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Biochemical characterization of individual and combined plant growth-promoting microorganisms[1]

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ABSTRACT

The increasing demand for using microorganisms in agriculture to improve food production requires constantly assessing microbial diversity. This study aimed to investigate the biochemical properties of individual and combined multifunctional microorganisms, as well as to identify potential applications in biotechnology or agriculture. The experiment comprised 29 treatments, with 7 single and 21 combined microorganisms: M01 (*Serratia marcescens*), M02 (*Bacillus toyonensis*), M03 (*Phanerochaete australis*), M04 (*Trichoderma koningiopsis*), M05 (*Azospirillum brasilense*), M06 (*Azospirillum* sp.), M07 (*Bacillus* sp.), M08 to M28 (combination among these microorganisms) and M29 (control - no microorganisms). All the single and combined treatments assimilated nitrogen, produced siderophores and indoleacetic acid and solubilized phosphate. Only the treatments M04, M13 and M26 produced HCN. Additionally, all treatments, except for M03, produced biofilm. Only M03, M07, M09, M10, M12 and M13 solubilized potassium.

KEYWORDS: Bacteria isolates, multifunctional microorganisms, nitrogen assimilation.

RESUMO

Caracterização bioquímica de micro-organismos promotores de crescimento de plantas individuais e combinados

A crescente demanda pelo uso de micro-organismos na agricultura para melhorar a produção de alimentos exige uma avaliação constante da diversidade microbiana. Objetivou-se investigar as propriedades bioquímicas de micro-organismos multifuncionais individuais e combinados, bem como identificar aplicações potenciais em biotecnologia ou agricultura. O experimento compreendeu 29 tratamentos, com 7 micro-organismos isolados e 21 combinações: M01 (*Serratia marcescens*), M02 (*Bacillus toyonensis*), M03 (*Phanerochaete australis*), M04 (*Trichoderma koningiopsis*), M05 (*Azospirillum brasilense*), M06 (*Azospirillum* sp.), M07 (*Bacillus* sp.), M08 a M28 (combinação entre esses micro-organismos) e M29 (controle - sem micro-organismos). Todos os tratamentos individuais e combinados assimilaram nitrogênio, produziram sideróforos e ácido indolacético e solubilizaram fosfato. Apenas os tratamentos M04, M13 e M26 produziram HCN. Adicionalmente, todos os tratamentos, exceto M03, produziram biofilme. Somente M03, M07, M09, M10, M12 e M13 solubilizaram potássio.

PALAVRAS-CHAVE: Isolados de bactérias, micro-organismos multifuncionais, assimilação de nitrogênio.

INTRODUCTION

Multifunctional microorganisms are also called plant growth-promoting microorganisms. They are an effective and environmentally sustainable alternative for replacing chemical fertilizers and pesticides (Khatri & Tyagi 2015, Cherif-Silini et al. 2021).

Over the last few years, due to the rising cost of fertilizers and solubilization issues, researchers have increasingly focused on microbial bio-inoculants, such as bio-fertilizers and bio-pesticides, for sustainable agriculture (Vurukonda et al. 2018). Plant growth-promoting microorganisms interact with plants and promote their growth due to the production of phytohormones and exopolysaccharides and the availability of nutrients, such as phosphorus and iron, in the soil solution (Isawa et al. 2010), in an eco-friendly and sustainable way (Gomes et al. 2014).

Plant growth-promoting microorganisms solubilize inorganic P and insoluble K by producing

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mineral compounds and organic acids, which reduce the soil pH to release P (Ahmad et al. 2020) and K (Figueiredo et al. 2016). Furthermore, they can solubilize nutrients by secreting extracellular enzymes into the soil (Chen et al. 2006). Moreover, they are used as biological control agents against phytopathogens. They can synthesize a variety of antibiotic and antifungal compounds, including lytic enzymes, siderophores and hydrogen cyanide (Srivastava 2017).

Multifunctional microorganisms can be a viable alternative for sustainable agriculture with decreased synthetic inputs (Nascente et al. 2017), and, since studies on the biochemical characterization of single microorganisms are fairly known, this study aimed to investigate the biochemical properties of individual and combined microorganisms, to identify potential applications of their multi-functionalities in biotechnology or agriculture.

MATERIAL AND METHODS

The tests were conducted at the Embrapa Arroz e Feijão (Santo Antônio de Goiás, Goiás state, Brazil), in 2022, where the multifunctional microorganisms used are deposited in the microorganisms collection. The experiment comprised 29 treatments, with single or combined microorganisms: M01 (*Serratia marcescens*), M02 (*Bacillus toyonensis*), M03 (*Phanerochaete australis*/fungi), M04 (*Trichoderma koningiopsis*/fungi), M05 (*Azospirillum brasilense*), M06 (*Azospirillum* sp.), M07 (*Bacillus* sp.), M08 to M28 (combination among these microorganisms), and M29 (control - no microorganisms).

Each bacteria isolate was previously grown in Petri dishes containing a solid medium (nutrient agar) for cell suspension preparation. The suspensions were prepared by transferring bacteria cells to Erlenmeyer flasks containing liquid medium 523 (Kado & Heskett 1970). Then, the flasks were transferred to a shaker for incubation, for 24 hours, at 28 ºC. The concentration was adjusted using a spectrophotometer to A540 = 0.5, corresponding to 1×10^8 colony-forming units (CFU) per mL. The fungi isolates (*Trichoderma koningiopsis* and *Phanerochaete australis*) were grown in a Petri dish containing potato-dextrose-agar (PDA) and incubated for five days, as described by França et al. (2015). For the combined microorganisms' treatments, the fungi isolates were grown separately and mixed just before each test. The same protocols were applied for either fungi or bacterial isolates, as it follows:

- Indoleacetic acid (IAA) production: 5 µL drops of bacterial suspension of each treatment (single or combined), or a 5-mm disc containing fungi mycelium (for fungi isolates), were placed in Erlenmeyer flasks (150 mL capacity). These flasks contained 50 mL of PD medium [potato (200 g L^{-1}) and dextrose (20 g L^{-1})], supplemented with L-tryptophan (100 mg L^{-1}) or in the absence of L-tryptophan (control). They remained for 8 days of growth in a rotary shaker at 150 rpm and 26 ± 2 ºC. Every two days, 1 mL of culture medium containing the microorganism treatment was transferred to the spectrophotometer for IAA detection. For treatments containing fungi, mycelium was separated by centrifugation at 12,000 rpm, for 15 min. The indoleacetic acid detection was quantified in a spectrophotometer at 540 nm. The concentrations, in µg mL^{-1}, were calculated from a standard curve with known concentrations of the synthetic form of the hormone (0-100 µg mL^{-1}) and used to calculate the IAA concentration in the samples (Oliveira et al. 2009). The evaluation was conducted in triplicate;

- Phosphate solubilization: 5 drops of bacterial suspension, or a 5-mm disc containing fungi mycelium (for fungi isolates), of each isolate or combined microorganism (put together at the beginning of the test) were placed in Petri dishes containing 30 mL of trypticase soy agar medium [TSA; (1/10 - w/v)]. This medium received $CaHPO_4$ and the pH was adjusted to 7.0. The experiment was conducted in triplicate. The Petri dishes were incubated at 28-30 ºC, until the control achieved full growth (dishes containing each isolate without adding the phosphorus source). The solubilization potential was determined by the formation of a clear halo around the colony in the culture medium (Cattelan 1999);

- Hydrogen cyanide (HCN) production: 1/10 of solid LB medium was supplemented with 4.4 g L^{-1} of glycine and 0.081 g L^{-1} of $FeCl_3.6H_2O$, stimulators of hydrogen cyanide production. The isolate or combined microorganism (put together at the beginning of the test) was grown for 7 days at 28 ºC on a separate dish. The dish lid was covered with filter paper that had been soaked in a mixture of 5 % (w:v) picric acid and 2 % (w:v) $NaCO_3$. The hydrogen cyanide production was indicated by the

filter paper's color change from yellow to brown (Felestrino et al. 2018);

- Potassium solubilization: the medium contained 0.05 g L^{-1} of yeast extract, 1.0 g L^{-1} of glucose, 0.5 g L^{-1} of KNO_3, 0.05 g L^{-1} of $(NH_4)_2.SO_4$, 0.02 g L^{-1} of KCl, 0.01 g L^{-1} of $MgSO_4$, 0.00001 g L^{-1} of $FeSO_4$, 0.0001 g L^{-1} of $MnSO_4$, 15 g L^{-1} of agar and 0.0026 g L^{-1} of bromocresol green. The medium used was Pikovskaya, with modifications. Agar was added into the medium after the pH was adjusted to 7. The evaluation of each isolate or combined microorganism (put together at the beginning of the test) was carried out after 5 days of incubation of the 29 treatments, in triplicate, in the medium. The acidification of the medium showed a translucent to yellowish halo, indicating the solubilization of the nutrient (Fernandes et al. 2020);

- Biofilm production: each isolate or combined microorganism (put together at the beginning of the test) was cultured on Congo red agar (CRA), modified according to Freeman et al. (1989) (nutrient agar: 28 g; sucrose: 50 g; Congo red dye: 0.8 g; deionized water: 1 L). The dishes were incubated for 48 hours, at 28 ºC. Biofilm-producing strains comprised those that produced rough and black colonies. Smooth and red colonies were thought not to produce biofilms when the adjacent medium's color changed to black (Freeman et al. 1989);

- Siderophores production: each isolate or combined microorganism (put together at the beginning of the test) was cultivated for 7 days in King B medium (King et al. 1954) at 28 ºC and under constant agitation of 150 rpm. The suspensions were centrifuged at 10,000 rpm for 5 min. Then, 150 µL of the supernatant were transferred and homogenized with 150 µL of chromium azurol S solution (CAS) in triplicate ELISA microplate wells. Finally, it was put in the dark for 30 min. The Gen5 software determined the absorbance in a spectrophotometer at a 630 nm wavelength (Alexander & Zuberer 1991);

- Nitrogen assimilation: according to the method described by Estrada de Los Santos et al. (2001), the medium used for detecting the nitrogen-fixing capability of the treatments comprised 5 g of mannitol, 5 g of sucrose, 0.4 g L^{-1} of K_2HPO_4, 0.4 g L^{-1} of KH_2PO_4, 0.2 g L^{-1} of $MgSO_4.7H_2O$, 0.02 g L^{-1} of $CaCl_2$, 0.002 g L^{-1} of $NaMoO_4.H_2O$, 0.01 g L^{-1} of $FeCl_3$, 0.075 g L^{-1} of bromothymol and 2.3 g L^{-1} of agar. The pH of the medium was adjusted to 5.7. It was autoclaved at 120 ºC, for 15 min. Each isolate or combined microorganism (put together at the beginning of the test) was pipetted into a tube after the medium was put into 5-mL tubes. After 7 days, if there was discoloration in the medium, it would be due to the bacterial interaction;

- Zinc solubilization: the medium used comprised 10 g L^{-1} of glucose, 1 g L^{-1} of $(NH_4)_2SO_4$, 0.2 g L^{-1} of KCl, 0.1 g L^{-1} of K_2PO_4, 0.2 g L^{-1} of $MgSO_4.7H_2O$, 1 g L^{-1} of ZnO and 15 g L^{-1} of agar adjusted to pH 6.0 and incubated at 30 ºC. Each isolate or combined microorganism (put together at the beginning of the test) was cultured in the medium for 7 days. The zinc solubilization evaluation included measuring the diameter of the translucent halo around the colonies (Berraquero et al. 1976);

- Microorganism compatibility test: conducted to identify one microorganism inhibiting the development of the other. Therefore, each microorganism's combination was placed in a Petri dish containing nutrient agar (for bacteria isolates) and potato-dextrose-agar (PDA) (for fungi isolates) (Harshita et al. 2018).

When considering the compatibility test data, siderophores and IAA, an analysis of variance was performed, and the means grouped using the Scott-Knott test ($\alpha \leq 0.05$). However, for the compatibility test, the T-test was applied. In addition, the SAS statistical package was used (SAS 1999).

RESULTS AND DISCUSSION

All the treatment combinations were compatible. However, they differed in their compatibility diameter (Table 1). The application of compatible mixtures of fungal and bacterial biocontrol agents with several mechanisms of pathogen suppression and growth promotion is a reliable and potential form of disease suppression (Mishra et al. 2013). Harshita et al. (2018) reported compatibility between *Pseudomonas fluorescens* and *Bacillus subtilis*. Regarding compatibility among components of a combination, more information concerning protocols that can detect the effect of each component is necessary. Thus, investigations are demanded to improve the knowledge regarding the combination of microorganisms, whether bacteria with bacteria or bacteria with fungi. Mishra et al. (2013) reported that *Pseudomonas*, in general, suppressed the growth of *Trichoderma* under *in vitro* conditions. However, in this study, among all the microorganisms,

Table 1. Compatibility test among multifunctional microorganisms grown in pairs.

Treatment tags	Treatment composition	Diameter (mm)	
		First microorganism	Second microorganism
M08	BRM 32114 + BRM 32110	12.93 a	13.33 a
M09	BRM 32114 + AbV5	27.38 a	15.73 b
M10	BRM 32114 + BRM 53736	12.76 a	13.89 a
M11	BRM 32110 + AbV5	10.61 a	10.88 a
M12	BRM 32110 + BRM 53736	56.80 a	58.60 a
M13	AbV5 + BRM 53736	56.86 a	58.68 a
M14	BRM 63574 + BRM 63573	7.44 a	7.85 a
M15	BRM 63574 + AbV5	13.19 a	12.33 a
M16	BRM 63573 + AbV5	8.09 a	8.52 a
M17	BRM 63574 + BRM 32114	15.24 a	12.01 a
M18	BRM 63573 + BRM 32114	10.85 a	6.47 b
M19	BRM 63574 + BRM 32110	18.01 a	14.40 b
M20	BRM 63573 + BRM 32110	7.30 a	7.32 a
M21	BRM 63574 + BRM 53736	56.65 a	56.83 a
M22	BRM 63573 + BRM 53736	6.30 a	5.95 a
M23	BRM 62389 + BRM 63573	7.31 a	7.20 a
M24	BRM 62389 + BRM 63574	13.50 a	12.40 a
M25	BRM 62389 + AbV5	26.06 a	28.12 a
M26	BRM 62389 + BRM 53736	51.75 a	56.75 a
M27	BRM 62389 + BRM 32114	15.80 a	13.07 a
M28	BRM 62389 + BRM 32110	13.98 a	9.94 b

* Means followed by the same letter in the row do not differ by the T test ($p \leq 0.05$).

T. koningiopsis was the most compatible with other microorganisms (Table 2), what is evidenced by the colony size in the treatments M12, M13 and M21, combinations involving *T. koningiopsis* and bacteria isolates.

All the tested treatments assimilated nitrogen (Table 2). Nitrogen fertilizer is a major input for the majority of crops. However, only 30-40 % of the applied N are used by the crop due to losses through volatilization, denitrification, leaching and runoff (Kumar et al. 2000). According to Wang et al. (2012), diazotrophic bacteria, such as those from the *Azospirillum* sp. and *Bacillus* sp. genera, promote nitrogen assimilation. Furthermore, other bacteria can assimilate nitrogen, as shown in this study. The biological nitrogen assimilation method can decrease the use of chemical nitrogen fertilizer, prevent the depletion of soil organic matter and reduce environmental pollution to a considerable extent (Azarpour et al. 2011).

All the treatments solubilized phosphorus (Table 2). According to Peix et al. (2001), *Azospirillium* sp. and *Bacillus* sp. play a role in improving the phosphorus-solubilizing capability. According to Backer et al. (2018), phosphorus-solubilizing microorganisms can help plants to access non-accessible phosphorus storage by releasing phosphorus from its recalcitrant forms. Khan et al. (2020) stated that phosphorus is the second most important macronutrient for plant development and growth. Moreover, *B. subtilis* produces enzymes that change nutrients in the soil to make them more accessible to plants, including the key minerals nitrogen and phosphorus (Hashem et al. 2019).

Potassium solubilization occurred only in M03, M07, M09, M10, M12 and M13 (Table 2). Potassium solubilizing plant growth-promoting microorganisms, such as *Acidothiobacillus ferrooxidans*, *B. edaphicus*, *B. mucilaginosus*, *Burkholderia*, *Paenibacillus* sp. and *Pseudomonas*, has already been reported (Liu et al. 2012). Fernandes et al. (2020) stated that the highest accumulation of K in the root system was obtained with plants treated with *Azospirillum* sp. + pool of *T. asperellum*, which differed significantly from the control treatment. Bhattacharjee et al. (2008) reported that potassium is essential for regulating cellular osmotic potential and raising the root system's specific surface area.

The treatments M03, M04, M13, M25, M26, M27 and M28 produced HCN (Table 2). *Trichoderma koningiopsis* could produce hydrogen cyanide alone or in combination with *Phanerochaete*

Table 2. Biochemical characterization of isolate and combined microorganisms.

Treatments	Nitrogen fixation	Hydrogen cyanide	Potassium	Zinc	Phosphate	Biofilm	IAA (μg mL^{-1})	Siderophore (%)
M01 BRM 32114 (*Serratia marcescens*)	+	-	-	-	+	+	7.14 a*	22.27 d
M02 BRM 32110 (*Bacillus toyonensis*)	+	-	-	-	+	+	10.92 a	86.28 b
M03 BRM 62389 (*Phanerochaete australiani*)	+	+	+	+	+	-	2.73 c	70.46 b
M04 BRM 53736 (*Trichoderma konigiopsis*)	+	+	-	-	+	+	1.71 d	78.23 b
M05 AbV5 (*Azospirillum brasilense*)	+	-	-	-	+	+	4.14 b	76.24 b
M06 BRM 63574 (*Azospirillum* sp.)	+	-	-	-	+	+	4.51 b	21.57 d
M07 BRM 63573 (*Bacillus* sp.)	+	-	+	-	+	+	3.50 b	84.13 b
M08 BRM 32114 + BRM 32110	+	-	-	-	+	+	1.93 d	55.80 c
M09 BRM 32114 + AbV5	+	-	+	-	+	+	2.28 c	87.73 b
M10 BRM 32114 + BRM 53736	+	-	+	-	+	+	2.36 c	84.53 b
M11 BRM 32110 + AbV5	+	-	-	-	+	+	2.25 c	95.42 a
M12 BRM 32110 + BRM 53736	+	-	+	-	+	+	2.59 c	107.27 a
M13 AbV5 + BRM 53736	+	+	+	-	+	+	2.02 d	77.10 b
M14 BRM 63574 + BRM 63573	+	-	-	-	+	+	3.88 b	76.83 b
M15 BRM 63574 + AbV5	+	-	-	-	+	+	5.81 b	84.33 b
M16 BRM 63573 + AbV5	+	-	-	-	+	+	3.41 b	93.51 a
M17 BRM 63574 + BRM 32114	+	-	-	-	+	+	2.92 c	75.81 b
M18 BRM 63573 + BRM 32114	+	-	-	-	+	+	6.77 a	107.42 a
M19 BRM 63574 + BRM 32110	+	-	-	-	+	+	3.85 b	27.39 d
M20 BRM 63573 + BRM 32110	+	-	-	-	+	+	2.70 c	47.56 c
M21 BRM 63574 + BRM 53736	+	-	-	-	+	+	4.93 b	99.37 a
M22 BRM 63573 + BRM 53736	+	-	-	+	+	+	4.15 b	23.60 d
M23 BRM 62389 + BRM 63573	+	-	-	+	+	+	3.57 b	31.18 d
M24 BRM 62389 + BRM 63574	+	-	+	+	+	+	2.86 c	67.80 b
M25 BRM 62389 + AbV5	+	+	-	+	+	+	2.35 c	68.03 b
M26 BRM 62389 + BRM 53736	+	+	+	+	+	+	2.97 c	69.91 b
M27 BRM 62389 + BRM 32114	+	+	+	+	+	+	5.95 b	97.03 a
M28 BRM 62389 + BRM 32110	+	+	+	+	-	+	2.92 c	41.50 c

IAA: indolacetic acid; +: the microorganism or the combination has the biochemical characteristic; -: the microorganism or the combination does not have the biochemical characteristic; * means followed by the same letter do not differ by the Scott-Knott test at $p < 0.05$.

australiani or *A. brasilense*. Besides, *Phanerochaete australiani* in combination with *Serratia marcescens* or *Bacillus toyonensis* also could produce hydrogen cyanide. Meanwhile, their combination with another microorganism led to a negative ability to produce HCN. Hydrogen cyanide is a volatile antimicrobial compound produced by numerous species of rhizobacteria involved in broad-spectrum biological control of root diseases, since they prevent the proliferation and development of pathogenic microorganisms (Ali et al. 2020). Many bacterial genera, such as *Rhizobium*, *Pseudomonas*, *Alcaligenes*, *Bacillus* and *Aeromonas*, produce HCN (Srivastava 2017).

The biofilm production occurred for single microorganisms and their combinations, except for M03 and M4 (Table 2). Biofilm protects the plant from potential phytopathogens by producing antibiotics or antifungals that kill invading bacteria or fungi (Hashem et al. 2019). Rezende et al. (2021) recorded *Bacillus* sp., *B. thunringiensis*, *Serratia* sp. and *A. brasilense* among microorganisms that produce biofilm.

Significantly different amounts of IAA were observed for all the treatments. It is worth noting that M01 (*S. marcescens*), M02 and M18 showed 7.14, 10.92 and 6.74 μg mL^{-1}, respectively (Table 2). None of the combined treatments produced more IAA than BRM 32114, 32110 and 63573 alone. IAA is one of the most physiologically active auxins, and a member of the group of phytohormones that is generally considered the most important native auxin (Mohite 2013). According to Kumar et al. (2015), IAA promotes root growth and development, which improve the nutrient intake. All isolate and combined microorganism treatments produced siderophores (Table 1), even though statistically different. The treatments M9, M10, M11, M12, M15,

M18 and M21 produced more siderophores than each component of the combination. Kumar et al. (2015) suggested that bacteria can produce siderophores and antibiotics to suppress phytopathogens, what is important in agronomy by indirectly increasing the plant growth and yield and making the micronutrient Fe available.

According to the results of the present study, the tested microorganisms (single and in combination) produce many substances that can improve plant development. Therefore, these characteristics are important when considering using these microorganisms to improve plant development in agricultural areas.

The tested microorganisms have a great potential for future studies as plant growth promoters, since they have important biochemical characteristics (such as nitrogen fixation, P, K and Zn solubilization, and indoleacetic acid, siderophores and biofilm production), what could improve plant development.

CONCLUSIONS

1. Usually, the treatments had the capacity of nitrogen assimilation, biofilm production and phosphorus solubilization;
2. Only the single *Trichoderma koningiopsis* or *Phanerochaete australiani* and the combination of *T. koningiopsis* with *Azospirillum brasilense* or *P. australiani*, and the combination of *P. australiani* with *Serratia marcescens* or *Bacillus toyonensis*, produced hydrogen cyanide;
3. All the treatments solubilize phosphorus, while, among them, *P. australiani*, *T. konongiopsis* and their combination did not produce biofilm;
4. *Bacillus toyonensis* produced the highest amount of indoleacetic acid, while *Bacillus* sp. + *Serratia marcescens* produced the greatest quantity of siderophores;
5. The treatment combinations of *A. brasilense* AbV5 + *T. konigiopsis* were the most compatible among all the other treatments.

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