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ORIGINAL PAPER

Impact of the *ahas* transgene for herbicides resistance on biological nitrogen fixation and yield of soybean

Mariangela Hungria · André Shigueyoshi Nakatani · Rosinei Aparecida Souza · Fernando Bonafé Sei · Ligia Maria de Oliveira Chueire · Carlos Arrabal Arias

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Abstract Studies on the effects of transgenes in soybean [*Glycine max* (L.) Merr.] and the associated use of specific herbicides on biological nitrogen fixation (BNF) are still few, although it is important to ensure minimal impacts on benefits provided by the root-nodule symbiosis. Cultivance CV127 transgenic soybean is a cultivar containing the *ahas* gene, which confers resistance to herbicides of the imidazolinone group. The aim of this study was to assess the effects of the *ahas* transgene and of imidazolinone herbicide on BNF parameters and soybean yield. A large-scale set of field experiments was conducted, for three cropping seasons, at nine sites in Brazil, with a total of 20 trials. The experiment was designed as a completely randomized block with four replicates and the following treatments: (T1) near isogenic transgenic soybean (Cultivance CV127) + herbicide of the imidazolinone group (imazapyr); (T2) near isogenic transgenic soybean + conventional herbicides; and (T3) parental conventional soybean (Conquista) + conventional herbicides; in addition, two commercial cultivars were included, Monsoy 8001 (M-SOY 8001) (T4), and Coodetec 217 (CD 217) (T5). At the R2 growth stage, plants were collected and BNF parameters evaluated. In general, there were

M. Hungria (✉) · A. S. Nakatani · R. A. Souza · C. A. Arias
Embrapa Soja, C.P. 231, Londrina, PR 86001-970, Brazil
e-mail: mariangela.hungria@embrapa.br; hungria@pq.cnpq.br; biotecnologia.solo@hotmail.com

A. S. Nakatani
e-mail: andrenakatani@yahoo.com.br

R. A. Souza
e-mail: rosysouza@iapar.br

C. A. Arias
e-mail: carlos.arias@embrapa.br

A. S. Nakatani
Fundação Araucária, Av. Comendador Franco 1341, Curitiba, PR 80215-090, Brazil

A. S. Nakatani
CAPES, Setor Bancário Norte, Q2, Bl L, Lote 06, Brasília, DF 70040-020, Brazil

Present Address:
R. A. Souza
IAPAR, C.P. 481, 86001-970 Londrina, PR, Brazil

F. B. Sei
Universidade do Estado de Santa Catarina (UDESC/FAC), C.P. 281, 88520-000 Lages, SC, Brazil
e-mail: fernandobs@totalbiotecnologia.com.br

Present Address:
F. B. Sei
Total Biotecnologia Indústria e Comércio Ltda, Rua Emílio Romani 1190, CIC, 81460-020 Curitiba, PR, Brazil

L. M. de Oliveira Chueire
CNPq, SHIS QI 1, Cj B, Lago Sul, 71605-001 Brasília, DF, Brazil
e-mail: ligia.chueire@embrapa.br

no effects on BNF parameters due to the transgenic trait or associated with the specific herbicide. Similarly, at the final harvest, no grain-yield effects were detected related to the *ahas* gene or to the specific herbicide. However, clear effects on BNF and grain yield were attributed to location and cropping season.

Keywords Biological nitrogen fixation · *Glycine max* · Imidazolinones · Transgenic soybean · Environmental monitoring

Introduction

In 2012, genetically modified (GM) crops occupied 170 million hectares worldwide, in Brazil reaching 36.6 million ha, ranking second of 28 countries (James 2012). The leading transgenic crop globally is soybean at 75.4 million hectares (James 2011).

A transgenic soybean containing the *ahas* gene was developed in 1997. This soybean cultivar is tolerant of the imidazolinone class of herbicides due to the introduction of an imidazolinone-tolerant acetohydroxyacid synthase (AHAS) from *Arabidopsis thaliana* via biolistics (Chukwudebe et al. 2012). Herbicides in the imidazolinone group (imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz and imazaquin) control a wide range of weeds, by acting at the growing points after absorption by roots and leaves, and translocation via phloem and xylem. This group of herbicides inhibits the synthesis of the enzyme AHAS (E.C. 4.1.3.18, AHS or AHAS, also known as acetolactate synthase and ALS), responsible for the biosynthesis of the amino acids valine, leucine and isoleucine (Souza et al. 2013).

The use of herbicide-resistant transgenic plants and crop management with specific herbicides have raised concerns of whether the symbiotic nitrogen fixation process is negatively affected by the transgene and associated management practices. The adoption of these biotechnologies might affect biological nitrogen fixation (BNF) directly through effects on the diazotrophic bacteria (Kremer and Means 2009) and/or on the functioning of the symbiosis (Montero et al. 2001), thus jeopardizing the contribution of BNF to the soybean plant (Bohm et al. 2009). Indirect effects could also represent a limitation, through the effects on the legume host, by changes in root exudates or in the plant physiology (Lynch et al. 2004; Kremer et al. 2005; Powell et al. 2007; Garcia-Villalba et al. 2008; Zobiole et al. 2010; Moldes et al. 2012).

The BNF process is of great importance for soybean cropping in Brazil. It is estimated that, annually, BNF contributes N equivalent to approximately US$ 15 billion of synthetic fertilizers, in addition to decreasing environmental pollution and mitigating production of greenhouse gases associated with N-fertilizers (Hungria and Mendes 2014). Therefore, it is important that deployment of GM soybean biotechnologies are accompanied by extensive and rigorous field studies with monitoring of biosafety to ensure minimal impacts on the environment and maintenance of benefits provided by BNF. For example, concerns were recently raised over the contribution of BNF in Brazil by transgenic glyphosate-resistant soybean (Hungria et al. 2014).

The aim of this study was to evaluate the effects of *ahas*-transgenic soybean and of the herbicide imazapyr on BNF parameters and grain yield, in a large-scale set of experiments, involving 20 field trials over three growing seasons at nine locations in Brazil.

Materials and methods

Sites

The experiments were carried out over three growing seasons (summer of 2006/2007, short-season of 2007 and summer of 2007/2008), at nine sites in Brazil, with a total of 20 experiments, cropped with the Conquista MG/BR 46 conventional soybean cultivar (genealogy: Lo76-4484 × Numbaíra; G.8.1; determinate growth habit) and its near isogenic transgenic Cultivance. All details about the sites, geographic localization, type of soil, climate classification, previous cropping, rainfall and temperatures have been previously described by Souza et al. (2013).

Treatments and experimental design

In all field locations, three treatments were set up: (T1) Cultivance soybean (GM soybean, near isogenic event CV127), containing the *ahas* gene, and herbicide of the imidazolinone group (imazapyr) as the sole agent for weed control; (T2) Cultivance and conventional herbicides for weed control; (T3) Conquista (conventional soybean), the parental non-transgenic cultivar, and conventional herbicides for weed control. In addition, two reference soybean cultivars were included as comparison in these field studies, Monsoy 8001 (M-SOY 8001) (T4), and Coodetec 217 (CD 217) (T5), both also treated with conventional herbicides. Both of these are common non-transgenic Brazilian commercial cultivars with a similar maturity classification and determinate growth habit as Conquista.

The experimental design was a completely randomized block with four replicates. The plots consisted of four rows of 5 m long, spaced at 0.5 m between lines, with eight lines per plot. Seeds were sown manually, in November and beginning of December (summer growing seasons of 2006/2007 and 2007/2008) and in March (short-season of 2007). At sowing time seeds were always inoculated with the commercial strains *Bradryhizobium japonicum* SEMIA 5079 (= CPAC 15) and *Bradyrhizobium diazoefficiens* SEMIA 5080 (= CPAC 7), applied to supply 1.2 million cells seed^{-1}.

The herbicides used in the present study consisted of: (1) imazapyr, herbicide of the imidazolinone group, sprayed at a rate of 70 g ai ha^{-1} and; (2) the conventional herbicide Volt, a combination of Bentazon (400 g ai ha^{-1}) and Acifluorfen (170 g ai ha^{-1}), sprayed at a rate of 570 g ai ha^{-1}. Fertilizing, irrigation and pest control were carried out uniformly in each experimental area, according to the soybean cropping recommendations for each region.

Biological nitrogen fixation evaluations

At the R2 stage, ten plants were randomly collected per replicate (avoiding a central area of about 6 m^2 established for harvesting grains) for evaluation of nodulation and plant growth. In the laboratory, shoots were separated from roots and the latter were carefully washed and placed in a forced-air dryer at 65 °C until constant weight was obtained ($\sim$72 h). Nodules were removed from the roots and dried again. Nodulation [nodule number (NN) and dry weight], shoot dry weight (SDW) and shoot total N (Kjeldahl digestion and determination of N concentration using a Tecator automatic N analyzer) were determined as described before (Hungria et al. 2006). BNF associated with the soybean plant was estimated using the N-ureide technique in dry tissues of plants harvested at R2 stage, as described by Hungria et al. (2006). Analysis was performed with stems including the petioles, according to Herridge and Peoples (1990). Dry stems (generated by incubation at 65 °C until a constant weight) of ten plants per replicate were ground (20 mesh), extracted with 0.1 M phosphate buffer (pH 7.0) and 2.5 mL of ethanol (2:1, v:v, buffer: ethanol), and heated at 80 °C for 5 min. Samples were filtered in cheesecloth, centrifuged at 10,000*g* for 5 min. and stored at −5 °C. Concentration of N-ureides (allantoin and allantoic acid) was determined based on the method of Vogels and van der Drift (1970), as described before (Hungria 1994; Hungria et al. 2006). Nitrate was evaluated in the same extracts by the salicylic acid technique (Cataldo et al. 1975) that was adapted for smaller sample sizes as described by Hungria (1994). N-ureides were expressed as a percent of total N (N-ureides plus nitrate) in the stem + petiole tissues and also transformed to total N-ureide content in shoots (TNU) for treatment comparison purposes.

Yield

At physiological maturity, soybean was harvested to estimate grain yield. All seeds from the harvested plants were collected, cleaned, weighed, and seed moisture was determined and weight corrected to 13 %.

Statistical analysis

Statistical analyses were conducted using Analysis of Variance Procedures (ANOVA), and means comparisons across treatments at each site were determined using the Tukey's test (Steel and Torrie 1980).

Results

Effect of transgene *ahas* on BNF

In the crop season of 2006/2007, in general there were no changes on BNF parameters due to transgenic trait or related to transgenic plant + imidazolinone-group herbicide management in the seven sites evaluated (Table 1). Only at Londrina the parameters of total N in shoots (TNS) (−34 %) and total N as ureides (TNU) (−40 %) decreased in T1 (Cultivance + imazapyr) compared to T2 (Cultivance + conventional herbicide) and T3 (non-transgenic Conquista + conventional

Table 1 Biological nitrogen fixation parameters evaluated at R2 growth stage of soybean in the summer cropping season of 2006/2007 in seven sites in Brazil

T[a]	Parameters[b]						
	NN	NDW	SDW	SNC	TNS	%NU	TNU
Santo Antonio da Posse							
T1	28.9 ab[c]	138	23.2	56.6 ab	1,317 ab	72.1 a	949 a
T2	42.3 a	199	24.0	54.9 ab	1,320 ab	73.3 a	967 a
T3	42.9 a	184	26.6	56.9 ab	1,520 a	76.1 a	1,156 a
T4	22.0 b	106	19.4	59.5 a	1,160 b	63.2 b	733 b
T5	19.2 b	89	20.4	50.7 b	1,037 b	60.0 b	622 b
CV (%)[d]	27.5	41.1	14.8	5.6	22.3	8.2	20.2
Ponta Grossa							
T1	32.8	94 b	35.0	55.7	1,953 ab	76.8	1,500 a
T2	54.3	143 ab	37.4	51.8	1,938 ab	75.0	1,453 a
T3	45.4	138 ab	35.9	57.1	2,052 a	79.9	1,640 a
T4	57.8	194 a	27.3	59.9	1,637 b	69.1	1,131 b
T5	41.8	106 b	33.4	58.1	1,943 a	73.9	1,436 a
CV (%)	24.9	26.6	20.8	7.2	19.2	7.7	23.1
Londrina							
T1	44.4 ab	104 ab	15.4	47.0	725 c	67.9	492 d
T2	58.4 a	123 a	19.9	48.0	957 ab	73.1	699 ab
T3	43.5 ab	111 ab	20.2	54.5	1,102 a	74.4	820 a
T4	25.6 b	75 b	17.2	54.1	936 ab	68.8	644 bc
T5	32.8 b	70 b	16.1	51.2	829 bc	67.2	557 cd
CV (%)	21.3	19.4	15.4	9.5	21.1	8.1	17.7
Uberaba							
T1	50.7 ab	167	11.2	36.2	407	68.9	280
T2	56.0 ab	160	10.2	42.3	434	73.3	318
T3	72.3 a	236	10.7	42.6	456	72.8	332
T4	35.1 b	171	7.8	43.4	342	61.1	209
T5	37.6 ab	176	7.2	40.9	296	60.7	180
CV (%)	32.4	25.4	31.0	8.0	27.7	6.6	32.3
Sete Lagoas							
T1	27.2	117	15.7	41.1	646	78.2	505
T2	43.2	189	13.4	44.7	599	77.1	461
T3	29.8	149	14.3	40.9	586	76.7	450
T4	33.5	238	12.0	52.8	635	75.0	476
T5	32.0	193	12.8	49.3	634	74.4	472
CV (%)	36.0	32.4	24.8	11.7	33.6	9.2	27.7
Santo Antônio de Goiás							
T1	30.8	79	19.3 a	44.6 ab	861	80.2 a	690 a
T2	70.1	154	15.2 ab	42.6 ab	651	77.3 a	503 b
T3	56.0	162	14.5 abc	47.9 a	697	76.9 a	536 b
T4	48.3	204	12.6 bc	43.4 ab	549	70.2 b	385 c
T5	51.9	158	9.4 c	36.3 b	342	65.5 b	224 d
CV (%)	36.9	49.1	18.1	8.7	32.1	9.0	11.9
Brasília							
T1	28.5	187 ab	27.4 ab	53.5 ab	1,471 ab	74.2a	1,091 ab
T2	43.8	207 a	25.0 ab	52.5 b	1,316 bc	76.2a	1,003 abc
T3	33.3	163 ab	30.4 a	54.4 ab	1,658 a	80.1a	1,328 a
T4	34.8	122 ab	21.9 b	56.4 a	1,240 bc	65.0b	806 bc
T5	21.3	74 b	20.5 b	54.6 ab	1,124 c	62.1b	698 c
CV (%)	31.4	36.7	14.5	2.8	25.2	8.2	16.6

[a] Treatments: T1 = Cultivance (transgenic cultivar) + imazapyr; T2 = Cultivance (transgenic cultivar) + conventional herbicide; T3 = Conquista (non-transgenic cultivar) + conventional herbicide; T4 = Monsoy 8001 + conventional herbicide; T5 = Coodetec 217 + conventional herbicide

[b] Parameters: *NN* nodule number (nodule number plant^{-1}), *NDW* nodule dry weight (mg plant^{-1}), *SNC* shoot N concentration (mg N g plant^{-1}), *TNS* total N in shoot (mg N plant^{-1}), *%NU* percentage of total N as N-ureides, *TNU* total N as ureides (mg N-ureides plant^{-1})

[c] Means followed by the same letter for each column within the same site do not differ by the Tukey's test ($p < 0.05$); in the absence of letters there was no statistical difference

[d] *CV* (%) coefficient of variation

herbicide). In Brasília, the TNS decreased in T2 (−21 %) compared to T3. Only at Santo Antonio de Goiás the TNU in T1 presented a higher value compared to T2 (+37 %) and T3 (+29 %) (Table 1).

In the short-season of 2007, in general no effects were observed due to the transgenic trait on BNF parameters (Table 2). Only in Uberaba the transgenic treatments (T1 and T2) decreased NN (−51 and −40 %, respectively) and dry weight (NDW) (−51 and −46 %, respectively) and SDW (−41 and −63 %, respectively) compared to the T3 (non-transgenic cultivar + conventional herbicide). Also, TNS (−64 %) and TNU (−71 %) decreased in T2 compared to T3.

No effects related to the transgene on BNF parameters were observed in the crop season of 2007/2008 (Table 3).

Effect of herbicides on BNF

In the crop season of 2006/2007 in general no differences between the herbicides were observed (Table 1). Only in Londrina, the Cultivance with the imidazolinone-group (T1) presented lower values of TNS (−24 %) and TNU (−30 %) than with the conventional herbicide. Contrarily, in Santo Antonio de Goiás the imidazolinone-group herbicide resulted in higher TNU (+37 %) compared to conventional herbicide.

During the short-season of 2007, no differences between herbicides on BNF attributes were recorded in any of the seven field trials (Table 2). Similarly, in 2007/2008, with exception of the shoot nitrogen content (SNC) in Sete Lagoas, where the transgenic treatment with imazapyr was higher (+34 %) in comparison with the transgenic treatment with conventional herbicides, no differences were found between the herbicides on BNF parameters (Table 3).

Effects of season and sites on BNF

There were clear differences on BNF parameters according to the site within each crop season (Table 4), but without a defined pattern. E.g., Santo Antonio de Goiás presented the highest values of NN in the crop season of 2006/2007, but in the season of 2007/2008 the lowest nodulation.

Table 2 Biological nitrogen fixation parameters evaluated at R2 growth stage of soybean in the short-season of 2007 in six sites in Brazil

T[a]	Parameters[b]						
	NN	NDW	SDW	SNC	TNS	%NU	TNU
Uberaba							
T1	12.8 b[c]	51 b	2.6 b	39.4	103 ab	68.5	71 ab
T2	15.7 b	58 b	1.6 b	40.5	69 b	60.0	43 b
T3	26.1 a	106 a	4.4 a	43.1	191 a	77.2	150 a
T4	5.7 b	21 b	3.2 ab	39.8	123 ab	68.8	90 ab
T5	8.6 b	31 b	3.2 ab	39.8	132 ab	72.5	100 ab
CV (%)[d]	48.8	49.2	45.5	10.9	42.5	9.2	52.2
Sete Lagoas							
T1	23.0	147	8.2	42.9	354	85.2 ab	303
T2	17.2	99	7.2	42.4	306	81.2 ab	251
T3	20.0	131	8.5	45.8	395	89.0 a	354
T4	11.5	82	7.2	41.4	300	81.0 ab	245
T5	8.0	66	5.8	40.0	230	74.8 b	176
CV (%)	64.3	59.0	26.4	15.7	28.5	5.8	31.7
Santo Antonio de Goiás							
T1	32.0	125	14.9 a	47.0	699 a	86.2	605 a
T2	23.2	137	14.0 a	47.8	666 a	78.2	526 ab
T3	36.7	193	13.9 a	46.6	652 a	84.5	552 ab
T4	20.3	126	12.3 ab	41.1	517 ab	81.0	431 ab
T5	26.6	149	9.7 b	37.3	363 b	71.0	276 b
CV (%)	47.9	29.2	13.9	17.2	19.1	12.0	27.5
Brasília							
T1	45.0	319 a	14.2	49.9	720	76.2	567
T2	39.6	313 a	14.9	42.9	660	73.8	516
T3	36.1	253 ab	14.8	44.2	648	74.8	496
T4	32.2	187 b	14.5	46.6	718	73.2	582
T5	30.7	180 b	12.3	43.0	529	63.5	345
CV (%)	38.6	38.4	24.5	19.9	38.8	20.1	55.8
Teresina							
T1	30.8	121	9.8	30.5	302	56.3	174
T2	27.0	94	9.2	37.6	351	58.3	210
T3	25.6	99	11.0	28.0	306	57.0	173
T4	33.4	155	10.7	33.1	382	60.0	245
T5	26.8	117	9.4	32.5	308	58.3	180
CV (%)	45.6	61.0	29.5	11.8	29.5	11.8	50.6
Vilhena							
T1	48.4 ab	154 ab	6.8	44.0	304	60.8	186
T2	53.6 a	196 ab	7.3	42.7	314	60.2	190
T3	62.9 a	209 a	7.4	41.1	304	59.0	179
T4	25.2 c	147 ab	7.0	41.7	292	59.0	173
T5	30.2 bc	131 b	7.5	42.0	314	59.5	187
CV (%)	27.8	25.6	16.0	8.4	14.5	6.8	19.5

[a,b,c,d] As described in Table 1

Table 3 Biological nitrogen fixation parameters evaluate at R2 growth stage of soybean in the summer cropping season of 2007/2008 in seven sites in Brazil

T[a]	Parameters[b]						
	NN	NDW	SDW	SNC	TNS	%NU	TNU
Santo Antonio da Posse							
T1	99.6[c]	314 ab	34.0 a	29.3	999	81.5	809
T2	116.4	419 a	32.8 ab	31.7	1,043	84.6	888
T3	94.5	294 ab	32.5 ab	31.2	1,015	83.5	850
T4	69.9	210 b	21.6 bc	35.6	771	85.1	663
T5	85.2	195 b	25.1 c	34.0	840	80.2	675
CV (%)[d]	22.3	30.0	12.8	15.3	19.4	4.94	23.4
Londrina							
T1	66.4	228 ab	20.8 a	32.1	692	68.6	490
T2	74.9	235 ab	15.6 ab	35.6	526	72.2	379
T3	102.0	307 a	16.8 ab	35.0	589	70.4	422
T4	49.1	157 b	12.4 ab	36.4	461	69.2	320
T5	54.1	161 b	10.0 b	32.9	330	70.5	237
CV (%)	46.3	22.8	25.7	17.2	38.8	10.2	47.2
Uberaba							
T1	122.9	172	22.9	26.9	604	72.8	439
T2	110.7	165	25.6	28.7	741	70.3	519
T3	137.0	201	22.6	31.9	734	73.8	539
T4	101.1	172	20.8	29.6	618	72.8	450
T5	84.6	135	19.9	27.7	555	71.6	396
CV (%)	34.0	34.9	27.5	10.4	30.8	8.95	30.3
Sete Lagoas							
T1	21.4	68 ab	21.5	36.6 a	790	88.0	693
T2	31.3	77 ab	22.6	27.4 b	610	83.1	507
T3	39.6	127 a	20.8	34.6 ab	719	87.3	632
T4	22.4	59 b	21.9	34.6 ab	753	87.7	663
T5	18.2	43 b	19.1	41.2 a	790	88.6	698
CV (%)	31.0	38.2	25.6	11.7	25.0	6.58	27.0
Santo Antonio de Goiás							
T1	15.7	88	12.0	34.5	419	62.0	263
T2	11.4	57	10.0	32.1	313	61.0	190
T3	19.2	86	10.6	36.3	398	61.3	246
T4	18.6	114	9.9	42.6	421	59.8	251
T5	11.9	41	8.6	39.7	341	61.0	208
CV (%)	36.0	51.5	17.8	13.5	25.9	4.98	29.7
Brasília							
T1	41.5	64	16.8	33.9	565	61.4	353
T2	39.9	45	16.6	33.5	558	56.2	312
T3	35.7	51	16.8	35.6	595	60.4	363
T4	42.7	63	14.4	36.0	518	55.3	286
T5	33.9	51	16.8	38.2	638	58.8	380
CV (%)	23.4	35.5	14.6	15.1	18.6	9.10	24.7
Vilhena							
T1	109.4 a	262 a	10.0	37.4	374	65.0	242
T2	99.0 ab	246 ab	12.2	37.5	454	65.6	299
T3	97.5 ab	241 ab	12.9	34.0	458	65.0	299
T4	78.6 ab	206 ab	10.8	41.1	436	64.7	282
T5	68.0 b	159 b	10.2	37.9	386	67.2	260
CV (%)	16.9	18.9	20.8	15.6	27.7	6.32	27.6

[a,b,c,d] As described in Table 1

Effect of the *ahas* transgene and herbicides on soybean grain yield

Considering each crop season (Table 5), in general no effects of transgene *ahas* or of the herbicides across the three seasons (20 field trials) on soybean grain yield were observed. In the crop season of 2006/2007 no effects were detected. In the short-season of 2007 only in Vilhena the conventional treatment (T3) showed higher yield than T1 and T2 (both transgenic treatments), of +39 and +32 %, respectively. In the crop season of 2007/2008, Londrina presented higher grain yield (+20 %) in the T2 (Cultivance) compared to the T3 (conventional cultivar). Contrarily, in Brasília the T3 recorded grain yield 20 % higher than the T2.

There were changes on yield related to year (E.g. season 2006/2007 and 2007/2008 presented higher yields than in the short-season 2007—although the data were not analyzed statistically) (Table 5). We also observed differences in grain yield according to the site, e.g., in the crop season of 2006/2007 higher grain yield was recorded at Londrina (+238 %) in comparison to Brasília (Table 5).

It is important to mention that the symbiotic performance of both Conquista and Cultivance was, in most trials, better than the performance of the varieties used as controls, M-Soy 8001 and CD 217 (Tables 1, 2, 3), confirming the good symbiotic capacity of the Conquista and the near isogenic transgenic Cultivance. However, when all sites were analyzed together in each crop season, no differences between any of the treatments were observed (Table 5).

Discussion

Issues of environmental biosafety and impacts on human health are frequently raised associated with the use of transgenics (Cerdeira et al. 2007), and long-term field-monitoring studies are needed to address such aspects. One critical concern is about the effect of transgenes on BNF in legume root nodules and raises apprehensions, as BNF is key to the competitive success of Brazilian soybean as a commodity (Hungria and Mendes 2014). In the present study, in the great majority of the field trials evaluated we did not find impacts of the *ahas* gene on BNF parameters in soybean across three consecutive cropping seasons at nine locations in Brazil.

Several studies have addressed impacts of herbicide-tolerant crops on soil microorganisms (Azevedo and Araújo 2003; Bruinsma et al. 2003; Dunfield and Germida 2004; Liu et al. 2005), but specific studies of imidazolinone-tolerant plants are still few. According to Lynch et al. (2004), transgenic plants may affect the soil microorganisms, among them the N_2-fixing bacteria, by altering root morphology, physiology and exudation, or by releasing transgene products with antimicrobial activity, or even by horizontal gene transfer amongst the microorganisms. However, most studies have reported no changes or only slight transient effects on soil microorganisms due to transgenic traits (Lamarche and Hamelin 2007; Liu et al. 2008; Bohm and Rombaldi 2010; Weinert et al. 2010), including studies considering the effects of *ahas* transgene introduced in sugarcane (*Saccharum* spp.) (Dini-Andreote et al. 2010) and soybean (Souza et al. 2013).

Specifically regarding the impacts of transgenic soybean on BNF, the trait most extensively studied is glyphosate tolerance (RR® soybean). Many reports argue that the RR trait does not affect symbiotic nitrogen fixation by soybean (Powell et al. 2009; Masoud et al. 1996; Suarez et al. 2003). Negative impacts on bacterial growth are frequently associated with high dosage of glyphosate (Malty et al. 2006) rather than with the transgenic host (Bohm and Rombladi 2010). On the other hand, negative effects of glyphosate-resistance in soybean on BNF parameters have been recently reported (Hungria et al. 2014). Concerns are that the transgenic trait may impair bacterial growth (Kremer and Means 2009) or functioning of the symbiosis (Montero et al. 2001; Hungria et al. 2014), resulting in lower contributions of BNF to the soybean crop (Bohm et al. 2009; Hungria et al. 2014).

We found that the location and cropping season had more impact on BNF than the transgenic trait itself, and our findings are in agreement with other studies that attribute more evident effects related to soil type, site, climate, time of the year, tillage practice and stage of crop development (Gyamfi et al. 2002; Dunfield and Germida 2004; Fang et al. 2005; Griffiths et al. 2007; Lamarche and Hamelin 2007). When changes in a soil microbial community associated with transgenic crop are recorded, they are usually transient and not

Table 4 Biological nitrogen fixation parameters at the R2 stage of soybean growth across three growing seasons in Brazil (without distinguishing the treatments)

Site[a]	Parameter[b]																				
	NN			NDW			SDW			SNC			TNS			%NU			TNU		
	S1[c]	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3
SAP	31.0 c[d]	–	93.1 a	143 ab	–	286 a	22.7 b	–	29.2 a	55.7 a	–	32.4 ab	1,271 b	–	93 a	68.9	–	83.0 b	886 b	–	777 a
PGR	46.4 abc	–	–	135 ab	–	–	33.8 a	–	–	56.5 a	–	–	1,904 a	–	–	74.9	–	–	1432 a	–	–
LON	41.0 abc	–	69.3 b	96 b	–	218 ab	17.8 c	–	15.1 cd	51.0 ab	–	34.4 a	910 c	–	52 cde	70.2	–	70.2 c	642 c	–	370 cd
UBE	50.3 ab	13.8 b	111.3 a	182 a	53 c	169 b	9.4 d	3.0 d	22.4 b	41.1 c	40.5 a	29.0 b	387 e	124 c	65 bc	67.3	69.4 c	72.3 c	264 d	91 c	469 c
SLG	33.1 bc	15.9 b	26.6 cd	177 a	105 bc	75 c	13.6 c	7.4 c	21.2 b	45.8 bc	42.5 a	34.9 a	620 d	317 b	73 b	76.2	82.2 a	86.9 a	473 c	266 b	639 b
SAG	51.4 a	27.8 ab	15.4 d	151 ab	146 bc	77 c	14.2 c	14.1 a	10.2 e	43.0 c	44.0 a	37.0 a	620 d	580 a	37 e	74.0	80.2 ab	61.0 e	468 cd	487 a	232 e
BRA	32.3 c	36.7 a	38.8 c	151 ab	251 a	55 c	25.1 b	14.1 a	16.3 c	54.3 a	45.3 a	35.5 a	1,362 b	655 a	57 bcd	71.5	72.3 bc	58.4 e	985 b	501 a	339 cde
TER	–	28.7 ab	–	–	117 bc	–	–	10.0 b	–	–	32.4 b	–	–	330 b	–	–	58.0 d	–	–	196 bc	–
VIL	–	44.1 a	90.5 ab	–	167 ab	223 ab	–	7.23 c	11.2 de	–	42.3 a	37.6 a	–	305 b	42 de	–	59.7 d	65.5 d	–	184 bc	276 de

[a] Sites: *SAP* Santo Antonio de Posse, *PGR* Ponta Grossa, *LON* Londrina, *UBE* Uberaba, *SLG* Sete Lagoas, *SAG* Santo Antonio de Goiás, *BRA* Brasília, *TER* Teresina, *VIL* Vilhena

[b] Parameters: *NN* nodule number (nodules $plant^{-1}$), *NDW* nodule dry weight (mg $plant^{-1}$), *SNC* shoot N concentration (mg N g $plant^{-1}$), *TNS* total N in shoot (mg N $plant^{-1}$), *%NU* percentage of total N as N-ureide; *TNU* total N as ureide-N (mg ureide-N $plant^{-1}$)

[c] Crop Seasons: S1 = Season 2006/2007; S2 = Short-season 2007; S3 = Season 2007/2008

[d] Means followed by the same letter for each column do not differ by Tukey test ($p < 0.05$); in the absence of letters there was no statistical difference

detected during subsequent evaluations (Griffiths et al. 2000; Dunfield and Germida 2003).

In our study, we also found no differences in BNF traits that could be associated with the herbicides of the imidazolinone-group or the conventional herbicides. In agreement with our results, Gonzalez et al. (1999), under field condition in Argentina, found no differences among eight herbicides applied (including imazethapyr and imazaquin) on soybean nodule number or dry weight at the V2, V6 and R5 growth stages.

Considering soybean grain yield, in general we did not observe any effect due to the transgenic trait across three cropping seasons. Only in two trials (Vilhena in the short season of 2007 and Brasília in the 2007/2008 season) the conventional cultivar (T3) presented higher yields (+20 to +39 %), while in another trial (Londrina, season 2007/2008) the transgenic cultivar (T2) recorded higher yield (+20 %). As for BNF parameters, differences in yield in our study were related to year and site. Burke et al. (2008), at three locations, and Thomas et al. (2007), at four locations, both in north Carolina (USA), also did not observe differences in corn yield between an imidazolinone-tolerant hybrid and a conventional hybrid, except for one location where the imidazolinone-tolerant corn showed a better performance (+15 %). These authors found differences only among sites, related to differences in edaphoclimatic conditions, in agreement with our results. Darmency (2013), in a review on herbicide-resistant crop varieties, also found no effects of imidazolinone-resistance in wheat (*Triticum aestivum* L.) or sunflower (*Helianthus annuus* L.) yield. Regarding RR® soybeans, Bohm et al. (2009) and Bohm et al. (2011) did not find negative effects of cropping transgenic soybean (glyphosate resistance) on yield in southern Brazil, nor did Qaim and Traxler (2005) at 59 farms in Argentina, or Hungria et al. (2014) in six sites in Brazil during three consecutive cropping seasons.

Our study contributes with the first report of evaluation of BNF parameters and yield of an imidazolinone-tolerant cultivar of soybean in comparison with the near isogenic conventional cultivar. Twenty field trials at nine Brazilian sites—differing in edaphoclimatic conditions, covering different biomes, over three cropping seasons—were investigated and did not produce differences on BNF parameters or grain yield that could be associated with the *ahas* transgene or the specific herbicide.

Table 5 Soybean yield (kg ha^{-1}) across three growing seasons in different sites of Brazil

Treatments[a]	Sites[b]								
	SAP	LON	UBE	SLG	SAG	BRA	VIL	TER	Treatment average
Summer season of 2006/2007[c]									
T1	3,159 a[d]	4,721 ab	3,432 a	4,114 a	3,313 a	1,411 b	–	–	3,748 a
T2	3,202 a	5,171 a	3,404 a	4,028 a	3,275 a	1,553 b	–	–	3,816 a
T3	2,788 a	4,463 ab	3,107 a	3,829 a	3,131 a	1,435 b	–	–	3,463 a
T4	3,182 a	3,799 b	3,303 a	3,830 a	3,397 a	2,622 a	–	–	3,502 a
T5	3,392 a	4,143 ab	3,276 a	4,033 a	3,007 a	2,347 ab	–	–	3,570 a
Site average[e]	3,144 c	4,459 a	3,304 c	3,967 b	3,224 c	1,873 d			
Short season of 2007									
T1	–	–	ND	ND	2,018 a	3,343 ab	1,701 b	1,865 a	2,256 a
T2	–	–	1,864 a	2,446 a	1,929 a	3,386 a	1,801 b	2,279 a	2,284 a
T3	–	–	2,594 a	2,743 a	2,180 a	2,898 ab	2,371 a	2,197 a	2,510 a
T4	–	–	2,019 a	2,510 a	1,986 a	2,874 ab	1,519 b	2,046 a	2,159 a
T5	–	–	2,092 a	2,940 a	1,632 a	2,772 b	2,488 a	2,447 a	2,395 a
Site average[e]			2,142 bc	2,659 ab	1,949 c	3,055 a	1,976 c	2,176 bc	
Season 2007/2008									
T1	3,372 a	3,856 ab	3,329 ab	4,205 a	2,584 a	3,160 ab	3,185 a	–	3,384 a
T2	3,375 a	4,002 a	3,464 ab	4,507 a	2,598 a	3,037 b	3,154 a	–	3,448 a
T3	2,706 ab	3,328 bc	3,200 b	4,402 a	3,098 a	3,660 a	3,484 a	–	3,411 a
T4	2,468 ab	2,903 cd	4,272 a	4,424 a	2,744 a	3,695 a	3,104 a	–	3,373 a
T5	2,337 b	2,580 d	4,263 a	4,943 a	3,267 a	3,284 ab	2,545 b	–	3,317 a
Site average[e]	2,852 b	3,334 b	3,705 ab	4,496 a	2,858 b	3,367 b	3,094 b		

[a] Treatments: T1 = Cultivance (transgenic cultivar) + imazapyr; T2 = Cultivance (transgenic cultivar) + conventional herbicide; T3 = Conquista (non-transgenic cultivar) + conventional herbicide; T4 = Monsoy 8001 + conventional herbicide; T5 = Coodetec 217 + conventional herbicide

[b] Sites: *SAP* Santo Antonio de Posse, *LON* Londrina, *UBE* Uberaba, *SLG* Sete Lagoas, *SAG* Santo Antonio de Goiás, *BRA* Brasília, *VIL* Vilhena, *TER* Teresina

[c] Due to a very dry season grains were not harvested in Ponta Grossa (PGR) in the summer season of 2006/2007

[d] Means followed by the same letter for each column (except for "Site average" line values) within the same season do not differ by the Tukey's test ($p < 0.05$). *ND* not determined

[e] Means followed by the same letter along the line do not differ by the Tukey's test ($p < 0.05$)

Conclusions

Investigation of a near isogenic transgenic and the non-transgenic pair of soybean cultivars in 20 field trials performed in three cropping seasons in Brazil revealed no effects on BNF parameters or grain yield that could be attributed to the introduction of the *ahas* transgene or to the use of the specific herbicide.

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