Applied Soil Ecology 61 (2012) 147–157

Contents lists available at SciVerse ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Growth and biochemical responses of soybean to double and triple microbial associations with *Bradyrhizobium*, *Azospirillum* and arbuscular mycorrhizae

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ARTICLE INFO

Article history:
Received 8 December 2011
Received in revised form 3 April 2012
Accepted 2 May 2012

Keywords:
Bradyrhizobium
Azospirillum
Arbuscular mycorrhizae
Rhizosphere interactions
Soybean
Biochemical responses

ABSTRACT

Bradyrhizobium (B), *Azospirillum* (A) and arbuscular mycorrhizal fungi (M) are plant beneficial rhizospheric microorganisms often applied as biofertilizers. The objectives of the present work were to study the effects of double (B + A and B + M) and triple (B + A + M) microbial combinations on soybean yield (*Glycine max* [L.] Merr.), nodulation, mycorrhization and plant metabolites concentrations, as compared to *Bradyrhizobium* alone, after 28 and 56 days of growth under controlled conditions. All microbial combinations increased soybean root biomass, but not shoot biomass. The highest total biomass of soybean (root + shoot) was observed with the double association *Azospirillum* and *Bradyrhizobium* (B + A). This treatment reduced the total number of nodules but seems to increase their capacity to fix nitrogen, as shown by their high starch concentration during establishment. The presence of mycorrhizae (B + M) had a transitory adverse effect on depth of nodulation as compared to *Bradyrhizobium* alone, which could indicate competition between these two symbionts during establishment (day 28). The triple association (B + A + M) reduced shoot growth, as well as the number of small nodules. The higher concentration of the stress-induced amino acid proline in nodules and leaves in response to B + A + M indicates that soybean plants are under stress when in presence of the combination of three symbionts. During establishment, the concentration of coumestrol in soybean roots was lower with microbial combinations than with *Bradyrhizobium* alone, which could indicate a common regulating signal between soybean and both *Azospirillum* and mycorrhiza. Our results show that complex interactions and competition between the three microorganisms induced differential growth and nodulation responses, which can be linked to metabolic changes.

1. Introduction

Legumes live in symbiosis with two major rhizosphere microorganisms: nodule-forming bacteria capable to fix atmospheric N_2 to provide the plant with nitrogen (N) (Prévost and Antoun, 2007), and arbuscular mycorrhizal fungi forming external hyphal networks which transport P and water to their host (Smith and Read, 2008; Fortin et al., 2008; Khasa et al., 2009). Numerous studies have explored the common features of these two symbioses, which share common biochemical plant symbiotic signals (Vierheilig, 2004; Hause and Schaarschmidt, 2009). For instance, flavonoids found in root exudates of host plants are known as key signaling compounds in a number of plant–microbe interactions (Steinkellner et al., 2007). Flavonoids act both as chemoattractants for rhizobial bacteria and elicitors of fungal hyphal growth in mycorrhiza establishment (Steinkellner et al., 2007). Recent major discoveries of common symbiotic genes activation (Gherbi et al., 2008; Hayashi et al., 2010) have definitively established that the rhizobium symbiosis has evolved from the ancestral arbuscular mycorrhizal symbiosis (Parniske, 2008; Oldroyd et al., 2009). Moreover, the recent description of symbiotic signals that stimulate the formation of arbuscular mycorrhizae, the MYC factors, confirmed that they are ancestral molecular analogs of the lipo-chitooligosaccharidic NOD factors of the rhizobial symbiosis (Maillet et al., 2011). Numerous authors have studied the interaction between legumes, rhizobial and arbuscular mycorrhizal partners (reviewed by Chalk et al., 2006). With soybean cultivated in pots, earlier studies have reported both antagonistic (Bethlenfalvay et al., 1985) and synergistic effects of the two symbionts (Bethlenfalvay et al., 1988).

Other rhizospheric microbes, such as plant growth promoting rhizobacteria (PGPR) can also interact with plants and improve their growth (Hayat et al., 2010). Among them, diazotrophic rhizobacteria from the genus *Azospirillum* are able to reduce atmospheric N_2 into ammonia, and have been shown to promote plant growth. Associations of rhizobium and *Azospirillum* species with several legumes, including soybean, generally promote seed

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http://dx.doi.org/10.1016/j.apsoil.2012.05.006

germination, plant growth, root ramification and nodulation (Khan et al., 2010). In addition, microbial associations of *Azospirillum* with AM fungi have been shown to improve yields of legumes (Toro et al., 1996; Saini et al., 2004), as well as those of gramineous crops like maize (Miyauchi et al., 2008), sorghum (Saini et al., 2004) and barley (Negi et al., 1990). In this non-symbiotic association, the role of the plant is to provide the constituents of their root exudates to these rhizospheric bacteria. The bacteria can then utilize these as source of energy and in counterpart provide NH_3 to the plant (Saharan and Nehra, 2011). Furthermore, *Azospirillum* can colonize root surface and induce root morphological changes that give the plant a better access to nutrients (Okon and Kapulnik, 1986) or promote plant growth through diverse mechanisms (Dobbelaere et al., 2003).

Biochemical interactions between plants and microorganisms are complex and the accumulation and translocation of metabolites between stems and roots or nodules are indicators of the status of the symbiosis (Bertrand et al., 2011). In soybean in symbiosis with *Bradyrhizobium*, photosynthates are transported from the leaves to the nodules through the phloem under the form of sucrose. In the nodule, carbon (C) from sucrose is made available to the bacteroids to sustain N_2 fixation (Crawford et al., 2000). In response, the bacteroids release to the plant the ammonia they produce from N_2 fixation. In soybean, nitrogen is exported from the nodules to the other plant parts through the xylem under the form of ureides, which are degraded in the leaves to release ammonia and then assimilated by the plant into amino acids (Crawford et al., 2000). The regulation of this pathway is complex and involves ammonia availability to the plant and feedback inhibition of N_2 fixation by high concentration of nitrogenous compounds in the leaves (Serraj et al., 1999a,b). Many reports confirm that a major portion of photosynthates are translocated to the nodules and utilized there in support of N_2 fixation, nodule growth and maintenance, and assimilation of ammonia (Streeter, 1980). On the other hand, N_2 fixation in nodules is rapidly inhibited under conditions that reduce C availability (Curioni et al., 1999). The mutualistic biochemical relationship between soybean and mycorrhiza is not fully understood. It is however known that, in return to enhanced mineral nutrition by the fungi, the host plant channels between 4 and 20% of its net photosynthetic C to its mycobionts through the arbuscules that are the sites of exchange for phosphorus, carbon, water, and other nutrients (Lerat et al., 2003).

In field studies, synergistic relationships between *Rhizobium*, *Azospirillum* and/or arbuscular mycorrhizal fungi have been shown to increase biomass of legume and of non-legume crops (Saini et al., 2004), including soybean (Bagyaraj et al., 1979; Hayat et al., 2010). However, in laboratory experiments, synergistic as well as antagonistic effects have been observed between the three rhizospheric partners (Requena et al., 1997; Valdenegro et al., 2001). From a practical point of view, the three plant beneficial microbes *Rhizobium*, *Azospirillum* and arbuscular mycorrhizal fungi are used as bio-inoculants all over the world (Brockwell and Bottomley, 1995; Lucy et al., 2004; Hamel and Plenchette, 2007). Considering the expanding use of these microorganisms in agriculture and the general commercial importance of soybean, there is a need to better understand the interactive effects of *Bradyrhizobium*, *Azospirillum* and arbuscular mycorrhizal fungi on soybean growth and physiology.

The objectives of the present work were to study the effects of double and triple inoculations with *B. japonicum*, *Azospirillum* and mycorrhizal fungi on soybean yield, nodulation and mycorrhization. To better characterize soybean response to these microorganisms, C and N metabolism were investigated by the assessment of carbohydrates, amino acids and ureides contents in nodules and in leaves. Furthermore, measurements of three flavonoids in soybean roots, daidzein, genistein and coumestrol, will help to understand plant–microbe signaling when multiple microorganisms are involved.

2. Materials and methods

2.1. Microbial inoculation and plant growth conditions

Two bacterial microorganisms were used for soybean inoculation: *Bradyrhizobium japonicum*, strain 14M2b, isolated from Québec agricultural soil under soybean–maize rotations (Agriculture and Agri-Food Canada (AAFC) culture collection), and *Azospirillum canadense*, strain DS2, a new species isolated from corn rhizosphere in London, Ontario (AAFC, Mehnaz et al., 2007). Liquid bacterial inocula were prepared by growing *B. japonicum* cells in standard yeast mannitol broth (YMB) (Vincent, 1970), and *A. canadense* cells in standard malate N-free medium (Mehnaz and Lazarovits, 2006). Dilutions of individual or combined bacterial inocula were used to inoculate soybean seed at a rate of 10^6 cells per seed (Prévost et al., 2010). About two hundred soybean seeds were soaked in 2 mL of appropriate bacterial inocula overnight.

The arbuscular mycorrhizal fungus used was *Glomus irregulare* strain DAOM 197198 (AAFC Ottawa, Canada) obtained from Premier Tech, Rivière-du-Loup, QC, in the form of sterile liquid inoculum containing 400 spores mL^{-1}. The mycorrhizal inoculum was mixed to the plant growth substrate at the concentration of 2 mL L^{-1} (i.e. 800 spores L^{-1}) before sowing.

Ten seeds of soybean [*Glycine max* (L.) Merr.] cv. *Korus*, an early cultivar (2550 Crop Heat Units) recommended for cultivation in the province of Québec, Canada (CRAAQ, 2003), previously inoculated according to the bacterial treatments, were sown in 24-cm deep pots (Treepots TPOT9, 10 cm-diam. × 24 cm-depth, Stuewe & Sons Inc., Tangent, OR, USA). Pots were previously filled with a mixture of sterilized silice–vermiculite (1:1 (v/v)) inoculated or not with spores of G*lomus irregulare*. Plants were grown under controlled environmental conditions in a growth chamber (Conviron Model PGR15, Controlled Environments Limited, Winnipeg, Canada) at 22/17 °C day/night temperatures with a 16 h-photoperiod and a photon flux density of 500 $\mu mol\ m^{-2}\ s^{-1}$. Two weeks after seeding, seedlings were thinned to three plants per pot. Two hundred milliliters of N-free Hoagland's solution at 1/2 P concentration was added weekly to each pot. Plants were watered daily.

2.2. Treatments and experimental design

The experiment was set up as a complete randomized design with four replicates, two dates of harvest (28 and 56 days) and four inoculation treatments: *B. japonicum* (B); *B. japonicum* + *A. canadense* (B + A); *B. japonicum* + *G. irregulare* (B + M); *B. japonicum* + *A. canadense* + *G. irregulare* (B + A + M). Biochemical measurements were made on plants from four pots for each microbial treatment while growth and nodulation characteristics were measured on plants from four other pots. The experiment thus included a total of 64 pots: 4 replicates × 2 (1 pot for growth and nodulation measurements + 1 pot for biochemical analyses) × 4 microbial treatments × 2 dates of harvest.

2.3. Plant biomass, nodulation, Azospirillum and mycorrhizal evaluation

At the two harvest dates, roots and shoots from four pots for each treatment were separated and roots were gently washed under a stream of cold water to remove the substrate. Nodules were separated from roots by hand. Three parameters were measured to assess nodulation characteristics: (1) number of nodules; (2) nodules size; and (3) root depth for nodulation (Prévost et al., 2010). Nodules were separated in two classes according to their size