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Co-inoculation of Azospirillum brasilense and AMF in the development and copper content in maize and sorghum grown in contaminated soil

Coinoculação de Azospirillum brasilense e FMAs no desenvolvimento e teores de cobre em sorgo e milho cultivados em solo contaminado

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Highlights

Arbuscular mycorrhizal fungi optimize sorghum roots in copper-contaminated soil.
Acaulospora scrobiculata boosted mycorrhizal colonization and root volume in sorghum.
Co-inoculation of AMF and Azospirillum brasilense reduced copper in sorghum.
Rhizoglomus clarum has optimized maize leaf area in copper-contaminated soil.
Co-inoculation of AMF and A. Brasilense boosted maize growth in copper-free soil.

Abstract

Copper contamination has increased in soils subjected to the application of fungicides and organic fertilizers, including areas under maize and sorghum cultivation. Arbuscular mycorrhizal fungi (AMF) and *Azospirillum brasilense* bacteria have shown promise in promoting plant growth and could be an alternative for plant development in copper-contaminated soils. This study aimed to determine the influence of co-inoculation with AMF and *Azospirillum brasilense* on the development and copper levels in sorghum and maize plants cultivated in contaminated soil. The experimental design was completely

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randomized with a factorial arrangement (6x2), involving six inoculum sources (*Acaulospora scrobiculata; Rhizoglomus clarum; Azospirillum brasilense; Acaulospora scrobiculata + Azospirillum brasilense; Rhizoglomus clarum + Azospirillum brasilense;* and a control without inoculation) in soil with and without the addition of 400 mg kg^{-1} of Cu, with seven replications. The following parameters were evaluated: Plant height, culm diameter, number of tillers, root volume, chlorophyll a and b, leaf area, shoot dry mass, root dry mass, copper content in grains, shoot, and roots, mycorrhizal colonization, and the most probable number of *A. brasilense* in the roots. According to the results, the inoculum sources exhibited different significant interactions in the evaluations, depending on the crop and the Cu dose applied to the soil. Inoculation with *A. scrobiculata* in Cu-contaminated soils favored root growth, dry mass, and mycorrhizal colonization in sorghum. In contrast, the use of *R. clarum* resulted in better development of maize shoots. Regarding isolated inoculation and co-inoculations between *A. brasilense* and AMF, we found that these reduced the Cu content in the sorghum shoots to levels below Brazilian legislation, thus representing a potential for cultivation in soils with excess copper.
Key words: Contamination. Microorganisms. *Sorghum bicolor. Zea mays.*

Resumo

A contaminação com cobre tem aumentado nos solos submetidos a aplicação de fungicidas e fertilizantes orgânicos, incluindo áreas sob cultivo de milho e sorgo. Os fungos micorrízicos arbusculares (FMAs) e bactérias *Azospirillum brasilense* evidenciaram-se promissores para promover o crescimento vegetal e poderão ser uma alternativa para o desenvolvimento das plantas em solo contaminado com cobre. Objetivou-se determinar a influência da coinoculação com FMAs e Azospirillum brasilense na promoção do desenvolvimento e nos teores de cobre em plantas de sorgo e milho cultivados em solo contaminado. O delineamento experimental foi inteiramente casualizado com arranjo fatorial (6x2), com seis fontes de inóculo (*Acaulospora scrobiculata; Rhizoglomus clarum; Azospirillum brasilense; Acaulospora scrobiculata + Azospirillum brasilense; Rhizoglomus clarum + Azospirillum brasilense,* testemunha sem inoculação), solo sem e com a adição de 400 mg kg^{-1} de Cu, com sete repetições. Avaliou-se: altura de planta, diâmetro de colmo, número de perfilhos, volume de raiz, clorofila a e b, área foliar, massa seca de parte aérea, raiz, teor de cobre no grão, parte aérea e raiz, colonização micorrízica e o número mais provável de *A. brasilense* nas raízes. De acordo com os resultados, as fontes de inóculos exibiram diferentes interações significativas nas avaliações, dependendo da cultura e da dose de Cu aplicada no solo. A inoculação com *A. scrobiculata* em solos contaminados com Cu favoreceu o crescimento de raiz, massa seca e colonização micorrízica no sorgo. Já o uso de *R. clarum* possibilitou melhor desenvolvimento na parte aérea do milho. Com relação a inoculação isolada e as coinoculações entre *A. brasilense* e FMAs verificou-se que estas reduziram o teor de Cu na parte aérea de plantas de sorgo para valores abaixo da legislação brasileira, representando assim uma possibilidade de ser cultivada em solo com excesso de cobre.
Palavras-chave: Contaminação. Microrganismos. *Sorghum bicolor. Zea mays.*

Introduction

Copper (Cu) is mainly present in the soil in the form of Cu^{2+} and, when in excess, it induces problems in plant development (Alghamdi & Alasmary, 2022). The use of Cu-based fertilizers and fungicides, mining and waste disposal areas have increased the concentration of this metal in the soil, which can cause environmental contamination (Cipoleta et al., 2019). In high concentrations in the soil, Cu inactivates cytoplasmic enzymes, compromises photosynthesis, and interferes with nutrient metabolism, affecting plant growth (Li et al., 2023; Mir et al., 2021; Rodrigues et al., 2016).

The increase in Cu levels in the soil has led to questions about its effect on agricultural crops, such as maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.). Maize is a cereal with high energy value, as it has a large amount of starch in the endosperm (Blandino et al., 2023), while sorghum is used for the production of grains, forage, alcohol, and biomass. It is an alternative to maize cultivation in regions with water deficit. Also, because it does not contain gluten, its use for human food has increased in several countries (Menezes et al., 2021). These plants, when exposed to excess copper in the soil, show inhibition of enzymatic activity and protein functions, resulting in a decrease in roots, shoots, photosynthesis, and leaf length (Mishra et al., 2020; Benimeli et al., 2010). Therefore, it is necessary to develop alternatives that allow the continued cultivation of these plants in soils that have been contaminated with Cu.

Among the soil microorganisms with the potential to stimulate plant development in soil contaminated with Cu, there are arbuscular mycorrhizal fungi (AMF) that increase plant growth and stress tolerance (Cahyaningtyas & Ezawa, 2023; Paiva et al., 2022; Zhang et al., 2014). This occurs through processes such as nutritional improvement of the plant, retention of metals in the fungal mycelium, and their dilution in plant tissues due to greater biomass production (Bourles et al., 2022; Andreazza et al., 2013). There are also bacteria of the Azospirillum genus that colonize both the external and internal surfaces of plants in association with the roots (Giatti & Piza, 2022) and are capable of stimulating plant development through the synthesis of substances that promote plant growth, such as auxins, gibberellins, and cytokinins, acting on the mechanisms of inducing plant resistance to stress and in the phosphate solubilization (Ribeiro et al., 2022; Fukami et al., 2018).

Co-inoculation of AMF with soil bacteria can induce greater nitrogen accumulation and plant growth, increased nodulation, and improved root variables (Primieri et al., 2021). The literature reports the beneficial effect of co-inoculation for several agricultural crops, including maize (Dhawi et al., 2015) and sorghum (Brasil et al., 2006). However, doubts still remain as to whether co-inoculation with AMF and *Azospirillum* promotes the development and reduces Cu levels in maize and sorghum plants grown in soil contaminated with this metal.

The purpose of this study was to determine the influence of co-inoculation with AMF and *Azospirillum brasilense* in promoting the development and copper levels in maize and sorghum plants grown in contaminated soil.

Material and Methods

The experiments were carried out in a greenhouse belonging to the Forestry Engineering Department of the Federal University of Santa Maria, Frederico Westphalen campus, Rio Grande do Sul, Brazil. The soil used was collected in the 0-20 cm layer and characterized as Red Latosol (Santos et al., 2018). After collection, the soil was air-dried, crushed, and sieved through a 2 mm mesh; mixed with medium sand in a 50% (v/v) proportion to obtain a 35% approximate texture, whose purpose was to facilitate root analysis. A sample from the prepared soil was taken to determine its physical and chemical attributes, presented in Table 1. Subsequently, sterilization was carried out in an autoclave at 121ºC, in 3 cycles of 30 min, according to the standard methodology of the research group (unpublished data).

Table 1
Physical and chemical attributes of the soil used for growing sorghum and maize

------- Physical attributes -----		--------------------------- Chemical attributes ---------------------------						
Clay	OM	pH	P	K	Cu	Zn	Mg	Al+H
%	%	1:1	-----------------mg/L---------------				----Cmolc/L---	
46	1.0	5.3	4.6	27.5	4.2	1.3	1.0	4.9

* OM: Organic matter; P: Phosphorus; K: Potassium; Cu: Copper; Zn: Zinc; Mg: Magnesium; Al+H: Potential acidity.

After chemical analysis of the soil, dolomitic limestone was added to raise the pH to 6.5. Fertilization was also carried out with chemical fertilizer formula NPK 10-20-10, according to the recommendation of the Manual de Calagem e Adubação para os Estados do Rio Grande do Sul e de Santa Catarina (2016), for maize and sorghum crops with expected yields of 9 t ha^{-1} and 4.2 t ha^{-1}, respectively, waiting 45 days for pH stabilization. After this period, part of the soil was contaminated with 400 mg of Cu kg^{-1} in the form of Cu sulfate ($CuSO_4.5H_2O$), with agitation in a plastic bag, which also remained at rest for 45 days to stabilize the chemical reactions. The experimental units were cultivation pots with 5-liter capacity, which were filled with 5 kg of soil, containing one plant.

The AMF isolates (*Acaulospora scrobiculata* and *Rhizoglomus clarum*) were obtained from Embrapa Agrobiologia, Seropédica, Rio de Janeiro (RJ). The bacterial inoculant used was Azo Total Max, composed of bacteria from the *Azospirillum brasilense* species, strains AbV5 and AbV6, at a 2,000,000 Colony-Forming Units (CFU) concentration, obtained from the Total Biotecnologia Indústria e Comércio Ltda company in Curitiba, Paraná (PR). The maize cultivar used was MORGAN 20A78, and the sorghum cultivar was BRS 330.

The crops were sown in the first half of September 2021, with 4 seeds sown per pot. The seeds were previously disinfected with 2% sodium hypochlorite for 15 min and then washed in running water. At the time of sowing, inoculations were carried out according to their treatments, with 30 spores of each AMF isolate being deposited, and the *A. brasilense* bacteria was directly inoculated with a 0.5 mL dose and without adding additives to the seeds before sowing. After 10 days of sowing, thinning was carried out, leaving only one plant per pot. Plant irrigation was carried out using an automatic drip system, providing 8-mm irrigation six times a day.

The experimental design was completely randomized, with a (6 x 2) factorial arrangement, with six inoculum sources (Acaulospora scrobiculata; *Rhizoglomus clarum; Azospirillum brasilense; A. scrobiculata + A. brasilense; R. clarum + A. brasilense* and control without inoculation), without Cu, and with the addition of 400 mg of Cu kg^{-1} of soil, with seven replicates. This Cu dose addition was based on the results of J. C. Silva (2019), who, working with this same soil, with a 35% clay texture, showed that the 400 mg of Cu kg^{-1} of soil dose results in a toxic content for plants.

During the plant physiological maturation period, the chlorophyll a and b relative index was determined using a portable ClorofiLOG Falker® Chlorophyllmeter, measuring the fully expanded upper leaf in both cultures, with three replicates performed on each leaf. The leaf area (LA) was also assessed using the triangle/trapezoid method proposed by Sousa et al. (2015), with three measurements taken on each plant leaf: total length, blade base width and width of the blade middle width, obtained with a millimeter ruler. To determine the Leaf Area in grasses, Equation 1 was used, according to the methodology proposed by Sousa et al. (2015).

Leaf Area = Triangular leaf area [(C)*(A/2)/2] + trapezoidal leaf area [((C + B)/2)*(A/2)]

Equation 1

With:

A: Measurement of the leaf blade total length;

B: Measurement of the blade base width;

C: Measurement of the blade middle width.

Subsequently, in order to obtain the total leaf area, the result from Equation 1 must be multiplied by the number of leaves on the plant.

At the end of the crop cycle, the following evaluations were carried out: Plant height (PH), measured from the plant collar to the flag leaf, using a measuring tape; culm diameter (CD) using a digital caliper; number of tillers (sorghum). After drying the plants in an oven at 65±1°C until constant weight, shoot dry mass (SDM) and root dry mass (RDM) were quantified. Subsequently, the plants were ground in a Willey-type mill with a 10-mesh sieve to determine the Cu content in the shoot (CuSH), root (CuR), and grain (CuG), through nitro-perchloric digestion (3:1) and determination in atomic absorption spectrophotometry, as proposed by Miyazawa et al. (2009).

The percentage of mycorrhizal colonization was determined using the root clarification and staining technique with 0.05% (m/v) Trypan Blue and was estimated in three replicates per plant using the grid line method (Giovanetti & Mosse, 1980).

To determine the endophytic diazotrophic (Azospirillum brasilense) bacteria population, 1 gram of root from each replicate was used, which were disinfected in a 5% (m/v) NaOCl solution for two minutes. Subsequently, the roots were macerated, and serial dilution was performed with 0.85% (m/v) saline solution of each sample in dilutions of 10^{-3}, 10^{-4}, 10^{-5}, which were inoculated in semi-solid NFb culture medium and incubated at 30°C for 14 days. The bacterial population estimate was made based on the Most Probable Number (MPN) method using the McCrady Table, whose values were expressed in colony forming unit/gram of root (CFU/g) (Dobereiner et al., 1995).

The results were subjected to analysis of variance and, when significant, the effects of the inoculations within each copper dose were unfolded, with the inoculation means compared by the Scott-Knott test at 5% probability of error ($p \leq 0.05$) and the copper dose treatment through of the T-test ($p \leq 0.05$) and, when not significant, the simple effects of inoculation and copper dose were also analyzed via the Scott-Knott test and the T-test with the aid of the SISVAR statistical program (Ferreira, 2011).

Results and Discussion

Soil characterization prior to the implementation of sorghum and maize crops revealed 46% clay, 1% organic matter, 5.3 pH, and low levels of phosphorus (4.6 mg/L), potassium (27.5 mg/L), zinc (1.3 mg/L), and magnesium (1.0 Cmolc/L), while the copper content was considered high, reaching 4.2 mg/L, according to the parameters established in the Liming and Fertilization Manual for the States of Rio Grande do Sul and Santa Catarina (Manual de Calagem e Adubação para os Estados do Rio Grande do Sul e Santa Catarina, 2016) (Table 1). This information was essential to determine the amount of chemical fertilizer needed to meet the nutritional requirements of sorghum and maize plants.

The results demonstrated significant interaction between Cu doses applied to the soil and inoculum sources for mycorrhizal colonization in sorghum and maize (Figure 1). Mycorrhizal colonization in sorghum did not differ statistically between inoculation treatments. However, in soil with copper, the co-inoculation of *A. scrobiculata + A. brasilense* and in soil without Cu, inoculation of *R. clarum,* induced a higher percentage of mycorrhizal colonization. Species of mycorrhizal fungi belonging to the *Acaulosporaceae* family have a certain tolerance to stress factors and have a greater capacity to colonize plants with this same characteristic (Cahyaningtyas & Ezawa, 2023).

The percentage of mycorrhizal colonization in maize did not show a significant difference between the inoculum sources in uncontaminated soil; however, with the application of 400 mg kg^{-1} of Cu, the co-inoculation of *A. scrobiculata + A. brasilense* resulted in a 58.4% colonization (Figure 1). Some bacteria have the ability to stimulate mycelial growth, increasing the number of hyphae and consequently mycorrhizal infection in plants, resulting in a beneficial effect from the mycorrhizal fungi and rhizobacteria co-inoculation (Brasil et al., 2006).

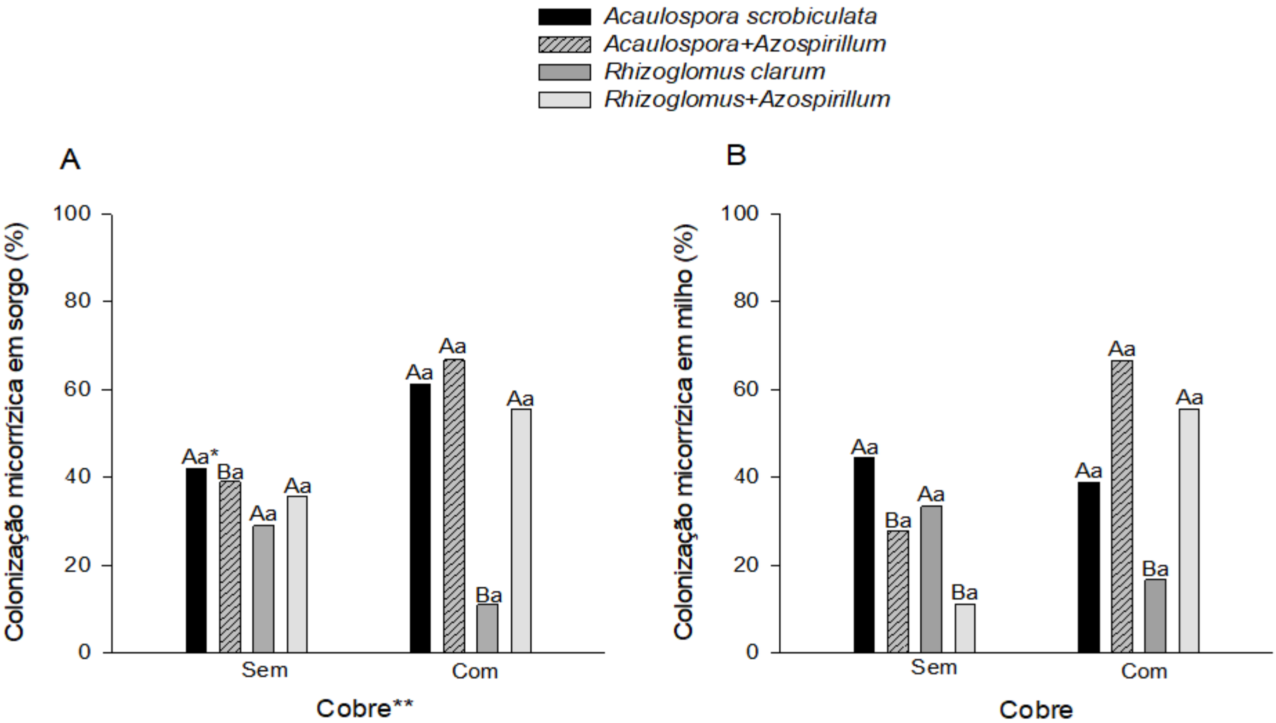

Figure 1. Percentage of mycorrhizal colonization in sorghum (A) and maize (B) grown in soil without and with Cu addition, inoculated with *Acaulospora scrobiculata, Rhizoglomus clarum, Acaulospora+Azospirillum, Rhizoglomus+Azospirilum.*
*Means followed by the same capital letter between treatments with and without copper and lowercase in the inoculation treatment do not differ from each other by the Scott-Knott test at 5% probability of error (p≤0.05). **Without = natural Cu content in the soil; With = 400 mg of Cu kg^{-1} of soil.

The Azospirillum brasilense density showed significant interaction between Cu doses and inoculum sources for sorghum and maize (Table 2). The amount of Azospirillum present in the sorghum roots was greater in the soil without copper and when inoculated in isolation, and in maize it was also greater in the soil without copper, when inoculated in isolation, not differing from the co-inoculations. Thus, this behavior can be attributed to the copper content influencing the density of microorganisms, indicating that high concentrations of this element can affect both microbial cell viability and metabolite synthesis, as already observed by Alemneh et al. (2022), which tends to reduce the population of Azospirillum in soils contaminated with copper.

Table 2
Most Probable Number (MPN) of *Azospirillum brasilense* (CFU/g) in sorghum and maize roots grown in soil with and without copper addition and different inoculum sources

	-------------Sorghum------------		----------------Maize--------------	
	Copper**		Copper	
Inoculum sources	Without	With	Without	With
Acaulospora+Azospirillum	$7.5x10^3$ Ab*	$9.5x10^2$ Ba	$3.0x10^2$ Aa	$6.0x10^2$ Ab
Rhizoglomus+Azospirillum	$2.1x10^3$ Ac	$6.5x10^2$ Ba	$3.5x10^3$ Aa	$7.3x10^3$ Aa
Azospirillum brasilense	$1.1x10^4$ Aa	$6.0x10^2$ Ba	$5.5x10^3$ Aa	$4.5x10^2$ Bb

*Means followed by the same lowercase letter in the column and uppercase letter in the row, within each plant, do not differ from each other by the Scott-Knott test at 5% probability of error (p≤0.05). **Without = natural Cu content in the soil; With = 400 mg of Cu kg^{-1} of soil.

On the other hand, the results did not show a significant interaction between the sources of mycorrhizal inoculum and copper doses for culm diameter (CD), number of tillers, leaf area (LA), chlorophyll a and b for sorghum, and plant height (PH), culm diameter (CD), shoot dry mass (SDM), chlorophyll a and b for maize. The CD and number of tillers of sorghum were not affected by the Cu dose; however, the other variables, both for sorghum and maize, showed a reduction with the addition of 400 mg of Cu kg^{-1} of soil (Table 3). As already reported by Mir et al. (2021), Eid et al. (2021), Peternella et al. (2021), Krämer (2010), high doses of Cu affect plant morphological parameters, reducing height, stem diameter, shoot dry mass, and root dry mass.

Table 3
Significant simple effect of copper (Cu) on culm diameter (CD), number of tillers, leaf area (LA) and chlorophyll a and b of sorghum, on plant height (PH), culm diameter (CD), shoot dry mass (SDM), chlorophyll a and b of maize

	--Sorghum------------------------------------				
Copper*	CD (mm)	No. of tillers	LA (cm2)	Chlorophyll a	Chlorophyll b
Without	15.29 a**	1.22 a	2180.5 a	35.22 a	14.47 a
With	15.89 a	1.31 a	1815.1 b	31.46 b	12.51 b
CV %	12.73	29.64	26.31	13.78	22.46
	--Maize------------------------------------				
	PH (m)	CD (mm)	SDM (g)	Chlorophyll a	Chlorophyll b
Without	1.64 a	18.35 a	67 a	28.69 a	8.49 a
With	1.49 b	15.40 b	49 b	25.08 b	6.49 b
CV %	14.22	15.82	27.58	19.85	35.78

*Without = natural soil content; With = 400 mg of Cu kg^{-1} of soil. **Means followed by the same lowercase letter in the column do not differ from each other by the Scott-Knott test at 5% probability of error (p≤0.05).

The lower maize SDM production in soil with 400 mg of Cu may be related to the reduction in chlorophyll levels. High doses of Cu compromise photosynthesis by reducing the levels of chlorophyll and carotenoids induced by the inactivation of enzymes responsible for the pigment biosynthesis and, consequently, negatively affect the plant morphological parameters (Rodrigues et al., 2016).

Regarding the isolated effect of the inoculum sources, sorghum CD was higher with the inoculations of A. scrobiculata and R. clarum + A. brasilense, however, there was no significant difference for the maize (Table 4). Increased stem diameter, height, fresh and dry mass were observed by Zhang et al. (2014) in Zenia insignis, a plant belonging to the Fabaceae family, and by Bisca et al. (2023) and Damin et al. (2020) in maize crops inoculated with AMF.

Sorghum LA was higher with A. scrobiculata and A. brasilense inoculation, with 2470.65 cm2 and 2413.25 cm2 means, respectively (Table 4). According to the literature, AMF are capable of improving plant nutrition, increasing leaf area and photosynthetic pigment content (Tristão et al., 2006), while inoculation with A. brasilense promotes an increase in LA in plants, increasing the efficiency of solar energy capture due to the greater amount of photosynthetically active cells (Longhini et al., 2016).

Inoculation with R. clarum, A. brasilense and co-inoculation with A. scrobiculata + A. brasilense allowed greater maize SDM in relation to the other treatments (Table 4). This fact corroborates some studies, which also observed an increase in SDM of plants with the inoculation of AMF, bacteria, and the combination of both, providing a synergistic effect between them and the development of plants (Primieri et al., 2021; Dhawi et al., 2015; Brasil et al., 2006).

Chlorophyll a and b values were significantly higher with the inoculations regarding the control treatment for sorghum and without significant effect for maize (Table 4). This fact was also observed by Fukami et al. (2018) during inoculation with A. brasilense, which promoted improvements in plant photosynthetic parameters, such as chlorophyll content, greater biomass production, and plant height. While symbiosis with AMF can increase the absorption of P and Mg in the plant, inducing an increase in chlorophyll content, improving the performance of mycorrhizal plants under stress conditions (Sheng et al., 2008).

Table 4

Significant simple effect of inoculum sources for culm diameter (CD), number of tillers, leaf area (LA), chlorophyll a and b of sorghum and plant height (PH), culm diameter (CD), shoot dry mass (SDM), chlorophyll a and b of maize

	----------Sorghum----------				
Inoculum source	CD (mm)	No. of tillers	LA (cm^2)	Chlorophyll a	Chlorophyll b
A. scrobiculata	16.2 a*	1.42 a	2470.6 a	34.6 a	15.5 a
R. clarum	14.9 b	1.28 a	1959.5 b	32.8 a	14.7 a
A. brasilense	14.9 b	1.24 a	2413.3 a	36.1 a	13.6 a
Acaulospora+Azosp.	14.8 b	1.23 a	1887.1 b	33.7 a	13.3 a
Rhizoglomus+Azosp.	16.1 a	1.15 a	1776.5 b	35.9 a	14.6 a
Control	13.8 b	1.24 a	1479.7 b	26.7 b	9.1 b
CV %	12.73	29.64	26.31	13.78	22.46
	----------Maize----------				
	PH (m)	CD (mm)	SDM (g)	Chlorophyll a	Chlorophyll b
A. scrobiculata	1.62 a	17.17 a	55 b	28.22 a	8.51 a
R. clarum	1.61 a	17.57 a	65 a	26.99 a	8.03 a
A. brasilense	1.63 a	17.67 a	66 a	28.38 a	7.07 a
Acaulospora+Azosp.	1.66 a	15.69 a	65 a	24.80 a	6.05 a
Rhizoglomus+Azosp.	1.67 a	17.21 a	54 b	29.16 a	7.63 a
Control	1.22 b	15.93 a	46 b	23.77 a	7.34 a
CV %	14.22	15.82	27.58	19.85	35.78

*Mean values followed by the same lowercase letter in the column do not differ from each other using the Scott-Knott test at a 5% probability of error ($p \leq 0.05$).

There was a significant interaction between Cu doses and inoculum sources for PH and shoot and root dry mass (RDM) for sorghum, LA and RDM for maize (Table 5). Inoculation with *A. brasilense* provided greater sorghum height, in the soil without addition of Cu, while in the treatment with 400 mg kg^{-1} of Cu applied to the soil, the greatest PH was with *R. clarum* inoculated alone and together with *A. brasilense.* This is related to the production of phytohormones by microorganisms that neutralize the disturbance caused by toxic metals, consequently increasing the plant's tolerance, optimizing the plant's physiological development (Eid et al., 2021).

The sorghum SDM in soil without Cu was significantly higher with inoculations and co-inoculations compared to the control; however, it was higher with the inoculation of *A. scrobiculata* in soil with 400 mg kg^{-1} of Cu in the soil (Table 5). This result demonstrates the ability of these microorganisms to survive in environments with high doses of metals, by retaining high concentrations of metals in mycorrhizal structures (mycelium and fungal vesicles), consequently preventing mobilization to the shoot, resulting in improvements in plant development (Dhalaria et al., 2020).

Table 5

Plant height (PH), shoot dry mass (SDM) and root dry mass (RDM) of sorghum and leaf area (LA), root dry mass of maize inoculated with *A. scrobiculata, R. clarum, A. brasilense, A. scrobiculata + A. brasilense, R. clarum + A. brasilense* and control (without inoculation), grown in soil without and with addition of Cu

Inoculum source	Cu *		Cu		Cu	
	Without	With	Without	With	Without	With
	----------------------------------Sorghum--					
	---------- PH (m) ----------		-------- SDM (g) ---------		-----RDM (k)----	
A. scrobiculata	0.93 Ab**	0.91 Ab	64 Ba**	97Aa	41 Bb	64 Aa
R. clarum	0.84 Bb	1.05 Aa	54 Aa	45 Ac	32 Ab	26 Ab
A. brasilense	1.12 Aa	0.92 Bb	54 Ba	75 Ab	65 Aa	57 Aa
Acaulospora+Azosp.	1.08 Aa	0.97 Ab	60 Ba	84 Ab	22 Bb	56 Aa
Rhizoglomus+Azosp.	0.86 Bb	1.12 Aa	56 Ba	74 Ab	28 Bb	56 Aa
Control	0.97 Ab	0.93 Ab	48 Ab	35 Ac	22 Ab	16 Ab
CV %	15,65		23,66		32,13	
	---Maize-------------------------------------					
	---------- LA (cm^2) ---------		---------- RDM (g) -------			
A. scrobiculata	2894.7 Aa	2293.8 Ab	25 Aa	40 Aa		
R. clarum	1438.6 Bb	3364.2 Aa	42 Aa	35 Aa		
A. brasilense	3359.3 Aa	1678.9 Bb	28 Aa	33 Aa		
Acaulospora+Azosp.	2801.9 Aa	1986.2 Bb	29 Ba	53 Aa		
Rhizoglomus+Azosp.	2747.0 Aa	1970.9 Ab	11 Bb	42 Aa		
Control	1280.6 Ab	932.3 Ac	10 Ab	15 Ab		
CV %	21.55		31.04			

*Without = natural soil content; With = 400 mg of Cu kg^{-1} of soil. **Means followed by the same lowercase letter in the column and uppercase letter in the row, within each parameter, do not differ from each other by the Scott-Knott test at 5% probability of error (p≤0.05).

Inoculation of *A. brasilense* resulted in higher sorghum RDM in soil without Cu, while at the 400 mg kg^{-1} dose, the highest values were obtained with the inoculation of *A. scrobiculata, A. brasilense* and co-inoculations. In maize, inoculation of A. scrobiculata, *R. clarum* and *A. brasilense* promoted greater RDM in soil without Cu, and with 400 mg kg^{-1} the inoculations were superior to the control (Table 5). Rhizobacteria have the ability to increase the mycorrhizal effect by regulating physical-chemical parameters such as release of root exudates, changes in soil pH, secretion of phytohormones and consequently affect plant growth (Giatti & Piza, 2022). While AMF induce an increase in root development, enabling better nutrient acquisition by plants (Primieri et al., 2021).

Maize LA was higher with *A. scrobiculata, A. brasilense* inoculation and co-inoculations in soil without Cu and with *R. clarum* in soil with 400 mg of copper kg^{-1} of soil (Table 5). It is noteworthy that AMF produce phytohormones that regulate plant growth (Zhang et al., 2014), while *A. brasilense* acts on the host's metabolism, leading to a significant increase in the formation of roots, stems, and leaves (Ribeiro et al., 2022). These microorganisms are capable of protecting plants against Cu toxicity through mechanisms that retain these metals in the fungal mycelium and also through the nutritional improvement of plants (Andreazza et al., 2013).

There was no significant interaction between the Cu variation factors and inoculum source for the Cu content in the sorghum shoot and root and Cu content in the grain, shoot and root of maize, presenting only a significant simple effect for the treatments. The Cu content in the sorghum and maize shoot was higher with the application of 400 mg of Cu kg-1 of soil, with no significant difference in the Cu content in the maize grain, being lower than the other parts of the plant (Table 6). This result is related to the low mobility of Cu in the plant (I. R. Silva & Mendonça, 2007), as this element has an affinity with ligands found in the roots, resulting in accumulation in this organ, in addition to being especially concentrated in the cell wall of xylem cells (Rodrigues et al., 2016). The values found in maize grain are below the maximum acceptable limit of 10 mg Cu kg^{-1} for cereal grains, as determined by the Agência Nacional de Vigilância Sanitária [ANVISA] (1998). In the maize shoot, a content close to 15 mg kg^{-1} was identified, consistent with the value found by Peternella et al. (2021), and in forage sorghum, the content was 10 mg kg^{-1}, similar to that mentioned by Krämer (2010).

Table 6

Simple effect of Cu treatment and simple effect of inoculum sources (A. scrobiculata, Rhizoglomus clarum, Azospirillum brasilense, A. scrobiculata. + A. brasilense, Rhizoglomus c. + Azospirillum b., control without inoculation) on Cu content in the sorghum shoot (CuSH) and root (CuR) and in the maize shoot (CuSH), root (CuR), and grain (CuG)

Cu*	-------------Sorghum----------		---------------------Maize---------------------		
	CuSH (mg kg^{-1})	CuR (mg kg^{-1})	CuSH (mg kg^{-1})	CuR (mg kg^{-1})	CuG (mg kg^{-1})
Without	8.48 b**	89.35 b	12.93 b	130.92 b	3.13 a
With	11.89 a	327.75 a	16.47 a	409.22 a	3.52 a
CV (%)	27.3	26.5	28.3	33.4	30.1
	------------Sorghum-----------		--------------------Maize----------------------		
Inoculum source	CuSH (mg kg^{-1})	CuR (mg kg^{-1})	CuSH (mg kg^{-1})	CuR (mg kg^{-1})	CuG (mg kg^{-1})
A. scrobiculata	10.23 b	166.77 b	14.44 b	272.31 b	2.91 b
R. clarum	9.55 b	220.37 b	14.01 b	225.39 b	3.24 b
A. brasilense	8.95 b	223.62 b	12.26 b	216.18 b	3.16 b
Acaulospora+Azosp.	9.31 b	167.01 b	13.16 b	252.24 b	3.39 b
Rhizoglomus+Azosp.	9.87 b	198.89 b	14.69 b	281.30 b	2.57 b
Control	13.21 a	274.64 a	19.65 a	372.97 a	4.68 a
CV %	27.3	26.5	28.3	33.4	30.1

*Without = natural Cu content in the soil; With = 400 mg of Cu kg^{-1} of soil. **means followed by the same lowercase letter in the column do not differ from each other according to the Scott-Knott test (p<0,05) probability of error.

The inoculation and co-inoculation treatments allowed for a lower Cu content in the sorghum shoot and root, in the maize shoot, root, and grain compared to the control, which, without inoculation, resulted in sorghum shoot values above the 10 mg kg^{-1} limit that is established by ANVISA (2019) for use in the composition of animal feed, consequently, the microorganisms showed a potential in reducing copper levels in the sorghum shoot, meeting the ANVISA (2019) regulations. These microorganisms have the ability to transform contaminants, such as heavy metals, into less toxic forms, via mobilization reactions, which can be through autotrophic and heterotrophic leaching, chelation and methylation, as well as through immobilization reactions, via biosorption, bioaccumulation, and precipitation processes (Bourles et al., 2020).

Conclusions

According to the results, the inoculation with *Acaulospora scrobiculata* in copper-contaminated soils promoted a significant increase in dry biomass, root volume, and mycorrhizal colonization in sorghum plants. Furthermore, *Rhizoglomus clarum* favored the shoot development in maize plants. The co-inoculation of *Azospirillum brasilense* with arbuscular mycorrhizal fungi was effective in reducing copper concentrations in the sorghum shoot to levels below the limits established by Brazilian legislation. These results indicate the potential of microbial inoculations as a promising strategy for the management of copper-contaminated soils.

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