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Tripartite fungus-yeast-bacteria consortium improves agronomic performance in early maize growth

Consorcio tripartito de hongo-levadura-bacteria mejora el desempeño agronómico en el crecimiento temprano del maíz

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ABSTRACT

The formulation and evaluation of microbial consortia remain key challenges in developing sustainable agricultural technologies. This study assessed the potential of microbial consortia to promote early-stage maize growth using a rapid and efficient *in vitro* approach. Four germination methods were compared to identify the most suitable for evaluating fungal effects. The compatibility of fungal strains with previously isolated bacteria and yeasts was analyzed to formulate consortia based on mutual compatibility. The selected method was used to evaluate consortium efficacy, and the functional mechanisms of the most effective combination were characterized. The between-paper germination system proved optimal for plant growth promotion (PGP) studies with fungi, as it produced handleable seedlings displaying architecture closely resembling field-grown plants. Thirteen treatments were developed from compatible strain combinations. Among them, the tripartite consortium composed of *Aureobasidium* sp. VFRB, *Clavispora* sp. Y35, and *Pantoea* sp. CanP2-19b significantly enhanced germination rates, seedling architecture, and biomass accumulation. Functionally, the fungus mobilized nutrients and produced siderophores; the yeast exhibited high indole production; and the bacterium effectively solubilized phosphorus and potassium. These results highlight the agrobiotechnological potential of this consortium for use in technologies that improve maize establishment. Additionally, the study underscores the value of germination assays as essential tools in the bioprospecting of plant growth-promoting microorganisms, contributing to the advancement of sustainable agriculture.

Keywords: Consortia design, compatibility test, germination test, plant growth promoting yeast, plant architecture.

RESUMEN

La formulación y evaluación de consorcios microbianos representa un reto clave para el desarrollo de tecnologías agrícolas sostenibles. Este estudio evaluó la capacidad de consorcios microbianos para promover el crecimiento de maíz en etapas tempranas mediante una metodología *in vitro* rápida y eficiente. Se compararon cuatro métodos de germinación para identificar el más adecuado para evaluar el efecto de microorganismos fúngicos. Se evaluó la compatibilidad de las cepas fúngicas con bacterias y levaduras previamente aisladas para conformar consorcios por compatibilidad. Mediante el método seleccionado se evaluó la eficacia de los consorcios y se caracterizaron los mecanismos de acción del consorcio sobresaliente. El sistema de germinación en rollos de papel fue ideal para estudios de promoción de crecimiento vegetal (PCV) con hongos al obtener plántulas manipulables con arquitectura similar a las germinadas en condiciones naturales. Se obtuvieron trece consorcios de las combinaciones compatibles. El consorcio tripartito: *Aureobasidium* sp. VFRB, *Clavispor*a sp. Y35 y *Pantoea* sp. CanP2-19b mejoró significativamente la tasa de germinación, la arquitectura de las plantas y la acumulación de biomasa. A nivel funcional, el hongo mostró capacidad para movilizar nutrientes y producir sideróforos; la levadura una alta producción de indoles, y la bacteria habilidad para solubilizar fósforo y potasio. Estos hallazgos respaldan el potencial agrobiotecnológico del consorcio para su integración en tecnologías orientadas a optimizar el establecimiento del cultivo de maíz. Asimismo, el estudio destaca la utilidad de los ensayos de germinación como herramientas clave en la bioprospección de microorganismos PCV, aportando información relevante para el desarrollo de prácticas agrícolas sostenibles.

Palabras clave: Diseño de consorcios, pruebas de compatibilidad, pruebas de germinación, levaduras promotoras de crecimiento vegetal, arquitectura vegetal.

INTRODUCTION

Microbial consortia comprising microorganisms from diverse phylogenetic groups have demonstrated superior capability in optimizing plant nutrition, development, and health processes compared to individual populations. This characteristic renders them a promising alternative with significant potential to enhance agricultural productivity, conserve, and promote soil biodiversity (Maciag *et al.*, 2023). These multifaceted consortia reinforce the diversity of soil metabolic pathways, thereby fostering plant growth, inducing systemic resistance, suppressing diseases, and enhancing tolerance to abiotic factors, ultimately leading to increased crop productivity (Luziatelli *et al.*, 2023). Typically, these consortia consist of various plant growth-promoting bacteria (PGPB); while in other cases, they are comprised of plant growth-promoting fungi (PGPF) that similar to bacteria promote vigorous plant development. Although less common, there are instances where growth-promoting yeasts (PGPY) have been studied; despite the limited presence of PGPY in research, some evidence suggests that they serve as excellent biofertilizers and plant stimulants (Carvajal *et al*., 2024).

The germination stage and early seedling development play a crucial role in achieving an optimal plant population per unit area, thereby ensuring favorable crop performance (Mandić *et al*., 2023). During these stages, a complex network of regulatory processes, influenced by growth regulators, nutrient availability, and symbiotic associations, significantly impacts both germination and seedling development (Xue *et al*., 2021). However, these processes can be disrupted by abiotic stressors, nutrient deficiencies, and phytopathogen presence, resulting in poor germination, diminished vigor, and reduced yields (Khaeim *et al*., 2022).

The La Frailesca region in Chiapas is characterized by intensive agricultural and livestock activities, including corn, beans, coffee, and peanuts. However, excessive use of agrochemicals has led to soil fertility degradation. This has caused land abandonment and the conversion of forested areas into farmland. In response, the non-profit association "The Nature Conservancy" has funded collaborative projects with INIFAP to establish agroforestry systems as an alternative for restoring degraded soils. Among these recovery strategies, studies have focused on diagnosing the phytosanitary status of these soils, resulting in the identification of various strains of both phytopathogens and beneficial fungi (Chávez-Díaz *et al*., 2022). Additionally, bacteria and yeasts with capabilities to promote plant growth have been isolated, demonstrating physiological capacities that positively influence soil fertility (Resendiz-Venado *et al*., 2022). These studies have confirmed the antagonistic capacity of fungi, yeast and bacteria against plant disease-causing agents. Furthermore, bacteria have been found to have the potential to enhance plant growth, leading to the formulation of consortia based on their compatibility (Gayosso-Barragán *et al*., 2023; Resendiz-Venado *et al*., 2022). Microorganisms derived from these studies (primarily bacteria) have been evaluated under both *in vitro* and in field conditions. However, due to limitations in time and resources, there is a critical need to implement practical and efficient methodologies for assessing multi-strain microbial consortia. Such approaches should reliably predict their effects on crop performance, with the aim of accelerating the development and formulation of effective bioinoculants for sustainable agricultural applications.

Laboratory germination tests have proven to be excellent predictors of different plant genotypes' field performance, facilitating the exploration of treatments that enhance seed and seedling physiological processes, thereby helping to develop strategies that ensure their establishment in the field (Calvillo-Aguilar *et al*., 2023; Khaeim *et al*., 2022; Xue *et al*., 2021). Building upon this knowledge, the present study investigates the integration of fungal strains into consortia with bacteria and yeasts based on their compatibility to promote plant growth, and evaluates their effectiveness using an adapted in vitro method designed to robustly predict the agronomic performance of maize during its early growth stages.

MATERIALS AND METHODS

Microbial strains, inocula, and plant material

Microbial strains isolated from La Frailesca, Chiapas, during 2021 and 2022 were utilized in this study. Fungal strains comprised *Aureobasidium* sp. VFRB, associated with bean plants; *Cordyceps* sp. SVD and *Trichoderma* sp. SVA, isolated from jungle soil;

Purpureocillium sp. BOF, isolated from forest soil; and *Trichoderma* sp. LCCG, associated with peanut plants, all previously identified by morphometry and molecularly (Chávez-Díaz *et al*., 2022). Preexisting microbial consortia were used. The 2021 consortium consisted of *Stenotrophomonas* sp. LIMN, *Rhizobium* sp. WFRFC, and *Enterobacterium* sp. LCMG. The 2022 Consortium comprised *Pantoea* sp. CanP2-19b, *Pseudomonas* sp. 37L, and *Pseudomonas* sp. P1.1-2b (Ruiz-Ramírez *et al*., 2024). Consortium E1 integrated by bacteria from the 2021 consortium in addition to *Serratia* sp. 385 isolated from mining tailing (Zelaya-Molina *et al*., 2016), while E2 Consortium included *Pantoea* sp. CanP2-19b and the yeast *Clavispora* sp. Y35 isolated from conical maize race from Hidalgo state (Zelaya-Molina *et al*., 2021). The plant growth-promoting capacity of all microorganisms was previously known, except for *Aureobasidium* sp. VFRB. Fungal cultures were maintained on potato dextrose agar (PDA) medium at 26±2°C until conidia formation, harvested, and adjusted to a concentration of $1\text{x}10^4$ conidia/mL using a hemocytometer. Bacterial and yeast strains were cultured on tryptone soy agar (TSA) medium, then suspended in sterile water to achieve an optical turbidity according to 0.5 in the McFarland scale. To obtain crude extracts, all microorganisms were cultured in tryptone soy broth (TSB) for 9 days at 28 ± 2 °C under constant agitation, and subsequently filtered using Whatman No. 4 paper to remove cellular biomass. In the experiments, plants were inoculated with 10 mL of cell suspension or crude extract, depending on the type of microorganism applied. For the consortia, equal volumes of each microorganism were combined at the concentrations previously described to obtain the final inoculum volume. Maize seeds of the hybrid INIFAP H391, cultivated in Tepatitlan de Morelos during the spring-summer cycle of 2023, served as the plant model.

Selection of a fungal-assisted seed germination method

Four maize seed germination methods were evaluated to identify the most effective technique for producing handleable seedlings for further analysis. The methods included: the between paper method (BP), germination in acrylic boxes (AB) (Calvillo-Aguilar *et al*., 2023), peat moss seedbeds (PM), and sand seedbeds (SN). Each method involved 10 seeds as experimental unit inoculated with *Aureobasidium* sp. VFRFB propagules as descibed (X.1) or a crude fungal extract (X.2), across four replicates arranged in a randomized complete block design. After 15 days of incubation at 26 ± 2 °C under a 14:10 h light:dark photoperiod and 6000 lux in a bioclimatic chamber, seedling handleability was assessed based on elongated, non-deformed growth resembling natural soil development, and ease of substrate removal. Additionally, plant length, fresh weight, and dry weight were assessed to determine the optimal method for the study. These characteristics facilitate improved manipulation and ensure accuracy in subsequent analyses.

Compatibility test among fungi, bacteria and yeast

Dual culture confrontations were established in triplicate between individual fungi and bacteria on PDA Petri plates to assess compatibility or incompatibility of interactions. Dual cultures were incubated at 26±2°C in darkness. After 7 days, subculturing was conducted from the confrontation line to assess the effects of one microorganism on another. An arbitrary evaluation scale, structured based on observational data, was developed to assess the interactions: (+1) indicated a bactericidal effect by the fungus or yeast; (0) indicated coexistence of fungi and bacteria or yeast on the medium without causing damage to each other, signifying compatibility; (-1) indicated a fungicidal effect exerted by the bacteria or yeast. Decimal values between 0 and +1 indicated a bacteriostatic gradient, while those between 0 and -1 indicated a fungistatic gradient. Combinations deemed compatible in the compatibility tests were used as consortia in subsequent experiments. In the case of *Trichoderma* sp. SVA, it exhibited a mild bacteriostatic effect against two of the bacterial strains comprising the 2021 consortium. Nevertheless, the treatment was included based on scientific interest, aiming to observe the effect of the metabolites generated during their interaction on the plant. Treatments appear in table 1.

Table 1. Treatments based on compatibility among Fungi and Bacteria

Treatment	Fungi	Bacteria
T1	Control	
T2	*Aureobasidium* sp. VFRB	-
T3		*Pantoea* sp. CanP2-19b
T4		*Clavispora* sp. Y35
T5		*Pseudomonas* sp. 37L
T6		*Pseudomonas* sp. IngP1.1-2b
T7		Consortium 2022
T8		Consortium E2
T9	*Trichoderma* sp. SVA	-
T10		*Stenotrophomonas* sp. LIMN
T11		*Clavispora* sp. Y35
T12		*Pseudomonas* sp. IngP1.1-2b
T13		Consortium 2021

Control: Non, inoculated plants. Consortium 2021 comprised LIMN, WFRFC, and LCGM. Consortium 2022 comprised CanP2-19b, 37L, and IngP1.1-2b. E2 Consortium comprised Y35 and CanP2-19b.

Effect of fungi alone and in consortium on maize seedlings performance

Following the selected germination method, 25 seeds were organized as an experimental unit per treatment and inoculated with fungal conidia suspensions (1X10^4 conidia/mL), either individually or combined with bacteria and yeast. Treatments were stablished according to treatments formulated in table 1; each treatment comprised four replicates arranged in randomized complete blocks. The experiment was conducted twice. In the control groups, sterile distilled water replaced the inoculum. The experiment was conducted in a bioclimatic chamber for 18 days at 26±2°C, with a relative humidity of 60%, and a photoperiod of 14:10 h light:dark at an intensity of 6000 Lux. Upon completion, plants were harvested, and various parameters including germination percentage, plumule length, root length, number of secondary roots, fresh weight, and dry weight of the plumule and root were recorded.

Plant growth-promoting traits in the fungi-yeast-bacteria consortium

Plant growth-promoting traits were assessed to explain the effectiveness of the tripartite consortium. Nitrogen fixation was determined in N-free-BB medium, with positive strains indicated by yellow (NH_4^+) or blue (NO_3^-) color change (Baldani *et al*., 2014). Phosphate and potassium solubilization were evaluated by yellowish halo formation in NBRIP-BPB and Aleksandrow's media, respectively (Mehta & Nautiyal, 2001; Parmar & Sindhu, 2019). Zinc mobilization was identified by clear zones in Zn-enriched medium (Kamran *et al*., 2017), and siderophore production by yellow-orange halos in CAS-Agar (Schwyn & Neilands, 1987). Salinity and water stress tolerance were determined by microbial biomass production in saline and low water activity media (Arminjon & Lefort, 2025; Liu *et al*., 2022). Indole production was assessed by culturing microorganisms on YPD agar (yeast peptone dextrose agar) supplemented with L-tryptophan. After 7 days of incubation, 10 mL of Salkowski reagent (35% HCl, 0.5 M $FeCl_3$) was added, and a reddish-pink coloration indicated a positive result (Gordon & Weber, 1951; Szkop *et al*., 2012). Media were inoculated with 1×10^4 conidia/mL for fungi, and 0.5 McFarland turbidity cell suspensions for yeast and bacteria. Responses were recorded as intense (+++), medium (++), low (+), or negative (-).

Data processing and statistical analysis

Compatibility data, handleable seedlings data for method selection and plant-growth promotion traits data were analyzed using a Chi-square test, followed by Fisher's F test to determine significance levels. Seedling length, fresh weight, and dry weight data were transformed using a base-10 logarithm. After confirming that statistical assumptions were met, the data were subjected to two-way ANOVA followed by Tukey's post hoc test. Graphs were generated using raw data. To assess the impact of fungi alone and in consortium with bacteria and yeast, the response ratio (RR) was calculated to evaluate the effect of inoculation on seed germination. The response ratio was determined using the formula RR = (Ln(Xa/Xb) * 100), where Xa represents the experimental data, and Xb denotes the mean of the control group (seeds without fungal or bacterial inoculation) (Luo *et al*., 2022). This data and crude data from root architecture and biomass accumulation went through a two-way ANOVA was performed, followed by a Tukey test for mean separation. The experiments were replicated at least twice, and data were analyzed collectively. All data processing and statistical analyses were conducted using R software version 4.3.1 (R Core Team, 2024) and RStudio version 2023.06.0 Build 421 (RStudio Team, 2024).

RESULTS

Selection of fungal-assisted germination method

Among the fungal-assisted germination methods, the between paper method (BP.1 and BP.2) produced the highest number of handleable seedlings, with greater seedling length, fresh weight, and dry weight, regardless of whether it was inoculated with propagules or crude extract. In contrast, the germination method in acrylic boxes (AB.1 and AB.2) resulted in deformed plants that were difficult to manipulate (Figure 1). Meanwhile, methods PM.1, PM.2, SN.1, and SN.2 produced seedlings with substrate residue that was difficult to remove. Additionally, in the PM method, mycelial growth was observed on the surface of the seedbeds. The seedlings exhibited clear visual differences, with slightly better development observed in those inoculated with conidial suspensions compared to those treated with cell-free crude extracts (Figure 2).

Compatibility among fungi, bacteria, and yeast, and treatments design

The *Aureobasidium* sp. VFRB strain demonstrated compatibility with the three bacterial strains comprising the 2022 Consortium, and the yeast included in the E2 Consortium. The *Trichoderma* sp. SVA strain exhibited a slight bacteriostatic effect against the bacteria of the 2021 Consortium but showed compatibility with independent strains, such as *Pseudomonas* sp. IngP1.1-2b, *Stenotrophomonas* sp. LIMN and the yeast *Clavispora* sp. Y35. In contrast, *Trichoderma* sp. LCCG, *Purpureocillium* sp. BOF, and *Cordycep*s sp. SVD exhibited compatibility with fewer than three bacterial strains. The yeast *Clavispora* sp. Y35 showed a gradient of bacteriostatic activity against independent bacteria as *Enterobacterium* sp. LCMG, *Pseudomonas* sp. IngP1.1-2b, and *Serratia* sp. 385. Meanwhile, the bacterial strains *Pseudomonas* sp. 37L, *Rhizobium* sp. WFRFC, and *Stenotrophomonas* sp. LIMN exerted a certain degree of antagonistic activity against the yeast (Figure 3). Based on these findings, 13 treatments were defined to use in the subsequent experimentation (Table 1).

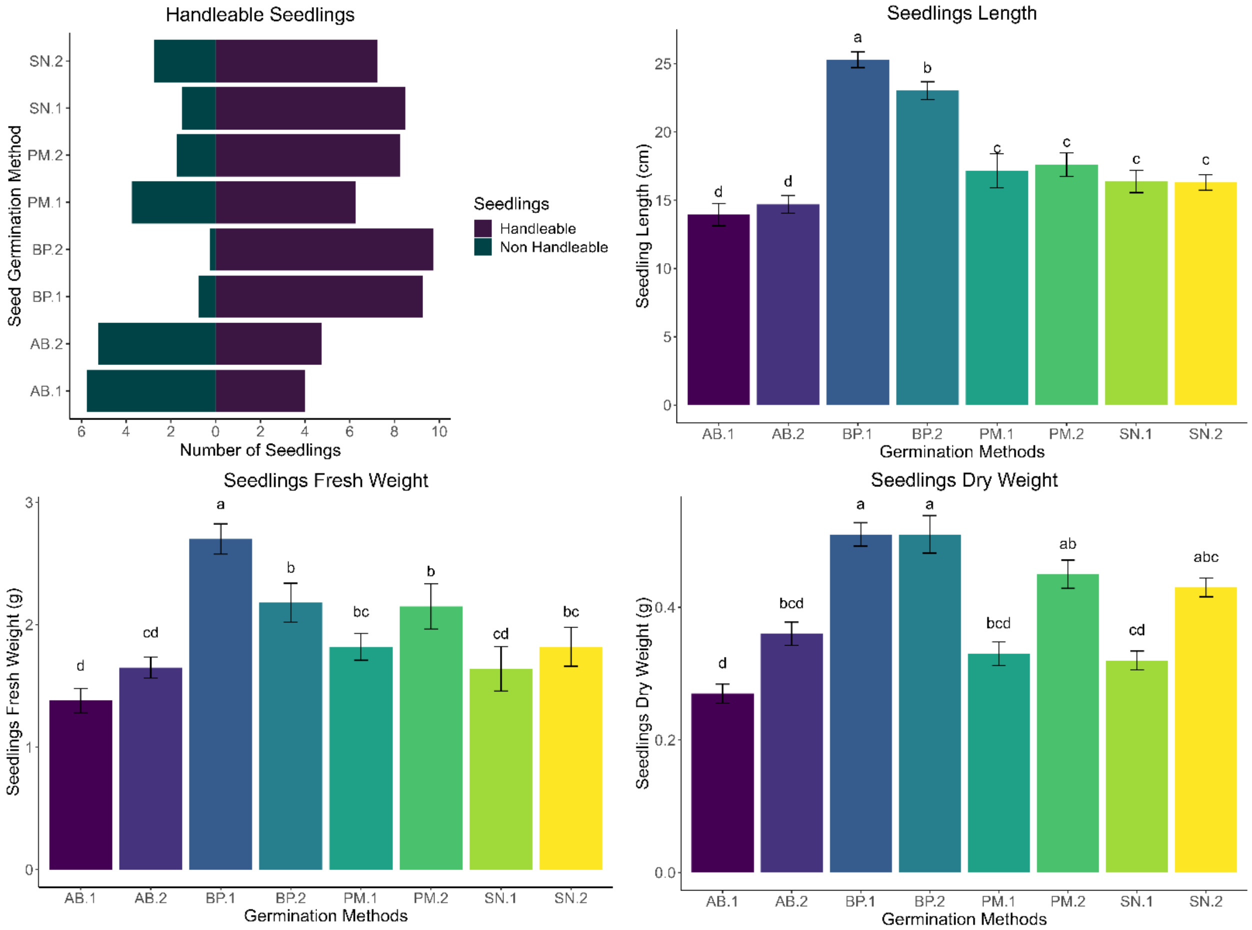

Figure 1. Performance of fungal-assisted germination methods using fungal inoculum and crude extract. Different letters indicate statistically significant differences (n=10, Chi-square, Fisher's test p ≤ 0.01 for viable seedlings; Tow-way ANOVA, Tukey p ≤ 0.01 for seedling length, fresh weight, and dry weight). AB: Acrylic box method; BP: Between paper method; PM: Peat moss seedbed method; SN: Sand seedbed method. X.1: inoculated with microbial cell suspension; X.2: amended with crude extract (cell-free).

Effect of fungi-bacteria-yeast inoculum on maize seed germination

The results indicate that the best response ratios on seed germination were obtained from the combination of *Aureobasidium* sp. VFRB with the E2 Consortium as well as with the independent yeast *Clavispora* sp. Y35, increasing germination rates up to to 6% and 5.5% respectively. In contrast *Trichoderma* sp. SVA reduced germination rates when were independently or inoculated in combination with *Pseudomonas* sp. IngP1.1-2b or *Stenotrophomonas* sp. LIMN, with decreases ranging from 1.3% to 12 % in respect to control plants, except when combined with the yeast Y35 (Figure 4).

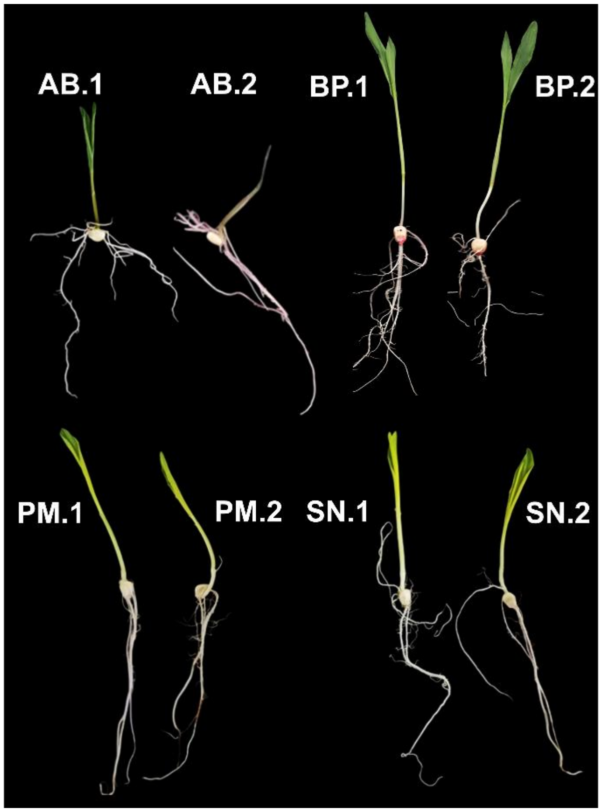

Figure 2. Maize seedlings obtained through germination assisted by the fungus *Aureobasidium* sp. VFRB. The images show representative seedlings selected from a consensus of 40 observations. AB: Acrylic box method; BP: Between paper method; PM: Peat moss seedbed method; SN: Sand seedbed method. X.1: inoculated with microbial cell suspension; X.2: amended with crude extract (cell-free).

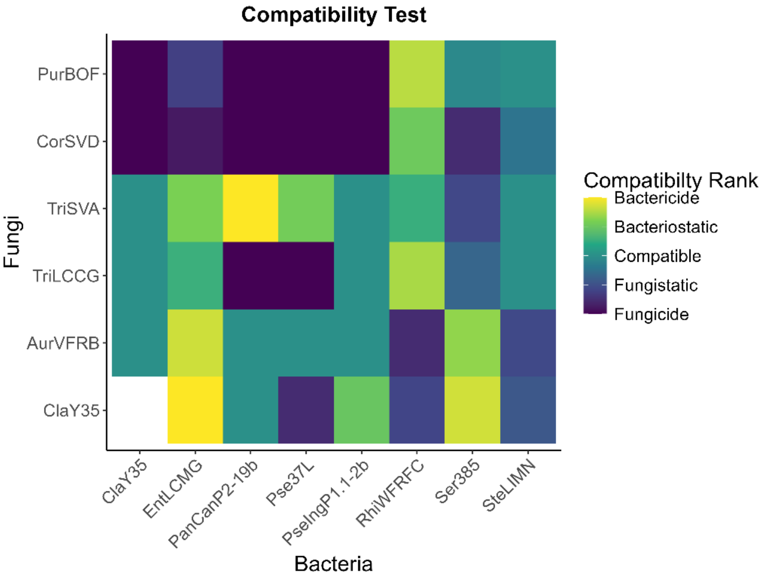

Figure 3. Heatmap showing the levels of compatibility in the interactions among fungi and bacteria isolated from La Frailesca, Chiapas. Data represent the mean of three replicates. (n=6, Chi-square, Fisher’s test p≤0.01).

Effect of fungal-bacterial-yeast Inoculum on maize seedling architecture

The plumule of the seedlings was primarily enhanced in treatments containing *Aureobasidium* sp. VFRB combined with *Pseudomonas* genus bacteria, such as *Pseudomonas* sp. 37L, *Pseudomonas* sp. EngP1.1-2b, and the 2022 Consortium, which includes both along with *Pantoea* sp. CanP2-19b. In the presence of *Pseudomonas* sp. 37L, the plumule increased by up to 30.26%. Conversely, *Trichoderma* sp. SVA, when applied alone, led to a reduction in plumule length compared to control plants. However, when combined with *Pseudomonas* sp. IngP1.1-2b, it facilitated growth by 24.5% over control plants (Table 2).

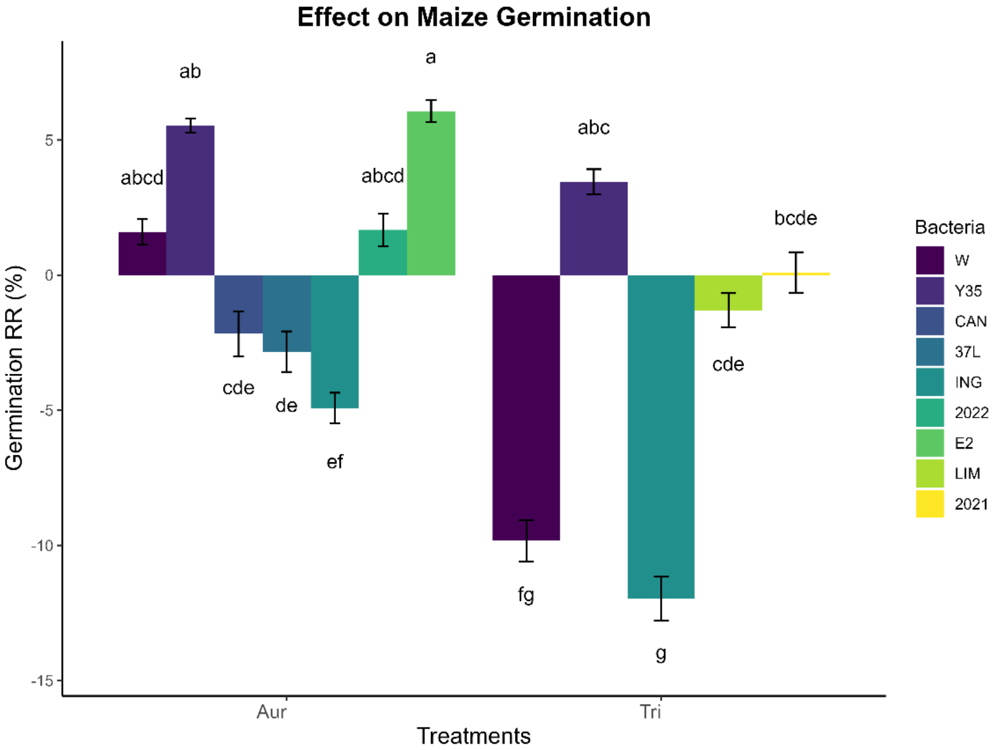

Figure 4. Response ratio of seed germination following inoculation with either fungi alone or in combination with bacteria and yeast. Bars represent the mean of four replicates (n = 25; Two-way ANOVA, Tukey p ≤ 0.01). W: Distilled water; Aur: *Aureobasidium* sp. VFRB; Tri: *Trichoderma* sp. SVA; CAN: *Pantoea* sp. CanP2-19b; Y35: *Clavispora* sp. Y35; 37L: *Pseudomonas* sp. 37L; ING: *Pseudomonas* sp. IngP1.1-2b; 2022: Consortium 2022 (CAN + 37L + ING); E2: Consortium E2 (CAN + Y35); LIM: *Stenotrophomonas* sp. LIMN; 2021: Consortium 2021 (LIMN + WFRFC + LCGM).

Biomass accumulation in maize seedlings in response to fungal-bacterial-yeast inoculation

Overall, treatments with positive effects resulted in a greater increase in dry weight compared to fresh weight. Notably, the combination of the fungus *Aureobasidium* sp. VFRB, the yeast *Clavispora* sp. Y35, and the bacterium *Pantoea* sp. CanP2-19b significantly enhanced biomass accumulation. Relative to control plants, root dry weight increased by 44.63% and plumule dry weight by 36.23%, while root fresh weight increased by 32.92% and plumule fresh weight by 20.76%. In contrast, treatments involving the fungus *Trichoderma* sp. SVA led to a substantial reduction in seedling fresh weight, with decreases of up to 24.69% in the plumule and 32.31% in the roots. However, when *Trichoderma* sp. SVA was combined with *Clavispora* sp. Y35 or *Stenotrophomonas* sp. LIMN, root dry weight increased significantly by 32.69% and 20.99%, respectively, while plumule dry weight increased by 29.51% and 9.01%, compared to control plants. (Figure 5).

Table 2. Seedling architecture response of maize seedlings to fungal-bacterial-yest inoculation

Fungi	Bacteria / Yeast	Plumule Length (cm)			Root Length (cm)			Number of Roots		
	Control	88.13	d	-	128.93	e	-	4.7	h	-
AurVFRB	-	97.67	c	+10.3%	141.63	bcd	+9.4%	6.82	e	+37%
	ClaY35	96.73	c	+9.3%	153.47	a	+17.4%	9.54	a	+71%
	PanCAN	92.44	cd	+4.8%	143.88	b	+11%	8.66	b	+61%
	Pse37L	119.27	a	+30.3%	135.47	cde	+5%	5.57	g	+17%
	PseING	107.5	b	+19.9%	134.31	e	+4.1%	6	f	+27%
	Con2022	111.87	b	+23.9%	135.33	cde	+4.9%	7.66	d	+49%
	ConE2	98.73	c	+11.36%	160.8	a	+22.1%	9.94	a	+75%
TriSVA	-	73.44	e	-18.2%	113.94	f	-12.4%	4.47	h	+0.8%
	ClaY35	92.94	cd	+5.3%	142.63	bc	+10.1%	6.91	e	+39%
	SteLIMN	89.19	d	+1.2%	135.13	cde	+4.7%	6.15	f	+27%
	PseING	112.6	ab	+24.5%	130.33	e	+1.1%	6.93	e	+39%
	Con2021	78.2	e	-12%	139.93	bcd	+8.2%	8.19	c	+56%

For each variable, values followed by the same letter are not significantly different (n=25 Two-way ANOVA, Tukey's test $p \leq 0.01$). The third column shows the percentage response ratio of plants to each variable in the presence of microorganisms, relative to the control plants. The experiment was conducted twice, and data from both trials were analyzed collectively. AurVFRB: *Aureobasidium* sp. VFRB; TriSVA: *Trichoderma* sp. SVA; Control: Non inoculated seedlings; ClaY35: *Clavispora* sp. Y35; PanCAN: *Pantoea* sp. CanP2-19b; Pse37L: *Pseudomonas* sp. 37L; PseING: *Pseudomonas* sp. IngP1.1-2b; Con2022: Consortium 2022 (PanCAN+Pse37L+PseING); ConE2: Consortium E2 (PanCAN+ClaY35); SteLIM: *Stenotrophomonas* sp. LIMN; Con2021: Consortium 2021 (SteLIMN+RhiWFRFC+EntLCGM)

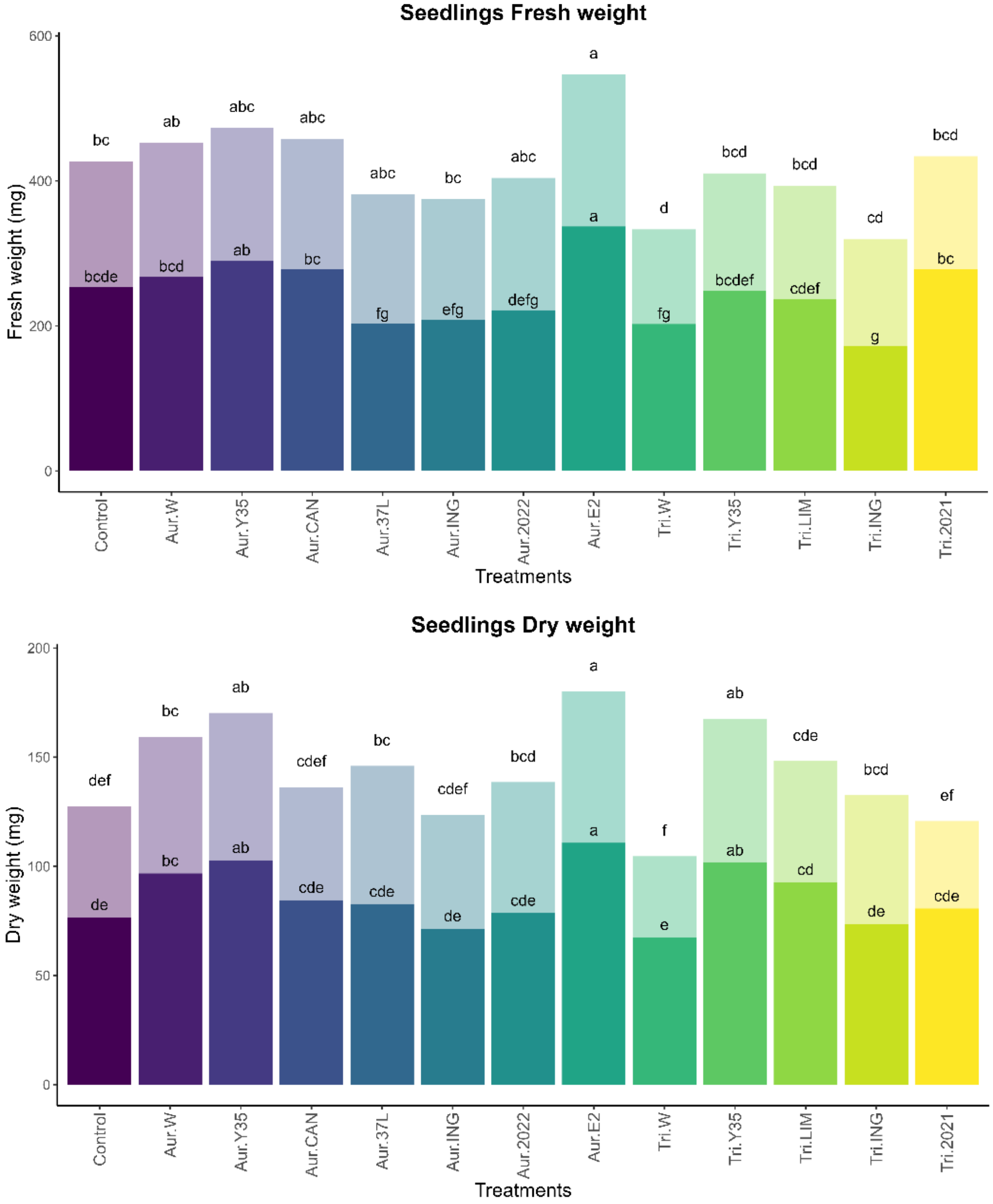

Figure 5. Seedling biomass accumulations expressed as fresh and dry weight in response to inoculation with either fungi alone or in combination with bacteria and yeast. Bright bars represent root fresh or dry weight; dim bars represent plumule fresh or dry weight. Bars represent the mean of four replicates (n=25; Two-Way ANOVA, Tukey $p \leq 0.05$). W: Distilled water; Aur: *Aureobasidium* sp. VFRB; Tri: *Trichoderma* sp. SVA; CAN: *Pantoea* sp. CanP2-19b; Y35: *Clavispora* sp. Y35; 37L: *Pseudomonas* sp. 37L; ING: *Pseudomonas* sp. IngP1.1-2b; 2022: Consortium 2022 (CAN+37L+ING); E2: Consortium E2 (CAN+Y35); LIM: *Stenotrophomonas* sp. LIMN; 2021: Consortium 2021 (LIMN+WFRFC+LCGM).

Plant-growth promotion traits in the fungi-yeast-bacteria consortium

The three microorganisms comprising the consortium exhibited differential levels and intensities of expression of plant growth-promoting traits. *Aureobasidium* sp. VFRB demonstrated the highest number of beneficial traits, with siderophore production being its most prominent feature, while the remaining traits were expressed at medium or low intensity. *Clavispora* sp. Y35 showed a high capacity for indole production and was the only strain capable of producing NH_4^+. In contrast, *Pantoea* sp. CanP2-19b exhibited the greatest capacity for phosphorus solubilization (Figure 6).

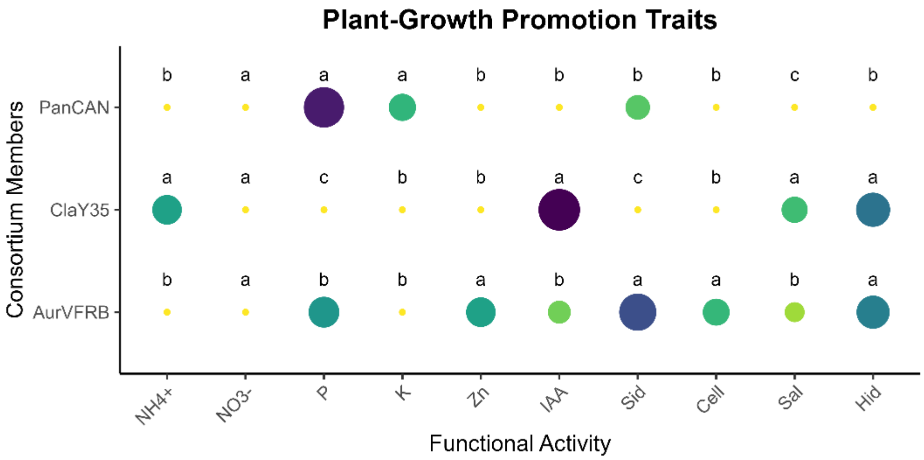

Figure 6. Enzymatic profile and intensity of plant growth-promoting traits expressed by the members of the tripartite consortium. Bubbles size represent the intensity of expression of the plat-growth promotion traits. Bubbles followed by different letters represent statistical differences (n=6, Chi-square, Fisher's test p≤0.05).

DISCUSSION

Microorganism-assisted agriculture is gaining relevance, particularly in staple crops like maize due its importance to food sovereignty in diverse countries (Guzmán *et al*., 2025; Zelaya-Molina *et al*., 2024). Bioprospecting enables the development of effective formulations using native microbial agents and helps predict their colonization efficiency and impact on plant phenotype (Sethi, 2024; dos Reis *et al*., 2024; Agbowuro *et al*., 2021). To support this, consistent evaluation methods are essential for distinguishing treatment effects and guiding informed decision-making on the selection of individual plant growth-promoting agents, microbial consortia, or treatment strategies (Calvillo-Aguilar *et al*., 2023).

Most standardized methods focus on evaluating germination in response to agrochemicals, natural extracts, or PGPRs *in vitro,* aiming to directly assess the interaction between microorganisms and plants, excluding other intervening factors (Calvillo-Aguilar *et al*., 2023; Mandić *et al*., 2023). This enables observation of whether the microorganism can enhance the plant's inherent biological fitness (Khaeim *et al*., 2022; Xue *et al*., 2021). This study evaluated four different germination methods (acrylic boxes, between paper, peatmoss beds, and sand beds) to identify the most suitable for producing handleable maize seedlings, thereby enabling the collection of robust data to inform the selection of optimal microbial consortia. Seedlings inoculated with conidial suspensions showed superior development compared to those treated with crude extracts, likely due to the direct interaction and colonization by PGPF. Such colonization supports adaptive responses to environmental conditions and enhances the production of beneficial metabolites that improve plant physiology (Hossain *et al*., 2020; Chen *et al*., 2021; Wang *et al*., 2024). The between-paper method produced handleably seedlings with easily removable mycelium; although root tips were prone to breakage, this method outperformed the others across all evaluated criteria. In contrast, peatmoss and sand beds resulted in substrate adhering to the roots due to mycelial growth, complicating seedling handling. Additionally, sporulation observed on these substrates may increase the risk of contaminating the growth chamber. Acrylic boxes produced poorly developed and irregularly shaped seedlings (Figure 2). These results indicate that the method by Calvillo-Aguilar *et al*. (2023) is adaptable for evaluating not only bacteria and yeasts but also other microorganisms like fungi, enabling straightforward assessment of crop physiological responses to plant growth-promoting agents.

Designing microbial consortia for agricultural purposes necessitates thorough research. This involves understanding the interactions among the microorganisms constituting the consortium, as their persistence, viability upon application, colonization ability, and physiological attributes crucially depend on these interactions (Maciag *et al*., 2023; Prigigallo *et al*., 2023). Dual *in vitro* cultures provide an efficient evaluation method to assess whether these microorganisms can coexist in the same environment, thus playing a crucial role in synthetic microbial consortia formulation (Tabacchioni *et al*., 2021). While dual culture compatibility assays simplify the complex environmental factors influencing microbial establishment, they effectively identify microorganisms exhibiting fundamental incompatibility. In this study, a significant fungistatic activity of *Clavispora* sp. Y35, *Pseudomonas* sp. IngP1.1-2b, *Pseudomonas* sp. 37L, and *Pantoea* sp. CanP2-19b was observed against the fungi *Purpureocillium* sp. BOF and *Cordyceps* sp. SVD. This preliminary screening prevented the allocation of resources to assess combinations where initial spatial coexistence is not probable.

Based on these principles, consortia comprising fungi, bacteria, and yeasts were established. However, in the case of T13, the bacteriostatic action of *Trichoderma* sp. SVA against *Rhizobium* sp. WFRFC and *Enterobacterium* sp. LCMG (included in the 2021 consortium) was overlooked. The decision to include this treatment was driven by scientific interest in exploring the effects of a partially incompatible interaction and observing its impact when the microorganisms came into contact with the plant. This decision was supported by the presence of various metabolites involved in this bacteriostatic interaction, which could potentially benefit the plant (Tabacchioni *et al*., 2021). Although *Trichoderma* sp. SVA is a PGPF, it limited maize seedling development when applied alone, underscoring the need for germination tests, as some *Trichoderma* strains may be crop-specific or even detrimental for some other crops (Bailey *et al*., 2006). Interestingly, in combination with Consortium 2021 (despite its bacteriostatic effects) the bacteria appeared to stabilize the system, improving root development and restoring germination and biomass levels comparable to the control.

Numerous studies have demonstrated that early association between microorganisms and seeds enhances germination rates, plant architecture, and other agronomical traits (Calvillo-Aguilar *et al*., 2023; Cardarelli *et al*., 2022). Mechanisms such as the production of growth regulators, secretion of volatiles, and enzymatic activity are employed by microorganisms to stimulate early crop performance (Metwally *et al.*, 2022). While these capabilities are desirable in plant growth-promoting microorganisms (PGPM), it is crucial to conduct germination tests to evaluate the interaction between the crop and the PGPM, as they could potentially display adverse effects (Luziatelli *et al.*, 2023). The outcome of the interaction (whether beneficial or detrimental to the crop) depends on the speed, intensity, and duration of the mechanisms expressed by both the plant growth-promoting microorganism (PGPM) and the crop. A balanced activity-response dynamic between the PGPM and the plant is essential for establishing a positive symbiotic association (Chávez-Díaz & Zavaleta-Mejía, 2019).

Plant architecture is shaped by both genetic factors and environmental influences. During early crop development, the presence of plant growth-promoting microorganisms (PGPM) directly impacts a complex regulatory network, initiating differential gene expression that alters processes such as phosphorus metabolism, sugar formation, growth regulation, and lipid metabolism (Chen *et al*., 2021). In our study, all consortia significantly influenced the architecture of maize seedlings. Specifically, the fungus-yeast-bacteria association (*Aureobasidium* sp. VFRV, *Clavispora* sp. Y35, *Pantoea* sp. CanP2-19b) resulted in plants with extended, robust root systems, discreet plumules and favorable fresh-dry weight ratios (Table 2, Figure 5). Similar results were reported in a study involving the inoculation of the native bacterial consortium from the K-25 maize variety, composed of *Lysinibacillus* sp. ZM1 and *Paenibacillus dendritiformis* ZM2. This consortium enhanced germination rates and improved seedling architecture, including both the root system and plumule. Conversely, seeds treated with antibiotics to eliminate native bacteria exhibited significant reductions in these parameters, underscoring the crucial role of native microorganisms in the early development of maize (Pal *et al.*, 2022). Other report revealed that inoculation with a consortium composed of the fungi

Serendipita indica and *Rhizophagus intraradices*, along with the bacterium *Azotobacter chroococcum*, enabled maize plants to maintain optimal development under water stress without major alterations to leaf or root structure. This microbial combination promoted vegetative growth, enhanced biomass accumulation, supported osmolyte balance, and sustained the regulation of antioxidant enzyme activity (Tyagi *et al*., 2023). To our knowledge, most studies on microbial consortia in maize have primarily focused on combinations of plant growth-promoting bacteria and fungi. However, a study in strawberry demonstrated that a consortium composed of the fungus *Claroideoglomus claroideum*, the yeast *Naganishia albida*, and the bacterium *Burkholderia caledonica* significantly influenced crop physiology. Inoculated plants exhibited increased biomass accumulation, relative water content, and chlorophyll levels, which correlated with enhanced antioxidant system activity and higher fruit yields, even under water stress conditions (Pérez-Moncada *et al*., 2024).

The influence of microorganisms on the architecture of the rhizosphere and phyllosphere is mediated by various metabolites involved in the complex communication network between the microorganism and the plant. These metabolites also participate in biogeochemical processes and shape the population dynamics associated with the crop (Wu *et al*., 2023). For this reason, traits associated with plant growth promotion are highly desirable in microorganisms comprising a consortium. In this study, *Clavispora* sp. Y35 exhibited a strong capacity for indole production, *Pantoea* sp. CanP2-19b demonstrated notable phosphorus-solubilizing ability, and *Aureobasidium* sp. VFRB showed high potential for siderophore production among other traits (Figure 6). Together, these three traits are critical for optimizing maize performance by enhancing soil nutrient availability and contributing to improved crop health and yield (Marzban *et al*., 2016; de Oliveira-Paiva *et al*., 2024; Luo *et al*., 2024). The yeast *Clavispora* sp. has been reported as part of the native microbiota of Mexican maize landraces and as an excellent producer of indoles (Ramos-Garza *et al*., 2023). Our research group has contributed to documenting the plant growth-promoting capacity of this yeast in maize (Resendiz-Venado *et al*., 2022; Calvillo-Aguilar *et al*., 2023), and the present study adds to the growing body of evidence supporting *Clavispora* sp. as a plant growth-promoting agent. The genus *Pantoea* is widely distributed and commonly associated with cereal crops. Although some species are known to be plant pathogens, others have demonstrated significant potential as plant growth and health promoters, owing to their ability to fix nitrogen, solubilize phosphorus, synthesize siderophores, and produce indoles, which can induce beneficial physiological changes in plants (Lv *et al*., 2022). The fungus genera *Aureobasidium* is considered controversial due to its ability to present a yeast-like morphology under certain conditions (Slepecky & Starmer, 2009). However, the VFRV strain has consistently exhibited a filamentous growth form since its isolation. *Aureobasidium* has been primarily utilized as a biological control agent, attributed to its production of a wide range of lytic enzymes, siderophores, and volatile compounds (Di Francesco *et al*., 2023). This study highlights several traits associated with plant growth promotion; however, it does not exclude the possibility that additional, untested mechanisms may contribute to the observed effects on maize seedlings. Collectively, the fungus *Aureobasidium* sp. VFRFB, the yeast *Clavispora* sp. Y35, and the bacterium *Pantoea* sp. CanP2-19b were bioprospected as a consortium with strong potential to support maize development during its early growth stages.

CONCLUSIONS

The synthetic consortium composed of *Aureobasidium* sp. VFRB, *Clavispora* sp. Y35, and *Pantoea* sp. CanP2-19b (fungi-yeast-bacteria consortium) demonstrated high agrobiotechnological potential, significantly enhancing germination rates, seedling architecture, and biomass accumulation. Similarly, the fungus-yeast (*Aurobasidium* sp. VFRFB and *Clavispora* sp. Y35) combination yielded favorable results. In contrast, *Trichoderma* sp. SVA, when applied alone, negatively affected seedling development, underscoring the importance of a well-structured bioprospecting protocol that includes germination test to assess compatibility between plant growth-promoting agents and crop seeds.

The paper-assisted germination method proved to be a valuable tool for microbial bioprospecting, enabling the development of maize seedlings with architecture comparable to field-grown plants. Its advantages include the generation of easily manageable seedlings, adaptability for evaluating bacteria, yeasts, and fungi, and the clear differentiation between plant genotype effects and microbial interactions.

Overall, the study highlights the relevance of germination tests in PGPM bioprospecting and supports the use of microbial consortia as a sustainable strategy to enhance crop productivity and soil health.

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