UNIVERSITÀ DI PARMA
ARCHIVIO DELLA RICERCA

University of Parma Research Repository

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

Publisher copyright

note finali coverpage

(Article begins on next page)

05 July 2024

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

Cristian Dal Cortivo[a], Giuseppe Barion[a], Giovanna Visioli[b], Monica Mattarozzi[b], Giuliano Mosca[a], Teofilo Vamerali[a],[*]

[a] Department of Agronomy, Food, Natural Resources, Animals and the Environment, University of Padua, Viale dell'Università 16, 35020 Legnaro, Padua, Italy
[b] Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy

ARTICLE INFO

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 bio-fertiliser 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.

1. 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 bio-fertilisers (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 phytormones (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 biofertiliser 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 Fendrik, 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.

2. Materials and methods

2.1. 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$_2$O). 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.

2.2. 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 h 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 biofertiliser 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).

2.3. 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 bio-fertiliser 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:

$$\text{NDVI} = \frac{\text{ref}_{\text{NIR}} - \text{ref}_{\text{RED}}}{\text{ref}_{\text{NIR}} + \text{ref}_{\text{RED}}}$$

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).

2.4. 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).

3. Results

3.1. 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 (Fig. 1 B, D), as well as abundant colonisation of inner root tissues (Fig. 1F, H), whereas no bacteria were detected in non-inoculated control plants (Fig. 1A, C, E, G). The leaves of treated plants were also appreciably colonised (Fig. 2 B), with the bacteria penetrating the intercellular spaces of the epidermis and crowding, in particular, around the stomata complexes (Fig. 2 D). There was also considerable internal mesophyll colonisation (Fig. 2H). 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.

3.2. 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) (Fig. 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).

3.3. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4).

3.4. 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.
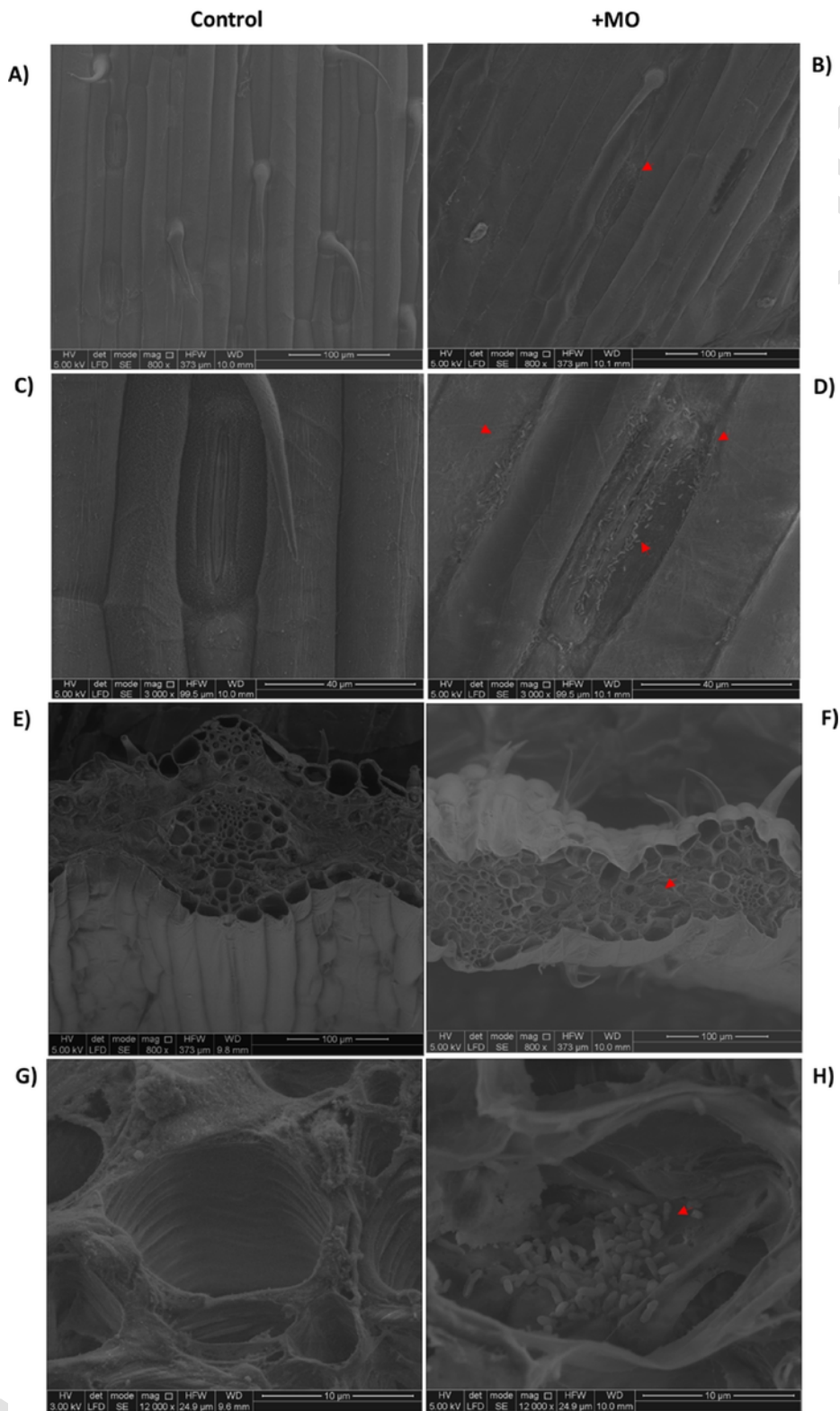
Control

+MO
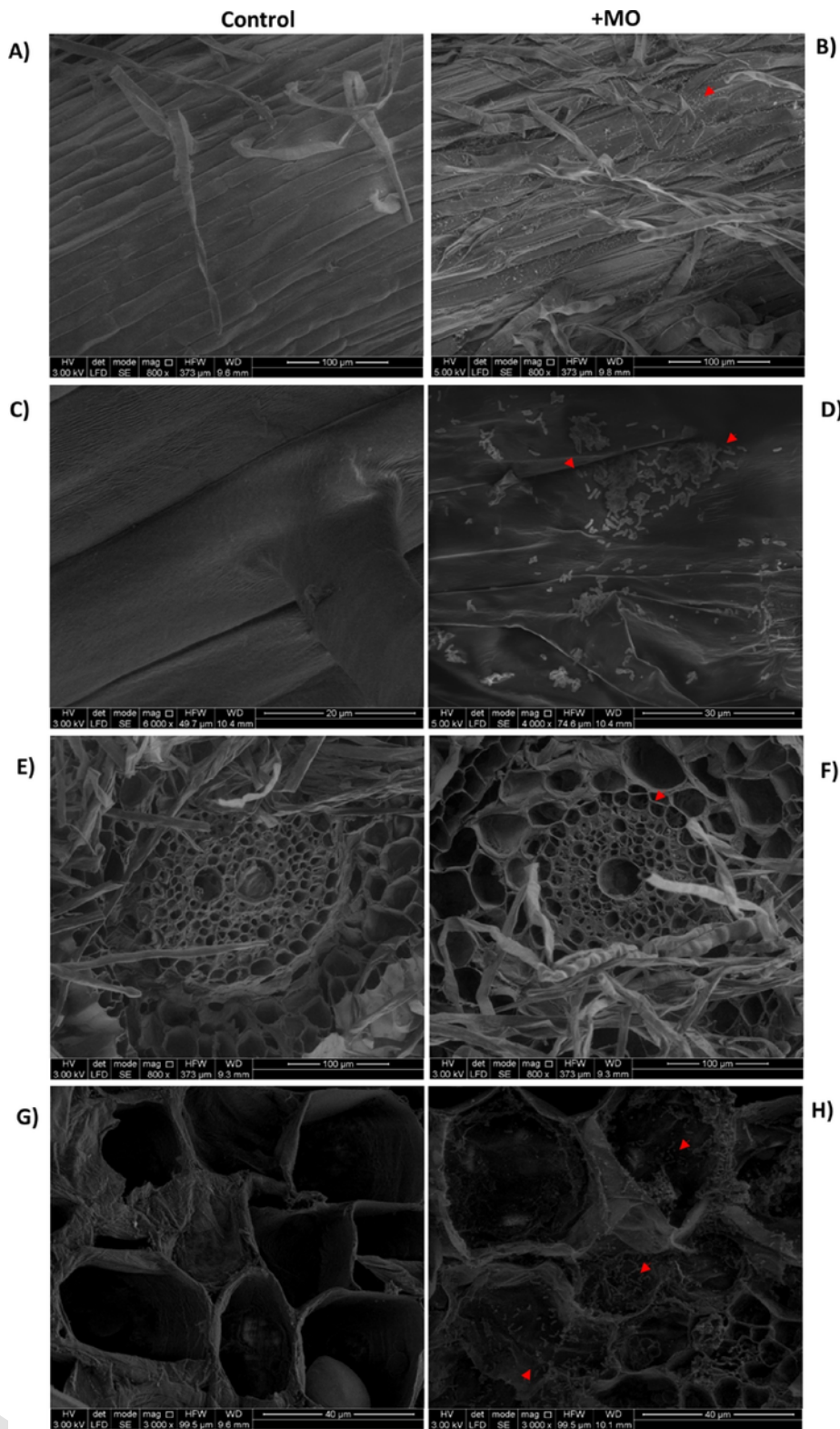
Fig. 1. ESEM micrographs of root surfaces (A, B, C, D) and transversal sections (E, F, G, H) in control (non-inoculated, left) and inoculated (+MO, right) 7-day-old wheat seedlings. Note abundant bacteria colonisation on right side only. Red arrows indicate the points of the root tissues magnified to observe bacterial colonisation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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

Fig. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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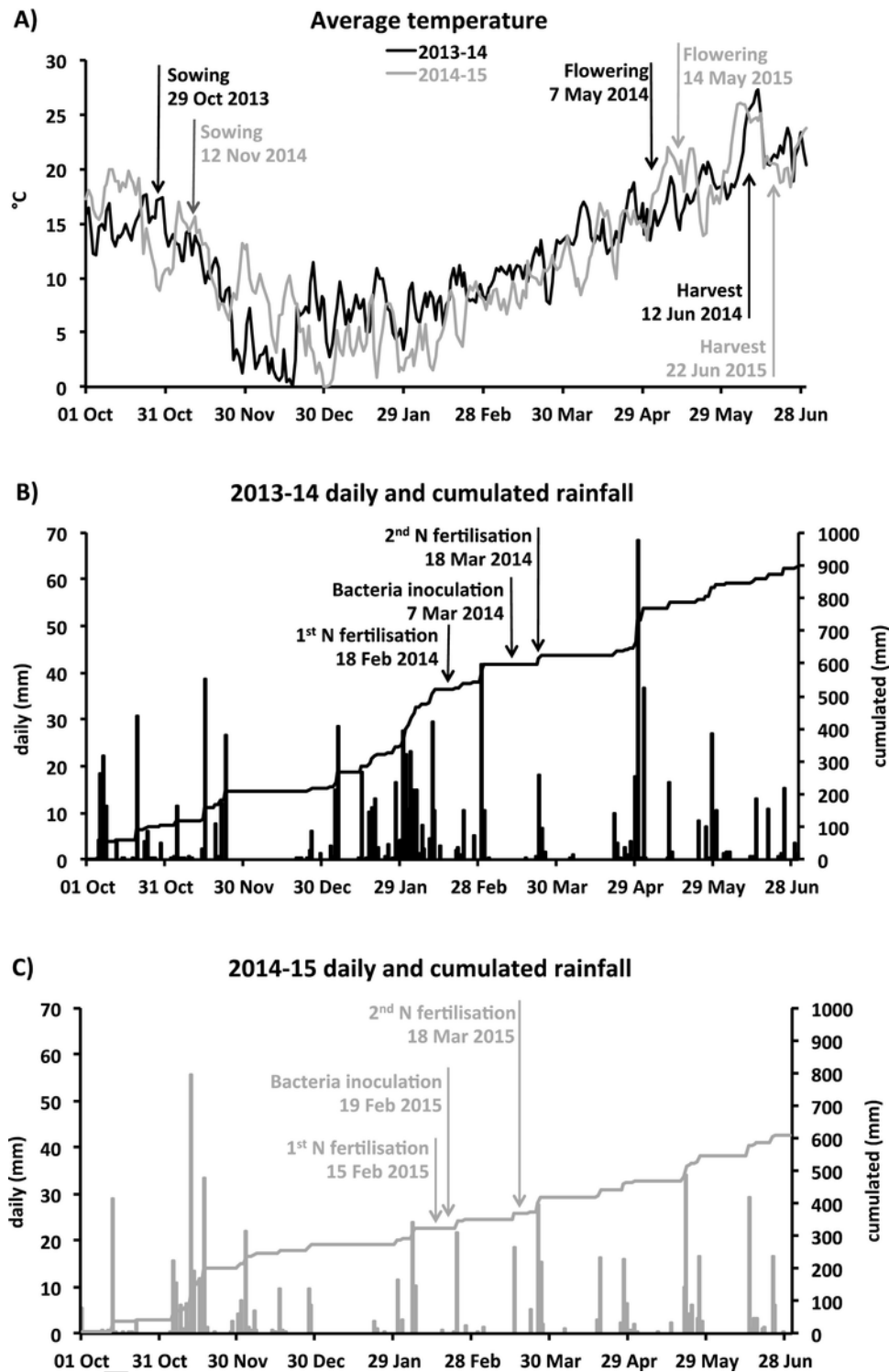
Fig. 3. Dynamics of seasonal daily mean temperatures (A), and daily and cumulated rainfall across the wheat crop cycle in the first-year (B) and second-year trials (C) at the Legnaro experimental site (Padua, Italy).

5.6 t ha^{-1}, +14%), and the role of N fertilisation in driving productivity (Table 3).

3.5. 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%) (Fig. 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

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.

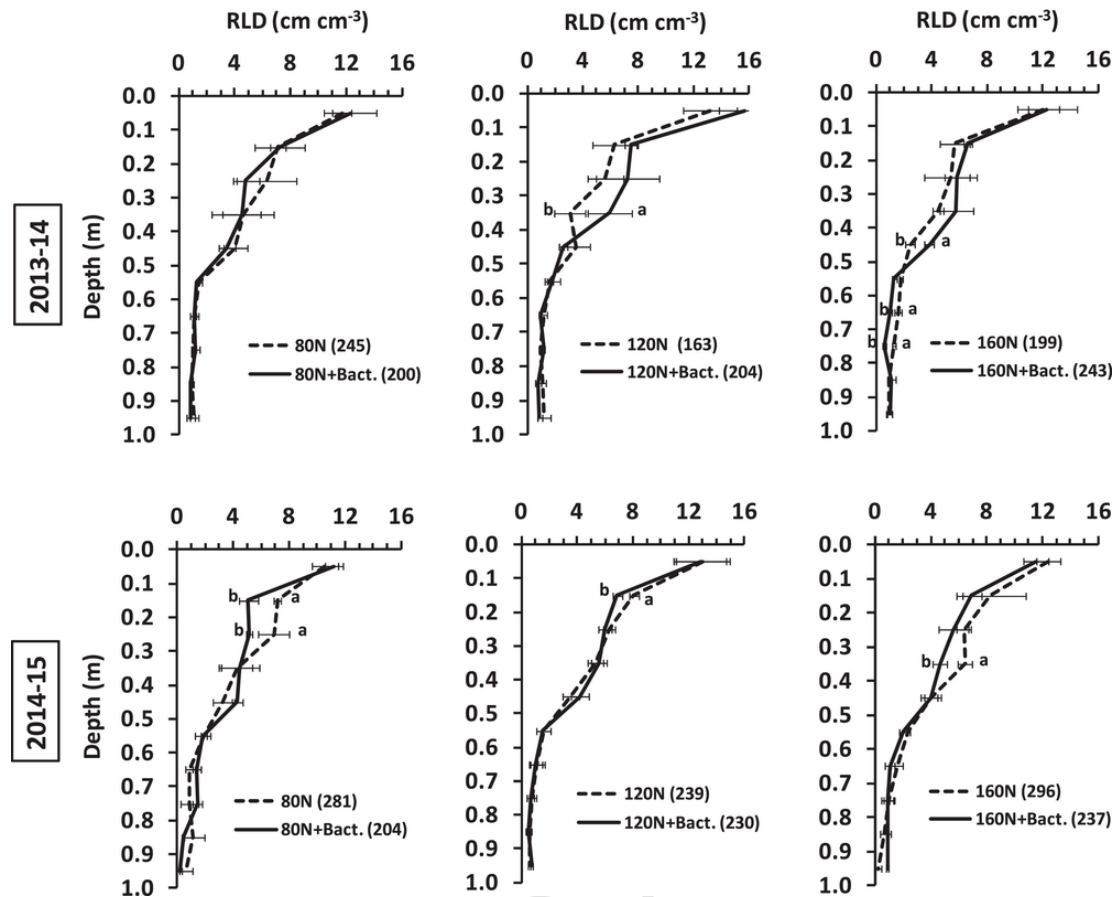| Treatment	Length		Surface area		Diameter		Root tips		Ramification index	
	(m plant^{-1})		(m^2 plant^{-1})		(μm)		(no. plant^{-1})		(no. forks m^{-1})	
Untreated	35.6 ± 4.64	a	0.37 ± 0.05	a	344 ± 19.4	b	7559 ± 1178	b	555 ± 33	b
Seed application	29.7 ± 4.87	a (−17)	0.41 ± 0.07	a (+10)	443 ± 15.1	a (+29)	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)

Fig. 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.

position and cluster overlap shown in Fig. 7 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.

4. 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. Bio-fertilisers 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 eas-

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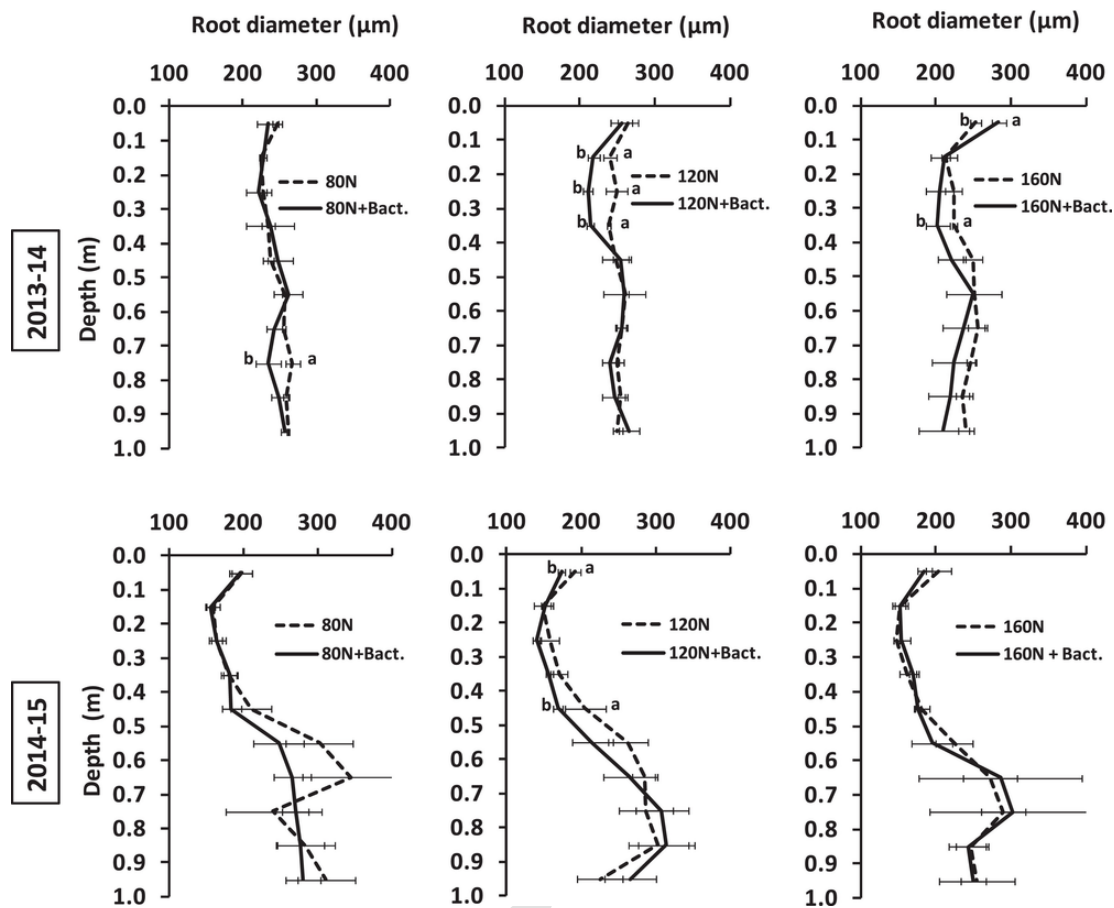
Fig. 5. Root diameter (mean ± se; n = 3) patterns in bacteria-inoculated *Triticum aestivum* L. plants (continuous line) *vs.* non-inoculated controls (dashed line) with decreasing N fertilisation levels (160, 120 and 80 kg ha^{-1}) at the flowering stage in a two-year field trial. Letters: statistically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

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

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
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
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 (−)
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
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 (−)
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
160 N + Bact.	0.94 ± 0.06 a (−10)	0.56 ± 0.02 a (+24)	2.30 ± 0.05 ab	2.03 ± 0.06 a (−4)	48.5 ± 5.74 b (−18)	67.5 ± 0.77 a (−6)	6353 ± 36.3 a (−)
Fertilisation	ns	ns	**	**	ns	ns	ns
Bact. application	ns	ns	ns	ns	ns	ns	ns
Fert × Bact. 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.
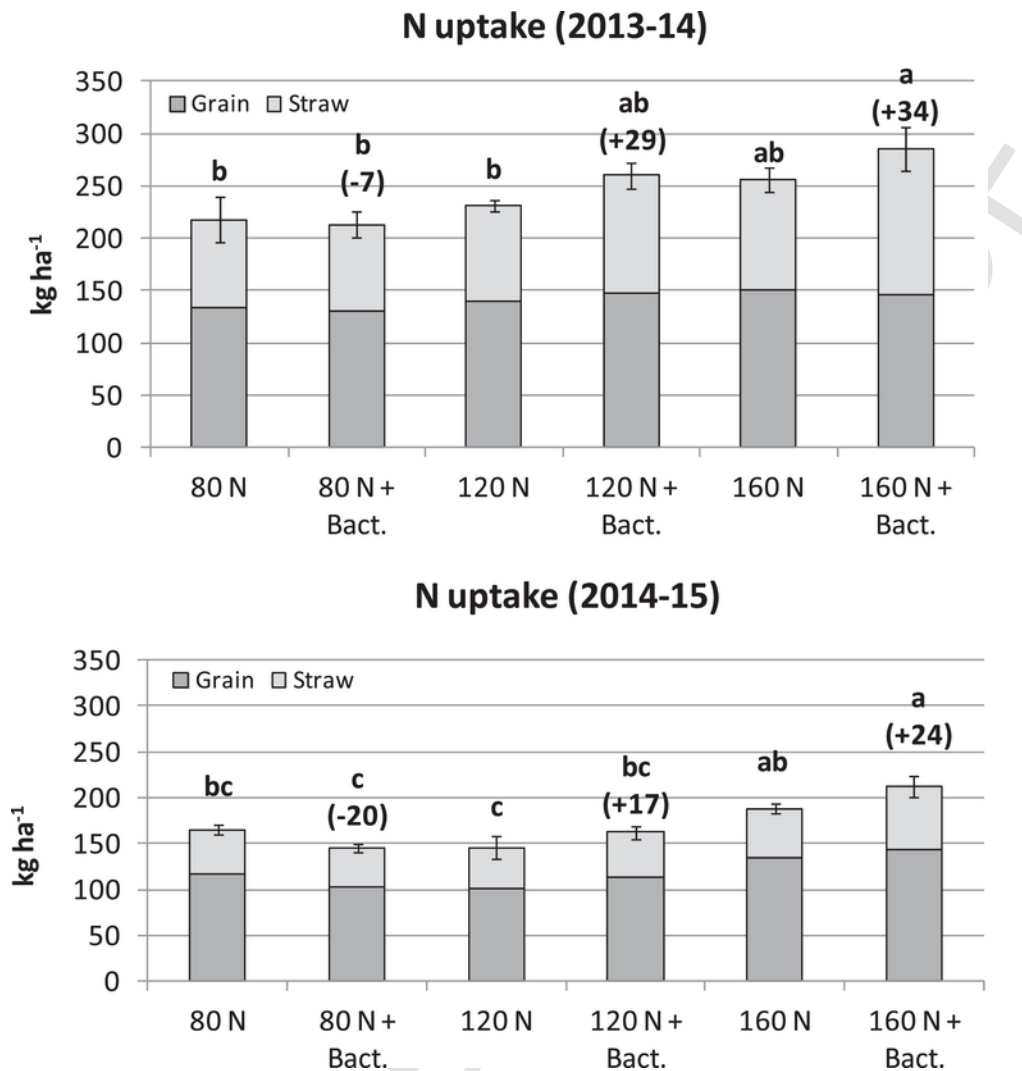
Fig. 6. Overall (grain + straw) nitrogen uptake on a per hectare basis by bacteria-inoculated *Triticum aestivum* L. plants *vs.* non-inoculated controls with decreasing N fertilisation levels (160, 120 and 80 kg ha^{-1}) at plant harvest in a two-year field trial. Letters: statistically significant 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.

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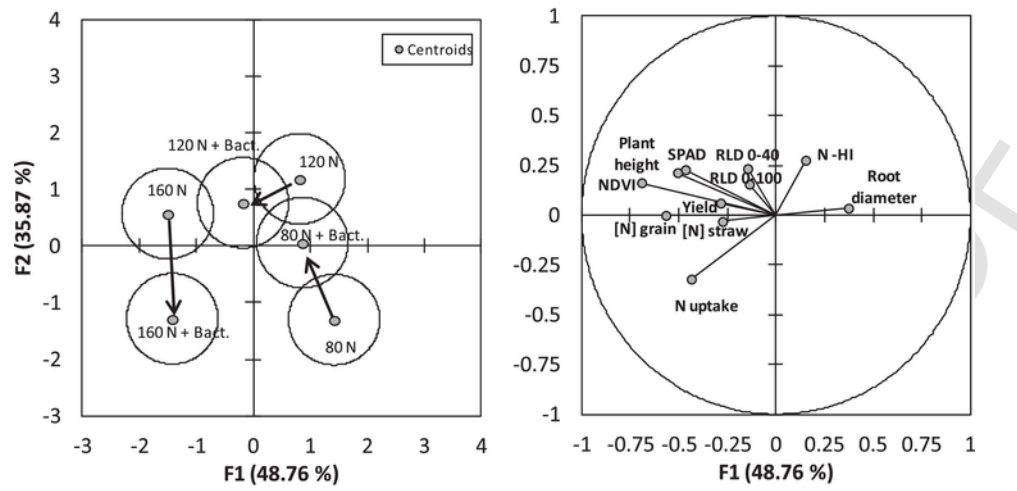
	F1	F2
RLD 0-100 cm	-0.137	0.155
RLD 0-40 cm	-0.145	0.233
Root diameter	0.374	0.034
N uptake	**-0.435**	**-0.322**
SPAD	**-0.465**	0.228
NDVI	**-0.691**	0.162
Plant height	**-0.506**	0.213
[N] grain	**-0.566**	-0.002
[N] straw	-0.275	-0.032
N-HI	0.153	0.275
Yield	-0.284	0.060

Fig. 7. Principal component analysis (PCA; top right) with variable loadings (highlighted values > |0.4|; bottom) 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 PCA comprise 70% of cases.

tial 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 bio-fertilisers.

References

Almaghrabi, O.A., Abdelmoneim, T.S., Albishri, H.M., Moussa, T.A.A., 2014. Enhancement of maize growth using some plant promoting rhizobacteria (PGPR) under laboratory conditions. Life Sci. J. 11, 764–772.

Araújo, A.E.S., Baldani, V.L.D., Galisa, P.S., Pereira, J.A., Baldani, J.I., 2013. Response of traditional upland rice varieties to inoculation with selected diazotrophic bacteria isolatedfrom rice cropped at the Northeast region of Brazil. App. Soil Ecol. 64, 49–55.

Arrese-Igor, C., González, E.M., Marino, D., Ladrera, R., Larrainzar, E., Gil-Quintana, E., 2011. Physiological response of legumes nodules to drought. Plant Stress 5, 24–31.

Basaglia, M., Casella, S., Peruch, U., Poggiolini, S., Vamerali, T., Mosca, G., Vanderleyden, J., De Troch, P., Nuti, M.P., 2003. Field release of genetically marked Azospirillum brasilense in association with Sorghum bicolor L. Plant Soil 256, 281–290.

Benmati, M., Le Roux, C., Belbekri, N., Ykhlef, N., Djekoun, A., 2013. Phenotypic and molecular characterization of plant growth promoting Rhizobacteria isolated from the rhizosphere of wheat (Triticum durum Desf.) in Algeria. African J. Microbiol. Res. 23, 2893–2904.

Bhattacharyya, P., Jha, D., 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28, 1327–1350.

Braccini, A.L., Dan, L.G.M., Piccinin, G.G., Albrecht, L.P., Barbosa, M.C., Ortiz, A.H.T., 2012. Seed inoculation with Azospirillum brasilense, associated with the use of bioregulators in maize. Rev. Caatinga 25, 58–64.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol. 71, 4951–4959.

Dalla Santa, O.R., Hernández, R.F., Alvarez, G.L.M., Ronzelli Junior, P., Soccol, C.R., 2004. Azospirillum sp. inoculation in wheat: barley and oats seeds greenhouse experiments. Braz. Arch. Biol. Technol. 47, 843–850.

Dashadi, M., Khosravi, H., Moezzi, A., Nadian, H., Heidari, M., Radjabi, R., 2011. Co-inoculation of Rhizobium and Azotobacter on growth indices of faba bean under water stress in the green-house condition. Adv. Stud. Biol. 3, 373–385.

Depret, G., Houot, S., Allard, M.R., Breuil, M.C., Nouaim, R., Laguerre, G., 2004. Long-term effects of crop management on Rhizobium leguminosarum biovar viciae populations. FEMS Microbiol. Ecol. 51, 87–97.

Dobbelaere, S., Vanderleyden, J., Okon, Y., 2003. Plant growth promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant. Sci. 22, 107–149.

Dreyfus, B., Garcia, J.L., Gillis, M., 1988. Characterization of Azorhizobium caulinodans sp. a stem nodulating nitrogen-fixing bacterium isolated from Sesbania rostrata. Int. J. Syst. Bacteriol. 38, 89–98.

Elekhtyar, N.M., 2015. Efficiency of Pseudomonas fluorescens as plant growth-promoting rhizobacteria (PGPR) for the enhancement of seedling vigor, nitrogen uptake, yield and its attributes of rice (Oryza sativa L.). Int. J. Sci. Res. Agric. Sci. 2, 57–67.

Fallik, E., Sarig, S., Okon, Y., 1994. Morphology and physiology of plant roots associated with Azospirillum. In: Okon, Y. (Ed.), Azospirillum/Plant Associations. CRC Press, Boca Raton, pp. 77–86.

Fang, Y., Du, Y., Wang, J., Wu, A., Qiao, S., Xu, B., Zhang, S., Siddique, K.H.M., Chen, Y., 2017. Moderate Drought stress affected root growth and grain yield in old, modern and newly released cultivars of winter wheat. Front. Plant Sci. 8, 672.

Faruq, G., Shamsuddin, Z., Nezhadahmadi, A., Prodhan, Z.H., Rahman, M., 2015. Potentials of Azospirillum spp. for improving shoot and root of a Malaysian sweet corn variety (J58) under in vitro condition. Int. J. Agric. Biol. 17, 395–398.

Fentahun, M., Akhatar, M.S., Muleta, D., Limmese, F., 2013. Isolation and characterization of nitrogen deficit Rhizobium isolates and their effect on growth of haricot bean. Afr. J. Agric. Res. 46, 5942–5952.

Gaskins, M.H., Albrecht, S.L., Hubbell, D.H., 1985. Rhizosphere bacteria and their use to increase plant productivity: a review. Agric. Ecosyst. Environ. 12, 99–116.

Glick, B.R., Penrose, D.M., Li, J.P., 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190, 63–68.

Hungria, M., 2010. Inoculation with selected strains of Azospirillum brasilense and A. lipoferum improves yields of maize and wheat. Plant Soil 331, 413–425.

Kaushal, M., Wani, S.P., 2016. Rhizobacterial-plant interactions: strategies ensuring plant growth promotion under drought and salinity stress. Agric. Ecosyst. Environ. 231, 68–78.

Kennedy, I.R., Tchan, Y., 1992. Biological nitrogen fixation in non-leguminous field crops: recent advances. Plant Soil 141, 93–118.

Khalid, A., Arshad, M., Zahir, Z.A., 2004. Screening plant growth promoting rhizobacteria for improving growth and yield of wheat. J. Appl. Microbiol. 96, 473–480.

Kumar, A., Maurya, B.R., Raghuwanshi, R., 2014. Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (Triticum aestivum L.). Biocatal. Agric. Biotechnol. 3, 121–128.

Ma, W., Zalec, K., Glick, B.R., 2001. Biological activity and colonization pattern of the bioluminesence-labeled plant growth promoting bacterium Kluyvera ascorbata SUD165/26. FEMS Microbiol. Ecol. 35, 137–144.

Mahanta, D., Rai, R.K., Mishra, S.D., Raja, A., Purakayastha, T.J., Varghese, E., 2014. Influence of phosphorus and biofertilizers on soybean and wheat root growth and properties. Field Crops Res. 166, 1–9.

Marques, A.P.G.C., Pires, C., Moreira, H., Rangel, A.O.S.S., Castro, P.M.L., 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using Zea mays as indicator plant. Soil Biol. Biochem. 42, 1229–1235.

Millet, E., Avivi, Y., Feldman, M., 1984. Yield response of various wheat genotypes to inoculation with Azospirillum brasilense. Plant Soil 80, 261–266.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant 15, 473–497.

Nain, L., Rana, A., Joshi, M., Jadhav, S.D., Kumar, D., Shivay, Y.S., Paul, S., Prasanna, R., 2010. Evaluation of synergistic effects of bacterial and cyanobacterial strains as biofertilizers for wheat. Plant Soil 331, 217–230.

Okon, Y., Bloemberg, G.V., Lugtenberg, B.J.J., 1998. Biotechnology of biofertilization and phytostimulation. In: Altman, A. (Ed.), Agricultural Biotechnology. Marcel Dekker, New York, pp. 327–349.

Pérez-Montaño, F., Alías-Villegas, C., Bellogín, R.A., del Cerro, P., Espuny, M.R., Jiménez-Guerrero, I., López-Baena, F.J., Ollero, F.J., Cubo, T., 2014. Plant growth promotion

in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. Microbiol. Res. 169, 325–336.

Panwar, J.D.S., Singh, O., 2000. Response of Azospirillum and Bacillus on growth and yield of wheat under field conditions. Indian J. Plant Physiol. 5, 108–110.

Pedraza, R.O., Bellone, C.H., Carrizo de Bellone, S., Boa Sorte, P.M.F., dos Santos Teixeira, K.R., 2009. Azospirillum inoculation and nitrogen fertilization effect on grain yield and on the diversity of endophytic bacteria in the phyllosphere of rice rainfed crop. Eur. J. Soil Biol. 45, 36–43.

Piccinin, G.G., Dan, L.G.M., Braccini, A.L., Mariano, D.C., Okumura, R.S., Bazo, G.L., Ricci, T.T., 2011. Agronomic efficiency of Azospirillum brasilense in physiological parameters and yield components in wheat crop. J. Agron. 10, 132–135.

Qiang, L., Hua-wei, L., Wei-ling, W., 2014. Colonization of Azorhizobium caulinodans in wheat and nutrient-related miRNA expression. J. Plant Nutr. Fertil. 20, 930–937.

Rafi, M.M., Varalakshmi, T., Charyulu, P.B.B.N., 2012. Influence of Azospirillum and PSB inoculation on growth and yield of foxtail millet. J. Microbiol. Biotech. Res. 2, 558–565.

Roesti, D., Gaur, R., Johri, B.N., Imfeld, G., Sharma, S., Kawaljeet, K., Aragno, M., 2006. Plant growth stage, fertiliser management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields. Soil Biol. Biochem. 38, 1111–1120.

Rosas, S.B., Avanzin, G., Carlier, E., Pasluosta, C., Pastor, N., Rovera, M., 2009. Root colonization and growth promotion of wheat and maize by Pseudomonas aurantiaca SR1. Soil Biol. Biochem. 41, 1802–1808.

Sørensen, J., Nicolaisen, M.H., Ron, E., Simonet, P., 2009. Molecular tools in rhizosphere microbiology − from single-cell to whole-community analysis. Plant Soil 321, 483–512.

Sabry, S.R.S., Saleh, S.A., Batchelor, C.A., Jones, J., Jotham, J., Webster, G., Kothari, S.L., Davey, M.R., Cocking, E.C., 1997. Endophytic establishment of Azorhizobium caulinodans in wheat. Proc. R. Soc. Lond. B 264, 341–346.

Saharan, B., Nehra, V., 2011. Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res. 21, 1–30.

Stabentheiner, E., Zankel, A., Pölt, P., 2010. Environmental scanning electron microscopy (ESEM) − a versatile tool in studying plants. Protoplasma 246, 89–99.

Steenhoudt, O., Vanderleyden, J., 2000. Azospirillum, a free living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. 24, 487–506.

Sylvia, D.M., Furhrmann, J.J., Hartel, P., Zuberer, D.A., 2005. Principles and Applications of Soil Microbiology. Pearson Prentice Hall Upper Saddle River, (pp. 35–38.).

Tahir, M., Mirza, M.S., Zaheer, A., Dimitrov, M.R., Smidt, H., Hameed, S., 2013. Isolation and identification of phosphate solubilizer Azospirillum, Bacillus and Enterobacter strains by 16SrRNA sequence analysis and their effect on growth of wheat (Triticum aestivum L.). Aus. J. Crop. Sci. 7, 1284–1292.

Tahir, M., Mirza, M.S., Hameed, S., Dimitrov, M.R., Smidt, H., 2015. Cultivation-based and molecular assessment of bacterial diversity in the rhizosheath of wheat under different crop rotations. PLoS One 10 (6), e0130030.

Turan, M., Gulluce, M., Şahin, F., 2012. Effects of plant-growth-promoting rhizobacteria on yield, growth, and some physiological characteristics of wheat and barley plants. Commun. Soil Sci. Plant Anal. 43, 1658–1673.

Turan, M., Ekinci, M., Yildirim, E., Güneş, A., Karagöz, K., Kotan, R., Dursun, A., 2014. Plant growth-promoting rhizobacteria improved growth, nutrient and hormone content of cabbage (Brassica oleracea) seedlings. Turk. J. Agric. For. 38, 327–333.

Turner, T.R., James, E.K., Poole, P.S., 2013. The plant microbiome. Genome Biol. 14, 209.

Vamerali, T., Guarise, M., Ganis, A., Bona, S., Mosca, G., 2003. Analysis of root images from auger sampling with a fast procedure: a case of application to sugar beet. Plant Soil 255, 387–397.

Venieraki, A., Dimou, M., Vezyri, E., Kefalogianni, I., Argyris, N., Liara, G., Pergalis, P., Chatzipavlidis, I., Katinakis, P., 2011. Characterization of nitrogen-fixing bacteria isolated from field-grown barley, oat, and wheat. J. Microbiol. 49, 525–534.

Videira e Castro, I., Fareleira, P., Ferreira, E., 2016. Nitrogen fixing symbiosis in a sustainable agriculture. In: In: Hakeem, K.R., Akhtar, M.S., Abdullah, S.N.A. (Eds.), Plant, Soil and Microbes: Implications in Crop Science, vol. 1, Springer International Publishing, Switzerland, pp. 55–91.

Visioli, G., Vamerali, T., Mattarozzi, M., Dramis, L., Sanangelantoni, A.M., 2015. Combined endophytic inoculants enhance nickel phytoextraction from serpentine soil in the hyperaccumulator Noccaea caerulescens. Front. Plant Sci. 6, 638.

Wieland, G., Fendrik, I., 1998. In: In: Elmerich, C., Kondorosi, A., Newton, W.E. (Eds.), Colonization of Wheat by Azoarcus Sp. and Interaction with Other Diazotrophs, vol. 31, Springer Netherlands, Dordrecht, (of the series, pp. 406-406).

Yadav, B.K., Akhtar, M.S., Panwar, J., 2015. Rhizospheric plant microbe interactions: a key factor to soil fertility and plant nutrition. In: Arora, N.K. (Ed.), Plant Microbe Symbiosis: Applied Facets. Springer, Cham, pp. 127–145.