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Article

Use of a microbial consortium from the southeast of Coahuila with potential for its application as a biofertilizer

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Abstract

Environmental sustainability is becoming increasingly important, and in the case of agriculture, the aim is for resources to be economically sustainable, maximizing production and minimizing costs. Among the current options, biofertilizers have gained relevance as they are a promising alternative by improving plant nutrition and strengthening defenses with the use of beneficial microorganisms in the rhizosphere. Although biofertilizer production traditionally focuses on the selection, characterization, and formulation of individual isolates (strains) with desired traits to promote plant growth, evidence suggests that bioinoculants increase efficacy when using microbial communities (consortia). This work aimed to evaluate the biotechnological potential of a microbial consortium obtained from southeastern Coahuila, which was identified via massive sequencing of the 16S rRNA gene and the 18S rRNA gene and was made up mainly of the yeast genera *Meyerozyma* spp., *Debaryomyces* spp., and *Kurtzmaniella* spp., as well as bacteria of the genus *Bacillus*. The evaluation as a biofertilizer was carried out with three formulations, culture medium with consortium [Med+C] and two alternatives, molasses with consortium and culture medium plus molasses with consortium [Mel+C and Med+Mel+C]; they were evaluated under greenhouse conditions in *Spinacia oleracea* (spinach), and there were also a control (water) and a commercial product as treatments. The application of the different formulations, in particular Med+C, tends to increase the agronomic variables of the crop (height, stem diameter, leaf length and width, fresh weight and dry weight) and the amount of minerals (Fe, K and Cu) compared to the control treatment. The results obtained indicate that the application of microbial consortia significantly reduces the use of chemical fertilizers.

Keywords:

Bioinoculant, Bacillus, *Debaryomyces*, *Kurtzmaniella*, yeasts, *Meyerozyma*.

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Introduction

Environmental sustainability has become important, especially in agriculture, with economically sustainable resources maximizing production and minimizing costs. Among the options currently available, biofertilizers have become relevant, as they include live microorganisms and chemicals that regulate plant growth and provide benefits to the soil, which makes them increasingly recognized (Ding *et al*., 2024). A promising alternative to improve plant nutrition and strengthen their defenses is the use of beneficial microorganisms from the rhizosphere, such as 'rhizobacteria and fungi that promote plant growth' (Sevillano-Cano, 2024).

This is mainly due to their varied mechanisms of action and because they are less risky to health and the environment. The use of biofertilizers even improves soil conditions and increases the capacity to provide nutrients, with a significant impact on plant growth and crop production (Mashatleh *et al*., 2024). Undoubtedly, a double advantage with the application of microbial inoculants to the soil is, first, that they increase the direct advantages of the crop in nutrition, and second, that they help soil health and recovery (Bhattacharyya and Jha, 2012). On the other hand, co-inoculation (plant-microorganisms) has been shown to stimulate growth, nodulation, and nitrogen fixation in plants (Muthusamy *et al*., 2023).

Although the role and importance of these microorganisms are well known, and despite the great diversity that can be found in the soil with characteristics of interest, there are only a few microorganisms that are used and marketed as biofertilizers, barely ~50 functional strains that are applied in agriculture (Pirtila *et al*., 2021).

In the production of biofertilizers, the selection, isolation and characterization of microbial species, also known as bioinoculants, with characteristics that promote, among other things, greater plant growth, is highlighted; however, there is evidence that suggests an increase in the efficacy of the bioinoculant when it is composed of several microorganisms instead of individual species (Hernández-Álvarez *et al*., 2023).

The latter is interesting since one of the leading causes of having few functional strains is the complexity in individual isolation and characterization, so a consortium can be an efficient alternative, even considering the synergy produced in a consortium derived from the different mechanisms of action in the microorganisms present, sometimes superimposed on plant protection mechanisms (Vassilev *et al*., 2006).

The use of biofertilizers made up of microbial consortia of multiple characteristics offers a great opportunity, since such consortia can provide benefits to crops, including increased nutrient absorption, disease resistance, and stress tolerance (Yadav and Yadav, 2024). The purpose of this work was to evaluate the biofertilizer potential in greenhouse tests of a consortium obtained from the soil in the southeast of Coahuila.

Materials and methods

Isolation: root samples were collected from a tomato crop that grew in calcareous soil in General Cepeda, Coahuila, the coordinates of which are 25° 32' 00" north latitude and 102° 32' 00" west longitude; the isolation was performed by the method of serial dilutions and plate counting (Hoben and Somasegaran, 1982). The isolated consortia correspond to the horticultural microbiology laboratory of the Department of Horticulture at the Antonio Narro Autonomous Agrarian University (UAAAN), for its acronym in Spanish.

Culture conditions and molecular characterization. The consortium sample used was grown in 500 mL Erlenmeyer flasks with modified Rennie medium (Rennie, 1981), with temperature controlled at 28 °C, orbital shaking at 120 rpm for 48 h. To understand the diversity of species and correlate with their role in the microbial consortium, a metagenomic taxonomy analysis was carried out; therefore, DNA extraction was obtained using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions.

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Subsequently, the sample was amplified by PCR with the universal primers for ribosomal genes 16SrRNA (27F; AGAGTTTGATCCTGGCTCAG and 1492R; GGTTACCTTGTTACGACTT) and 18SrRNA (18SF; AACCTGGTTGATCCTGCCAGT and 18SR; TTGATCCTTCTGCAGGTTCACC). The amplicons obtained were purified using the QIAquick® PCR Purification Kit according to the manufacturer's instructions. The quality and quantity of amplicons obtained were determined by their concentration and purity using a NanoDrop One((Thermo) and visualized in a 1% agarose gel, TBE 0.5x.

The sequencing and analysis of taxonomic diversity were performed with Oxford Nanopore sequencing at Secoya Labs SC, Mexico City. Treatments. The consortium used as a biofertilizer was grown in three formulations: modified Rennie culture medium (Med+C) and two alternative media -molasses (Mel+C) and culture medium+molasses (Med+Mel+C). The growths obtained were used in the greenhouse experiments.

A spinach (*Spinacia oleracea* L.) crop was used, to which 6 ml L^{-1} with 6.3 x 10^{10} CFU ml^{-1} of biofertilizer was applied. These doses were established with previous experimentation of the formulations in seed priming (imbibition for 24 h). In addition to the three formulations, a commercial treatment (Inobac, BioJal() and a control treatment with water alone were used.

Experimentation in the field. It was carried out from April to June 2023; the experiment was established in a tunnel-type greenhouse with a wet wall and polyethylene cover in the Department of Horticulture of the UAAAN, located in Buenavista, south of the municipality of Saltillo, in the state of Coahuila de Zaragoza, Mexico, whose geographical coordinates are 25° 21' 20.79" north latitude and 101° 1' 52.87" west longitude, at an altitude of 1 779 m.

A spinach crop was established (HortaFlor, Rancho Los Molinos, % germination of 92) with direct sowing in soil in furrows with previous addition of compost, and a drip irrigation system with drippers spaced 10 cm apart. Seeds were primed (24 h) with each treatment prior to direct sowing, and two drench applications of the treatments were performed at 15 and 30 days following seedling emergence.

Sampling of agronomic variables. All agronomic variables were obtained by manual measurement with a tape measure (Truper 3 m) and with an analog vernier (Scienceware(). Leaf width and length measurements were taken from the first undamaged adult leaf at the base of the plant, using the same position for all the experimental units. In the case of fresh weight and dry weight, they were obtained with a gravimetric balance (Ohaus(), and the percentage of moisture was determined by the difference between these two measurements.

Elemental and mineral analysis. The biomass obtained from the different experiments was used to perform the analysis of the elemental content: carbon (C), hydrogen (H), oxygen (O), nitrogen, and sulfur. This was measured in an elemental analyzer (Elemental Analyzer Flash 2000, Thermo Scientific() using methionine as standard. Each sample was dried and pulverized to weigh 2 to 4 µg of biomass.

The relative percentage of oxygen was calculated by difference from the relative percentage of CHNS (Kumar, 2014). Mineral analysis of K, Mg, Cu, Fe and Zn contents in spinach leaf tissue was evaluated on dried, ground samples with particle size homogenization and digested with 1:1 HCl, volumetrically adjusted to 50 ml (Kavanagh, 1981), for analysis with an atomic absorption spectrophotometer (GBC, Scientific Equipment() calibrated using standard curves for each mineral evaluated.

Statistical analysis

A randomized complete block design was used. Due to a previous observation of normality of the obtained data using a Shapiro-Wilk test, it was decided to perform the data analysis of the agronomic variables using the non-parametric statistical test of Kruskal-Wallis with *post hoc* analysis with a statistical significance level of $p \leq 0.05$. The above tests and the analysis of principal components to establish the relationship of variables with treatments were carried out with Infostat software,

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version 2020. The study of mineral variables, with Pearson's correlation plots, was obtained with Minitab 19 Statistical Software.

Results and discussion

Metagenomic identification. The amplicons obtained were used to carry out a metagenomic taxonomy analysis of the consortium. The analysis of the diversity of species presents in the consortium used indicates that some of the most representative species, due to their abundance, have already been reported in previous studies to promote plant growth (Robas-Mora *et al*., 2022).

Among the species reported by the metagenomic analysis are *Meyerozyma guilliermondii*, *M. athensensis*, *M. caribbica*, *M. smithsonii*, and *M. carpophila*, *Debaryomyces udenii*, *D. mycophilus*, and *D. hansenii*, as well as the species *Kurtzmaniella* sp. Nonetheless, the presence of organisms of the genus *Bacillus* (*B. toyonensis* and *B. mobilis*) should also be highlighted.

This implies that, in the consortium, although primarily made up of yeasts, there are also bacteria in coexistence. Although biofertilizers are currently focused on plant growth-promoting bacteria, there is an initiative to explore various yeasts with biofertilizer potential (bioprospecting). Among these microorganisms, the following stand out: plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF), and mycorrhiza helper bacteria (MHB), among others, which work together in consortium biofertilizer formulations (Odoh *et al*., 2020).

The genus *Meyerozyma guilliermondii* has been linked to improved phosphate solubility and improved productivity of corn (*Zea mays* L.) (Nakayan *et al*., 2013). In this sense, de Robador *et al*. (2023) studied this genus and reported it as a biological control agent (BCA) in the induction of defensive responses in vine plants.

On the other hand, in 2024, Sevillano-Cano *et al*. reported that the species *Debaryomyces hansenii* not only improves nutrient absorption and stimulates plant growth and flower development but could also amplify induced systemic resistance (ISR) in cucumber (*Cucumis sativus* L.) crops; this species is also recorded in the consortium reported here.

Some of the characteristics that make yeasts a potential source of biofertilizers include their ability to be abundant in soil due to their metabolism and life cycle. Yeasts are facultative anaerobes that reproduce by budding or cell fission, which allows them to adapt to a wide variety of environments (Hernández-Fernández *et al*., 2021).

Yeasts have great potential as promoters of plant growth; in soils, they are important for processes related to nutrient transformations and maintenance of structure, as they improve the stability of soil aggregates, which affects the water retention capacity and fertility of the substrate (Botha, 2011). On the other hand, there are reports of the ability of some yeasts to produce indole-3-acetic acid, ammonium, polyamines, as well as the solubilization capacity of calcium phosphate and zinc oxide (Fu *et al*., 2016).

Among the species found in the genus *Bacillus*, *B. toyonensis* stands out, which has the potential to produce several antimicrobials, making it potentially useful for the development of new strategies for controlling microbial diseases in plants (Luo *et al*., 2021). *Bacillus mobilis* was also present, which is an endophytic bacterium that has been reported with antifungal activity against pathogenic species of Botryosphaeriaceae (Romero-Cuadrado *et al*., 2024). In this way, it can be inferred that these microorganisms, although to a lesser extent, also have potential as biopesticides.

Field tests. A determining factor in the process of selection or bioprospection of promising microorganisms for use as biofertilizers is field crop tests, particularly in agronomic variables, which are the most visible aspects for the end user. In this case (Table 1), it was observed that, although there are no statistically significant differences for all variables, there is a tendency to obtain higher values in treatments where the consortium is included.

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Table 1. Statistical results by the Kruskal-Wallis test of the agronomic variables evaluated in *Spinacia oleracea* (spinach).

Treatment	Height (cm)		Stem diameter (cm)		Leaf length (cm)		Leaf width (cm)		Fresh weight (g)		Dry weight (g)		Moisture (%)	
	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD
Mel+C	32	1.79	4.3	0.61	19	3.16	14	1.94	303.34^{AB}	59.09	17.01^{AB}	4.04	94.07	0.89
Med +Mel+C	34	3.74	3.9	0.74	18	3.27	13	3.3	218.29^{BC}	67.59	11.34^{B}	5.49	94.81	0.91
Med+C	30	4.9	4.8	0.79	21	4.19	13	2.92	334.52^{A}	73.9	22.68^{A}	4.28	93.22	2.11
Commercial	30	5.67	4.4	0.66	19	4.88	12	1.42	175.77^{C}	67.65	11.34^{B}	5.07	93.75	1.84
Control	32	4.24	3.9	0.72	17	2.95	11	2	280.66^{AB}	123.75	17.01^{B}	9.74	94.17	1.44
H-value	4.68		8.46		2.73		4.81		13.4		12.14		6.55	
p-value	0.3125		0.0748		0.5989		0.2967		0.0095		0.0149		0.1613	

Treatments: mel+C (molasses+consortium), med+mel+C (medium+molasses+consortium), med+C (medium +consortium), commercial (Inobac, BioJA®), control (water). $^{A, B\ y\ C}$= they represent significant differences by Kruskal-Wallis comparison ($p< 0.05$); SD= standard deviation; H-value= Kruskal-Wallis Chi-square value.

In particular, the variables of DW and FW are the ones that benefit the most, with higher medians compared to the control treatment, especially in the Med+C treatment. Field results are higher in several of the agronomic variables compared to similar studies (Shafeek *et al*., 2021; Safdar *et al*., 2022).

Correlation of variables and treatments. A biplot (Figure 1) was created using the results of the principal component analysis, with the sum of PC1 and PC2, to examine the interrelationship between agronomic variables and treatments, they have a range of 90.5%, which implies that this percentage of the information is represented.

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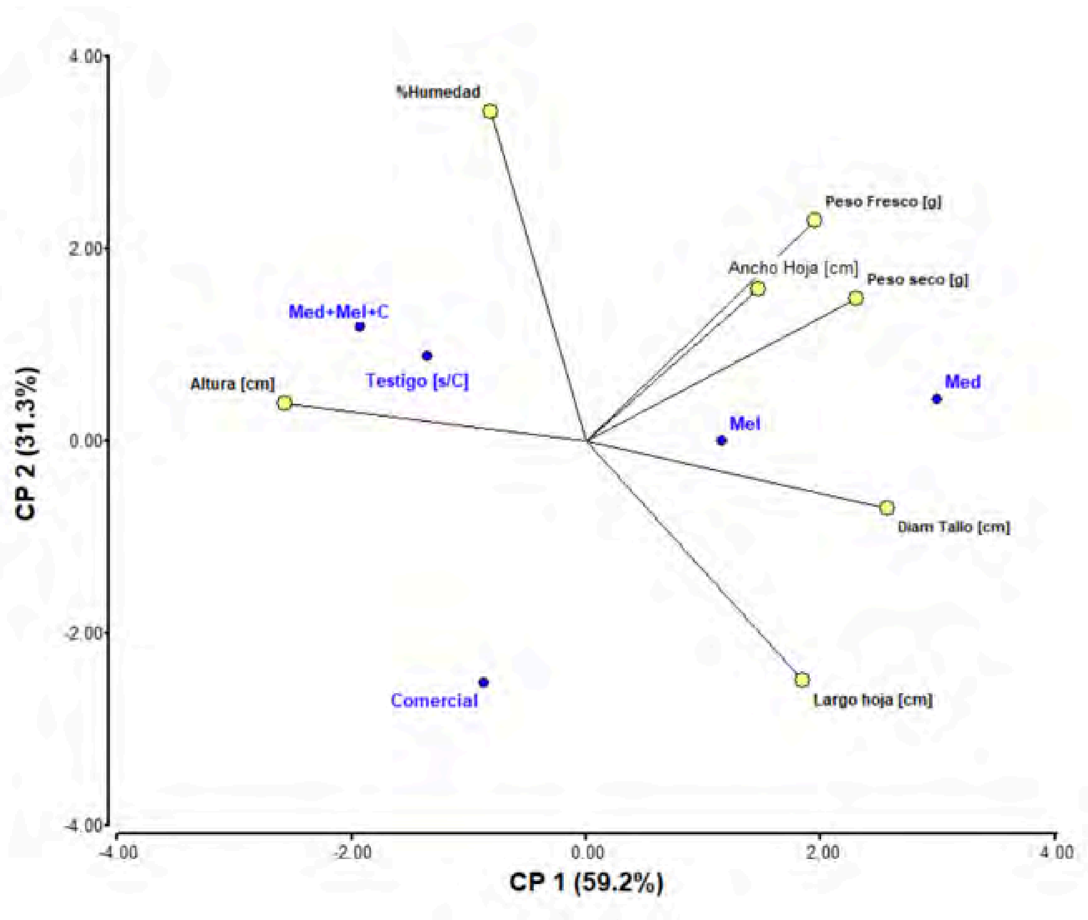
Figure 1. Principal components PC1 and PC2, using agronomic variables and the formulations Med+C, Mel+C, Med+Mel+C, commercial, and control (water) as treatments.



When the angle between the vectors in the figure is <90°, there is a positive relationship; if it is >90°, there is a negative relationship, and if the angle between the vectors is 90°, there is no significant relationship (Seymen, 2021); height and leaf length show a weak relationship between variables; on the other hand, the variables dry weight, fresh weight, and leaf width, when considering the amplitude of the angles between them and their proximity in the quadrant, are more associated with the Mel+C and Med+C treatments.

Elemental analysis and empirical formula of biomass. When performing the elemental analysis of the spinach biomass obtained in triplicate of the different treatments (Table 2), values >30% carbon were determined for all treatments, which aligns with the description by Méndez *et al*. (2023), who explain that herbaceous biomass species have a lower carbon content than woody biomass species, which is consistent with spinach as it is a herbaceous species.

Table 2. Elemental analysis of the biomass of *Spinacia oleracea* at harvest, presented as a percentage of dry biomass weight (DW) and expressed in the different formulations applied.

Biomass of Spinacia oleracea in formulations	Elemental analysis (%) DW					Empirical biomass formula	C/N ratio
	C	H	O	N	S		
Med+C	32.23	4.33	58.67	4.2	0.57	$CH_{1.601}O_{1.387}N_{0.110}S_{0.006}$	7.67

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Biomass of Spinacia oleracea in formulations	Elemental analysis (%) DW					Empirical biomass formula	C/N ratio
	C	H	O	N	S		
Mel+C	32.6	4.36	58.15	4.35	0.35	$CH_{1.595}O_{1.341}N_{0.114}S_{0.006}$	7.5
Med+Mel+C	30.99	4.17	60.12	4.24	0.49	$CH_{1.603}O_{1.458}N_{0.117}S_{0.005}$	7.31
Commercial	32.13	4.29	58.79	4.46	0.32	$CH_{1.592}O_{1.376}N_{0.118}S_{0.003}$	7.2
Control (water)	31.77	4.27	59.35	4.2	0.41	$CH_{1.600}O_{1.406}N_{0.113}S_{0.004}$	7.56
SE	1.17	0.15	1.63	0.3	0.13		

Treatments= mel+C (molasses + consortium), med+mel+C (medium+molasses+consortium), med+C (medium +consortium), commercial (Inobac, BioJal®), control (water). SE= standard error.

Among the results to be highlighted is sulfur (S), since this is an element that must be added in nutrition; the largest amount is present in the Med+C treatment, which implies a greater absorption of this nutrient; sulfur is essential for the proteins that plants require as part of their structure (Milera-Rodríguez *et al*., 2024).

The molecular formula of *S. oleracea* biomass was determined based on the relative percentage of C, H, O and N, according to formulas described by Kumar (2014). Similarly, the C/N ratio (which can be associated with the efficiency of nitrogen use) presents values #7.2; it must be kept in mind that lower C/N values imply that the plant uses the available nitrogen to prioritize its growth; this is particularly congruent with herbaceous species that prioritize the formation of proteins, especially those involved in photosynthesis (Zhang *et al*., 2020).

Correlation analysis between minerals. The contribution of minerals in fertilization is an important aspect that significantly influences the achievement of better yields (Coraspe-León, 2009). Nevertheless, understanding how they correlate with each other in the plant is also of utmost importance, especially when explaining how the microbiome intervenes in the absorption of nutrients, both those naturally present in the soil and those used in nutrition.

In the correlograms presented (Figure 2A, 2B, 2C, 2D, and 2E), the correlation between nutrients can be better analyzed, and although correlation does not necessarily imply causality, it serves as an approximation between the nutrients that the plant has obtained throughout its life cycle. Undoubtedly influenced by biofertilization, the interaction of oxygen with other elements is particularly noticeable when compared with the control treatment, since they can form oxides of different types that can intervene in the plant's development, as in the case of Cu, where the control presents its only positive correlation.

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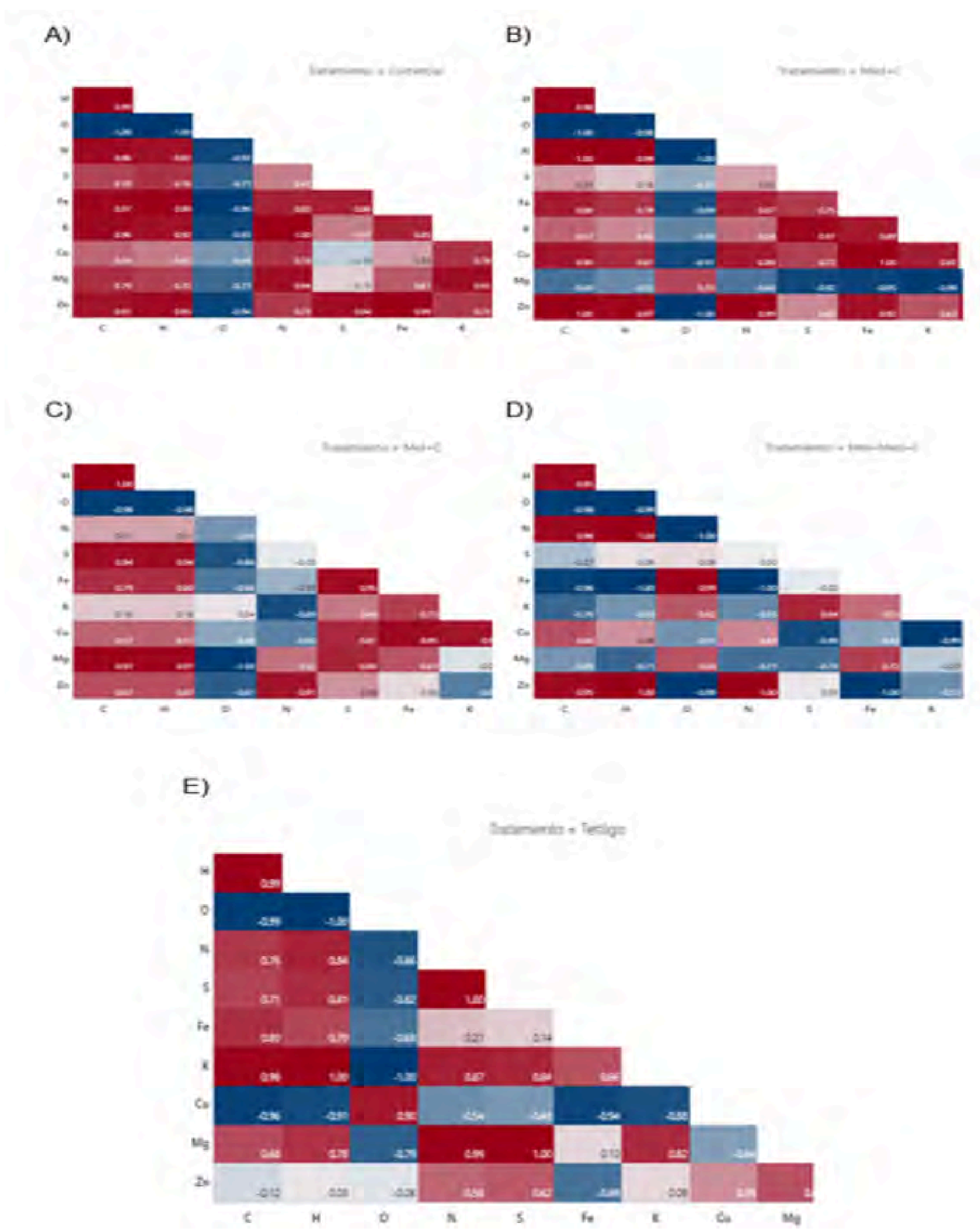
Figure 2A, 2B, 2C, 2D and 2E Correlation between important elements (C, H, O, N, S, Fe, Cu, K and Mg) for each treatment evaluated (Med+C, Mel+C, Med+Mel+C, commercial and control).



Using mixtures of microorganisms with biofertilizer potential, such as *Bacillus amyloliquefaciens*, *Nostoc muscorum*, and *Saccharomyces cerevisiae* (Omer *et al*., 2023), has resulted in improvements in the histological structures of plants, demonstrating the ability of the different microorganisms present in a consortium to work together.

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In addition to the above, the species *Meyerozyma guilliermondii*, *Debaryomyces hansenii*, and *Rhodotorula mucilaginosa* have also been reported with the capacity to produce IAA, solubilize zinc, produce NH_3, catalase, and protease, and solubilize phosphorus (Bilek *et al*., 2020). In particular, yeasts of the genus *Meyerozyma* that are capable of synthesizing indole compounds and probably have the capacity for the production of siderophores (De Lima *et al*., 2022), which are primarily involved in the absorption of Fe, Cu and Zn.

Conclusions

The use of consortia of microorganisms is a way to enhance the effectiveness of a biofertilizer, especially when they have varied functions. This research used a consortium from a region where environmental conditions are primarily adverse due to extreme temperatures and low rainfall, characteristic of the southeast of Coahuila, and which is also made up of yeasts and bacteria, where the former have an important effect by providing the necessary CO_2 to the plant, as well as helping the assimilation of nutrients, such as the minerals presented (especially Zinc, Cu, and Fe).

Probably because of the presence of siderophores, and that in the case of the species of bacteria obtained in the sampling, they can be complementary as they are antagonists with several phytopathogenic microorganisms (subsequent analyses are suggested to evaluate this capacity), it also helps to enrich the natural microbiota of the soil, due to the predominant use of microorganisms isolated from the same region. Similarly, by testing the different formulations, it can be concluded that they also have an important role in field application, in addition to their growth in the laboratory.

Although the culture medium plus the consortium [Med+C] seems to be the best treatment compared to the different variables evaluated, we must emphasize that the other formulation treatments also have a positive impact on the plants, and that the intention of using other growth media for microorganisms was proposed to make larger-scale production easier and more affordable. Although there is still work to be done, these findings make this microbial consortium a promising candidate for use in sustainable and environmentally friendly integrated crop management.

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