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Strategies to promote early nodulation in soybean under drought

Paula Cerezini[a], Biana Harumi Kuwano[a], Michele Barbosa dos Santos[b], Fernanda Terassi[c], Mariangela Hungria[d], Marco Antonio Nogueira[d,*]

[a] *Department of Agronomy, Universidade Estadual de Londrina, Londrina, PR, Brazil*
[b] *Department of Chemistry, Universidade Estadual de Londrina, Londrina, PR, Brazil*
[c] *Universidade Norte do Paraná, Londrina, PR, Brazil*
[d] *Embrapa Soja, Londrina, PR, Brazil*

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ABSTRACT

Biological nitrogen fixation (BNF) is a sustainable process that dismisses the use of supplemental N-fertilizers in soybean [*Glycine max* (L.) Merrill]. Strategies to provide early nodulation may increase the effectiveness of BNF under stressing conditions like drought. We assessed the effects of inoculation of *Bradyhizobium*, co-inoculation of *Bradyrhizobium* and *Azospirillum*, and addition of microbial secondary metabolites (MSM) on nodulation parameters and soybean yield in four field experiments in two growing seasons, 2013/14 and 2014/15, in Southern Brazil. The treatments were: non-inoculated (Ni) control; Ni + N-fertilizer (100 kg ha^{-1} at sowing and 100 kg ha^{-1} at full flowering, as urea); Inoculated with *Bradyrhizobium* (I); Co-inoculated with *Bradyrhizobium* + *Azospirillum brasilense* (Co-I); Co-I + microbial secondary metabolites (MSM) and I + MSM. All trials were rainfed and the second trial in 2014/15 was severely affected by drought and high temperatures. The co-inoculation with *Azospirillum* increased the soybean nodulation at early developmental stages and resulted in higher shoot N accumulation and plant growth, especially under drought. The addition of MSM attenuated the effect of drought on nodulation and in one trial increased the grain yield by 15% and 7% in relation to the N-fertilizer and sole inoculation with *Bradyrhizobium*, respectively. The strategy of co-inoculation stimulates an early nodulation and helps the maintenance of nodulation under drought; moreover, the addition of MSM improves nodulation and may increase the grain yield.

1. Introduction

Soybean [*Glycine max* (L.) Merrill] is the most widely grown oilseed worldwide, having reached a global 318.25 million metric ton in 2014/15 crop season (USDA, 2015). Currently, Brazil is the second largest producer, with 96.04 million metric ton in 2014/15 (CONAB, 2015). The success of the soybean crop in Brazil is in part attributed to the symbiosis with *Bradyrhizobium* and the biological nitrogen fixation (BNF) process, which provides nearly all N required and ensures high yields without supplemental mineral N (Alves et al., 2003; Hungria et al., 2006a).

However, drought and high temperatures (Hungria and Vargas, 2000; Sinclair et al., 2007; Chalk et al., 2010), or incompatibility of the microsymbiont with chemicals in the seed treatment (Hungria et al., 2015) may impair the BNF effectiveness. This biological process is negatively affected by drought even before the transpiration rate and photosynthesis (King and Purcell, 2005; Sinclair et al., 2007; Arrese-Igor et al., 2011). The impact of drought on plant and microsymbiont depends on the intensity, duration, and the plant developmental stage (Christophe et al., 2011). More drastic effects on nodulation and grain yield generally occur during the establishment of the symbiosis and formation of nodules (V2), full flowering (R2), or grain filling (R5) (Streeter, 2003; González-Dugo et al., 2010; Christophe et al., 2011).

Strategies to stimulate the early establishment of the symbiosis may result in increases in nodulation, nodule occupancy and more effective BNF, which might result in more even yields under drought (Hungria et al., 2006a; Chibeba et al., 2015). The co-inoculation of *Bradyrhizobium* with plant growth-promoting rhizobacteria like *Azospirillum* has been a beneficial strategy to promote early nodulation, BNF, and improve the crop's performance

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Abbreviations: BNF, biological nitrogen fixation; CFU, colony forming units; DAE, days after emergence; LCO, lipo-chitooligosaccharides; NDVI, normalized difference vegetation index; PET, potential evapotranspiration; MSM, microbial secondary metabolites.

* Corresponding author at: P.O. Box 231, 86001-970 Londrina, PR, Brazil.
E-mail address: marco.nogueira@embrapa.br (M.A. Nogueira).

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and grain yield (Hungria et al., 2013; Chibeba et al., 2015). These results are attributed to the ability of plant growth-promoting rhizobacteria to produce phytohormones that stimulate the root system for exploring the surrounding soil (Saharan and Nehra, 2011), and may increase tolerance to abiotic stresses like drought. Field experiments performed on Oxisols in different Brazilian regions have shown that co-inoculation with *Bradyrhizobium* and *Azospirillum* improves the soybean grain yield in relation to the sole inoculation with *Bradyrhizobium* or supplemental mineral N (Hungria et al., 2015).

Lipo-chitooligosaccharides (LCOs) are microbial secondary metabolites (MSM) essential for communication and establishment of the *Bradyrhizobium*-legume symbiosis (Cullimore et al., 2001; Gough, 2003). Although not acting directly on the growth and development of the host plant, MSM stimulate the symbiosis and promote the microbial growth, among other effects (Davies, 1992). Under greenhouse conditions, the use of MSM of rhizobia increased the nodulation of soybean by 21% in number and by 12% in mass of nodules, whereas under field conditions, MSM associated with rhizobial inoculant increased the number of nodules by 23.6% and the grain yield by 4.8% as compared with the sole inoculation with *Bradyrhizobium* (Marks et al., 2013).

There are few studies on the combination of these sustainable technologies with the inoculation of *Bradyrhizobium* in soybean limited by drought. The aim of this study was to evaluate the effect of co-inoculation of *Bradyrhizobium* with *Azospirillum* and addition of LCOs on traits related to BNF and grain yield of soybean under different hydric regimes in field experiments.

2. Material and methods

2.1. Experimental sites

Four field experiments were conducted in 2013/14 and 2014/15 cropping seasons. In 2013/14, two trials were installed in Londrina, PR, Brazil in a clay soil (23°11′S, 51°11′W, 620 m a.s.l., Cfa Köpen-Geiger climate, Rhodic Eutrudox soil type, USDA), sown at different dates. The first, 14 Oct. 2013 is considered early sowing, while the second, 23 Nov. 2013 is considered late sowing in the regular local sowing calendar (EMBRAPA, 2010). In 2014/15, the trials were repeated in Londrina (sown on 4 Nov. 2014), and in a different edafoclimatic condition in Ponta Grossa in a sandy-loam soil (25°13′S, 50°01′W, 880 m a.s.l., Cfb Köpen-Geiger climate type, Typic Hapludox soil type, USDA) sown on 18 Nov. 2014. Both soils have previously established and naturalized populations of *Bradyrhizobium*, since they have been cropped with soybean for more than 30 years.

Before sowing, topsoil samples (0–20 cm) were collected for chemical, granulometric, and microbiological analyses (Table 1). The soil rhizobial populations were estimated by the most probable number (MPN) technique (Vincent, 1970), using soybean cultivar BRS 360 RR as trap plant.

2.2. Experimental design and treatments

The experimental design was in randomized blocks with six replications, and the following treatments: non-inoculated (Ni) control; Ni + N-fertilizer (100 kg ha^{-1} at sowing and 100 kg ha^{-1} at full flowering, as urea); Inoculated with *Bradyrhizobium* (I); Co-inoculated with *Bradyrhizobium* + *Azospirillum brasilense* (Co-I); Co-I + Microbial secondary metabolites (MSM); I + MSM. In 2013/14, the soybean cultivar was BMX Potência RR; in 2014/15, the cultivar was BRS 359 RR in both sites. Plots consisted of eight lines of 6.0 m in length spaced 0.50 m apart and density of 280,000 plants ha^{-1}.

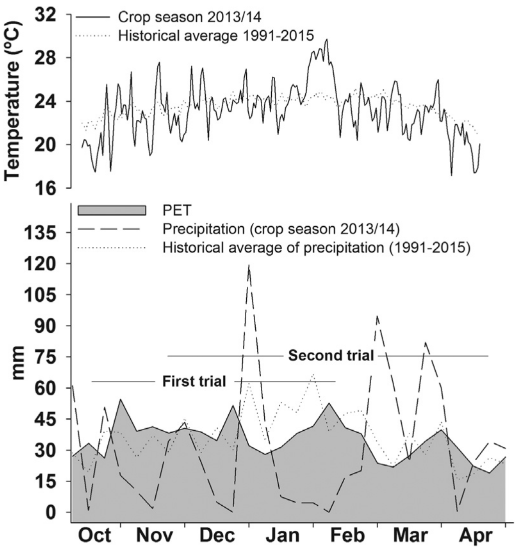

Fig. 1. Daily average temperature (°C), potential evapotranspiration (PET), precipitation (mm) every 7 days during the crop season period (2013/14), and historical (1991–2015) average temperature and precipitation in Londrina. Data on historical averages were adapted from Sibaldelli and Farias (2015).

The *Bradyrhizobium* inoculants contained the strains SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7) of *B. japonicum* and *B. diazoefficiens*, respectively (with 1.2×10^9 colony forming units (CFU) mL^{-1}). The dose of inoculant was calculated to deliver 1.2×10^6 cells per seed. Treatments with co-inoculation received *Azospirillum brasilense* strains Ab-V5 and Ab-V6 (1×10^8 CFU mL^{-1}) in a dose to deliver 1.2×10^5 cells per seed. Treatments with MSM received the LCOs at 100 mL 50 kg^{-1} seeds, obtained as described in Marks et al. (2015).

2.3. Field management

Plants received 250 or 300 kg ha^{-1} of N–P–K (0–20–20) in furrow, simultaneously the sowing in both trials, in 2013/14 and 2014/15, respectively. No N-fertilizer was applied, except for the N-fertilized control plots. Seeds were not treated with fungicides or insecticides. At V4 stage (Fehr and Caviness, 1977), plants were leaf-sprayed with 20 g ha^{-1} of Mo ($Na_2MoO_4 \cdot 2H_2O$) and 2.5 g ha^{-1} of Co ($CoCl_2 \cdot 6H_2O$).

The potential evapotranspiration (PET), water balance for Londrina (calculated by Pan Evaporation Method, based on the average daily values for 2013/2014) and the mean daily temperatures and rainfall during the 2013/14 crop season trials, in addition to the historical average for rainfall and air temperature, are shown (Fig. 1). During the crop cycle in 2014/15, the mean temperature ranged between 19.1–29.0 °C and 15.1–27.5 °C, and the rainfall was 718 mm and 889 mm in Londrina and Ponta Grossa, respectively.

2.4. Plant sampling, analysis, and grain yield

The dynamics of nodulation were evaluated in the trials performed in 2013/14 crop season. Five plants were collected on the second and on the seventh line of the plot, i.e., 2 or 3 plants each side, within 0.5 m in the line, and the next sampling performed after 0.4 m forward to avoid effects of compensatory growth caused by gaps resulting from the previous sampling. Samples were taken at 10, 15, 25, 45, 65, and 95 days after emergence (DAE) for the first trial and at 12, 18, 27, 40, 70, and 115 DAE for the second trial, corresponding to V1, V2, V4, R2, R4-5, and R6-7 growth stages (Fehr and Caviness, 1977). The shoots were separated from roots, and dried at 65 °C for 72 h for assessment of shoot and root dry weights. The

Table 1
Chemical characteristics, granulometric fractions, and population of *Bradyrhizobium* at the 0–20 cm topsoil layer in the experimental field sites before sowing.

Site	Crop season	pH	Al + H	K	Ca + Mg	P	N-NH$_4$$^+$	N-NO$_3$$^-$	Total-N	C	Clay	Silt	Sand	*Bradyrhizobium* cells
			cmol$_c$ dm^{-3}			mg dm^{-3}	mg kg^{-1}		g kg^{-1}	g dm^{-3}	g kg^{-1}			MPN g $^{-1}$
Londrina	2013/14	5.7	3.83	0.95	7.55	22.0	11.61	2.38	1.57	14.01	753	166	80.5	2.4×10^5
Londrina	2014/15	5.4	3.53	0.61	6.40	20.0				12.70				3.5×10^4
Ponta Grossa	2014/15	5.1	3.89	0.45	4.42	15.0	9.26	4.33	1.55	18.70	238	30.0	732	2.9×10^4

pH (CaCl$_2$); P (Mehlich I); H + Al (SMP); Al, Ca, Mg, K (KCl). MPN = Most Probable Number.

nodules from the root crown were counted and weighed for assessment of the number and dry weight of nodules. Shoots were ground (<10 mesh) and total shoot N content was determined in sulfuric extracts by the green salicylate colorimetric method (Searle, 1984).

Physiological parameters were measured with a portable gas-exchange meter (LCpro-SD, ADC BioScientific Ltd., UK) during the full flowering of plants (R2) for the two trials in 2013/14 crop season. Determinations included stomatal conductance, net photosynthetic rate, transpiration rate and intercellular CO$_2$ concentration. SPAD units were determined in the recently expanded trifoliolates with a portable meter, model SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan), and converted into leaf-chlorophyll concentration (μg cm^{-2}) (Kaschuk et al., 2010). These analyses were performed in the same trifoliolates, in two plants per plot, in the morning (9–11 a.m.). The NDVI (normalized difference vegetation index) was measured using a 505 Handheld device GreenSeeker® sensor (Ntech Industries, Inc., USA) in the same day. The NDVI values range from −1 to 1 so that the closer to 1 the greener is the vegetation.

Grain yield at maturation (R8) was determined by harvesting a central area of each plot (8 m^2). Harvests occurred on 26 Feb. 2014 and on 23 Apr. 2014, for the first and second trial, respectively. Cleaned seeds were weighed and the values corrected to 13% of moisture, after determination of humidity in a grain moisture tester (Gehaka Agri model G800, São Paulo, Brazil).

In the 2014/15 crop season, plants were sampled only at 35 DAE in Londrina and at 54 DAE in Ponta Grossa for assessments of nodulation (number and dry weight), and for analysis total shoot N content. At R8 stage (12 Mar. 2015 in Londrina, and 31 Mar. 2015 in Ponta Grossa), plants were harvested for analysis of grain yields, as previously described.

2.5. Statistical analyses

Data were submitted to tests of normality and homogeneity of variances for each trial, followed by analysis of variance (ANOVA) with application of F test ($P \leq 0.05$), and means comparison by Tukey's test ($P \leq 0.05$).

3. Results

The physiological responses of plants measured at the second trial in 2013/14 confirmed the stressing conditions caused by drought associated to high temperatures. There was reduction in gas exchanges, photosynthesis (−32%) and transpiration rates (−23%), stomatal conductance (−50%) and intercellular CO$_2$ (−10%) compared with the first trial, in the average of all treatments. The treatments had only minor effects on the physiological measurements (Table 2). In the first trial, the non-inoculated plants showed higher net photosynthesis (17%), transpiration rate (8%), stomatal conductance (40%), and intercellular CO$_2$ (8%) in relation to the other treatments. In the second trial, there was significant difference only for stomatal conductance, which was 26% higher in plants inoculated with *Bradyrhizobium*; and chlorophyll content, 7% higher in plants inoculated with *Bradyrhizobium* + MSM compared with the non-inoculated plants.

3.1. Nodulation in 2013/14 crop season

In the first trial, the control plants that received mineral N showed a consistent reduction in number and dry weight of nodules, in all samplings (Fig. 2A and 2C). There was more number of nodules in plants co-inoculated with *Bradyrhizobium* + *Azospirillum*, than the non-inoculated plants at 10 (33%) and 65 DAE (38%); compared with plants that received N fertilization, nodulation was higher (78% in mass and 67% in number) at 10 DAE. At 25 DAE, plants inoculated with *Bradyrhizobium* + MSM showed 30% more nodules than plants only inoculated with *Bradyrhizobium* and twice as more than plants that received mineral N. The total shoot N content in plants showed similar trend as shoot dry weight (not shown), wherein plants with mineral N showed higher initial N concentration in leaves in most of the sampling dates, followed by a sharp decrease at 90 DAE (Fig. 2E).

For the second trial, there was a general decrease in nodulation (-52% in mass, −31% in number) compared with the first trial (Fig. 2B and 2D), but the negative effect of mineral N on nodulation was less evident. At 12 DAE, plants inoculated with *Bradyrhizobium* and *Bradyrhizobium* + MSM had 20% more mass and 31% more number of nodules than the non-inoculated plants. At 40 and 115 DAE plants inoculated with *Bradyrhizobium* + MSM stood out for number of nodules, showing 28% and 21% more than plants inoculated only with *Bradyrhizobium*, respectively. However, higher dry weight of nodules (37%) at 40 DAE was observed in plants co-inoculated with *Azospirillum*. Considering the total shoot N at 27 DAE (Fig. 2F), the plants co-inoculated with *Azospirillum* accumulated 97% more N than the N-fertilized plants. At 18 and 40 DAE, non-inoculated plants and inoculated with *Bradyrhizobium* stood out for total shoot N. At 70 DAE higher values were observed in non-inoculated and N-fertilized plants, but both followed a decrease at 115 DAE, when the plants inoculated with *Bradyrhizobium* and co-inoculated with *Azospirillum* accumulated, respectively, 38% and 21% more N than N-fertilized plants (Fig. 2F).

3.2. Nodulation in 2014/15 crop season

The application of N-fertilizer reduced the number and dry weight of nodules in both trials (Table 3), by −35% and −42%, respectively, in Londrina, and by −28% and −19% in Ponta Grossa, compared with the average of the other treatments. The total shoot N content in plants grown in Londrina was 20% higher when inoculated only with *Bradyrhizobium* compared with plants of treatments non-inoculated, co-inoculated + MSM and inoculated with *Bradyrhizobium* + MSM. In Ponta Grossa, there was 30% more total shoot N content in plants with mineral N, compared with plants non-inoculated and co-inoculated with *Azospirillum* + MSM (Table 3).

Table 2
Net photosynthetic and transpiration rates, stomatal conductance, intercellular CO_2 concentration, NDVI (normalized difference vegetation index), and chlorophyll content in soybean analyzed in full flowering (R2) period of plants in two trials in Londrina in 2013/14 crop season.

Treatments	Photosynthetic rate μmol CO_2 m^{-2} s^{-1}	Transpiration rate mmol H_2O m^{-2} s^{-1}	Stomatal conductance mol H_2O m^{-2} s^{-1}	Intercellular CO_2 μmol CO_2 mol^{-1}	NDVI	Chlorophyll μg cm^{-2}
	First trial					
Non-inoculated	15.78 a	3.85 a	0.340 a	227.9 a	0.878 a	19.68 a
Non-inoculated + N-fertilizer (200 kg ha^{-1})[a]	13.32 b	3.61 a	0.217 b	211.9 abc	0.889 a	20.04 a
Inoculated with *Bradyrhizobium*	12.59 b	3.38 a	0.210 b	213.6 abc	0.877 a	19.56 a
Co-inoculation with *Bradyrhizobium* + *Azospirillum*	13.43 b	3.83 a	0.186 b	202.9 bc	0.881 a	20.51 a
Co-inoculation with *Bradyrhizobium* + *Azospirillum* + MSM	12.35 b	3.49 a	0.212 b	219.0 ab	0.878 a	20.15 a
Inoculated with *Bradyrhizobium* + MSM	13.80 b	3.33 a	0.191 b	200.2 c	0.881 a	20.63 a
p-value	<0.01	0.0305	<0.01	<0.01	0.4869	0.2001
CV (%)	7.21	8.82	20.51	4.48	1.27	4.16
	Second trial					
Non-inoculated	9.43 a	2.62 a	0.106 b	184.3 a	0.645 a	19.46 b
Non-inoculated + N-fertilizer (200 kg ha^{-1})	9.67 a	2.77 a	0.106 b	192.8 a	0.658 a	20.04 ab
Inoculated with *Bradyrhizobium*	10.80 a	2.80 a	0.138 a	196.4 a	0.610 a	19.56 ab
Co-inoculation with *Bradyrhizobium* + *Azospirillum*	9.02 a	2.79 a	0.114 ab	193.0 a	0.632 a	20.51 ab
Co-inoculation with *Bradyrhizobium* + *Azospirillum* + MSM	9.84 a	2.78 a	0.099 b	200.4 a	0.643 a	19.71 ab
Inoculated with *Bradyrhizobium* + MSM	9.40 a	2.50 a	0.087 b	187.9 a	0.638 a	20.85 a
p-value	0.0652	0.6661	<0.01	0.1143	0.0688	0.0269
CV (%)	9.88	13.66	14.40	5.19	4.02	3.88

MSM – Microbial secondary metabolites; CV – Coefficient of variation; [a]N-fertilizer (100 kg ha^{-1} at sowing and 100 kg ha^{-1} at full flowering, as urea); Means (n = 6) followed by the same letters in the same column do not differ one another (Tukey, $P \leq 0.05$).

Table 3
Number of nodules, nodule dry weight, total shoot N content, at 35 days after emergence (DAE) in Londrina and at 54 DAE in Ponta Grossa, in soybean plants (BRS 359 RR) cultivated in 2014/15 crop season.

Treatments	Londrina			Ponta Grossa		
	Number of nodules (n° $plant^{-1}$)	Nodule dry weight (mg $plant^{-1}$)	Total shoot N content (mg N $plant^{-1}$)	Number of nodules (n° $plant^{-1}$)	Nodule dry weight (mg $plant^{-1}$)	Total shoot N content (mg N $plant^{-1}$)
Non-inoculated	21.4 a	45.70 a	138.8 b	115 ab	430.1 ab	206.5 b
Non-inoculated + N-fertilizer (200 kg ha^{-1})[a]	12.4 b	17.15 b	163.4 ab	86.0 b	356.2 b	299.5 a
Inoculated with *Bradyrhizobium*	17.5 a	40.22 a	174.5 a	108 ab	392.1 ab	259.7 ab
Co-inoculation with *Bradyrhizobium* + *Azospirillum*	18.4 a	43.34 a	155.9 ab	114 ab	425.3 ab	248.9 ab
Co-inoculation with *Bradyrhizobium* + *Azospirillum* + MSM	19.0 a	33.76 a	141.7 b	135 a	496.4 a	209.3 b
Inoculated with *Bradyrhizobium* + MSM	19.5 a	39.99 a	141.6 b	123 ab	460.1 ab	277.2 ab
p-value	<0.01	<0.01	<0.01	0.017	0.0423	<0.01
CV (%)	15.04	19.07	9.83	19.00	17.13	16.94

MSM – Microbial secondary metabolites; CV – Coefficient of variation. [a]N-fertilizer (100 kg ha^{-1} at sowing and 100 kg ha^{-1} at full flowering, as urea); Means (n = 6) followed by the same letters in the same column do not differ one another (Tukey, $p \leq 0.05$).

Table 4
Soybean grain yield in trials carried out in Londrina during 2013/14, and in Londrina and Ponta Grossa during 2014/15.

Treatments	Trials			
	1 – Londrina (2013/14)	2 – Londrina (2013/14)	3 – Londrina (2014/15)	4 – Ponta Grossa (2014/15)
	(kg ha^{-1})			
Non-inoculated	3505 a	1322 ab	3052 bc	2825 b
Non-inoculated + N-fertilizer (200 kg ha^{-1})[a]	3453 a	1454 a	3126 bc	3126 a
I (Inoculated with *Bradyrhizobium*)	3433 a	1119 abc	3360 ab	2927 ab
I + Azo (co-inoculation with *Bradyrhizobium* and *Azospirillum*)	3364 a	768 c	2930 c	2830 b
I + Azo + MSM (microbial secondary metabolites)	3393 a	991 abc	2937 c	2878 b
I + MSM	3433 a	888 bc	3596 a	3003 ab
p-value	0.9457	<0.01	<0.01	<0.01
CV (%)	7.25	23.9	5.73	4.58

MSM – Microbial secondary metabolites; CV – Coefficient of variation. [a]N-fertilizer (100 kg ha^{-1} at sowing and 100 kg ha^{-1} at full flowering, as urea); Means (n = 6) followed by the same letters in the same column do not differ one another (Tukey, $p \leq 0.05$).

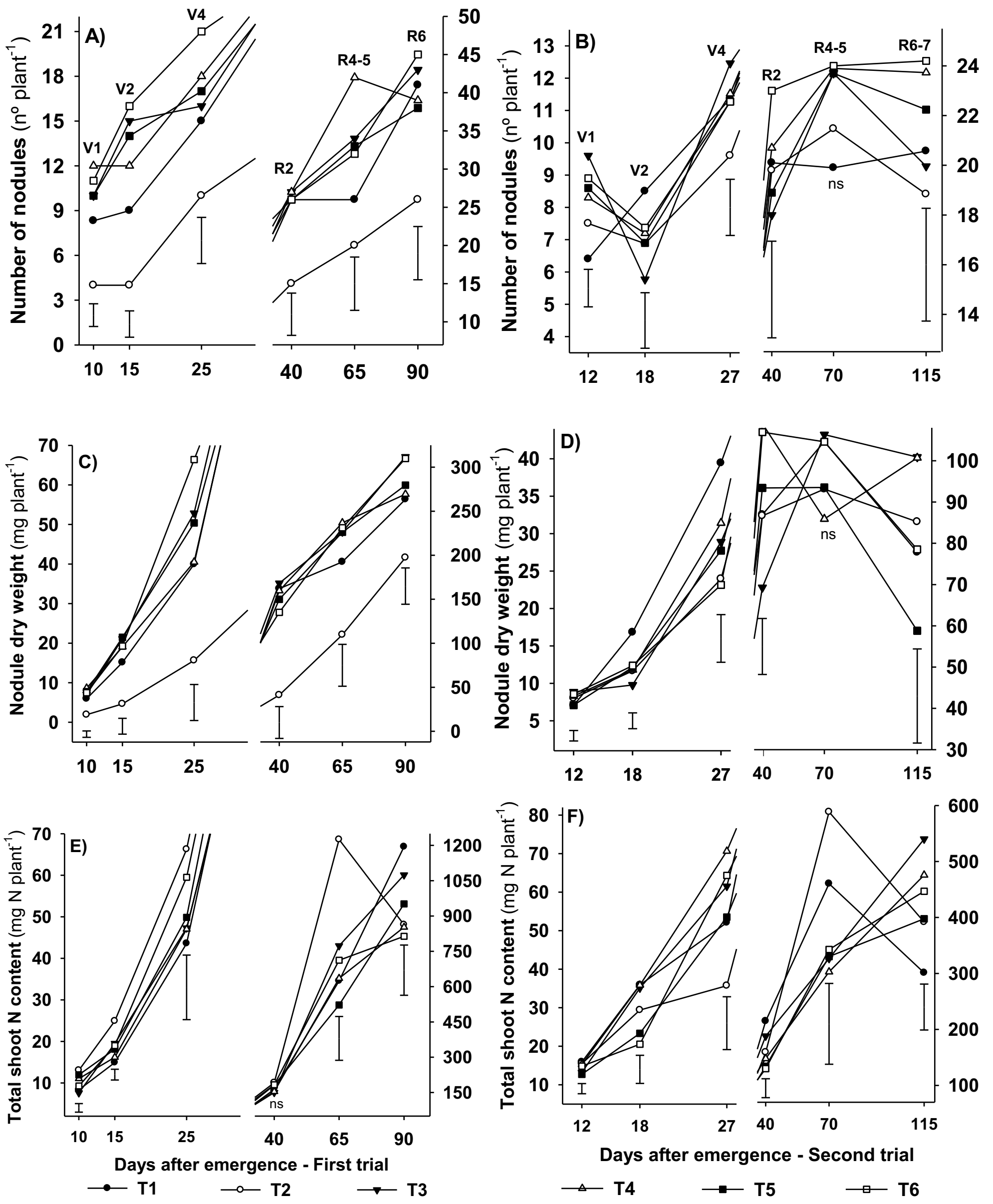

Fig. 2. Number of nodules (A and B); nodule dry weight (C and D) in root crown in different days after emergence (DAE), and total shoot N content (E and F) in soybean plants in trials performed in Londrina in crop 2013/14: first trial (Oct/2013–Feb/2014) and second trial (Nov/2013–Apr/2014). —●— T1 – non-inoculated (Ni) control; —○— T2 – Ni + N-fertilizer (100 kg ha^{-1} at sowing and 100 kg ha^{-1} at full flowering, as urea); —▼— T3 – Inoculated with *Bradyrhizobium* (I); —△— T4–Co-inoculation with *Bradyrhizobium* spp. + *Azospirillum brasilense* (Co-I); —■— T5–Co-I + Microbial secondary metabolites (MSM); —□— T6–I + MSM. Vertical bars represent the standard deviation (SD) for Tukey's test ($P \leq 0.05$) ($n = 6$).

3.3. Grain yield

In 2013/14, there was no effect of treatments on grain yield in the first trial, which averaged 3430 kg ha^{-1} (Table 4). In the second trial, the grain yield dropped severely as result of drought during the initial developmental stage and at full flowering, and averaged 1090 kg ha^{-1}, 68% less than the average of first trial (Table 4). In this case, plants that received mineral N had higher yield, but not differing from plants non-inoculated, inoculated with *Bradyrhizobium*, and co-inoculated with *Azospirillum* + MSM.

In 2014/2015, plants inoculated with *Bradyrhizobium* + MSM showed the highest yield in Londrina, but did not differ from plants inoculated only with *Bradyrhizobium*. In Ponta Grossa, the N-fertilized plants increased the grain yield by 10% compared with non-inoculated plants, co-inoculated with *Azospirillum* and co-inoculated + MSM (Table 4).

4. Discussion

The use of biofertilizers and biomolecules in the soybean production system helps to reduce costs and minimize environmental impacts caused by mineral N-fertilizers. Biofertilizers benefit plants by means of BNF (Alves et al., 2003; Hungria et al., 2006a), production of phytohormones (Saharan and Nehra, 2011), phosphate solubilization (Saikia et al., 2010), and induction of plant resistance to biotic and abiotic stresses (Gurska et al., 2009). Studies have confirmed the effectiveness of co-inoculation with *Bradyrhizobium* and *Azospirillum* in increasing soybean grain yield (Hungria et al., 2013, 2015) and stimulating earlier nodulation (Chibeba et al., 2015), and also positive effects of MSM on soybean and corn yields (Marks et al., 2013, 2015).

In the present work, there was a clear influence of the climate on the performance of soybean and on attributes related to BNF, when a dry period and high air temperatures that reached 36.6 °C occurred in the 2013/14 crop season and impaired mainly the second trial. During this season, the air temperature was far above the historical averages, whereas the rainfall was lower during some critical stages of the second trial, i.e., just after the sowing (V2) and during the flowering (R1-R3). Water stress in the second trial was confirmed by physiological data like photosynthesis and transpiration rates, which decreased in comparison with the first trial. According to Flexas et al. (2004), the stress level can be quantified by the stomatal conductance, wherein values ≥ 0.2 mol H_2O m^{-2} s^{-1} represents well watered plants; between 0.1–0.2 mol H_2O m^{-2} s^{-1} moderate drought stress, and ≤ 0.1 mol H_2O m^{-2} s^{-1} severe drought stress. Considering this classification, the plants in the second trial were under moderate drought stress at R2 stage, what is corroborated by lower NDVI indices. This environment also resulted in a strong decrease in nodulation and yield.

The soybean crop reaches the maximum hydric needs between flowering and grain filling, 7–8 mm day^{-1} (EMBRAPA, 2010). In studies performed in Londrina along 15 cropping seasons, the maximum grain yield was obtained with 650–700 mm of water sheet, well distributed throughout the crop cycle (Farias et al., 2010). In our study during the 2013/14 the crop season, the total rainfall was 496 mm in the first trial, and 695 mm in the second. However, there was an uneven distribution during the second trial, with only 18 mm in 20 days during V2–V4, a critical period of plant development, when the symbiosis is being established. Water restriction persisted in the R1-R5 (early-flowering and beginning of grain formation), with only 30 mm accumulated in 40 days. In this period, the plants of the first trial were at advanced reproductive stages (R6-R7), and were not as severely affected by drought as the plants in the second trial.

The nodulation in the first trial of 2013/14 increased at initial phases in plants co-inoculated with *Azospirillum*, which is critical for an early establishment of the symbiosis (Chibeba et al., 2015). There was also a positive effect of the MSM, as previously observed (Marks et al., 2013). In the second trial, the early stages of plant development and the establishment of the symbiosis occurred under dry conditions that severely decreased the nodulation in comparison with the first trial, leading to a consistently lower nodulation along all over the crop cycle. Even so, the inoculation with *Bradyrhizobium*, either as sole inoculation, co-inoculated with *Azospirillum*, or added with MSM, provided earlier nodulation. However, co-inoculation with *Azospirillum* stimulated more the mass of nodules, whereas the MSM stimulated the number. Such differences seem to be related to the mechanisms of actions, in which *Azospirillum* mainly act by hormonal effect, increasing the nodule size and number (Chibeba et al., 2015; Hungria et al., 2013), whereas MSM intermediate the signaling between *Bradyrhizobium* and the host plant via LCOs, increasing the number of nodules (Cullimore et al., 2001; Marks et al., 2013). The stimulation of nodulation by MSM confirms its key role in the formation and establishment of *Bradyrhizobium*-Legumes symbiosis, as observed in *Medicago* spp. (Cullimore et al., 2001).

The co-inoculation provided more accumulation of N in shoots at the early stages of plant development. *Azospirillum* is known for improving plant traits that may help to cope with water deficit, like branching of roots and increased density of root hairs (Cassán and Garcia De Salomone, 2008; Hungria et al., 2015). Consequently, there are also benefits to the BNF and plant growth. Chibeba et al. (2015) observed that the beneficial effects of *Bradyrhizobium*-*Azospirillum* co-inoculation were more evident in the field than in greenhouse, suggesting that *Azospirillum* may help plants to overcome environmental stresses in the field. Hungria et al. (2015) also confirmed this effect, wherein *Azospirillum*-*Bradyrhizobium* co-inoculation increased the grain yield of soybean under moderate water restriction.

The addition of N-fertilizer in our study decreased the nodulation when the availability of water was not restrictive, corroborating Hungria et al. (2006b) wherein the application of 200 kg ha^{-1} of N-fertilizer reduced the nodulation and the benefits of BNF, and did not increase yields. The non-inoculated plants, on the other hand, showed a similar or even higher nodulation than the inoculated treatments. This is because all experimental areas have been cropped with soybean for more than 30 years and have a high population of pre-established bradyrhizobia in the soil, as revealed the analysis for bradyrhizobial population. However, although the population established is able to colonize and promote nodulation, they might not be as effective as the strains just added via inoculants. Moreover, the nodulation process is usually delayed when relying only on the rhizobial population established in the soil, which may affect the plant performance and grain yield. In the case of rainfed crops, an early establishment of the symbiosis is very important if any restrictive factor like drought occurs thereafter. In fact, inoculation in areas with established bradyrhizobial population have shown an average increase in yields of about 8% (Hungria et al., 2013).

The negative effect of water restriction on soybean depends on its phenological stage (Avila et al., 2013; Ku et al., 2013). Drought severity on grain yield may be alleviated when it occurs at early phases, because plants would have time to recover, but occurrence at reproductive stages may lead to deeper drops in yields, depending on the intensity (Chalk et al., 2010). Soybean plants assimilate about 20% of total N until early flowering stage (R1 stage), and 80% of N during the reproductive stage after R2 (Ohyama et al., 2013). Therefore, the continuous assimilation of nitrogen via BNF during the vegetative stage and after initial reproductive stage is essential for plant performance and seed yield (Ohyama et al., 2013).

In the second trial of 2013/14, the occurrence of drought at two critical stages of soybean development (V2–V4 and R1–R5) affected the BNF process, with reduction of N accumulation, and expressive reduction of yield by 68% less than the first trial. In this case, the treatment with mineral N showed some gain in yield over two inoculated treatments. As BNF is vulnerable to drought (Sinclair et al., 2007; Arrese-Igor et al., 2011; Cerezini et al., 2014), some studies have shown that supplemental mineral N could increase grain yield under drought (Purcell and King, 1996; Ray et al., 2006; Salvagiotti et al., 2008). However, this gain did not compensate the costs of using N fertilizer (Hungria et al., 2006b), including environmental costs associated to N production and losses. Even 'starter' doses of mineral N have shown no significant effect on grain yield (Alves et al., 2003), and may even impair the establishment of the symbiosis and its capacity for N supply at later stages like pod filling. Even with a gain in yield under drought, N-fertilizer was not economically viable (Hungria et al., 2013; Salvagiotti et al., 2008). Considering the most contrasting yield gain of 686 kg ha^{-1} with N-fertilizer, the approximate soybean (US$ 0.25 per kg) and urea (US$ 1.00 per kg of N) prices in the Brazilian market, the N-application was not profitable.

Despite positive effects on nodulation, inoculation, co-inoculation, or use of MSM not necessarily resulted in higher yields, which increased only in the second growing season in Londrina. However, there were clear effects in terms of N accumulated in shoot biomass. In the first trial of 2013/14, when the climate conditions (temperature and rainfall) were less severe, the N-fertilized plants accumulated more N in the shoots. However, as the sink strength increased with the pod filling, the accumulated N in N-fertilized plants decreased sharply, whereas the BNF-based plants kept the accumulated N levels, probably because plants that received N mineral entered in senescence earlier than plants inoculated with *Bradyrhizobium*. Under the severe weather conditions observed in the second trial of 2013/14 growing season, both N-fertilized and BNF-based on soil bradyrhizobial population stood out in terms of N accumulation in shoots at early reproductive developmental stages. However, both decreased sharply at the pod filling stage, whereas the inoculated plants kept higher N contents in the biomass that could have supported higher grain yields if mild climate conditions had occurred thereafter. Despite higher accumulation of N in N-fertilized plants at vegetative and early reproductive stages, there was a drop in accumulated N at pod filling, probably because the decreased nodulation by N-fertilizer was not enough to supply the demanded N at later stages (Kaschuk et al., 2016). Under a more severe climatic condition as in the second trial, both N-supplied and BNF-based on the soil bradyrhizobial population led to a drop in N accumulation in plant shoots at pod filling stages. Even sometimes inoculation does not result in higher grain yields, it is the most sustainable way to supply N for soybeans and assure that among several factors comprising the yield components, at least N be as less restrictive as possible (Alves et al., 2003; Chalk et al., 2010). In addition, the N remaining in the crop residues can be an important source of N for the subsequent crop, increasing the sustainability of the production system.

In conclusion, severe drought associated to high temperatures sharply decrease nodulation and yield of soybean. Even under such condition, supplemental mineral N does not result in an economically viable increase of grain yield. On the other hand, estimates show that the soybean crop in Brazil saves about US$ 7 billion year^{-1} with the BNF process (Hungria et al., 2013). The strategy of inoculation, co-inoculation or use of MSM, even in soils with established rhizobial population, increase the nodulation at initial stages and help its maintenance under drought. These practices assure higher N accumulation at later reproductive stages and may eventually increase grain yield.

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