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Agronomic performance of maize under nitrogen fertilization and inoculation with *Azospirillum brasilense*

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Abstract

Given the potential of *Azospirillum brasilense* as an alternative to improve availability and use of N by maize, it is critical to conduct research in different regions to assess the results of inoculation under several different conditions. The study aimed to analyse the influence of *Azospirillum brasilense* on the growth and maize yield, as well as its interaction with nitrogen (N) fertilization. The experiment was conducted in two trials (Palotina, PR – trial 1; Ariquemes, RO – trial 2) in Brazil. The experimental design was randomized blocks in a factorial 2x4 arrangement. First factor was application of N fertilization (with or without application), while the second factor consisted of *A. brasilense* inoculation such as: no inoculation (control), via seed treatment (ST), via leaf application (LA) and ST + LA. The N contents of leaf and the agronomic performance were assessed. Analysis of variance was conducted with the F test (p<0.01). The mean levels of the inoculation factor with *A. brasilense* were compared via Tukey's test (p<0.01). The F-test was conclusive in comparing the means of the levels of N application factor. The analysis was conducted by means of orthogonal contrasts to compare the treatment of *A. brasilense* with those treated with N fertilization. In trial 1, the applying N fertilization associated to *A. brasilense* via ST and LA may contribute to increase yield. In trial 2, inoculation with *A. brasilense* seems to be a technology with good prospects for application, contributing to improve production conditions and increase maize yield. Inoculation with *A. brasilense* is important to supply N to plants, with an increase in agronomic performance of maize. However, inoculation with *A. brasilense* was not sufficient to meet the demand for N fertilization.

Keywords: Biological fixation, Fertilization, Maize yield, Plant nutrition, *Zea mays* L.
Abbreviations: N_nitrogen; ST_seed treatment; LA_leaf application; PR_Paraná; RO_Rondônia; cp_commercial product; ANOVA_analysis of variance

Introduction

Providing appropriate nutrition is essential to achieve high maize yields, and nitrogen (N) is a key nutrient to increase yield and ensure expression of the full productive potential of the maize hybrid being used (Melo et al., 2011; Galindo et al., 2019). The soil can make N available to crops from the mineralization of organic matter. Therefore, the higher the content of organic matter in the soil, the maize will have a greater amount of available N. In no-tillage crops in the state of Paraná (PR) in Southern Brazil, the yield reached 9,000 to 10,000 kg ha^{-1}, while in specific situations the maximum yield reached up to 18,000 kg ha^{-1} (Fontoura and Bayer, 2009).

In the no-till system, there may be less N available for maize when plants start to grow due to microbial biomass that may reduce N availability by immobilizing mineral N (Oliveira and Caires, 2003; Dai et al., 2021). Urea is the most commonly source used as topdressing fertilizer since it is more affordable. However, the volatilization contributes to low N recovery by maize plants (Oliveira and Caires, 2003; Zhang et al., 2019).

Inoculation with bacteria of the genus *Azospirillum* is an interesting alternative to make the best use of N available in the soil, since it can increase the yield of important crops such as maize under various conditions of climate and soil by N biological fixation and increasing the volume of soil exploited with the increase in the root system (Hungria et al., 2010; Santos et al., 2012; Zeffa et al., 2019). Inoculation with *Azospirillum* can make an important contribution to supplement of nitrogen supply in maize plants through biological fixation and it can also boost growth of these plants due to production of phytohormones. Thus, as maize requires large amounts of N to achieve high yields, biological fixation can be used as an additional source of N (França et al., 2011; Gavilanes et al., 2020).

It must be noted that factors such as plant genotype, strains and edaphoclimatic conditions influence the benefits that inoculation in maize can provide. In addition, the different systems of cultivation and soil should be considered when analysing the gains that can be achieved with biological fixation of N (Oliveira et al., 2018; Enrico et al., 2020). Given the potential of *Azospirillum brasilense* as an alternative to improve availability and use of N by maize, it is critical to conduct research in different regions to assess the results of inoculation under several different conditions. Therefore, this investigation aims to analyse the influence of *A. brasilense* on the development and yield of maize, and its interaction with N fertilization.

Results

Agronomic performance of maize at Palotina, PR (trial 1)

The application of N did not affect the leaf N content compared to the control (non-application of N). Without inoculation, the leaf N content was 23.89 g kg^{-1}, a value lower than the one seen when N was not applied, with 27.17 g kg^{-1} of content of leaf N. For the inoculation factor of *A. brasilense*, no significant effect (p>0.01) was seen. For the contrast, a significant effect was seen (p<0.01). The values obtained in leaf N content with inoculation of *A. brasilense* were 2.72 g kg^{-1} higher in comparison to the one seen for application of N (Table 2).

No significant effect on plant height was observed for the inoculation factor of *A. brasilense*. For the N application, greater plant height was seen in comparison to non-application of N within the following levels: without inoculation, ST, and ST + LA. In contrast, a significant effect was observed for treatments with inoculation of *A. brasilense*, while a 0.07 m reduction in height was seen in comparison to the height that observed for application of N (Table 2).

For the height of ear insertion, a difference was observed only for application of N compared to non-application, within the inoculation of *A. brasilense* ST + LA, with greater height for the application of N. No significant effects were seen for inoculation and contrast (Table 2). Likewise, no significant effect was observed for stem diameter and number of rows of grains per ear for both factors and for the contrast (data not shown).

In the absence of *A. brasilense*, the number of grains per row increased by 6.01% with N application, while it was 10.45% in ST + LA inoculation. No significant effect was observed in the number of grains per row for the inoculation factor of *A. brasilense* and the contrast (Table 3). For 100-grains weight, no significant effect was observed for both factors and for contrast (data not shown). For yield, a significant effect was verified only for the N application, with an increase of 1,455 kg ha^{-1} under inoculation of *A. brasilense* ST + LA, compared to non-application of N fertilization (Table 3).

Agronomic performance of maize at Ariquemes, RO (trial 2)

For leaf N content in maize plants, application of N fertilization resulted in an increment in all levels of inoculation of *A. brasilense*. A significant effect for the contrast was also observed. The group of treatments that got inoculation with *A. brasilense* provided smaller content of N compared to the group that got application of N fertilization with a difference of 2.26 g kg^{-1} (Table 4).

A significant effect was observed on plant height for both factors, with increase of N application without inoculation of *A. brasilense*, whereas for all forms of inoculation, greater height was also observed in comparison to non-inoculation. For the height of cob insertion and stem diameter, a significant effect was observed for the application of N compared to N control (no N applied) also without inoculation of *A. brasilense*. The contrast was also significant for stem diameter, with a 1.29 mm reduction for inoculation of *A. brasilense* compared to the application of N fertilization (Table 4).

A significant effect was observed for both factors on the number of rows per ear and number of grains per row. In general, increment happens due to the application of N within the level without inoculation or increment due to inoculation of *A. brasilense* in the level without N application. In contrast, a significant effect was seen only for the number of grains per row, with reduction for the group with inoculation of *A. brasilense*, compared to the group with application of N. However, for 100-grains weight, a significant effect was observed only for N application, with an increase in the inoculation level of *A. brasilense* ST + LA (Table 5).

For yield, a significant effect was seen for both factors. Within the level without N application, inoculation with *A. brasilense* ST, LA, and ST + LA provided increments of 27, 26 and 25%, respectively. Within the inoculation level of *A. brasilense* ST + LA, there was an increase of 18% when N was applied, as well as in the non-inoculation level, with an increase of 39% when N was applied. In contrast, a significant effect was observed: the group that received only one application of N fertilization was higher by 1,115 kg^{-1} compared to the group of those which were inoculated only with *A. brasilense* (Table 5).

Discussion

The smallest content of leaf N was detected at trial 1 for application of N without *A. brasilense*. This can be explained by the diluting effect, since the plants that got N application were taller compared to those that did not get N. The development of dense leaf canopies may result in decreased concentration of N on leaves, even if this nutrient is not limiting. To increase photosynthesis, the plants transfer N from shaded leaves to leaves that get more light. As for the leaf/stem ratio, when the maize plant grows, the stem grows more than leaf, leading to a reduction in N concentration in the entire plant, because the stem has lower N concentration (Riedell 2010; Zhao et al., 2018), as opposed to what was observed at trial 2, where higher leaf N content was seen for application of N fertilization.

The greater plant growth and the increase in leaf area are explained by the greater amount of available N, since plants that are in good nutritional condition have greater capacity to assimilate CO_2 and synthesize carbohydrates, thus generating greater growth and durability of the leaf area in the plant. Deficiency of N decreases the active photosynthetic area, since leaf senescence can take place earlier due to transfer of N from older leaves to the growth spots, leading to reduced cob size and grain weight (Repke et al., 2013). Generally, in this study, inoculation with *A. brasilense* alone was not enough to justify the results obtained for application of N.

Table 1. Results of chemical and physical analysis of soil from experimental areas.

Trial 1 - Palotina, PR										
pH - $CaCl_2$	P - Mehlich	OM	Ca	Mg	K	Al	H+Al³	SB	CEC	V
	(mg dm^{-3})	(g dm^{-3})	------------- (cmolc dm^{-3}) -------------							(%)
5.1	14.2	36.0	6.5	1.8	0.8	0.1	4.1	9.1	13.2	69.0
Sand			Silt			Clay				
-- % --										
7.4			24.9			67.7				
Trial 2 - Ariquemes, RO										
pH - $CaCl_2$	P - Resin	OM	Ca	Mg	K	Al	H+Al³	SB	CEC	V
	(mg dm^{-3})	(g dm^{-3})	------------- (cmolc dm^{-3}) -------------							(%)
4.3	10.0	18.0	0.8	0.3	0.1	0.3	3.8	1.2	5.0	24.0
Sand			Silt			Clay				
-- % --										
13.0			21.2			65.8				

OM: organic matter, SB: sum of bases, CEC: cation exchange capacity, V: base saturation.

Table 2. Leaf N content, plant height, and ear height insertion of maize plants under *A. brasilense* inoculation and N application. Palotina, PR, Brazil, 2014/2015 (trial 1).

Leaf N content (g kg^{-1})				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	23.89 b	25.98 a	26.15 a	25.60 a
Without	27.17 a	27.00 a	26.57 a	26.27 a
Mean:	26.08	F (*A. brasilense*):		NS
CV:	7.04	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	*		Estimate:	2.72
Plant height (m)				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	2.02 a	1.98 a	1.95 a	2.03 a
Without	1.93 b	1.92 b	1.97 a	1.94 b
Mean:	1.97	F (*A. brasilense*):		NS
CV:	2.78	F (N):		*
Contrast (inoculation with *A. brasilense* - application of N)				
F:	*		Estimate:	-0.07
Ear insertion height (m)				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	0.99 a	1.00 a	0.99 a	1.02 a
Without	0.98 a	0.97 a	0.99 a	0.95 b
Mean:	0.99	F (*A. brasilense*):		NS
CV:	5.71	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	NS			

*Significant (p<0.01). Means followed by same letter (lowercase) for N application do not differ each other, by F-test at level of 10%. NS: non-significant (p>0.01), means do not differ from each other. ST: seed treatment, LA: leaf application.

Table 3. Number of grains per row and yield of maize plants under *A. brasilense* inoculation and N application. Palotina, PR, Brazil, 2014/2015 (trial 1).

Number of grains per row				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	27.45 a	27.16 a	26.56 a	28.32 a
Without	25.80 b	26.80 a	27.08 a	25.36 b
Mean:	26.81	F (*A. brasilense*):		NS
CV:	5.01	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				

F:	NS			
Yield (kg ha^{-1})				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	12,346 a	12,213 a	11,610 a	13,024 a
Without	11,470 a	12,464 a	12,735 a	11,569 b
Mean:	12,179	F (*A. brasilense*):		NS
CV:	12.92	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	NS			

*Significant (p<0.01). Means followed by same letter (lowercase) for N application do not differ each other, by F-test at level of 10%. NS: non-significant (p>0.01), means do not differ from each other. ST: seed treatment, LA: leaf application.

Table 4. Leaf N content, plant height, ear height insertion, and stalk diameter of maize plants under *A. brasilense* inoculation and N application. Ariquemes, RO, Brazil, 2015/2016 (trial 2).

Leaf N content (g kg^{-1})				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	22.19 a	23.62 a	23.97 a	23.64 a
Without	18.09 b	21.03 b	19.14 b	19.60 b
Mean:	21.41	F (*A. brasilense*):		NS
CV:	9.88	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	*		Estimate:	-2.26
Plant height (m)				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	1.95 Aa	1.94 Aa	1.95 Aa	1.94 Aa
Without	1.78 Bb	1.94 Aa	1.94 Aa	1.94 Aa
Mean:	1.91	F (*A. brasilense*):		*
CV:	2.83	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	NS			
Ear height insertion (m)				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	0.91 a	0.89 a	0.89 a	0.89 a
Without	0.81 b	0.88 a	0.88 a	0.89 a
Mean:	0.91	F (*A. brasilense*):		NS
CV:	2.83	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	NS			
Stalk diameter (mm)				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	18.55 a	17.34 a	17.33 a	18.40 a
Without	16.47 b	17.36 a	17.01 a	17.42 a
Mean:	17.48	F (*A. brasilense*):		NS
CV:	5.48	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	*		Estimate:	-1.29

*Significant (p<0.01). Means followed by same letter (lowercase) for N application do not differ each other, by F-test at level of 10%. Means followed by same letter (uppercase) for *A. brasilense* inoculation do not differ each other by Tukey's (1949) test, by 10% level.NS: non-significant (p>0.01), means do not differ from each other. ST: seed treatment, LA: leaf application.

Table 5. Number of grain rows per ear, number of grains per row, 100-grain weight, and yield of maize plants under *A. brasilense* inoculation and N application. Ariquemes, RO, Brazil, 2015/2016 (trial 2).

Number of grain rows per ear				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	14.49 Aa	14.12 Aa	14.03 Aa	14.54 Aa
Without	13.45 Bb	14.20 ABa	14.20 ABa	14.33 Aa
Mean:	14.17	F (*A. brasilense*):		*
CV:	3.13	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	NS			
Number of grains per row				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	29.20 ABa	27.66 ABa	27.03 Ba	30.12 Aa
Without	23.54 Bb	27.03 Aa	27.41 ABa	27.24 Aa
Mean:	27.28	F (*A. brasilense*):		*
CV:	6.52	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	*		Estimate:	-2.31
100-grain weight (g)				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	22.61 a	23.87 a	22.68 a	25.57 a
Without	21.76 a	21.87 a	23.60 a	22.91 b
Mean:	23.11	F (*A. brasilense*):		NS
CV:	7.98	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	NS			
Yield (kg ha^{-1})				
	A. brasilense			
N	Without	ST	LA	ST + LA
With	6,622 Aa	6,580 Aa	6,086 Aa	6,612 Aa
Without	4,055 Bb	5,586 Ab	5,501 Aa	5,432 Ab
Mean:	5,809	F (*A. brasilense*):		*
CV:	12.92	F (N):		*
Contrast (inoculation *A. brasilense* - application of N)				
F:	*		Estimate:	-1,115

*Significant (p<0.01). Means followed by same letter (lowercase) for N application do not differ each other, by F-test at level of 10%. Means followed by same letter (uppercase) for *A. brasilense* inoculation do not differ each other by Tukey's (1949) test, by 10% level. NS: non-significant (p>0.01), means do not differ from each other. ST: seed treatment, LA: leaf application.

However, it improved many parameters of maize plants as a supplement to application of N. Dartora et al. (2013) assessed inoculants such as *A. brasilense* (*Ab-V5* strain) and on *Herbaspirillum seropedicae* (*SmR1* strain) together with nitrogen fertilization and observed that stem diameter was increased due to inoculation. The increase in the stem diameter is a key component for plant physiology because, in addition to playing the role of supporting the leaves, it acts as a storage structure for soluble solids, which are essential for kernels to develop (Carmo et al., 2012). Therefore, the greater the stem diameter, the greater the capacity of the plant to store photo-assimilates, which will later be used for grain filling.

The factors that interfere in plant responses are not fully understood, and the results found are largely influenced by factors from the bacterium itself, such as the strain used and the number of cells per seed (Repke et al., 2013). The positive effects provided by *A. brasilense* are observed in other studies. Different strains of this bacterium increased maize yield (Hungria et al., 2010; Lana et al., 2012; Skonieski et al., 2019).

The greater yield can probably be explained by the benefits provided by inoculation with *Azospirillum* spp. which could stimulate growth of the root system and increase the rate of development of side roots (Radwan et al., 2004; Rozier et al., 2019). Other factors that cause these microorganisms to produce positive effects on plants are the production of substances that promote growth, increase protection against phytopathogens, increase resistance of plants to stress and biological fixation of N. The effectiveness of these bacteria can be impaired due to their free association with plants in the rhizosphere or inside the tissues, which increases their vulnerability to the environment. In addition, diazotrophic bacteria are impacted by the genotype of the host plant and by soil characteristics (Dartora et al., 2013).

In leguminous plants biological fixation can be used as an additional source of N to mineral fertilization and, since this nutrient is not available in sufficient quantity to nourish grasses such as maize. To ensure high yield in maize cultivation without extra costs, it is necessary to implement more modern and efficient management techniques for soil protection, such as straw no-tillage, planting vegetables to develop topdressing and promoting nutrient fixation or recycling (França et al., 2011).

To achieve this goal, and with higher costs of production inputs, particularly fertilizers, there is a growing interest in using inoculants with bacteria to boost plant growth, particularly plants that demand large nitrogen fertilization (Hungria et al., 2010). Thus, to supply the part of N that the maize crop needs, biological fixation is an suitable option (Lana et al., 2012).

The results obtained in trial 1 indicate that applying topdressing N associated to inoculation of *A. brasilense* via ST and PA, which can contribute to increase yield, even in a region with advanced production technology, and possibly prevent increased use of fertilizers. Moreover, the results indicate that *A. brasilense* showed positive effects on soil condition and crop management in the tested environment. Given the positive results produced in trial 2, inoculation with *A. brasilense* is presented as a technology with good prospects of application in the region to improve production conditions and increase yield for maize crop.

Inoculating *A. brasilense* with application of N fertilization produced promising results compared to non-inoculation. Therefore, the potential exists to use inoculation to supplement nitrogen fertilization to operate as productive components of maize in both sites where the study was performed.

Inoculating with *A. brasilense* analysis did not differ from applying N in most of the parameters analysed for maize. In some situations, the performance was inferior and in others it was superior, but still with very close values. The association of the increase in many parameters, when inoculation + N application was performed, reinforced the inoculation with *A. brasilense*. It is important to supply N to plants, with an increase in agronomic performance of maize. However, inoculation with *A. brasilense* was not sufficient to meet the demand for N fertilization. Further studies are required to improve this technique and reduce the use of nitrogen fertilizers and provide further benefits to crops.

Materials and Methods

Site description

The experiment was conducted in two sites: trial 1 located in Palotina, PR state, Brazil (24º34'S 53º86'W) and trial 2 located in Ariquemes, Rondônia (RO) state, Brazil (9º95'S 62º96'W). The climate of the region of trial 1 is the Cfa (mesothermal humid subtropical), per Koppen-Geiger climate classification, with average temperatures of 15 to 37 ºC and annual rainfall of approximately 1,650 mm (Aparecido et al., 2016). The climate of the region of trial 2 is the Aw (equatorial); the average annual temperature is 26.1 °C, with annual average precipitation of 1,928 mm (Alvares et al., 2013).

On September 11, 2014, no-tillage seeding of DKB-390 PRO3 (early cycle) maize hybrid was conducted in trial 1, an area which had been previously cultivated with wheat. Conventional seeding of GNZ 2005 (early cycle) maize hybrid was conducted in trial 2 on August 20, 2015. The chosen hybrids are recommended for each respective region, being among the most cultivated. Different hybrids were used since the locations are different in climatic and environmental conditions. Before being seeded, the area was ploughed and harrowed, and remained fallow. The pest management and base fertilization followed the usual technical recommendations prescribed for the regions. In trial 1, fertilization was performed at sowing with 300 kg ha^{-1} of N-P-K fertilizer (08-20-20), supplying 24 kg of N ha^{-1}, 60 kg of P_2O_5 ha^{-1} and 60 kg of K_2O ha^{-1}. In trial 2, fertilization was performed at seeding with 400 kg ha^{-1} of N-P-K fertilizer (04-30-16), supplying 16 kg N ha^{-1}, 120 kg P_2O_5 ha^{-1} and 64 kg K_2O ha^{-1}, besides using 51.7 kg ha^{-1} of KCl fertilizer, which supplied another 30 kg ha^{-1} of K_2O.

Experimental design

The experimental design was randomized blocks, with 5 (trial 1) or 4 (trial 2) repetitions in a 2 x 4 factorial arrangement. The first factor consisted of applying N fertilization (120 kg ha^{-1}) (with or without application). The second factor consisted of inoculating *A. brasilense*: without inoculation, via ST, via LA, and ST + LA. The experimental units included plots with 6 rows spaced at 0.6 m and 4 m long. The two centre meters of the two rows were considered usable area.

N fertilization was conducted with urea in stage V_4-V_5 of maize plants. For inoculation with *A. brasilense*, a commercial product (cp) containing *Abv5* and *Abv6* strains was used, with a guarantee of 2 x 108 colony-forming units. It used 100 mL cp ha^{-1} in ST and 200 mL cp ha^{-1} in LA in V_4-V_5 stage of the maize plants. For inoculation, a CO_2 pressurized backpack sprayer was used with constant 2-bar pressure at a flow rate of 0.65 L min^{-1}, equipped with 6 fan-type nozzles XR110.02 (Teejet®). The application was carried out 50 cm from the target at a speed of 1 m s^{-1}, thus supplying a spray volume of 200 L ha^{-1}.

Traits measured

The content of N leaf was determined by sulfuric solubilization following the Kjeldahl method (Sáez-Plaza et al., 2013). To collect leaf samples, the central third of the leaf at the base of the ear was removed, during the R_1 stage, for each sampled plant (Malavolta et al., 1997). To assess the height of insertion of the ear and total height of the plant, eight plants from each plot were measured. The measurement was made from the ground level to the ear node (insertion height) and up to the flag leaf ligule (plant height). The stem diameter was measured at the height of the second visible internode, in the R_4 stage.

Yield was determined by weighing the kernels in the usable area of each plot and moisture correction to 14%. The results were preseented in kg ha^{-1}. The number of grains per ear was obtained by counting the number of rows per ear and grains per row. Five ears per plot were considered for these assessments. To 100-grains weight, two subsamples per plot were measured. The weight obtained was corrected for 14% moisture.

Statistical analysis

Analysis of variance (ANOVA) was conducted with the F-test (p<0.01). The mean levels of the inoculation factor with *A. brasilense* were compared via Tukey's (1949) test (p<0.01). The F-test was conclusive in comparing the means of the levels of N application factor. Analysis by means of orthogonal contrasts was conducted to compare the treatment with inoculation of *A. brasilense* (without N application) with the treatment in which the application with N fertilization was performed (without inoculation of *A. brasilense*). The Sisvar 5.6 software was used for the analyses (Ferreira, 2011).

Conflict of interests

The authors appoint that there is not any conflict of interests.

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