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A strain of *Bacillus subtilis* stimulates sunflower growth (*Helianthus annuus* L.) temporarily

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ABSTRACT

Preliminary studies showed that a *Bacillus subtilis* strain stimulates plant growth. We investigated how inoculating seeds of a sunflower cultivar (*Helianthus annuus* L.) with this strain stimulated plant growth, soil properties and emissions of greenhouse gasses, i.e. carbon dioxide (CO_2) and nitrous oxide (N_2O), when cultivated in a greenhouse. Unfertilized sunflowers or fertilized with urea served as controls. After one month, root length and fresh and dry root weight of the sunflower was significantly higher in the bacteria amended plant than in the urea and unfertilized plants. However, at harvest, no positive effect was observed. The number of seeds per plant and seed weight was not significantly different between the treatments, but total plant N was significantly higher in urea-amended plants than in unfertilized plants. The CO_2 production rate was not affected by treatment, but the N_2O emission rate was significantly higher in soil amended with urea plus bacteria soil compared to the unfertilized treatments. It was found that the *B. subtilis* strain used in this study had a positive, but only temporarily effect on growth of the sunflower cultivar used.

1. Introduction

Bacteria are the most abundant microorganisms in soil and up to 6×10^8 cells g^{-1} can be found (Kilian et al., 2000). The most representative genera are *Pseudomonas*, *Clostridium*, *Arthrobacter*, *Achromobacter*, *Micrococcus*, *Flavobacterium*, *Azospirillum*, *Azotobacter* and *Bacillus*, with the latter being the most common group of bacteria isolated from soil and other environments (Kilian et al., 2000; Vessey, 2003; Forchetti et al., 2007; Felici et al., 2008; Swain and Ray, 2009). It is well known that *Bacillus* stimulates plant growth. For instance, Yildrim et al. (2008) isolated several *Bacillus* strains from saline soils in Turkey and found that they increased development of radish. However, percentage of emergence and mean emergence time decreased. Siddiqui and Akhtar (2009) found that *B. subtilis* 7612 inhibited galling and nematode reproduction, while promoted plant growth.

Bacillus sp. shows a wide range of mechanisms that can stimulate plant development. First, *Bacillus* sp. produces structurally diverse antibiotics (Ongena et al., 2005; Forchetti et al., 2007; Lee et al., 2008; Liu et al., 2009; Swain and Ray, 2009). Second, it rapidly colonizes plant roots and has the capacity to multiply on the roots (Dijkstra et al., 1987). It remains close the root tip by passive displacement on the elongating cells. Third, *Bacillus* sp. induces systemic resistance by producing volatile organic compounds (Ryu et al., 2004) and promotes plant and root growth through the production of phytohormones and extracellular enzymes (Yao et al., 2006; Forchetti et al., 2007; Lee et al., 2008; Swain and Ray, 2009). Fourth, it decreases the ethylene levels in plants by deamination of 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor of ethylene (Penrose and Glick, 2003). As a result of this reaction (ACC plus ACC deaminase), α-ketobutyrate and ammonia are obtained reducing the ethylene production (Penrose and Glick, 2003). Reduced ethylene production is known to affect fruit ripening, leaf senescence, flower abscission, germination, cell elongation and proliferation, nodulation and response to pathogenic attack (Kende and Zeevaart, 1997; Glick et al., 1998).

Sunflower (*Helianthus annuus* L.) is an important crop and ornamental plant in the world. It is easily cultivated and is grown mainly under rainfed conditions on a wide range of soils (Shehata and El-Khawas, 2003). It is used for animal feed and is the second most important crop producing edible oil after soybean (Shehata and El-Khawas, 2003; Fairless, 2007). Recently, sunflower has also been cultivated to produce biodiesel (Demirbas, 2007).

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In a previous study, it was found that a strain of *B. subtilis* increased yield and marketable yield of tomato (*Lycopersicon esculentum* Mill, cv Rio Fuego) (Mena-Violante and Olalde-Portugal, 2007). In this study we investigated if a strain of *B. subtilis* affected sunflower development and its yield. Unfertilized sunflowers or fertilized with urea served as controls. Sunflower was cultivated in soil amended with or without urea or urea plus *B. subtilis* under greenhouse conditions. Soil properties, emission of CO_2 and N_2O, plant growth parameters and yields were monitored in three experiments. The objective of this study was to investigate the effect of a *B. subtilis* strain on growth of sunflower, soil properties and emission of CO_2 and N_2O.

2. Materials and methods

2.1. Sampling site, collection and soil properties

Alcholoya is a hamlet in Acatlán, State of Hidalgo, (2120 m above sea level, 20°09′N and 98°26′W) at a distance of 147 km from México City. The climate is temperate with dry winters (Cwb) (Peel et al., 2007), a mean annual temperature of 14 °C and mean annual rainfall of 600 mm. Maize, beans, barley, and maguey are the main crops in the area.

Three different 400 m^2 areas were defined in a field. The 0–15 cm top-layer of each plot was spade-sampled until 800 kg were obtained, pooled separately and 5-mm sieved. As such, three different soil samples were obtained. This field based replication was maintained in the greenhouse experiments. The soil was classified as Typic Fragiudepts with pH 6.5 and electrical conductivity (EC) 0.7 dS m^{-1}, had a water holding capacity (WHC) 846 g kg^{-1} soil, an organic C content 11.1 g C kg^{-1} soil and total N content of 1.0 g N kg^{-1} soil.

2.2. Bacillus subtilis

Bacillus subtilis was isolated from the rhizosphere of potato (identified by 16S ribosomal RNA, rRNA, gene sequencing) and selected for its antagonistic activity against phytopathogens. Its phosphate solubilizing activity was determined as described by Burr et al. (1984), 1-aminocyclopropane-1-carboxilate deaminase enzymatic activity as reported in Penrose and Glick (2003), indole-3-acetic acid production by the colorimetric method (Azcón et al., 2009) and root colonization on maize and sunflower in a Petri dish using Phytagel (Sigma Co.) as medium (Dijkstra et al., 1987). The strain was stored in glycerol at −80 °C.

Before inoculation, the bacterium was reactivated and cultured in Potato-Dextrose (PD) and grown at 180 rpm on a rotary shaker and 37 °C until the exponential phase (1×10^7 colony forming units, CFU mL^{-1}). *Bacillus* strains grew easily in potato dextrose and showed antagonistic activity towards fungi in this medium (Singh and Deverall, 1984; Dass and Teyegaga, 1996). The culture was washed twice in 0.9% NaCl by centrifugation (15 min at 10,000 rpm and 4 °C) and resuspended in sterile phosphate buffer (pH 7.0) to a density of approximately 10^7 CFU mL^{-1} before the seed dressing treatment or/and soil application (Shishido et al., 1996).

2.3. Seed treatment and cultivation of sunflower in the greenhouse

The seeds of *H. annuus* were obtained from "*Departamento de Fitotecnia de la Universidad Autónoma de Chapingo, Texcoco, Estado de México*". Seeds were disinfected by cleaning them 5 times with 1.5% NaOCl, washing 5 times with distilled water for 5 min, submerging in 70% ethanol for 3 min and washing them 5 times with sterile distilled water for 5 min.

Thirty-six sub-samples of 6.5 kg soil from each plot ($n = 3$) were added to polyvinyl chloride (PVC) tubes (length 50 cm and ⌀ 16 cm) filled at the bottom with 7 cm of gravel topped up with 3 cm sand (Bellini et al., 1996). As such, a layer of 30 cm soil was obtained.

Four different treatments were applied to soil of the three sampled plots. In a first treatment, 27 soil columns (nine columns for each of the three soils sampled) were left unfertilized and uncultivated (considered the CONTROL treatment). In a second treatment, 27 soil columns were planted with three sunflower seeds, but left unfertilized (considered the PLANT treatment). In a third treatment, 27 soil columns were planted with three sunflower seeds and fertilized with 0.5 g urea (considered the UREA treatment). In a fourth treatment, 27 soil columns were planted with three sunflower seeds dressed with *B. subtilis* and fertilized with 0.5 g urea (considered the BACTERIA treatment). The seeds used in the fourth treatment were disinfected, dried, and dressed with a suspension of 1% carboxymethylcellulose and 1×10^7 CFU mL^{-1} of *B. subtilis*. The amount of urea added was such that the plants were fertilized with 75 kg N ha^{-1}.

The PVC tubes were placed on a plastic recipient to collect water leached out from the soil columns. Plants were irrigated with tap water as needed by the plants. As such, plants were irrigated 20 times with 1 L tap water. The tap water used in the experiment was analyzed for mineral N and contained 0.45 mg NO_2^--N L^{-1} and 1.92 mg NO_3^--N L^{-1}, but no NH_4^+. As such, approximately 19 kg mineral-N ha^{-1} was additionally added to the sunflower plant with the tap water over the growing season. During the experiment, no water leached out of the soil columns.

Eight days after emergence two plantlets were discarded. Twelve days after emergence, plantlets in the UREA and BACTERIA treatment were amended with another 0.5 g urea. As such, sunflower plantlets in those two treatments were fertilized with a total amount of 150 kg N ha^{-1} (Scheiner et al., 2002). Three weeks after sowing, plantlets were drenched with bacteria by applying a 4 mL bacteria suspension (at the same concentration as described above) near the plantlet roots of the BACTERIA treatment at a depth of 3 cm. At the onset of the experiment and approximately every two days for the next 30 days, the columns were closed air-tight and the atmosphere analyzed for CO_2 and N_2O at time 0 and after 3, 15 and 30 min.

After 37 days, 60 days and at the end of the vegetation period, three PVC tubes were selected at random from each treatment. The entire soil column was removed from the PVC tube and soil samples were collected from 0–15 cm to 15–30 cm layers by taking care not to damage the root structure. Roots were separated from shoots and the length of both was measured. Roots and shoots were air-dried, weighted and analyzed for total N. The first experiment started on August 13th and lasted until December 13th, 2008. The whole experiment was repeated twice, i.e. a second experiment that run from October 8th, 2008 to March 17th, 2009 and the third running from April 1st to July 17th, 2009.

2.4. Production of CO_2 and N_2O

A cylindrical PVC chamber (length 50 cm and ⌀ 16 cm) was placed on the PVC tube and air-tied sealed. Zero, 3, 15 and 30 min after the upper cylindrical chamber was sealed, 20 cm^3 air was injected into the PVC chamber headspace, the gas was mixed by flushing at least 3 times with the air inside the chamber and finally was collected for the analysis. A 20 cm^3 air sample was taken and injected into 17-mL air-evacuated vials. The amount of CO_2 and N_2O was determined with an Agilent 4890D gas chromatograph fitted with an electron capture detector (ECD). A J&W Scientific GS-Q column (30 m, 530 μm I.D.) was used to separate CO_2 and N_2O from the other gasses. The carrier gas N_2 was flowing at 5 mL min^{-1}. The temperature of detector, injector and oven was set up at 225, 100