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ORIGINAL PAPER

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Benefits of inoculation of the common bean (*Phaseolus vulgaris*) crop with efficient and competitive *Rhizobium tropici* strains

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Abstract Cropping in low fertility soils, especially those poor in N, contributes greatly to the low common bean (*Phaseolus vulgaris* L.) yield, and therefore the benefits of biological nitrogen fixation must be intensively explored to increase yields at a low cost. Six field experiments were performed in oxisols of Paraná State, southern Brazil, with a high population of indigenous common bean rhizobia, estimated at a minimum of 10^3 cells g^{-1} soil. Despite the high population, inoculation allowed an increase in rhizobial population and in nodule occupancy, and further increases were obtained with reinoculation in the following seasons. Thus, considering the treatments inoculated with the most effective strains (H 12, H 20, PRF 81 and CIAT 899), nodule occupancy increased from an average of 28% in the first experiment to 56% after four inoculation procedures. The establishment of the selected strains increased nodulation, N_2 fixation rates (evaluated by total N and N-ureide) and on average for the six experiments the strains H 12 and H 20 showed increases of 437 and 465 kg ha^{-1}, respectively,in relation to the indigenous rhizobial population. A synergistic effect between low levels of N fertilizer and inoculation with superior strains was also observed, resulting in yield increases in two other experiments. The soil rhizobial population decreased 1 year after the last cropping, but remained high in the plots that had been inoculated. DGGE analysis of soil extracts showed that the massive inoculation apparently did not affect the composition of the bacterial community.

Keywords Common bean · DGGE · Nitrogen fertilizer · Nitrogen fixation · *Rhizobium*

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Introduction

Today Brazil is the largest producer of common bean (*Phaseolus vulgaris* L.) worldwide and the grains represent the most important source of protein for the population. The crop occupied 3 million ha in 2002, but the country is characterized by one of the lowest yields in the world, only 728 kg ha^{-1} (CONAB 2002). A poor technology level and cropping in low fertility soils, especially with low N content, contribute greatly to this scenario. Therefore an adequate supply of N through symbiosis with N_2-fixing rhizobia should increase yield at a low cost as well as preserving water resources from pollution by NO_3^-.

Poor nodulation and lack of responses to inoculation in field experiments have been frequently reported worldwide, raising doubts about the efficiency of bean inoculation (Graham 1981; Pereira et al. 1984; Buttery et al. 1987; Ramos and Boddey 1987; Hardarson 1993). The explanation for the failure in some trials mainly cited a high but inefficient population of indigenous common bean rhizobia in both soils (Graham 1981; Thies et al. 1991) and seeds (Andrade and Hungria 2002). Furthermore, the common bean-rhizobia symbiosis is quite sensitive to environmental stresses, such as high temperatures and soil dryness, leading to low N_2 fixation efficiency (Graham 1981; Hungria et al. 1997; Hungria and Vargas 2000).

Nevertheless, there are reports of positive responses to inoculation in Brazilian soils, e.g. Peres et al. (1994) observed, in well-drained oxisols of Cerrados without irrigation, that the yield gains due to inoculation ranged from 63 to 290 kg ha^{-1} in relation to the non-inoculated treatments. More recently, successful strain selection programs performed in Brazil and aimed at increases in nodulation and N_2 fixation rates have also been reported (Hungria et al. 2000; Mostasso et al. 2002), encouraging further studies with improved strains. The selected rhizobial strains from those programs belong to *Rhizobium tropici* species, which seem to be the most adequate for tropical acid soils (Martínez-Romero et al. 1991;

Graham et al. 1994; Hungria et al. 2000; Mostasso et al. 2002). As a result of this selection program, strain PRF 81 is now recommended for use in Brazilian commercial inoculants (Hungria et al. 2000), together with CIAT 899. However, it still remains to be confirmed whether the combination of inoculation with selected strains and N fertilizer is necessary for maximum yields, as reported by Vargas et al. (2000).

The objectives of this work were therefore: (a) to evaluate the symbiotic effectiveness of new *R. tropici* strains isolated from the Cerrados soils (Mostasso et al. 2002); (b) to assess the effects of reinoculation on rhizobial soil population and on common bean yield; and (c) to verify the effects of supply with N fertilizers.

Materials and methods

R. tropici strains used in this study included: type strain CIAT 899^T (= UMR 1899, = USDA 9030, = TAL 1797, = HAMBI 1163, = ATCC 49672), which has the designation of SEMIA 4077 in the Brazilian germplasm bank; PRF 81 (= SEMIA 4080), isolated from a soil of the Paraná State (Hungria et al. 2000); and strains H 12, H 20, H 53, H 54 and H 57, isolated from Brazilian Cerrados soils (Mostasso et al. 2002). All strains came from the Embrapa Soja culture collection.

Six field experiments were performed between 1999 and 2001 in oxisols of Paraná State, southern Brazil, in the districts of Londrina and Ponta Grossa. The main chemical characteristics of the soils before sowing in 1999 were as follows, for Londrina and Ponta Grossa, respectively: acid soils, with pH in $CaCl_2$ of 5.19 and 4.95 and low contents of N (0.15 and 0.12 g kg^{-1}), C (2.13 and 2.08 g kg^{-1}) and P (6.9 and 4.1 mg kg^{-1}). Soil textures were, for Londrina and Ponta Grossa, respectively: clay 72% and 44%, lime 17% and 8%, sand 11% and 48%. The experimental plots measured 5.0×4.0 m, with 0.5 m between lines, and plots were separated by 2.0 m and small terraces. Five days before each sowing, plots received 84 kg ha^{-1} P and 60 kg ha^{-1} K. The cultivar Pérola (colored seeds) was used in Ponta Grossa and Diamante Negro (black seeds) in Londrina.

The rhizobia soil population was evaluated at the depth of 0–10 cm before each sowing, and at 20–25 (early vegetative growth), and 30–38 (early flowering) days after emergence (DAE) in each treatment, using the most probable number (MPN) counting technique (Vincent 1970) with bean plants of cultivar Pérola and the statistical tables of Andrade and Hamakawa (1994). Soil population was also evaluated 1 year after the last harvest, in 2002. The experiments were always performed in the same plots to verify the effects of reinoculation. Non-inoculated controls with or without N fertilizers (30 kg of N ha^{-1} as urea at sowing and 30 kg of N ha^{-1} at 35 days after sowing, spread) were always included.

In 2001 two other experiments were performed in Londrina and Ponta Grossa to verify the effects of N fertilizer addition. The treatments included inoculation with strains PRF 81, H 12 and H 20. For the inoculation with PRF 81 additional N fertilizer treatments were: 15 kg N at sowing and 15 kg N at early flowering; 15 kg N at sowing and 30 kg N at early flowering; and 30 kg N at sowing and 30 kg N at early flowering. These N fertilizer treatments were also included without inoculation using the bean cultivar IAPAR 14 (black seeds).

The inoculants were prepared with sterile peat and at a final concentration of 10^9 cells g^{-1} peat. Inoculant was added at a rate of 500 g 50 kg^{-1} seeds with 300 ml 10% (w/v) sucrose solution to increase adherence.

At early flowering (30–38 DAE) 12 plants were randomly chosen for the evaluation of nodulation and plant growth. Shoots were removed, roots were washed and nodules were also removed. Plant material was placed in a forced-air dryer at 65°C until constant weight was reached (approximately 72 h). Nodulation (nodule number and dry weight), shoot and root dry weight, total N (Kjeldahl digestion and determination of N concentration using a N automatic analyzer; Tecator) and N-ureide (Hungria and Neves 1987) contents in shoots were determined. Yield was evaluated at the final harvest, considering an area of 3.0×2.0 m from the four central rows of each plot. Seeds were cleaned and weighed and values were corrected to 13% moisture content, after determination of the humidity level in a grain moisture tester.

Nodule occupancy by the inoculant strains was evaluated in 40 randomly chosen nodules from each treatment, in plants collected at early flowering. Strains were isolated from the nodules and purified using standard microbiological procedures (Vincent 1970). The DNA of each strain was extracted and 50 ng were used for amplification by the PCR (Polymerase Chain Reaction) technique with ERIC (Enterobacterial Repetitive Intergeneric Consensus) primer, as described before (Mostasso et al. 2002). Each of the strains used as inoculant has a different ERIC-PCR profile (Mostasso et al. 2002), therefore it was possible to estimate nodule occupancy by the inoculated strains by analyzing the profiles obtained.

The experiments were performed in a complete randomized block design with six replicates. Data were submitted to analysis of variance (SAS Institute 1999) and statistically significant differences were determined by the Duncan's test. After 6 years, all data were submitted to a multivariate analysis, considering the effects of treatments, sites and the cropping seasons. Statistically significant differences between means were also determined by the Duncan's test (SAS Institute 1999).

Soil bacteria diversity was evaluated 1 year after the last experiment. Soil samples of the 0- to 10-cm layer were taken from each plot in Londrina and Ponta Grossa and microbial DNA was extracted using the Ultraclean soil DNA kit (Mobio Laboratories, United States). The DNA extracted was amplified using the bacterial primer sequences described by Kozdrój and van Elsas (2001). Amplification was performed using the following cycles: 2 min at 94°C; 30 cycles of 1 min at 94°C, 2 min at 55°C, 2 min at 72°C; followed by 10 min at 72°C. Gel electrophoresis (1.5% w/v agarose gel) confirmed one band of approximately 700 bp. DNA of soil extracts (200–500 ng) were loaded onto a 6% (w/v) polyacrylamide gel with a denaturing gradient ranging from 20% to 70%, in a DGGE (Denaturating Gradient Gel Electrophoresis) apparatus (Bio-Rad DCode) as described elsewhere (Kozdrój and van Elsas 2001). Gels were photographed and the lanes detected were analyzed using the Bionumerics program (Applied Mathematics, Kortrijk, Belgium).

Results and discussion

The indigenous rhizobial population before the first sowing was estimated at 1.1×10^3 and 3.2×10^3 cells g^{-1} soil in Ponta Grossa and Londrina, respectively. However, despite the high population of rhizobia, inoculation allowed an increase in rhizobial population. Figure 1 shows the results obtained in non-inoculated or inoculated plots with strain H 12 in Ponta Grossa, where the rhizobial population increased slightly with the presence of the common bean plant, but a further expressive increase was obtained by inoculation with strain H 12. Similar results were obtained with the other strains, in both Londrina and Ponta Grossa (data not shown). After the last common bean harvest, in 2001, the population of H 12 in Ponta Grossa was estimated at 4.9×10^3 cells g^{-1} soil and, 1 year after, at 2.1×10^3 cells g^{-1}, while in the non-inoculated treatment it was estimated at 0.9×10^3 cells

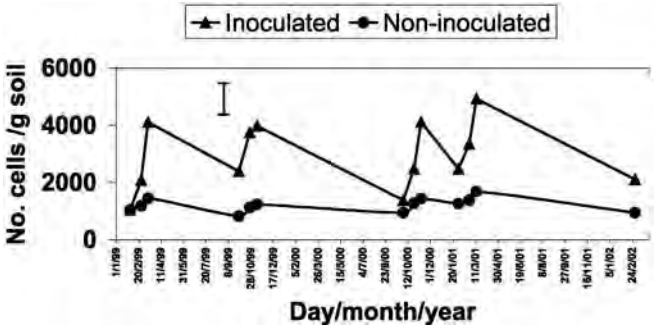

Fig. 1 Rhizobia soil population evaluated at the depth of 0–10 cm before each sowing, at 20 (early vegetative growth), and 35 (early flowering) days after emergence using the MPN counting technique with bean plants of cultivar Pérola. Soil samples taken in Ponta Grossa, from plots non-inoculated or inoculated with *Rhizobium tropici* strain H 12. The vertical bar indicates the LSD value at $p \leq 0.05$ (Duncan's test)

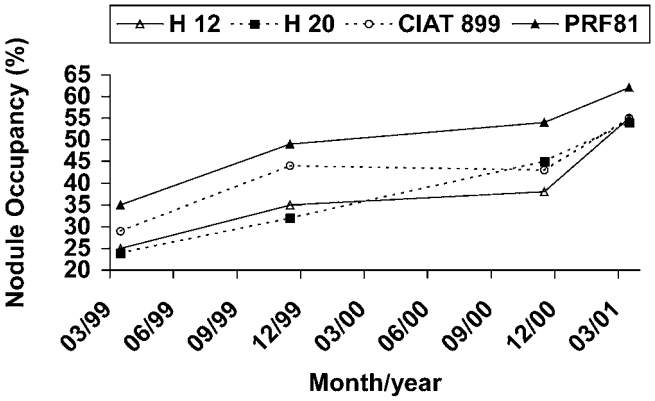

Fig. 2 Nodule occupancy (% of nodules) by strains H 12, H 20, CIAT 899 and PRF 81 in 3 years of field experiments in Ponta Grossa, PR, Brazil. Occupancy was evaluated by the ERIC-PCR profile of strains isolated from 40 nodules per treatment and the data represent the means of six replicates

g^{-1}. Therefore the populations decreased 1 year after the last cropping, but remained higher in plots that had been inoculated, and similar results were obtained in Londrina as well as in both sites for the other strains (data not shown).

Apparently, no differences were detected in the DGGE profiles 1 year after the last experiment in both Londrina and Ponta Grossa (data not shown). There are limitations to the DGGE technique, e.g. non-culturable bacteria can be detected, but clearly not all species are represented (Müller et al. 2002). Furthermore, the profiles obtained with this technique only show the dominant species and bands from more than one species may be hidden behind one band, thus underestimating bacterial diversity (Heuer et al. 2001). However, despite those limitations, the method seems to be the most sensitive for detecting differences in community diversity (Müller et al. 2002). Finally, one should also consider that in our study we have used universal primers for the 16S rRNA region (Kozdrój and van Elsas 2001) as a first approach to evaluate changes in bacteria community diversity. However, for more detailed studies, other specific primers, such as those proposed by Gomes et al. (2001), would be more appropriate since they can distinguish different phylogenetic groups of bacteria. In our study, the DGGE profiles obtained with the soil DNA of the different treatments amplified with universal primers were similar; therefore the massive introduction of rhizobial strains apparently did not affect other bacteria in the community. However, the method did not allow for the detection of the increase in the rhizobial population.

In general, inoculation with strains H 12, H 20, PRF 81 and CIAT 899 also increased nodule dry weight, and resulted in higher N_2 fixation rates, expressed by both the higher content of total N in shoots and the higher percentage of N as ureides. Statistically significant increases in nodulation and N contents were obtained for all experiments performed, except for those in Londrina in October of 2000 and February of 2001 due to a very dry season, and as an example Table 1 displays the results obtained in Ponta Grossa in February of 2001.

Table 1 Effects of inoculation with selected *Rhizobium tropici* strains on nodulation and N accumulation in shoots of bean cultivar Pérola at early flowering (35 days after emergence). Experiment performed in Ponta Grossa, PR, in February of 2001

Treatment	Nodulation		N content	
	Number (no./pl)[c]	Dry weight (mg/pl)	Total N (mg N/pl)	N-ureides (%)
H 12	62 a[a]	70 bc	113 ab	58 ab
H 20	55 a	68 bc	102 abc	55 ab
H 53	47 a	55 c	87 b	49 b
H 54	51 a	58 c	67 cd	50 b
H 57	52 a	62 c	74 cd	52 b
CIAT 899	68 a	88 ab	120 ab	60 ab
PRF 81	70 a	92 a	138 a	65 a
C[b] − N	45 a	25 d	44 d	25 c
C[b] + N	22 a	12 d	38 d	15 c
CV(%)	38	25	11	13

[a] *Values followed by the same letter, in the same column*, did not show statistical difference ($p \leq 0.05$, Duncan's test)
[b] Non-inoculated control, with or without N fertilizer (30 kg N ha^{-1} as urea at sowing and 30 kg N ha^{-1} at 35 days after sowing, spread)
[c] *pl* in an plant

An increase in nodule occupancy by inoculated strains was confirmed after the first inoculation, with a further increase with reinoculation and cropping, as shown in Fig. 2 for strains H 12, H 20, CIAT 899 and PRF 81 in Ponta Grossa. For strain H 12, nodule occupancy increased from 25% in 1999 to 55% in 2001. Considering the mean of the four strains with the better performances, H 12, H 20, PRF 81 and CIAT 899, nodule occupancy increased from 28% in the first experiment to 56% after four inoculation procedures. In a previous study with PRF 81, it was not possible to evaluate nodule occupancy by serology under field conditions because of cross reactions with indigenous bean rhizobia (Hungria et al. 2000). Therefore, in this paper, the use of ERIC-PCR profiles has proven to be a useful technique to follow competitiveness

Table 2 Grain yield (kg ha^{-1}) of common bean inoculated with *R. tropici* strains or non-inoculated receiving N fertilizer or not. Experiments performed in Ponta Grossa and Londrina, State of Paraná, Brazil, in soils with an initial population estimated as 1.1×10^3 and 3.2×10^3 cells *Rhizobium* g^{-1} soil, respectively

Treatment	Ponta Grossa (kg ha^{-1})				Londrina (kg ha^{-1})		Mean yield (kg ha^{-1})
	02/99 Pérola	10/99 Pérola	10/00 Pérola	02/01 Pérola	02/01 D.Negro	10/01 D.Negro	
H 12	2,301 ab[a]	2,663 a	544 a	755 b	1,985 abc	1,751 a	1,667 a
H 20	2,321 a	2,582 a	578 a	1,046 a	2,049 ab	1,594 ab	1,695 a
H 53	1,954 c	2,103 bc	534 a	647 b	1,923 bc	1,198 c	1,394 cd
H 54	2,013 bc	2,112 bc	579 a	760 b	1,893 bc	1,413 bc	1,462 bc
H 57	1,970 c	2,002 cd	554 a	808 b	2,104 b	1,417 bc	1,476 bc
CIAT 899	2,211 abc	2,480 ab	538 a	930 ab	2,240 a	1,468 b	1,644 ab
PRF 81	2,328 a	2,650 a	599 a	809 b	2,168 b	1,435 bc	1,665 a
C[b] – N	1,321 d	1,612 d	530 a	809 b	1,734 c	1,375 bc	1,230 d
C[b] + N	2,455 a	2,771 a	607 a	801 b	1,902 bc	1,629 ab	1,694 a
CV (%)	11	9	18	15	11	12	13

[a] *Values followed by the same letter, in the same column*, did not show statistical difference ($p \leq 0.05$, Duncan's test)
[b] Non-inoculated control, with or without N fertilizer (30 kg N ha^{-1} as urea at sowing and 30 kg N ha^{-1} at 35 days after sowing, spread)

and persistence of strains in the presence of a wide diversity of indigenous rhizobia.

Even though yield of two out of the six experiments was drastically affected by a dry season, it was possible to verify the benefits related to the inoculation with selected strains in both Londrina and Ponta Grossa (Table 2). Considerable increases in yield were obtained in both places and, as an average of the six experiments, the strains H 12 and H 20 showed gains of 437 kg grains ha^{-1} and 465 kg ha^{-1}, respectively, over the indigenous rhizobia population. The inoculation with the two strains used in the Brazilian commercial inoculants, CIAT 899 and PRF 81, also increased yield in relation to the indigenous population, confirming the usefulness of their recommendation in commercial inoculants (Hungria et al. 2000). The yields obtained by inoculation with the four *R. tropici* strains were comparable to that of the control receiving 60 kg N ha^{-1} (Table 2).

Failures in inoculation of the common bean crop have often been reported and usually attributed to the lack of competitiveness against indigenous rhizobia, and to environmental and plant genetic factors (Graham 1981; Pereira et al. 1984; Buttery et al. 1987; Hardarson 1993). However, the results described in this paper show that through a selection program of rhizobial strains it is possible to increase nodulation, N_2 fixation rates and yield. Adding to other reports (Mendes et al. 1994; Peres et al. 1994; Hungria et al. 1997, 2000; Mostasso et al. 2002), there is thus evidence that N_2 fixation in Brazilian cultivars can support high yields even in soils poor in N. The Brazilian strain selection program has decided to work with *R. tropici* species (Hungria and Araujo 1995) due to their higher tolerance to acidity and high temperature and to their higher genetic stability in comparison to other common bean species (Martínez-Romero et al. 1991; Hungria et al. 1993, 2000; Michiels et al. 1994). Indeed, the genetic instability of strains belonging to *R. leguminosarum* bv. *phaseoli* used in Brazilian commercial inoculants has already been reported (Hungria and Araujo 1995), but the efficacy of *R. etli* selected strains still remains to be determined.

The competitiveness among common bean rhizobial species has been discussed and there are reports indicating that *R. tropici* would be less competitive than *R. etli* or *R. leguminosarum* (Martínez-Romero and Rosenblueth 1990; Oliveira and Graham 1990; Streit et al. 1992). More recently, the effects of soil pH on competitiveness were reported (Anyango et al. 1995), giving advantage to *R. tropici* in acid soils. In this study, a considerable increase in nodule occupancy of *R. tropici* even in the presence of a high indigenous population was observed, confirming previous results obtained by our group (Hungria et al. 2000; Mostasso et al. 2002), and showing the importance of developing an efficient symbiosis of local cultivars with adapted strains selected from the indigenous population.

In the other two experiments performed in 2001 with inoculation and N fertilizers, the rhizobial soil population was estimated at 1.1×10^3 and 1.1×10^4 cells g^{-1} soil, for Ponta Grossa and Londrina, respectively. In Ponta Grossa, although no differences were observed in yield, the addition of N fertilizer resulted in higher yields, with the higher values being observed with PRF 81 as inoculant, and 15 kg N at sowing and 30 kg N at early flowering stage. The application of 30 kg N at sowing decreased early nodulation (data not shown), without bringing further benefits in yield (Table 3). In Londrina, the dry season limited yield but PRF 81 increased yield, and the supply of 30 kg N either at sowing or at flowering stage without inoculation decreased nodulation (data not shown) and did not result in yield increases (Table 3). A combined analysis of both experiments showed the synergistic effect between the low level of N fertilizer and the N_2 fixation-N. Thus on average, inoculation with strain PRF 81 increased yield by 178 kg ha^{-1} in relation to the indigenous population, and a further increase of 132 kg ha^{-1} was obtained with a supplement of 15 kg N at sowing and 15 kg N at the early flowering stage. On the other hand, the addition of the same amount of N in the absence of inoculation only increased the yield by 10 kg ha^{-1}, and the addition of 30 kg N at sowing and 30 kg N at early flowering increased yield by 238 kg ha^{-1}, a lower

Table 3 Effects of inoculation and addition of N fertilizer on shoot N content at the flowering stage and yield of common bean cultivar IAPAR 14. Experiments performed in Ponta Grossa and Londrina, State of Paraná, in 2001, in soils with 1.1×10^3 and 1.1×10^4 cells *Rhizobium* g^{-1} soil, respectively

Treatment	Ponta Grossa		Londrina		Mean yield (kg ha^{-1})
	N (mg g^{-1})	Yield (kg ha^{-1})	N (mg g^{-1})	Yield (kg ha^{-1})	
Non-inoculated control (C)	31.3 a[a]	2,104 a	34.5 a	689 e	1,397 c
C +15 kg N sow. +15 kg N e.f.[b]	26.6 a	2,106 a	36.0 a	708 e	1,407 bc
C +15 kg N sow. +30 kg N e.a.	29.4 a	2,376 a	35.9 a	720 e	1,548 abc
C +30 kg N sow. +30 kg N e.f.	31.9 a	2,356 a	37.3 a	913 bc	1,635 ab
H 12	26.8 a	2,181 a	34.0 a	724 de	1,453 bc
H 20	29.5 a	2,307 a	33.6 a	648 e	1,478 abc
PRF 81	30.3 a	2,183 a	36.9 a	966 ab	1,575 abc
PRF 81+15 kg N sow. +15 kg N e.f.	31.0 a	2,342 a	36.2 a	1,072 a	1,707 a
PRF 81+15 kg N sow. +30 kg N e.f.	31.1 a	2,404 a	36.4 a	834 cd	1,619 abc
PRF 81+30 kg N sow. +30 kg N e.f	30.5 a	2,339 a	37.7 a	1.043 a	1,691 a
CV (%)	10	12	8	13	14

[a] *Values followed by the same letter, in the same column*, did not show statistical difference ($p \leq 0.05$, Duncan's test)
[b] *sow.* sowing stage, *e.f.* early flowering stage

value in comparison with the inoculation with half of that N dosage (310 kg ha^{-1}) (Table 3). Thus low levels of N fertilizer and inoculation with superior strains can increase yield, at a low cost, improving the supply of this important source of protein for the Latin America population.

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