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The effect of plant-associative bacteria (*Azospirillum* and *Pantoea*) on the fruit quality of sweet pepper under limited nitrogen supply

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ABSTRACT

This study investigates the influence of a commercial product, Biopron®, consisting of the bacteria *Azospirillum brasilense* and *Pantoea dispersa* on sweet pepper fruits (*Capsicum annuum* L.) under limited N supply. When the N supply was reduced from 12 (control) to 7 mmol L^{-1}, the concentration of total-N in the fruits was significantly reduced in both inoculated and non-inoculated plants. The N supply or inoculation did not affect the dry matter content or fruit firmness, but non-inoculated fruit with low N showed a decrease in pericarp thickness and a significant increase in the color parameter a^* compared with the control. Under limited N, inoculation increased the concentration of citric, ascorbic and succinic acids in green fruit compared with non-inoculated fruit, which showed lower values than control fruit. At a later (yellow) stage of development, only succinic acid showed a response to inoculation. Fruit peroxidase (EC 1.11.1.7) activity in fruit of inoculated plants was lower than that observed for non-inoculated fruit grown at both high- and low-N. In contrast, in yellow fruit, total phenolic compounds were increased under N limitation, with no inoculation effect. Our study shows that the effect of plant associative bacteria is not directly related with the increased potential availability of nutrients for uptake, especially for fruit quality characteristics.

1. Introduction

The region around the root is relatively rich in nutrients, due to the loss of as much as 40% of plant photosynthates from the roots (Lynch and Whipps, 1991). Consequently, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth (Nelson, 2004). Plant-growth-promoting rhizobacteria (PGPR) was first described by Kloepper and Schroth (1978), as soil bacteria that colonize the roots of plants following inoculation onto seeds and that enhance plant growth. *Azospirillum* and *Pantoea* are defined as free-living, plant-growth-promoting bacteria, capable of affecting the growth and yield of numerous plant species, many of agronomic and ecological significance (Bashan et al., 2004). These PGPR have no preference for crop plants or weeds, or for annual or perennial plants, and can be successfully applied to plants that have no previous history of PGPR in their roots (Dobbelaere et al., 2003).

PGPR enhance plant growth by direct and indirect means, but despite intensive studies on the physiology and molecular biology of these bacteria, the exact mode of action or the specific mechanisms involved have not been well characterized (Bashan et al., 2004; Glick, 1995). Direct mechanisms of plant growth promotion by PGPR can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while indirect mechanisms involve the ability of PGPR to reduce the deleterious effects of plant pathogens on crop yield (Nelson, 2004). PGPR have been reported to enhance plant growth directly by a variety of mechanisms: fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones (Glick, 1995; Dobbelaere et al., 2001). Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported (Bashan and Levanony, 1991; Bertrand et al., 2000).

Azospirillum sp. are known mainly for their ability to produce plant hormones as well as polyamines and amino acids in culture (Thuler et al., 2003), but they are also involved in the biological fixation of nitrogen and the increased activity of glutamate

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dehydrogenase and glutamine synthetase (Ribaudo et al., 2001). *A. brasilense* produces high quantities of extracellular indole acetic acid—increasing root elongation, root surface area, and root dry matter (El-Khawas and Adachi, 1999; Molla et al., 2001).

The effects of these microorganisms are influenced greatly by the species and genotype (Sensoy et al., 2007), soil type, and cultural practices such as fertilizer application (Gryndler et al., 2001), while the growth and fruit quality response of sweet pepper are also affected by the cultivation method (del Amor, 2006, 2007).

Nowadays, high-value crops are often cultivated in greenhouses in soilless conditions, which provide high levels of nutrients. This practice prevents any shortage or imbalance of nutrients in the rhizosphere but intensifies the release of nutrients into the environment. This is especially important for nitrate, the major anion contributing to crop productivity (Mohr and Schopfer, 1995). The reduced use of N-fertilizers will decrease costs and pollution, which is a consequence of the methods of synthesis and of the inefficient application to and uptake by crops; achievement of these goals is urgently required (Ter Steege et al., 2001). To reduce wastage, N-fertilization of soilless cultures needs to be reduced and plant-growth-promoting rhizobacteria, also called biofertilizers, could help to improve nutrient-use efficiency.

Although different studies have correlated N assimilation and plant growth and yield response with PGPR inoculation, so far little is known about its specific effect on fruit quality, especially in sweet pepper. We hypothesized that the PGPR effect on fruits may not always be associated with these reported increases in N uptake efficiency, and that physical and chemical composition as well as enzymatic characteristics could be significantly affected by these bacteria.

2. Material and methods

2.1. Plant growth conditions

Plants of sweet pepper (*Capsicum annuum* L.), cultivar Cierva, California type were transplanted from a commercial nursery on 18 January 2006. Plants were grown in a commercial plastic greenhouse, located in San Javier (Murcia, Spain), in plastic bags containing coconut coir fiber as substrate. Each bag had three 4 L h^{-1} drippers and three plants. Plants were irrigated with nutrient solution of composition (meq L^{-1}) 12NO_3^-, 1.5$H_2PO_4^-$, 6SO_4^{2-}, 7K^+, 8.5Ca^{2+}, and 4Mg^{2+} (control) or with a low-nitrogen solution: 7NO_3^-, 2$H_2PO_4^-$, 8SO_4^{2-}, 6K^+, 7.5Ca^{2+}, and 3.5Mg^{2+}. Irrigation management was according to local commercial soilless cultivation and the drainage percentage was maintained at 30% for both treatments throughout the crop cycle. A commercial microbial inoculant (Biopron®), consisting of cells of *A. brasilense* strain M3 and *P. dispersa* strain C3 immobilized in a solid support, was obtained from Probelte S.A. (Murcia, Spain). This product guarantees and certifies biological composition of both cell strains with 10^9 ufc g^{-1} (ufc: unit forming colonies). It was applied at 15 g per plant (45 g per substrate bag) of inoculant three days before transplanting. The most notable phenomenon in *Azospirillum* inoculation is that it is more successful and more profitable when other microorganisms are co-inoculated with *Azospirillum* (Bashan and Holguin, 1997). In the present study, *Pantoea* sp. was also added to the substrate, because Muthukumar and Udaiyan (2006) reported that this combination stimulated growth and nutrient uptake to a greater extent than when inoculated alone. However, a nutritional (N) effect of *Pantoea* can be disregarded as it does not have an effect on N nutrition and P is added in soilless conditions.

2.2. Total-N determination

The fruit dry weight was determined after at least 72 h at 70 °C. The total-N concentration was measured in the dry matter, using a LECO FP-528 (Leco Corporation, St. Joseph, MI, USA).

2.3. Fruit firmness

The fruit firmness was determined on fruit with intact skin, using a Bertuzzi FT011 penetrometer, fitted with an 8 mm diameter probe.

2.4. Color

Fruit color was determined with a Konica-Minolta CR-300 calorimeter, making three measurements along the equatorial perimeter.

2.5. Organic acids determination

The concentrations of organic acids were determined in fruit juice. Six fruits were cut and liquefied, centrifuged at 15,000 rpm for 20 min, at 5 °C, filtered (0.45 μm), and injected (10 μL) onto a heated (30 °C) Supelcogel C610H column (30 cm × 7.8 mm) that was protected with a Supelcogel C610H guard column (5 cm × 4.6 mm) (Supelco, Inc., Bellefonte, PA). The HPLC system was an HP 1100 series model with an autosampler and a UV detector set at 210 nm (Hewlett-Packard, Palo Alto, CA). The mobile phase was 0.1% phosphoric acid. The flow rate was 0.5 mL/min and the run time was 40 min per sample. Peaks were quantified by area integration and calibrated by injection of external standards.

2.6. Partial purification of pepper peroxidase

Pepper peroxidase was extracted according to Núñez-Delicado et al. (1996) but with some modifications. Fresh peppers (green and yellow) were washed, and the seeds and peduncle were removed. A 50 g sample was homogenized with 100 mL of sodium phosphate buffer (pH 7.3) for 5 min in an Ultraturrax. Ten milliliters of the homogenate were used for the extraction of phenolic compounds. The rest of the homogenate was filtered through four layers of cheesecloth. This filtrate was subjected to temperature-induced phase partitioning by adding TX-114 at 4 °C so that the final detergent concentration was 4% (w/v). The mixture was kept at 4 °C for 15 min and then warmed to 35 °C for 15 min. At this time, the solution became spontaneously turbid due to the formation, aggregation and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins and phenolic compounds. This turbid solution was centrifuged at 10,000 g for 15 min, at 25 °C. After discarding the pellet and detergent-rich phase, the clear, detergent-poor supernatant, which contained the soluble persimmon PPO, was brought to 30% saturation with $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60,000 g for 30 min, at 4 °C, and the pellet was discarded. $(NH_4)_2SO_4$ was added to the clear supernatant to give 80% saturation and the mixture was stirred for 1 h at 4 °C. The precipitate obtained between 30% and 80% was collected by centrifugation at the same rotor speed and dissolved in a minimum volume of 100 mM sodium phosphate buffer, pH 7.3. The salt content was removed by dialysis and the enzyme stored at −20 °C.

2.7. Enzymatic activity

The peroxidase activity was measured spectrophotometrically in a Shimadzu model S33 spectrophotometer, at the absorption