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Department of Agronomy, Food, Animals, Natural resources and Environment

Ph.D. COURSE IN CROP SCIENCE

XXX SERIES

IMPROVING ROOT GROWTH IN CEREALS FOR SUSTAINABLE CULTIVATION

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SUMMARY

The topic of my PhD research addresses the study on the effects that some new sustainable crop management practices have on enhancing root growth in two globally important cereal crops, wheat and maize.

The structure of the thesis consists of five chapters, corresponding to as many manuscripts already published (2 of 5) or on the way to be published in international scientific journals.

In detail, in the first and second chapter were studied the application effects of plant growth promoting microorganisms (PGPMs) on common wheat. The aspects taken under study after their inoculation were: the ability of selected endophityc bacteria and mycorrhizal fungi to colonize and interact with plant organs, the effects on root growth, the promotion of shoot development, the nutitional improvement by increasing N fixation and nutrient availability, the effects on grain yield and other agronomic parameters and finally the environmental significance by evaluating the possibility of cutting chemical fertilization.

Microscope investigations revealed an excellent ability of bacteria to adhere to the surface of intact leaves and roots, and to colonize both leaf mesophyll and root vascular tissues in aseptic conditions. Also the mycorrhizal fungus was able to colonize wheat roots efficiently.

Bacteria increased the number of root tips and ramifications in sterilized rhizobox soil, regardless of the method of application, and the volumetric root length density in the open field with medium and high N supply, resulting in greater N accumulation. Although the N dose had clear positive effects, no significant variations in grain yield or other agronomic parameters could be ascribed to bacteria inoculation.

The arbuscular mycorrhizal fungus allowed remarkable increases in volumetric root length density and root area density at flowering stage at medium and high N fertilization rates in both years, but not a low N rate. While inoculation had a negligible effect on grain yield, which followed a typical N dose-response model, improved uptake of N and other nutrients, particularly P and Zn were recorded at any fertilization rate, although only seldom significant.

Last three chapters aimed at investigating the potential rooting activity on maize plants of the recently market-introduced seed-applied fungicide Sedaxane®, a novel succinate dehydrogenase complex II inhibitor (SDHI) against soil-borne pathogenic fungi.

Laboratory investigations demonstrated that sedaxane has significant auxin-like and gibberellin-like effects, which induced marked morphological and physiological root changes according to an approximate saturation dose-response model. Sedaxane enhanced leaf and root glutamine synthetase (GS) activity resulting in greater protein accumulation, particularly in the shoot, while glutamate synthase (GOGAT) activity remained almost unchanged. Sedaxane also improved leaf phenylalanine ammonia-lyase (PAL) activity, which may be responsible for the increase in shoot antioxidant activity (phenolic acids), mainly represented by p-coumaric and caffeic acids.

Under controlled conditions in a greenhouse we evaluated the root growth enhancement side-effect of sedaxane, regardless of the main protective action against pathogenic fungi, in pot-cultivated maize plants subjected to both biotic and abiotic stress conditions in the early phenological stages.

The secondary effect of sedaxane on root growth was detectable both in absence and with high *Rhizoctonia solani* pressure, but it was more evident in the last situation. Significant

enhancements in root biomass, length, area and tips quantity were observed, but also shoot parameters, like leaf chlorophyll content and total biomass.

Seed-treated plants highlighted positive responses to both adverse growing conditions performed in the experiments reported in the last chapter, a decreasing soil fertility and a drought stress during early phenological stages. Results showed increased growth of shoot, with higher SPAD values, nutrient uptake and antioxidant activity (phenolic acids), as well as a greatly enhanced root growth than controls. Particularly under severe water stress condition, these parameters did not show marked differences from controls, but plants from treated seeds with fungicide were able to maintain a higher rate of transpiration to lower value of transpirable water in the soil.

INTRODUCTION OVERWIEW

The human population, accordingly to the Food and Agriculture Organization of the United Nations (FAO), is projected to reach 9.5 billion by the year 2050 and will demand at least a 50% additional increase in global food production. In addition to that, climate change may also induce various biotic and abiotic stresses in the near future. This may lead to an increase in the use of chemical fertilizers and pesticides to protect the crops under such adversaries. However, the indiscriminate use of agrochemicals would further result in the widespread pollution of the biosphere and they are not affordable to farmers in low-resource environments.

Therefore, it is an imperative to develop more and more sustainable agricultural practices to meet requirements for a global food security, maximizing crop yield while considerably reducing environmental pollution.

In recent years there is a considerable growing interest in trying to improve crop yields through better understanding of roots and the way they grow. Especially for annual crops an adequate root development is an essential factor from germination until reproductive stages as the ability to withstand stress depends mostly on the health of the roots. The depth and extent of the root system are so important for cereal that, for example, adequate pollination and grain fill depend on the plant ability to mine the soil for moisture and nutrients. For these reason breeders are very focused on enhancing rooting through genetics but also recently-developed sustainable agronomic practices allow annual crops to explore the soil by roots more efficiently, leading to very consistent yield increases and reducing, at the same time, the amount of fertilizers needed.

The topic of my PhD research addresses the study of the effects that some new sustainable crop management practices have on enhancing root growth in two globally important cereal crops, wheat and maize.

One possible way to improve sustainability of wheat cultivation is the application of plant growth promoting microorganisms (PGPMs), such as arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria (PGPR). These microorganisms, as association with rhizospheric, epiphytic and endophytic bacteria or symbiosis instauration with mycorrhizal fungi, can have a significant impact on plant growth and development. They are able to protect their hosts by acting as bioprotectants (via induced systemic resistance) and biopesticides (via antibiotic functions) but, moreover, they promote plant growth producing phytostimulators. Phytohormones-induced changes affect particularly root growth, enhancing morphological features (length, surface area, density).

On the other hand, seed treatments have played and are still playing a central role in sustainable crop production as, in term of environmental benefits, fungicides delivery via seed coating allows to introduce much less agrochemical substances per hectare compared with the amount needed by other application methods such as soil drench or injection. Typically chemical seed treatments do not offer benefits associated with root growth so there is an increasing focus today on introducing root enhancement proprieties to seed-applied active ingredients, in addition to their protection effects.

AIMS AND STRUCTURE OF THE THESIS

The research carried out during the PhD activities aimed to investigate:

a. the ability of plant growth promoting microorganisms (PGPR and VAM), included in commercial biofertilizers, to colonize plant tissues of slected wheat varieties;
b. the root growth enhacement due to their inoculation;
c. the effects on shoot growth, nutrient accumulation and agronomic parameters;
d. the possibility to reduce the chemical N fertilization;
e. the potential biostimulant activity of seed-applied Sedaxane fungicide on maize plants, monitoring morphological variations at increasing active ingredient doses;
f. the response of enzymes involved in nitrogen and phenylpropanoid metabolism, and of the protein, sugar and total phenol contents in both maize leaves and roots as consequence of seed treatments;
g. the effects of root growth enhancement by seed applied Sedaxane and consequent improvement of plant tolerance to different biotic and abiotic stress conditions.

All chapters of the present thesis meet these aims and correspond to scientific papers already published, submitted or conceived to be submitted to international scientific journals as follows:

Chapter 1: Dal Cortivo C., Barion G., Visioli G., Mattarozzi M., Mosca G., Vamerali T., 2017. Increased root growth and nitrogen accumulation in common wheat following PGPR inoculation: assessment of plant-microbe interactions by

ESEM. Published in *Agriculture, Ecosystems and Environment* 247: 396-408. doi: 10.1016/j.agee.2017.07.006;

Chapter 2: Dal Cortivo C., Barion G., Ferrari M., Visioli G., Dramis L., Mosca G., Vamerali T. Arbuscular mycorrhizal fungi and diazotrophic bacteria cover the gap in root growth under medium-high nitrogen supply with benefits for nutrient uptake in common wheat. Submitted to *Frontiers in Plant Science,* accepted after major revisions, re-submitted and currently under evaluation;

Chapter 3: Dal Cortivo C., Conselvan G.B., Carletti P., Barion G., Sella L., Vamerali T., 2017. Biostimulant effects of seed-applied sedaxane fungicide: morphological and physiological changes in maize seedlings. *Frontiers in Plant Science* 8: 2072. doi: 10.3389/fpls.2017.02072;

Chapter 4: Dal Cortivo C., Barion G., Ferrari M., Sella L., Vamerali T. Protection and growth enhancement effects of seed-applied sedaxane fungicide on maize seedlings under high *Rhizoctonia solani* pressure. Conceived to be submitted to *Crop Protection*;

Chapter 5: Dal Cortivo C., Barion G., Ferrari M., Vamerali T. The new SDHI fungicide sedaxane improves root growth and tolerance to nutrient and water deficiency in early growth stages of maize. Conceived to be submitted to *Journal of Agronomy and Crop Science.*

CHAPTER I

Increased root growth and nitrogen accumulation in common wheat following PGPR inoculation: assessment of plant-microbe interactions by ESEM

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Abstract

The use of plant growth-promoting rhizobacteria (PGPR) meets the current need to reduce nitrogen input in order to attain greater sustainability in the production of crops, particularly cereals. This study investigated whether a commercial biofertilizer containing a consortium of PGPR and N-fixing bacteria (*Azospirillum* spp., *Azoarcus* spp. and *Azorhizobium* spp.) affects shoot and root growth, N accumulation and grain yield in common wheat (*Triticum aestivum* L.).

Trials were conducted in a fertile, silty loam soil, firstly in rhizoboxes, by applying bacteria either as a seed-coating inoculum or by foliar+soil spraying, and then in the field by spraying the canopy at the tillering stage with decreasing levels of N fertilisation (160, 120 and 80 kg ha^{-1}) in two consecutive years. Culm height, leaf chlorophyll content, nitrogen accumulation and yield were recorded above ground, while below ground Root Length Density (RLD) patterns were investigated by soil coring and image analysis at the flowering stage. Environmental Scanning Electron Microscope (ESEM) imaging revealed an excellent ability of bacteria to adhere to the surface of intact leaves and roots, and to colonise both leaf mesophyll and root vascular tissues in aseptic conditions. Bacteria increased the number of root tips and ramifications (+65% *vs*. non-inoculated controls) in sterilised rhizobox soil, regardless of the method of application, and the volumetric root length density in the open field with medium (+29%) and high (+11%) N supply, resulting in greater N accumulation (about +25 kg ha^{-1}). Although the N dose had clear positive effects, no significant variations in grain yield (only + 1-3% *vs*. non-inoculated controls) or other agronomic parameters could be ascribed to bacteria inoculation.

The conclusion drawn is that the use of a combination of PGPR and N-fixing bacteria offers an opportunity to improve root growth in wheat and increase plant resilience to

environmental stresses, and helps to reduce N losses from agricultural ecosystems thereby offering partial fertiliser savings within crop rotations.

Keywords: Bio-fertilisers; common wheat; nitrogen accumulation; endophytic bacteria; root growth; vegetation indices.

Introduction

Chemical fertilisers are commonly used to supply essential nutrients to soil-plant systems in a wide range of cultivated crops. However, the use of high amounts of chemical fertilisers, especially nitrogen, has raised environmental concerns in the current agricultural systems of industrialised countries. There is an urgent need to find safe, alternative fertilisation strategies in order to improve the sustainability of agro-ecosystems, especially in cereal cultivation, while at the same time retaining competitive crop yields. One potential method of attenuating the negative environmental impact of chemical fertilisers, herbicides and pesticides is to apply plant growth-promoting rhizobacteria (PGPR) (Pérez-Montaño et al., 2014). The use of beneficial microorganisms is now widely accepted in intensive agriculture in many parts of the world (Bhattacharyya and Jha, 2012), and when they improve nutrient uptake they are called biofertilizers (Pérez-Montaño et al., 2014).

These bacteria play a role in plant nutrition by exerting non-symbiotic N fixation, enhancing the availability of nutrients in the rhizosphere, such as phosphorus and iron, and increasing the root surface area through the production of phytohormones (e.g., indole acetic acid IAA, cytochinins, gibberellins) (Dobbelaere et al., 2003; Kumar et al., 2014; Marques et al., 2010). PGPR can also produce a deaminase capable of cleaving ACC (1-

aminocyclopropane-1-carboxylate), an immediate precursor of ethylene, which generally inhibits root growth (Glick et al., 1998). The production of siderophores and synthesis of antibiotics, enzymes or fungicidal compounds by these bacteria can additionally protect plants against phytopathogenic microorganisms (Compant et al., 2005). There is growing interest in the use of PGPR with cereals and various studies are demonstrating their beneficial role in the growth and yields of several crop species. For example, N-fixing PGPR have been found to increase plant growth and productivity in both wheat and maize (Gaskins et al., 1985; Rosas et al., 2009; Turan et al., 2012). Significant yield increases in wheat and barley have resulted from application of a consortium of PGPR, especially when these have differing and complementary abilities (Turan et al., 2012). A combination of various PGPR strains has been shown to be effective in increasing growth and yield in wheat in both pot and field experiments under conditions of drought and salinity (Kumar et al., 2014; Kaushal and Wani, 2016).

However, several factors, such as plant genotype, bacteria species and strain, and agricultural practices, may affect plant responses and the success of inoculation (Khalid et al., 2004; Roesti et al., 2006; Tahir et al., 2015). To avoid these negative interrelations and increase biofertilizer effectiveness, scientists have recently developed new microbial associations. Consortia of PGPR with mycorrhizal fungi (Pérez-Montaño et al., 2014) or algae (Nain et al., 2010) can deliver better crop performance as a consequence of synergistic or cumulative interactions between the beneficial mechanisms of different microorganisms.

A large number of PGPRs, including isolates from the genera *Azospirillum, Azotobacter, Bacillus, Enterobacter, Pseudomonas, Klebsiella* and *Paenibacillus*, have been obtained from the rhizosphere of various crops, including wheat (Saharan and Nehra, 2011; Tahir et

al., 2013;). Of the plant-associated diazotrophic bacterial genera, *Azospirillum* was found to be highly abundant in the rhizosphere of wheat (Benmati et al., 2013; Venieraki et al., 2011), and *Azoarcus* sp. in that of rice and sorghum. *Azoarcus* sp. can also invade roots and spread into the shoots of wheat: microscope analyses show that it can locate in the intra- and inter-cellular parenchymatic and cortical root cells, resulting in better plant growth and nitrogen accumulation. In wheat, co-inoculation with *Azospirillum brasilense* Sp245, a natural associative bacterium, also aids the colonisation ability of *Azoarcus* (Wieland and Fredick, 1998).

Azorhizobium caulinodans is a stem- and root-nodulating N-fixing bacterium isolated from the stem nodules of *Sesbania rostrata* Bremek. & Oberm. (Dreyfus et al., 1988). Some studies have shown endophytic colonisation of non-legume roots, such as wheat, where it stimulates root growth and increases N content and yield (Qiang et al., 2014; Sabry et al., 1997).

Against this background, this work aimed at studying the effects on common wheat (*Triticum aestivum* L.) of a mixture of PGPR and free-living N-fixing bacteria, namely *Azospirillum* spp., *Azoarcus* spp. and *Azorhizobium* spp., provided as a commercial formulation suitable for use in a wide variety of field crops and trees. Seed inoculum was first studied *in vitro* by monitoring the colonisation and survival of bacteria in seedling shoots and roots using advanced *in situ* electron scanning microscopy techniques. Wheat plants were then grown in rhizoboxes with bacteria applied as a seed-coating inoculum or by soil+foliar spraying, and later in a two-year field trial at various N fertilisation levels with bacteria sprayed at the tillering stage, in order to examine the effects on the root characteristics of young and mature plants, N accumulation, and the expected influence on yield.

Materials and Methods

Environmental scanning electron microscope (ESEM) imaging of bacteria-root and bacteria-leaf interactions

Survival of the bacteria inoculum contained in the commercial product and its ability to colonise plant organs was assessed by Environmental Scanning Electron Microscope (ESEM). This is a powerful technique for observing biological specimens *in situ* without histological treatment of samples, which allows a real picture of bacterial colonisation outside and inside plant cells and tissues to be obtained (Stabentheiner et al., 2010).

A bacterial suspension of the commercial formula TripleN® (Mapleton Agri Biotec, Mapleton, Australia) containing *Azorhizobium* spp., *Azoarcus* spp. and *Azospirillum* spp., proposed for wheat and other crop species, was plated in Luria-Beltrami (LB) broth and incubated at 28°C for 3 days. Isolates were then grown overnight in 100 mL of LB medium at 30°C on a rotary shaker. Bacterial cells were collected by centrifugation and suspended in LB medium to obtain a final inoculum density of 5×10^8 CFU mL^{-1}.

Three pools of 30 seeds of common wheat *Triticum aestivum* L. var. Bologna (SIS, Bologna, Italy) were sterilised in 50% (v/v) commercial bleach for 15 min then given three rinses of 5 min each in sterile water. Seed sterility was ascertained by incubating one seed pool on LB agar plates at 30° C for 4 days and checking for the absence of bacterial contamination. One seed pool was kept for 2 h in the bacterial solution (5×10^8 CFU mL^{-1}) then briefly rinsed in sterilised water to remove non-adherent bacteria, while a second pool was left untreated and used as non-inoculated controls. Both inoculated and non-inoculated seeds were plated on MS agar medium (Murashige and Skoog, 1962) and incubated in a vertical position under controlled environmental conditions (22°C; 16 h/8 h light/dark; 120 μmol m^{-2} s^{-1} photosynthetically active radiation; 75% relative humidity) for

germination and root elongation. Seven-day-old fresh roots and 14-day-old fresh leaves of seedlings from inoculated and non-inoculated wheat seeds were collected directly from the plates and washed briefly in sterile water for ESEM imaging. Root fragments and leaf sections 5 mm in length were excised with a sterile lancet and fixed overnight in a 3% v/v glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.0 and 4°C. The samples were then thoroughly rinsed in 0.1 M phosphate buffer at pH 7.0 and dehydrated in acetone solution (25, 50, 75 and 100% v/v in deionised H_2O). Lastly, the samples were dried with a Critical Point Dryer (CPD 020, Balzers Union Limited, Balzers, Liechtenstein) in a CO_2 atmosphere and placed directly on aluminium stubs with double-sided, adhesive, conductive carbon tape.

In accordance with Sørensen et al. (2009), morphological analyses were carried out using a Quanta™ 250 FEG ESEM (FEI, Hillsboro, OR, USA) operating in low vacuum mode (pressure chamber set at 100 Pa) and with a beam accelerating voltage of 3 or 5 kV.

Root observations in rhizoboxes

To assess whether the TripleN® bacterial inocula had direct effects on the early root growth of common wheat (var. Bologna), an experiment was carried out in controlled conditions using rhizoboxes inside a greenhouse at the experimental farm of the University of Padua (NE Italy). The rhizoboxes were 45 cm high, 30 cm wide and 2.5 cm thick with transparent plexiglass sides, and were positioned at a 45° angle during plant growth to allow roots to be observed through the lower transparent wall. The boxes were filled with ~3.8 kg of a mix of sterilised sand and silty loam soil (1:1 w/w). Sterilisation was carried out at 105°C for 72 hours in a large oven. A ternary N-P-K fertiliser (8% N, 24% P_2O_5 and 24% K_2O) at a rate of 0.1 g kg^{-1}, roughly corresponding to 30 kg N ha^{-1} and 90 kg ha^{-1} of P_2O_5 and K_2O,

was added as pre-sowing fertilisation in order to mimic on-farm practices. Three seeds per rhizobox were sown at a depth of 3 cm and plants were grown for 50 days during February and March.

Two methods of bacteria application were examined, a seed-coating treatment before sowing (a widely-used inoculation option) and foliar+soil spraying after emergence, the method of application recommended by the biofertilizer manufacturer; these were compared with non-inoculated controls. With the seed-coating treatment, 0.1 g of freeze-dried TripleN product (containing 1×10^{10} CFU g^{-1}) was diluted in 2 mL of ultrapure water and added to 1000 seeds (i.e., ~38 g) just before sowing in order to facilitate bacterial adherence and reach a final concentration of 10^6 CFU per seed. Seeds had previously been sterilised in 15% v/v sodium hypochlorite for 15 min and then given three rinses of 5 min each in sterile water. The same amount of inoculum was given in the post-emergence treatment by spraying each plant with 10 mL of inoculum solution (0.01 g freeze-dried TripleN in 1 L water) on the leaves and on the ground surface at the 3-leaf (unfolded) stage (23 DAS, days after sowing).

The trial included 3 replicates/rhizoboxes per treatment, each with 3 plants.

At the end of the experiment, when the root system had more or less reached the bottom of the rhizoboxes, the plants were harvested and the root system gently washed so that it could be collected in its entirety. Roots were stored in ethanol solution (15% v/v) at 4°C until image acquisition and processing with WinRhizo software (Regent Instruments Inc., Ville de Québec, QC, Canada). The main root parameters, i.e., length, surface area, diameter, and number of tips and forks, were measured in 1-bit 400-DPI TIFF format root images acquired with a flatbed scanner (Epson Expression 11000 XL, Suwa, Japan).

Two-year field trial

In order to evaluate the effectiveness of bacteria inoculation under real cultivation conditions, an open-field trial was carried out during the 2013-14 and 2014-15 growing seasons with autumn sowing at the University of Padua's experimental farm at Legnaro, Padua (45° 21' N, 11° 58' E, 12 m a.s.l.), on the Po plain (NE Italy). Wheat was cultivated in a silty loam soil (fulvi-calcaric-cambisol; USDA classification) at pH 8.0, with 1.7% organic matter, a CEC of 11.4 cmol(+) kg^{-1}, and a total N content of 1.1 g kg^{-1} (arable layer, beginning of experiment). At the experimental site, the depth of the water table generally fluctuates between 0.8 m (winter) and 1.8 m (summer), mainly depending on rainfall, and annual precipitation is ~830 mm (30-year historical mean). The experimental design consisted of a completely randomised block with 3 replicates and 30-m^2 plots (10×3 m) containing 24 plant rows 12 cm apart. In both years, the previous crop had been sugar beet; the soil was ploughed to a depth of 0.3 m and harrowed twice at 0.2 m. Fertilisation consisted of 32 kg ha^{-1} of N, 96 of P_2O_5 and 96 of K_2O incorporated into the soil through harrowing. The high-yielding wheat var. Africa (SIS, Bologna, Italy) was cultivated in the first year, and the high-quality var. Bologna (SIS, Bologna, Italy), the most widespread in the region, in the second. In the first year, sowing took place on 29 October 2013, harvesting on 12 June 2014, and in the second year on 12 November 2014 and 22 June 2015, respectively. The crop was protected against weeds, insects and fungal diseases by specific treatments, following local agricultural recommendations.

Plants treated with the TripleN biofertilizer and the non-inoculated controls were factorially combined with nitrogen fertilisation at three decreasing levels: 160, 120 and 80 kg ha^{-1}. After pre-sowing fertilisation (with 32 kg of N for each level), half of the

remaining N dose was supplied at the tillering stage and half at the onset of stem elongation as ammonium nitrate (34% N).

The bacterial inoculum was applied following the manufacturer's instructions at a label dose of 4 g of commercial product (bacterial concentration of 1×10^{10} CFU g^{-1}) per hectare at the tillering stage (7 March 2014 in the first year, 19 February 2015 in the second). The microbial product (4 g) was rehydrated for one hour in 200 mL pure water then mixed with 600 L ha^{-1} of non-chlorinated water and sprayed mechanically onto the wheat using farm-scale technologies. The treatment was carried out in the late afternoon in order to minimise UV light interference with bacteria survival. The volume applied ensured good leaf and soil wetness and an expected bacterial density of 4×10^6 CFU per m^2 of ground and >400 CFU per cm^2 of leaves.

Leaf chlorophyll content was monitored during the growing cycle, from the beginning of stem elongation until the heading stage, with a SPAD 502 chlorophyll meter (Konica-Minolta, Hong Kong) on the last fully developed leaf (10 leaves per plot) at 10-day intervals throughout April and May of both years. Culm height was also measured on the same plants.

At the same time as the SPAD measurements, the Normalised Difference Vegetation Index (NDVI) of the canopy of each plot was calculated with an active handheld Greenseeker spectrometer (NTech Industries, Ukiah, CA, USA) linked to a GPS. The sensor measures canopy reflectance at wavelengths of 590 nm (ref_{RED}) and 880 nm (ref_{NIR}) and provides a ratio value as follows:

$$NDVI = \frac{ref_{NIR} - ref_{RED}}{ref_{NIR} + ref_{RED}}$$

Yield was measured at maturity in the central area of each plot (n=3) by collecting the grains with a plot combine harvester. Straw and grain weights and the harvest index were calculated in a check area of 1 m^2 in each plot. N concentrations were determined from these sample materials according to the Kjeldahl method.

The root system was investigated down to a depth of 1 m at full flowering in each year (on 16 May 2014 and 5 May 2015) using the coring method, with 3 replicates per treatment. The soil cores were split into 0.1 m sub-samples, which were frozen at -18°C until washing. Roots were separated from soil particles by a hydraulic sieving-centrifugation device on a 500-µm mesh, and coarse sand was removed by flotation. Roots were stored in a 15% v/v ethanol solution at 4°C until digitalisation as 1-bit 400-DPI TIFF format images using a flatbed scanner. The images were processed by KS 300 Rel. 3.0 software (Karl Zeiss, Munich, Germany), with adoption of a minimum area of 40 pixels for thresholding background noise and an elongation index ($perimeter^2$/area) > 40 to exclude round extraneous objects (e.g., organic debris, weed seeds). Root length was determined by the FbL (fibre length) algorithm, and the mean root diameter as the area-to-length ratio of root objects in a sample (Vamerali et al., 2003).

Statistical analysis

An ANOVA was carried out on the data for all the parameters examined using the Statgraphics Centurion XI software (Adalta, Arezzo, Italy). Separation of means was set at $P \leq 0.05$ with the Newman-Keuls test. In order to assess whether bacteria inoculation affected variability in root colonisation, the coefficient of variation (i.e., standard error-to-mean ratio) was calculated for each treatment over the 0-0.4 m profile (arable layer) at depth intervals of 0.1 m.

To facilitate interpretation of the large dataset from the two-year field trial, a factorial discriminant analysis (MDA, Multigroup Discriminant Analysis with Wilks' lambda and Pillai's trace tests) and a principal component analysis (PCA) were carried out to describe above- and below-ground plant behaviour in relation to the bacteria inoculum and N fertilisation. Multivariate data normality was first verified by the Shapiro test. Before analysis, data were standardised by subtracting the mean and dividing by the standard deviation within each variable. All analyses were performed with MS Excel XLSTAT (Addinsoft, Paris, France).

Results

ESEM analysis of bacteria-root and bacteria-shoot interactions

Bacterial colonisation and survival was investigated in roots and leaves of wheat seedlings grown *in-vitro*, at 7 and 14 days after seed inoculation, respectively. Good physical association with tissues of treated plants was found, with excellent colonisation of root cavities and deep bacterial biofilm formation (Figure 1 B, D), as well as abundant colonisation of inner root tissues (Figure 1 F, H), whereas no bacteria were detected in non-inoculated control plants (Figure 1 A, C, E, G).

The leaves of treated plants were also appreciably colonised (Figure 2 B), with the bacteria penetrating the intercellular spaces of the epidermis and crowding, in particular, around the stomata complexes (Figure 2 D). There was also considerable internal mesophyll colonisation (Figure 2 H). As for the roots, no bacteria were found on the shoots of non-inoculated control seedlings, either externally or internally, which confirmed the aseptic experimental conditions.

Climatic conditions in field trials

The climatic conditions in the two experimental seasons (2013-14 and 2014-15) differed greatly: in the first year the recorded rainfall was higher than in the second year (890 *vs*. 610 mm, October-June, +46%) as were winter/spring temperatures, although overall seasonal mean temperatures were similar (11.1°C) (Figure 3).

Different environmental conditions were observed around the time of bacterial application. In the first year, inoculum was supplied almost at the beginning of stem elongation, a slightly more advanced growth stage compared with the second year (end of tillering) when mechanical spraying was delayed because of the extremely rainy winter.

In the 10 days before inoculation, 58 mm of precipitation was recorded in the first year, but none in the second, whereas the opposite occurred after treatment, there being no precipitation for two weeks in the first year (18 mm only after 16 days) but 22 mm after 2 days in the second.

Temperatures at the time of bacterial treatment also differed in the two experiments: maximum and minimum daily temperatures on the inoculation day were 17.3 °C and 4.4 °C in the first year, and 11.2 °C and -2.6 °C in the second. In the first year, the average temperature for the 3 days following bacterial treatment was 10.6 °C, much higher than that recorded in the second year (4.3°C).

Effects on root morphology

Data from both the rhizobox and open-field trials showed the applied bacteria to have a bio-stimulating effect on plant growth. This was evident in the root growth of young plants

in the sterilised soil in the rhizoboxes, but was also appreciable in some soil layers in open fields.

In the rhizoboxes, all analysed root parameters were positively affected by the application of bacteria, whether as seed coating or foliar+soil spraying (Table 1). Compared with non-inoculated controls, root length remained almost unchanged, but root surface area increased by 10% with seed coating and 25% with foliar+soil spraying, although these improvements were not statistically significant ($P > 0.05$). The root parameters most affected were diameter (+26% average of the two treatments) and those related to root architecture, such as the number of root tips (+60% and +69%, respectively) and branches (+68% and +54%, respectively) ($P \leq 0.05$).

In the more complex field conditions, root analyses revealed some positive effects of the inoculum on mature plants (flowering stage), according to N fertilisation level and year. In the first trial, increases in Root Length Density (RLD, cm cm^{-3}) due to bacteria were observed at medium (120 kg ha^{-1}) and high (160 kg ha^{-1}) nitrogen fertilisation rates, mainly in the top 0.4 m of soil depth (Figure 4), with RLDs +29% and +11%, respectively, *vs*. non-inoculated controls. At these two N levels, the average RLD increase over the whole 0-1 m profile was still appreciable (+8% and +18%, respectively). At 80 kg N ha^{-1} of fertilisation, however, no effect of the inoculum was found at any depth (-4% RLD in the whole root pattern). With respect to the effects of N fertilisation (main effect), as expected, RLD progressively decreased with N supply (3.9, 3.8 and 3.6 cm cm^{-3} at 80, 120 and 160 kg N ha^{-1}, respectively). In the first year, a generally smaller root diameter was observed in bacteria-treated plots, the average values in the whole root pattern being -2%, -4% and -5% at 80, 120 and 160 kg N ha^{1}, respectively, *vs*. non-inoculated controls (Figure 5).

Unfortunately, many of these effects were not found in the second year trial, where the mean RLDs of the root profile in bacteria-treated plots was even slightly lower than those of the non-inoculated controls: -8%, -2% and -11% (not significant, $P > 0.05$) at 80, 120 and 160 kg N ha^{-1} of chemical fertilisation, respectively (Figure 4). Nevertheless, improvements in root densities due to bacteria were occasionally observed, e.g., only below a depth of 0.4 m at the lowest fertilisation level, below 0.8 m at maximum fertilisation, and in the 0.4-0.5 m depth interval at intermediate fertilisation. Root diameter followed the same trend as in the first year, with treated plants on average 7%, 3% and 1% smaller than non-inoculated controls (Figure 5).

In both years, a more stable rooting profile (RLD) under bacterial treatment was found, at least in the arable layer (0-0.4 m depth interval), with coefficients of variation generally lower than in controls at all N supply levels, meaning that bacteria may reduce the differences in rooting between soil layers (Figure 4).

Vegetation indices and N uptake of wheat in field trials

In both years, the wheat plants showed improved growth and leaf chlorophyll contents, due mainly to the level of nitrogen supply but also to bacterial inoculation. As expected, plant height, SPAD and NDVI were significantly increased by N fertilisation, particularly in the second year (Table 2). The positive effects of bacterial inoculum on the optical properties of the canopy at medium and low N fertilisation were clearly discernible, although only seldom significant (e.g., SPAD in 2014-15 at 120 kg N ha^{-1}), whereas there was a slight worsening of these vegetation indices with bacteria at the maximum fertilisation level. There were no significant interactions between bacteria and N supply on canopy parameters. Nor was any positive correlation found between the seasonal SPAD and NDVI

means in the first year (2013-14), as unexpectedly greater NDVI values were recorded in non-inoculated plants, probably because this index reveals both canopy greenness and soil covering.

Plant height was affected only by N fertilisation - the higher the dose, the taller the plants - whereas there was no influence of bacterial inoculum at any fertilisation level (Table 2).

Regarding N accumulation, the most noticeable result was the marked differences between the two years due to variety – high-yielding Africa in the first year and high-quality Bologna in the second – and probably to different climatic conditions.

Nitrogen concentration in the straw benefited slightly from bacterial treatment at medium-low N fertilisation in the first year, and significantly at maximum fertilisation in the second year ($P \leq 0.05$) (Table 3). A similar trend was also observed with grain N concentration, with a more general improvement (range: 1-4%).

The N harvest index (i.e., grain-to-plant N content) varied significantly according to cultivated variety, 57% in Africa (first year) and 70% in Bologna (second year), but was very stable across treatments. In this regard, a positive effect of bacterial treatment was found, in that it improved N accumulation at medium-high N fertilisation levels compared with non-inoculated controls, 25 kg ha^{-1} (+12%) on average in both years (Figure 6). Wheat yield was also very stable across treatments, particularly in var. Africa (first year), with 1-3% increases, according to fertilisation level, due to bacteria treatment. These results confirm the better yielding potential of Africa compared with Bologna (6.4 vs. 5.6 t ha^{-1}, +14%), and the role of N fertilisation in driving productivity (Table 3).

Principal component analysis (PCA) and cluster analysis (CA)

PCA conducted on the whole dataset of the two-year experiment identified two dummy variables, which explain an overall variability of 84.63%, attributed almost equally to each (F1 = 48.76%; F2 = 35.87%) (Figure 7). Relevant variables (loadings > |0.4|) were assigned to the F1 variable: NDVI, grain N concentration, plant height, SPAD and N uptake. The lodging value of the root diameter was very close to the threshold (+0.374).

Following the vector direction of each variable, generally good correlations were established among variables, particularly those very close together in the graph quadrants, i.e., NDVI, SPAD, plant height, root density, yield, and plant N concentration and uptake. The centroid position and cluster overlap shown in Figure 8 led to the conclusion that the bacteria mainly enhanced root growth at low N fertilisation, canopy greenness, N uptake and yield at medium N fertilisation, and N uptake at maximum fertilisation. At medium-low N supply, root diameter was also generally reduced by the bacterial inoculum. As expected in wheat, grain yield and N accumulation improved in accordance with the classical N dose-response model.

Discussion

Finding environmentally sustainable methods of improving productivity and reducing the use of chemical fertilisers on cereals is a current challenge in the field of agricultural research globally. Biofertilizers represent an interesting solution, and the commercial product tested here was found to have a positive impact on wheat cultivation. In aseptic conditions, ESEM analysis revealed very good survival rates and colonisation of external and internal leaf and root tissues of wheat by *Azospirillum* spp., *Azoarcus* spp. and *Azorhizobium* spp. bacterial species after artificial application, a mandatory basis for

successful application in open fields. It is recognised that physical, chemical and biological activities taking place in the open field can affect microbial growth and distribution in crop plants, even totally. The ability of PGPR to colonise internal tissues is essential for their translocation to root and shoot organs, to promote plant growth or protect against pathogens (Turner et al., 2013), thus increasing resilience against biotic and abiotic stresses.

Colonisation of the root surface is not expected to be uniform, and microscope investigations here showed no bacteria present on the root tips, in agreement with Ma et al. (2001). However, these bacteria easily enter and colonise internal tissues, as they were observed in the hollow root spaces of the conduction vessels, from where they can also translocate above ground (Turner et al., 2013). ESEM investigations also show that these bacteria adhere efficiently to leaves and, in particular, crowd in the intercellular spaces of the leaf epidermis and around the stomata openings, through which they can enter and colonise the mesophyll.

The most appreciable effect of bacteria inoculation in controlled conditions was root stimulation, regardless of the method of application, i.e., seed inoculum or foliar+soil spraying. The ability of these bacteria to promote root growth was evidenced here as a change in the root architecture related to a marked increase in the number of root tips and ramifications, suggesting that wheat plants can take advantage of a more complex root system, at least in the early stages of growth. In this way, faster root establishment would allow the plant to explore greater soil volumes and have access to greater amounts of nutrients and water. Other authors' studies of seed inoculation have shown that various PGPRs have positive effects on the shoot development and yield of wheat (Mahanta et al., 2014; Piccinin et al., 2011) and maize (Almaghrabi et al., 2014; Braccini et al., 2012;

Faruq et al., 2015), and on the early root growth, N uptake and yield of rice (Araújo et al., 2013; Elekhtyar, 2015) and cabbage (Turan et al. 2014), but little information is available on root growth in adult plants.

In the more complex situation of open fields, the interactions between PGPR and the resident soil microbioma, fertility, and soil and climatic conditions should all be considered to gain a better understanding of their true role. In trials carried out in the fertile, silty loam soil of Legnaro, encouraging root length enhancements were found in the arable layer at medium-high N fertilisation levels in the first year, whereas small benefits in the deep soil at low N input only appeared in the second year. Similarly, unstable effects due to PGPR were found with seed inoculation of sorghum with various strains of *Azospirillum brasilense* in open fields by Basaglia et al. (2003), who reported considerable root enhancement in one year, but no effect/colonisation in the second year of the trial, ascribing the failure to abundant rainfall after sowing in clay soil.

The key factor for successful PGPR-plant association is survival and reproduction on host plants (Steenhoudt and Vanderleyden 2000). The PGPR community is highly influenced by several factors, such as genotype (different wheat varieties were used in the two years of this trial), plant age, soil properties and agronomic management (Roesti et al., 2006). The association requires plant and microorganisms to be compatible and a soil environment favourable to the onset of the signals which precede colonisation (Videira e Castro et al., 2016). Extreme temperatures, pH, salinity and metal pollution are all critical factors in root colonisation (Fentahun et al., 2013). Other factors are soil water and mineral contents (Arrese-Igor et al., 2011) and synthetic agricultural inputs, such as nitrogen fertilisers, herbicides and pesticides, which can negatively affect microbiological populations (Depret et al., 2004).

Various possible reasons for root enhancement failure in the second-year trial were considered, such as the late phenological stage of the wheat at bacterial supply (end of tillering) when the root system in the arable layer was already well developed and bacterial PGPSs (plant growth-promoting substances) may have had a negligible effect, which was not the case in the first year. Root colonisation of young plants in rhizoboxes was undoubtedly facilitated by prior soil sterilisation, while in open fields applied bacteria must face hundreds of millions to billions of resident bacteria cells per gram of farmland soil (Yadav et al., 2015). The roots of wheat and other crop species are also naturally colonised by specific rhizospheric bacterial strains (Kennedy and Tchan, 1992), which can limit the association of selected PGPR.

With respect to climatic conditions, water is essential for bacterial survival on the canopy after application and for migration from the soil surface to the rhizosphere. A high soil water content stimulates anaerobic bacteria species, whereas an excessively low content slows down bacterial activity, leading to spore formation (Sylvia et al., 2005). In this study, the rooting power of bacteria in the first year was associated with abundant rainfall before inoculation and the absence of precipitation after inoculation, while low temperatures and high rainfall within a few days of inoculation characterised the second-year trial. The negative impact of low temperatures was excluded, as a very high rate of bacteria survival was measured in inoculum samples kept in the open for 2 days after inoculation, but excessive rainfall may have hampered root colonisation by the bacteria after application, as in the experiment carried out by Basaglia et al. (2003).

Wheat growth and yield are highly dependent on input level and particularly on the efficiency of nitrogen use. Chemical fertilisation was confirmed as a key factor in obtaining sustainable yields with the high quality required by the bakery industry. The maximum N

dose tested (160 kg ha^{-1}) is the recommended rate in northern Italy for a yield target of 6-7 t ha^{-1}, which seems, unexpectedly, to be compatible with the positive role of bacteria; even reduction to the medium dose (120 kg N ha^{-1}) still preserves the effects of the bacteria. As root growth is reduced by external N supply, it may be that applied bacteria provides more evident root stimulation and better agronomic performance at a medium-high N level, in accordance with existing literature (Dalla Santa et al., 2004; Millet et al., 1984).

Several authors have reported improved N accumulation in inoculated plants as a result of biological N-fixation of PGPRs (Dalla Santa et al., 2004; Panwar and Singh, 2000), but in the temperate climate of Legnaro root growth enhancement is presumed to play a major role in nutrient acquisition (Okon et al., 1998; Steenhoudt and Vanderleyden, 2000). More efficient N fixation probably occurred in the second-year trial with no apparent root stimulation, although the occurrence of root hair proliferation is not excluded (Fallik et al., 1994). *Azoarcus* and *Azospirillum* have been found to colonise plant leaves (Steenhoudt and Vanderleyden, 2000), where they apparently find a favourable environment for N fixation and for obtaining nutritional resources from the host in the absence of strong competition from the natural rhizospheric microbioma (Pedraza et al., 2009). The absence of a real symbiosis may still be crucial for widespread exploitation of PGPR in cereals, and bacteria associations can only partially satisfy the plant's nutrient needs (Hungria, 2010).

The main result of this study was the finding that the crop exhibited greater N accumulation, so some beneficial environmental effects in terms of reduced N losses from agricultural ecosystems can be expected from the application of PGPRs. Possible agronomic benefits consist in savings on fertilisers, but it does not seem that significant improvements in vegetation indices and yield are currently achievable in the fertile agro-ecosystem of northern Italy. Indeed, greater root growth may compete with grain filling for

plant resource allocation under optimal growing conditions (Fang et al., 2017), although the negligible root stimulation in the second year and the fact that root expansion generally falls drastically after flowering would seem to contradict this. One important issue to explore and develop further in this research field is exploitation of the synergistic action of various bacteria with different characteristics and/or associations with mycorrhizal fungi or algae and the mechanisms of plant interaction (Dashadi et al., 2011; Nain et al., 2010; Pérez-Montano et al., 2014; Rafi et al., 2012), as recently demonstrated by Visioli et al. (2015) in the metal hyperaccumulator *Noccaea caerulescens*, where co-inoculation of two specific strains greatly improved growth and nickel uptake and translocation.

The use of mixed PGPR and N-fixing bacteria in conventional wheat cultivation could be a means of improving root growth and possibly coping better with environmental stresses. Integrating the mechanisms of rooting power and N-fixation in a consortium of bacteria may help reduce N losses from agricultural ecosystems and therefore make partial savings on chemical fertilisers, although external N supply is still essential to maintaining crop yield and quality standards.

Fine-tuning research in this area to permit selection of microorganisms for specific crops and varieties will probably be the main future challenge in successful exploitation of PGP bacteria and the development of commercial biofertilizers.

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Figures and Tables

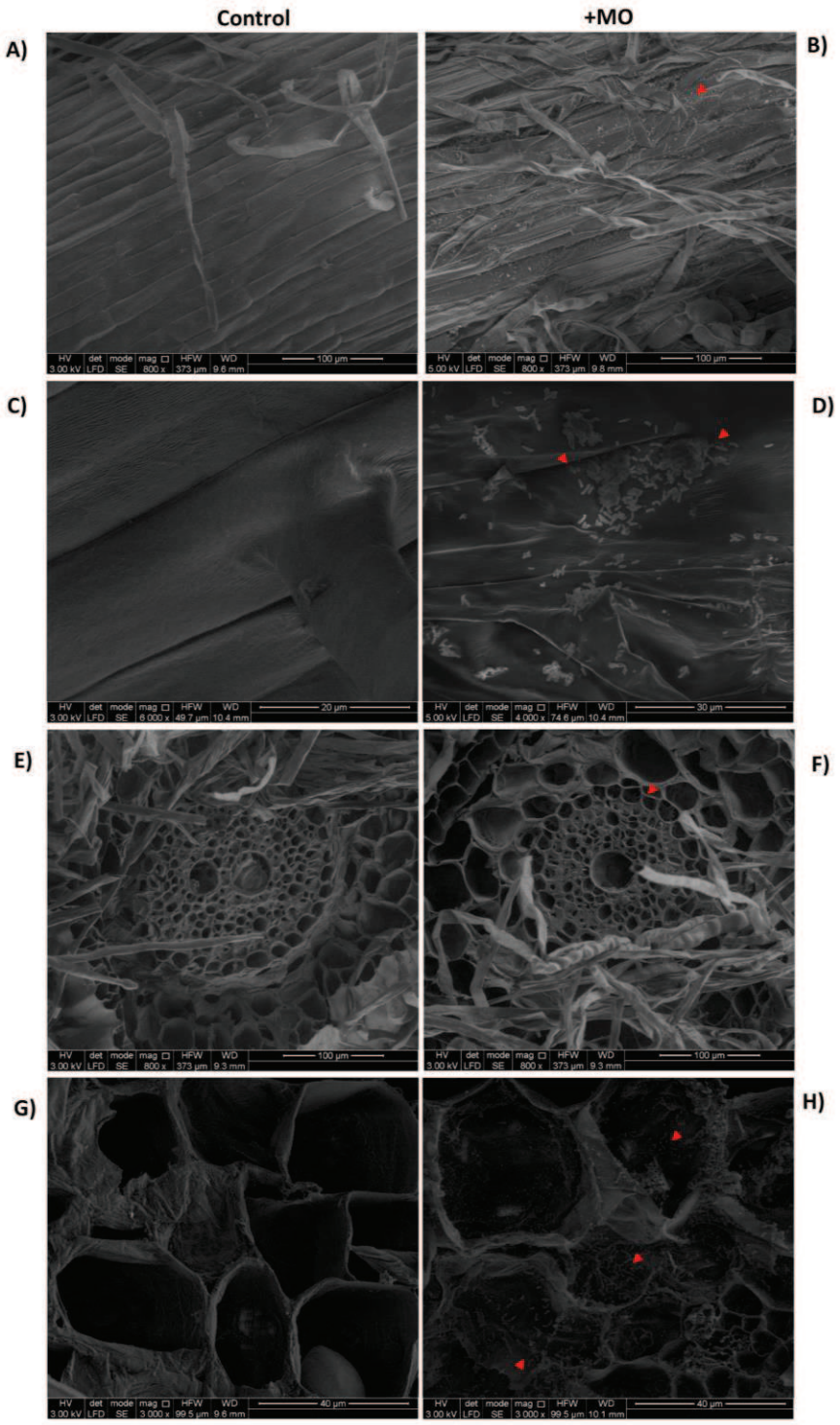


Figure 1. ESEM micrographs of root surfaces (A, B, C, D) and transversal sections (E, F, G, H) in control (non-inoculated, left) and inoculated (+MO, right) 7-day-old wheat seedlings. Note abundant bacteria colonisation on right side only. Red arrows indicate the points of the root tissues magnified to observe bacterial colonisation.

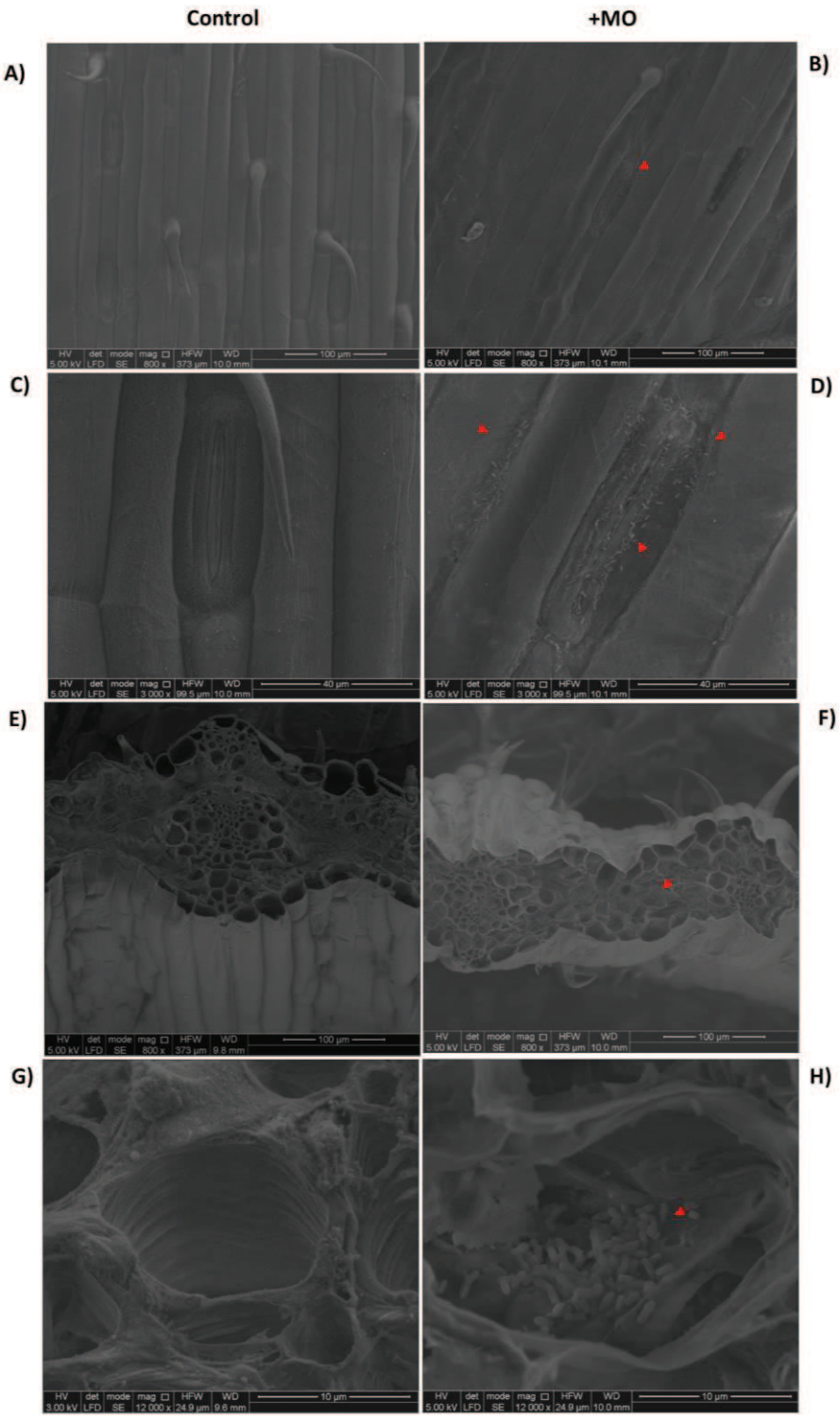


Figure 2. ESEM micrographs of leaf surfaces (A, B, C, D) and transversal sections (E, F, G, H) of control (non-inoculated, left) and inoculated (+MO, right) 14-day-old wheat seedlings. Note abundant bacteria colonisation on right side only, particularly around the stomata (D, 3000× magnification). Red arrows indicate the points of the leaf tissues magnified to observe bacterial colonisation.

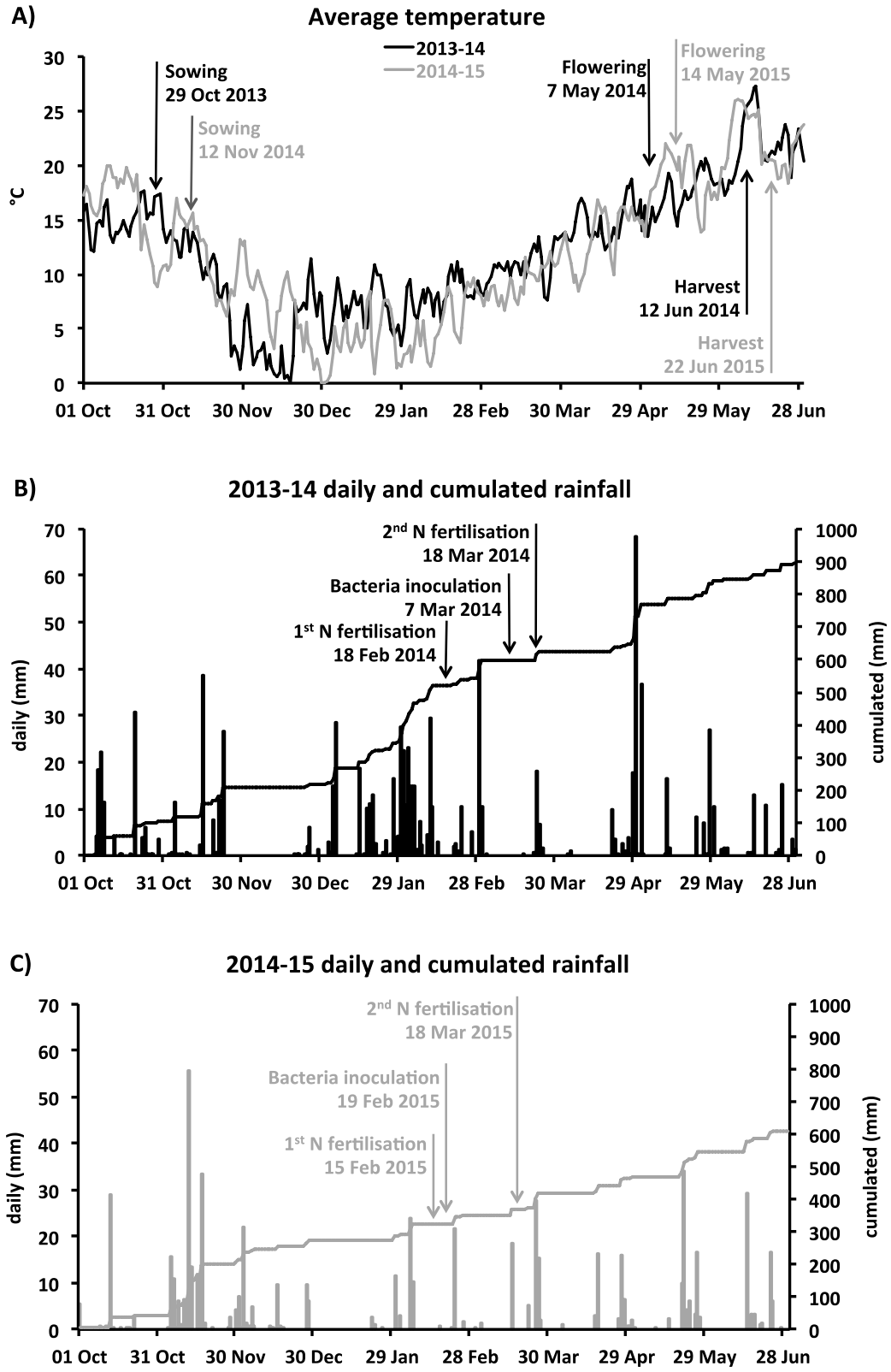


Figure 3. Dynamics of seasonal daily mean temperatures (A), and daily and cumulated rainfall across the wheat crop cycle in the first-year (B) and second-year trials (C) at the Legnaro experimental site (Padua, Italy).

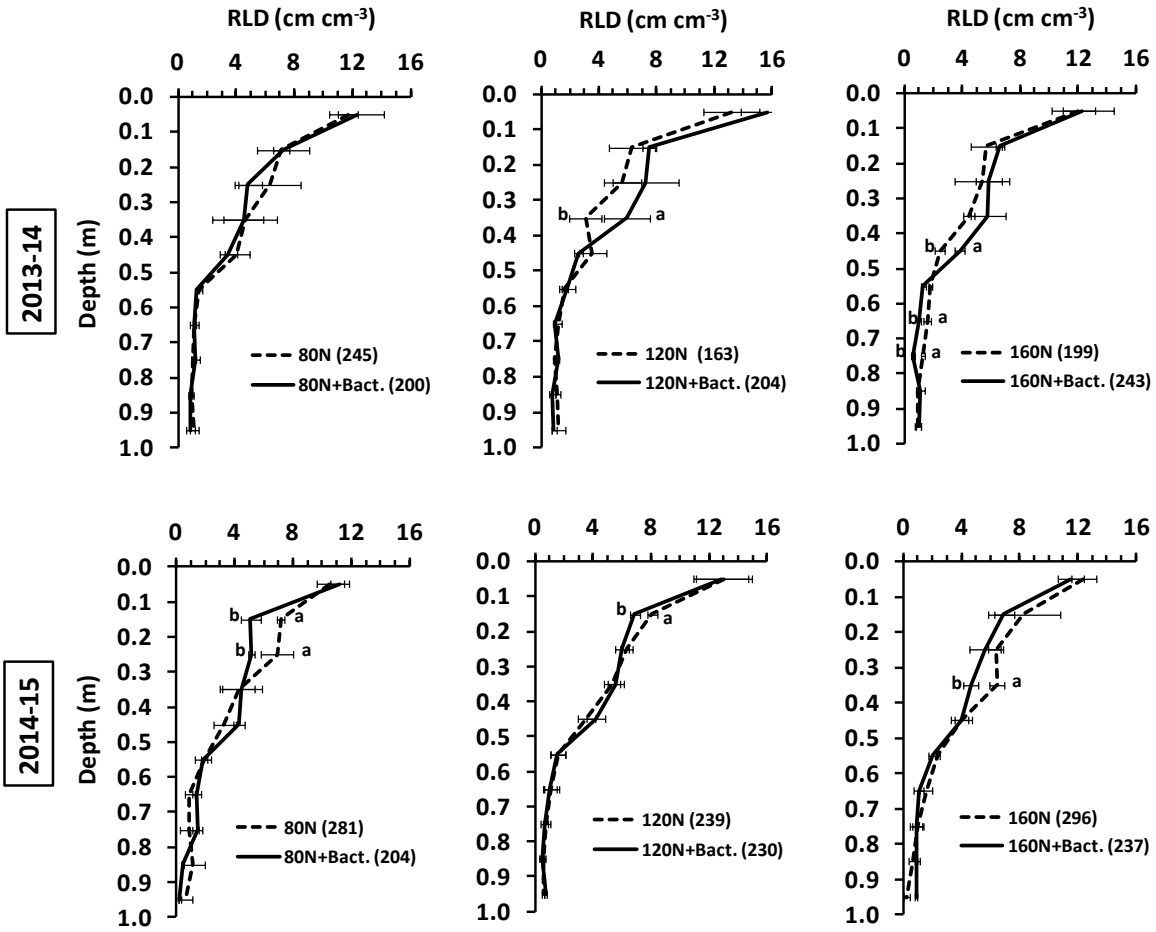


Figure 4. Root length density (RLD, mean±SE; n=3) patterns in bacteria-inoculated *Triticum aestivum* L. plants (continuous line) *vs*. non-inoculated controls (dashed line) with decreasing N fertilisation levels (160, 120 and 80 kg ha^{-1}) at the flowering stage in a two-year field trial. Letters: statistically significant differences among treatments at each depth interval (Newman-Keuls test, $P \leq 0.05$). In brackets in the graphs: coefficients of variation (CV %) of each treatment across the 0-0.4 m depth interval.

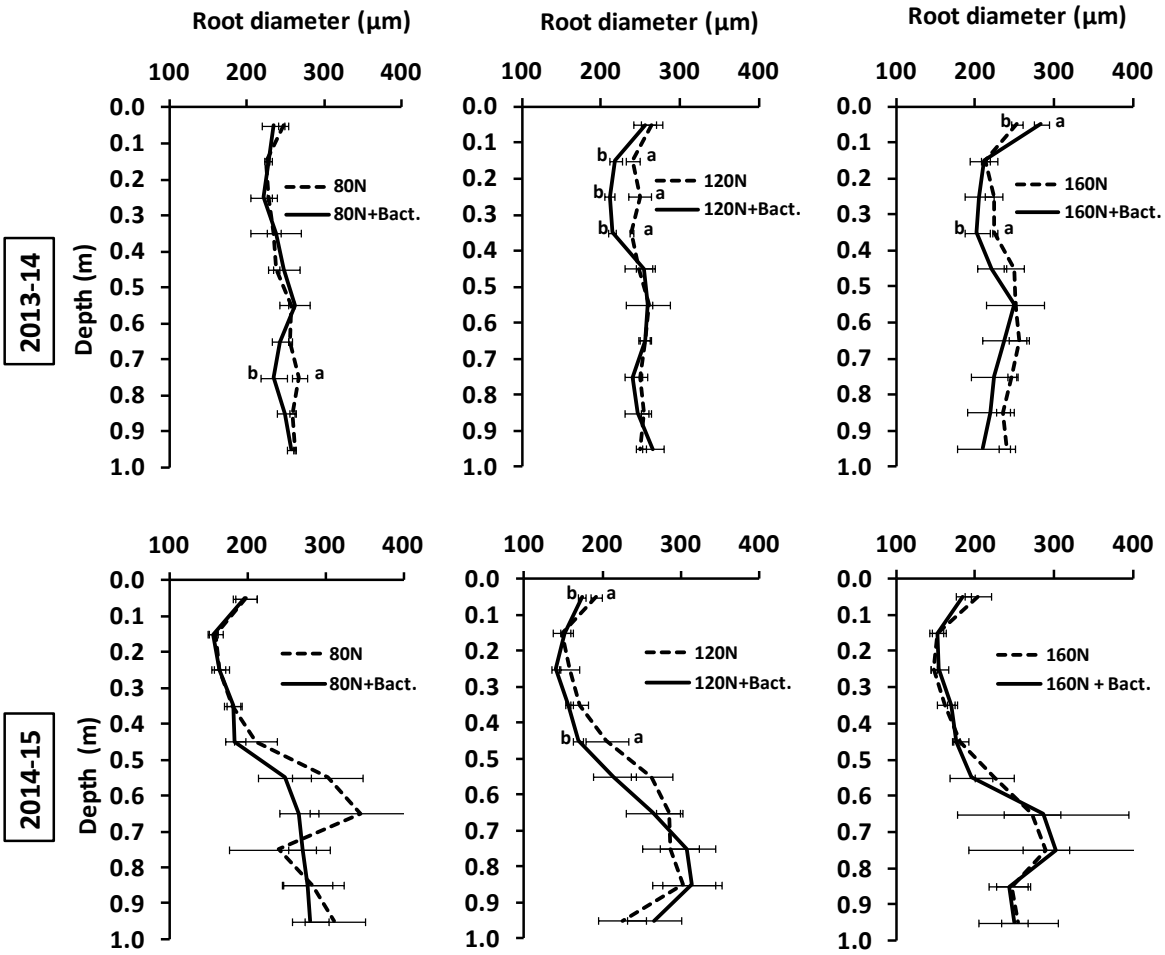


Figure 5. Root diameter (mean±SE; n=3) patterns in bacteria-inoculated *Triticum aestivum* L. plants (continuous line) *vs*. non-inoculated controls (dashed line) with decreasing N fertilisation levels (160, 120 and 80 kg ha^{-1}) at the flowering stage in a two-year field trial. Letters: statistically significant differences among treatments at each depth interval (Newman-Keuls test, $P \leq 0.05$).

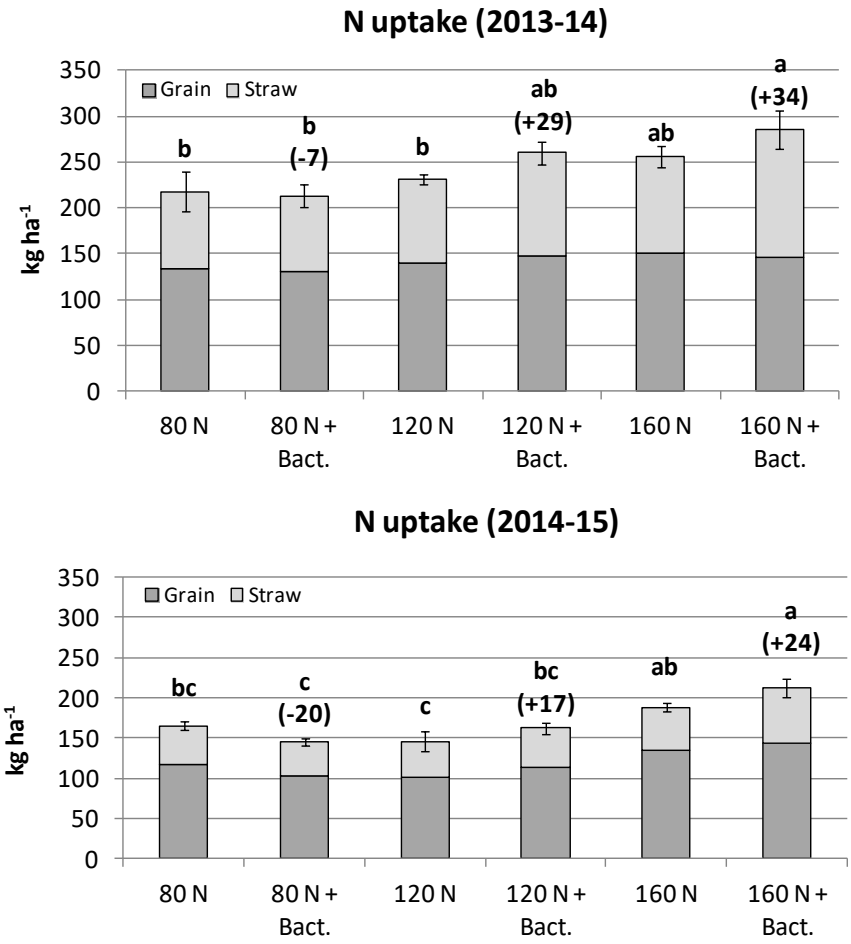
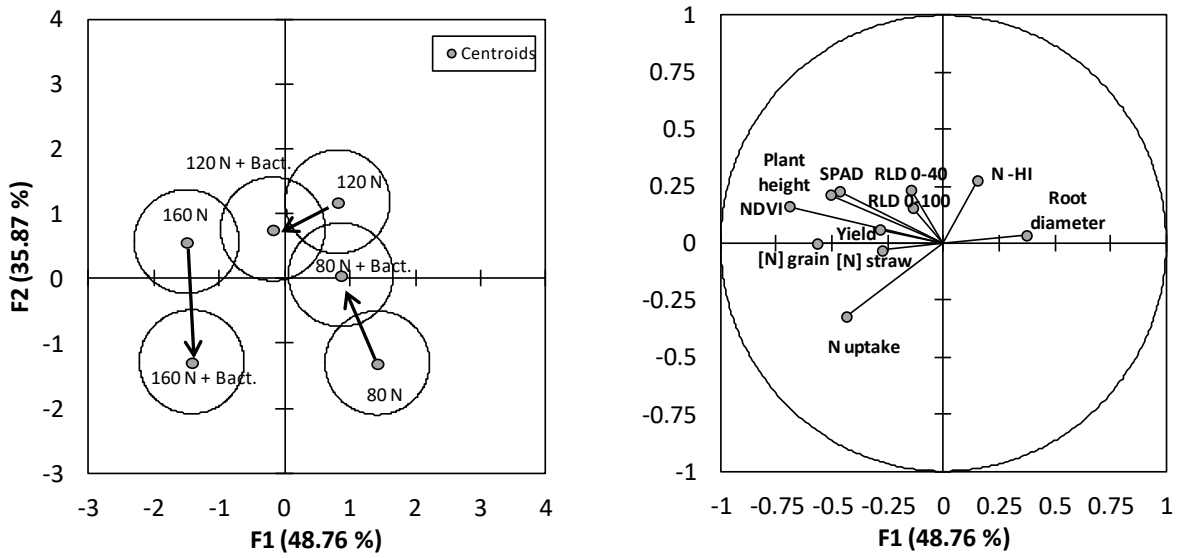


Figure 6. Overall (grain + straw) nitrogen uptake on a per hectare basis by bacteria-inoculated *Triticum aestivum* L. plants *vs*. non-inoculated controls with decreasing N fertilisation levels (160, 120 and 80 kg ha^{-1}) at plant harvest in a two-year field trial. Letters: statistically significant differences among treatments for multiple comparisons (Newman-Keuls test, $P \leq 0.05$). In brackets: variations (kg ha^{-1}) in bacteria-treated plants *vs*. non-inoculated controls at each N fertilisation level.



	F1	F2
RLD 0-100 cm	-0.137	0.155
RLD 0-40 cm	-0.145	0.233
Root diameter	0.374	0.034
N uptake	**-0.435**	-0.322
SPAD	**-0.465**	0.228
NDVI	**-0.691**	0.162
Plant height	**-0.506**	0.213
[N] grain	**-0.566**	-0.002
[N] straw	-0.275	-0.032
N-HI	0.153	0.275
Yield	-0.284	0.060

Figure 7. Principal component analysis (PCA; top right) with variable loadings (highlighted values > |0.4|; bottom table) and discriminant analysis (DA; top left) for nitrogen fertilisation level (160, 120 and 80 kg ha^{-1}) and bacterial inoculum in a two-year trial. Circles in the DA comprise 70% of cases.

Table 1. Root parameters (mean±SE; n=3) in bacteria-inoculated *Triticum aestivum* L. plants (two methods of application) *vs*. non-inoculated controls at 50 days after sowing in sterilised soil in rhizoboxes. Letters: significant differences among treatments within the same parameter (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in bacteria-treated plants *vs*. non-inoculated controls.

Treatment	Length (m plant^{-1})		Surface area (m^2 plant^{-1})		Diameter (µm)		Root tips (no. plant^{-1})		Ramification index (no. forks m^{-1})	
Untreated	35.6 ± 4.64	a	0.37 ± 0.05	a	344 ± 19.4	b	7559 ± 1178	b	555 ± 33	b
Seed application	29.7 ± 4.87	a (-17)	0.41 ± 0.07	a (+10)	443 ± 15.1	a (+29)	12102 ± 2136	a (+60)	933 ± 77	a (+68)
Soil + foliar spraying	36.2 ± 5.84	a (+2)	0.47 ± 0.08	a (+25)	422 ± 13.3	a (+23)	12758 ± 2610	a (+69)	856 ± 60	a (+54)

Table 2. Optical parameters (SPAD and NDVI) and culm height (mean±SE; n=3) in bacteria-inoculated *Triticum aestivum* L. plants *vs*. non-inoculated controls at increasing N fertilisation levels (80, 120 and 160 kg ha^{-1}) in a two-year field trial. NDVI and SPAD: average seasonal values (stem elongation - heading). Letters: significant differences among treatments within the same parameter (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in bacteria-treated plants *vs*. non-inoculated controls at each N fertilisation level.

Treatment	SPAD				NDVI				Culm height (cm)			
	2013-14		2014-15		2013-14		2014-15		2013-14		2014-15	
80 N	45.8 ± 0.64	c	39.7 ± 0.19	d	0.71 ± 0.004	bc	0.66 ± 0.02	c	71.0 ± 1.36	b	68.1 ± 0.52	b
80 N + Bact.	48.0 ± 0.53	bc (+5)	40.2 ± 0.85	d (+1)	0.70 ± 0.004	c (-1)	0.67 ± 0.03	bc (+1)	72.9 ± 0.86	ab (+3)	67.9 ± 1.02	b (-1)
120 N	49.4 ± 0.43	ab	41.5 ± 0.35	c	0.72 ± 0.004	b	0.70 ± 0.02	abc	73.4 ± 1.05	ab	69.5 ± 0.58	ab
120 N + Bact.	49.5 ± 0.45	ab (+1)	42.9 ± 0.59	b (+3)	0.71 ± 0.004	b (-1)	0.72 ± 0.02	ab (+3)	73.1 ± 0.95	ab (-1)	69.9 ± 0.75	ab (+1)
160 N	50.8 ± 0.52	a	44.2 ± 0.27	a	0.76 ± 0.004	a	0.73 ± 0.01	a	74.6 ± 0.79	a	71.1 ± 0.41	a
160 N + Bact.	49.7 ± 0.51	ab (-2)	43.7 ± 0.55	ab (-1)	0.72 ± 0.004	b (-5)	0.74 ± 0.02	a (+1)	73.8 ± 1.10	ab (-1)	72.0 ± 1.38	a (+1)
Fertilisation	**		***		ns		*		ns		**	
Bact. application	ns		ns		ns		ns		ns		ns	
Fert × Bact. app	ns		ns		ns		ns		ns		ns	

n.s. = not significant; *, ** and *** = significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Table 3. Nitrogen concentrations in straw and grain tissues, nitrogen harvest index and yield (mean±SE; n=3) in bacteria-inoculated *Triticum aestivum* L. plants *vs*. non-inoculated controls at increasing N fertilisation levels (80, 120 and 160 kg ha^{-1}) in a two-year field trial. Letters: significant differences among treatments within the same parameter (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in bacteria-treated plants *vs*. non-inoculated controls at each N fertilisation level.

Treatment	Straw [N] (% d.w.)				Grain [N] (% d.w.)				N harvest index (%)				Yield (kg ha^{-1})			
	2013-14		2014-15		2013-14		2014-15		2013-14		2014-15		2013-14		2014-15	
80 N	0.81 ± 0.09	a	0.43 ± 0.02	b	2.06 ± 0.07	c	1.83 ± 0.05	b	57.3 ± 3.83	ab	70.1 ± 1.77	a	6507 ± 149	a	5158 ± 97.7	b
80 N + Bact.	0.84 ± 0.08	a (+4)	0.43 ± 0.03	b	2.13 ± 0.07	bc (+3)	1.85 ± 0.03	b (+1)	56.5 ± 4.80	ab (-1)	70.8 ± 0.71	a (+1)	6356 ± 152	a (-2)	5252 ± 281	b (+2)
120 N	0.83 ± 0.07	a	0.46 ± 0.04	b	2.17 ± 0.07	bc	1.84 ± 0.05	b	60.7 ± 1.05	a	69.6 ± 2.77	a	6469 ± 23.3	a	5604 ± 95.7	ab
120 N + Bact.	0.86 ± 0.06	a (+4)	0.45 ± 0.02	b (-2)	2.21 ± 0.06	abc (+2)	1.90 ± 0.01	b (+3)	57.0 ± 3.73	ab (-6)	70.2 ± 1.34	a (+1)	6688 ± 282	a (+3)	5745 ± 136	a (+3)
160 N	1.04 ± 0.12	a	0.45 ± 0.03	b	2.39 ± 0.02	a	1.95 ± 0.04	ab	59.0 ± 1.92	ab	71.6 ± 0.96	a	6294 ± 141	a	5982 ± 100	a
160 N + Bact.	0.94 ± 0.06	a (-10)	0.56 ± 0.02	a (+24)	2.30 ± 0.05	ab (-4)	2.03 ± 0.06	a (+4)	48.5 ± 5.74	b (-18)	67.5 ± 0.77	a (-6)	6353 ± 36.3	a (+1)	6057 ± 140	a (+1)
Fertilisation	ns		ns		**		**		ns		ns		ns		***	
Bact. application	ns		ns		ns		ns		ns		ns		ns		ns	
Fert × Bact. app	ns		ns		ns		ns		ns		ns		ns		ns	

n.s. = not significant; *, ** and *** = significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

CHAPTER II

Arbuscular mycorrhizal fungi and diazotrophic bacteria cover the gap in root growth under medium-high nitrogen supply with benefits for nutrient uptake in common wheat

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Abstract

Arbuscular mycorrhizal fungi and diazotrophic bacteria stimulate plant growth and crop yield through several mechanisms that include improved nutrient uptake and water use efficiency, and soil pathogen inhibition. These benefits are mostly related to changes in root morphology and physiology, although they vary considerably with crop species and growth conditions.

This study investigated the effects of a biofertilizer containing the mycorrhizal fungus *Rhizophagus irregularis* and the diazotrophic N-fixing bacterium *Azotobacter vinelandii* on root and shoot growth, yield, and nutrient uptake in common wheat (*Triticum aestivum* L.). An open field trial was carried out over two years to investigate the interaction between inoculation and three decreasing doses of nitrogen fertilization (160, 120 and 80 kg ha^{-1}). A rhizobox experiment with sterilized soil was carried out to assess root mycorrhizal colonization using microscopic techniques, and early root growth following seed inoculum or foliar + soil spraying after emergence.

Efficient root colonization by *R. irregularis* was observed at 50 days after sowing in seed-inoculated rhizobox plants, together with improved root tip density and branching (+ ~30% *vs*. controls), while the effects of post-emergence inoculation were not yet observable at plant sampling. In contrast, in the open field trial, following late (end of tillering) soil/canopy spraying we were able to detect notable increases in volumetric root length density (+22% *vs*. controls) and root area density (+18%) after about two months (flowering stage) in both years under medium and high N fertilization doses, but not at the low N dose. While inoculation had a negligible effect on grain yield, which followed a typical N dose-response model, slightly improved uptake of N and other nutrients, particularly Zn, P, and K, were recorded at all fertilization rates.

We conclude that the highest N fertilization dose is still required to achieve high yield and standards of quality (protein contents) in wheat cultivation. In light of this, the use of mixed mycorrhizae-PGPR biofertilizers appears to be a sustainable means for minimizing the adverse effects of N supply on root expansion and for improving the uptake of low-mobility nutrients.

Keywords: *Rhizophagus irregularis*; *Azotobacter vinelandii*; common wheat; nitrogen fertilization; root growth; nutrient uptake.

Introduction

In last decades, agronomic management developed from scientific research has focused on increasing crop productivity through intensive use of fertilizers and chemicals (Tilman et al., 2002). In order to provide sufficient food for humans in a context of fast population growth and new concepts of sustainability, agriculture is now called upon to reduce the use of non-renewable resources and address the negative impact of conventional agricultural practices on climate and of environmental pollution (Foley et al., 2011).

Wheat is one of the world's most widely-grown crops, providing staple food for one third of human population. It is cultivated under a great number of climatic conditions and agricultural systems, and consequently with variable yield responses (Marris, 2008). The current average global grain yield is 3.3 t ha^{-1}, a value that should be almost doubled to meet future food demands (Dixon et al., 2009). In many countries, this target has already been met, and even exceeded, as a result of the intensive use of agro-chemicals, as wheat yield responds well to input level. Large applications of chemical fertilizers, particularly

nitrogen, are required to maintain high wheat yields, although this can have negative consequences for the environment and human health (Sutton, 2011).

In order to address these critical issues and deal more effectively with environmental stresses, research is now focusing on exploring plant root-microbial associations (Berg, 2009). Plant-aiding microorganisms are currently becoming available as commercial biofertilizers, and are claimed to have beneficial effects on cereal growth and productivity as a result of interaction with several rhizosphere processes (Vessey, 2003; Lesueur et al., 2016). Plant growth-promoting rhizobacteria (PGPR) are known to stimulate plant growth by fixing N, producing auxins and other phytohormones that enhance root growth, and by synthesizing siderophores, that enhance low-available nutrient uptake from the soil, and other substances that help plants withstand adverse conditions (Bishnoi, 2015).

Vesicular-arbuscular mycorrhizal (VAM) fungi are non-pathogenic microorganisms that establish symbioses with many spontaneous and cultivated species, and have the ability to boost water and nutrient uptake, especially in poor, arid soils, and to protect the plant against root pathogens (Solaiman and Mickan, 2014). Application of PGPR and mycorrhizal fungi consortia has also been shown to improve plant growth as a result of synergistic interactions between microorganisms (Singh and Kapoor, 1999; Artursson, 2005; Behl et al., 2007; Najafi et al., 2012; Pérez-de-Luque et al., 2017).

The literature documents a positive influence on the shoot growth, nutrient uptake and grain yield of wheat after inoculation with arbuscular mycorrhizal fungi (Pellegrino et al., 2015; Berruti et al., 2016), PGPR (Pérez-Montaño et al., 2014) or VAM-PGPR consortia (Behl et al., 2007). However very few studies, mostly in laboratory and only seldom in field conditions, have investigated the effects of microbial inoculation on root growth, and they have not taken into account the type of agricultural management applied and the

constrains imposed by abiotic and biotic factors (Vacheron et al., 2013; Fusconi, 2014; Hakeem et al., 2016, Dal Cortivo et al., 2017).

A vigorous root system is fundamental for the development of healthy crops, and, consequently, for obtaining high yields (Fageria and Moreira, 2011). Root colonization by VAM and bacteria can increase root length and surface area, allowing a large soil volume to be explored, and thereby enhancing water and nutrient uptake, particularly under drought conditions (Marulanda et al., 2006; 2009).

In this context, the aim of our study was to investigate the response of common wheat (*Triticum aestivum* L.) to inoculation with a biofertilizer containing the VAM *Rhizophagus irregularis* and the diazotrophic bacterium *Azotobacter vinelandii* in both controlled and open field conditions. At a preliminary stage, the microorganisms were applied as a seed inoculum or by foliar spraying young seedlings cultivated in rhizoboxes in order to assess early root growth and the effectiveness of mycorrhizal colonization. Over a two-year trial in open fields, the inoculum was applied by foliar-spraying at the end of tillering in combination with decreasing N fertilization doses in order to assess whether these microorganisms are able to improve root growth and alleviate nitrogen deficiency. Root growth patterns down to a depth of 1 m, nutrient uptake, and yield performances were assessed in two wheat varieties.

Materials and Methods

Rhizobox trial and root observations

A rhizobox trial was set up in order to study the effects on the early root growth of wheat after inoculation with the commercial biofertilizer Rhizosum N® (Biosum Technology,

Madrid, Spain), which contains the VAM *Rhizophagus irregularis* (2% w/w) and the diazotrophic bacterium *Azotobacter vinelandii* (1×10^{10} CFU g^{-1}). Transparent, plexiglass-sided rhizoboxes, measuring 30 × 2.5 cm and 45 cm high, were randomly positioned inside a greenhouse at the University of Padua's experimental farm at a 45° angle to allow root observation through the transparent underside during growth. They were filled with a mixture of sand and silty-loam soil (1:1 w/w), previously sterilized at 105 °C for 72 hours in a large oven. About 30 kg of N ha^{-1} and 90 kg ha^{-1} of P_2O_5 and K_2O were incorporated into the soil to mimic basic pre-sowing field fertilization. Three seeds of wheat var. Bologna (SIS, Bologna, Italy) were sown at a depth of 3 cm in each rhizobox, and plants were grown for 50 days from February to March 2014.

Inoculum was applied as a seed-coating treatment or by foliar spraying, and results were compared with not inoculated controls. In the former case, just before sowing, 0.1 g of freeze-dried formulation was diluted in 2 mL of ultrapure water and added to 1000 seeds (i.e., ~38 g) in order to facilitate adherence; the final inocula contained 0.19 µg of *R. irregularis* and 10^6 CFU of *A. vinelandii* per seed, consistent with the recommended application rate of 50 g ha^{-1} of commercial formulation. All seeds had been previously sterilized in 15% v/v sodium hypochlorite solution for 15 min, then rinsed for 5 min in ultrapure water three times. In the second case, the same amount of product per plant was applied post-emergence by spraying 10 mL inoculum solution (0.01 g Rhizosum N in 1 L of water) on the leaves and the ground surface at the 4-leaf (unfolded) stage (23 DAS, days after sowing). Three rhizoboxes (replicates) per treatment were examined (nine in total).

At the end of the experiment, determined as when the roots had more or less reached the bottom of the rhizobox at the growth stage Z14 (Zadoks et al., 1974), i.e., four leaves on main shoot, the plants were harvested and the root system was gently washed so it could be

collected in its entirety. Roots were stored in ethanol solution (15% v/v) at 4 °C until processing. The main parameters, i.e., length, surface area, diameter, and number of tips and forks, were measured from 1-bit 400-DPI TIFF-format images of the roots acquired with a flatbed scanner (Expression 11000 XL, Epson, Suwa, Japan). Image analysis was carried out with the WinRhizo® software (Regent Instruments Inc., Ville de Québec, Canada).

Pot trial and mycorrhizae detection

In order to assess the viability of the commercial inoculum and the extent of possible root mycorrhization in wheat, simultaneously as the rhizobox experiment, we carried out a similar trial with seed-inoculated plants grown in 3.1-L pots (9 cm diameter, 50 cm height) following the same inoculation procedure as for the rhizoboxes (i.e., substrate and seed sterilization, dose of inoculum, number of replicates). We collected 40-day-old roots of inoculated and non-inoculated control wheat plants from the pots and washed them briefly in sterile water before optical microscope and ESEM (Environmental Scanning Electron Microscopy) imaging.

For the microscopic analysis, 5-mm long root fragments were excised and fixed in ethanol 70% v/v. Following the procedure of Trouvelot et al. (1986) modified, root fragments were incubated in KOH 10% at 60 °C for 30 min., briefly rinsed in distilled water, and incubated for 1 min in methyl blue 1% w/v in lactic acid. The excess of methyl blue was removed with adsorbent paper, and the roots fixed in lactic acid on a microscope slide for 5 minutes. The latter step was repeated twice more, allowing the roots to dry. Root fragments were then placed in a Falcon tube and immersed in lactic acid for 1 h. Finally, root samples were mounted on a microscope slide, gently pressed with a cover slide, and examined with a

microscope (Nikon eclipse E600) mounted with a DS-FIZ camera to detect mycorrhizae. Forty-five root pieces, coming from the three rhyzoboxes/replicates (15 each), were visually analyzed and each of them assigned to a specific class of mycorrhization a follows: absence of infection (class 0); traces of infection (class 1); less than 10% infection (as root length) (class 2); from 11 to 50% of infection (class 3); from 51 to 90% of infection (class 4); more than 90% of infection (class 5). An index of mycorrhizal colonization (M) was calculated as follows:

$M\% = (95 \times n5 + 70 \times n4 + 30 \times n3 + 5 \times n2 + n1) / N$

where n1, n2, n3, n4 and n5 are the number of root pieces belonging to classes 1, 2 ,3, 4 and 5, respectively, and N is the total number of root pieces examined.

ESEM analyses was used as further tool to ascertain root mycorrhization. One-mm long root fragments were excised with a sterile lancet and fixed overnight in 3% v/v glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.0 and 4 °C. The samples were then extensively rinsed in 0.1 M phosphate buffer pH 7.0, and dehydrated in acetone solution (25, 50, 75 and 100% v/v in deionised H_2O). Lastly, the samples were dried with a Critical Point Dryer (CPD 020, Balzers Union Limited, Balzers, Liechtenstein) in a CO_2 atmosphere, and attached to aluminum stubs with double-sided adhesive conductive carbon tape. Root mychorrization was observed with an Environmental Scanning Electron Microscope (ESEM) Quanta™ 250 FEG (FEI, Hillsboro, OR, USA), operating in low vacuum mode (pressure chamber set at 100 Pa), and with a beam accelerating voltage of 3 or 5 kV.

Two-year field trial

We conducted a field trial during the 2013-14 and 2014-15 growing seasons at the University of Padua's experimental farm at Legnaro (45° 21' N, 11° 58' E, 12 m a.s.l) on the Po plain (NE Italy). Wheat was cultivated in a silty-loam soil (fulvi-calcaric-cambisol; USDA classification) with a pH of 8.0, an OM content of 17 g kg^{-1}, CEC of 11.4 cmol (+) kg^{-1}, and a total N content of 1.1 g kg^{-1} (arable layer at the beginning of the experiment). Annual precipitation at this site is ~830 mm (30-year historical mean).

We used a completely randomized block experimental design with 3 replicates; each plot measured 10 × 3 m (30 m^2) and comprised 24 crop rows 12 cm apart. In both years, the previous crop had been sugar beet. The soil was ploughed to a depth of 0.3 m and harrowed at 0.2 m, and prior to sowing the fertilizers N, P_2O_5, and K_2O were incorporated in doses of 32, 96 and 96 kg ha^{-1}, respectively. The high-yielding wheat variety Africa (APSOV, Voghera - Italy) was grown in the first year, and the high-quality Bologna (SIS, Bologna, Italy) in the second, as two of the main varieties cultivated in the region. First year sowing took place on October 29, 2013, harvesting on June 12, 2014; second year sowing was on November 12, 2014, harvesting June 22, 2015. Crops were protected against weeds and pests by specific treatments, in accordance with local management recommendations. To offset possible interactions between fungicides and mycorrhizal fungi, plants were protected from fungal pathogens at the heading stage using compatible active ingredients (Plant Health Care Incorporation, 2009).

Inoculated plots and non-inoculated controls were factorially combined with three decreasing rates of N fertilization, i.e., 160 (conventional rate in NE Italy for a target grain yield of 6-7 t ha^{-1}), 120 (75% of the conventional rate) and 80 kg N ha^{-1} (50% of the conventional rate). In addition to pre-sowing fertilization, half the remaining N dose at

each fertilization level was applied at the tillering stage and half at the onset of stem elongation with ammonium nitrate.

The microbial inoculum (Rhizosum N) was applied at a label dose of 50 g of freeze-dried product ha^{-1}, following the manufacturer's instructions, at tillering (March 7, 2014 in the first year, February 19, 2015 in the second). The commercial formulation was rehydrated for one hour in 200 mL pure water, mixed with 600 L ha^{-1} of non-chlorinated water, and sprayed mechanically onto the wheat using farm-scale technologies. The treatment was performed late afternoon in order to minimize the interaction of UV light with bacterial survival. The application volume ensured good soil and canopy wetness, with expected inoculation rates of about 0.1 mg VAM m^{-2}, and 5×10^7 CFU m^{-2}.

During the growing cycle, from the beginning of stem elongation to the booting stage, the relative chlorophyll content in leaves was monitored with a SPAD 502 chlorophyll meter (Konica-Minolta, Hong Kong) on the last fully-developed leaf (10 leaves per plot randomly chosen) at 10-day intervals. Culm height was also measured on the same plants.

At the same time as the SPAD measurements were taken, the Normalized Difference Vegetation Index (NDVI) of the canopy of each plot was measured with an active handheld Greenseeker spectrometer (Ntech Industries, Ukiah, CA, USA). The sensor measures canopy reflectance at wavelengths of 590 nm (ref_{RED}) and 880 nm (ref_{NIR}), and provides a ratio value as follows:

$$NDVI = \frac{ref_{NIR} - ref_{RED}}{ref_{NIR} + ref_{RED}}$$

At maturity, yield was measured in the central area of each plot by collecting the grains with a combine harvester. Straw and grain weights, and the harvest index were measured in a checking area of 1 m^2. These sample materials were used to measure N concentration according to the Kjeldahl method, while Ca, K, P, Fe, Mg and Zn concentrations were

measured by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (SPECTRO CirOS Vision EOP, SPECTRO Analytical Instruments GmbH & Co. KG, Kleve, Germany). Oven-dried samples of grains and straw of about 0.4 g were mixed with 7 mL HNO_3 (65% v/v) and 1 mL H_2O_2 (30% v/v), then microwave acid-digested (Mileston ETHOS 900, Bergamo, Italy) following EPA method 3052. The samples were then diluted to 25 mL with distilled water, filtered (0.45-µm CA), and analyzed by ICP-OES. Measurement accuracy was assured with certified reference materials (ERM-CD281 and BRC-402, JRC-IRMM, Belgium).

At full flowering (May 16, 2014 in year 1, May 5, 2015 in year 2), the root system was investigated down to a depth of 1 m using the coring method, and with 3 replicates per treatment. The soil cores (70 mm diameter) were split into 0.1-m sub-samples, which were frozen at -18 °C until washing. Roots were cleaned of soil particles using a hydraulic sieving-centrifugation device on a 500-µm mesh sieve, and stored in a 15% v/v ethanol solution at 4 °C until digitalization. Root images were processed in the KS 300 Rel. 3.0 software (Karl Zeiss, Munich, Germany), with a minimum area of 40 pixels for thresholding background noise. Root length was determined by the FbL (fiberlength) algorithm, and the mean root diameter was calculated as the area-to-length ratio of root objects in a sample (Vamerali et al., 2003).

At the same time as the root examination, 100 root tips per treatment were observed through a stereomicroscope (×10-50 magnification) to assess the extent of root mycorrhizal colonization. In the first year only, due to the laboriousness and time-consuming procedure, roots were collected from 0-0.3 m depth soil cores after gently washing. Fungal structures in the root tips were blue-colored after staining with ink (Vierheilig et al., 1998), which revealed the presence of arbuscules and/or vesicles in each root fragment.

Statistical analysis

The data from all examined parameters were subjected to an ANOVA performed in the Statgraphics Centurion XI software (Adalta, Arezzo, Italy). Separation of means was set at $P \leq 0.05$ with the Newman-Keuls test.

To facilitate interpretation of the large dataset from the two-year field trial, factorial discriminant analysis (MDA, Multigroup Discriminant Analysis with Wilks' lambda and Pillai's trace tests), and principal component analysis (PCA) were carried out to describe above- and below-ground plant behavior in response to the microbiological inoculum and N fertilization. Multivariate data normality was first verified by the Shapiro test. Before analysis, data were standardized by subtracting the mean and dividing the result by the standard deviation within each variable. All analyses were performed in MS Excel XLSTAT (Addinsoft, Paris, France).

Results

Climatic conditions in field trials

The climatic conditions differed greatly between the two growing seasons (2013-14 and 2014-15). Seasonal rainfall was higher in the first than in the second year (October-June: 890 *vs*. 610 mm, +46%), as were winter/spring temperatures (Figure SI1).

Climatic conditions at the time of biofertilizer application also differed in the two years. In the first year, inoculum was applied at the beginning of stem elongation, a slightly more advanced growth stage compared with the second year (end of tillering), because of the extremely rainy winter, which had delayed mechanical spraying.

In the 10 days before inoculation, 58 mm of precipitation was recorded in the first year, but none in the second, whereas after treatment there was no precipitation for two weeks in the first year (18 mm after 16 days) compared with 22 mm after 2 days in the second.

Temperatures at the time of bacterial treatment also differed in the two years: maximum and minimum daily temperatures on the inoculation day were 17.3 °C and 4.4 °C in the first year, 11.2 °C and -2.6 °C in the second. In the first year, the average temperature over the 3 days following inoculation was 10.6 °C, much higher than the 4.3 °C recorded in the second year.

Effects of inoculum on root growth

In controlled conditions (rhizoboxes), plants developed from inoculated seeds showed a more complex root system than untreated controls, as revealed by the higher root tip and fork densities (about +30%). Due to slight improvements in root length (+3%) and diameter (+11%), treated plants also exhibited improved root surface area (+20% *vs*. controls), although this was not statistically significant (Table 1). At the end of the experiment, maximum root depth was similar across treatments ($P > 0.05$).

In contrast, plants inoculated by foliar+soil spraying developed a smaller root system than untreated controls, with significantly reduced maximum root depth and branching (both –28%, $P \leq 0.05$), it being shorter (-16%, not significant) and having a smaller surface area (–21%) (Table 1).

In sterilized pot soil, microscopic observations revealed an abundance of fungal structures in excided root fragments of 40-day old seedlings grown from inoculated seeds, while no coloration was found in root tissues of non-inoculated controls, as expected (Fig. 1 A, B). The mycorrhization index calculated on 45 root pieces was 47%, revealing a sufficient

viability of the commercial inoculum and effectiveness in root colonization. These findings were confirmed by ESEM imaging of micro-dissected mycorrhized roots previously disinfected and processed under sterile conditions, which showed the presence of intraradical VAM propagules (Fig. 1D), whereas none were detected in non-inoculated control plants (Fig. 1C).

In the more complex field situation, analysis was made of the root system collected at the flowering stage from the 0-1 m depth profile. Root length density (RLD) of var. Bologna (second-year trial) was higher than that of var. Africa (first year), but the effects of nitrogen fertilization and inoculation were stable across years. Without inoculation, plants fertilized with the lowest nitrogen dose (80 kg ha^{-1}) had the greatest RLD values in the entire 0-1 m soil profile (averages of 3.78 and 5.48 cm cm^{-3}, in 1^{st} and 2^{nd} years, respectively), and in the top 0-0.4 m soil layer (7.23 and 10.16 cm cm^{-3}, respectively) (Fig. 2, Table SI1). The average RLD was negatively correlated with N fertilization dose, particularly in the second year (whole profile R^2 = 0.64 1^{st} year, 0.99 2^{nd} year): the higher the N dose the lower the root length density (Fig. 2). In the 1^{st} year, the RLD of var. Africa was 35% lower at 120 kg N ha^{-1} and 31% lower at 160 kg N ha^{-1} than the lowest N dose (80 kg ha^{-1}) in the whole soil profile, and the reductions were even greater in the arable layer (-44% and -33%, respectively). In the 2^{nd} year, there were smaller reductions in the RLD of var. Bologna: on average -12% and -26% in the 0-1 m profile, and -8% and -21% in the top 0.4 m depth layer at 120 and 160 kg N ha^{-1}, respectively, compared with the 80 Kg N ha^{-1} dose.

Root length reductions due to increases of N fertilization were not observed in both varieties with inoculation with Rhizosum N, so that plants fertilized with 120 or 160 kg N ha^{-1} attained the same or even greater RLD as those fertilized at 80 kg N ha^{-1} without inoculation (Fig. 2). As a result, the root length density of inoculated plants was poorly

correlated with nitrogen dose (low regression coefficients), revealing a reduction in variability across fertilization levels.

Comparison between inoculated and non-inoculated plants at the same N fertilization dose, revealed that biofertilization increased root growth at medium and high N supply (120 and 160 kg ha^{-1}), while the opposite generally occurred at the lowest dose (80 kg N ha^{-1}). Although inoculated and control plants did not differ significantly in overall mean RLD at 0-1 m, application of *Rizophagus irregularis* and *Azotobacter vinelandii* led to evident root enhancements in specific soil layers, mainly in the arable profile ($P \leq 0.05$) (Fig. 3).

As regards the root surface density (cm^2 of roots per cm^3 of soil), despite a trend towards greater values in inoculated plants than in controls at 120 and 160 kg N ha^{-1} fertilization rates in the top 0-0.4 m soil and in the whole profile (0-1 m average), we could not detect any significant difference between inoculation and controls (Table SI1).

On the other hand, contrasting responses were found in the two years/varieties with regard to root diameter: in the 1st year (var. Africa) there was a general small increase in root diameter following inoculation, but a slight decrease ($P > 0.05$) in the 2nd year (var. Bologna) (Table SI1).

The "N fertilization × inoculation" interaction was not statistically significant for any of the investigated root parameters.

Microscopic assessments carried out in the 1st year on 100 root apexes of var. Africa in the arable layer showed that mycorrhizal root colonization increased (+3%) in inoculated plants at low and medium N fertilization rates, but decreased (-4%) at high N supply, compared with non-inoculated controls. Natural occurrence of mycorrhization (no inoculation) was relatively high under medium–low N supply (32-35%), but markedly decreased at 160 kg N ha^{-1} (23%). However, it seems that mycorrhization increases the

number of root apexes containing fungal arbuscules, and reduces the number containing vesicles, particularly at 80 kg N ha^{-1} (Fig. 4).

Vegetation indices and shoot growth

Vegetation indices, such as SPAD and NDVI, were affected mainly by fertilization, except for NDVI in the first year, as increased N supply led to significant improvements in chlorophyll contents and canopy greenness ($P \leq 0.05$) (Table 2). The effect of inoculation with *R. irregularis* + *A. vinelandii* was not significant. Plant height was very stable across treatments, on average ~74 cm in var. Africa (1st year), and ~55 cm in var. Bologna (2nd year) (data not shown).

Nutrient uptake and grain yield

Nitrogen concentrations in straw and grains, N removal and yield were often significantly affected by N fertilization (main effect, $P \leq 0.05$), and the higher the dose the greater the effects, while inoculation and the interaction between the two factors resulted always not significant. However residual N concentration in straw was reduced, although not significantly, in inoculated plots with maximum effects at 80 kg N ha^{-1} fertilization (-12% in year 1, -21% in year 2) (Table 3). The average N concentration in straw differed between years, being 7.6 g kg^{-1} DW in the 1st year, and 4.9 g kg^{-1} DW in the 2nd, likely due to varietal differences and climatic conditions.

On the other side, biofertilizer application only seldom increased N concentration in the harvested grains, mainly in the 2nd year with var. Bologna, with the better response observed at the medium N fertilization rate (+10%; $P \leq 0.05$), although the major driver of N accumulation (i.e., proteins) was fertilization (Table 3).

As a result of the combination of biomass production and nutrient concentration in plant tissues, inoculation generally slightly enhanced N removal (straw + grains), although without statistical significance. Maximum improvements were detected at 160 kg N ha^{-1} fertilization: +32 kg ha^{-1} (+12%) for Africa, and +23 kg ha^{-1} (+13%) for Bologna *vs*. controls (Table 3).

Microbial inoculation did not have any significant effect on grain yield, while nitrogen fertilization significantly improved grain yield, particularly in the second year with the high-quality var. Bologna (Table 3).

The biofertilizer also improved the concentrations in straw tissue and grains of other nutrients, like K, P, and Zn, but seldom significantly (Table 4). In particular, straw had significantly higher concentrations of Zn at 80 kg N ha^{-1} in the 1st year (+ 44%), and of K at 160 kg N ha^{-1} in the 2nd year ($P \leq 0.05$). The major positive effects of inoculation on plant nutrition were found at the highest N dose in straw, and at the lowest and medium N doses in grains, although the latter were never significant. The response of var. Bologna at 80 kg N ha^{-1} was interesting, there being a general reduction in the concentrations of all nutrients in the straw, and an increase in the grains, meaning that they tended to have increased nutrient harvest indices (i.e., the ratio between nutrient accumulation in grains and total above-ground nutrient accumulation).

Principal component analysis (PCA) and discriminant analysis (DA)

PCA conducted on the whole dataset of the two-year trial identified two synthetic variables, which explain an overall variability of 73.3%, attributed mostly to the first variable (F1 = 51.5%; F2 = 21.8%) (Fig. 5). Relevant (loadings > |0.4|) nitrogen-related variables were assigned to the F1 variable (shoot N uptake, SPAD, NDVI, and grain N

concentration), while yield and grain P concentration were assigned to F2. Following the vector direction of each variable, generally good correlations were established among the variables plotted very closely together, i.e., NDVI, SPAD, yield, and grain and straw N concentrations, and these were negatively correlated with the nitrogen harvest index (N-HI, i.e., the ratio between N accumulation in grains and total above-ground N accumulation). P and Zn concentrations in grain were correlated with root length density. The centroid position and cluster separation in the discriminant analysis (Fig. 5) summarizes wheat response to inoculation and fertilization, and shows that the mycorrhizal fungus + diazotrophic bacteria consortium can enhance root growth and nutrient allocation in the grains at medium and high fertilization, while increased fertilization levels limit root growth and N-HI, but increase yield and N uptake.

Discussion

The use of plant-aiding microorganisms to improve crop nutrition and health while reducing agricultural inputs in support of sustainable and/or organic agriculture is receiving increasing attention worldwide. Although they can be applied individually, it has been suggested that mycorrhizal fungi and bacteria consortia can be used together to cover different functions and exploit synergistic effects in order to cope better with abiotic (e.g., nutrient and water deficiency) and biotic stresses (Nadeem et al., 2014). *Rhizophagus irregularis* is a widespread species in biofertilizer formulation for many crops. In the present study, microscopic observation and ESEM imaging revealed appreciable root colonization by this mycorrhizal fungus of wheat grown in sterilized pot soil, suggesting its potential use with this crop in the open field. By excluding the activity of natural

microflora, we ascertained that *R. irregularis* can promote root growth after seed application, while increased N availability might at least be expected from the free-living diazotrophic *Azotobacter vinelandii* through N-fixation (Sethi and Adhikary, 2012) and transportation from soil to roots (Bücking et al., 2012).

The literature confirms the substantial benefits to plant growth when VAM fungi and PGPR are able to survive after application and to colonize the rhizosphere (Singh et al., 2004). Seed inoculation with VAM fungi is reported to be as effective as soil inoculation (Oliveira et al., 2016), because mycorrhizal spores can survive and develop fungal structures during seed germination and root expansion, which would support the root enhancements found with foliar+soil application in our field trials. Bacterial inoculants are generally successful when applied as seed coatings in leguminous and other species, but they can also be beneficial when applied to the soil (Bashan, 1998).

In early growth stages (rhizobox trial), we documented marked root morphological changes due to seed inoculation, which mainly concerned the promotion of root initiation and branching, but there was also a trend for greater length and surface area, enabling the plants to access more soil resources. Similar effects with *R. irregularis* were found in rice by Gutjahr et al. (2009), who detected increased root length and branching of the main roots, and a greater number of first- and second-order lateral roots. A more branched root system induced by VAM germinating spores was also found in *Medicago truncatula* (Oláh et al., 2005), but this effect is common in many other species, probably as a means to increase the root sites available for mycorrhizal colonization (Harrison, 2005).

Root morphological changes may be also a consequence of PGP rhizobacteria inoculation (Zahir et al., 2003), as they can produce plant growth regulators and phytohormones (Tsavkelova et al., 2006; Hayat et al., 2010), like indole-3-acetic acid, which is involved in

root initiation and cell division (Salisbury, 1994; Barazani and Friedman, 1999). However, we unexpectedly found a negative impact on shoot and root growth in wheat under foliar+soil inoculation in its early growth stages, when we might simply expect a delayed effect. In this regard, we may hypothesize that for a short period after inoculation an excess of phytohormones had reduced plant growth (Dobbelaere et al., 2002).

Cooperation among microorganisms is probable with microorganism consortia, as *Azotobacter* spp. bacteria have been reported to stimulate mycorrhization of wheat roots (Diederichs and Manske, 1991), and many studies report enhanced root and shoot growth, and nutrient uptake, and improved yield, due to positive interactions among plant-aiding microorganisms (Behl et al., 2007).

For practical applications, attention should be drawn to the finding that our biofertilizer encouraged stable root growth across years and varieties under medium-high nitrogen fertilization doses (120 and 160 kg ha^{-1}) in the field, but unexpectedly reduced it at the low N rate (80 kg ha^{-1}), despite improved mycorrhization. There is evidence that soil nutrient availability, whether excessive or severely deficient, significantly affects root growth (Hodge, 2006; Fageria and Moreira, 2011). Nitrogen availability is a strong conditioning factor of root morphology and growth (Eghball et al., 1993), and high, homogeneous soil nitrate concentration can inhibit lateral and primary root growth under laboratory conditions (Walch-Liu et al., 2006). In our trial, the reduction in root length density with increased amounts of chemical fertilizer is in agreement with many reports in the literature (Comfort et al., 1988; Robinson et al., 1994; Svoboda and Haberle, 2006), suggesting that root growth impairment can occur at extremely high and extremely low N supply (Costa et al., 2002). Despite species-specific regulation, an excess of nitrogen can reduce lateral root formation through hormone-mediated mechanisms related to reduced shoot-to-root auxin

transport (Dubrovsky et al., 2011), and ethylene accumulation at the root level (Mohd-Radzman et al., 2013). *Azotobacter* is capable of synthetizing biologically active substances, particularly auxins, that directly affect root growth and morphology (Tsavkelova et al., 2006), and many PGPRs lower root ethylene levels through production of the ACC-deaminase enzyme (Zahir et al., 2008; Visioli et al., 2014) and toxins, like rhizobitoxine, capable of inhibiting ethylene synthesis (Nadeem et al., 2014). Mycorrhizal colonization can modify plant hormone levels as well, thereby affecting root growth responses (Fusconi, 2014).

Inoculation clearly helped to minimize the negative impact of chemical fertilization on root growth, although there was no clear relationship with mycorrhization level, which tended to decrease with increasing fertilizer doses. Good root mycorrhization generally occurs under low fertility conditions (Johnson, 2010), as these symbioses are negatively affected by chemical fertilizers (Kahiluoto et al., 2001; Aghili et al., 2014), but there are many factors affecting AM structure density in roots, like soil management and other cropping practices (Behl et al., 2007). The effects of N fertilization on PGPR establishment and survival is not yet well understood (Martínez et al., 2011), but some studies show that high levels of N fertilizers reduce the number of PGPR species associated with crop roots (Yuan et al., 2011) or minimize their effects on grain yield and N uptake (Ali et al., 2005).

Inoculation exerts a major effect on roots in the arable layer, although in many cases there were appreciable root improvements at greater depths (Fig. 3), which may play a role in water and nutrient uptake under drought conditions if RLD is maintained above the critical threshold of 1 cm cm^{-3} for water uptake (White et al., 2015). In this regard, inoculation may play a relevant role in coping with abiotic stresses in adverse environments or during grain

filling and maturation (Gooding et al., 2003) in a perspective of climate change (reduced precipitation, water-table lowering) (Baruffi et al, 2012; Brunetti et al., 2000).

In our study, there was no stable positive correlation between root expansion and canopy greenness, grain yield and quality. Inoculation resulted in improved accumulation of low-mobile nutrients, like K and Zn, particularly in the straw, and of N, although to a lesser extent as it is less dependent on root expansion, in agreement with existing literature (Fusconi, 2014; Vessey, 2003). Undoubtedly, the agronomic success of these microorganisms in field applications depends on climatic conditions (Augé, 2001), the plant genome (An et al., 2010; Turner et al., 2013) and competition with resident microbioma (Roesti et al., 2006), but larger root systems have also a key environmental advantage in reducing N losses from agricultural ecosystems.

We conclude that the association between the VAM *Rhizophagus irregularis* and the free-living diazotroph *Azospirillum vinelandii* can benefit wheat cultivation by improving root exploration in both laboratory and field conditions. There is no evidence of a negative interaction between inoculation response and N fertilization at medium-high doses (120-160 kg ha^{-1}), which are still necessary to maintain a high protein content (>120 g kg^{-1}, i.e., >21 g N kg^{-1}). In fertile soils, like those of our trials, moderate improvements in the uptake of some nutrients may be expected as a result of inoculation. Further investigations on the use of this inoculum are needed to verify whether yield improvements can be achieved in marginal soils or in organic agriculture. As for future perspectives, the possibility to replace post-emergence inoculation with seed application of microorganisms to reduce application costs should also be verified.

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Figures and Tables

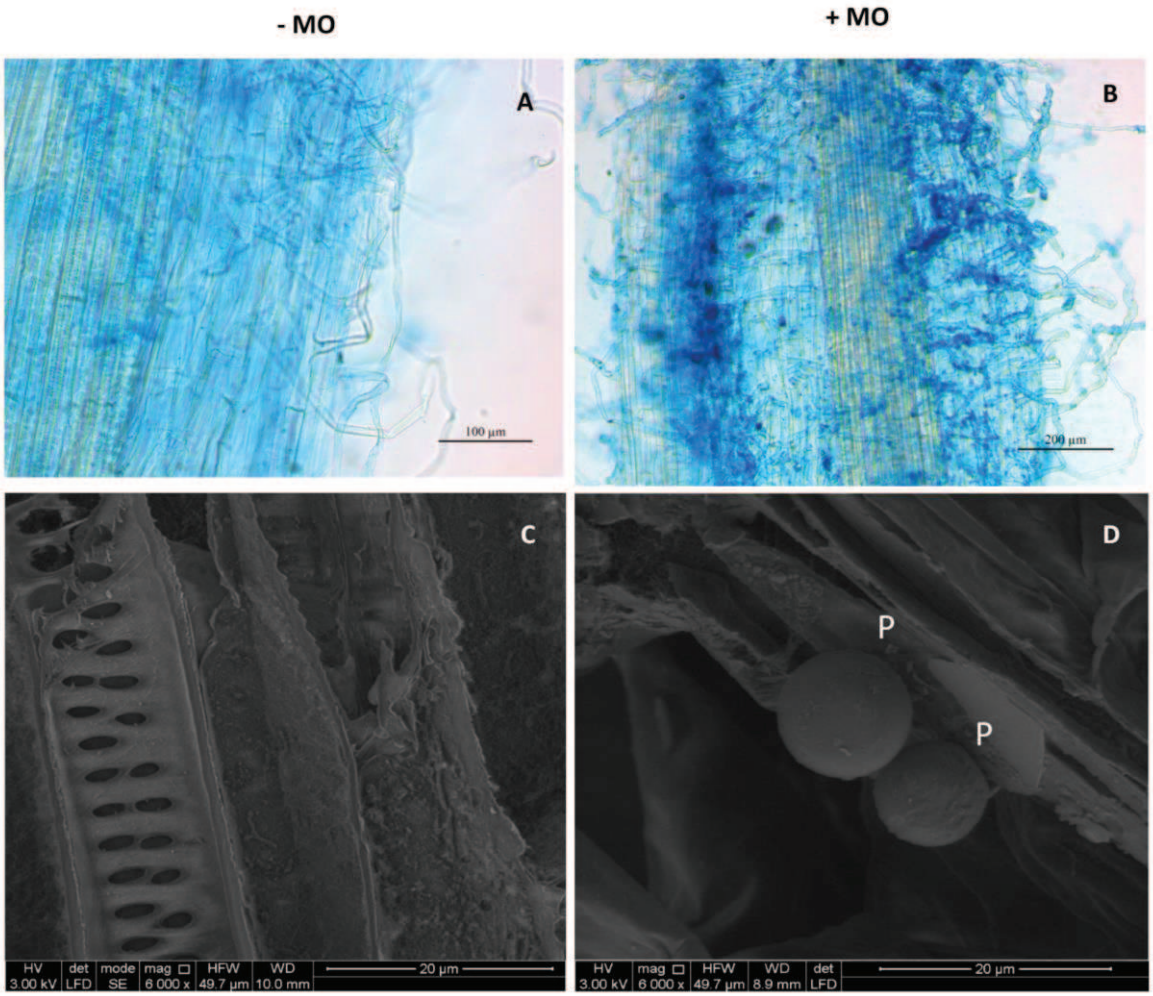


Figure 1. Optical microscope micrographs of root surfaces (A, B) and microdissected longitudinal root sections analyzed by ESEM (C, D) in non-inoculated controls (A, C) and seed-inoculated (B, D) 40-day-old *Triticum aestivum* L. (var. Bologna) seedlings. Note abundant colonization and mycorrhizal structures (P = propagules) on right-hand images only.

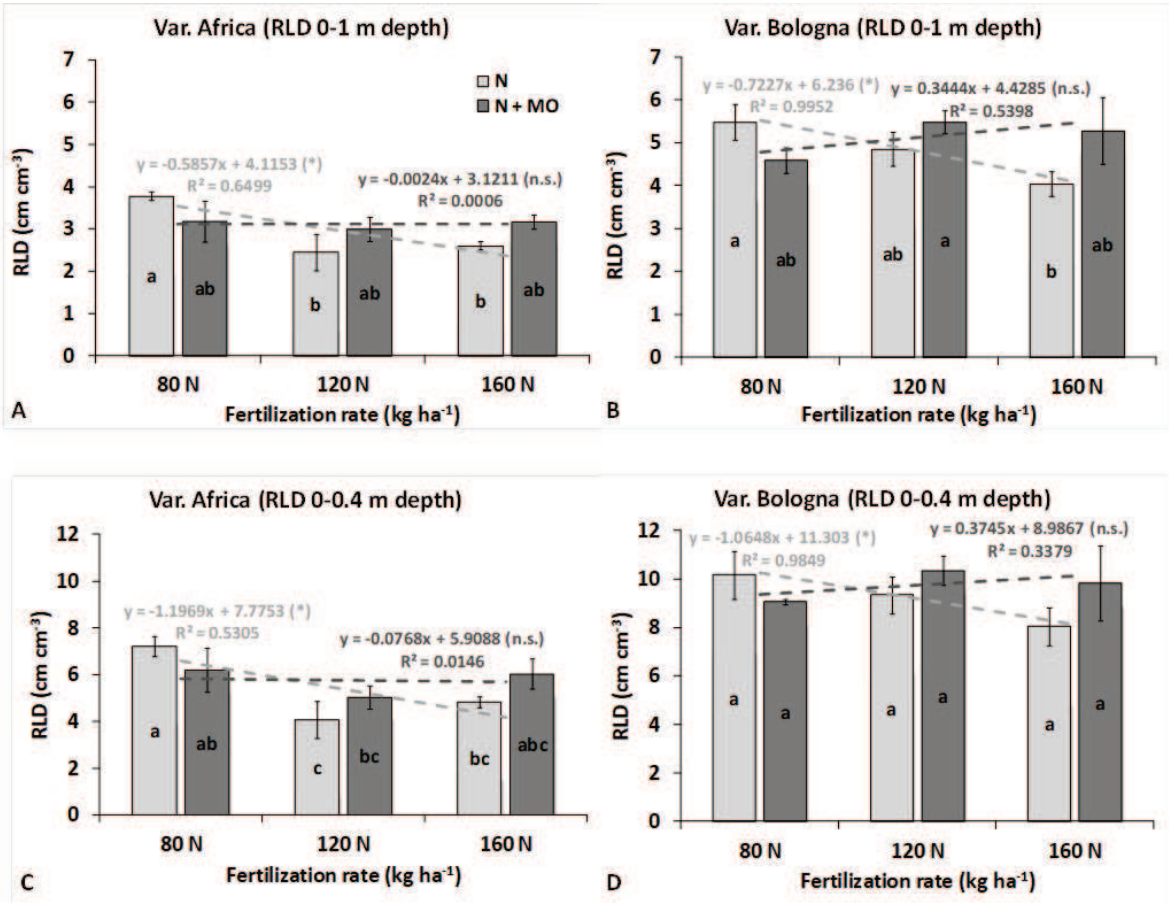


Figure 2. Root length densities (RLD; mean±SE; n=3) (bars) in the whole profile (A, B) and in the arable layer (C, D) of inoculated (+MO) *Triticum aestivum* L. plants and non-inoculated controls at the flowering stage with increasing N fertilization doses (80, 120 and 160 kg ha^{-1}) in the 1st year (var. Africa; A, C) and 2nd year (var. Bologna; B, D) of the field trial. Dashed line: linear regressions of RLD over N dose in inoculated and non-inoculated treatments (for each regression n = 9). Letters: statistical significant difference among treatments (multiple comparisons) (Newman-Keuls test, $P \leq 0.05$).
Significance level in regressions: * = $P \leq 0.05$
; n.s. = not significant

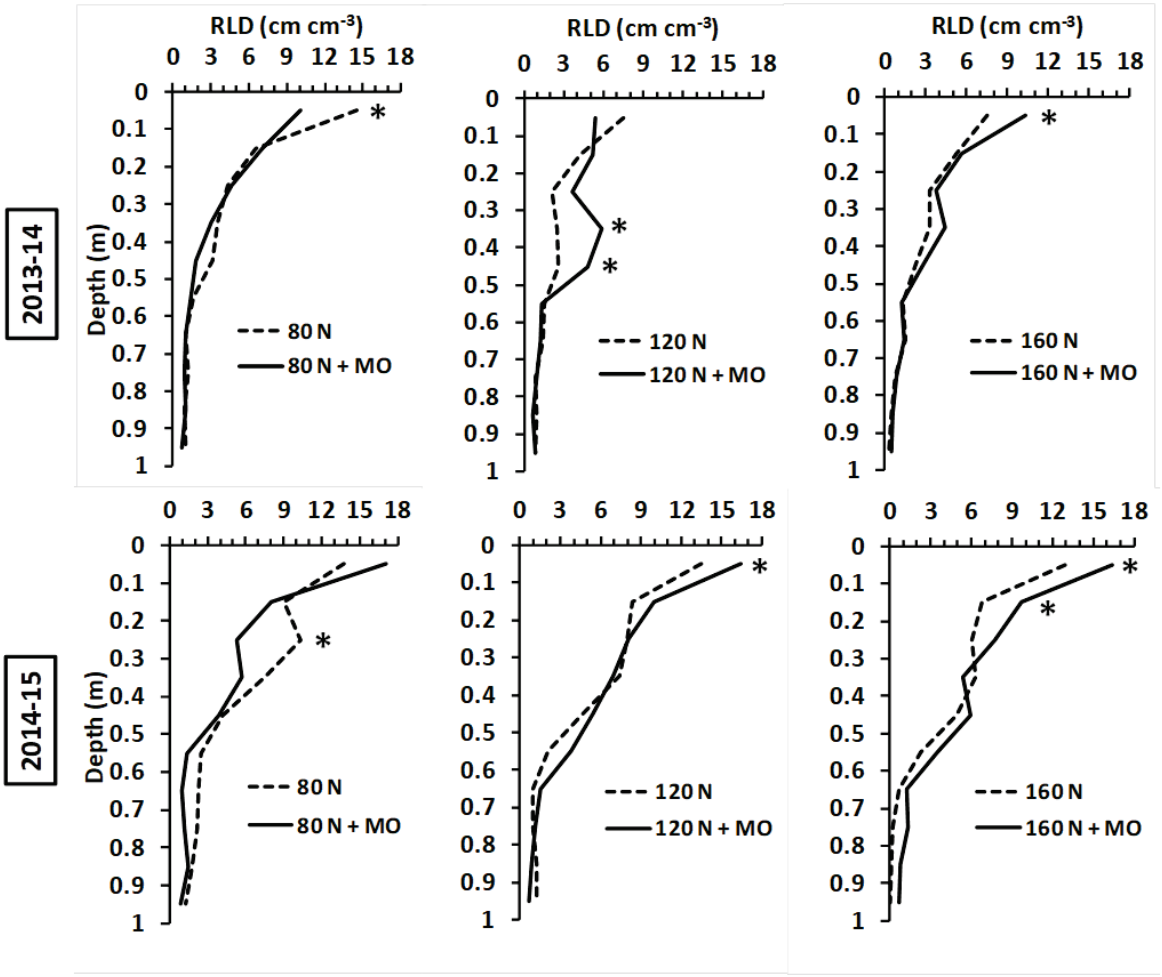


Figure 3. Root length density (RLD) patterns in inoculated (+MO) *Triticum aestivum* L. plants (continuous line) *vs*. non-inoculated controls (dashed line) with increasing N fertilization doses (80, 120 and 160 kg ha^{-1}) at the flowering stage in a two-year field trial. Asterisks mean significant difference between treatments at specifics soil depths ($P \leq 0.05$).

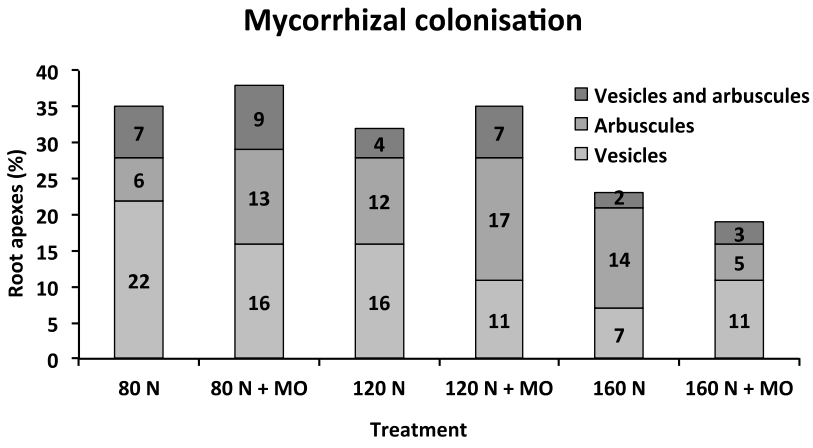
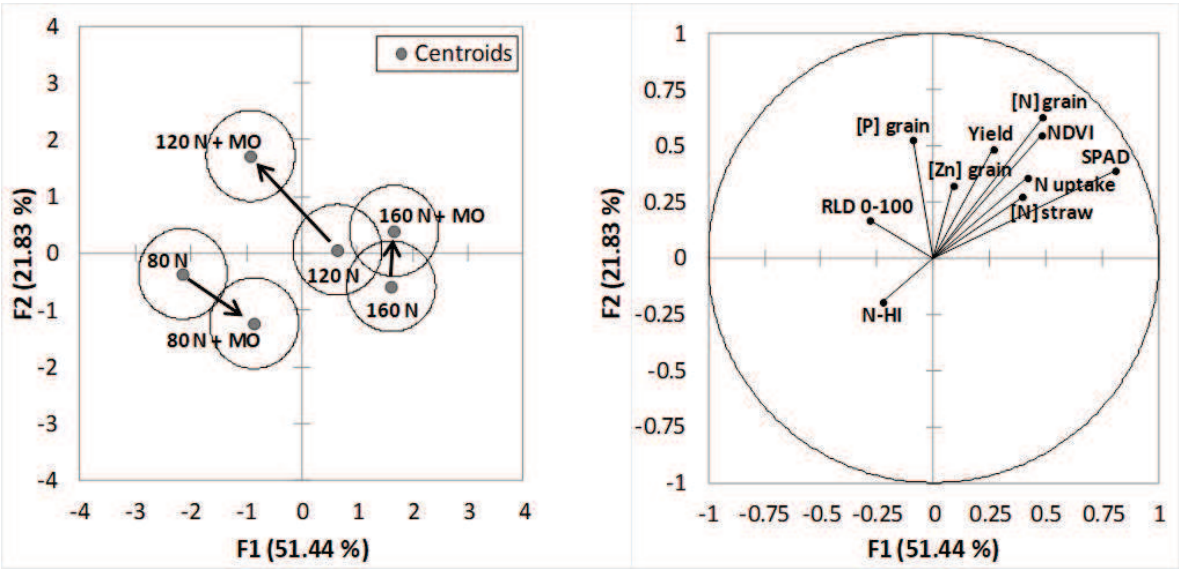


Figure 4. Rates of mycorrhized (% on 100 root apexes) in the 0-0.3 m soil depth interval in inoculated (+MO) *Triticum aestivum* L. plants of var. Africa (1st year field trial) vs. non-inoculated controls at the flowering stage at increasing N fertilization doses (80, 120 and 160 kg ha-1), with detected fungal structures specified.



	F1	F2
RLD 0-100	-0.283	0.169
N uptake	**0.417**	0.358
SPAD	**0.805**	0.390
NDVI	**0.479**	**0.547**
[N] grain	**0.481**	**0.628**
[N] straw	0.392	0.274
N-HI	-0.226	-0.195
Yield	0.265	**0.484**
[Zn] grain	0.088	0.323
[P] grain	-0.093	**0.527**

Figure 5. Principal component analysis (PCA; top right) with variable loadings (values > |0.4| in bold; bottom table) and discriminant analysis (DA; top left) for nitrogen fertilization level (160, 120 and 80 kg ha^{-1}) and microbiological inoculation (+MO) in the two-year field trial. In the DA circles contain 70% of cases, and arrows indicate the variation from non-inoculated to inoculated treatments within the same fertilization dose.

Table 1. Root parameters (mean±SE; n=3) in inoculated *Triticum aestivum* L. plants (two methods of application) *vs*. non-inoculated controls at 50 days after sowing (DAS) in sterilized soil in rhizoboxes. Letters: significant differences among treatments within the same parameter (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in inoculated plants *vs*. non-inoculated controls.

Treatment	Depth (cm)	Length (m plant^{-1})	Surface area (m^2 plant^{-1})	Diameter (µm)	Tips (No. plant^{-1})	Branching index (No. forks m^{-1})
Untreated	41.4 ± 2.33 a	35.6 ± 4.64 a	0.037 ± 0.005 a	344 ± 19 ab	7559 ± 1178 b	555 ± 33 b
Seed application	39.9 ± 3.21 a (-4)	36.7 ± 6.88 a (+3)	0.045 ± 0.009 a (+20)	383 ± 12 a (+11)	9723 ± 1113 a (+29)	720 ± 63 a (+30)
Soil + foliar spraying	29.9 ± 5.31 b (-28)	29.9 ± 7.62 a (-16)	0.029 ± 0.009 a (-21)	309 ± 13 b (-10)	5003 ± 1637 c (-34)	399 ± 59 c (-28)

Table 2. Vegetational parameters (SPAD and NDVI, mean seasonal values from stem elongation to heading stage, ±SE; n=3) in inoculated (+MO) *Triticum aestivum* L. plants *vs*. non-inoculated controls at increasing N fertilization doses (80, 120 and 160 kg ha^{-1}) in a two-year field trial. Letters: significant differences among treatments within the same parameter and year (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in inoculated plants *vs*. non-inoculated controls at each N fertilization dose.

Treatment	SPAD		NDVI	
	2013-14	2014-15	2013-14	2014-15
80 N	40.8 ± 1.05 b	38.3 ± 1.48 b	0.71 ± 0.004 a	0.63 ± 0.012 b
80 N + MO	40.6 ± 1.08 b (-0.4)	38.3 ± 1.44 b	0.69 ± 0.003 a (-2)	0.62 ± 0.022 b (-1)
120 N	43.3 ± 1.04 a	41.4 ± 0.91 a	0.70 ± 0.004 a	0.72 ± 0.030 a
120 N + MO	41.4 ± 1.14 b (-4)	41.9 ± 0.43 a (+1)	0.70 ± 0.005 a (-0.5)	0.73 ± 0.014 a (+1)
160 N	44.5 ± 1.02 a	41.8 ± 0.42 a	0.70 ± 0.005 a	0.72 ± 0.006 a
160 N + MO	45.4 ± 0.93 a (+2)	43.6 ± 0.49 a (+4)	0.69 ± 0.004 a (-0.5)	0.74 ± 0.005 a (+3)
Fertilization	*	**	ns	***
MO application	ns	ns	ns	ns
Fert. × MO app.	ns	ns	ns	ns

n.s. = not significant; *, ** and *** = significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Table 3. Nitrogen concentrations in straw and grain tissues, shoot nitrogen removal and yield (mean±SE; n=3) at harvest in inoculated (+MO) *Triticum aestivum* L. plants *vs*. non-inoculated controls at increasing N fertilization levels (80, 120 and 160 kg ha^{-1}) in a two-year field trial. Letters: significant differences among treatments within the same parameter (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in inoculated plants *vs*. non-inoculated controls at each N fertilization dose.

Treatment	Straw [N] (g kg^{-1} d.w.)		Grain [N] (g kg^{-1} d.w.)		Shoot N (grain + straw) (kg ha^{-1})		Yield (kg ha^{-1})	
	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15
80 N	6.3 ± 0.2 bc	5.0 ± 0.2 ab	19.4 ± 0.3 b	18.3 ± 0.07 cd	214 ± 17 b	135 ± 9 bc	5991 ± 376 a	4963 ± 252 b
80 N + MO	5.5 ± 0.1 c (-12)	4.0 ± 0.3 b (-21)	19.5 ± 0.5 ab (+1)	17.7 ± 0.01 d (-4)	227 ± 8 b (+6)	120 ± 1 c (-11)	6566 ± 258 a (+10)	4494 ± 140 b (-9)
120 N	8.5 ± 1.4 a	5.0 ± 0.7 ab	20.9 ± 0.8 ab	19.3 ± 0.05 bcd	264 ± 23 ab	163 ± 20 ab	6607 ± 103 a	5882 ± 194 a
120 N + MO	7.9 ± 0.4 ab (-7)	4.5 ± 1.1 ab (-11)	20.4 ± 0.7 ab (-2)	21.1 ± 0.06 a (+10)	267± 39 ab (+1)	171 ± 10 ab (+5)	6412 ± 377 a (-3)	5939 ± 116 a (+1)
160 N	8.7 ± 0.8 a	4.9 ± 0.3 ab	21.4 ± 0.7 a	19.5 ± 0.03 abc	257 ± 10 ab	172 ± 7 ab	6303 ± 235 a	6021 ± 32 a
160 N + MO	8.4 ± 0.6 ab (-3)	5.7 ± 0.4 a (+16)	21.3 ± 0.8 ab (-1)	20.1 ± 0.04 ab (+3)	289 ± 19 a (+12)	195 ± 13 a (+13)	6413 ± 71 a (+2)	5959 ± 157 a (-1)
Fertilization	**	ns	*	**	Ns	**	ns	***
MO application	ns	ns	ns	ns	Ns	ns	ns	ns
Fert. × MO app.	ns	ns	ns	ns	Ns	ns	ns	ns

n.s. = not significant; *, ** and *** = significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Table 4. Mineral concentrations (mean±SE; n=3) in straw and grains at harvest in inoculated (+MO) *Triticum aestivum* L. plants *vs*. non-inoculated controls at increasing N fertilization doses (80, 120 and 160 kg ha^{-1}) in a two-year field trial. Letters: significant differences among treatments within the same nutrient (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in inoculated plants *vs*. non-inoculated controls at each N fertilization level.

STRAW

Treatment	[K] (mg kg^{-1} dw)		[P] (mg kg^{-1} dw)		[Zn] (mg kg^{-1} dw)	
	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15
80 N	9288 ± 194 a	8756 ± 926 b	870 ± 44 b	852 ± 113 A	8.8 ± 0,33 d	8.3 ± 0.88 b
80 N + MO	9454 ± 430 a (+2)	6939 ± 220 c (-21)	930 ± 7 b (+7)	792 ± 44 a (-7)	12.7 ± 0.93 a (+44)	8.1 ± 0.26 b (-3)
120 N	10183 ± 928 a	8951 ± 855 b	957 ± 35 b	821 ± 44 a	9.3 ± 0.82 cd	9.1 ± 0,16 b
120 N + MO	10911 ± 915 a (+7)	9055 ± 450 b (+1)	1135 ± 49 a (+19)	836 ± 51 a (+2)	11.2 ± 0.79 abc (+21)	9.8 ± 0.23 a (+7)
160 N	10980 ± 556 a	8857 ± 398 b	1001 ± 40 ab	804 ± 14 a	10.3 ± 0.29 bcd	7.9 ± 0.85 b
160 N + MO	11149 ± 626 a (+2)	10706 ± 968 a (+21)	1006 ± 76 ab (+1)	883 ± 19 a (+10)	11.5 ± 0.62 ab (+12)	9.9 ± 0.34 a (+25)

GRAINS

Treatment	[K] (mg kg^{-1} dw)		[P] (mg kg^{-1} dw)		[Zn] (mg kg^{-1} dw)	
	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15
80 N	3733 ± 12 b	2878 ± 61 a	3094 ± 60 a	3032 ± 96 b	19.6 ± 0.35 b	15.8 ± 2.03 a
80 N + MO	3731 ± 25 b (-0.5)	3018 ± 99 ab (+5)	3154 ± 19 a (+2)	3228 ± 76 ab (+6)	20.8 ± 0.47 ab (+6)	17.4 ± 0.85 a (+10)
120 N	3807 ± 19 ab	3030 ± 150 ab	3142 ± 32 a	3295 ± 133 ab	20.9 ± 0.56 ab	18.4 ± 0.57 a
120 N + MO	3872 ± 24 a (+2)	3412 ± 167 a (+13)	3169 ± 63 a (+1)	3607 ± 181 a (+9)	22.5 ± 1.09 a (+8)	19.2 ± 1.27 a (+4)
160 N	3791 ± 43 ab	2998 ± 130 ab	3110 ± 38 a	3183 ± 112 ab	21.7 ± 0.30 ab	16.5 ± 0.82 a
160 N + MO	3813 ± 39 ab (+1)	2855 ± 69 b (-5)	3114 ± 55 a (+0.5)	3091 ± 79 ab (-3)	21.2 ± 1.26 ab (-2)	17.0 ± 1.31 a (+2)

Supplementary information

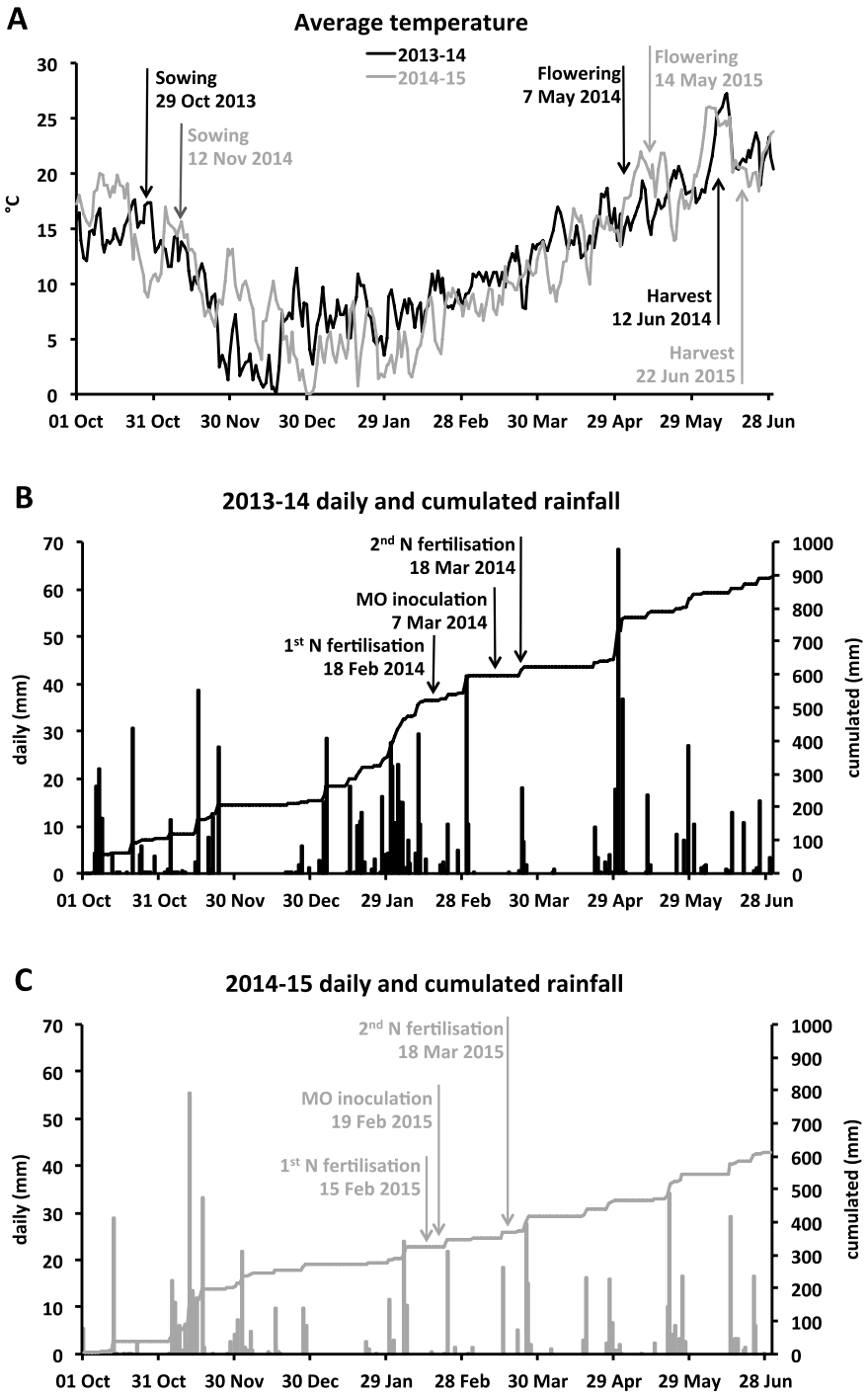


Figure SI1. Dynamics of seasonal daily mean temperatures (A), daily and cumulated rainfall in first-year (B) and second-year trials (C) over the crop cycle of wheat at the Legnaro experimental site (Padua, Italy).

Table SI1. Main root parameters, i.e., root length density (RLD), root surface density (RSD) and average diameter (AD) (mean; n=3) at flowering stage in the whole soil profile (0-1 m depth), in the arable layer (0-0.4 m depth) and in the subsoil (0.4-1 m) of inoculated (+MO) *Triticum aestivum* L. plants *vs*. non-inoculated controls at increasing N fertilization doses (80, 120 and 160 kg ha^{-1}) in a two-year field trial. Letters: significant differences among treatments within the same parameter (Newman-Keuls test, $P \leq 0.05$). In brackets: % variation in inoculated plants *vs*. non-inoculated controls at each N fertilization dose.

Variety (year)	Treatment	RLD (cm cm^{-3})			RSD (cm^2 cm^{-3})			AD (µm)		
		0-1 m	0-0.4 m	0.4-1 m	0-1 m	0-0.4 m	0.4-1 m	0-1 m	0-0.4 m	0.4-1 m
Africa (2013-14)	80 N	3.78 a	7.23 a	1.48 Ab	0.30 a	0.57 a	0.13 ab	261 a	240 a	275 ab
	80 N + MO	3.18 ab (-16)	6.20 ab (-14)	1.16 b (-21)	0.26 ab (-14)	0.50 ab (-13)	0.10 bc (-19)	270 a (+4)	253 a (+5)	282 a (+2)
	120 N	2.45 b	4.08 c	1.36 ab	0.19 c	0.32 c	0.11 abc	253 a	248 a	257 c
	120 N + MO	3.00 ab (+23)	5.03 bc (+23)	1.65 a (+22)	0.25 abc (+29)	0.41 bc (+28)	0.14 a (+31)	270 a (+7)	258 a (+4)	279 ab (+8)
	160 N	2.61 b	4.83 bc	1.12 b	0.21 bc	0.38 bc	0.09 c	251 a	244 a	255 c
	160 N + MO	3.17 ab (+22)	6.04 abc (+25)	1.26 ab (+12)	0.25 abc (+21)	0.48 ab (+25)	0.10 bc (+12)	256 a (+2)	245 a (+1)	263 bc (+3)
	Fertilization	ns	*	ns	*	*	ns	ns	ns	*
	MO application	ns	ns	ns	ns	ns	ns	ns	ns	*
	Fert. × MO app.	ns	ns	ns	ns	ns	ns	ns	ns	ns
Bologna (2014-15)	80 N	5.48 a	10.16 a	2.37 a	0.47 a	0.82 a	0.24 a	304 ab	261 a	332 b
	80 N + MO	4.59 ab (-16)	9.06 a (-11)	1.61 ab (-32)	0.41 ab (-12)	0.77 a (-6)	0.18 ab (-26)	333 a (+10)	276 a (+6)	372 a (+12)
	120 N	4.85 ab	9.33 a	1.86 ab	0.43 ab	0.80 a	0.19 ab	319 ab	273 a	350 ab
	120 N + MO	5.48 a (+13)	10.34 a (+11)	2.25 ab (+21)	0.46 ab (+5)	0.82 a (+3)	0.21 ab (+10)	295 b (-8)	254 a (-7)	322 b (-8)
	160 N	4.04 b	8.03 a	1.38 b	0.36 b	0.69 a	0.14 b	314 ab	270 a	343 ab
	160 N + MO	5.28 ab (+31)	9.81 a (+22)	2.26 ab (+64)	0.43 ab (+18)	0.75 a (+9)	0.21 ab (+46)	292 b (-7)	243 a (-10)	325 b (-5)
	Fertilization	ns	ns	ns	ns	ns	ns	ns	ns	ns
	MO application	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Fert. × MO app.	ns	ns	ns	ns	ns	ns	*	ns	*

n.s. = not significant; *, ** and *** = significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

CHAPTER III

Biostimulant side-effects of seed-applied sedaxane fungicide at increasing doses: morphological and physiological changes in maize seedlings

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Abstract

Most crops are routinely protected against seed-born and soil-borne fungal pathogens through seed-applied fungicides. The recently-released succinate dehydrogenase inhibitor (SDHI), sedaxane®, is a broad-spectrum fungicide, used particularly to control *Rhizoctonia* spp., with documented growth-enhancement effects on wheat. This study investigates the potential biostimulant secondary-effects of sedaxane and related physiological changes in disease-free maize seedlings (3-leaf stage) at increasing application doses (25, 75 and 150 µg a.i. seed^{-1}) under controlled sterilized conditions.

Here it is demonstrated that sedaxane has significant auxin-like and gibberellin-like effects, which induced marked morphological and physiological changes according to an approximate saturation dose-response model. Maximum benefits were attained at the intermediate dose, which significantly increased root length (+60% *vs*. untreated controls), area (+45%) and forks (+51%), and reduced root diameter. Sedaxane enhanced leaf and root glutamine synthetase (GS) activity resulting in greater protein accumulation, particularly in the above-ground compartment, while glutamate synthase (GOGAT) activity remained almost unchanged. Sedaxane also improved leaf phenylalanine ammonia-lyase (PAL) activity, which may be responsible for the increase in shoot antioxidant activity (phenolic acids), mainly represented by *p*-coumaric and caffeic acids.

It is concluded that, in addition to its protective effect, sedaxane can facilitate root establishment and intensify nitrogen and phenylpropanoid metabolism in young maize plants, and may be beneficial in overcoming biotic and abiotic stresses in early growth stages.

Keywords: biostimulant; hormone-like activity; nitrogen metabolism; phenolic acids; root branching; succinate dehydrogenase inhibitor (SDHI).

Introduction

In intensive agriculture, seed coating is a technique whereby several compounds, such as pesticides, fertilizers and biostimulant substances, are applied to the seed surface so they can start to act on the seedlings during germination and/or at the seed-soil interface immediately after sowing (Ehsanfat and Modarres-Sanavy, 2005).

Protecting field crop plants from soil- and seed-borne pathogens during germination and in early growth stages is crucial to ensuring safe, fast establishment (Mathre et al., 2001). Fungicides are chemical and biological compounds that kill pathogenic fungi or inhibit fungal spores germination (McGrath, 2004) and, together with insecticides, are the molecules most frequently used in the seed coatings of many crops.

A fungicidal seed treatment is commonly composed of a trace quantity of fungicide evenly distributed among the seeds along with the adhesive substances needed to bind them to the seed surface (Sharma et al., 2015). Modern seed dressing fungicide formulations are often a mixture of several active ingredients with different modes of action (systemic and contact), which broadens the spectrum of control to include a wide range of pathogens and reduces the likelihood of resistance onset. Common fungicide combinations for cereals are triticonazole + prochloraz, both sterol-inhibiting fungicides, and fludioxonil + metalaxyl-M, the former a non-systemic phenylpyrrole, which inhibits transport-associated phosphorylation of glucose, the latter an acilalanine RNA synthesis inhibitor.

Substances on the seed surface can affect germination, as the degree to which they attract or repel moisture may vary considerably (Scott, 1989). When applied at high concentrations, fungicides have been reported to have potential direct negative effects on seed germination, rootlet growth, and emergence (Minamor, 2013). In many cases, the effects of seed-applied fungicides on plants vary according to growing conditions: under

low pathogen pressure, they do not improve crop emergence and grain yield of wheat, but under high pressure from *Fusarium graminearum* they do (May et al., 2010). Environmental factors may also play a role (Cox and Cherney, 2014). Seed coating is expected to suppress arbuscular mycorrhizal fungi, thereby hampering root colonization and consequently reducing the beneficial effects on plant growth (Chiocchio et al., 2000; Channabasava et al., 2015).

In the search for highly effective active ingredients, attention is currently focused on useful secondary effects of fungicides on seedling development, regardless of genotype and growing conditions. Several fungicides have been found to have positive side-effects on plant physiology (Berdugo et al., 2012). The ubiquinol oxidase inhibitor (Qol) strobilurin family is known to increase several morphological traits of maize, such as leaf number and area, and shoot and root biomasses (Lazo and Ascencio, 2014). Strobilurins have also been found to increase tolerance to abiotic stresses, in that they can delay senescence of the photosynthetic leaf area, change the balance of the phytohormones and increase CO_2 assimilation in wheat (Wu and von Tiedemann, 2001; Köhle et al., 2002). The azole fungicide class also influences the physiology of treated plants by increasing the chlorophyll content in winter wheat plants, delaying leaf senescence, and protecting plants from several abiotic stresses (Fletcher et al., 2010).

Recent studies have demonstrated the influence on plant physiology of pyrazole-carboxamide succinate dehydrogenase inhibitors (SDHIs) (Ajigboye et al., 2014; Ajigboye et al., 2016). These are a relatively new class of fungicides (since 2000) and now include various active ingredients, such as boscalid, bixafen, isopyrazam and sedaxane, which are able to disrupt fungal respiration causing a breakdown in energy/ATP production (Avenot and Michailides, 2010). Of these SDHIs, sedaxane (Syngenta Crop Protection, Basel,

Switzerland) has recently been released for use as a treatment for local and systemic protection of cereal seeds, seedlings and roots against pathogenic fungi, both seed-borne (*Ustilago nuda, Tilletia caries, Monographella nivalis, Pyrenophora graminea*) and soil-born (*Rhizoctonia solani, R. cerealis, Gaeumannomyces graminis*, *Typhula incarnata*) (Zeun et al., 2013; Ajigboye et al., 2016). When sedaxane moves from the seed to the soil and into the plant tissues, it has been found to improve the development of the roots and lower stems of cereals (Swart, 2011). Previous research has described wheat responding positively to sedaxane in terms of greater biomass, better growth and drought resistance (Ajigboye et al., 2016). These morpho-physiological reactions are also known to be induced by biostimulants (Calvo et al., 2014), defined as substances that at low doses are able to enhance hormones biosynthesis, nutrient uptake from the soil, resistance to biotic/abiotic stresses, crop quality, and root growth (Kauffman et al., 2007).

Against this background, the present study aimed at investigating the potential biostimulant activity of seed-applied sedaxane on maize plants. To this end, it was: i) carried out a bioassay of biostimulants, ii) monitored morphological variations at increasing fungicide doses, and iii) studied the response of enzymes involved in nitrogen and phenylpropanoid metabolism, and of the protein, sugar and total phenol contents in both leaves and roots. The trials were carried out in the lab and the greenhouse on pot-cultivated, disease-free maize plants in order to distinguish biostimulant effects from plant protection properties.

Materials and Methods

Characteristics of sedaxane

In this study, the fungicide formulation Vibrance® 500 FS, a commercial flowable concentrate for seed treatment containing 500 g sedaxane® L^{-1}, i.e., 43.7% w/w of AI (density 1.17 g mL^{-1}; pH 6.39) was used. Sedaxane is the ISO common name for a mixture of two cis-isomers, 2'-[(1RS,2RS)-1,1'-bicycloprop-2- yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide and two trans-isomers 2'-[(1RS,2SR)-1,1'- bicycloprop-2-yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide (IUPAC). The minimum purity of sedaxane is 960 g kg^{-1}, with ranges of 820-890 g kg^{-1} for the 2 trans-isomers (SYN508210 - 50:50 mixture of enantiomers), and 100-150 g kg^{-1} for the 2 cis-isomers (SYN508211 - 50:50 mixture of enantiomers) (EFSA, 2012).

Pot trial set-up and plant analysis

Plants of the maize hybrid Hydro (Syngenta, Basel, Switzerland) were grown in cylindrical PVC pots (50 cm high, 9 cm diam, 3.1 L volume) in a greenhouse in the L. Toniolo experimental farm of the University of Padua (Legnaro, NE Italy). The pots were filled with a sterilized (36 h in an oven at 120 °C) mixture of silty-loam soil collected from a field on the experimental farm (pH 8.4) and fine sand (1:1 w/w) in order to facilitate water drainage and root collection, and supplemented with a standard dose of pre-sowing fertilization (about 100 kg N ha-1, 150 kg P2O5 ha-1 and 300 kg K2O ha-1). Maize seeds were treated with three increasing doses of sedaxane: 25, 75 and 150 µg AI seed-1, corresponding to label doses of 2.5, 7.5 and 15 mL of the commercial product Vibrance® 500 FS (500 g AI L-1) in 50,000 seeds. Treated seeds were compared with untreated controls. The experimental design was completely randomized with 6 replicates.

Three seeds per pot were sown at the end of June, and immediately after emergence plants were thinned to one per pot. At harvest, growth measurements were taken from three

pots/plants, and enzymatic activity assays were carried out from a further three.

Water stress was avoided throughout the experiment by regularly watering the plants. Before plant harvest, which took place 20 days after sowing (DAS) at the 3-leaf stage, leaf chlorophyll content was measured in the last fully developed leaf with a SPAD (Soil Plant Analysis Development) 502 chlorophyll meter (Konica-Minolta, Hong Kong). Three replicate samples of shoots were taken to measure fresh and dry (after 24 h oven-drying at 105 °C) weights, and roots were collected, gently washed of soil and stored in a 15% v/v ethanol solution until morphological characterization. Root length, surface area, diameter, and number of tips and forks were measured by analysis of 1-bit 400-DPI images of the roots acquired with a flatbed scanner (Epson Expression 11000XL, Epson, Suwa, Japan) using the WinRhizo software (Regent Instruments, Ville de Québec, Canada).

Three replicates were stored at -80 °C until analysis, then shoot and root tissue samples were taken from them for enzymatic activity assays. Each plant was analyzed in triplicate in each enzymatic assay (n = 9).

A further trial using the same sand-soil mixture (1:1 w/w), but not sterilized, was performed following the same procedure and timing of the main experiment. Plants grown in non-sterilized soil were measured for SPAD, fresh and dry weights, and root morphological parameters, as reported above (Supplementary Material).

Bioassay to test the biological activity of sedaxane

We assessed the biological activity of sedaxane by measuring the reduction in root growth in the model plant watercress (*Lepidum sativum* L.) to ascertain auxin-like activity, and the increase in shoot length in lettuce (*Lactuca sativa L.*) to ascertain giberellinic-like activity (Audus, 1972).

Watercress and lettuce seeds were surface-sterilized by immersing them in 8% hydrogen peroxide for 15 minutes. After rinsing 5 times with sterile distilled water, 20 seeds were aseptically placed on filter paper in a Petri dish. In the case of watercress, the filter paper was moistened with 1.2 mL of H_2O (controls), or with 1.2 mL of 0.1, 1, 10 and 20 mg L^{-1} indoleacetic acid (IAA, natural auxin) (Sigma-Aldrich, St. Louis, MO, USA) to obtain the calibration curve, or with 1.2 mL of a serial dilution of the tested product Vibrance containing 500 g L^{-1} of AI sedaxane. The experimental design for lettuce was the same as for watercress, except that the sterile filter paper was moistened with 1.4 mL of the above solutions, while the calibration curve was a progression of 0.0001, 0.001, 0.01 and 0.1 mg L^{-1} gibberellic acid (GA) (Sigma-Aldrich, St. Louis, MO, USA).

Seeds were germinated in the dark at 25 °C. After 48 h for watercress and 72 h for lettuce, the seedlings were removed from the dishes and the lengths of the watercress roots and the lettuce shoots were measured with a digital gauge.

A linear regression model ($y = a + bx$) was used to describe the dose-response relationship after logarithmic transformation of IAA, GA and the sedaxane doses (Conselvan et al., 2017).

Protein extraction and determination

Fresh leaf and root samples, previously stored at -80 °C, were ground to a homogenous powder with liquid N_2. Proteins were extracted homogenizing 0.5 g of roots or shoot materials with 5 mL of 38 mM KH_2PO_4 and 62 mM K_2HPO_4 pH 7, at 4 °C temperature. After 2 minutes, the extract was filtered through three layers of muslin and centrifuged at 15,000 g for 20 min at 4 °C. A 50-µL supernatant sample was incubated with 50 µL of milliq water and 2.5 mL of 0.00117 M Bradford reagent. After 15 min the protein

concentration in the extract was determined according to Bradford (1976), using a Jasco V-530 UV/vis spectrophotometer (Jasco Corporation, Tokyo, Japan) at 595 nm wavelength. The protein concentration was expressed as mg of proteins per g of fresh root or shoot weight.

Enzyme extraction and assay conditions

To extract the enzymes involved in N reduction and assimilation pathways, fresh shoot and root samples were ground to a homogeneous powder with liquid N_2. Each activity assay was carried out in triplicate and with 3 biological repetitions using specific buffers for enzyme extraction.

Glutamine synthetase (GS; EC 6.3.1.2) was extracted by homogenizing 0.6 g of root or shoot material at 4 °C with 2.4 mL of a solution of 1 mM Tris(hydroxymethyl)aminomethane HCl (Tris-HCl), 25 mM KH_2PO_4, 10 mM L-cysteine hydrochloride monohydrate and 3% (w/v) bovine serum albumin at pH 7.8 (Baglieri et al., 2014). After 10 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 g for 25 min at 4 °C. A 200-µL sample of supernatant was incubated with 200 µL of reaction buffer (50 mM Tris-HCl, 20 mM $MgSO_4$, 80 mM L-glutamate, 30 mM NH_2OH, 24 mM ATP, pH 7.8) at 37 °C for 25 min. Reaction was blocked with a stopping solution (0.5 mL of 370 mM $FeCl_2$ $6H_2O$ and 670 mM HCl). Samples were centrifuged at 15,000 g for 15 min. The amount of γ-glutamyl hydroxamate in the supernatant was determined photometrically (wavelength 540 nm) against an immediately-stopped parallel sample (Jezek et al., 2015). A standard curve was made using authentic γ-glutamyl hydroxamate (GHA) proportional to absorbance intensity. Enzyme activity was expressed

as μmol of GHA produced per g of fresh root or leaf tissue per minute (Conselvan et al., 2017).

Glutamate synthase (GOGAT; EC 1.4.7.1) was extracted by homogenizing 0.5 g of root or shoot material with 2 mL of a solution of 100 mM Tris-HCl pH 8.2, 10 mM $MgCl_2$ $6H_2O$, 2 mM β-mercaptoethanol, 10% (v/v) glycerol and 1 mM Na_2EDTA. After 15 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was centrifuged a second time at 15,000 g for 15 min at 4 °C. For the enzyme assay, 100 μL of extract was added to 900 μL of reaction buffer (41.6 mM HEPES pH 7.5, 1 mM NADH, 10 mM EDTA, 20 mM glutamine) and 300 μL (for leaf extract) or 900 μL (for root extract) of 10 mM α-ketoglutaric acid. The reaction time was 2 min for the shoot extract and 1.5 min for the root extract at 30 °C. GOGAT was assayed spectrophotometrically by monitoring NADH oxidation at wavelength 340 nm according to Avila et al. (1987). GOGAT activity was expressed as nmol NADH reduced per g of fresh root or shoot per minute.

For the phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) assay, 1 g of shoot material was homogenized with 0.1 g of poly(vinylpolypyrrolidone) (PVPP) and 5 mL of 100 mM potassium phosphate buffer (pH 8.0) containg 1.4 mM β-mercaptoethanol. After 10 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 g for 20 min at 4 °C. A 60-μL sample of supernatant was incubated with 400 μL of 100 mM Tris-HCl buffer (pH 8.8), 140 μL of 100 mM phosphate buffer and 200 μL of 40 mM phenylalanine at 37 °C for 30 min. Reaction was stopped with 200 μL 6N HCl (El-Shora, 2002). After centrifuging at 10,000 g for 15 min, the absorbance of the supernatant was measured at 280 nm against an immediately-stopped parallel sample. A standard curve was made using authentic cinnamic acid at increasing dilutions. PAL activity was expressed as nmol

cinnamic acid produced per mg of protein in the sample per minute.

Soluble phenols extraction and determination

Soluble phenolic acids were extracted homogenizing 200 mg of leaf material with 600 mL of pure methanol. The extract was kept in ice for 30 min and then centrifuged at 15,000 g for 30 min at 4 °C. Total phenols were measured according to the procedure described by Arnaldos et al. (2001). In brief, 1 mL of 2% Na_2CO_3 and 75 µL of Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA) were added to 50 µL of the phenolic extract. After 15 min of incubation in the dark at 25 °C, the absorbance was measured at 725 nm. A standard curve was made using authentic gallic acid. The soluble phenols content was expressed as mg of gallic acid equivalent (GAE) per g of fresh shoot material.

Free phenolic acid concentrations were revealed on 0.1 g shoot samples treated with 5 mL 80% (v/v) acetonitrile (ACN) in 10-mL tubes for 5 min at room temperature with agitation (70 rpm). After centrifugation (5 min, 10,000 RCF), clear supernatant was filtered at 0.2 µm (Acrodisc syringe filters with GHP membranes) and kept in clean tubes at -20 °C until processing. HPLC analysis was carried out according to the method described by Adom et al. (2003) with modifications. Samples were manually shaken, then 200 µL was extracted and placed in vials for HPLC auto-sampling. The mobile phase was 0.25% (v/v) trifluoroacetic acid (TFA, solvent A) and pure ACN (solvent B). The HPLC gradient was linear: after 2 µL sample injection, solvent B was kept at 4% for 1.16 min, then increased gradually to 12% in 1.16 min, to 23% in 4.63 min, to 95% in 1.85 min, to 100% in 1.16 min, and the final rate was maintained for a further 2.78 min. Analysis had a duration of 11.58 min at a solvent flow rate of 1.1 mL min^{-1}. The HPLC equipment (Shimadzu, Kyoto, Japan) had a UV diode array detector (SPD-M20A) at wavelength 282 nm, and an Ultra

Tech sphere C18 analytical column (33 mm × 4.6 mm i.d., 1.5 μm particle size; Cil Cluzeau, Sainte-Foy-La-Grande, France) kept at 36 °C. Control sample solutions of shoots containing known phenolic acid concentrations were analysed at the beginning of each new batch analysis, and measurement accuracy was verified by checking expected concentrations.

Each peak was identified by analysing the retention time and absorbance spectrum of each pure compound (i.e., *p*-Coumaric, caffeic, syringic, vanillic and *t*-ferulic acids). The coefficients of determination of all calibration curves were >99%.

Quantitative determination of sugars

Shoots (5 g) were homogenised in methanol (20 mL) with an Ultra Turrax T25 at 13,500 rpm for 30 s until they attained uniform consistency. Samples were filtered once through filter paper (589 Schleicher) and a second time through cellulose acetate syringe filters (0.45 μm). The extract was then ready for HPLC analysis.

For this analysis, we used a Jasco X-LC liquid chromatography system (Jasco Inc., Easton, MD, USA) consisting of a pump model PU-2080, a multiwavelength detector model MD-2015, an auto-sampler model AS-2055 and a column oven model CO-2060, and the ChromNAV (Jasco Inc., Easton, MD, USA) chromatography data system software.

Sugars were separated in a HyperRez XP Carbohydrate Pbþþ analytical column (7.7 mm × 300 mm; ThermoFisher Scientific, Waltham, MA, USA), operating at 80 °C. Isocratic elution was carried out with water at a flow rate of 0.6 mL min^{-1}. D-(β)-glucose and D-(β)-fructose were quantified by a calibration method. Standards were dissolved in water and the calibration curves were generated with concentrations ranging from 100 mg L^{-1} to 1,000 mg L^{-1} (Nicoletto et al., 2013).

Statistical analysis

The data are the means of measurements from three different pots per treatment and. An analysis of variance (ANOVA) was performed in the SPSS 23 (IBM Corp) software, and was followed by pairwise post-hoc analyses (Student-Newman-Keuls test) to determine significant differences among means at $P \leq 0.05$.

Results

Audus test and effects of sedaxane on shoot and root growth

The Audus test was carried out beforehand to determine the biostimulant properties of the active ingredient sedaxane. As with the natural auxin IAA, which reduces root elongation in the model plant watercress and exhibits a proportional dose-response trend, increasing concentrations of sedaxane led to a progressive reduction in root length, suggesting an auxin-like effect (Figure 1). We also found sedaxane to exhibit gibberellic-like activity, as it enhanced the shoot growth of lettuce and had a dose-response trend similar to that of exogenous gibberellic acid (Figure 2). Both regression curves were significant ($P < 0.02$ for root responses, $P \leq 0.05$ for shoot responses), revealing the hormone-like activity of this fungicide.

Under sterile conditions, fungicide treatment did not significantly enhance plant growth, although the medium dose of sedaxane (75 µg seed^{-1}) resulted in an appreciable increase in shoot (+21%) and root (+10%) biomasses compared to untreated controls (Table 1). The effects of the seed treatments were more evident on other root features: root length increased by 60% and root area by 45% at the intermediate fungicide dose. While root

diameter was slightly smaller ($P > 0.05$), there was increase in the number of root tips and forks, with the intermediate and maximum doses having the greatest effects (tips +27% and +17%, forks +51% and +48%, respectively), although only the increase in root branching was significant. These results show that root stimulation by sedaxane may be dose-dependent with a saturation trend.

Effects of sedaxane on leaf chlorophyll, protein and sugar contents

The greenness of leaves, as measured by SPAD values, was very stable across treatments at the end of the trial (Table 1), while protein content was significantly influenced by sedaxane ($P < 0.001$), increasing by 14% at the intermediate and highest AI doses (Table 2). A similar effect was found in the roots, with protein abundance increasing significantly at the highest AI dose (+20% *vs*. untreated controls).

Fungicide treatment did not affect the shoot and root glucose content, the former having an average concentration of 3374 μg g^{-1} FW, the latter 3766 μg g^{-1} FW. The only variation found with regard to fructose was a significant reduction in the shoot at the lowest and highest sedaxane doses (-21% and -15%, respectively, *vs*. untreated controls) (Table 3).

Variations of GS and GOGAT activities with sedaxane

Both glutamine synthetase (GS) activity and glutamate synthase (GOGAT) activity were higher in the shoot than in the roots, on average by 3.8 times and 2.1 times, respectively. Seed treatment with sedaxane induced a significant increase in GS activity in both shoot ($P < 0.01$) and root ($P < 0.001$) (Table 2), with increases above ground at the lowest and intermediate AI doses (+145% and +45%, respectively, *vs*. controls), while the highest

increases in the roots were recorded at the intermediate and highest AI doses (both +66%, $P \leq 0.05$).

As regards GOGAT activity, sedaxane treatments did not induce any changes in the shoots, while slight, but non–significant, reductions were observed in the roots.

Effect of sedaxane on the leaf phenyl propanoid metabolism

A significant increase in soluble phenolic acids in the shoot was observed at the lowest sedaxane dose (+14% *vs*. untreated controls), while values similar to controls were detected at greater AI doses ($P \leq 0.05$) (Table 4). However, when individual compounds were analyzed, large differences were detected among treatments for caffeic acid, and, to a lesser extent, for syringic and *p*-coumaric acids ($P \leq 0.05$). A significantly higher concentration of caffeic acid was found in all treated plants compared with untreated controls ($P \leq 0.05$). Sedaxane increased caffeic acid by 41-58%, depending on the applied dose, and *p*-coumaric acid, the most abundant phenolic compound, at the lowest (+23%) and intermediate (+19%) doses. There were only slight differences in the vanillic and ferulic acid contents in treated plants compared with controls ($P > 0.05$).

ANOVA revealed a significant increase ($P \leq 0.05$) in PAL enzyme activity in the shoot with the lowest and highest fungicide doses (+29% and +43%, respectively) compared with untreated controls (Table 4).

Discussion

Sedaxane belongs to the new class of succinate dehydroganase inhibitors and is currently used as seed coating fungicide on various crops in several countries with increasing

worldwide registration approval. It has a broad antifungal spectrum and is of particular interest in combatting *Rhizoctonia solani* and *Mycosphaerella reliana* in maize.

In light of previous results on root stimulation in wheat (Barchietto et al., 2012), we investigated the side-effects of sedaxane in maize, over and above its protective capacity, and found that seed treatment induced significant modifications to morphological traits and physiological activities in disease-free plants grown in sterile soil.

The Audus test is considered to be the most reliable bioassay in terms of reproducibility and repeatability for verifying and quantifying the biostimulant activity of molecules in plants, and can be used to ascertain whether an exogenous compound has auxin- and/or gibberellin-like activity (Conselvan et al., 2017). Auxin (IAA) the most important hormone in plants, it being involved in several plant growth and development phases, such as embryogenesis, organogenesis, tissue patterning and tropisms (Davies, 2010). In particular, molecular genetic studies have brought to light the central role of auxin in primary root elongation, lateral root initiation, and root hair development (De Smet et al., 2006; Overvoorde et al., 2010). The phytohormone gibberellin (GA) also modulates plant development by lengthening roots and stems and expanding leaves (Fleet and Sun, 2005). We used an Audus bioassay to demonstrate that sedaxane has both auxin- and gibberellin-like activity, thereby confirming its biostimulant properties.

Although the improvements in aerial and root biomasses detected in this trial were not significant, we found that root length and area, and the number of root tips and branches increased almost in proportion to the dose of sedaxane, consistent with results reported by Colla et al. (2014) on maize coleoptile elongation with protein hydrolysates. All these modifications on root morphology modifications are known responses to biostimulant compounds (Calvo et al., 2014). Root development is essential for plant survival as it is

crucial for water and nutrient acquisition for growth, the synthesis and accumulation of secondary metabolites, and interaction with soil organisms (Saini et al., 2013).

The data collected from this trial are consistent with those of Barchietto et al. (2012) on the stimulation of wheat shoots and roots by seed-applied sedaxane. At 30 days after sowing (DAS), they observed significant increases in root length in treated plants compared with controls, and no differences in root biomass, as in our case study at 20 DAS. Interestingly, they also found that at 60 DAS root length was unaffected by sedaxane seed treatment, whereas root biomass increased significantly (+39-87%, according to variety).

In the sterile soil conditions of our trial, the leaf chlorophyll content remained very stable across treatments, but this was not the case in the supplementary trial we carried out in unsterile soil conditions to investigate the potential effect of sedaxane in field-like conditions, where we found a slight but significant increase in chlorophyll (up to 7%) (Table SI1). This result is in line with practical expectations in the field given the correlation between chlorophyll content and photosynthetic activity, the N status of the plant and protein contents (Prost and Jeuffroy, 2007; Sim et al., 2015).

It should be noted that sedaxane may affect not only fungal mitochondria but also the SDH complex II of plants, partially inhibiting its activity (Avenot and Michailides, 2010). Fuentes et al. (2011) reported that *Arabidopsis* plants with compromised expression of the flavoprotein subunit of SDH had better photosynthetic performance than wild-type plants. Inhibition of the SDH subunit also resulted in an increase in leaf stomatal number and aperture, which significantly increased CO_2 assimilation, in turn enhancing growth and protein production. Araújo et al. (2011) obtained similar results with tomato plants with antisense inhibition of the iron-sulphur subunit of SDH. However, the higher leaf chlorophyll content of sedaxane-treated maize observed in our supplementary study with

unsterilized soil may also be related to a slowing down of chlorophyll molecule degradation, as reported for fungicides of the strobirulin class (Grossmann and Retzlaff, 1997; Xu and Huang, 2009).

The higher protein content in sedaxane-treated seedlings may be ascribed to better nitrogen metabolism through the activity of the enzymes involved. In fact, the GS/GOGAT metabolic pathway is the main route of N assimilation in higher plants (Mokhele et al., 2012), allowing ammonium taken up directly or originating from nitrate to be assimilated into amino acids (Xu et al., 2012). The GS enzyme is also critical for the re-assimilation of the NH_4^+ constantly released in large amounts via photorespiration, phenylalanine consumption for lignin biosynthesis and protein catabolism (Lea and Miflin, 2011). GS activity, which increased significantly following sedaxane application, therefore plays a pivotal role in many aspects of plant development (Seabra and Carvalho, 2014), as it is a key component in nitrogen use efficiency (NUE) and plant yield (Thomsen et al., 2014).

GS and GOGAT enzyme activities have been previously reported to be affected by biostimulants (Baglieri et al., 2014). Our data are consistent with those of Ajigboye et al. (2016), who found that improvements in the photosynthetic efficiency, growth, and biomass of sedaxane-treated wheat plants were associated with up- or down-regulation changes in gene expression, and consequent modifications to physiological processes, particularly under drought stress conditions. In particular, sedaxane is reported to induce transcriptional regulation of genes and transcriptional factors resulting in an altered flavonoids and phenolic synthesis metabolism (Ajigboye et al., 2016). Our study confirmed that sedaxane stimulates phenylpropanoid metabolism in maize as we found an increase in PAL enzyme activity, although the effect unexpectedly was not observed at the intermediate dose. The PAL enzyme catalyzes the first metabolic step from primary to

secondary metabolism (Douglas, 1996), deaminating phenylalanine to produce cinnamic acid. As a consequence, there was an increase in the total content of phenolic compounds in shoot tissues from seedlings treated with the lowest concentration of sedaxane, but not at the highest dose. However, there were substantial changes in the concentrations of individual phenolic acids in relation to fungicide application, in particular, a considerable increase in caffeic acid in treated plants, which may be of interest in view of its weak auxin-like effect (Lavee et al., 1985; Ishikura et al., 2001; Nagasawa et al., 2016). The main precursor of lignin in the cell wall of gramineous plants is *p*-coumaric acid, and its increased abundance in sedaxane-treated plants could give rise to more intense cell activity and division. Vanillic and *p*-coumaric acids are also reported to be antifungal phenolics, meaning that sedaxane may also contribute indirectly to plant defence (Lattanzio et al., 2006; Zabka and Pavela, 2013; Pusztahelyi et al., 2015). In any case, the stimulation of the secondary metabolism can also be justified by the enhanced activity of the primary metabolism activity.

As with other SDHIs studied in wheat, all the physiological changes brought about by sedaxane may also delay senescence and improve the yield and protein content of maize plants (Bayles, 1999; Dimmock and Gooding, 2002; Zhang et al., 2010), but this requires further investigation in actual field conditions.

It is concluded that sedaxane has a considerable effect on rooting power, particularly on the length, surface area and number of lateral roots of maize. This study indicates that sedaxane displays biostimulant activity in maize seedlings due to its hormone-like activities, corroborated by the fact that most of the observed effects are saturated at moderate doses, as with phytohormones. There are high expectations that seed treatment with this fungicide will facilitate plant establishment, and may provide particular benefits

under adverse soil and climatic conditions. Stimulation of the enzyme activities involved in N assimilation and phenilpropanoid metabolism is in agreement with previous findings on this active ingredient and other SDHI fungicides, and is consistent with improved N status and antioxidant activity.

As the fungicide doses tested here are within the recommended label range, the biostimulant activity of sedaxane is an additional benefit, over and above its protective role against seed- and soil-borne diseases, which could be exploited in the cultivation of maize. Although further studies are needed to see whether these improvements also influence final growth and yield, our preliminary results suggest that currently roots may be enhanced in the early growth stages, even in non-sterile soil.

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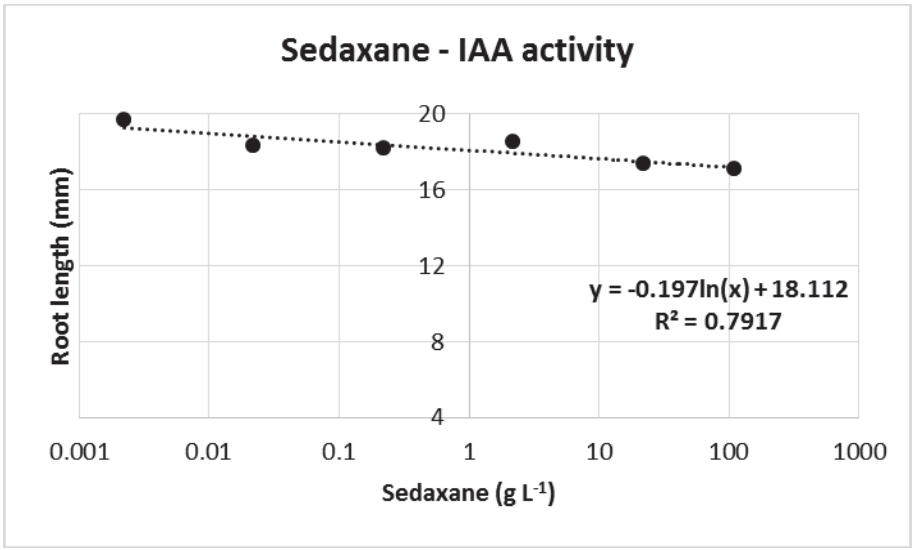
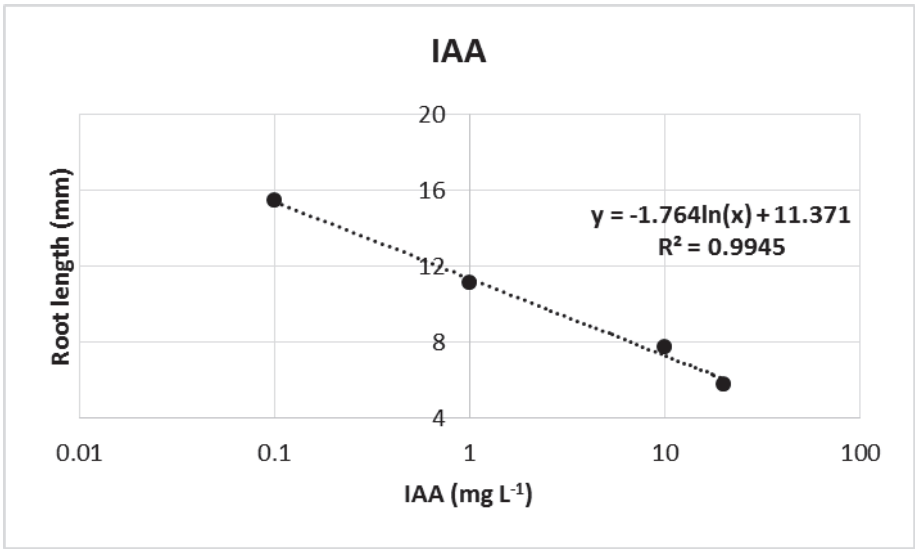
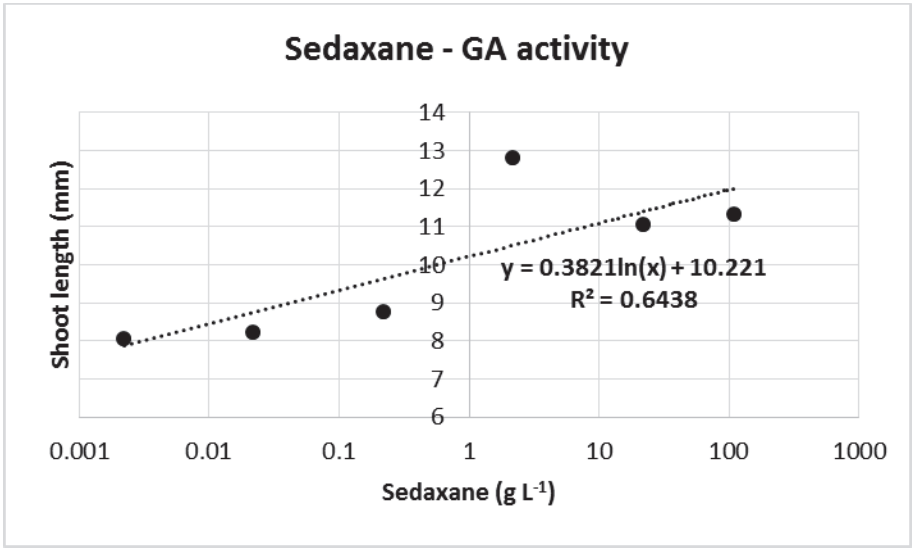
Figures and Tables

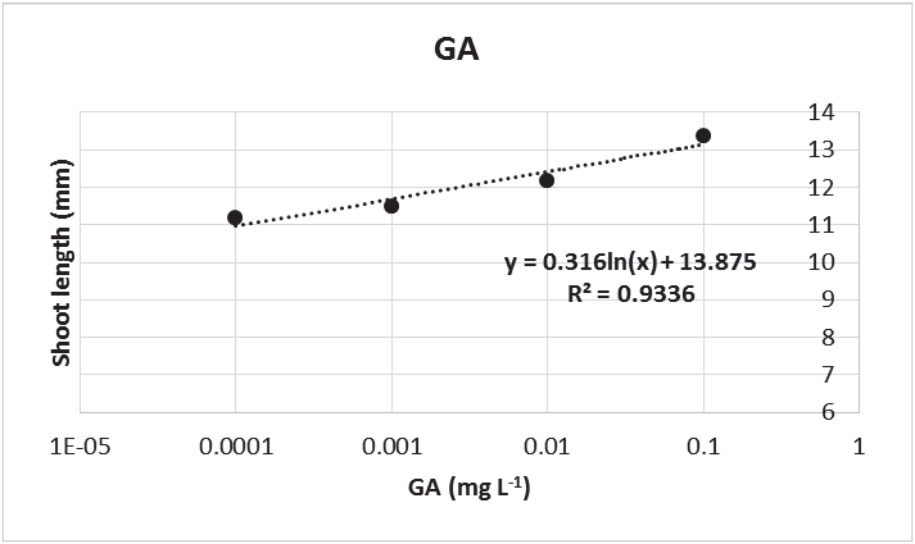




	R^2	b	*P*
IAA	0.99	-1.76	0.00
Sedaxane	0.79	-0.197	0.02

Figure 1. Audus test: auxin-like activity of sedaxane measured as root length variations in watercress. Linear regression analysis (below) performed on 20 samples and averaged over 5 replicates. Note that the x axis has a logarithmic scale.





	R^2	b	*P*
GA	0.93	0.72	0.04
Sedaxane	0.644	0.382	0.05

Figure 2. Audus test: gibberellin-like activity of sedaxane measured as variation in shoot length in lettuce. Linear regression analysis (below) was performed on 20 samples and averaged over five replicates. Note that the x axis has a logarithmic scale.

Table 1. Main shoot and root parameters (mean ± SE; n = 3) in *Zea mays* at 20 days after sowing (DAS) in sterilised pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

	Shoot		**Root**					
Sedaxane rate (µg seed^{-1})	**DW** (g plant^{-1})	**SPAD**	**DW** (g plant^{-1})	**Length** (m plant^{-1})	**Area** (m^2 plant^{-1})	**Diameter** (mm)	**Tips** (n plant^{-1})	**Forks** (n plant^{-1})
0	0.50±0.07^{a}	34.6±0.8 ^{a}	0.24±0.03^{a}	130.2±35^{b}	0.23±0.04^{b}	1.81±0.13^{a}	6657±1769^{a}	10594±2280^{a}
25	0.52±0.04^{a} (+3)	34.1±0.2^{a} (-1)	0.25±0.03^{a} (+3)	167.4±6^{ab} (+29)	0.26±0.01^{ab} (+14)	1.54±0.03^{a} (–15)	6180±957^{a} (–7)	12649±1681^{ab} (+19)
75	0.61±0.04^{a} (+21)	34.7±0.9^{a} (+0.5)	0.26±0.01^{a} (+10)	208.0±24^{a} (+60)	0.33±0.03^{a} (+45)	1.59±0.10^{a} (–12)	7784±994^{a} (+17)	15985±1849^{b} (+51)
150	0.53±0.04^{a} (+6)	34.3±0.8^{a} (–0.5)	0.24±0.01^{a} (+1)	184.9±16^{ab} (+42)	0.31±0.02^{ab} (+37)	1.68±0.10^{a} (–7)	8467±405^{a} (+27)	15711±718 ^{b} (+48)

Table 2. Shoot and root protein content, and glutamine synthetase (GS) and glutamate synthase (GOGAT) activities (mean ± SE; n = 9) in *Zea mays* at 20 days after sowing (DAS) in sterilised pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

	Shoot			**Root**		
Sedaxane rate (µg seed^{-1})	**Proteins** (mg g^{-1} FW)	**GS** (µmol GHA g^{-1} FW min^{-1})	**GOGAT** (nmol NADH g^{-1} FW min^{-1})	**Proteins** (mg g^{-1} FW)	**GS** (µmol GHA g^{-1} FW min^{-1})	**GOGAT** (nmol NADH g^{-1} FW min^{-1})
0	5.6±0.1^{b}	0.11±0.01^{c}	0.15±0^{a}	1.5±0^{b}	0.03±0^{b}	0.08±0.01^{a}
25	5.9±0.1^{ab} (+5)	0.27±0.01^{a} (+145)	0.15±0.01^{a}	1.5±0.1^{b}	0.04±0^{a} (+33)	0.06±0.01^{a} (-25)
75	6.4±0.2^{a} (+14)	0.16±0.01^{b} (+45)	0.15±0.01^{a}	1.5±0.1^{b}	0.05±0^{a} (+66)	0.07±0.01^{a} (-12)
150	6.4±0.2^{a} (+14)	0.11±0.01^{c}	0.15±0.01^{a}	1.8±0^{a} (+20)	0.05±0^{a} (+66)	0.07±0.01^{a} (-12)

Table 3. Shoot and root glucose and fructose (mean ± SE; n = 9) in *Zea mays* at 20 days after sowing (DAS) in sterilised pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

	Shoot		**Root**	
Sedaxane rate (µg seed^{-1})	**Glucose** (µg g^{-1} FW)	**Fructose** (µg g^{-1} FW)	**Glucose** (µg g^{-1} FW)	**Fructose** (µg g^{-1} FW)
0	3328±170^{a}	1005±24^{a}	3819±128^{a}	1322±56^{a}
25	3310±33^{a} (–1)	792±35^{b} (–21)	3724±163^{a} (–2)	1418±109^{a} (+7)
75	3340±113^{a}	1012±59^{a} (+1)	3665±140^{a} (–4)	1476±125^{a} (+12)
150	3518±70^{a} (+6)	855±14^{b} (–15)	3859±384^{a} (+1)	1313±191^{a} (–1)

Table 4 Shoot phenylalanine ammonia-lyase activity (PAL), soluble phenol content and phenolic acid profile (mean ± SE; n = 9) of *Zea mays* at 20 days after sowing (DAS) in sterilised pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

Sedaxane rate (µg seed^{-1})	**PAL** (nmol cinn. acid mg^{-1} prot. min^{-1})	**Soluble phenols** (as mg gallic acid g^{-1} FW)	**Vanillic acid** (µg g^{-1} FW)	**Caffeic acid** (µg g^{-1} FW)	**Syringic acid** (µg g^{-1} FW)	**p-Coumaric acid** (µg g^{-1} FW)	**Ferulic acid** (µg g^{-1} FW)
0	3.1±0.12^{b}	36.4±1.5^{b}	0.78±0.06^{a}	2.88±0.24^{b}	11.7±0.6^{b}	21±0.9^{b}	0.72±0.04
25	3.99±0.28^{a} (+29)	41.4±0.8^{a} (+14)	0.63±0.06^{a} (-19)	4.14±0.24^{a} (+44)	14.6±0.9^{a} (+25)	25.8±1.9^{a} (+23)	0.83±0.07 (+15)
75	3.08±0.22^{b} (-1)	35.1±1^{b} (-4)	0.63±0.03^{a} (-19)	4.55±0.43^{a} (+58)	13.8±0.6^{ab} (+18)	24.9±0.6^{a} (+19)	0.82±0.03 (+14)
150	4.42±0.26^{a} (+43)	35.2±2^{b} (-3)	0.63±0.05^{a} (-19)	4.06±0.34^{a} (+41)	12.4±0.6^{ab} (+6)	21.9±1^{b} (+4)	0.74±0.03 (+3)

Supplementary information

Table SI1. Main shoot and root parameters (mean ± SE; n = 3) in *Zea mays* at 20 days after sowing (DAS) in unsterilised pot soil at increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

	Shoot		**Root**					
Sedaxane rate (μg seed^{-1})	**DW** (g plant^{-1})	**SPAD**	**DW** (g plant^{-1})	**Length** (m plant^{-1})	**Area** (m^2 plant^{-1})	**Diameter** (mm)	**Tips** (n plant^{-1})	**Forks** (n plant^{-1})
0	0.35±0.03^{a}	27.9±0.7^{b}	0.17±0.01^{a}	137.4±9^{b}	0.21±0.01^{b}	1.55±0.05^{a}	4285±215^{b}	9798±1010^{a}
25	0.34±0.05^{a} (-3)	28.4±0.6^{ab} (+2)	0.17±0.01^{a}	155.5±21^{ab} (+13)	0.23±0.03^{ab} (+10)	1.49±0.01^{ab} (-4)	3804±618^{b} (-11)	10111±1277^{a} (+3)
75	0.42±0.02^{a} (+20)	28.4±0.7^{ab} (+2)	0.19±0.005^{a} (+12)	156.6±11^{ab} (+14)	0.23±0.02^{ab} (+10)	1.50±0.04^{ab} (-3)	4190±399^{b} (-2)	10298±796^{a} (+5)
150	0.42±0.04^{a} (+20)	29.9±0.6^{a} (+7)	0.20±0.01^{a} (+18)	189.1±19^{a} (+38)	0.27±0.02^{a} (+29)	1.43±0.03^{b} (-8)	5479±131^{a} (+28)	11628±958^{a} (+19)

CHAPTER IV

Protection and growth enhancement effects of seed-applied sedaxane fungicide on maize seedlings under high *Rhizoctonia solani* pressure

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Abstract

Seed-applied fungicides are commonly used to prevent or suppress soil- and seed-borne fungal pathogens, that can reduce survival, growth and yield of several crops in conventional agriculture. However, currently there is much interest on the secondary effects of new seed-applied fungicides, regardless of their protection role.

This work aimed at evaluating possible co-effects on shoot and root growth of maize (*Zea mays* L.) of the new SDHI active ingredient fungicide sedaxane (Syngenta Crop Protection, CH), in early plant stages with and without *Rhizoctonia solani* infection.

The commercial formulation Vibrance ® containing 500 g a.i. L^{-1} was applied to seeds of the hybrid Hydro (Syngenta) at three increasing Vibrance doses (2.5, 7.5, 15 mL for 50,000 seeds), and plants cultivated in pots absence or in presence of high *Rhizoctonia solani* AG 2-2IIIB pressure in the substrate, that was added as mycelium at sowing. A second trial was set up in order to evaluate the rate of seed germination.

Here we demonstrate that at aerial level the SPAD index and shoot biomass at the 3-leaf stage were improved compared to controls by all the fungicide doses, particularly at the intermediate one, allowing to increase the germination rate and to fully recover shoot injury owing to *Rhizoctonia*. The main effect of sedaxane was observed belowground at intermediate dose under fungal infection, with significant root biomass (+54%), length (+63%), and tip (+56%) and fork (+78%) densities improvements compared to untreated controls, although significant was also the groth enhancement in disease-free soil possibly due to its auxin-like effect.

Although a full plant biomass recovery under high *R. solani* pressure can occur at the smallest seed-applied sedaxane dose, a fully exploitation of the biostimulant co-effect of

sedaxane would require the application of intermediate doses, thus allowing to cope better against unfavorable climatic and soil conditions in the open field.

Keywords: biostimulant; root growth; seed protection; soil-borne pathogens; succinate dehydrogenase inhibitor (SDHI).

Introduction

Soil- and seed-borne fungal pathogens are the main cause that compromise seed germination, emergence and early development of many plant species cultivated at open field scale. Maize (*Zea mays* L.) is one of the most relevant cereal in the world for human and animal feeding (FAO, 2017) and very susceptible to infections by several fungal and *Oomycete* pathogens, it being cultivated in different environments and conditions (Agarwal and Sinclair, 1996). Fungal pathogens affect in particular the initial root growth, and early damage affect plant development in later stages and compromise yield potential (Turkington et al., 2016). Among fungal pathogens, *Rhizoctonia solani* has became in the last years more widespread in European soils (Goll et al., 2014) causing severe damages in cropping systems where cereal monoculture and minimum to no till practices are usually adopted (Cook et al., 2002). In maize, this pathogen causes 'crown and brace root rot' attacking all the below-ground plant parts, including seeds and hypocotyls. On seminal, adventitious and crown roots, *R. solani* mainly causes reddish-brown to black sunken lesions but also yellowing or necrosis of the cortex, determining a delayed development and often stunting or damping off of young plants (Mazzola et al., 1996; Pfähler and Petersen, 2004). As consequence of the weak root health, the susceptibility of plants to

drought stress, nutrient deficiency and secondary infections by other root diseases is largely increased (Cook, 2001).

Currently, *R. solani* is divided into 14 anastomosis groups (AG) based on hyphal anastomosis, cultural morphology, host range, pathogenicity and other biochemical or molecular characters. In particular, hyphal anastomosis occurs only between isolates of the same groups (Ogoshi, 1987; Sneh et al., 1991; Carling et al., 2002; Dorrance et al., 2003). Maize can host several *R. solani* anastomosis groups and, most specifically, *R. solani* AG 2-2IIIB was demonstrated to have a particularly high pathogenicity (Ithurrart et al., 2004), favored also by its strong ability to survive on plant residues of different hosts in crop rotations, particularly on sugar beet (Sumner and Bell, 1986).

To control early pathogenic infections almost the whole of the hybrid maize seed is usually treated with fungicides before sowing (Sharma et al., 2015). Seed coating is a process that consists to apply several chemical substances to the seed surface, with the possibility to add also living microorganisms and biostimulant materials (Ehsanfat and Modarres-Sanavy, 2005). A considerable advantage of this technique is the use of small and very precise amounts of active substances on seeds, which makes it grower-friendly and environment-friendly, as it minimizes farmer exposure to chemicals, and soil, water or air contamination compared to other conventional protection practices (Baudet and Peres, 2004).

High efficient fungicide active substances against *R. solani*, like azoxystrobin, fludioxonil and thiabendazole are lately available for seed coating in cereals (Hamada et al., 2011; Almasudy et al., 2015). Until recently, fungicides focused on control of phytopathogens with the purpose of reducing their inoculum only, because they typically do not offer any relevant and sure benefit in term of root or shoot development, resistance to abiotic stresses or crop yield enhancement (Minamor, 2013; Cox and Cherney, 2014). In addition to the

protection activity, there is nowadays an increasing interest on developing new aspects of fungicides, in order to uniform and accelerate crop emergence and stimulate growth immediately after. At this regard, in the past years it has been reported that the application of strobilurins, a class of fungicide molecules, can induce physiological changes in crops, like increased tolerance against abiotic stresses and improved morphological parameters like total leaf area, number of leaves per plant and both total shoot and root biomass in maize (Habermeyer, 1998; Lazo and Ascencio 2014). Other fungicide classes are involved in these positive secondary effects, like the recently released succinate dehydrogenase inhibitor (SDHI). Sedaxane® (Syngenta Crop Protection, Basel, Switzerland). This fungicidal active substance is commercialized under the seed treatment trademark Vibrance, and it has already been proven to improve several physiological processes in wheat (Ajigboye et al., 2016) together with increased emergence and seedlings vigor, and particularly a strong stimulation of lateral root initiation (Barchietto et al., 2012; Swart, 2011). Goll et al. (2014) showed that different *Rhizoctonia* isolates are sensitive to sedaxane, which shows an excellent activity in seedling protection during early stages of plant development.

At this time in Europe, sedaxane is licensed only for seed coating of small-grain cereals, and there is an interest in extending the use of this active substance to maize, by including it in the currently most used product, i.e., Maxim® XL (Syngenta Crop Protection, Basel, Switzerland) which contains Metalaxyl-M and Fludioxonil as active substances.

In this framework, the objective of this work was to investigate the effects of increasing doses of the seed-applied fungicide formulation Vibrance® 500 FS (Syngenta Crop Protection, Basel, Switzerland), a commercial flowable concentrate containing 500 g sedaxane L^{-1}, in reducing *Rhizoctonia solani* severity and its detrimental effect on maize

germination and growth in the early growth stages. A pot-trial was arranged in greenhouse with a soil artificially infected with a liquid fungal mycelium inoculum applied at maize sowing, while possible root growth enhancement co-effects of sedaxane were assessed in *Rhizoctonia*-free soil.

Materials and Methods

Pot trial set-up

In this experiment three different seed-application doses of vibrance were tested (low, 2.5 mL 50,000 seeds^{-1}; medium, 7.5 mL 50,000 seeds^{-1} and high, 15 mL 50,000 seeds^{-1}) in comparison with untreated control. The maize hybrid SY HYDRO (Syngenta, Basel, Switzerland), provided already coated with vibrance by Syngenta France CETAPP (Gaillon, France), was sown into 3.1-L pots (height 50 cm, diameter 9 cm) positioned inside a greenhouse-tunnel at the Experimental Farm of the University of Padova (Legnaro, Italy). Pots were filled with silty-loam soil collected from a field in the farm (pH 8.4) mixed with fine sand (1:1 w/w), in order to facilitate root sampling, and supplemented with a standard dose of pre-sowing fertilisation (about 100, 150 and 300 kg ha^{-1} of N, P_2O_5 and K_2O respectively). Three seeds per pot were sown, but immediately after emergence plants were thinned to 1 per pot and regularly irrigated with the same amount of water throughout the cultivation period (13 days). Pots were positioned following a completely randomized experimental design with 4 replicates for each treatment, maintaining those inoculated with *R. solani* separated and distant from non-inoculated ones in order to avoid the pathogen widespread.

Rhizoctonia solani inoculation

The above treatments were tested in both soil inoculated with *R. solani* and in un-inoculated soil as control. The inoculum of *R. solani* anastomosis group AG 2-2IIIB was obtained from infected barley seeds sent from EAME - Syngenta (Basel, Switzerland). The fungus was isolated from seeds and propagated in Potato Dextrose Agar (PDA, Difco) plates at the Plant Pathology section of the TESAF Department (University of Padova, Italy). The inoculum for infecting the pot substrate was produced by homogenizing the *Rhizoctonia* colonized PDA with a Waring blender in presence of an equal volume of ultrafiltered sterilized water. Inoculation was made just after sowing by applying 100 mL of the *Rhizoctonia* inoculum solution per pot on soil surface, followed by irrigation with 120 mL of deionised water in order to favour the distribution of inoculum in the upper volume of soil around seeds. A similar solution (water + PDA) without *R. solani* mycelium was added to un-inoculated control pots.

In order to identify the real effect of the seed-applied vibrance against the selected pathogen, the previous conditions were tested in a sterilized substrate (heated for 36 h in an oven at 120 °C), avoiding in this way any other soil-borne microbial or fungal influence on plant growth.

However, a parallel experiment quite similar to the one just described, except for the unsterilised substrate used, was performed to evaluate the effect of different vibrance application doses on plants grown in soil conditions in more similar to the real ones.

Plant analysis

The chlorophyll content was measured three times, at 7 DAS (days after sowing), 10 DAS and the day before plant harvest at three-leaf stage (12 DAS), on the last fully developed

leaf with a SPAD 502 chlorophyll meter (Konica-Minolta, Hong Kong). Shoots were collected and weighed to obtain fresh and dry (after 24 h oven-drying at 105 °C) weights. Roots were separated from shoots and after gently soil washing they were stored in a 15% ethanol solution until processing. Root length, surface area, diameter, number of tips and forks were measured by automatic analysis using WinRhizo software (Regent Instruments, Ville de Québec, Canada) of 1-bit 400 DPI root images acquired through a flatbed scanner (Epson Expression 11000XL, Epson, Suwa, Japan).

Free phenolic acid concentrations were revealed on 0.1 g shoot and root samples treated with 5 mL 80% (v/v) acetonitrile (ACN) in 10-mL tubes for 5 min at room temperature with agitation (70 rpm). After centrifugation (5 min, 10,000 RCF), clear supernatant was filtered at 0.2 µm (Acrodisc syringe filters with GHP membranes) and kept in clean tubes at -20 °C until processing. HPLC analysis was carried out according to the method described by Adom et al. (2003) with modifications. Samples were manually shaken, then 200 µL was extracted and placed in vials for HPLC autosampling. The mobile phase was 0.25% (v/v) trifluoroacetic acid (TFA, solvent A) and pure ACN (solvent B). The HPLC gradient was linear: after 2 µL sample injection, solvent B was kept at 4% for 1.16 min, then increased gradually to 12% in 1.16 min, to 23% in 4.63 min, to 95% in 1.85 min, to 100% in 1.16 min, and the final rate was maintained for a further 2.78 min. Analysis had a duration of 11.58 min at a solvent flow rate of 1.1 mL min^{-1}. The HPLC equipment (Shimadzu, Kyoto, Japan) had a UV diode array detector (SPD-M20A) at wavelength 282 nm, and an Ultra Tech sphere C18 analytical column (33 mm × 4.6 mm i.d., 1.5 µm particle size; Cil Cluzeau, Sainte-Foy-La-Grande, France) kept at 36 °C. Control sample solutions of shoots containing known phenolic acid concentrations were analysed at the beginning of each new batch analysis, and measurement accuracy was verified by checking

expected concentrations.

Each peak was identified by analysing the retention time and absorbance spectrum of each pure compound (i.e., *p*-Coumaric, caffeic, syringic, vanillic and *t*-ferulic acids). The coefficients of determination of all calibration curves were >99%.

Germination test

To evaluate the germination rate of maize with different seed applied vibrance doses a specific trial was set-up, in accordance to the ISTA (International Seed Testing Association) protocol. Fifty seeds (n = 4) for each treatment and control were sown in sterile sand (4 cm thick) placed in plastic trays considering both inoculated (*R. solani* AG 2-2IIIB) and un-inoculated treatments. The fungal inoculum was applied by spraying the solution to the sand (100 mL for 3.8 kg of sand) immediately after sowing, as for the pots. All trays were placed in a climatic chamber for 7 days, by setting the temperature at 25 °C with dark conditions throughout the experiment. At the end (7 DAS) only well-germinated seeds were considered.

Statistical analysis

Statistical analysis was carried out by the Statgraphics Centurion XI software (Adalta, Arezzo, Italy). ANOVA and the Student Newman-Keuls test ($P \leq 0.05$) were used to evaluate differences among means for all analyzed parameters.

Results

Germination rate

The germination rate of the tested maize hybrid SY HYDRO was generally high, above 95 % for all treatments, and was not affected by the pathogen infection or improved by seed treatments ($P > 0.05$). Under *R. solani* pressure the maximum germination was at 15 mL 50K seeds^{-1} of vibrance dosage, reaching a rate next to 100% (99.4 %) (Table 1), appreciably higher than that of seeds without fungicide treatment. On the contrary, without the pathogen infection, the presence of the medium or higher seed-applied vibrance dose determined a slight decrease of germination rate. In this situation, however, both radicles and coleoptiles fresh and dry weights were improved by the fungicide (except for the lowest dose of vibrance), whereas in infected plants the effect was not detectable at this early stage (data not shown).

Shoot parameters

Leaf chlorophyll content is an important index strongly correlated to the nutritional status of the plant, that was indirectly measured as SPAD units. On Table 2 the average values of 3 observation times (7, 10 and 12 DAS) are reported. In both sterilised and unsterilised soils, when treated plants were grown in pathogen-free conditions, there were no significant differences among treatments comparing different seed doses. Despite the absence of any statistically significant effect, when soil was infected by *R. solani*, SPAD improved as the vibrance dose increased (+2%, +2% and +7% compared to controls, at 2.5, 7.5 and 15 mL 50K seeds^{-1}, respectively).

In absence of *R. solani* infection, plants developed from treated seeds showed an increased shoot fresh biomass (FW, fresh weight), although the difference with the untreated control was not statistically significant, with better increments (+18%) at 7.5 mL 50K seeds^{-1} (sterilised soil) and +27% at 15 mL 50K seeds^{-1} (unsterilised soil). A similar trend was

established in terms of dry biomass (DW, dry weight) (Figure 1, 2). Under *R. solani* infection, the biostimulating effect of Vibrance was even more marked, reaching a maximum at the 7.5 mL 50K seeds^{-1} dose (+70% and +80% in FW and DW, respectively), and these differences were statistically different ($P \leq 0.05$) from the correspondent nil fungicide treatment. At 7.5 and 15 mL 50K seeds^{-1} doses, vibrance allows to recover the biomass losses caused by the pathogen infection, whereas in absence of any fungicide treatment, damages by *Rhizoctonia* markedly affected dry and fresh weights (both -27%, infected vs. uninfected controls) (Figure 1).

Root parameters

The seed treatments clearly influenced root growth, with significant effects on some relevant parameters particularly in sterilized pot soil with *Rhizoctonia* inoculum, but, despite this, the biostimulant effect of vibrance on root growth can be appreciated by analyzing only the increase in roots biomass (DW). The presence of the pathogen affected the root weight by 28% (difference between the healthy and the infected untreated plants) but the application of vibrance to the seeds allows to recover this biomass loss, in particular with the 7.5 mL dose that allows the plants to reach the highest dry weight (a significant +54% vs. controls) (Figure 3A). The positive effect of vibrance is also confirmed comparing the biomass values of plants grown on unsterilised soil without any pathogen pressure, even though in this case no significant variations were measured (Figure 4A).

The total root length of maize plants was positively affected by seed-dressing fungicide treatment. Regardless of soil sterilisation, all vibrance doses had a marked enhancement effect, in particular in not inoculated sterilised conditions. The maximum and significant

effect was observed at 7.5 mL Vibrance 50K seeds^{-1} dose in sterilised soil (+60% vs. controls) (Figure 3B) and at 15 mL 50K seeds^{-1} in unsterilised soil (+38%) ($P \leq 0.05$) (Figure 4B). The presence of the *R. solani* inoculum in the sterilized soil was found to be detrimental for root development, the total length of all treatments in fact resulted always smaller compared to the same treated plants grown in absence of pathogen. As expected, untreated seed sown in the infected soil developed plants with the smallest radical apparatus among all treatments (113 m plant^{-1}, -13% vs. untreated plant in *Rhizoctonia*-free condition). For this reason, the effect of vibrance was even more evident under *R. solani* soil infection; in these conditions root growth was greatly improved, reaching a significant enhancement with the 7.5 mL 50K seeds^{-1} vibrance dose (+63 % vs. control) ($P \leq 0.05$) (Figure 3B).

Plant responses in terms of root area reflected the trend of root length. In absence of the *R. solani* pressure, the greatest improvement was found at 7.5 mL 50K seeds^{-1} dose of vibrance in sterilised soil (+45%) and at 15 mL of vibrance in the unsterilised one (+26%), the latter difference being significant compared to correspondent controls ($P \leq 0.05$) (Figure 3C, Figure 4C).

Positively seed-treated plants showed slightly reduced root diameter, regardless of *R. solani* infection presence or absence in the sterilised soil (Figure 3D), but these decreases were not significant ($P > 0.05$). Otherwise, in the unsterilized soil without pathogen inoculum the plants treated with the highest dose of vibrance applied to the seed (15 mL) developed significantly thinner roots than those of control (-8%, $P \leq 0.05$) (Figure 4D).

In addition to the root extension enhancement, seed-applied vibrance also determined structural changes of root system. An evident effect of vibrance was an increase in the number of root tips at 15 mL 50K seeds^{-1} in the unsterilised soil (+28%) and at 15 mL in

sterilised one without *Rhizoctonia* inoculum (Figure 3E, Figure 4E). Indeed, in plants infected with the fungal pathogen the maximum effect was recorded at 7.5 mL of vibrance, allowing these plants to recover the negative effect due to the *R. solani* presence. In particular, the difference at this dose was statistically different from the untreated plants grown in the same biotic stress condition (+56%) (Figure 3E).

Finally, the number of forks within the root system of individual plants was stimulated in all experimental conditions, and increased progressively with the vibrance dosage. The maximum response, in both *Rhizoctonia*-infected and uninfected plants grown in sterilized soil was at 7.5 mL dose level (Figure 3F), with a significant increase compared to the correspondent untreated control only under the biotic stress condition ($P \leq 0.05$).

Discussion

Infestation of the potting soil with the aggressive *Rhizoctonia solani* AG 2-2IIIB anastomosis group negatively affected all shoot and root morphological growth parameters of young maize plants measured in this study. It can be hypothesized that, by performing the inoculation at the same time of sowing, it was possible for the fungus to begin an early infection process on the host plant (Sneh et al., 1991), which produced clearly visible symptoms at the trial end after just 13 DAS. Root image analysis with WinRhizo software, in fact, provided data related to evident injuries caused by *R. solani* mostly on plants without the seed fungicidal protection. In this case (infected control seeds) all root features were significantly reduced by the fungus. Previous studies report similar results confirming the aggressive nature of *R. solani* on roots of maize (Pfähler and Petersen, 2004) and other cereal crops like wheat and barley (Paulitz et al., 2003; Schroeder and Paulitz, 2008). On

the contrary, when vibrance, containing the new active ingredient sedaxane, was added as seed-dressing application, appreciated reductions of pathogen damages in terms of shoot and root growth were observed. The biological activity of sedaxane, that consists in a highly potent inhibition of the fungal succinate dehydrogenase enzyme, was ascertained by Zeun et al. (2012) by using an enzymatic test on purified *R. solani* mitochondria.

Among the different seed application doses compared in this study, a slight plant protection of vibrance against the fungal disease was visible already at the lowest one (2.5 mL 50K seeds^{-1}) but we found the best growth enhancement effect for almost all the parameters considered at both shoot and root level with the intermediate vibrance dosage (7.5 mL 50K seeds^{-1}).

In particular, growth improvements obtained thanks to this application dose became significantly relevant compared to the untreated controls only under the *R. solani* infection pressure, while in absence of inoculum differences among the seed treatments were less important. The 7.5 mL vibrance treatment on *R. solani*-inoculated seeds also greatly enhanced the means of many of the parameters considered, that resulted higher than the untreated, non-inoculated control, and this suggests that the fungicide has a positive effect on plant growth and development in addition to its activity against *R. solani*.

Taken together, these results demonstrate the considerable role that vibrance, and in particular sedaxane, has in ensure a good plant growth in presence of high biotic stress pressure, with results quite comparable to those of normal growing conditions. Also in other studies performed on wheat this active substance showed high levels of protection against *Rhizoctonia* spp. and other fungal pathogens under greenhouse conditions, but also on appreciable yield increase in open field (Zeun et al., 2012).

The substrate sterilization, by reducing or eliminating the potentially influence effects of beneficial soil microorganisms, confirms the positive secondary-effect of sedaxane on plant growth stimulation, highlighting improvements in shoot and root variables not dependent on the pathogen suppression, compared to untreated plants. This additional biostimulant effect of sedaxane has been previously reported for seed treatment of wheat (Barchietto et al., 2012).

Previous studies showed that many fungicide active substances offer an efficient degree of control of diseases caused by *R. solani* on different host plants (Taneja and Grover, 1982; Smiley et al., 1990; Almasudy et al., 2015), but none of them demonstrates an added value in terms of growth enhancement stimulation like that of sedaxane.

Despite previous data indicate that fungicidal seed dressing can tend to depress seed germination (Mercer et al., 1989) and slow the next developmental stages (Scott,1989; Jin et al., 2013), in our trial the germination rate did not differ significantly among treatments in both *R. solani*-free and -infected conditions.

In conclusion, this study demonstrates that an improved control of the root fungal pathogen *Rhizoctonia solani* in maize can be obtained by adding the new fungicide active substance sedaxane to Maxim XL (fludioxonil and melaxil-m), a fungicide currently and effectively used against this and other fungal pathogens. In addition to the excellent fungicidal activity against this increasingly widespread pathogen, vibrance can guarantee seed vigor and strong early growth which are, in maize, fundamental preconditions for obtaining an optimal crop establishment (TeKrony and Egli, 1991), with probably positive effects also on final productivity.

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Figures and Tables

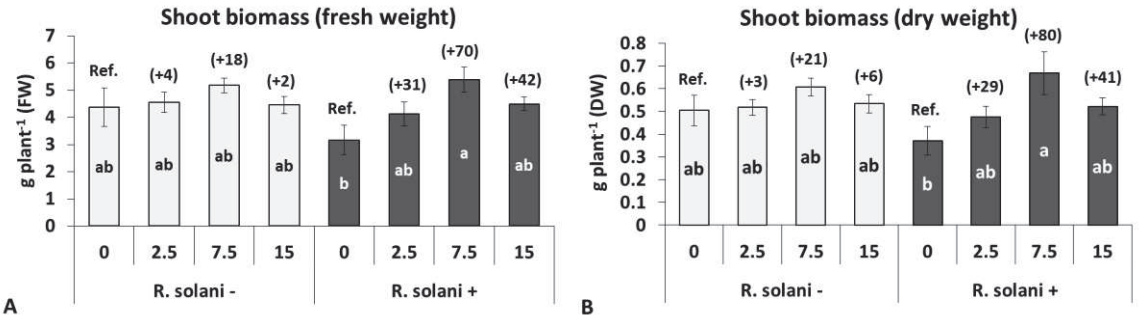

Figure 1. Shoot fresh weight (FW, A) and dry weight (DW, B) (mean ± SE; n = 4) in *Zea mays* at 13 days after sowing (DAS) in sterilised pot soil with (*R. solani* +) or without (*R. solani* -) *R. solani* inoculum and under increasing seed-applied doses of vibrance (0, 2.5, 7.5 and 15 mL 50K seeds^{-1}). Letters indicate significant differences among treatments within the same growing condition (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

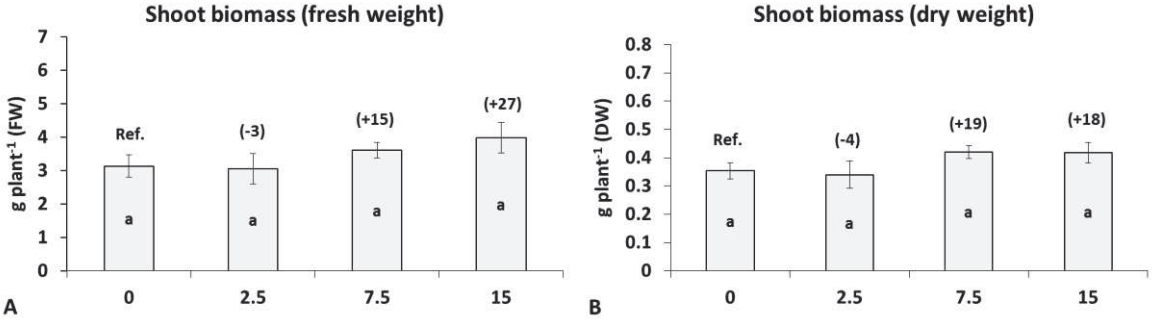

Figure 2. Shoot fresh weight (FW, A) and dry weight (DW, B) (mean ± SE; n = 4) in *Zea mays* at 13 days after sowing (DAS) in unsterilised pot soil under increasing seed-applied doses of vibrance (0, 2.5, 7.5 and 15 mL 50K seeds^{-1}). Letters indicate significant differences among treatments within the same growing condition (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

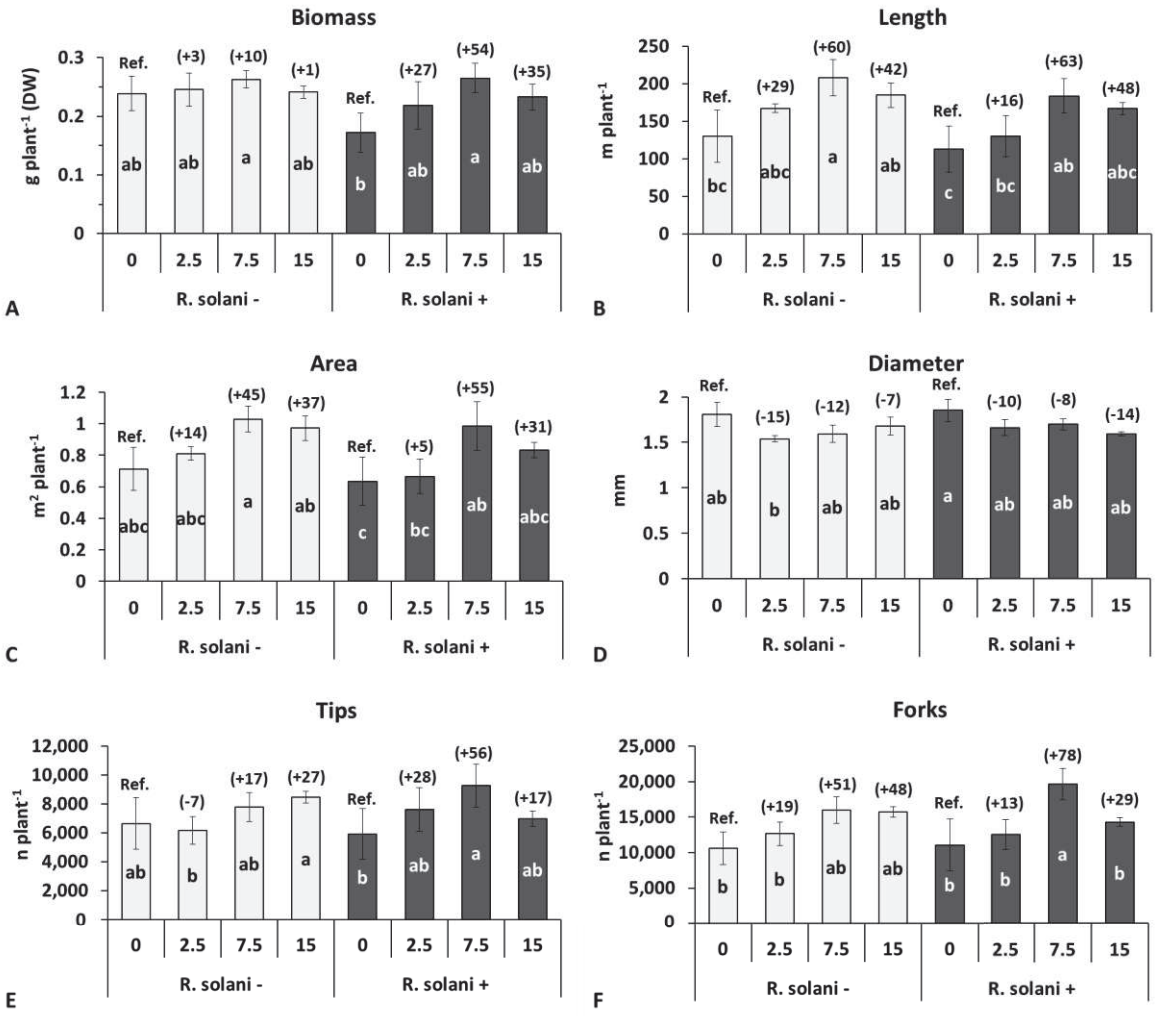

Figure 3. Main root parameters (mean ± SE; n = 4) in *Zea mays* at 13 days after sowing (DAS), in sterilised pot soil with *R. solani* inoculum absence (*R. solani* -) or presence (*R. solani* +), under increasing seed-applied doses of vibrance (0, 2.5, 7.5 and 15 mL 50K seeds^{-1}). Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

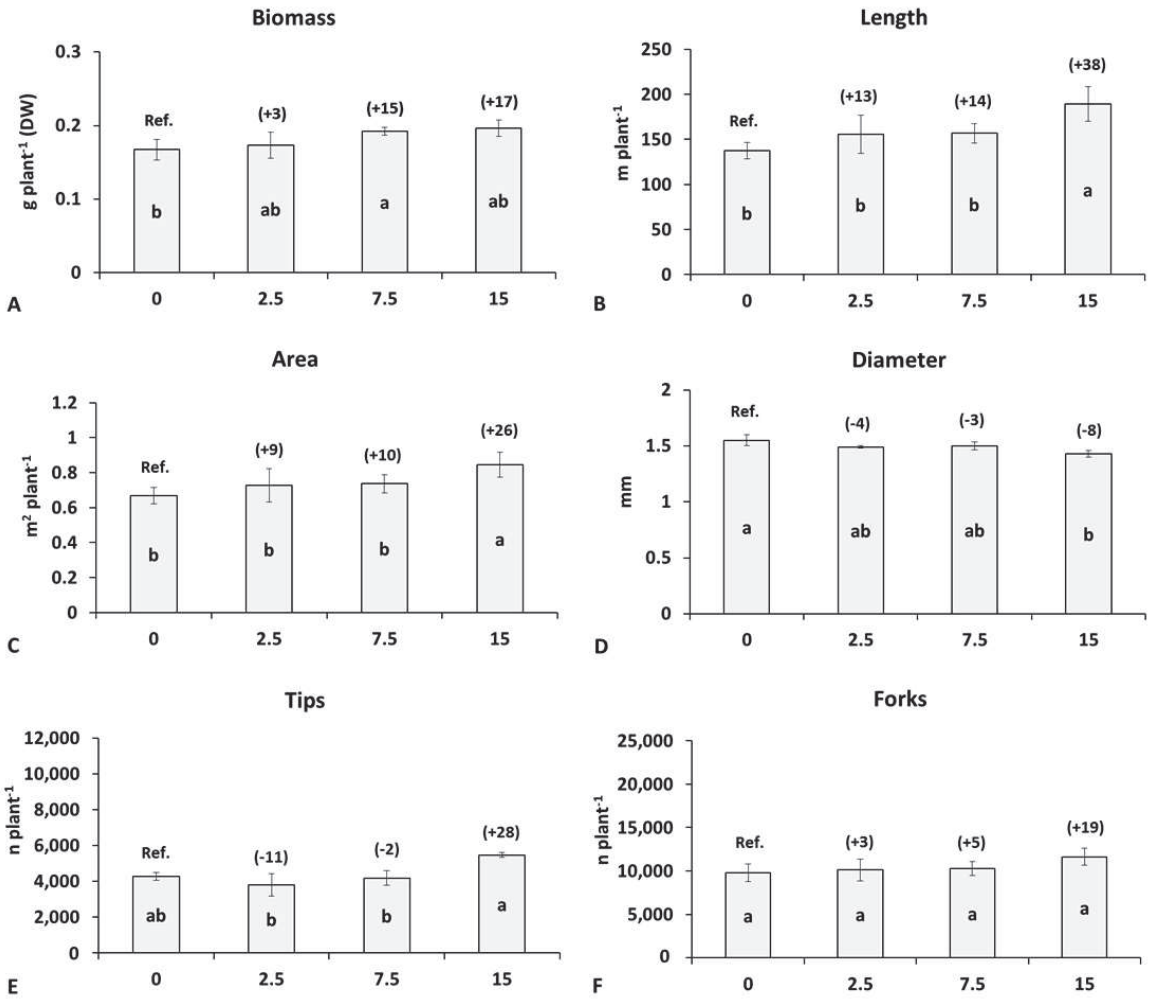

Figure 4. Main root parameters (mean ± SE; n = 4) in *Zea mays* at 13 days after sowing (DAS) in unsterilised pot soil under increasing seed-applied doses of vibrance (0, 2.5, 7.5 and 15 mL 50K seeds^{-1}). Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

Table 1. Germination rate (mean ± SE; n = 4) of *Zea mays* seeds at 7 days after sowing (DAS) in sterilised soil with or without *R. solani* inoculum and treated with increasing seed-applied doses of vibrance. Letters indicate significant differences among treatments within the same germination condition (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

Vibrance dose	**Germination rate**	
(mL 50K seeds^{-1})	***R. solani* -**	***R. solani* +**
0	98.9±1.1^{a}	96.7±1.1^{a}
2.5	98.9±1.1^{a} (=)	96.1±2.8^{a} (-1)
7.5	95.6±0.0^{a} (-3)	98.3±1.7^{a} (+2)
15	97.2±1.7^{a} (-2)	99.4±0.6^{a} (+3)

Table 2. SPAD (mean ± SE; n = 4) of *Zea mays* in sterilised with or without *R. solani* inoculum and unsterilised pot soil under increasing seed-applied doses of vibrance. Values are a mean of three observations at 7, 10 and 12 days after sowing (DAS). Letters indicate significant differences among treatments within the same growing condition (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

	SPAD		
Vibrance dose	**Sterilised soil**		**Unsterilised soil**
(mL 50K seeds^{-1})	***R. solani* -**	***R. solani* +**	***R. solani* -**
0	34.6±0.78^{a}	34.4±0.58^{a}	30.4±0.73^{a}
2.5	34.1±0.23^{a} (-1)	35.2±0.73^{a} (+2)	28.4±1.21^{a} (-7)
7.5	34.7±0.93^{a} (+1)	35.1±0.95^{a} (+2)	29.3±0.63^{a} (-4)
15	34.3±0.84^{a} (+1)	36.8±0.46^{a} (+7)	30.8±1.06^{a} (+1)

CHAPTER V

The new SDHI fungicide sedaxane improves root growth and tolerance to nutrient and water deficiency in early growth stages of maize

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Abstract

During the last few years, besides plant protection, an increasing interest has been addressed to stimulation of shoot and root systems by seed-coating fungicides. The aim of this work was to evaluate the biostimulating effects of the new fungicidal a.i. sedaxane, applied in addition to the widespread commercial product Maxim® XL (fludioxonil + metalaxil-m; Syngenta Crop Protection) as seed coating to maize grown under abiotic stress conditions in the early stages. In the first experiment, plants cultivated in pots were subjected to different soil nutrient availability performed trough three different levels of pre sowing fertilization rates while, in the second one, to two water availability conditions, one at optimal water supply and the other at a progressive water stress (down to wilting point). Maxim XL and Maxim XL + 2.5 mL Vibrance (containing 500 g L^{-1} sedaxane) 50K $seeds^{-1}$ were compared with the untreated controls, highlighting positive responses of seed-treated plants under both adverse growing conditions.

Results showed increased growth of shoot, with higher SPAD values, nutrient uptake and antioxidant activity (phenolic acids synthesys), as well as a greatly enhanced root growth than controls. Particularly in water stress conditions, these parameters did not show marked differences from controls, but plants from treated seeds with fungicide were able to maintain a higher rate of transpiration to lower value of transpirable water in the soil. Those treatments allow maize to increase its resilience to fluctuating soil water and nutrients availability which may occur during early growth stages, thus protecting yield potential of this crop.

Keywords: biostimulant; root growth; seed coating; abiotic stress; succinate dehydrogenase inhibitor (SDHI).

Introduction

Due to climate change, together with the drastic increasing in world population, there is more and more a considerably attention focused on the global need of arable land which, on the contrary, is beginning to shrink dramatically (Barrow et al., 2008). Expansion of crop cultivation on marginal, arid and less fertile areas and predicted increase in drought and high temperatures-related stresses are expected to reduce the productivity of many crops (Grover et al., 2010; Larson, 2013). Furthermore without considering adverse lands, negative factors like extreme temperatures, poor nutrient and water availability are beginning to be serious problems also in vocated areas (Yang et al., 2009).

Maize (*Zea mays* L.) is one of the most widespread crops in the world used as food, forage, and the raw material source for industry, which is currently playing a crucial role in global food security (FAO, 2017). This crop is very sensitive to environmental constraints and it can greatly reduce its grain yield particularly under drought and nutrient limitations (Mueller et al., 2012; Rossini et al., 2016). Therefore it is easy to realize how roots are the most important plant organs in this regard, absorbing water and nutrients from the soil and translocating them to top parts (Sainju et al., 2005; Stone et al., 2001).

Historically the approach to mitigate the negative impact of abiotic stress on plant growth and crop yield has been the selection of tolerant cultivars (Eisenstein, 2013) but nowadays there is an increasing focusing on agronomic practices and sustainable treatments that can help to achieve this goal (Olesen and Bindi, 2002).

Seed coating is the practice of covering seeds of most crops with external materials to improve handling, protection, and, to a lesser extent, germination enhancement and plant establishment (Sharma et al., 2015). Several previous studies have reported satisfactory effects of seed coating on seed germination, seedling emergence, root and shoot growth, leaf area, dry biomass and increase in yield (Zelonka et al., 2005; Gevrek et al., 2012; Tavares et al., 2013). Also several seed-applied fungicides, strobilurins in particular, have been found to have positive side-effects on plant physiology (Köhle et al., 2002; Berdugo et al., 2012). Agrochemical companies are actually interested in developing new fungicide molecules, more similar to bio-stimulating substances, able to protect and, at the same time, stimulate plant growth and development (Gomes Bezerra et al., 2015).

The fungicidal active ingredient Sedaxane® (Syngenta Crop Protection, Basel, Switzerland) has recently been released for use as a treatment for local and systemic protection of cereal seeds, seedlings and roots against pathogenic fungi, both seed-borne and soil-born (Zeun et al., 2013). When sedaxane moves from the seed to the soil and into the plant tissues, it has been found to improve the development of the roots and lower stems of cereals (Swart, 2011). Previous research has described wheat responding positively to sedaxane in terms of greater biomass, better shoot and root growth (Barchietto et al., 2012; Dal Cortivo et al., 2017) and drought resistance (Ajigboye et al., 2016).

Within this framework, this work compared the widely used commercial fungicide Maxim® XL (fludioxonil + metalaxyl-m), which is nowadays the most widespread seed-coating product for corn for maize, in combination or not with the new a.i. sedaxane (added as Vibrance, containing 500 g a.i. L^{-1}) in relation to the control (treated with any fungicide), with the aim to evaluate their effects of root growth enhancement and improvement of plant tolerance to two different abiotic stress conditions. In the first trial seed-treated plants

were grown under decreasing soil fertility while, in the second one, under two opposite soil water regimes (optimal availability vs. severe drought stress).

Materials and methods

Experimental set-up

All the trials were performed in 2015 and 2016 under a greenhouse-tunnel at the experimental farm "Lucio Toniolo" of the University of Padova (Legnaro, Italy), maintaining the climatic parameters (air temperature and relative humidity) stable across different experiments. Plants were grown in pots and rhizoboxes filled with a silty-loam soil collected from a field in the experimental farm and mixed, after air drying and grinding, with fine sand to obtain a 1:1 (w/w) mixture. Both experiments followed a completely randomized experimental design with 4 replicates of each treatment, resulting from a factorial combination of seed treatments and growing stress conditions. The seeds of corn hybrid SY HYDRO (Syngenta, Basel, Switzerland) were provided already coated with Maxim XL and Maxim XL + 2.5 mL Vibrance 50,000 seeds^{-1} (commercial flowable concentrate containing 500 g sedaxane L^{-1}), both compared with any seed treatment fungicide application (untreated control).

Nutrient stress trial

This experiment aimed at studying the effect of these seed applied fungicides on plant growth at three decreasing levels of soil fertility obtained by different rates of fertilization

supply, a full rate corresponding to 300 kg K_2O ha^{-1}, 150 kg P_2O_5 ha^{-1} and 100 kg N ha^{-1}; a halved rate and an unfertilized control.

Five seeds were sown at 3 cm of depth in large pots (21.2 L, 30cm large and 30cm high) filled with 8.5 kg of field soil and sand mixture, leaving only one plant after emergence. Pots were regularly watered at 3-day intervals with 300 mL of deionized water per pot up to trial end 30 days after sowing (DAS).

Shoot and root analysis

Leaf chlorophyll content was measured six times, at 12, 15, 19, 22, 25 and 28 DAS, using the a SPAD (Soil Plant Analysis Development) 502 chlorophyll meter (Konica-Minolta, Hong Kong). At 30 DAS, the root system was gently washed in order to separated soil particles and keep the apparatus intact and avoid as much as possible root breakings. The root system was cut at crown level, weighed immediately (for fresh weight) and after drying (105 °C, 24 hours, for dry weight).

Shoot biomass was also recorded at plant harvest. On dry shoot tissues, the total content of phenolic acids (i.e., *t*-Ferulic, *p*-Coumaric, vanillic and caffeic acids) was revealed through HPLC (High Performance Liquid Chromatography) after extraction with methanol, according to the method described by Adom et al. (2003).

Shoot nitrogen concentration was revealed through the Kjeldahl method, whereas P and K through Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (SPECTRO CirOS Vision EOP, SPECTRO Analytical Instruments GmbH & Co. KG, Kleve, Germany).

At root level, a thorough characterization of root parameters, i.e., length, area and diameter were measured by automatic analysis with the KS300 software (Carl Zeiss Vision GmbH,

München, Germany) of 1-bit 400 DPI TIFF root images acquired through a flatbed scanner (Epson Expression 11000XL, Epson, Suwa, Japan), according to the method of Vamerali et al. (2003a). In view of the high root biomass collected at harvest, only 2 replicates were thoroughly characterized for the above parameters, whereas fresh and dry root biomass were detected in all the replicates.

Water stress trial

This experiment, that aimed at evaluating the rooting power effect of these seed-applied treatments under different soil water availability conditions, was performed in two steps. The first was carried out in rhizoboxes comparing two water supply regimes (optimal and moderate deficiency) in the early growth stages of maize (until 3-4 leaves) while, in the second, maize cultivated in large pots was subjected to a progressive water stress, starting from the 4-leaf stage down to wilting point.

Plant observations in rhizobox

This trial was made up for obtain a preliminary characterization of all specific root parameters.

Rhizobox, 45 cm high, 30 cm wide and 2.5 cm thick containers with transparent plexiglass sides, were filled with 3.8 kg of soil/sand substrate and positioned at a 45° angle during plant growth to allow roots to be observed. Four seeds were sown at 3 cm of depth in each rhizobox, leaving only two plants after emergence. We considered 2 rhizoboxes for each treatment with 2 plants each. An amount of 150 mL rhizobox^{-1} of water was regularly applied at 3-day interval under the optimal water supply, while in the moderate water stress conditions rhizoboxes were irrigated only once after sowing.

Root deepening was measured three times, at 10, 13 and 15 DAS, using a millimetric ruler applied at the lower transparent wall of rhizoboxes. Before plant harvesting, the leaf chlorophyll content was measured as SPAD units and, at the end of the experiment (20 DAS), shoots were cut at the crown level and weighed immediately to obtain the fresh weight (FW) and after drying (105 °C, 24 hours), for obtaining the dry weight (DW). The root system was gently washed to remove soil particles keeping it intact and afterwards stored at 4°C in a 15% v/v ethanol solution until later processing. Root images were acquired as describe for the previous trial but this time analyzed with WinRhizo software (Regent Instruments Inc., Ville de Québec, QC, Canada) to obtain the main root parameters, i.e., length, surface area, diameter, and number of tips.

Plant observations in pots

In this experiment were used pots with a volume of 7.5 L (22 cm large and 20 cm high) filled with 5.8 kg of mixture substrate. Three seeds of maize per rhizobox were sown 3 cm deeply but only one plant was leaved after the emergence. Up to the 3 leaf-stage (16 DAS) all pots were 2 days-interval regularly irrigated with 150 mL of deionized water. At 17 DAS, the soil in all pots was fully saturated with 1.5 L of water, allowing the excess to be percolated in the following 24 hours. After complete percolation the substrate reached the field capacity and pots, at this point were carefully sealed using plastic dishes and mastic to avoid any water loss by evaporation. Maize culms leaked out from a hole made in center of each dish. From 17 DAS up to 45 DAS (trial end at 7-leaf stage), all pots were daily weighed to determine water daily transpiration. For plants subjected to unstressed water regime (optimal supply), this amount of water was restored daily through a tube connected directly to the pot soil, while for plants under progressive water stress, no water was

supplied to the soil from 21 DAS until the end of experiment. When daily transpiration in stressed plant reached minimum values (about 10 g per day, or lower) and were visibly suffering (dried and rolled leaves) appeared, the experiment ended. Then the root system was gently washed in order to separate all soil particles. The root system was cut at crown level, weighed immediately (fresh root weight) and after drying (105 °C, 24 hours, dry root weight). Aboveground biomass was also measured immediately at plant harvest (fresh shoot weight) and after drying (dry shoot weight) as done for roots. Also leaf chlorophyll content (SPAD values) was measured, at the beginning (17 DAS) and at the end (45 DAS) of the transpiration monitoring. Moreover, in laboratory, it was measured the concentration of free phenolic acids in shoots as described for the fertilization experiment.

Based on good correlation between daily transpiration of plants irrigated with the optimal water supply and the average transpiration of the first 4 days in all the days of experiment, it was possible to estimate the potential daily transpiration of stressed plant based on the average transpiration of their first 4 days. Then by dividing the actual transpiration of each plant with the corresponding potential transpiration plant by plant it was possible to estimate the relative transpiration (RT%) (Bona et al., 2000). Regression curves of the relative transpiration (RT) were determined as a function of the fraction of transpirable soil water (FTSW) (Ray and Sinclair, 1998). The linear plateau model used has the following equation: "$y = a + b \times (x - c) \times (x \leq c)$", where a, b and c are empiric coefficients indicating, respectively, the intercept on the y axis (a), the linear coefficient (b) and the intersection between the linear response and the declining transpiration trend (c), in accordance with what reported by Vamerali et al., 2003*b*.

Statistical analysis

Statistical analysis was carried out by the Statgraphics Centurion XI software (Adalta, Arezzo, Italy). ANOVA and the Student Newman-Keuls test ($P \leq 0.05$) were used to evaluate differences among means for all analyzed parameters.

Regardless of the rhizobox experiment, it was carried out in addition a factorial discriminant analysis (MDA, Multigroup Discriminant Analysis with Wilks' lambda and Pillai's trace tests) and a Principal Component Analysis (PCA) to facilitate interpretation and understand correlations between the various root parameters analyzed in both water conditions. Before analysis, data were standardized by subtracting the mean and dividing by the standard deviation within each variable. All analyses were performed with MS Excel XLSTAT (Addinsoft, Paris, France).

As only two replicates were processed for root parameters (length, area and diameter) in the nutrient stress trial, statistical analysis was not performed on these data.

Results

Nutrient stress trial

Although little different responses among various shoot and root parameters analyzed, plant growth was, as expected, significantly affected by the fertilization rate supplied in the pot medium before sowing ($P \leq 0.05$). To confirm this, for example, the leaf chlorophyll content markedly decreased as N supply was reduced. SPAD values of fungicide-treated plants were all slightly higher (by 2 or 3%) than untreated controls, although values were not statistically significant ($P > 0.05$), at any fertilization level (data not shown). Both fresh and dry shoot biomass showed a trend similar to that of SPAD, with generally improved values with Maxim XL but particularly when 2.5 mL of Vibrance 50K seeds^{-1} were added,

under high and null fertilization rates (+18% and +32% DW, respectively). Only Maxim XL had a slight (not statistically significant) negative impact on shoot biomass under lack of any fertilization (Figure 1).

From Kjeldahl analysis we could reveal N concentration in shoot tissues and from ICP that of P and K. Total element uptake was then calculated by multiplying tissue concentration by shoot biomass. For supplied nutrients (N, P and K) tissue concentration tended to increase with the fertilization rate, except nitrogen, which showed minimum values at intermediate fertilization rate. Although the effects of fungicides was variable in terms of element concentration, they generally increased the amount of nutrients accumulated by the plants thanks to shoot growth improvements, with generally better results with Maxim XL + Vibrance 2.5 mL, even if not statistically significant ($P > 0.05$), with the highest fertilization level but particularly under the worst nutrient supply condition. In this latter situation, Maxim XL-treated plants shown small decreases in N, P and K shoot accumulations (Figure 2).

The biosynthesis of antioxidant compounds (phenolic acids) in the shoot tissues was related to the seed-coating treatment. At both maximum and minimum fertilization rates, plants treated with Maxim XL + Vibrance 2.5 mL significantly increased the concentration of phenolic acids compared with controls (+36% and +53%, respectively) ($P \leq 0.05$). Among all detected compounds, caffeic acid, which is recognized to be a natural auxin, was the most abundant and reached the highest level with Maxim XL, both added with Vibrance or not (Figure 3).

Figure 4 highlights the results of seed-dressing fungicides about their effects on root growth. Clearly, Maxim XL shows a growth enhancement effect compared with untreated controls, although maximum rooting was observed with the addition of 2.5 mL of

Vibrance, especially in the absence of any fertilizer supply. As regards the effects of fertilization factor, under high and medium nutrient supply, plants generally expanded more their root system than under null fertilization.

The total root length of corn plants was positively affected by fungicide treatments, particularly in conditions of medium to low soil fertility. Maxim XL showed the maximum effect on root length at medium fertilization rate (+64% vs. controls), whereas the addition of Vibrance was appreciable in conditions of low fertility (+65%) (Figure 4B). At the maximum fertilization rate, treated plants developed a total length similar of that of untreated ones.

Root area reflected the trend in root length: again the greatest enhancement was shown by Maxim XL under medium-fertilization level (+61%) and also by Vibrance adding in unfertilized soil (+56%) (Figure 4C).

Plants derived from treated seeds also showed a positively reduced root diameter only when cultivated without any fertilizer supply (Figure 4D).

The root enhancement effect of seed applied fungicides was also evident in terms of dry biomass of the root system, at any fertilization regime. The maximum increases were observed at moderate fertilization rate for both fungicides (Figure 4A), although the benefit by the addition of Vibrance was appreciable again under the null fertilization.

Water stress trial

As regards the study related to this abiotic stress, some positive effects by seed-applied fungicides on plants grown were already observed on the preliminary-set rhizobox experiment.

Only the Maxim XL + Vibrance 2.5 treatment tendency improved both shoot fresh and dry weights while the Maxim XL alone generally decreased these parameters. A significant difference from untreated control was detected only for the first one in the water deficiency condition ($P \leq 0.05$) (Table 1). SPAD values resulted almost unchanged between different treatments and water soil content conditions (data not shown). Also belowground biomass was slightly, and never significantly, greater in plants where Maxim XL + Vibrance was applied ($P > 0.05$).

Positive feedbacks, on the contrary, were highlighted analyzing in details others root growth features. From graphs in Figure 5 is possible to appreciate the biostimulant effect of seed-applied Maxim XL + Vibrance treatment moreover when limiting of the water availability occurred. The total root length was, in fact, significantly improved (+63% vs. controls), ($P \leq 0.05$) and consequently also the root surface area showed a considerable increase, despite not statistically appreciable (+ 21% vs. controls; $P > 0.05$). Not only the root expansion was enhanced by seed-coating but also its structure changed, as the number of root terminations (tips) resulted significantly higher than that of the untreated plants (+41%, $P \leq 0.05$). Treated plants with both fungicides also developed thinner roots, the average diameter was reduced by -21% and -26% compared to controls (Maxim XL and Maxim XL + Vibrance, respectively) ($P \leq 0.05$).

When irrigation has been regularly carried out until the end of the experiment some positive effects due to treatments application were observed, but not as marked as before ($P > 0.05$).

The principal component analysis conducted on data obtained from irrigated rhizoboxes identified two dummy variables which explain the 89.61% (F1) and 10.39% (F2) of the total variability. Relevant variables (loadings > |0.5|) were assigned to the F1 variable:

length, number of tips, fresh and dry weight. The loading value of the surface area was very closed to the threshold (|0.483|). By graphs, it was possible to establish good correlations among variables, in particular the Maxim XL + Vibrance 2.5 treatment increases of root length, area, number of tips, fresh and dry weights and decreases the root diameter (Figure 6).

About rhizoboxes subjected to water stress condition, the two dummy variables developed by the PCA represent the 92.86% and the 7.14% of data variability (F1 and F2, respectively). The relevant variables (loadings > |0.5|) were assigned to the F1 variable: length, surface area, diameter, number of tips and fresh weight. The correlations between the various parameters resulted similar to the previous condition, as the diameter changes indirectly proportionally to others variables, particularly comparing the untreated control to the Maxim XL + Vibrance 2.5 treatment (Figure 7).

In the pot experiment a severe water stress was reached by plants (wilting point), but also in this case seed treatments promoted plant growth and tolerance against this abiotic condition. At pots closure (17 DAS), before monitoring the dynamics of leaf transpiration, plants treated with Maxim XL showed SPAD values equal to controls while those with Maxim XL + Vibrance 2.5 had a significant higher leaf chlorophyll content (+ 6% vs. control, $P \leq 0.05$) (Figure 8A). At end trial (45 DAS), plants which have been irrigated showed decreased values from 17 DAS but again the Maxim XL + Vibrance 2.5 treatment obtained the best result (+14% vs. untreated controls, $P > 0.05$). Under drought, instead, differences among untreated and treated plants were higher and particularly, adding Vibrance to Maxim XL, allowed to increase by 34% the SPAD mean value ($P \leq 0.05$) (Figure 8B).

Maxim XL + Vibrance 2.5 treatment has showed a relevant and significant increase of both fresh and dry shoot weights in the optimal water supply condition ($P \leq 0.05$), but also Maxim XL alone positively increases aboveground biomass (Table 2). Under water deficiency, on the contrary, seed treatments were not able to recover part of the shoot biomass decreasing due to the water lacking, showing even small reductions compared to relative controls. Root dry weight resulted statistically the same between control and treated plants ($P > 0.05$) but seed-applied fungicides slightly improved root biomass under optimal water supply and slightly decreased it in the opposite condition (Table 2).

Interesting are the results obtained from analysis of phenolic acids in shoot tissues. Figure 9 shows how seed-applied treatments led to an increase of these compounds, which perform many positive functions in plant physiology, particularly in water stress conditions. Under progressive water stress, as expected, these antioxidant compounds resulted more concentrated compared with optimal water supply. The treatment Maxim XL + Vibrance 2.5 had the maximum effect in both conditions of optimal water supply and water stress (+47% and +54% vs. controls, respectively; $P \leq 0.05$) by increasing particularly the syringic acid (Figure 9).

Under optimal water supply, all seed coating treatments led to higher total transpiration compared to controls (Figure 10A). Treatments made by fungicides increased, although not significantly, the amount of transpired water by 26% and 24% with Maxim XL and Maxim XL + Vibrance 2.5, respectively, reaching values up to ~1,700 g per plant over the 27 days. Under progressive water stress, no differences were found among treatments in terms of total transpiration, as the whole available water was taken up by the plants. However, the dynamics of leaf transpiration over the fraction of transpirable soil water (FTSW) differed among treatments. Seed treatments allowed to delay transpiration decline, meaning that

both seed-applied fungicides improve drought tolerance through some physiological mechanisms, perhaps involving stomata closure. Plants treated with Maxim XL and Maxim XL + Vibrance 2.5 undergone the decrease in transpiration (RT) at 28% and 30% of available water respectively, compared to 38% of untreated controls (Figure 10B).

Discussion

Maxim XL alone and Maxim XL added with Vibrance, applied as seed treatment. induced significant and evident increases both of morphological parameters and tolerance in stressed plants. In fact it was very interesting to understand if Vibrance, containing sedaxane, was able to contribute overcome adverse situations that may be very impactful at the beginning of maize cycle.

The improved root and shoot growth under nutrient deficiency and drought stress conditions in response to the sedaxane seed application confirm the biostimulant effect of this active ingredient. This beneficial role of sedaxane in early stages of wheat plants in absence of negative factors affecting their growth was reported by Barchietto et al. (2012), while Ajigboye et al. (2016) showed the same effects as response to drought tolerance.

Regarding the experiment with different nutritional conditions , plants treated with seed-applied fungicides generally had a lower concentration of nutrients in the shoot tissues in comparison with those untreated at the same fertilization level. This is due to the greater increase in the aerial biomass that, related to the unchanged N, P and K availability in the growing medium, led to a decreasing of those nutrients concentration in shoots. Despite this, multiplying the lower concentration with the greatest aboveground biomass developed, treated plants have reached the best uptake levels of nutrients.

The effects of a severe progressive water stress between four- and seven-leaf stages were evaluated by calculating a non-linear function, obtained by regressing values of relative transpiration over the fraction of transpirable soil water (FTSW). This function was used to represent the response of plants to decreased water availability, which appreciable differed in the maize treated and not-treated with seed-applied fungicides. Plants treated with Maxim XL and Maxim XL + Vibrance were able to maintain a high rate of transpiration to a low FTSW value (28 and 30%, respectively) while untreated plants started to show the decreasing of maximum transpiration level already with a 10% more of FTSW. The FTSW thresholds at which RT began to decline are included into those reported in literature for maize hybrids (30-40%) (Ray et al., 2002) but in this case, a difference of about 10% only induced by a fungicide seed-application, is very appreciable.

High shoot biomass values indicate that plants treated with fungicides have more turgid cells, with higher water content. For this reason, in progressive water stress conditions, plants can maintain a high rate of transpiration and delay stomata closure up to low values of soil water availability.

Sedaxane can therefore promote drought tolerance through some physiological mechanism, perhaps involving abscisic acid, which usually induces stomatal closure (Valerio et al., 2017). The reduce content of ABA may has been induced by high concentrations of phenolic acids, in particular by vanillic and *p*-Coumaric acid (Rai et al., 1986; Laloraya et al., 1986), especially in water stress conditions as seen in our experiments here reported. These results are interesting because these phenolic compounds perform many positive functions, including being precursor of lignin, constituent that make cell walls highly resistance against the attack of pathogens and insects, and giving to plant a mechanical support (Lattanzio et al., 2006).

Ajigboye et al. (2016) recently reported that sedaxane affects the expression of genes involved in defense, chlorophyll synthesis and cell wall modification determining an increased efficiency of PSII photochemistry, a reduced non-photochemical quenching and an improved biomass in plants grown under drought stress.

All the analyzed root parameters (total length, surface area, diameter, number of tips and dry weight) showed an increase and improvement of the root system between the seed-treatments.

Maize treated with Maxim XL + Vibrance, in particular, have fine roots with small diameter, high length and surface area values. An increase of these parameters together with a high number of tips are an essential assumption to ensure a better soil exploration capacity, thus improving water and nutrients uptake (Garnett et al., 2009; Buczko et al., 2009).

Plant recognizes Sedaxane like a hormonal-substance that promote root growth and development as generally the application of only Maxim XL had similar or slightly higher results of those of untreated control, as already reported in wheat by Turkington (2016); evidently this fungicide product provided a good protection against pathogens, but it didn't affect and improved plant physiological processes.

The results indicate that the application of sedaxane is associated with a positive rooting power effect and with a greater abundance of antioxidant compounds in shoots, conferring a tolerant response of maize plants grown under abiotic stress conditions. In conclusion, this new SDHI fungicide, applied as seed-coating, ensures an effective strategy to face nutrient limitations and drought, which are increasingly frequently injurious conditions, improving a better plant wellbeing during early growth stages.

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Figures and Tables

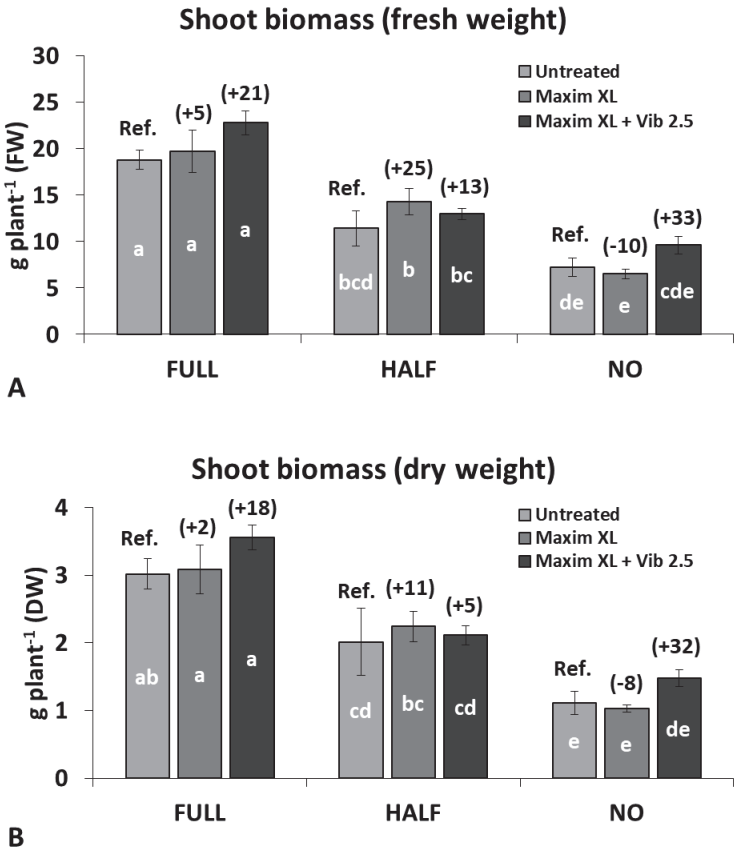


Figure 1. Shoot fresh weight (FW, A) and dry weight (DW, B) (mean ± se; n = 4) in *Zea mays* at 30 days after sowing (DAS) in pots with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) at decresing pre-sowing fertilisation rates (full, half and unfertilised). Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. correspondent untreated control.

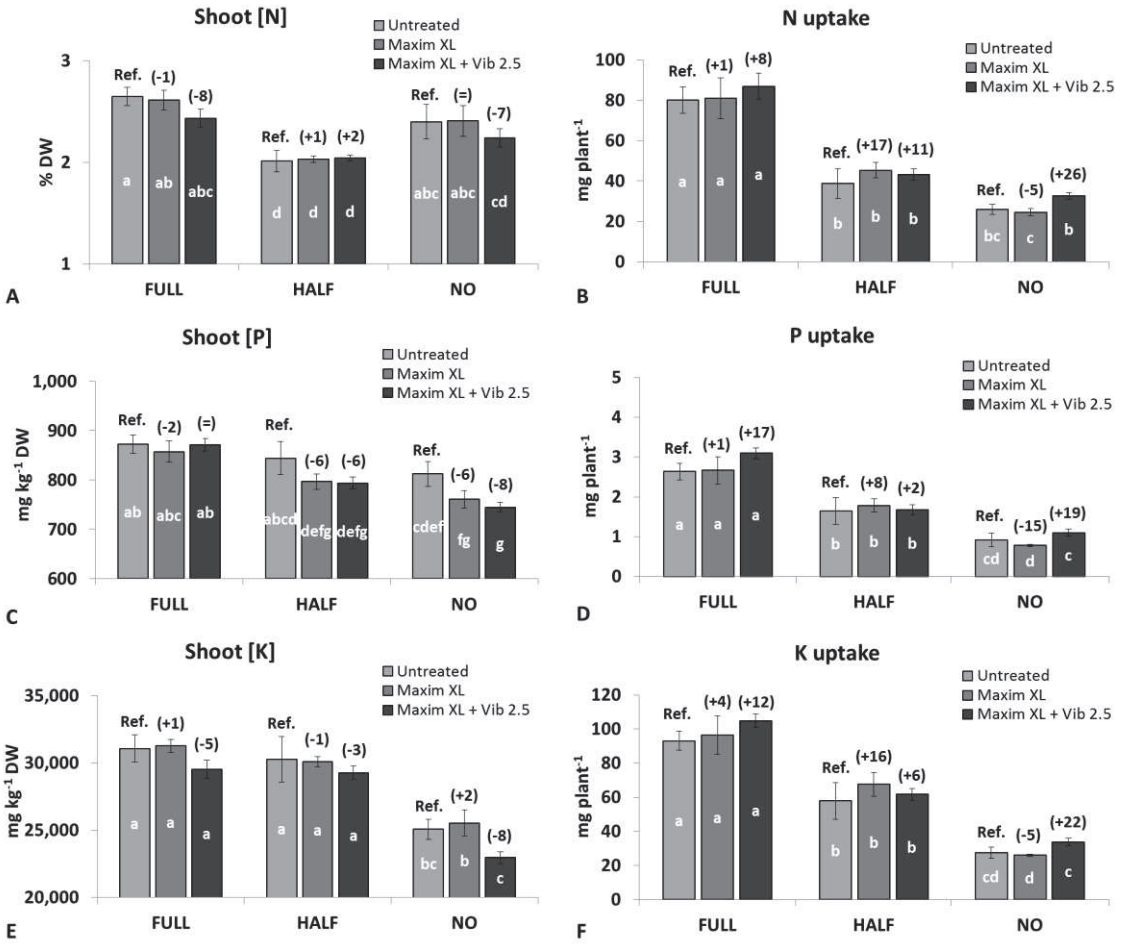


Figure 2. Shoot N, P and K concentrations (A, C, E) and uptake (B, D, F) (mean ± se; n = 4) in *Zea mays* at 30 days after sowing (DAS) in pots with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) at decresing pre-sowing fertilisation rates (full, half and unfertilised). Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. correspondent untreated control.

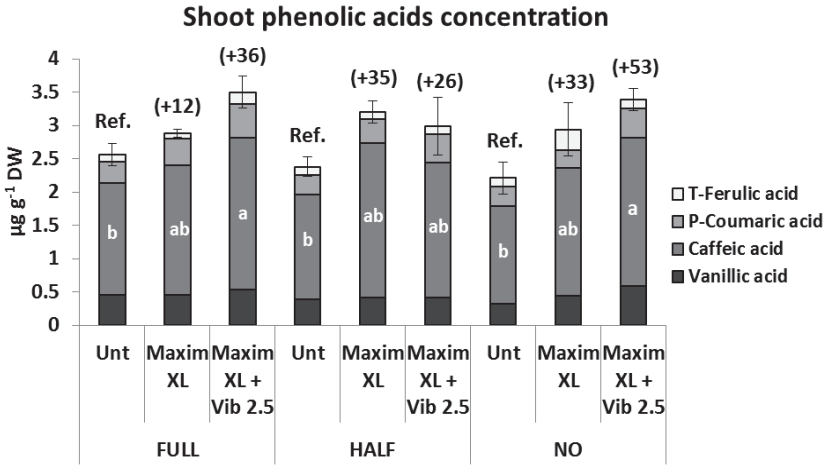


Figure 3. Phenolic acids concentration (mean ± SE; n = 4) in *Zea mays* shoots at 30 days after sowing (DAS) in pots with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) at decresing pre-sowing fertilisation rates (full, half and unfertilised). Letters indicate significant differences among treatments (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % total amounts variation *vs*. correspondent untreated control.

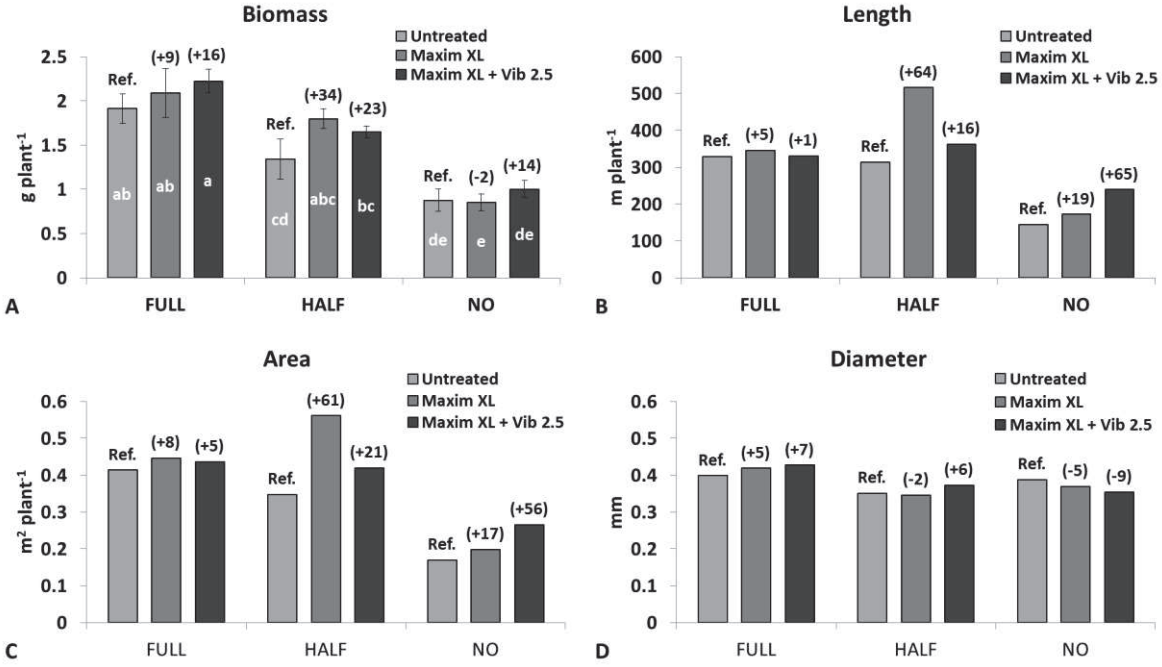


Figure 4. Main root parameters (mean ± se; n = 4) in *Zea mays* at 30 days after sowing (DAS) in pots with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) at decresing pre-sowing fertilisation rates (full, half and unfertilised). Letters for biomass values (mean ± se; n = 4) (A) indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$), for other parameters no statistical analysis was performed (mean; n = 2). In brackets: % variation *vs*. correspondent untreated control.

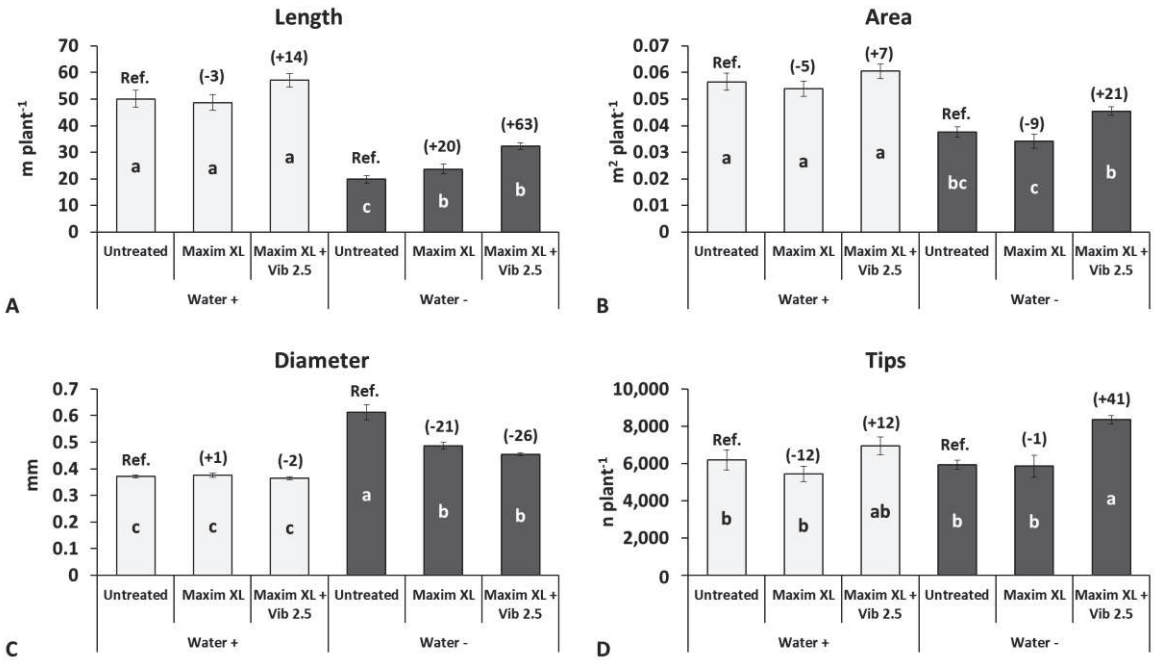
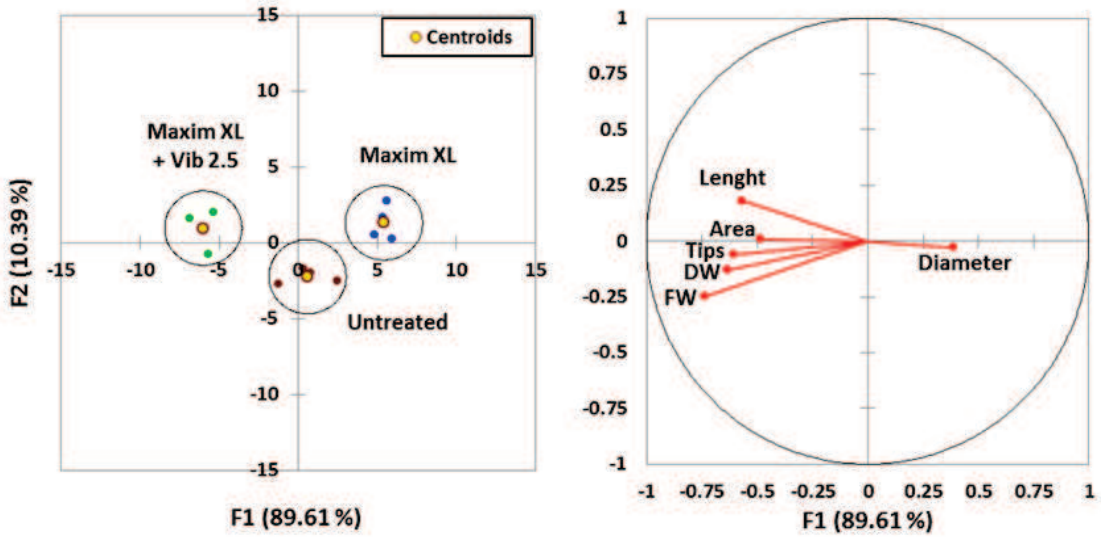
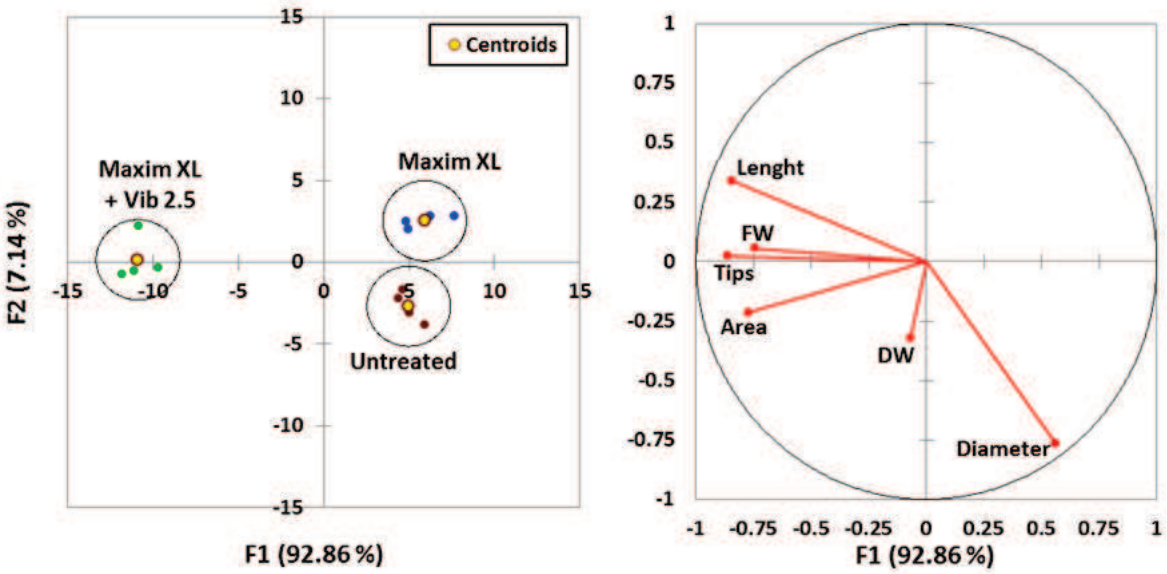


Figure 5. Main root parameters (mean ± se; n = 4) in *Zea mays* at 20 days after sowing (DAS) in rhizobox with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) in two water regimes (irrigated and not irrigated after sowing). Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. correspondent untreated control.



	F1	F2
Lenght	**-0.567**	0.181
Area	-0.483	0.010
Diameter	0.388	-0.028
Tips	**-0.605**	-0.059
Fresh weight (FW)	**-0.738**	-0.249
Dry weight (DW)	**-0.636**	-0.128

Figure 6. Principal component analysis (PCA; top right) with variable loadings (highlighted values > |0.5|; bottom table) and discriminant analysis (DA; top left) for different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) in maize cultivated in rhizobox with periodic irrigation from sowing to 20 DAS.



	F1	F2
Lenght	**-0.845**	0.339
Area	**-0.773**	-0.215
Diameter	**0.562**	**-0.765**
Tips	**-0.865**	0.023
Fresh weight (FW)	**-0.743**	0.055
Dry weight (DW)	-0.069	-0.324

Figure 7. Principal component analysis (PCA; top right) with variable loadings (highlighted values > |0.5|; bottom table) and discriminant analysis (DA; top left) for different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) in maize cultivated in rhizobox without irrigation from sowing to 20 DAS.

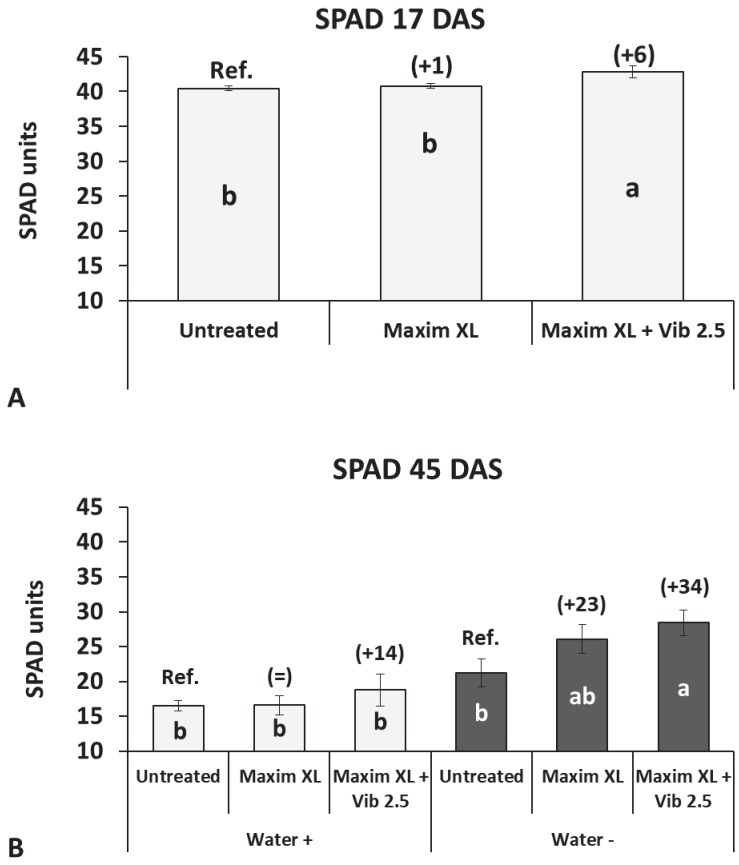


Figure 8. SPAD values (mean ± se; n = 4) in *Zea mays* with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}), at 17 days after sowing (DAS) (A), and at 45 DAS in pots under optimal water supply (Water +) or progressive water stress (Water -) (B). Letters indicate significant differences among treatments within the same time (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. correspondent untreated control.

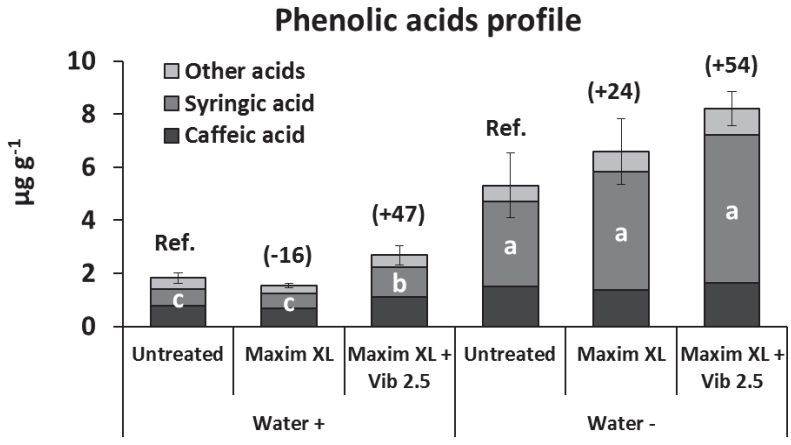


Figure 9. Phenolic acids concentration (mean ± SE; n = 4) in *Zea mays* shoots at 45 days after sowing (DAS) in pots with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) under optimal water supply (Water +) or progressive water stress (Water -) (B). Letters indicate significant differences among treatments (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. correspondent untreated control.

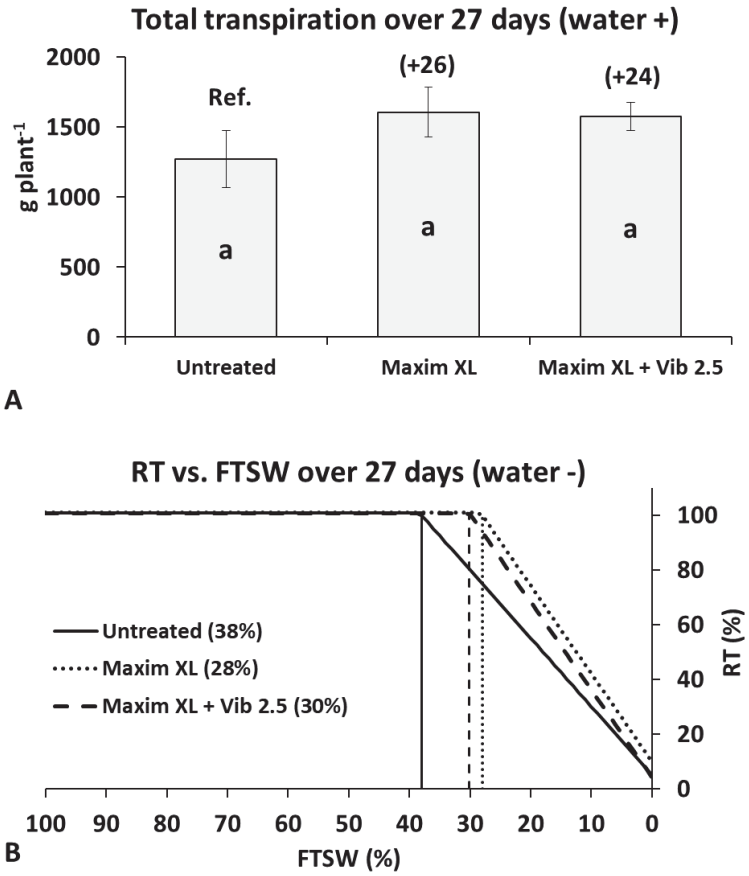


Figure 10. Total transpiration (mean ± SE; n = 4) over 27 days, from 17 days after sowing (DAS) to 45 DAS, under optimal water supply (Water +) (A) and linear plateau regressions of relative transpiration (RT) plotted vs. fraction of transpirable soil water (FTSW) under progressive water stress (Water -) (B), of *Zea mays* cultivated in pots with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}). In graph A letters indicate significant differences among treatments (Student-Newman-Keuls test, $P \leq 0.05$) and inside brackets numbers indicate the % variation *vs*. untreated control. In graph B coefficients of regression curves are: a = 100.76, b = 2.50 and R^2 0.98 for untreated plants; a = 100.99, b = 3.22 and R^2 0.93 for Maxim XL and a = 100.61, b = 3.20 and R^2 0.97 for Maxim XL + Vibrance 2.5.

Table 1. Shoot biomass (fresh weight, FW, dry weight, DW) and root biomass (dry weight, DW) (mean ± se; n = 4) of *Zea mays,* at 20 days after sowing (DAS), cultivated in rhizobox with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) in two water regimes (irrigated and not irrigated after sowing). Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. correspondent untreated control.

Water regime	Treatment	Shoot biomass Fresh weight (FW, g plant^{-1})		Shoot biomass Dry weight (DW, g plant^{-1})		Root biomass Dry weight (DW, g plant^{-1})	
Water +	Untreated	3.16 ±0.07 ^{a}		0.34 ±0.01 ^{ab}		0.23 ±0.01 ^{a}	
	Maxim XL	2.76 ±0.02 ^{b}	(-13)	0.30 ±0.01 ^{abc}	(-11)	0.22 ±0.01 ^{a}	(-4)
	Maxim XL + Vib 2.5	3.36 ±0.11 ^{a}	(+6)	0.37 ±0.02 ^{a}	(+8)	0.25 ±0.01 ^{a}	(+8)
Water -	Untreated	1.47 ±0.23 ^{d}		0.27 ±0.06 ^{bcd}		0.23 ±0.02 ^{a}	
	Maxim XL	1.48 ±0.05 ^{d}	(+1)	0.21 ±0.01 ^{d}	(-24)	0.22 ±0.03 ^{a}	(-2)
	Maxim XL + Vib 2.5	1.91 ±0.02 ^{c}	(+30)	0.25 ±0.01 ^{cd}	(-8)	0.27 ±0.01 ^{a}	(+16)

Table 2. Shoot biomass (fresh weight, FW, dry weight, DW) and root biomass (dry weight, DW) (mean ± se; n = 4) of *Zea mays,* at 45 days after sowing (DAS), cultivated in pots with different seed-dressing fungicide treatments (Untreated, Maxim XL and Maxim XL + Vibrance 2.5 mL 50K seeds^{-1}) under optimal water supply (Water +) or progressive water stress (Water -). Letters indicate significant differences among treatments within the same parameter (Student-Newman-Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. correspondent untreated control.

Water regime	Treatment	Shoot biomass Fresh weight (FW, g plant^{-1})		Shoot biomass Dry weight (DW, g plant^{-1})		Root biomass Dry weight (DW, g plant^{-1})	
Optimal supply	Untreated	16.17 ±2.84 ^{b}		3.01 ±0.52 ^{bc}		1.87 ±0.37 ^{a}	
	Maxim XL	19.38 ±2.48 ^{ab}	(+20)	3.68 ±0.46 ^{ab}	(+22)	2.44 ±0.33 ^{a}	(+31)
	Maxim XL + Vib 2.5	21.52 ±1.36 ^{a}	(+33)	3.99 ±0.18 ^{a}	(+32)	2.40 ±0.23 ^{a}	(+28)
Progressive water stress	Untreated	9.24 ±0.34 ^{c}		3.02 ±0.02 ^{bc}		2.57 ±0.07 ^{a}	
	Maxim XL	8.77 ±0.33 ^{c}	(-5)	2.70 ±0.13 ^{c}	(-11)	2.22 ±0.20 ^{a}	(-13)
	Maxim XL + Vib 2.5	8.89 ±0.31 ^{c}	(-4)	2.72 ±0.18 ^{c}	(-10)	2.23 ±0.15 ^{a}	(-13)

OVERALL CONCLUSIONS AND PERSPECTIVES FOR FUTURE RESEARCH

Integrating genetics, agronomic practices and new sustainable technologies is the priority solution to face modern agriculture challenges. Worldwide, more and more research is pointing to the optimization of root systems as the key for future crop productivity improvements.

By acting on the root zone directly, biofertilizers applications and new seed treatments such as those studied during my Ph.D. work have a great potentiality to ensure a strong and healthy plant growth minimizing, in the meantime, the environmental impact.

The conclusion drawn is that the use of a combination of PGPR and N-fixing bacteria offers an opportunity to improve root growth in wheat and increase plant resilience to environmental stresses, and helps to reduce N losses from agricultural ecosystems thereby offering partial fertilizer savings. Regarding appreciable agronomic parameters, medium-high N fertilization rates are still required to achieve high yield and quality standards in wheat cultivation. In this context, the use of mixed mycorrhizae-PGPR bacteria biofertilizers reveals to be a sustainable mean for minimizing the adverse effects N supply on root expansion.

Several beneficial mutualistic microorganisms have been discovered; however, their dependable utilization as biofertilizers in field conditions is still to be wholly harnessed. Like in the experiments reported in this thesis, for certain aspects, the results obtained in laboratory were not reproduced in the field. Therefore, such aspects need further research so that most effective strains or combinations of strains can be selected. As well, further work is needed to improve the understanding of the most effective types and application

methods of inoculants for specific crops, soils and management systems. Small-scale trials in order to test the suitability of commercial inocula previous to large- scale application, since the combination of several factors, including soil native microbial status, host-plant preference, and environmental conditions, can influence the success of inoculated PGPR and AMF in fully expressing their potential benefits in diffent environmental conditions.

Further studies are also need to clarify their effect on mineral fertilizer usage efficiency in cereal production and the possibility to replace post-emergence inoculation with seed application of microorganisms to reduce the application costs should be considered.

About fungicide seed treatments containing the new a.i. sedaxane, our studies demonstrate their evident effects in improving maize shoot and root growth thanks to physiological changes induced to plants. These benefits were most clearly documented with the presence of fungal pathogen pressure or moderate to high levels of abiotic stresses.

It is concluded that he biostimulant activity of sedaxane is an additional benefit, over and above its protective role against seed- and soil-borne diseases, which could be exploited in the cultivation of maize. Although further studies are needed to understand whether these improvements also influence final growth and yield, our preliminary results suggest that currently roots may be enhanced with benefits in overcoming biotic and abiotic stresses in early growth stages.

Sedaxane seed application, therefore, can represent a central role in sustainable maize production if additional research work confirms its effects in improving the crop performance, up to the influence on the final yield, under real cultivation conditions. Thanks to these treatments there is probably also wide scope for cultivation incentives in marginal soils as cereals can benefit by improved root exploration, thereby facilitating adaptation to adverse soil and climatic conditions, regardless of the species or variety.

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CURRICULUM VITAE

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I was born in Cittadella (Padova) on 5 October 1990.

In December 2012 I got the BSc degree in Agricultural Sciences and Technologies and in October 2014 the MSc degree in Agricultural Sciences and Technologies, both at the University of Padova.

In November 2014 I started the 3-years PhD course on Crop Science at the Department of Agronomy, Food, Natural Resources, Animals and the Environment of the University of Padova.

In the third year of the course I was visiting student for about 6 months (January 2017 - July 2017) at the Department of Field Crops, Forage and Grassland, Faculty of Agriculture of the University of Zagreb where I conducted research on sustainable agronomic practices for optimum wheat bread-making quality, under the supervision of Prof. Zlalko Svečnjak, PhD and Dario Jareš, PhD.

LIST OF PUBLICATIONS

Articles in scientific journals:

1. Visioli G., Bonas U., Dal Cortivo C., Pasini G., Marmiroli N., Mosca G., Vamerali T., 2018. Variations in yield and gluten proteins in durum wheat varieties under late-season foliar vs. soil application of nitrogen fertilizer in a northern Mediterranean environment. *Journal of the Science of Food and Agriculture*. In press. doi: 10.1002/jsfa.8727.
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