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RESEARCH ARTICLE

# Paraburkholderia atlantica is the main rhizobial symbiont of Mimosa spp. in ultramafic soils in the Brazilian Cerrado biome

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## Abstract

*Aims* To evaluate the occurrence, the characterization and identity of nodulating bacteria in symbiosis with *Mimosa* spp. in the ultramafic massif of Barro Alto, Goiás state, Brazil.

*Methods* Nodules from field grown *M. somnians* and *M. claussenii* were sampled for bacteria isolation and *in situ* detection using microscopy. Isolates were characterized for their nodulation capacity on *M. pudica* and common bean, and their tolerance to Ni in culture medium. Bacteria were also partially identified by their 16S rRNA gene sequences. In addition, *recA*, *gyrB*, *nodC* and *nifH* genes from five representative isolates were sequenced for phylogenetic studies.

*Results* *In situ* detection indicated the exclusive presence of *Paraburkholderia* sp. within the nodules. This identification was confirmed for most of the isolates by the analysis of their 16S rRNA gene sequences. All isolates identified as *Paraburkholderia* sp. were able to effectively nodulate *M. pudica*, but those tested in common bean produced ineffective

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nodules. Inoculation tests showed that these bacteria improved *M. pudica* growth in the absence of nitrogen. None of the isolates showed Ni tolerance. The concatenated 16S rRNA, *recA* and *gyrB*, and the *nodC* and *nifH* genes sequences demonstrated that the five selected isolates were closely related to *P. atlantica*.

*Conclusions* This is the first report of *Paraburkholderia* present in nodules from *Mimosa* plants naturally growing in ultramafic soils. These results suggest that this symbiosis may be a factor to be considered as part of programs to restore ultramafic soils in Barro Alto areas degraded by mining activity.

**Keywords** Beta-rhizobia · Biological nitrogen fixation · Cerrado · Serpentine soils

## Introduction

Ultramafic soils contain excesses of potentially toxic metals, such as cobalt (Co), chromium (Cr), copper (Cu), and mainly nickel (Ni), in addition to a low availability of nutrients, such as calcium (Ca), phosphorus (P) and nitrogen (N), and a high magnesium (Mg)/Ca ratio, resulting in a strong mineral imbalance (Echevarria 2018). In Brazil, ultramafic complexes in the Goiás state (GO), in the Cerrado biome, are amongst the largest Ni reserves in the world. Since Ni is a highly sought metal for stainless steel production, these areas are of great economic importance due to the intense mining activity that started in the late 1970s (Barbosa et al. 2013; Reeves et al. 2007). On the other hand, these mining operations result in the degradation of extensive areas of native vegetation, demanding the development of restoration technologies.

The use of native plant species from ultramafic soils for the restoration of areas impacted by Ni mining may be an appropriate strategy since levels of heavy metals in these soils affect the development of non-native species not adapted to this type of environment (Van der Ent et al. 2013; Whiting et al. 2004). Furthermore, the characteristics of ultramafic soils lead to the occurrence of a range of plant species adapted to extreme soil conditions with specific physiological mechanisms (Pędziwiatr et al. 2018). This characteristic flora is undoubtedly related to a close association with soil and rhizosphere microorganisms, which most likely contribute to the conditioning of adaptive characteristics (Pessoa-Filho et al. 2015). An essential group within this microbiota, and which are specifically related to the nitrogen cycle, is that comprised of bacteria capable of fixing atmospheric nitrogen either in a free-living state or in symbiosis with plants such as legumes (Fabaceae).

A previous study of the predominant species in the ultramafic soils of Barro Alto – GO, identified two species of *Mimosa* (*M. claussenii* and *M. somnians*) as effective pioneer plants, with good biomass production, and tolerance to high Ni concentrations (Andrade 2011). *Mimosa* is a large genus from the Fabaceae family, with approximately 500 species, most of which are native to the New World (Simon et al. 2011). The largest center of diversity of *Mimosa* is central Brazil, where many species can be observed in the vegetation of the Cerrado and Caatinga, and wherein it is the most diverse genus (Mendonça et al. 1998). The Cerrado biome is also an important endemism center for *Mimosa* (Simon and Proença 2000). Studies on the occurrence and characterization of diazotrophic bacteria in symbiosis with *Mimosa*, native to these environments, may be important for designing programs and strategies that aim at environmental conservation and the recovery of degraded areas. Chaer et al. (2011) considered that the use of $N_2$-fixing legumes for reclamation of severely degraded lands is a technique that can be applied in several situations, emphasizing its potential to restore soil organic matter levels, ecosystem biodiversity, and other environmental functions.

Until 20 years ago, it was thought that only Alphaproteobacteria ("rhizobia") were capable of forming nodules in symbiosis with legumes. This perception was altered by studies indicating that particular legumes could also be nodulated by Betaproteobacteria (see review by Gyaneshwar et al. 2011). In particular, these studies focused on the genus *Mimosa* as it appeared to be mostly nodulated by Betaproteobacteria, specifically by *Paraburkholderia, Cupriavidus* and *Trinickia* spp. (Bontemps et al. 2010; Chen et al. 2005a, b, 2006, 2007, 2008; Estrada-de los Santos et al. 2018; Mishra et al. 2012; Sheu et al. 2012, 2013). *Mimosa* species from the Cerrado and Caatinga biomes are associated with these diazotrophic bacteria, particularly *Paraburkholderia* spp., which probably play a prominent role in N cycling in these ecosystems (Bontemps et al. 2010; Dias et al. 2021; Pires et al. 2018; Reis Jr et al. 2010). These same studies suggested that environmental characteristics, rather than host species, were responsible for determining the distribution of *Paraburkholderia*

species. Therefore, it is logical to assume that in an environment as particular as the ultramafic soils, biological nitrogen fixation (BNF) is conducted by microsymbionts that could still be unknown and specific to these unique environments.

Alpha and betaproteobacterial symbionts can be associated with leguminous plants in metal-rich soils. Vincent et al. (2019) studied the isolates from root nodules of *Acacia spirorbis* subsp. *spirorbis*, a mimosoid tree legume endemic to New Caledonia that grows in ultramafic soils, and found bacteria from the genera *Paraburkholderia* and *Bradyrhizobium*. In another study, the bacteria isolated from nodules of *Serianthes calycina*, also an endemic mimosoid legume from New Caledonia, clustered together with *Bradyrhizobium elkanii* (Chaintreuil et al 2007).

With regard to *Mimosa* symbionts, Klonowska et al. (2012) showed that out of 96 strains isolated from nodules of *M. pudica* in ultramafic soils, again from New Caledonia, 4% were identified as *Rhizobium mesoamericanum* and 96% as *Cupriavidus taiwanensis*. In Uruguay, in an area with soils originated from metal-rich underlying strata and containing considerable concentrations of Zn, Cu, Co, Ni, and Fe, all the isolates from nodules of five native *Mimosa* species belonged to the genus *Cupriavidus*, closely related to *C. necator* and *C. pinatubonensis* (Platero et al. 2016).

The present study aimed to evaluate the occurrence, to characterize, and to identify the nodulating diazotrophic bacteria in symbiosis with plants of the genus *Mimosa* in the ultramafic massif of Barro Alto – GO, Brazil. It is anticipated that the results of this study will help advance our knowledge about the symbiosis between diazotrophic bacteria and legumes present in soils with high metal concentrations, and provide new information that could be used to support restoration programs of ultramafic soil areas impacted by mining.

## Materials and methods

### Sampling sites

Sites in the ultramafic complex of Barro Alto – GO, located within the Anglo American mining company area, were selected based on the presence of primary vegetation and on topographical, geochemical and geological observations (Andrade 2011; Pessoa-Filho et al. 2015). Based on these data, samples were collected from three areas; Site 1, named SAP (15°06'04.4"S; 49°00'38.4"W), is characterized by a loamy Cambisol, mainly composed of saprolites, with 603.53 mg $kg^{-1}$ of extractable Ni, and is a ''campo sujo'' vegetation type, which is defined as a grassland formation with grasses and small and sparse shrubs (Oliveira-Filho and Ratter 2002); Site 2, named LAT (15°06'31.1"S; 49°01'15.0"W), is characterized by a sandy clay loam Oxisol, mainly composed of laterites, with 134.66 mg $kg^{-1}$ of extractable Ni, and is a ''cerrado ralo'' vegetation type, a savanna with small trees whose trunks are characteristically twisted, mixed with shrubs and an herbaceous layer (Oliveira-Filho and Ratter 2002); and Site 3, an adjoining non-ultramafic cerrado (15°05'05.0"S; 48°58'54.6"W), that is characterized by a clay loam Oxisol, with 5.20 mg $kg^{-1}$ of extractable Ni, and is a ''cerradão'' vegetation type, a forest formation with 50-90 % tree coverage (Oliveira-Filho and Ratter 2002). Physicochemical properties of the soils of these sites are presented in Table 1.

Sites 1 and 2 are 1.37 km apart from each other. The "cerradão" (Site 3) is located 3.60 km from Site 1 and 4.97 km from Site 2 (Fig. S1). The local climate is Cwa according to the Köppen classification, which corresponds to a typical savanna climate with 1500 mm of mean annual precipitation and two well-defined seasons: dry, from May to September, and rainy, from October to April. The maximum and minimum annual average temperatures are 29.9 °C and 19.8 °C, respectively.

### Sampling of nodules

The sampling expeditions occurred during the rainy season, in December 2009, November 2012, and December 2013. *Mimosa somnians* Humb. & Bonpl. ex Willd. a short, low-lying subshrub widespread from northeastern Argentina to southern Mexico (Fig. S2a and Fig. S2b) and *M. claussenii* Benth. a treelet restricted to the Cerrado biome but widely distributed within it (Fig. S2c and Fig. S2d) were found in all three sampling sites. Root nodules were collected and preserved in silica gel for later bacterial isolation.

In the November 2012 sampling, some nodules (three per plant) collected from plants (five of each species) in sites 1 (SAP) and 2 (LAT) were cut in half to determine if they were potentially active and effective by the appearance of a pinkish coloration

**Table 1** Physicochemical properties of two ultramafic soils (Sites 1 and 2) and a non-ultramafic Cerrado soil (Site 3) from the area of the mining company *Anglo American do Brasil*, in Barro Alto, Goiás state

| Sites | pH | H+Al | Mg | Ca | P | K | Cu | Fe | Mn | Ni | Zn | SOM | Clay | Sand | Silt |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ---- cmolc $dm^{-3}$ ----- | | | mg $dm^{-3}$ | | ---------- mg $kg^{-1}$ ---------- | | | | | g $kg^{-1}$ | ---------- g $kg^{-1}$ ---------- | | |
| Site 1 (SAP) | 6.2 | 6.5 | 5.2 | 1.5 | 0.6 | 59.0 | 1.4 | 6.7 | 1.5 | 603.5 | 0.1 | 64.0 | 253 | 410 | 337 |
| Site 2 (LAT) | 6.5 | 3.2 | 6.9 | 0.8 | 0.3 | 31.0 | 1.9 | 23.8 | 19.7 | 134.7 | 0.4 | 18.0 | 260 | 477 | 263 |
| Site 3 (cerrado) | 5.9 | 9.8 | 0.1 | nd | 1.9 | 67.0 | 3.0 | 66.9 | 88.9 | 5.2 | 0.6 | 40.0 | 300 | 347 | 353 |

Soil samples collected from 0 to 10 cm depth; Values are means of three replicates; nd not detected

Ca, Mg and Al: extracted with 1N KCl; P and K: using the Mehlich 1 extractor ($H_2SO_4$ 0.0125 M + HCl 0.05 M); Cu, Fe, Mn, Ni, *Zn*, Extracted by diethylene triamine pentaacetic acid (DTPA); *SOM*, Soil organic matter, Walkley & Black

Adapted from Pessoa-Filho et al. (2015)

resulting from the presence of leghemoglobin (Lb), and were then immediately placed into vials containing 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.5) for microscopic analysis (Reis Jr et al. 2010).

### Microscopy and in situ detection of microsymbionts

The nodules collected in the field and preserved in glutaraldehyde (November 2012 sampling) were prepared and sectioned for light microscopy and analysis by *in situ* immunogold labeling (plus silver enhancement) using antibodies raised against *Paraburkholderia phymatum* STM815$^T$ and *Cupriavidus taiwanensis* LMG19424$^T$ according to Chen et al. (2005b) and Elliott et al. (2007). The reactions of the test samples were compared visually with the corresponding positive (*M. pudica* nodules infected with *P. phymatum* STM815$^T$ or *C. taiwanensis* LMG19424$^T$) controls to determine which of the antibodies had reacted with each nodule (Reis Jr et al. 2010). Negative controls were sections treated with non-immune serum substituted for the primary antibodies.

### Bacteria isolation and plant tests

Bacteria were axenically isolated from single nodules, purified from single colonies and cultivated in YMA medium with Congo red (Vincent 1970). Stock cultures were maintained on YMA at 4 °C, and for long-term storage strains were cryopreserved in YM plus 30% (v/v) glycerol at -80 °C.

Nodulation capacity was evaluated using *Mimosa pudica* L., which was chosen as a model host because it is a fast-growing species and has an ability to nodulate with a wide range of *Mimosa*-nodulating rhizobia (Bontemps et al. 2010; Pires et al. 2018). Seeds were surface-sterilized in 96% ethanol for 30 seconds, followed by three minutes in 25% sodium hypochlorite, then washed six times with sterile distilled water. After this, to break dormancy, seeds were scarified with concentrated sulfuric acid followed by several washes with sterile distilled water. Seeds were subsequently placed onto 1% water agar for germination at 28 °C in darkness. Fifty-millilitres tubes containing Jensen medium with 12 grams of agar per litre (Jensen and Collins 1985) were used for planting germinated seeds that were inoculated with 1.0 ml of a $10^8$ cells $ml^{-1}$ solution with each bacterial isolate; uninoculated plants were used as negative controls (Elliott et al. 2007). Three replications were used for each isolate. After one month, the plants were harvested, and the presence/absence of nodules was recorded. Comparisons between the vigor of inoculated and control plants, as well as observation of effective nodules, were used as qualitative evidence for the nodulation capacity of rhizobial isolates (Pires et al. 2018).

Common bean (*Phaseolus vulgaris* L.) was also used as an additional host since it is a promiscuous papilionoid legume (Moura et al. 2022). Fourteen bacterial isolates were selected for this experiment according to their origin (host and site). The experiment was carried out according to de Faria and de Lima (1998). The substrate used in this experiment was composed of a 2:1 mixture of sand and ground charcoal (Carvalho et al. 2008). Common bean seeds were surface-sterilized as described before followed

by inoculation with 1.0 ml of a $10^8$ cells $ml^{-1}$ solution and planted in 2 l capacity plastic pots (two seeds per pot). Plants inoculated with *Rhizobium freirei* strain PRF81 and uninoculated plants were used as positive and negative controls, respectively. Until 10 days after germination (DAG), the pots received only sterilized distilled water. After this period, N-free Norris nutrient solution (Norris and Date 1976) was then applied. Four replications were used for each isolate. The plants were harvested after one month, and the presence/absence of nodules was recorded.

Additionally, another experiment with *M. pudica* was conducted, in which 11 different bacterial isolates selected according to their origin (host and site) were tested. The substrate used in this experiment was composed of a 1:1 mixture of perlite and sand. *Mimosa pudica* was inoculated with 1.0 ml of a $10^8$ cells $ml^{-1}$ solution per seed and planted in Leonard jars (two seeds per jar). Uninoculated and nitrogen fertilized plants were used as negative and positive controls, respectively. All nutrients, except N, were supplied using Norris solution, and for the nitrogen fertilized control 0.3 ml of a 10% $NH_4NO_3$ solution was applied weekly. The dry mass of nodules, roots and shoots were evaluated at 60 DAG. The experiment was set up in a complete randomized block design, with three replications. Analysis of variance and the Scott-Knott mean range test were performed using the SAS statistical program (SAS Institute).

### Nickel tolerance

The tolerance of the bacterial isolates to increasing concentrations of Ni was evaluated in culture medium following the methodology of Klonowska et al. (2012). Plates of YMA medium (Vincent 1970) were used with concentrations from 0.0 to 5.0 mM (0.5 mM intervals) of Ni, prepared from a $NiCl_2$ stock solution (Raja et al. 2009). The plates with different concentrations of Ni were divided into three sections and in each subdivision an isolate was tested by inoculating three drops (10 µL) of a suspension with approximately $10^8$ cells $ml^{-1}$. The growth of colonies was evaluated for one week, with daily checks.

### DNA extraction, amplification, and sequencing

Each bacterium was cultured in plates with YMA medium for 72 h at 28 °C. Afterward, a purified colony was transferred into YM liquid medium for 24 h at 28 °C. Subsequently, bacterial DNA was extracted using the Pure Link Genomic DNA Kit (Invitrogen), following the manufacturer's instructions. The extracted DNA from each isolate was used as a template for PCR reactions and sequencing of the 16S rRNA gene. Additionally, five isolates were selected according to their origin for amplification and sequencing of the *recA*, *gyrB*, *nodC* and *nifH* genes, which are widely used in phylogenetic studies with symbiotic bacteria (e.g., Peix et al. 2015). The PCR products were generated using Dr. Max DNA polymerase (MGMED, Co.) and the forward and reverse strands sequenced (Macrogen, Korea). Primers used for DNA amplification and sequencing are listed in Table S1. In addition, the sequences were deposited in the GenBank database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/genbank/), and the accession numbers are available in the phylogenetic trees.

### Phylogenetic analyses

The partial sequences of the 16S rRNA gene were used to build a phylogenetic tree. In addition, phylogenetic trees based on concatenated sequences of 16S rRNA, *recA*, and *gyrB* genes, and a single gene tree of the *nodC* and *nifH*, were generated for five selected isolates. For all these genes, the sequences were aligned using the ClustalW Multiple Alignment algorithm in BioEdit. The phylogenetic trees were built using the maximum likelihood (ML) method using the Jukes-Cantor model in MEGA X software (Kumar et al. 2018). For comparison, the alignment included sequences of *Paraburkholderia* type strains.

## Results

### Microscopy and in situ detection of microsymbionts

Nodules sampled from *M. claussenii* (Fig. 1A) and *M. somnians* (Fig. 1B) growing in sites 1 (SAP) and 2 (LAT) on 11/2012 and examined by optical microscopy had a typical and apparently functional structure. The symbiotic bacteria present in all the examined nodules from both *M. claussenii* (Fig. 1C) and *M. somnians* (Fig. 1D) were identified as belonging to *Paraburkholderia* spp. by

immunostaining with a genus-specific antibody raised against *P. phymatum* STM815$^T$. When an antibody against *Cupriavidus* spp. was used for immunostaining, the bacteroids within the infected cells were unmarked (Fig. 1E), indicating that bacteria belonging to this genus were not present. A positive control section of a *M. pudica* nodule infected with *C. taiwanensis* LMG19424$^T$ is shown in Fig. 1F as a comparison to Fig. 1E.

## Bacteria isolation and plant tests

All *M. somnians* and *M. claussenii* sampled during the three sampling expeditions had root nodules. In total, 72 bacterial isolates were obtained, 48 from *M. somnians* and 24 from *M. claussenii*. Additional information and characterization of these bacteria are presented in Table S2.

In the first nodulation test, performed with *M. pudica* in tubes containing Jensen medium, the results were considered as positive when at least one nodule was present, and negative, when no nodules were formed. Among the 72 isolates, 69 tested positive and three tested negative (Table S2).

In the evaluations carried out with common bean, all the 14 bacterial isolates tested were able to form nodules (Table S2). However, when these nodules were compared to those observed on the positive control plants, inoculated with the *Rhizobium freirei* strain PRF81, they were smaller and did not show the

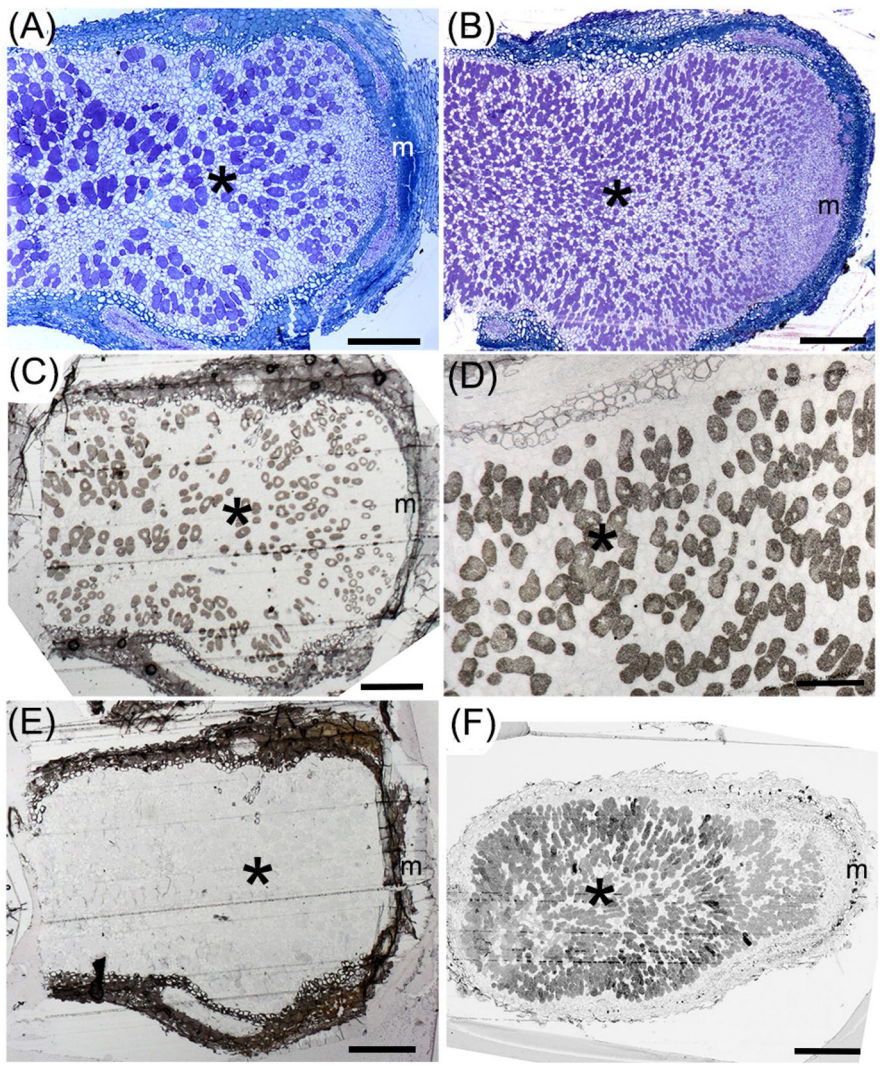


**Fig. 1** Light microscopy of sections of $N_2$-fixing nodules formed on *Mimosa claussenii* (A, C, E), *M. somnians* (B, D), and *M. pudica* (F). The sections were either stained with toluidine blue (A, B), immunogold labelled with an antibody against *Paraburkholderia phymatum* STM815$^T$ (C, D), or immunogold labelled with an antibody against *Cupriavidus taiwanensis* LMG19424$^T$ (E, F). Note the typical structure of an effective nodule in all three species, with an apical meristem (m) and a large infected zone which contains the $N_2$-fixing symbionts (*). The *M. claussenii* (C) and *M. somnians* (D) nodules gave a positive reaction with the *P. phymatum* antibody, but a negative reaction with the *C. taiwanensis* antibody (E); this contrast with a *M. pudica* nodule infected with *C. taiwanensis* LMG19424$^T$ (F). Bars, 200 µm (A, B, C, E, F), 50 µm (D). All nodules shown in these figures, except for (F), come from site 1 (SAP)

characteristic pinkish color within them. The observation of the development of plants inoculated with these isolates confirmed that these nodules were ineffective. While plants inoculated with *R. freirei* were healthy and well developed, those that received the *Mimosa* spp. isolates clearly showed strong nitrogen deficiency and had impaired growth (Fig. S3).

In the third test, performed with 11 isolates, again on *M. pudica*, in terms of root and shoot dry mass production (Table 2), it is clear that N limitation greatly influenced the development of the plants. The response to the application of N fertilizer in the closed system of Leonard jars was evident, and this treatment showed a better response in relation to the others (Table 2; Figure S4a). The inoculation with the bacterial isolates also had a determining effect on the growth of the plants, when compared to the control without inoculation and without N. There were differences between the tested isolates regarding the growth promotion of *M. pudica* plants. Isolates W38, W41 and W84 stood out, with increases of 264%, 295% and 278% in root dry mass, and 1191%, 1307% and 1275% in shoot dry mass, respectively, when compared to the control (Table 2; Figure S4b).

**Table 2** Dry mass of nodules, roots and shoots of *Mimosa pudica* plants inoculated with different isolates from nodules of two species of *Mimosa* (*M. somnians* and *M. claussenii*) collected in the area of the mining company *Anglo American do Brasil*, in Barro Alto, Goiás state

| Isolate | Nodules dry mass (mg) | Roots dry mass (mg) | Shoots dry mass (mg) |
|---|---|---|---|
| W2 | 42 b | 90 c | 310 d |
| W3 | 59 b | 103 c | 533 c |
| W31 | 74 a | 180 b | 597 c |
| W33 | 73 a | 97 c | 731 b |
| W38 | 93 a | 171 b | 736 b |
| W41 | 89 a | 186 b | 802 b |
| W45 | 51 b | 109 c | 378 d |
| W47 | 44 b | 84 c | 300 d |
| W48 | 65 b | 125 c | 500 c |
| W80 | 79 a | 130 c | 557 c |
| W84 | 65 b | 178 b | 784 b |
| Nitrogen | 0 c | 949 a | 2734 a |
| Control | 0 c | 40 c | 43 e |
| CV (%) | 30.5 | 24.2 | 19.2 |

Means followed by the same letter in the column do not differ by Scott-Knott's test at 5% probability

*CV,* Coefficient of variation

### Nickel tolerance

None of the bacterial isolates evaluated in this study could grow in the presence of Ni in any tested concentration (from 0.5 to 5.0 mM of Ni), indicating their sensitivity to this metal.

### Phylogenetic analyses

The 16S rRNA gene sequence was obtained for 54 of the 72 bacterial isolates evaluated in the present study. Among the sequenced isolates, 39 were isolated from *M. somnians* and 15 were from *M. claussenii*; all were clustered within the *Paraburkholderia* genus, closely related to *P. atlantica* (Fig. 2).

The analyses of the five isolates that were selected for a more robust phylogenetical study (W3, W33, W38, and W48, isolated from *M. somnians*, and W47 isolated from *M. claussenii*) using the concatenated sequences from 16S rRNA, recA, and *gyrB* genes, confirmed that these strains belong to *P. atlantica* (Fig. 3). The same was observed when the *recA* and *gyrB* genes were analyzed separately (Fig. S5 and Fig. S6).

Maximum likelihood trees constructed for nitrogen fixation (*nifH*) and nodulation (*nodC*) genes showed that the strains isolated from ultramafic soils grouped with the closely related species *P. youngii* and *P. atlantica* (Fig. 4a, b).

## Discussion

Species within the genus *Mimosa* have a known relationship with various types of nodulating $N_2$-fixing bacteria, from both Alpha and Betaproteobacteria classes (Barrett and Parker 2005, 2006; Bontemps et al. 2010, 2016; Chen et al. 2005a, b; Elliott et al. 2007; Gehlot et al. 2013; Platero et al. 2016; Pires et al. 2018). According to Bontemps et al. (2010), in the Brazilian biomes known as Cerrado and Caatinga, *Mimosa* spp. nodulation is mainly associated with beta-rhizobia of the genus *Paraburkholderia*. However, nodulation studies with these species have not been conducted on ultramafic soils in Brazil.

*Cupriavidus* spp., which seem particularly adapted to metal-rich environments and are commonly

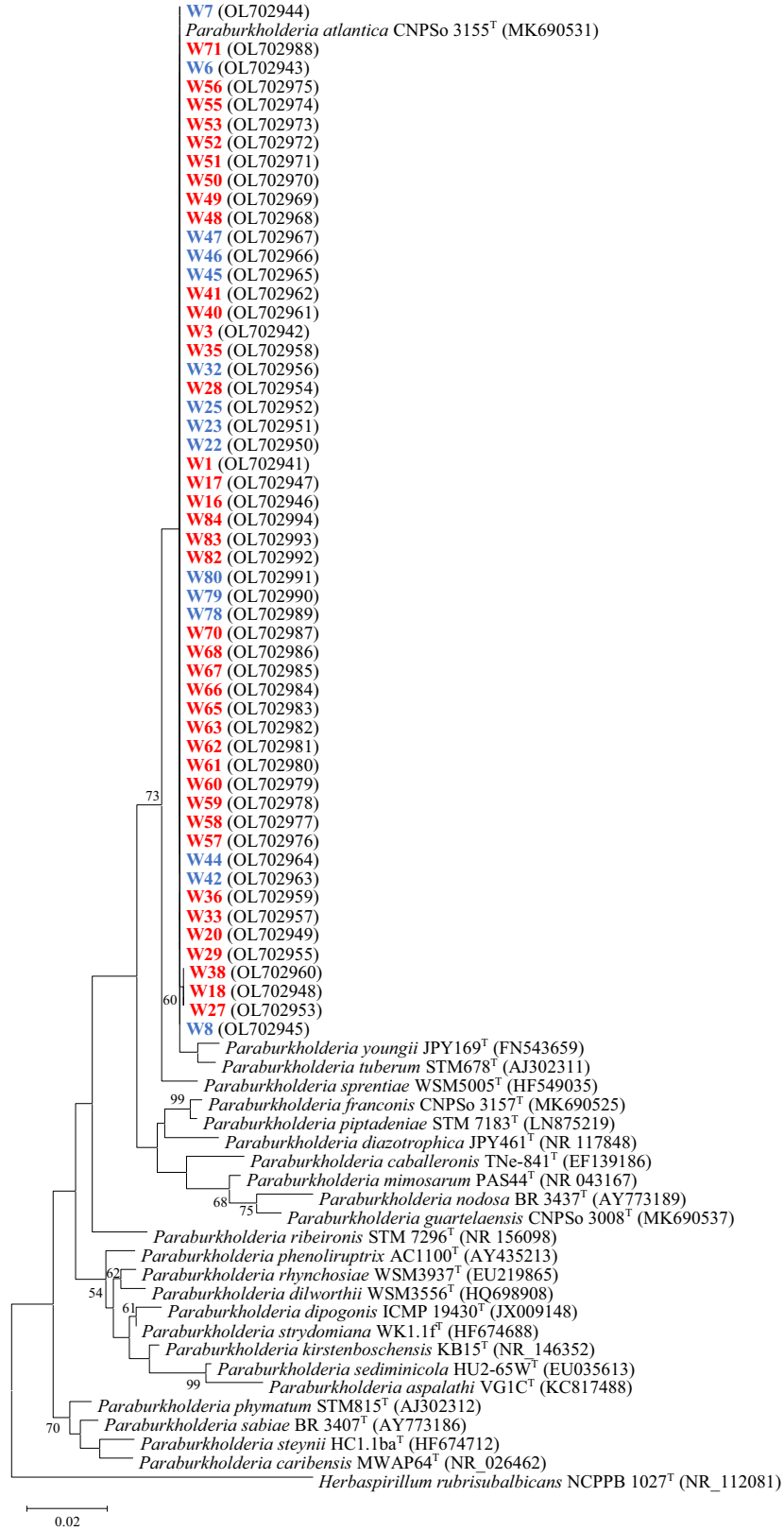


**Fig. 2** Maximum-likelihood phylogenetic tree of the 16S rRNA gene sequences of 54 *Paraburkholderia* isolates from root nodules of *Mimosa somnians* and *M. claussenii,* and 24 type strains (1119 nucleotides). The numbers in the branches are the bootstrap values > 50% (1000 replications). *Herbaspirillum rubrisubalbicans* NCPPB 1027$^T$ was included as outgroup. Strains under study are shown in boldface. The red and blue colors for "W" strains are from *Mimosa somnians* and *Mimosa claussenii*, respectively. In parenthesis: GenBank accession number. Jukes-Cantor model was used for phylogenetic reconstruction. Scale bar represents two substitutions per 100 nucleotide positions

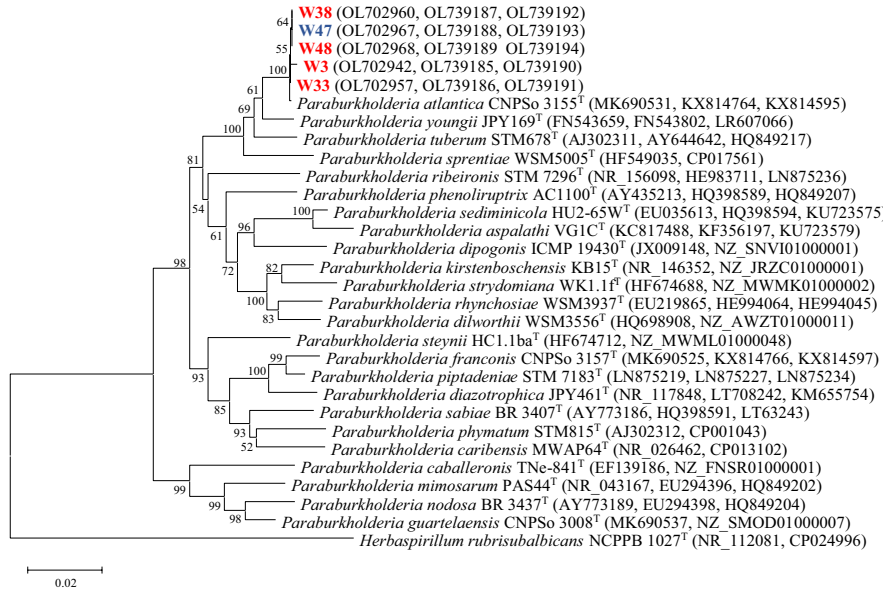


**Fig. 3** Maximum-likelihood phylogenetic tree of the 16S rRNA+*recA*+*gyrB* gene sequences of five *Paraburkholderia* isolates from root nodules of *Mimosa somnians* and *M. claussenii*, and 24 type strains (2041 nucleotides). The numbers in the branches are the bootstrap values > 50% (1000 replications). *Herbaspirillum rubrisubalbicans* NCPPB 1027$^T$ was included as outgroup. Strains under study are shown in boldface. The red and blue colors for "W" strains are from *Mimosa somnians* and *Mimosa claussenii*, respectively. In parenthesis: GenBank accession number. Jukes-Cantor model was used for phylogenetic reconstruction. Scale bar represents two substitutions per 100 nucleotide positions

isolated from nodules of *Mimosa* spp. growing in such soils (Klonowska et al. 2012; Platero et al. 2016), were not found in the ultramafic soil areas of Barro Alto. This means that they are not the preferred symbionts of the *Mimosa* species that occurs naturally in this environment. Indeed, despite their association with *Mimosa* spp., highlighted in several studies around the world, *Cupriavidus* spp. have not yet been isolated from nodules of *Mimosa* within its main centers of radiation, the Caatinga and Cerrado biomes in central Brazil (Bontemps et al. 2010; Reis Jr et al. 2010). Interestingly, previous reports on nodulation of *M. claussenii* (Reis Jr et al. 2010) and *M. somnians* (Elliott et al. 2007) showed that when inoculated with *C. taiwanensis* these species were ineffectively nodulated, contrary to what happened when the same species were inoculated with *P. phymatum*.

Our results indicate that *M. somnians* and *M. claussenii*, highly prevalent in the studied region, are preferably nodulated by *Paraburkholderia* spp. (Fig. 1 and Fig. 2; Table S2), both in ultramafic and non-ultramafic soils. Edaphoclimatic conditions are believed to have an important influence on the occurrence of these bacteria in the nodules of *Mimosa* spp. While *Cupriavidus* spp. have been reported in neutral-alkaline soils (Platero et al. 2016), *Paraburkholderia* spp. predominate in the more acidic soils (Bontemps et al. 2010; Dias et al. 2021; Reis Jr et al. 2010). Soils with higher N-content may also favor *Cupriavidus* spp. (Elliott et al. 2009). Pires et al. (2018) confirmed that soil factors such as pH, nutrients and organic matter influence the predominance of certain types of rhizobia and their establishment of symbiotic relationships with local legumes. The ultramafic soils of Barro Alto (sites 1 and 2) are characterized by slightly acidic pH, low nutrients, and high heavy metal content, especially Ni (Table 1). Except for the soil heavy metals content, these are characteristics that generally favor the presence of *Paraburkholderia*.

The inoculation tests on *M. pudica* confirmed the symbiotic character for most isolates, but three unidentified isolates (W30, W64, W81) were unable to nodulate (Table S2). Probably, they are contaminants or opportunistic non-rhizobial bacteria that were living in (or closely-adhered to) the nodules, as

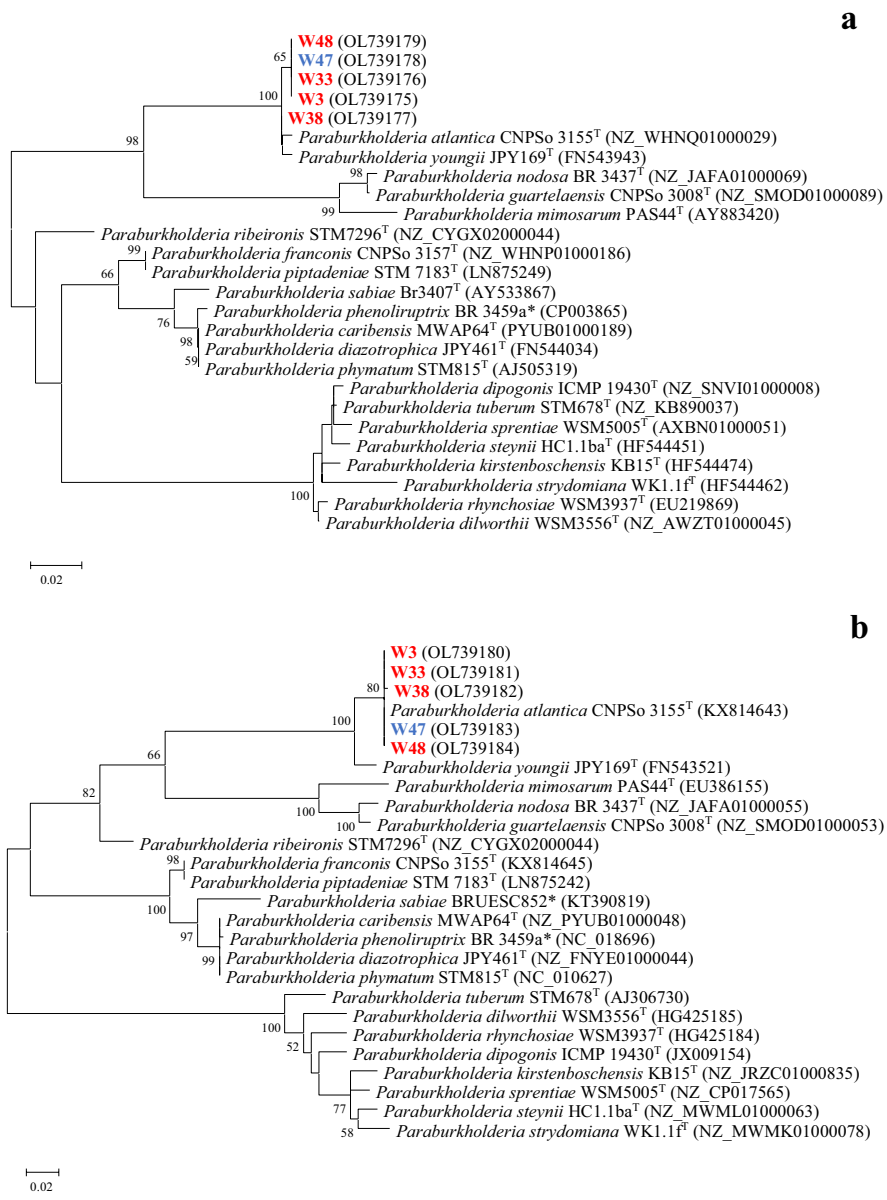


**Fig. 4** Maximum-likelihood phylogenetic trees of five *Paraburkholderia* isolates from root nodules of *Mimosa somnians* and *M. claussenii* and 20 type strains (a) *nifH* gene sequences (276 nucleotides) and (b) *nodC* gene sequences (419 nucleotides), isolated from root nodules of *Mimosa* spp. The numbers in the branches are the bootstrap values > 50% (1000 replications). Strains under study are shown in boldface. The red and blue colors for "W" strains are from *Mimosa somnians* and *Mimosa claussenii*, respectively. In parenthesis: GenBank accession number. Jukes-Cantor model was used for phylogenetic reconstruction. *= non-type strain. Scale bar represents two substitutions per 100 nucleotide positions

postulated by Martínez-Hidalgo and Hirsch (2017). In the trial conducted in greenhouse for 60 days, inoculation with the nodulating *Paraburkholderia* spp. improved plant growth, most likely through N fixation (Table 2; Fig. S4). Plants inoculated with the isolates W38, W41 and W84 produced approximately four times more roots and fourteen times more shoots than uninoculated control plants. It is known that nodulated *Mimosa* can fix $N_2$ within their native environments, and therefore might make a valuable contribution to the N-cycle of the fragile ecosystems of the Cerrado and the Caatinga biomes (Dias et al. 2021; Reis Jr et al. 2010). These results allow us a glimpse into the possibility of using inoculated native *Mimosa* spp. with selected strains of *Paraburkholderia* for ecological restoration of post-mining areas as part of degraded land recovery projects. After correction of the physical and chemical factors that restrict plant growth, the introduction of nodulating $N_2$-fixing legumes, with superior capacity to grow quickly in poor substrates and to withstand harsh local edaphoclimatic

conditions, constitutes an efficient strategy to accelerate soil reclamation and to initiate natural succession (Chaer et al. 2011).

All tested bacteria were also able to form nodules on common bean (Table S2). This plant is known for establishing promiscuous symbioses, since it can associate with a broad variety of rhizobia species (Moura et al. 2022). Indeed, previous studies have indicated the possibility of nodule formation by *Paraburkholderia* spp. in common bean (Dall'Agnol et al. 2016; Talbi et al. 2010), showing that nodulation by these mimosoid-associated bacteria extends beyond the subfamily Caesalpinioideae. In the present study, this association was ineffective, as can be clearly seen in Fig. S3, in which the plants inoculated with the isolates W3 and W32 have performed no better than the uninoculated controls, and are considerably less healthy than those inoculated with the *R. freirei* strain PRF81, recommended for common bean inoculation in Brazil. Dall'Agnol et al. (2016) used common bean plants to trap rhizobia from an undisturbed soil of the Brazilian Cerrado, and most of the isolates were identified as *Paraburkholderia*. These isolates were also evaluated to confirm their ability to nodulate common bean and almost all of them were either ineffective or showed low effectiveness in fixing $N_2$ (Dall'Agnol et al. 2016).

Contrary to what was expected, none of the isolates were able to grow in culture medium with added Ni, even in the lowest tested concentration. Strains resistant to Ni have already been described in species of the genus *Paraburkholderia* (Stoppel and Schlegel 1995; Vincent et al. 2019). Other examples of bacteria resistant to Ni originating from ultramafic soils are highlighted by Abou-Shanab et al. (2006, 2009) and Pal et al. (2004). Delorme et al. (2001) found many metal-resistant bacteria associated with a metal-hyperaccumulating plant (*Thlaspi caerulescens* J. & C. Presl.), but a lower number of tolerant bacteria was found associated with a non-accumulating plant (*Trifolium pratense* L.), growing in soils collected in the vicinity of a Zn smelter in Palmerton, Pennsylvania, USA. Despite the prevalence of *M. somnians* and *M. claussenii* in the Barro Alto ultramafic soils, these species are not considered to be hyperaccumulators.

Klonowska et al. (2012) evaluated 96 isolates from *M. pudica* nodules, growing in ultramafic soils of New Caledonia. The great majority of these isolates were identified as *Cupriavidus taiwanensis*, but only 16% of the isolates were resistant to Ni levels of up to 15 mM, while the other isolates were incapable of growth in the metal-enriched medium. Surprisingly, these tolerant bacteria came from areas with low bioavailable Ni.

These observations, together with the results obtained in the present study, raise questions about the survival strategies of legume symbionts, especially beta-rhizobial ones, in soils with high levels of heavy metals. A probable explanation would be the existence of different micro-niches in the soil wherein these bacteria could be concentrated, thereby avoiding metal toxicity (Klonowska et al. 2012), but also the soil organic matter which is a key factor in mitigating metal toxicity (Boteva et al. 2016; Pessoa-Filho et al. 2015). In addition, the production of siderophores by bacteria could bind metals in the extracellular environment, reducing the concentrations of free metals in their niches, probably by affecting their diffusion and consequently their toxicity (Rajkumar et al. 2010; Schalk et al. 2011). *Paraburkholderia* is known to be a genus rich in siderophore-producing bacteria (Pratama et al., 2020; Vargas-Straube et al 2016). Also, in the study of Sujkowska-Rybkowska et al. (2020) on the *Lotus corniculatus*-mesorhizobia symbiosis, it was shown that the accumulation of phenols and reorganization of the nodule apoplast can diminish the negative effects of Ni, Co and Cr on the symbiosis, and thus improve plant adaptation to metal stress occurring in ultramafic soils. Several members from the soil microbial community can use different mechanisms, such as biosorption, bioaccumulation or modification of the chemical state, to contribute to the alleviation of metal stress (Caracciolo and Terenzi 2021). On the other hand, plants growing in heavy metal rich environments produce exudates like enzymes, phytochelators, organic acids, flavonoids, etc. which can also decrease their toxicity in the soil (Khan 2021).

The phylogenetic analysis based on the 16S rRNA gene (Fig. 2) showed that all isolated strains grouped within the species *P. atlantica* that has recently been separated from the South African species *P. tuberum* (Paulitsch et al. 2020; Mavima et al. 2021, 2022). Five strains (W3, W33, W38, W47 and W48) were selected and phylogenetically analyzed by multilocus sequence analysis (MLSA) of the concatenated sequences from 16S rRNA, *recA*, and *gyrB* genes. The subsequent phylogeny confirmed that these strains belonged to *P. atlantica* (Fig. 3). Moreover, the ML trees constructed

for nitrogen fixation (*nifH*) and nodulation (*nodC*) genes also showed that for both genes the strains isolated from ultramafic soils were closely related to *P. atlantica* (Fig. 4a and Fig 4b). Mavima et al. (2021) presented a study with 30 strains, initially identified as *P. tuberum* (sv. mimosae) isolated from *Mimosa* spp. nodules. Using a polyphasic approach (MLSA, ANI, C+G DNA content, phenotypic characteristics), the authors concluded that 12 strains were conspecific with *P. atlantica* CNPSo 3155$^{T}$, while the others were placed in the new species *P. youngii*. *Paraburkholderia atlantica* was described by Paulitsch et al. (2020) in a study of strains from the Atlantic Forest isolated using *P. vulgaris* and *M. pudica* as trapping hosts (Dall'Agnol et al. 2017).

*Paraburkholderia atlantica* is among the most frequently found beta-rhizobia associated with different *Mimosa* species in Brazil, other South American countries and Mexico (Bontemps et al. 2010, 2016; Lammel et al. 2013; Mishra et al. 2012). *Mimosa somnians* is a widespread species that seems to prefer *P. atlantica* as its symbiont, as the present work and previous studies conducted in Brazil (Bontemps et al. 2010) and Mexico (Bontemps et al. 2016) have indicated. On the other hand, *M. claussenii* symbionts were previously described by Bontemps et al. (2010) and most were *P. nodosa*. Nevertheless, it is not surprising that *M. claussenii* can nodulate with both *P. atlantica* (present study) and *P. nodosa* (Bontemps et al. 2010), since their symbiotic genes are very similar, and both are the most common *Mimosa* symbionts found by far in the Cerrado biome (Bontemps et al. 2010).

This study showed, for the first time, symbiotic strains of *Paraburkholderia (P. atlantica*) in nodules from *Mimosa* species naturally growing in ultramafic soils, and that these bacteria are their dominant symbionts in this environment. Inoculation tests showed that *P. atlantica* was most likely essential for plant growth in the absence of nitrogen, and hence that they provide a vital ecological role in the maintenance of the native *Mimosa* populations. These data further suggest that these symbioses could make an important contribution to programs aimed at restoring ultramafic soils in Barro Alto areas degraded by mining activity.

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**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Clemente Batista Soares Neto, Paula Rose de Almeida Ribeiro, Jerri Edson Zilli, Euan Kevin James, and Fábio Bueno dos Reis Junior. The first draft of the manuscript was written by Clemente Batista Soares Neto, Paulo Ivan Fernandes-Júnior, Helson Mario Martins do Vale and Fábio Bueno dos Reis Junior and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Declarations**

**Competing interests** The authors have no relevant financial or non-financial interests to disclose.

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