Applied Soil Ecology 44 (2010) 176–180

Contents lists available at ScienceDirect

Applied Soil Ecology

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

Effect of glyphosate on symbiotic N_2 fixation and nickel concentration in glyphosate-resistant soybeans

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ARTICLE INFO

Article history:
Received 3 April 2009
Received in revised form 22 November 2009
Accepted 2 December 2009

Keywords:
Glyphosate
Nickel
Symbiosis
Photosynthesis
Glyphosate-resistant soybean

ABSTRACT

Decreased biological nitrogen fixation in glyphosate-resistant (GR) soybeans has been attributed directly to toxicity of glyphosate or its metabolites, to N_2-fixing microorganisms. As a strong metal chelator, glyphosate could influence symbiotic N_2 fixation by lowering the concentration of nickel (Ni) that is essential for the symbiotic microorganisms. Evaluation of different cultivars grown on different soil types at the State University of Maringá, PR, Brazil during the summer 2008 revealed, significant decreases in photosynthetic parameters (chlorophyll, photosynthetic rate, transpiration and stomatal conductance) and nickel content with glyphosate use (single or sequential application). This work demonstrated that glyphosate can influence the symbiotic N_2 fixation by lowering nickel content available to the symbiotic microorganisms.

1. Introduction

Glyphosate is a non-selective, broad-spectrum metal chelating herbicide that inhibits the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), which is necessary for the synthesis of aromatic amino acids (Jaworski, 1972). In some circumstances plant pathogens contribute to infection of roots of glyphosate-susceptible plants by soil-borne microorganisms due to decreased production of plant protection compounds known as phytoalexins (Kremer et al., 2005).

Glyphosate-resistant (GR) soybeans are developed biotechnologically by introducing of the *cp4* gene coding for resistant forms of EPSP synthase (Duke et al., 1991). The introduction of this type of glyphosate resistance may have unforeseen consequences for symbiotic microorganisms associated with soybeans due to the translocation of glyphosate to important metabolic sinks such as root nodules (Reddy and Zablotowicz, 2003) and the exudation of relatively large quantities of glyphosate into the rhizosphere of GR soybeans (Duke, 1996; Kremer et al., 2005). The soybean nitrogen fixing symbiont, *Bradyrhizobium japonicum*, possesses a glyphosate-sensitive EPSP synthase and accumulates shikimic, hydroxybenzoic and protocatechuic acids (PCA) upon exposure to glyphosate which inhibits growth and induces death at high concentrations (Moorman et al., 1992; De Maria et al., 2006). The toxic effect of glyphosate to *B. japonicum* also has been attributed to the inability of the organism to synthesize aromatic amino acids. The loss of energy and fixed N_2 provided by *B. japonicum* may be significant factors responsible for reduced growth and yield in GR soybean (Moorman et al., 1992; Hernandez et al., 1999).

Herbicides can influence nitrogen metabolism through direct effects on the rhizobial symbiont (Zobiole et al., 2007) or indirectly by effecting the physiology of the host plant (Moorman, 1989). In addition, glyphosate affects the balance of IAA in GR soybeans, which leads to lower root nodulation by *B. japonicum* (Kremer and Means, 2009). Several metabolites or degradation products of glyphosate have been identified or postulated (Rueppel et al., 1977; Sprankle et al., 1978). Among these compounds are aminomethylphosphonic acid (AMPA), sarcosine and glycine (Hoagland, 1980). Chlorotic symptoms in GR soybean following glyphosate application have been attributed to the accumulation of AMPA (Reddy et al., 2004).

Nickel (Ni) is directly related to N_2 fixation, and increases hydrogenase activity in bacteroids isolated from nodules (Klucas et al., 1983). Urease is the only known Ni-containing enzyme in higher plants, although N_2-fixing microorganisms require Ni for

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doi:10.1016/j.apsoil.2009.12.003

the hydrogen uptake hydrogenase that processes hydrogen gas generated during N_2 fixation (Evans and Sorger, 1966; Taiz and Zeiger, 1998) and low levels of Ni in agricultural soils can limit the symbiotic hydrogenase activity of *Rhizobium leguminosarum* (Ureta et al., 2005).

The roles of Ni in plant metabolism remain mostly unknown; however effects attributable to Ni deficiency suggest that it may be involved in the transport of nutrients to the seed or grain and movement of Fe into plant cells (Brown et al., 1987). In nature, substantial amounts of H^+ are reduced to H_2 gas that can compete with the reduction of N_2 by nitrogenase electrons. In *Rhizobium*, 30–60% of the energy supplied to nitrogenase can be lost as H_2, decreasing the efficiency of N_2 fixation. *Rhizobia* that have the hydrogenase enzyme to cleave H_2 formed during fixation can generate electrons for N_2 reduction and increase the efficiency of N_2 fixation (Marschner, 1995).

These hydrogenases play a specific role in maintaining the energy efficiency of symbiotic nitrogen fixation. The synthesis of hydrogenases is dependent on a supply of Ni and a mechanism to "sense" that the substrate H_2 is available (Maier and Triplett, 1996). Glyphosate is a phosphonic acid (Franz et al., 1997) chelator of metallic cations (Jaworski, 1972; Kabachnik et al., 1974; Bromilow et al., 1993; Coutinho and Mazo, 2005) that could affect the availability of Ni and may explain the direct effect of glyphosate on N_2 fixation by symbiotic microorganisms. The objective of the present study was to investigate the effect of glyphosate on nodule formation and its interrelation with Ni in GR soybean plants.

2. Material and methods

2.1. Soil and growing conditions

The experiment was conducted in the greenhouse equipped with an evaporative cooling system (25–35: 20–22 °C day/night) under natural daylight conditions at the State University of Maringá, Paraná State, Brazil, between October 14, 2007 and February 15, 2008 (location: 23°25′S, 51°57′W). 5 dm^{-3} polyethylene pots were, filled with either a Typic Hapludox (75% clay, 16% sand, pH $CaCl_2$: 5.40, Al: 0.0, Ca: 8.22, Mg: 3.03, K: 0.47 cmolc dm^{-3}, P: 10.90, S: 5.47, Fe: 88.02, Zn: 11.98, Cu: 32.38, Mn: 95.04 mg dm^{-3} and C_{org}: 7.82 g dm^{-3}) or a Rhodic Ferralsol (21% clay, 71% sand, pH $CaCl_2$: 5.10; Al: 0.0, Ca: 1.85, Mg: 1.24, K: 0.26 cmolc dm^{-3}, P: 18.10, S: 27.06, Fe: 264.30, Zn: 1.73, Cu: 3.08, Mn: 32.82 mg dm^{-3} and C_{org}: 7.82 g dm^{-3}) soil. Characteristics of the soils, organic matter (C_{org}) and pH in $CaCl_2$ were determined as described by Embrapa (1997). The soils were collected from the A horizon and sieved to pass a (10 mesh screen). Independent of chemical analyses, samples of 10 kg the Typic Hapludox soil were fertilized with 100 mg K_2O and 250 mg P_2O_5 per kg of soil and samples of 10 kg the Rhodic Ferralsol soil was amended with 80 mg K_2O, 80 mg P_2O_5 and 1 mg $ZnSO_4$ per kg of soil.

2.2. Seed and glyphosate treatments

Seeds of near-isogenic normal and GR soybean varieties of early (BRS 242 GR and Embrapa 58), medium (BRS 245 GR and BRS 133), and late (BRS 247 GR and BRS 134) maturity groups were treated with 40 g carboxim + 40 g thiram L^{-1} fungicides and 13.5 g Co + 135.0 g Mo L^{-1} 100 kg^{-1} seeds. Seeds were then inoculated with a double commercial rate at 300 mL 100 kg^{-1} of seeds of a culture of *Bradyrhizobium elkanii*, strains SEMIA 587 and SEMIA 5019 at a concentration of 5×10^9 *Rhizobia* per gram. Six seeds per pot were sown at 3 cm depth and thinned to three plants per pot at the one-leaf stage.

The commercially formulated isopropylamine salts of glyphosate (480 g a.e. L^{-1}) was applied to GR soybean: T1—single application of glyphosate (1200 g a.e. ha^{-1}) at the four-leaf stage (25 days after sowing, DAS); T2—sequential application (600 + 600 g a.e. ha^{-1}) at the four-leaf and five-leaf stage (25 DAS and 35 DAS); T3—without glyphosate; and T4—non-GR parental line. The non-GR parental line was considered the treatment control for each cultivar, and did not receive any glyphosate.

Plants were spraying outside the greenhouse, using a backpack sprayer with SF110.02 nozzles, under 2 kgf cm^{-2} of CO_2 at 190 L ha^{-1} to prevent runoff. Air temperature was between 25 and 29 °C, relative humidity was between 80% and 89%, wet soil and wind speed between 5 and 10 km h^{-1} under open sky without cloudiness during glyphosate treatment. After glyphosate application, plans were returned to the greenhouse and irrigated the following day to keep the soil moist, to ensure absorption of the herbicide. The pots were irrigated daily to keep the soil moist, and hand weeded for weed control.

2.3. Data collection

The last fully expanded trifolium (diagnostic leaf) was collected from three plants in each pot to at R1 growth stage to determine the nickel concentration. After dry digestion, Ni was measured by ICP (inductively coupled plasma spectrometry) spectrometry (AES PerkinElmer). The R1 growth stage was slightly different for the cultivars maturity pairs: BRS 242 GR (46 DAS); BRS 245 (54 DAS) and BRS 247 (65 DAS). Prior to collecting leaves, photosynthetic rates (A) of the diagnostic leaf of three different plants in each pot were determined between 7:00 and 11:00 am by infrared gas analysis (IRGA, ADC Model LC*pro+*, Analytical Development Co. Ltd, Hoddesdon, UK).

The chlorophyll index (CI) was measured with a Minolta SPAD-502 meter to measure absorption at 650 and 940 nm wavelengths to estimate chlorophyll concentration (Singh et al., 2002; Richardson et al., 2002; Pinkard et al., 2006). SPAD readings were taken randomly on leaf mesophyll tissue only (with veins avoided) of the terminal leaflet of the diagnostic leaf. Two leaves were chosen per plant in each pot and measurements were averaged to provide a single CI reading per pot. After CI and SPAD assessments, the shoots were clipped close to the ground and roots were carefully removed from soil, washed under running water, packed in paper bags to dry in an air circulation oven at 65–70 °C weighed after a constant dry weight was achieved. Nodules were counted from three plants in each pot immediately after the roots were washed and then placed in the air circulation oven to determine the nodule dry biomass.

2.4. Statistical analyses and experimental design

Main effects and two factor interactions accounted for 96 experimental units distributed in a completely randomized block experimental design. Treatments were combined in a $4 \times 3 \times 2$ factorial scheme with four replicates. The first factor was represented by four herbicide treatments (T1, T2, T3 and T4), the second factor was the cultivar maturity groups and the last factor was soil type. The three near-isogenic pairs of soybean cultivars consisting of the glyphosate-resistant and normal parent of each were selected from early, medium, and late maturity groups commonly grown in Brazil. Embrapa 58 and BRS 242 GR are early maturity cultivars, BRS 133 and BRS 245 GR are medium maturity cultivars, and BRS 134 and BRS 247 GR are late maturity cultivars.

The data errors passed the normality test of Shapiro and Wilk (1965). All data were subjected to analysis of variance and the