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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 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

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(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 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 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.

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 presowing 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)