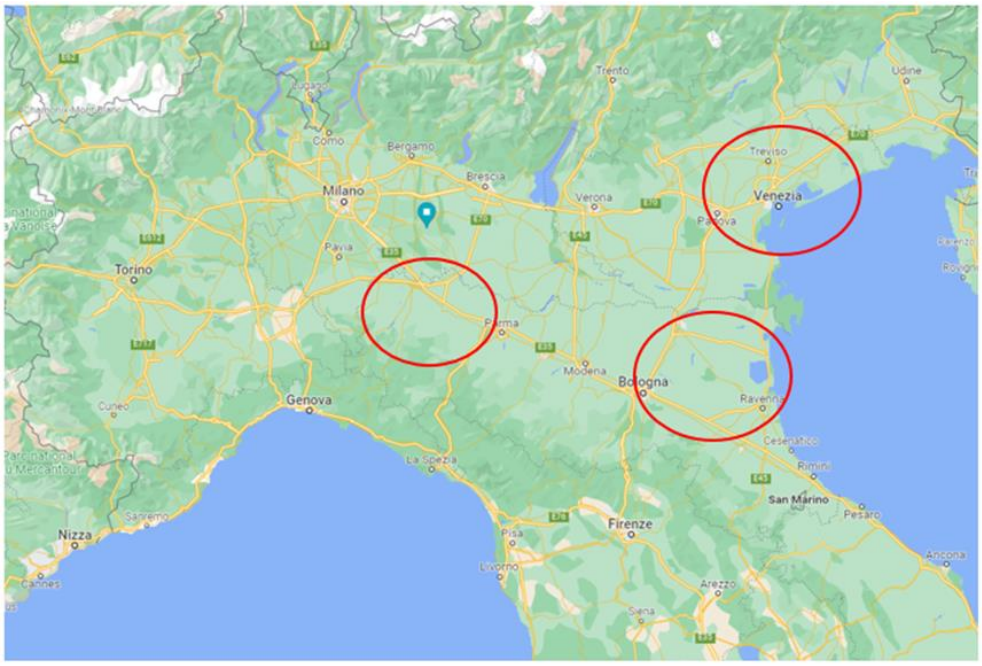


Figure 30: main seed corn production areas, where sampling was conducted

This regional and local variability allowed to explore several different soil types and environmental conditions in a rather limited region. The weather is a first important element to consider to better understand these differences: the 2020 growing season has been overall quite

favorable for corn production with a good level of precipitations all along the summer. As visible in Figure 31, the conditions in Western and Eastern production areas were comparable, with a very similar temperature pattern and a rather dry first part of the season, followed by some rainy events throughout July and August. Contrarily, the conditions were pretty different in the North-Eastern area, where the maximum temperatures were slightly lower and the amount of rain was higher, especially in June.

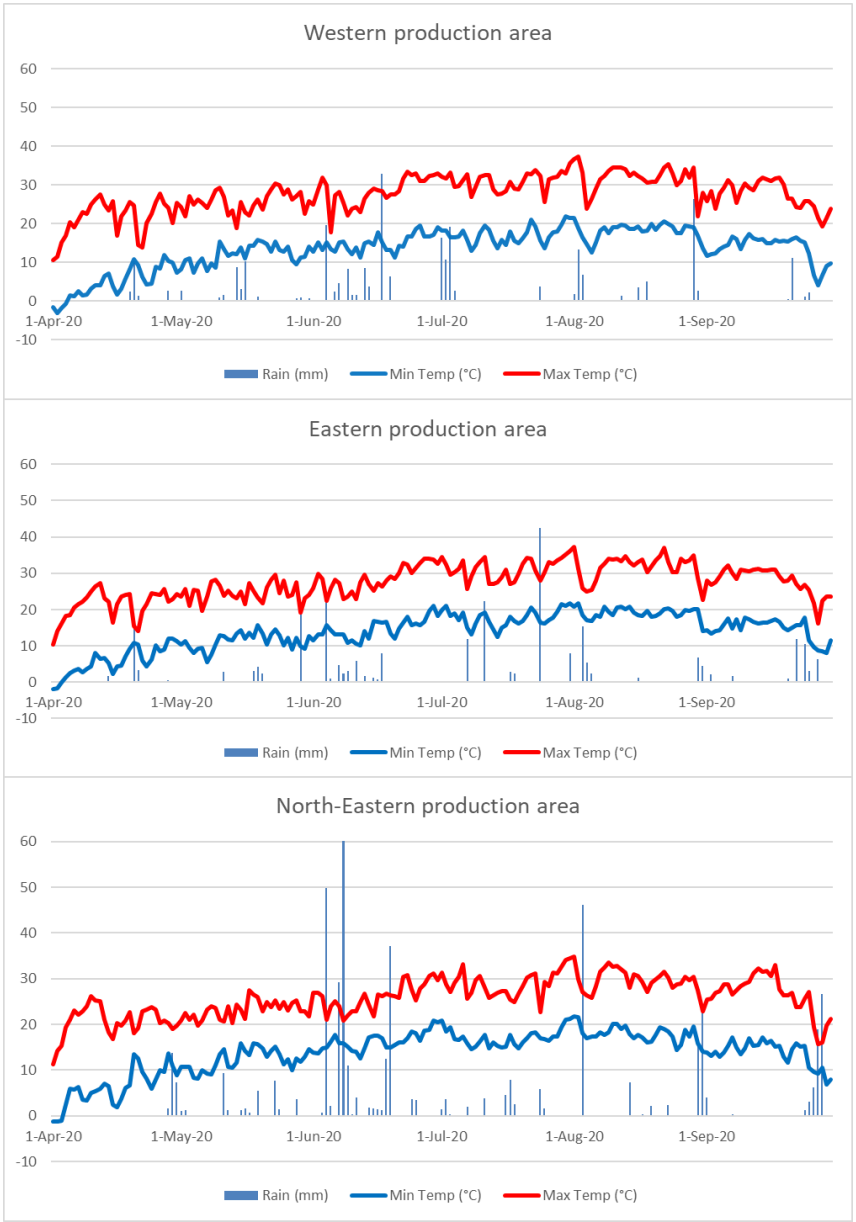


Figure 31: weather data from the three production areas as recorded in San Nicolò (PC), Castel San Pietro (BO) (ARPA Emilia-Romagna) and Eraclea (VE) (ARPA Veneto)

As shown in Table 4, locations selection allowed to explore quite a huge geographical variability and to ensure a good distribution of the selected genotypes into the three main production areas. In addition to that, a field conducted with organic management was included, to check eventual differences with the rest of the locations, all recurring to intensive agricultural practices.

Grower #	Area	City	Genotype	Fertile/Sterile	Female planting date	Row spacing	Agricultural management
1	West	Podenzano (PC)	A	Fertile	5-May	70 cm	Conventional
2	West	Podenzano (PC)	C	Sterile	4-May	70 cm	Conventional
3	West	San Pietro in Cerro (PC)	C	Sterile	20-May	70 cm	Conventional
4	West	San Pietro in Cerro (PC)	D	Fertile	27-May	70 cm	Conventional
5	West	Stagno Lombardo (CR)	D	Fertile	12-May	70 cm	Conventional
6	West	Roccabianca (PR)	D	Fertile	31-May	70 cm	Conventional
7	East	Castel Guelfo (BO)	A	Fertile	28-Apr	70 cm	Conventional
8	East	Medicina (BO)	A	Fertile	24-Apr	70 cm	Conventional
9	East	Castel San Pietro (BO)	C	Sterile	27-Apr	70 cm	Conventional
10	East	Imola (BO)	D	Fertile	25-May	70 cm	Conventional
11	East	Molinella (BO)	B	Fertile	22-May	70 cm	Conventional
12	East	Imola (BO)	A	Fertile	11-May	70 cm	Conventional
13	East	Faenza (RA)	D	Fertile	28-Apr	70 cm	Conventional
14	East	Ravenna (RA)	B	Fertile	11-May	70 cm	Conventional
15	East	Faenza (RA)	A	Sterile	25-Apr	70 cm	Conventional
16	East	Argenta (FE)	C	Fertile	18-May	70 cm	Organic
17	East	Comacchio (FE)	B	Fertile	15-May	70 cm	Conventional
18	East	Fiscaglia (FE)	B	Fertile	4-May	70 cm	Conventional
19	North-East	Caorle (VE)	C	Sterile	22-Apr	70 cm	Conventional
20	North-East	Eraclea (VE)	B	Fertile	3-May	70 cm	Conventional

Table 4: basic information about the locations included in the study

In each targeted field, samples of about 1 Kg of soil were collected in the range of 5 to 30 cm of depth, by joining subsamples collected in representative positions throughout the fields. While the first 5 centimeters of soil were removed as considered to be too affected by external environmental conditions, the remaining part was sampled with a dedicated soil test probe, up to 30 cm of depth. This ordinary soil analysis allows to get more precise data about each soil's characteristics and collect metadata to be used for statistical analysis and to be related to microbiota characterization results (Table 5).

Grower #	Texture	pH	CEC	Organic matter (%)	C/N	Sand (%)	Silt (%)	Clay (%)	Total N (g/Kg)	P_2O_5 (ppm)	K_2O (ppm)
1	Silty clay loam	7.6	29.2	3.17	9	19	41.7	39.4	2.05	741.3	173.5
2	Loam	7	15.3	2.2	7.9	38.4	45.3	16.3	1.61	93.9	168.2
3	Silty clay	7.9	27.7	2.2	8.2	12.3	47.2	40.5	1.55	19.6	309.8
4	Silty clay loam	7.7	25.5	1.8	7.4	17.1	45	38	1.42	99	244.1
5	Loam	7.9	14.8	1.59	10.9	35.8	46.3	17.9	0.84	26.6	178.8
6	Silt loam	7.9	19.8	2.01	8.4	17.6	56.4	26	1.39	33.3	409
7	Silty clay loam	7.8	26.9	2.27	8.2	18.9	42.5	38.6	1.61	25.8	364.6
8	Clay	7.9	30.9	2.87	8.7	19.1	36.7	44.1	1.92	130.8	501.8
9	Clay loam	8	22.7	1.55	11.5	22.2	44.3	33.5	0.78	14	305
10	Silty clay loam	7.9	27.5	2.74	8.8	18.7	43.4	38	1.8	35.8	393.4
11	Clay loam	8	21.6	2.54	11.4	27.9	45	27.1	1.3	75.7	344.2
12	Clay loam	8	20.2	1.94	9.6	24.4	48.6	27	1.17	26.7	305.8
13	Loam	8	18.8	1.92	10.2	31.4	44.2	24.3	1.09	35.4	264.2
14	Silty clay	8	31.1	2.42	8.9	6.5	47.2	46.3	1.58	43	303.6
15	Loam	7.9	17.6	2.52	10.8	37.8	42.8	19.4	1.35	38.4	176.9
16	Silty clay	7.9	31.1	3.92	10.8	14.1	45.7	40.2	2.12	104.4	421.9
17	Loam	7.8	21.9	2.75	12	23.3	49.9	26.8	1.33	44.4	287.8
18	Loam	7.9	19.9	2.13	9.9	26.5	48	25.5	1.24	71.6	229.2
19	Silty clay loam	7.9	24.7	3.74	10.4	13.9	57.9	28.2	2.08	75.1	339.6
20	Silt loam	7.9	21.1	2.82	10.2	15.1	59.9	25	1.61	65.1	281

Table 5: main soil characteristics at each location

In addition to this, additional agronomic data were collected all along the maize growing season: previous crops, agricultural practices (fertilization and pest management strategies), phytosanitary status (presence and incidence of leaves diseases and ear molds), grain/biomass yield (green yield, harvest moisture, dry grain yield), seed quality and germination parameters (seed size, warm and cold germination, occurrence of mechanical, insects and disease-related physical damages).

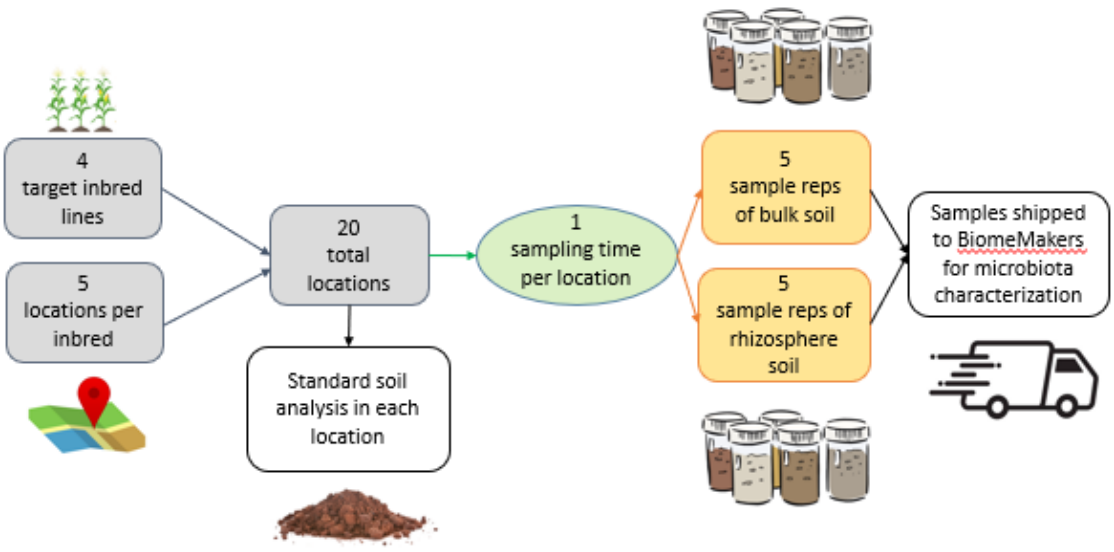


Figure 32: experimental and sampling scheme for soil microbiota characterization

To perform the microbiota characterization, five separate subsamples of bulk soil and five subsamples of rhizosphere soil were collected in each location (Figure 32) at R3 to R4 growth stages of corn, at between 10 and 20 cm of depth. While the bulk soil samples were collected with a probe, in areas with no plant coverage, the rhizosphere soil samples were taken from right below the inbred corn plants, from between the plant roots. 5 randomly selected plants were uprooted with the help of a shovel, in order to preserve most of the root system and the ground close to it. Plants where then shaken and the soil detaching from their roots was sampled.

Samples were collected into 50 mL tubes, stored at -20 °C and then immediately shipped for molecular analysis to Biome Makers laboratory. The characterization was performed by using BeCrop, a proprietary technology developed in-house by BiomeMakers, integrating genomics, AI and AgData to identify the microbial biomarkers and provide a meaningful explanation of soil function in agriculture. More in details, DNA extraction was performed by using the DNeasy, PowerLyzer and PowerSoil Kits from Qiagen. 16S rRNA and ITS marker regions were selected in order to characterize bacterial and fungal microbial communities, respectively, associated with bulk soil and rhizosphere samples. Libraries were defined according to the two-step PCR Illumina protocol. For this purpose, custom primers and amplification of the 16S rRNA V4 and ITS1 regions were adopted (US patent application 15779531). Sequencing was performed with an Illumina

MiSeq instrument, by taking advantage of a pair-end sequencing (2x300bp). Operational Taxonomic Unit (OTU) clustering was conducted at 97% sequence identity, then a quality filtering was performed through a *denovo* chimera removal by using the UCHIME algorithm (Edgar et al., 2011). The SINTAX algorithm (Edgar, 2016), which uses k-mer similarity, was used for taxonomic annotation: only results showing a species level score of at least 0.7 bootstrap confidence were retained. As a taxonomic reference, the SILVA database version 132 (Glöckner et al., 2017) and UNITE database version 7.2 (Nilsson et al., 2019) were used.

Data analysis was mainly conducted by using ANOVA, principal components analysis (PCA) and principal coordinate analysis (PCoA). Predictive models were generated by using mixed effects and elastic net regularization and variable selection method (Zou and Hastie, 2005). This method is a combination of Lasso and Ridge regression, used to overcome the limitations of both. Data elaboration was also performed through the BeCrop portal and its proprietary algorithms.

4.3 Results

A preliminary analysis was performed on grain yield results, to understand how they can be influenced by the production area and by the genotype. As reported in Figure 33, there are important differences related to the three production areas, but they are not significant, due to a high variability in the results in each area.

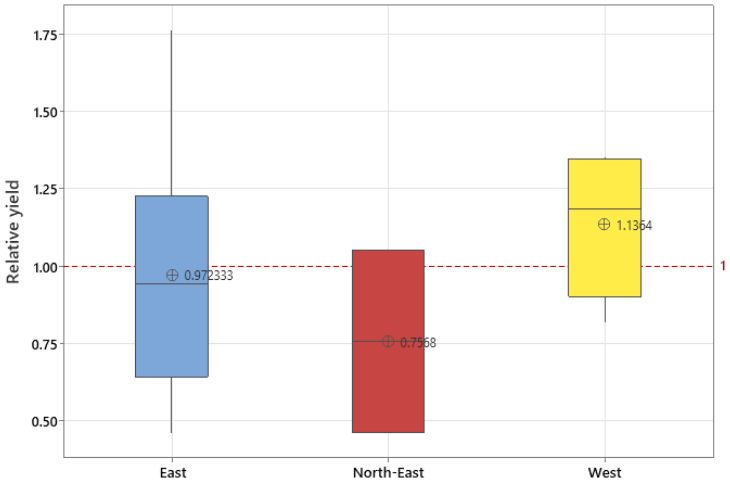


Figure 33: relative grain yield by production area

The analysis by genotype (Figure 34) allowed to identify some remarkable and significant differences in the results, clearly highlighting the different yield potential of the inbred lines considered in this study, especially for genotype D.

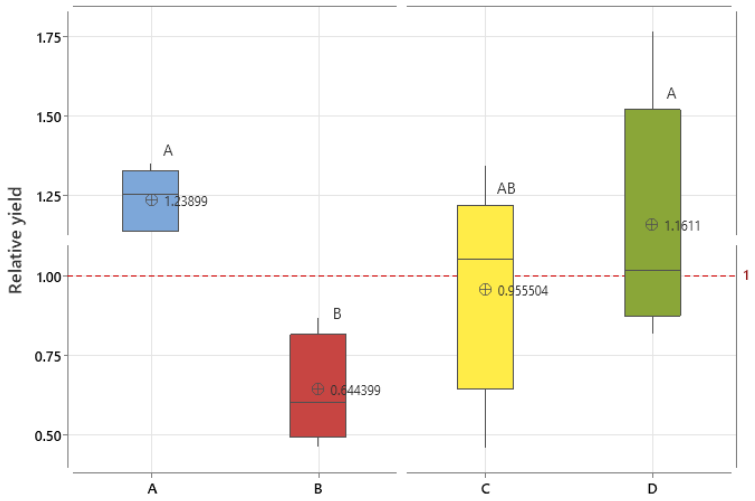


Figure 34: relative grain yield by genotype. Different letters indicate statistically significant differences among treatments.

Regarding microbiome, the first investigation was done on the base of the production areas, to evaluate differences in soil microbiota and its biogeographical pattern, tied to macro areas environmental conditions and independently of the specific single-field conditions. Microbial beta-diversity in each of the three main production areas was compared through a principal coordinate analysis, done separately for the 16S region (bacteria) and the ITS (fungi). Results related to the 16S showed the clear overlap of the North-Eastern and the Eastern production areas, while the Western area clustered well apart from the other ones (Figure 35). In this analysis the North-Eastern area, with only 2 locations, is actually under-represented, so it's difficult to highlight differences with other areas.

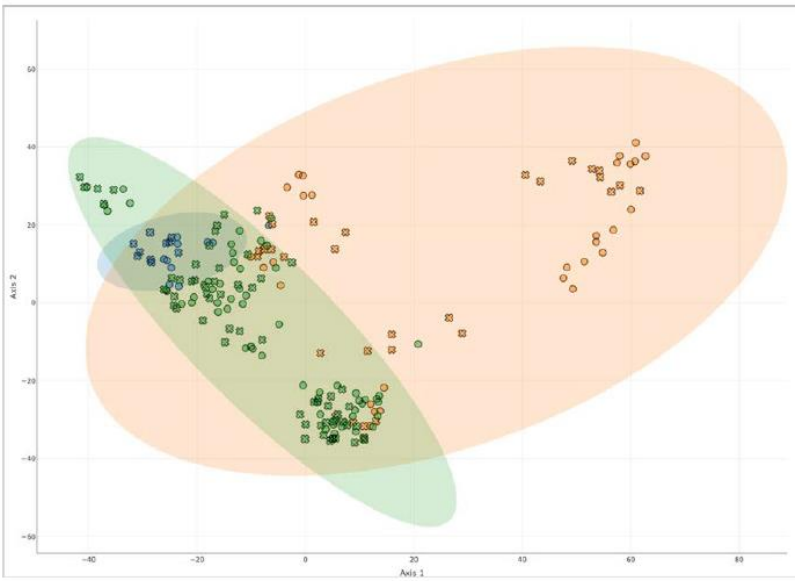


Figure 35: 16S principal coordinate analysis results by production area: blue=north-eastern; green=eastern; orange=western. Circles=rhizosphere samples; crosses=bulk soil samples.

As shown in figure 36, the same analysis was then repeated on ITS, to understand differences in terms of fungal microbiome, but it was not possible to observe clearly separated clusters.

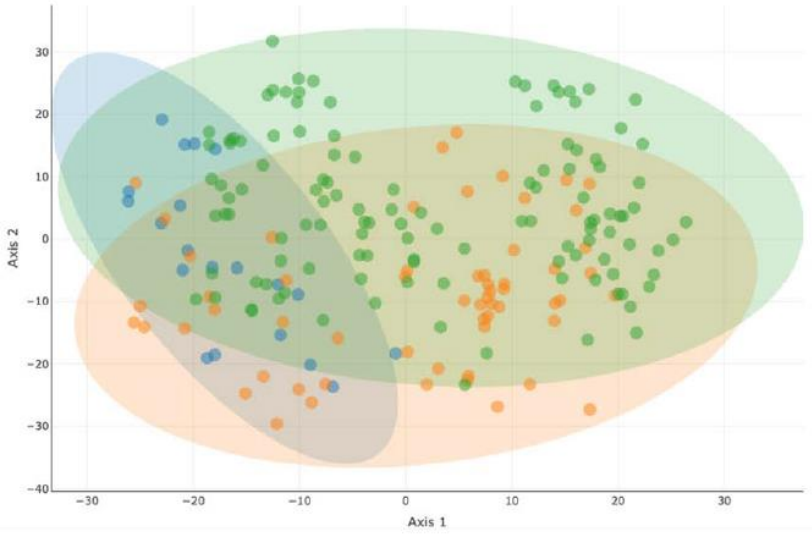


Figure 36: ITS principal coordinate analysis results by production area: blue=north-eastern; green=eastern; orange=western.

Focusing more on 16S beta diversity, the analysis was repeated on the single provinces where fields were located. Some parcels in Piacenza province showed a different profile, compared to the remaining data (Figure 37).

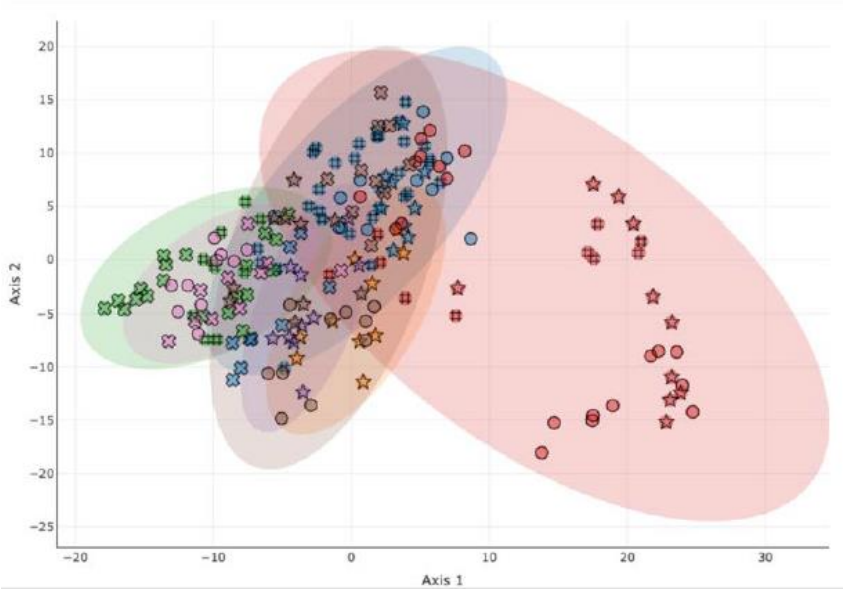


Figure 37: 16S principal coordinate analysis results by production area: red=Piacenza; blue=Bologna; orange=Cremona; green=Ferrara; purple=Parma; brown=Ravenna; pink=Venice. Different symbols represent different genotypes.

It was not possible to explain the precise reasons for this separation with the current dataset: to better explain these results additional and more specific samplings and analysis should be done, focusing on this issue.

A remarkable confirmation of an expected effect was about the agricultural management practices: an organic parcel was in fact included among the other fields. The analysis done with a proprietary BiomeMakers algorithm and reference thresholds, confirmed that the organic field had a higher score than the rest of the fields, as related to microbial biodiversity (Figure 38). As illustrated in the picture, it was in fact rated as “B level”: this level is normally typical of integrated farming management, more than organic management. As expected, the rest of the sampled

fields had a “C level” score, typical of intensive agriculture and then in line with their management practices. Since we only had one organic field, we cannot consider this comparison as statistically significant, but only as a first observation and confirmation of an expected shift in soil microbial biodiversity.

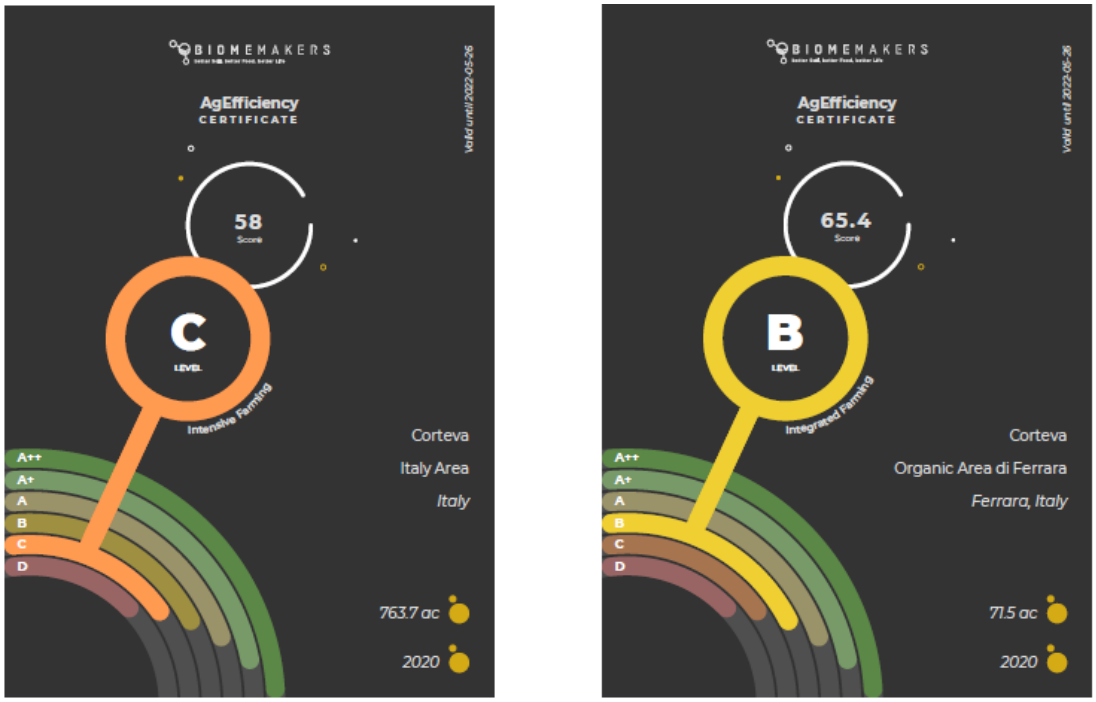


Figure 38: Scoring of microbial biodiversity in conventional vs. organic field management

The analysis of prevalence on 16S and ITS (Figures 39 and 40) highlighted the existence of a core microbiome that is independent from the genetic and the location. Therefore, a few key taxa tend to be preponderant in all the different samples. This is especially true for bacteria, where the prevalence heatmap shows very clear relative abundance thresholds, while for fungi the situation is a bit more confused, but still showing rather clear thresholds and main taxa.

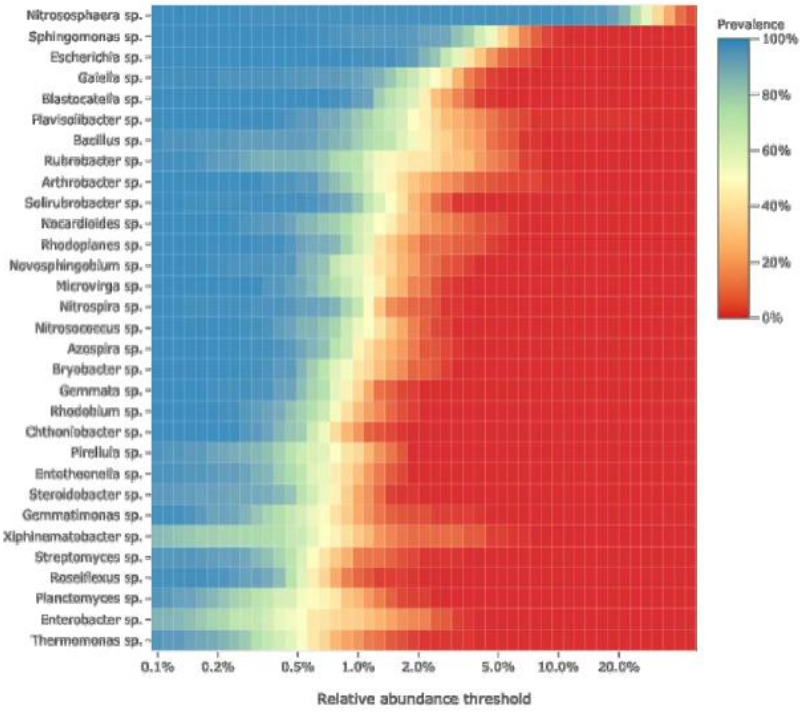


Figure 39. 16S relative abundance heatmap

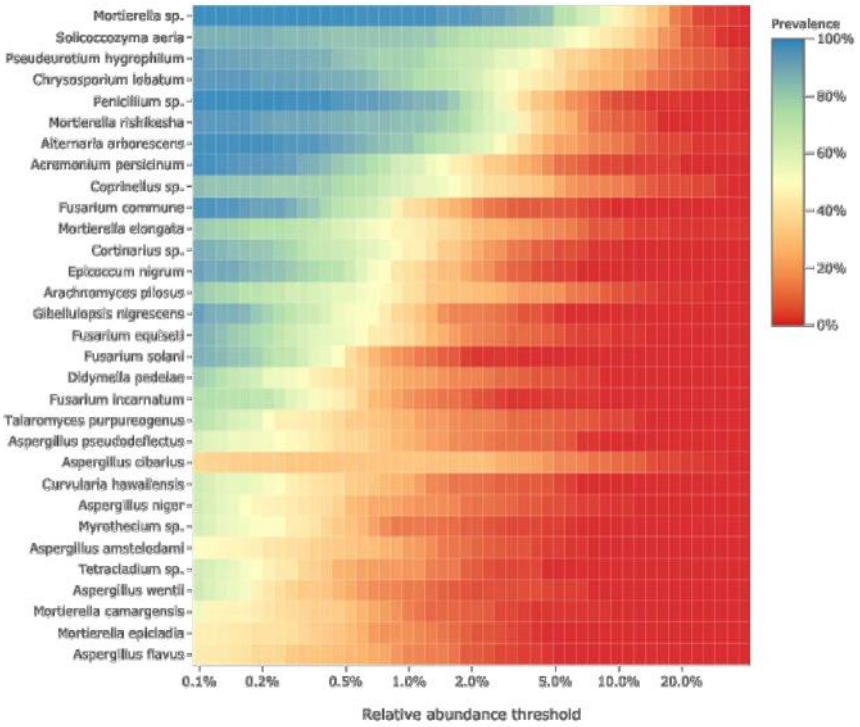


Figure 40. ITS relative abundance heatmap

Fields and their relative samples were then divided on the base of the final grain yield into two categories:

- Low yield fields
- Medium-high yield fields

Results from microbiota characterization, analyzed through a principal component analysis (PCA) (Figure 41), shows two clearly separated clusters from 16S results, indicating a clear relationship trend between bacterial microbiome and yield, even though results are still not significantly different. Those clusters appear then to be good drivers of yield.

The same analysis, repeated on ITS, showed much poorer results, since it was impossible to define clearly separated clusters.

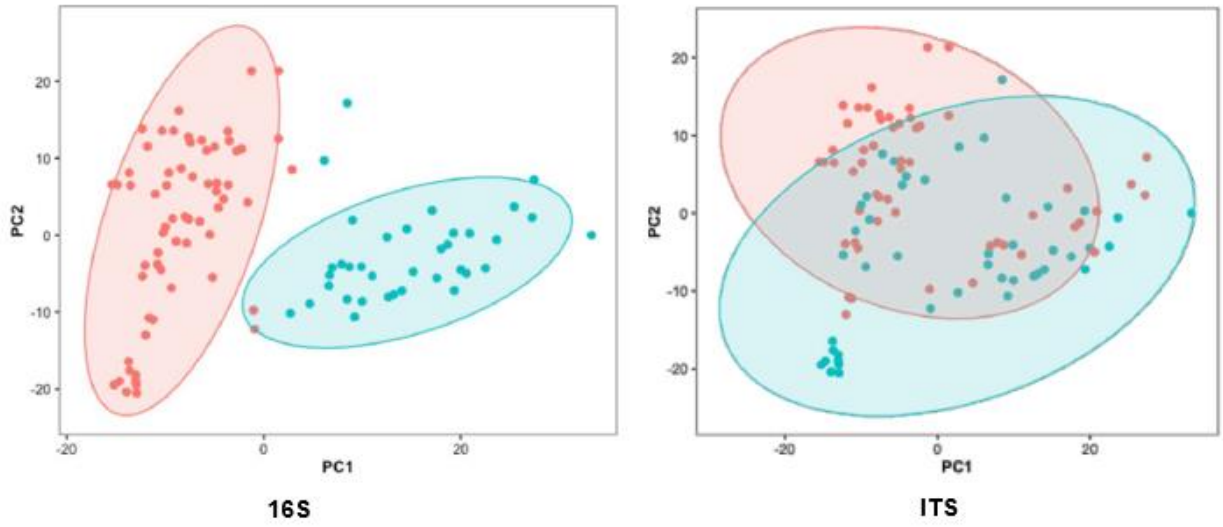


Figure 41. PCA graphical results on 16S/ITS, by yield results.
Blue = low yield fields; red = medium-high yield fields

Interestingly, some bacterial OTUs were differentially expressed into the two groups ($p < 10^{-4}$). *Actinoallomurus* sp. and *Acidicaldus* sp. were the OTUs more abundant into the medium-high yield

parcels, while *Neobacilus drentesis*, *Thiobacillus* sp., *Mariprofundus* sp. and *Clavibacter* sp. were the OTUs more abundant into the low yield fields.

In the light of the above findings, yield predictive models were created, taking into consideration different variables and their combinations. The first model that was created considered the genotype, the environment and their interactions. A mixed effects model was used in this case, since the design is not an equilibrium design and there are no replicates for all seeds in all environments. As from table 6, two models were designed:

- A model with no interactions ("fit2"): in such a model, the "best" genotype is the same in all environments.
- A model that considers the interaction between genotype and environment ("fit"): it estimates the effects of different genotypes in each environment.

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
fit2	6	245.8772	261.5082	-116.9386	233.8772	NA	NA	NA
fit	15	244.7272	283.8047	-107.3636	214.7272	19.15	9	0.02394729

Table 6. Yield predicting models based on genotype, environment and their interaction.

Comparing those two models, the one with interactions showed to be the most accurate one, as indicated by the lower AIC (Akaike's information criterion (Akaike, 1974)) results. This means that the seed yield clearly depends on the interaction between the environment and the genotype.

As a further step, a model was trained with different sets of variables, by using the elastic net regularization and variable selection method. Three sets of variables have been considered in this case:

- The microbiome
- A selection of the most important environmental variables (called "Other var")
- The genotype (called "Seed")

Model	Microbiome pcas	Other Var (PQ)	Seed	Accuracy
Model 1		X	X	0.739
Model 2	X			0.914
Model 2	X	X		0.903
Model 3	**X**	**X**	**X**	**0.980**

Table 7. Contribution of different sets of variables to yield predicting accuracy.

As shown in table 7, when only the genotype and the environment were included, the model explained the 73.9% of the yield variability. When the microbiome alone was considered, it explained 91.4% of the yield variability, while when all the three sets of variables were included, they explained 98% of the yield variability.

4.4 Discussion

The results show clear significant differences in terms of yield, as related to the genotype, while the production area, despite highlighting remarkable average yield differences, did not return significant results, due to the high intrinsic variability in the data, likely affected by the different genotype allocation in each area.

The microbial beta-diversity was instead clearly influenced by the production area, with clear differences in some of the areas affected by this study. Namely, the Western Po Valley area and especially some parcels in Piacenza province showed a quite clear segregation, compared to the other locations/parcels, in terms of 16S beta-diversity. Although it is not possible to explain the reasons for such separation, these results represent a very important baseline for further studies, aimed to better evaluate the role that soil microbiota plays on crop performances and to understand how the environmental conditions can drive to better production results by shaping the microbiota beta-diversity. This is even more important, given that the differences in beta-diversity were tied to an increased productivity in those areas that were clustering apart. Explaining these differences appear to be paramount for better driving products positioning and field/grower selection.

The comparison between conventional and organic management, despite not statistically significant, was perfectly in line with the expectations and literature (Hartmann et al., 2015; Lupatini et al., 2017), providing a confirmation of a higher microbial biodiversity for the organic parcel, even though more typical of integrated farming: this might be due to a relatively recent conversion of the field to organic management. This demonstrates that agricultural practices typical of organic agriculture can play a very important role in improving the soil microbial biodiversity. Moreover, the application of similar agricultural techniques, such as the use of organic fertilizers (Francioli et al., 2016), the use of cover crops during the non-productive season (Kim et al., 2020), the improvement of crop rotations (Venter et al., 2016), when feasible in a context of conventional farming, can be effective tools to improve soil health.

The existence of a core microbiome that is independent from the environment and the genetics was clearly highlighted by the analysis of prevalence. This might be tied to some ubiquitous microbial OTUs. However, it also spots a light on the capacity of plants to select the microbes to recruit, not being then just passively exposed to the microbiota of different soils, but working actively to choose the ones to cooperate with. This finding is in line with similar results obtained with other plant species (Pfeiffer et al., 2017; Simonin et al., 2020).

The relationship between yield and soil microbiota, despite still not significant is a remarkable finding, indicating that bacterial microbiota can be an important driver of crop yield. More investigations can be done on those species that are more abundant into the two field categories (low and medium-high yield) to better understand their role through dedicated experiments and to check if they can be used as a marker for field selection. Interestingly, among the two OTUs that are more abundant into medium-high yield parcels, *Actinoallomurus* sp. has already been reported as being a plant growth promoting rhizobacteria (PGPR) (Hamedi and Mohammadipanah, 2015), while *Acidicaldus* sp. was reported as having potential PGPR characteristics (Massena Reis and dos Santos Teixeira, 2015).

Yield predicting models demonstrated, at first, the importance of the interaction between genotype and environment (GxE) and then the importance to select the genetic materials that better fit a certain environment, in order to get the best yield results. The GxE effect on maize is

a well-known phenomenon, supported by a pretty consistent literature (Comstock and Moll, 1963; Kang and Gorman, 1989) and being considered in breeding programs (Kang, 1997). Despite this, the GxE evaluation in this study was important to demonstrate its existence inside the Italian seed corn production area, to better understand the characteristics and the impact of production locations on yield and for assessing its contribution on yield predictive models.

The impact of soil microbiome on explaining yield variability was huge and bigger than that of the combination of genotype and environment. Given that the environmental variables were not bringing additional contribution to that of microbiome, we can say that microbiome can capture very well the environmental variability and can be alone an excellent predictor of yield. This was even better when the predictive model also included the genotype factor: in this case, it was capable of almost completely explaining yield variability (98%). Similar results were reached by Imam et al. (2021) on potato.

4.5 Conclusions

Understanding how soil microbiota can affect the agricultural results and how it can be used as a reference to understand the complex processes and dynamics taking place in the agricultural ecosystems, is a critical step forward for the implementation of a more sustainable agriculture. This study was a preliminary investigation about the potential exploitation of soil microbiota for seed corn production in the Italian production environment, however it allowed to reach some very important basic learnings.

It was in fact demonstrated that the soil microbiota is a key and very important driver for yield and that it can be influenced by the agricultural management and by the location/environment.

Better understanding the details of this relationship between microbiome and agricultural results will offer an outstanding opportunity to improve yield and reliability of seed crops and of the general agriculture. For this reason, more specific studies will be needed to target and define those taxa and the characteristics microbial populations that can have a positive return in terms of yield and quality of the produce.

This will allow to understand the production potential of different locations and better guide the positioning of genotypes at both macro-area and field level. It will also offer the opportunity to develop microbial consortia, by selecting taxa that can have positive implication for the crop and for the different genotypes being grown. Both approaches can be a way to maximize the potential of each genetics and/or to minimize the constraints affecting seed corn and other crops results, in a sustainable way.

Chapter 5

General conclusions

This project returned some very important results about opportunities to improve seed production sustainability as well as yield and reliability. The study about the use of AMF, reported in chapter 3, allowed to test two very promising fungal species and their potential on improving seed corn production. We were also able to collect some important information about the interaction between corn plants and AMF. Firstly, we proved that AMF could put in place symbiosis with corn plants, not only in a controlled environment, but also in field conditions and at different times of application. Secondly, we observed some important physiological interactions that highlighted the activity of AMF as related to plant nutrition and development and that will be especially important for further studies.

The study of soil microbiome provided an overall picture of what is the impact of soil microbial communities on seed corn yield and how they can be used as a yield predictor. Additionally, we had first results about some microbial taxa that appears to be strongly connected with yield results. Despite not being immediately usable, these data represent a first important reference for better driving field selection and product positioning in order to reach the best productivity results by minimizing external inputs.

Overall, this PhD project represented a very important step forward for seed corn production sustainability and to improve yield and reliability in an eco-friendly way. It will serve as a guideline for better driving Corteva's field operations, as well as a reference document for the further research activities that are needed to move forward on some potential applications of these results, like for example better adjusting maize fertilization, thanks to AMF, and furtherly study those soil taxa that are mostly tied to seed corn yield.

Chapter 6

References

Chapter 1: General introduction

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