SYMBIOSIS (2009) 48, 154–163 ©2009 Balaban, Philadelphia/Rehovot ISSN 0334-5114

Nitrogen fixation with the soybean crop in Brazil: Compatibility between seed treatment with fungicides and bradyrhizobial inoculants

Rubens José Campo[1], Ricardo Silva Araujo[1,2], and Mariangela Hungria[1,2*]

[1]Embrapa Soja, Cx. Postal 231, 86001-970, Londrina, Paraná, Brazil, Tel. +55-4333716206, Fax. +55-4333716100,
Emails. hungria@cnpso.embrapa.br; rjcampo@cnpso.embrapa.br; rsarsa@gmail.com;
[2]Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-MCT), Brasilia, Federal District, Brazil

(Received June 1, 2008; Accepted October 3, 2008)

Abstract

Biological nitrogen fixation with the soybean crop can be improved by seed inoculation with superior *Bradyrhizobium* strains, but factors that reduce the population of inoculated bradyrhizobia on the seed will directly affect the efficiency of the process. Seed treatment with fungicides has been broadly practiced as cheap insurance against seed- and soil-borne pathogens, but toxicity of most fungicides to bradyrhizobia has often been underestimated. The compatibility between seed treatment with fungicides in single or mixed applications (including Benomyl, Captan, Carbendazin, Carboxin, Difenoconazole, Thiabendazole, Thiram, Tolylfluanid) and bradyrhizobial inoculants was examined in laboratory, greenhouse and field experiments during five crop seasons in Brazil. Bacterial survival on the seeds was severely affected by all fungicides, resulting in mortalities of up to 62% after only 2 h and of 95% after 24 h. Fungicides also reduced nodule number, total N in grains and decreased yield by up to 17%. The toxic effects of fungicides were more drastic in sandy soils without soybean inoculation and cropping history, reducing nodulation by up to 87%, but were also important in areas with established populations of soybean bradyrhizobia. Therefore, fungicides should be used only when the seeds or soil are contaminated with pathogens, otherwise biological N_2 fixation may be severely affected.

Keywords: *Bradyrhizobium*, inoculation, soybean, fungicides, toxicity

1. Introduction

Nitrogen (N) is the nutrient most required by the soybean [*Glycine max* (L.) Merr.] crop, such that an estimated 300–330 kg N are necessary for yielding 3,000 kg ha^{-1} grain. As hardly 50% of the N-fertilizer is taken up by the legume, it would be necessary to apply about 600 kg N ha^{-1} to satisfy the crop's demand for N. Considering the production costs in Brazil, the application of N-fertilizer would represent approximately 60% of the cropping expenses, making it economically unfeasible to grow soybeans in the country (Campo and Hungria, 2000; Hungria et al., 2005, 2006a, 2007). However, soybean can take advantage of the biological N_2 fixation process resulting from the symbiotic association with bacteria of the genus *Bradyrhizobium* – the bacteria invade the plant's root hairs and form nodules, where the triple bond of the molecular N_2 is broken down by the enzymatic complex of nitrogenase, producing ammonium, which will be taken up and metabolized by the plant.

Symbiotic associations with diazotrophic rhizobial strains occur with many legume species, but soybeans are amongst the most efficient at fixing N_2 (Unkovich and Pate, 2000; van Kessel and Hartley, 2000; Giller, 2001) and, in Brazil, it has been estimated that rates of N_2 fixation in soybean can exceed 300 kg of N ha^{-1}, providing up to 94% of total plant N and resulting in an economy to the country estimated at US$ 6 billion per year (Hungria et al., 2005, 2006a,b, 2007). Nevertheless, the efficiency of the biological process depends on several factors related to the host plant, to the bacteria and to the symbiosis, and in the tropics, edaphic constraints such as soil acidity, low soil fertility, high temperatures and drought very often limit the contribution of N_2 fixation (Hungria and Vargas, 2000).

---

[*] *The author to whom correspondence should be sent.*

Presented at the 15th International Congress on Nitrogen Fixation, January 21–26, 2007, Cape Town, South Africa

Progresses towards the genetic improvement of both the host plant and the bacterial strains have been reported for the soybean crop in Brazil, but any factor which may reduce the population of inoculated bradyrhizobia on the seed surface will have direct effects on the biological N_2 fixation process (Hungria and Vargas, 2000; Hungria et al., 2005, 2006a).

Diseases are amongst the most important and difficult factors affecting soybean yield, and about forty diseases caused by fungi, bacteria, nematodes and viruses are today major concerns for the crop in Brazil (Embrapa, 2006). The severity and economic importance of each disease vary from year to year, from region to region, and among cultivars, with annual losses estimated at 15 to 20% (Embrapa, 2006). Since most soybean pathogens are seed-borne, planting seeds with high physiological quality is important, as they may grow faster, be more tolerant to adverse conditions in the seedbed, and show higher tolerance to diseases (Campo and Hungria, 2000; Hungria et al., 2007). Seed treatment with fungicides has been broadly practiced as a cheap insurance against seed-borne and soil-borne seed rots and against seedling blights caused by fungi, such that today more than 90% of the soybean seeds in Brazil are treated with fungicides; furthermore, to avoid problems of seed emergence, combinations of systemic and contact fungicides have been employed and the number of active principles available has considerably increased (Campo and Hungria, 2000; Henning, 2004; Hungria et al., 2007; Embrapa, 2006).

Compatibility between seed treatment with fungicides and inoculants is now considered a major problem for the soybean crop in Brazil. In order to supply the N to more productive cultivars, it is mandatory to increase the population of selected bradyrhizobial strains on the seed surface. However, increasing phytosanitary problems mainly related to the monoculture system – the crop occupies today about 45% of all cropped land – are expected to result in heavy applications of fungicides (Campo and Hungria, 2000; Hungria et al., 2007). It has long been reported that fungicides may affect several steps of the symbiosis, from survival of the rhizobia on the seed to nodule formation and N_2 fixation efficiency (e.g., Curley and Burton, 1975; Gupta et al., 1988; Revellin et al., 1993; Cattelan and Hungria, 1994; Andrés et al., 1997; Dunfield et al., 2000).

Given the great variety of fungicides now available for seed treatment, it is necessary to assess their compatibility with *Bradyrhizobium*, as well as to delineate strategies to improve the compatibility of seed treatment with inoculation (Campo and Hungria, 2000; Hungria et al., 2005, 2007). As a first step, we report results of laboratory, greenhouse and field trials assessing the compatibility between bradyrhizobial inoculation and soybean seed treatment with fungicides during five crop seasons in Brazil.

2. Materials and Methods

Inoculant preparation and inoculation procedure

Single-strain inoculants were prepared in sterile peat, containing one of the four strains officially recommended for soybean in Brazil: *Bradyrhizobium elkanii* strains SEMIA 587 and SEMIA 5019 (=29W) and *Bradyrhizobium japonicum* strains SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7). The inoculants were prepared to contain approximately 10^9 cells g^{-1} inoculant and at sowing a proportional mixture of inoculants containing two strains was prepared. All four strains are highly effective in fixing N_2 with soybean, and according to the Brazilian inoculant legislation until 2006, the inoculants should contain two of the four recommended strains, in any combination (Hungria and Campo, 2007; Hungria et al., 2007).

A 10% sucrose (w/v) solution intended to increase adhesion of the peat inoculant was applied at a rate of 300 ml 50 kg^{-1} seeds for all experiments, as recommended for soybean (Embrapa, 2006; Hungria et al., 2007). Seed treatment consisted of first applying the sucrose solution to the seeds followed by the fungicides and then by the peat inoculant. After mixing, seeds were allowed to air-dry in the shade for 15 min. For the laboratory and greenhouse experiments, inoculation was performed with one-tenth of the recommended inoculant dose (500 g inoculant 50 kg^{-1} seeds) to make it easier to verify possible toxic effects of fungicides. For the field trials the full recommended inoculant dose was applied to the seeds.

Fungicides tested

Fungicides evaluated in these studies are named after the active ingredients and the doses and products used are those recommended for soybean in Brazil (Embrapa, 2004, 2006). The following active ingredients were tested: Captan, Thiram and Tolylfluanid (contact fungicides), and Benomyl, Carbendazin, Carboxin, Difenoconazole, Thiabendazole (systemic fungicides).

Laboratory experiments

Seeds were surface-sterilized (Vincent, 1970) and treatment with inoculants and fungicides was performed as described above. Soybean cultivars used were those recommended for each region in each crop season (e.g., Embrapa, 2004).

Sub-samples of 100 seeds (approximately 13 g) were taken 2 h after seed treatment and were transferred to 125-ml Erlenmeyer flasks containing 50 ml of sterile saline solution with two drops of Tween 80; flasks were then shaken for 5 min. Seed wash was repeated three times to remove fungicides and inoculant from the seeds. The material collected from the three washes was pooled in a

200-ml bottle and the volume made up to 200 ml with sterile saline solution. A 10-ml sample was then taken and serially diluted to obtain bacterial plate counts on yeast-mannitol medium (YEM) (Vincent, 1970). Control treatments, i.e. + inoculation-fungicide, -inoculation + fungicide and -inoculation-fungicide were always included. Bacterial counts on seed surface were done at 0, 2 and 24 hours after seed treatment. Plates were incubated at 28°C and colonies were counted on the 5th and 8th days.

Table 1. Localities where experiments were performed.

District (State)	Latitude	Longitude	Soil type	Area	Year[1]
Terra Roxa (Paraná)	24°09'S	54°05'W	Sandy	New	1998/99
Vera Cruz (Paraná)	25°03'S	53°52'W	Clay	New	1998/99
Ponta Grossa (Paraná)	25°05'S	50°09'W	Clay	New	1997/98
Taciba (São Paulo)	22°23'S	51°17'W	Sandy	New/ old	2002/03 2003/04
Cristalina (Goiás)	16°46'S	47°36'W	Clay	New/ old	2000/01
Luziânia (Goiás)	16°15'S	47°57'W	Clay	New	2000/01
Jaciara (Mato Grosso)	15°57'S	54°58'W	Clay	New	2000/01
Lucas do Rio Verde (Mato Grosso)	13°03'S	55°54'W	Clay	New	2000/01

[1]New areas are those which have never been cropped with soybean and show fewer than 10^2 cells g^{-1} soil; old areas have been cropped with soybean and inoculated before and show at least 10^4 cells of *Bradyrhizobium* g^{-1} soil.

Greenhouse experiments

Soybean seeds were treated with fungicides and inoculated as described above. Two hours after inoculation one seed was aseptically planted per Leonard jar (Vincent, 1970) containing a sterilized mixture (1:1) of sand and vermiculite. Control treatments as described in the previous section were included. Plants were maintained under axenic conditions in the greenhouse and received N-free nutrient solution (Hungria et al., 1996) until R2 stage (open flower at one of the two uppermost nodes on the main stem with a fully developed leaf) (Fehr and Caviness, 1977), when they were harvested 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 (approximately 72 h). Nodules were removed from roots and dried again. Nodulation (nodule number and dry weight), shoot dry weight and shoot total N (Kjeldahl digestion and determination of N concentration using a Tecator automatic N analyzer, Sweden) were determined.

Field experiments

Experiments were performed during five crop seasons in the summers of 1997/1998, 1998/1999, 2000/2001, 2002/2003 and 2003/2004, at different sites representative of the ecosystems where soybeans are grown in Brazil. The sites were localized in the southern, southeastern and central-western regions; all soils were in the category of oxisols, and in the Brazilian classification of "latossolos" (Table 1).

At the onset of experimentation, twenty soil subsamples (0–20 cm) were taken from each site to evaluate soil chemical characteristics, following the procedures of Pavan et al. (1992).

Table 2. Chemical properties of the soils (0–20 cm) before liming and fertility correction in the first year of experiments.

District	pH	Al	H+Al	K	Ca	Mg	P	C	CTC	BS[1]
	($CaCl_2$)	($cmol_c\ dm^{-3}$)					($mg\ dm^{-3}$)	($g\ dm^{-3}$)	($cmol_c\ dm^{-3}$)	(%)
Terra Roxa	5.1	0.00	4.4	0.07	2.10	1.19	4.5	13	7.8	43
Vera Cruz do Oeste	4.8	0.02	5.9	0.18	8.02	3.49	4.6	26	17.6	66
Ponta Grossa	4.7	0.08	5.7	0.30	2.49	1.00	10.2	24	9.5	40
Taciba (new)	4.3	0.26	4.9	0.32	1.05	0.51	12.4	14	6.8	28
Taciba (old)	4.8	0.07	3.8	0.25	1.81	0.45	18.1	14	6.3	40
Cristalina (new)	4.8	0.06	6.1	0.11	1.76	1.02	1.2	27	9.0	32
Cristalina (old)	4.9	0.04	5.8	0.14	1.96	1.28	1.2	27	9.1	37
Luziânia	4.9	0.07	6.2	0.19	1.76	0.49	2.5	30	8.7	28
Jaciara	4.8	0.06	5.1	0.08	1.21	0.36	9.7	21	6.8	25
Lucas do Rio Verde	5.0	0.03	5.7	0.05	1.88	1.02	1.7	25	8.7	34

[1]Base saturation = $(K + Ca + Mg)/T_{cec}) \times 100$, where $T_{cec} = K + Ca + Mg$ + total acidity at pH 7.0 (H + Al).

Before being analyzed, soil samples were dried (60°C for 48 h) and finely ground (2-mm sieve). Soil pH was determined in $CaCl_2$ 0.01 M (1:2.5; soil:solution), after agitation for 1 h. Exchangeable Ca, Mg and Al were determined in the extract obtained with 1 N KCl (1:10; soil:solution) after agitation for 10 min. P and K contents were evaluated in the Mehlich-1 (0.05 M HCl + 0.0125 M H_2SO_4) extract (1:10; soil:solution) after agitation for 10 min. Aluminum was determined by titration with 0.015 N NaOH, using bromothymol blue as indicator.

Concentrations of Ca and Mg were determined in an atomic absorption spectrophotometer, K in a flame photometer, P by colorimetry, using the molybdenum-blue method and ascorbic acid as reducing agent, C by the oxidation of dichromate and N by the Kjeldahl method. Soil chemical properties are shown in Table 2.

Soil populations of bradyrhizobia were estimated at the 0–10 cm layer by the most probable number (MPN) technique (Vincent, 1970) and the statistical tables of Andrade and Hamakawa (1994), with counts on soybean plants of the cultivar Embrapa 48. Field experiments were performed in areas with ("old") and without ("new") established populations of bradyrhizobia. As Brazilian soils are originally devoid of soybean bradyrhizobia (Hungria et al., 2006a), areas with established populations are those with previous soybean inoculation and cropping history.

Fifty days before starting the experiment, soil pH values were determined and lime was applied to alleviate acidity, estimated to reach a saturation of bases to 50%, aiming at increasing the pH approximately 5.5. Before planting, the areas received fertilizer according to the standard recommendation: 300 kg ha^{-1} of N-P-K (0-28-20); other macro and micronutrients were supplied when necessary, according to the soil analysis. In addition, plants received 20 g ha^{-1} of Mo and 2 g ha^{-1} of Co as a foliar spray at V4 stage (Fehr and Caviness, 1977). Field plots measured 4.0 m (length) x 5.0 m (width), with ten rows 0.5 m apart from one another, and were separated by 1.0 m wide laneways to prevent contamination by superficial run-off containing bacteria or fertilizer. A complete randomized block design with six replicates was adopted for all field trials. Insects were controlled with biological and chemical insecticides and weeds with herbicides, after the technical recommendation for the crop (e.g., Embrapa, 2004). None of the experiments was irrigated.

Treatments consisted of inoculation with mixtures of two recommended strains of *Bradyrhizobium*, and seed treatment with contact and/or systemic fungicides (active principles varied in some experiments, to include new recommendations). Two controls were included in all experiments: 1) non-inoculated and not treated with fungicides; 2) inoculated and receiving 200 kg of N ha^{-1}, split in 50% at sowing and 50% at R2 stage, to make sure that N deficiency was not limiting plant production. Seeds were treated with fungicides and inoculated as described above. Seeds were hand planted as soon as possible, after inoculation and seed treatment, usually within 4 h. Plant populations were around 300,000 plants ha^{-1}.

Table 3. Reduction (%) in the number of inoculated *Bradyrhizobium* cells on the soybean seed surface 2 h and 24 h after the treatment with fungicides, relative to the untreated control receiving only inoculant[1]. Means of two subsamples of 100 seeds, in six replicates.

Active ingredients	2 h	24 h
Benomyl+Captan	62	82
Benomyl+Thiram	41	64
Benomyl+Tolylfluanid	47	81
Carbendazin+Captan	60	87
Carbendazin+Thiram	64	83
Carbendazin+Tolylfluanid	71	95
Carboxin+Thiram	49	81
Difenoconazole+Thiram	62	90
Thiabendazole+Captan	28	90
Thiabendazole+Thiram	24	87
Thiabendazole+Tolylfluanid	20	95

[1] 300 ml 10% sucrose solution plus 50 g of inoculant 50 kg^{-1} seeds (one-tenth of the recommended dose); inoculant prepared with soybean *Bradyrhizobium* strains SEMIA 5019 and SEMIA 5079 and containing 3.7 10^9 cells g^{-1} of inoculant.

Table 4. Effects of seed treatment with bradyrhizobial inoculants and fungicides[1] on nodulation (nodule number $plant^{-1}$) and total N accumulated in shoots (TNS, mg N $plant^{-1}$) of soybean cultivar BR 37, and reduction (%) in those parameters relative to the inoculated control. Plants were grown in Leonard jars, under greenhouse conditions, and harvested at R2 stage (full flowering). Means of six replicates.

Treatment	Nodulation		TNS	
	No.	Reduction	N content	Reduction
Non-inoculated control	0.0	–	3.4	–
Inoculated[2] (I)	14.3	–	164.4	–
I+Benomyl+Thiran	10.5	26.6	148.2	9.9
I+Benomyl+Captan	12.0	16.1	162.8	1.0
I+Thiabendazole+Captan	12.5	12.6	132.6	19.3
CV[3] (%)	17.6	–	14.5	–
LSD[4] 5%	1.5	–	15.6	–

[1] Fungicides were applied according to recommended doses for the soybean crop. [2] 300 ml 10% sucrose solution plus 50 g of inoculant 50 kg^{-1} seeds (one-tenth of the recommended dose); inoculant prepared with strains SEMIA 5019 and SEMIA 5079 and containing 2.7 10^9 cells g^{-1} of inoculant. [3] Coefficient of variation [4] Least statistical difference between means of two treatments within each column, at $p \leq 0.05$, according to the "t" test.

At the V4 and, sometimes, R2 stages, ten plants were randomly collected per replicate (avoiding areas established for harvesting grains) for evaluation of nodulation and plant growth. At the laboratory, nodule number and dry weight, shoot dry weight and total N in the shoots were determined as described for the greenhouse experiments. Grain yield at physiological maturity was determined from the six central rows of each plot (6 m^2 area), and data were corrected for 13% moisture content, after determination of the humidity level in a grain moisture tester (Vurroughf 700). Total N content of the seeds was also evaluated as described for the shoots.

Data from all experiments were first submitted to the tests of normality of the variables and of homogeneity of variances, and then to the analysis of variance (ANOVA); when significant differences were detected by the ANOVA, means were compared by the *t* test ($p \leq 0.05$) (SAS, 2001).

3. Results

Laboratory experiments

In the laboratory assays, all fungicides decreased cell numbers on seed surface by at least 20% as shortly as 2 h hours after inoculation (Table 3). At this first evaluation, the least toxic combinations were those with Thiabendazole (systemic) with Tolylfluanid or Thiram (contact). However, when left in contact with the inoculated bacteria for 24 h, bacterial mortality over 60% was observed for all treatments, with up to 95% of mortality in the treatments of Carbendazin + Tolylfluanid and Thiabendazole + Tolylfluanid (Table 3). The toxic effects of fungicides were confirmed for all combinations of the four commercial strains (data not shown).

Greenhouse experiments

In the experiments performed in sterile substrate and under greenhouse conditions, the decrease in the number of *Bradyrhizobium* cells on the seeds due to the toxic effects of the fungicides resulted in decreases in nodulation and soybean growth. Table 4 shows the results of one of those experiments, in which three mixtures of contact and systemic fungicides were tested. Despite a possible decrease in the toxic effects due to the adsorption of the fungicides to the vermiculite, decreases in nodule number of up to 27% and in total N accumulated by soybean plants at R2 stage of up to 19.3% were observed (Table 4).

Field experiments

The effects of fungicides were evaluated in nine experiments conducted at eight different sites (Table 1) during five summer crop seasons. As the results of all experiments were very similar, we have chosen to present only the full set of data from representative experiments, namely those from Central Brazil (Cristalina and Luziânia) (Table 5) in this paper. Interestingly, in none of those field experiments seed treatment with fungicides improved seedling emergence, neither in new areas nor in areas previously cropped with soybeans, as shown in Table 5.

Seed treatment with fungicides almost always negatively affected bacterial survival on the seeds, resulting in decreased nodulation, but the severity of the effects varied according to soil characteristics and history of soybean cultivation. In new areas, devoid of soybean bradyrhizobia in the soil, seed treatment with fungicides always decreased nodulation, whereas in areas in which the soil contained an established population of bradyrhizobia, the effects were attenuated as shown in Table 5. Thus in the new areas of Cristalina and Luziania, nodule number (NN) was reduced by an average of 30 and 81% and nodule dry weight (NDW) by 20 and 55%, respectively, while in the old area of Cristalina the reduction was of 12 and 17% for NN and NDW, respectively (Table 5).

Yield was not always statistically reduced by the treatment with fungicides, however, differences were often observed and once more effects were more drastic in new areas (Table 5). Thus, in the new area of Cristalina, in seven out of the thirteen treatments (54%) with fungicides, yield was significantly lower than in the treatment in which seeds were just inoculated, while in the old area statistically significant yield reduction was observed for only two treatments (15%); an even more drastic effect was observed in the new area of Luziania, with significantly lower yields in eleven out of thirteen fungicide treatments (85%) (Table 5). Considering the average of all treatments with fungicides performed in the new and old areas of Cristalina and in the new area of Luziania, decreases in yield of 6.3, 4.6 and 13.4%, respectively, were observed when all treatments with fungicides were compared to the treatment receiving only inoculant (Table 5). Decreases in the total N accumulated in soybean grains due to the application of fungicides were also observed, and estimated in 13, 6 and 8%, for the new and old areas of Cristalina and the new area of Luziania, respectively (Table 5).

The results of nodulation in another region, the southern state of Paraná, are shown in Table 6. Neither the Terra Roxa nor the Vera Cruz do Oeste sites had neither received soybean inoculant, or had been cropped with soybean before, and toxic effects of the fungicides were observed. Effects were most drastic in the sandy soil. Thus, in Terra Roxa, nodule number was reduced by up to 87% (Thiabendazole + Captan) at R2 stage, while in the clay soil of Vera Cruz do Oeste a maximum decrease of 32% was related to the treatment with Thiabendazole + Thiram (Table 6).

The full sets of data from the other field experiments are not shown. Instead, we have decided to pool the data from all experiments in order to show the reduction in nodulation due to the presence of the fungicides applied singly or in mixtures (Tables 7 and 8).

Table 5. Effects of seed treatment with inoculant containing *Bradyrhizobium*^{1} and fungicides^2 on plant emergence (PE, %), nodule number (NN, number plant^{-1}) and nodule dry weight (NDW, mg plant^{-1}) at R2 stage and grain yield (Y, kg ha^{-1}) and total N in the grain (TNG, kg N ha^{-1}) of soybean cultivar Vitoria at the full maturity. Experiments performed at three sites of the central-western region of Brazil in the 2000/2001 crop season. Means of six replicates.

Treatment	Cristalina (new area)^3					Cristalina (old area)^4					Luziania (new area)^3				
	PE	NN	NDW	Y	NG	PE	NN	NDW	Y	NG	PE	NN	NDW	Y	NG
Non-inoculated	88	5	17	1626	98	93	32	56	1900	113	96	5	23	2211	150
Inoculated^1 (I)	86	14	45	1717	105	92	43	75	2120	123	96	13	44	2546	143
I+200 kg N	89	6	12	1693	101	91	26	38	2135	127	93	26	38	2462	130
I+Carbendazin	87	12	42	1604	95	91	40	58	1971	111	96	9	35	2151	127
I+Carboxin	80	10	34	1561	93	91	38	55	2006	116	96	10	33	2090	124
I+Thiabendazole	82	11	42	1596	92	89	40	60	1764	101	96	10	37	2245	131
I+Difenoconazole	86	7	22	1519	92	91	39	61	1998	115	93	7	31	2249	131
I+Captan	88	11	35	1751	105	92	37	66	1963	115	94	7	35	2260	134
I+Thiram	88	10	42	1595	94	93	39	67	2107	122	97	6	29	1979	117
I+Tolylfluanid	85	8	29	1657	99	91	36	64	1921	112	95	4	28	2163	128
I+Carboxin+Thiram	87	7	29	1574	94	90	36	55	2118	123	95	9	35	2100	122
I+Thiabendazole+Tolylfluan	89	10	41	1598	96	92	37	61	2086	120	94	7	28	2226	133
I+Carbendazin+Thiram	83	8	38	1443	87	91	38	69	1997	115	93	6	38	2119	128
I+Carbendazin+Captan	90	7	31	1670	102	90	42	71	2109	127	95	7	30	2254	137
I+Difenoconazole+Thiram	89	11	42	1682	98	96	32	60	2168	127	94	10	30	2453	149
I+ Carboxin+Thiram	88	10	34	1664	105	95	38	58	2090	101	97	9	35	2379	142
CV^5 (%)	7.0	16.7	19.1	9.2	9.2	4.4	14.6	16.4	10.7	15.3	3	12	43	10.3	10.4
LSD^6	6.1	1.3	5.3	118	7.3	4.1	4.5	8.2	178	14.6	2.9	2.6	6.3	187	11.2

^{1}300 ml 10% sucrose solution plus 500 g of inoculant 50 kg^{-1} seeds; inoculant prepared with strains SEMIA 5079 + SEMIA 5080 and containing 4.1 10^9 cells g^{-1} of inoculant. ^{2}Fungicides were applied according to recommended doses for the soybean crop. ^{3}Containing fewer than 10^2 cells g^{-1} soil in the MPN analysis. ^{4}Containing 3.7 10^4 cells g^{-1} soil in the MPN analysis. ^{5}Coefficient of variation. ^{6}Least statistical difference between means of two treatments within each column, at $p \leq 0.05$, according to the "t" test.

In general, all active ingredients tested showed some degree of toxicity to inoculated bradyrhizobia, reflected in a reduction of nodulation when compared with the treatment where seeds were only inoculated. Tolylfluanid, Difenoconazole, and Captan were the most toxic fungicides, reducing nodulation by 46, 38 and 38%, respectively, when applied as a single fungicide (Table 7). In the old areas, the toxic effects of those three fungicides were substantially reduced, but still decreased nodulation by 16, 10 and 14%, respectively (Table 7).

When seeds were treated with mixtures of contact and systemic fungicides, the combinations of Benomyl + Thiram, Carbendazin + Captan, and Carboxin + Thiram caused over 40% reduction in nodulation of soybeans grown in new areas with clay soils (Table 8). Fungicide mixtures were even more toxic when soybeans were grown in new areas with sandy soils, resulting in decreases in nodulation ranging from 70 to 88%; the most toxic mixtures were those of Thiabendazole + Tolylfluanid (88%), Thiabendazole + Captan (87%), and Carbendazin + Tolylfluanid (83%). Lower effects were observed in areas with a previous history of soybean inoculation, with Difecnoconazole + Thiram reducing nodulation by 26%. On average, mixtures of fungicides reduced nodulation by 14 (old), 33 (new, clay) and 73% (new, sandy) (Table 8).

Still considering the average of several experiments, seed treatment with single fungicides reduced yield by up to 14.5% (Thiram) and 16.8% (Thiabendazole) in new and old areas, respectively; averaging all treatments the mean decrease was estimated at 10.7 and 7.5% in new and old areas, respectively (Table 7). The application of mixtures of fungicides also decreased yield in new areas by up to 14.5 (sandy) and 11.0% (clay), with an average for all treatments of 6.1 and 7.0%, respectively (Table 8). In old areas, toxic effects were attenuated, with a maximum decrease of 5.8% (Carbendazin + Thiran) and estimated at 1.6% when all treatments were considered (Table 8).

4. Discussion

Plant diseases represent a major constraint to plant productivity, making it necessary to obtain genetically resistant cultivars or, when these are not available, to utilize pesticides. Seed- or soil-borne plant pathogenic fungi may greatly affect plant stands and thus reduce yields, and seed treatment with fungicides, in cases where genetic resistance is not available, is a generally accepted and recommended practice. Currently more than 90% of the soybean seeds in Brazil are treated with fungicides (Henning, 2004;

Table 6. Effects of seed treatment with inoculant containing *Bradyrhizobium*[1] and fungicides[2] on nodule number (NN, number plant^{-1}), and reduction (%) of nodulation relative to the inoculated control at R2 stage of soybean cultivar BR 37. Experiments performed in two new areas[3] of the southern region of Brazil in the 1999/2000 crop season. Means of six replicates.

Treatments	Terra Roxa (sandy)		Vera Cruz do Oeste (clay)	
	NN	Reduction (%)	NN	Reduction (%)
Non-inoculated	1	–	5	–
Inoculated[1] (I)	23	0	34	0
I+Benomyl+Captan	6	74	26	24
I+Benomyl+Thiram	5	78	27	21
I+Benomyl+Tolylfluanid	5	78	25	27
I+Carbendazin+Captan	11	52	33	3
I+Carbendazin+Thiram	5	78	28	18
I+Carbendazin+Tolylfluanid	4	83	26	24
I+Carboxin+Thiram	14	39	29	15
I+Difenoconazole+Thiram	13	43	30	12
I+Thiabendazole+Captan	3	87	25	27
I+Thiabendazole+Thiram	7	70	23	32
I+Thiabendazole +Tolylfluanid	5	78	32	6
CV[4] (%)	53		30	
LSD[5]	3.6		6.2	

[1] 300 ml 10% sucrose solution plus 500 g of inoculant 50 kg^{-1} seeds; inoculant prepared with strains SEMIA 587 and SEMIA 5019 and containing 1.9 10^9 cells g^{-1} inoculant. [2] Fungicides were applied according to recommended doses for the soybean crop. [3] Containing fewer than 10^2 cells g^{-1} soil in the MPN analysis. [4] Coefficient of variation. [5] Least statistical difference between means of two treatments within each column, at $p \leq 0.05$, according to the "t" test.

Table 7. Reduction (%) of soybean nodulation (nodule number plant^{-1}) and grain yield (kg ha^{-1}) caused by seed treatment with fungicides applied as single active ingredients, in comparison to the treatment where seeds were just inoculated with *Bradyrhizobium*. Data obtained from field experiments performed in clay soils of the southern and central-western regions were pooled to compose table.

Active ingredient	Old areas (two experiments)		New areas (six experiments)	
	Nodulation	Yield	Nodulation	Yield
Carbendazin	9.0	7.0	17.2	11.0
Carboxin	11.5	5.4	20.8	13.5
Thiabendazole	8.0	16.8	20.0	9.5
Difenoconazole	10.5	5.8	38.2	11.8
Captan	14.0	7.4	37.7	5.6
Thiram	11.0	0.6	34.3	14.5
Tolylfluanid	16.0	9.4	46.2	9.3
General mean	11.4	7.5	30.6	10.7

Embrapa, 2006; Hungria et al., 2007).

There are reports showing that even though some fungicides may have little or no effect on rhizobial survival, and on nodulation, as shown for *B. japonicum* strain G49 with soybean (Revellin et al., 1993), these findings cannot be generalized. In fact, soybean rhizobial strains may be very sensitive to fungicides (e.g., Cattelan and Hungria, 1994) and, therefore, their utilization must be very cautious. In Brazil, due to the tremendous expansion of the soybean crop, the incidence of plant pathogens has become more frequent resulting in the recommendation of an increasing number of active ingredients. Furthermore, it is now common practice to treat seeds with combinations of systemic and contact fungicides (Embrapa, 2004, 2006), potentially increasing the toxicity to the inoculated bacteria.

In this study, laboratory evaluations demonstrated that fungicides may cause up to 62% bacterial mortality in a period as short as 2 h after seed treatment, increasing to up to 95% after 24 h. Under greenhouse controlled conditions, nodulation and total N accumulated in shoots at R2 stage were reduced by up to 27 and 19%, respectively. Similar results have been reported for soybean rhizobia (Pudelko and Madrzak, 2004; Bikrol et al., 2005) and other legumes as common bean (*Phaseolus vulgaris*) (Guene et al., 2003) – where seed treatment with fungicides was so toxic that no nodulation or N_2 fixation was observed – and chickpeas (*Cicer arietinum*) (Kyei-Boahen et al., 2001; Aamil et al., 2005). The fungicide Mancozeb has been shown to cause biochemical alterations and to reduce growth rate and symbiotic properties of *Bradyrhizobium* sp. USDA 3187 (Fabra et al., 1998). Biochemical alterations in *B. japonicum* have also occurred when soybean seeds were treated with Captan and inoculated (Dunfield et al., 2000). These authors have also observed that Captan induced changes in the FAME and Biolog profiles of the inoculated bacteria. This can mean that nodulation is affected since FAME profiles are altered in response to changes in cellular metabolism (Rose, 1989), and cellular fatty acids are involved in nodulation (Pueppke, 1989).

In field trials performed during five crop seasons in the main Brazilian soybean producing regions, the negative effects of seed treatment with fungicides on nodulation were always observed, being more noticeable in new areas. In addition, effects were more drastic in sandy than in clay soils. For example, decreases in nodulation of up to 88% in a sandy soil and of 43% in a clay soil of Paraná state emphasize the highly toxic effects of fungicides on bradyrhizobial inoculant strains. Therefore in areas cropped for the first time, especially in sandy soils, seed treatment with fungicides should be avoided, in order to help the establishment of large bacterial populations in the soil. Finally, although first-year areas often have a supply of N in the soil, decreases in nodulation and N_2 fixation may result in lower yields, and in this study yield decreases of up to 15% were observed.

Table 8. Reduction (%) of soybean nodulation (nodule number plant^{-1}) and grain yield (kg ha^{-1}) caused by seed treatment with fungicides applied as mixture of active ingredients, in comparison to the treatment where seeds were just inoculated with *Bradyrhizobium*. Data obtained from field experiments in the southern, southeastern and central-western regions were pooled to compose table. Numbers in parenthesis mean the number of experiments in which the combination has been tested.

Active ingredients	Old areas		New areas			
			Clay soils		Sandy soils	
	Nodulation	Yield	Nodulation	Yield	Nodulation	Yield
Carboxin+Thiram	16.5 (02)	0.1 (02)	40.5 (08)	12.8 (05)	53.6 (03)	5.1 (03)
Thiabendazole+Tolylfluanid	14.0 (02)	1.6 (02)	32.7 (10)	9.8 (05)	88.0 (02)	8.2 (02)
Carbendazin+Thiram	10.0 (02)	5.8 (02)	36.2 (10)	14.5 (05)	57.5 (02)	11.0 (02)
Carbendazin+Captan	4.0 (02)	0.5 (02)	42.4 (10)	7.3 (05)	74.5 (02)	8.9 (02)
Difenoconazole+Thiram	26.0 (02)	0.0 (02)	23.4 (09)	3.9 (05)	69.5 (02)	5.8 (02)
Benomyl+Captan	NI[1]	NI	22.0 (02)	5.4 (02)	74.0 (01)	NI
Benomyl+Thiram	NI	NI	42.5 (02)	5.6 (02)	78.0 (01)	NI
Benomyl+Tolylfluanid	NI	NI	36.0 (02)	4.0 (02)	78.0 (01)	NI
Carbendazin+Tolylfluanid	NI	NI	33.0 (02)	0.0 (02)	83.0 (01)	NI
Thiabendazole+Captan	NI	NI	26.0 (02)	3.0 (02)	87.0 (01)	NI
Thiabendazole+Thiram	NI	NI	30.0 (02)	0.8 (02)	70.0 (01)	NI
Fludioxonil+Metalaxyl	NI	NI	NI	NI	57.7 (06)	NI
General mean	14.1	1.6	33.2	6.1	72.6	7.0

[1] Treatment not included in the field experiment.

As expected, effects of fungicides were less drastic in areas with established populations of soybean bradyrhizobia. Main effects of reinoculation are observed on the early nodulation at the root crown, very important in helping a healthy establishment of the soybean plant (Hungria et al., 2007). In old areas, when nodulation by inoculant strains fails, the population already established in the soil is capable of nodulating soybean as soon as the localized effects of fungicides are gone, allowing a recovery of the N_2 fixation process. Therefore, effects of fungicides are less drastic than in new areas, and in this study were estimated at up to 26% on nodule number and 17% on soybean yield. However, decreases of this magnitude will result in lower incomes to the farmers.

A critical aspect raised in this study is about the real benefit that can be obtained from soybean seed treatment with fungicides. Even though fungicide application is considered as a cheap insurance for the crop (Henning, 2004), the practice not always results in yield increases (Campo and Hungria, 2000). This statement is confirmed by the results from this study. In none of the field trials performed over five crop seasons was plant emergence from seeds treated with fungicides higher than in the inoculated treatment not treated with fungicides. Furthermore, no increases were observed in yield. Therefore simple but very important procedures can be adopted towards avoiding the use of fungicides, with an emphasis on the utilization of certified seeds with good physiological and phytosanitary qualities.

Other interesting and environmentally-friendly strategies should also be considered as alternatives for the seed treatment with fungicides. A recent report (Huang and Erickson, 2007) has demonstrated a fungicidal effect of rhizobia applied to seeds of peas (*Pisum sativum*) and lentils (*Lens culinaris*), with certain strains of specific rhizobia being as effective as seed treatment with Thiram to control seedling damping-off. Similar results were obtained by Bardin et al. (2004), with *Rhizobium leguminosarum* bv. *viceae* strain R5 controlling *Pythium* damping-off of peas and sugar beet (*Beta vulgaris*). Positive effects were also observed for the control of seedling rot caused by *Rhizoctonia solani* in cowpeas (Kataria et al., 1985). These results suggest an interesting research opportunity to combine the beneficial effects of inoculation with N_2-fixing bacteria which can also behave as agents of biological control, an approach that can be cheaper and effective in preventing diseases without damaging rhizobia. For example, *Pseudomonas fluorescens* F113 is an effective agent of biological control and has no impact on the resident population of rhizobia nodulating red clover (*Trifolium* sp.) (Walsh et al., 2003). For the soybean crop, promising results were also reported by the co-inoculation of *Bradyrhizobium* with selected strains of *Bacillus subtilis* or their supernatants, controlling important soybean seed pathogens (Araújo et al., 2005).

In conclusion, large-scale soybean cropping in Brazil has enhanced phytosanitary problems, demanding an increased utilization of pesticides. Those pesticides, particularly fungicides applied to the seeds, may drastically affect rhizobial survival on the seeds, rhizobial population in the soils and N_2 fixation rates. A careful analysis of the need of using seed fungicides must be always considered, as the alleged benefits on yield may not be a general rule. Therefore fungicides should be used only when the seeds or soil are contaminated with pathogens. Maintaining the benefits resulting from N_2 fixation of soybean in Brazil is essential to maintain the competitiveness of the country in the international market (Hungria et al., 2006, 2007).

Acknowledgments

This work was partially supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil)/MCT projects and PQ (300698/2007-0). The authors thank Leny M. Miura (Embrapa Soja) for help in several steps of this work, and to Dr. Maria Cristina Neves de Oliveira for support in the statistical analyses.

REFERENCES

Aamil, M., Zaidi, A., and Khan, M.S. 2005. Fungicidal impact on chickpea-*Mesorhizobium* symbiosis. *Journal of Environmental Science and Health*, Part B **39**: 779–790.

Andrade, D.S. and Hamakawa, P.J. 1994. Estimativa do número de células de rizóbio no solo e inoculantes por infecção em planta. In: *Manual de Métodos Empregados em Estudos de Microbiologia Agrícola*. Hungria, M. and Araujo, R.S., eds. Embrapa-SPI, Brasília, DF, pp. 63–94.

Andrés, J.A., Correa, N.S., and Rosas, S.B. 1997. Survival and symbiotic properties of *Bradyrhizobium japonicum* in the presence of thiram: isolation of fungicide resistant strains. *Biology and Fertility of Soils* **26**: 141–145.

Araújo, F.F., Henning, A.A., and Hungria, M. 2005. Phytohormones and antibiotics produced by *Bacillus subtilis* and their effects on seed pathogenic fungi and on soybean root development. *World Journal of Microbiology & Biotechnology* **21**: 1639–1645.

Bardin, S.D., Huang, H.-C., Pinto, J., Amundsen, E.J., and Erickson, R.S. 2004. Biological control of *Pythium* damping-off of pea and sugar beet by *Rhizobium leguminosarum* bv. *viceae*. *Canadian Journal of Botany* **82**: 291–296.

Bikrol, A., Saxena, N., and Singh, K. 2005. Response of *Glycine max* in relation to nitrogen fixation as influenced by fungicide seed treatment. *African Journal of Biotechnology* **4**: 667–671.

Campo, R.J. and Hungria, M. 2000. Compatibilidade do uso de inoculantes e fungicidas no tratamento de sementes de soja. Embrapa Soja, Londrina, 32p. (Embrapa Soja. Circular Técnica, 26).

Cattelan, A.J. and Hungria, M. 1994. Nitrogen nutrition and inoculation. In: *Tropical Soybean, Improvement and Production*. FAO, Org. Embrapa-CNPSo, Londrina. pp. 201–215. (FAO Plant Production and Protection Series, No. 27).

Curley, R.L. and Burton, J.C. 1975. Compatibility of *Rhizobium japonicum* with chemical seed protectants. *Agronomy Journal* **67**: 807–808.

Dunfield, K.E., Siciliano, S.D., and Germida, J.J. 2000. The fungicides thiram and captan affect the phenotypic characteristics of *Rhizobium leguminosarum* strain C1 as determined by FAME and Biolog analyses. *Biology and Fertility of Soils* **31**: 303–309.

Embrapa. 2004. Tecnologias de produção de soja – Paraná 2005. Londrina, Embrapa Soja. 224p. (Sistemas de Produção 5).

Embrapa. 2006. Tecnologias de produção de soja – Região Central do Brasil – 2007. Londrina, Embrapa Soja, Embrapa Cerrados, Embrapa Agropecuária Oeste. 225p. (Sistemas de Produção 11).

Fabra, A., Angelini, J., Donolo, A., Permigiani, M., and Castro, S. 1998. Biochemical alterations in *Bradyrhizobium* sp USDA 3187 induced by the fungicide Mancozeb. *Antonie van Leeuwenhoek* **73**: 223–228.

Fehr, W.R. and Caviness, C.E. 1977. Stages of soybean development. Ames, Iowa State University. (Special report, 80).

Giller, K.E. 2001. *Nitrogen Fixation in Tropical Cropping Systems*. Wallingford, UK, CABI Publishing. 448p.

Guene, N.F.D., Diouf, A., and Gueye, M. 2003. Nodulation and nitrogen fixation of field grown common bean (*Phaseolus vulgaris*) as influenced by fungicide seed treatment. *African Journal of Biotechnology* **2**: 198–201.

Gupta, S.B., Rawat, A.K., and Khare, A.K. 1988. Effect of pre- and post-inoculation seed treatment with fungicides on nodulation and grain yield of soybean. *Legume Research* **11**: 167–172.

Henning, A.A. 2004. Patologia e tratamento de sementes: noções gerais. Londrina, Embrapa Soja. 51p. (Embrapa Soja. Documentos, 235).

Huang, H.-C. and Erickson, R.S. 2007. Effect of seed treatment with *Rhizobium leguminosarum* on *Pythium* damping-off, seedling height, root nodulation, root biomass, shoot biomass, and seed yield of pea and lentil. *Journal of Phytopathology* **155**: 31–37.

Hungria, M. and Campo, R.J. 2007. Inoculantes microbianos: situação no Brasil. In: *Biofertilizantes en Iberoamérica: Visión Técnica, Científica y Empresarial*. Izaguirre-Mayoral, M.L., Labandera, C., and Sanjuan, J., eds. Montevideo, Cyted/Biofag. pp. 22–31.

Hungria, M., Campo, R.J., and Mendes, I.C. 2007. A importância do processo de fixação biológica do nitrogênio para a cultura da soja: componente essencial para a competitividade do produto brasileiro. Londrina, Embrapa Soja. 80p. (Embrapa Soja. Documentos, 283).

Hungria, M., Campo, R.J., Mendes, I.C., and Graham, P.H. 2006a. Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: the success of soybean (*Glycine max* L. Merr.) in South America. In: *Nitrogen Nutrition and Sustainable Plant Productivity*. Singh, R.P., Shankar, N., and Jaiwal, P.K., eds. Houston, Texas, Studium Press, LLC. pp. 43–93.

Hungria, M., Franchini, J.C., Campo, R.J., Crispino, C.C., Moraes, J.Z., Sibaldelli, R.N.R., Mendes, I.C., and Arihara, J. 2006b. Nitrogen nutrition of soybean in Brazil: contributions of biological N_2 fixation and of N fertilizer to grain yield. *Canadian Journal of Plant Science* **86**: 927–939.

Hungria, M., Franchini, J.C., Campo, R.J., and Graham, P.H. 2005. The importance of nitrogen fixation to soybean cropping in South America. In: *Nitrogen Fixation: Origins, Applications and Research Progress*. Newton, W.E., ed., v.4, Nitrogen fixation in agriculture, forestry, ecology and the environment. Werner, W. and Newton, W.E., eds. Dordrecht, Amsterdam, Springer. pp. 25–42.

Hungria, M., Nishi, C.Y.M., Cohn, J., and Stacey, G. 1996. Comparison between parental and variant soybean *Bradyrhizobium* strains with regard to the production of lipo-chitin nodulation signals, early stages of root infection, nodule occupancy, and N_2 fixation rates. *Plant and Soil* **186**: 331–341.

Hungria, M. and Vargas, M.A.T. 2000. Environmental factors impacting N_2 fixation in legumes grown in the tropics, with an emphasis on Brazil. *Field Crops Research* **65**: 151–164.

Kataria, H.R., Yadav, J.S., Garg, F.C., and Grover, R.K. 1985. Inactivation of seed-treatment fungicides by *Rhizobium*. *Pesticide Science* **16**: 337–340.

Kyei-Boahen, S., Slinkard, A.E., and Walley, F.L. 2001. Rhizobial survival and nodulation of chickpea as influenced by fungicide seed treatment. *Canadian Journal of Microbiology* **47**: 585–589.

Pavan, M.A., Bloch, M.F., Zempulski, H.D., Miyazawa, M., and Zocoler, D.C. 1992. Manual de análise química do solo e controle de qualidade. Londrina, Brasil, Instituto Agronômico do Paraná. 40 p. (Circular 76).

Pudelko, K. and Madrzak, C.J. 2004. Influence of fungicide Funaben on nodulation of soybean [*Glycine max* (L.) Merr.] in the field conditions. *Journal of Plant Protection Research* **44**: 155–159.

Pueppke, S.G. 1996. The genetic and biochemical basis for nodulation of legumes by rhizobia. *Critical Reviews in Biotechnology* **16**: 1–51.

Revellin, C., Leterme, P., and Catroux, G. 1993. Effect of some fungicide treatments on the survival of *Bradyrhizobium japonicum* and on the nodulation and yield of soybean [*Glycine max* (L) Merr.]. *Biology and Fertility of Soils* **16**: 211–214.

Rose, A.H. 1989. Influence of the environment on microbial lipid composition. In: *Microbial Lipids*. Ratledge, C. and Wilkinson, S.G., eds. Academic Press, London. pp. 525–535.

SAS Institute. 2001. SAS user's guide, statistics, version 8.2, 6th ed. Cary, NC, SAS Institute.

Unkovich, M.J. and Pate, J.S. 2000. An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. *Field Crops Research* **65**: 211–228.

van Kessel, C. and Hartley, C. 2000. Agricultural management of grain legumes; has it led to an increase in nitrogen fixation? *Field Crops Research* **65**: 165–181.

Vincent, J.M. 1970. *A Manual for the Practical Study of Root Nodule Bacteria*. Blackwell Scientific Publications, Oxford. 119 p. (IBP Handbook, 15).

Walsh, U.F., Moënne-Loccoz, Y., Tichy, H.-V., Gardner, A., Corkery, D.M., Lorkhe, S., and O'Gara, F. 2003. Residual impact of the biocontrol inoculant *Pseudomonas fluorescens* F113 on the resident population of rhizobia nodulating a red clover rotation crop. *Microbial Ecology* **45**: 145–155.