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Association of *Bacillus subtilis* 34 and soil conditioner for promoting growth in okra plants

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Spatial and temporal distribution of water resources is uneven in arid and semiarid regions, consequently affecting the growth and production of vegetables, mainly when combined with high temperatures. Therefore, improving water use efficiency is an urgent issue for growing crops in regions with such characteristics. The use of cellulose-based water retainer polymers combined with growth-promoting rhizobacteria can induce the plant tolerance to water deficit and promote plant growth. The objective of this study was to evaluate the effect of the combined application of *Bacillus subtilis* isolate 34 and a cellulose-based water-retaining polymer (WRP) on the growth of okra plants. *B. subtilis*-34 was grown in a rice culture medium with and without WRP. A greenhouse experiment was conducted in a randomized block design with four treatments (*B. subtilis*-34 + WRP, *B. subtilis*-34, WRP, and control) and eight replications. No significant difference was found for growth of *B. subtilis*-34 in rice culture medium with and without WRP ($p>0.05$). All analyzed variables in okra plants subjected to application of *B. subtilis*-34 + WRP ($p<0.05$) showed higher results. The combined application of *B. subtilis*-34 and WRP promoted higher development of okra plants.

Key words: *Abelmoschus esculentus*, rhizobacteria, sustainability, water-retaining polymer.

INTRODUCTION

Okra (*Abelmoschus esculentus*), an annual crop species, is among the most grown vegetables in arid and semiarid regions worldwide (Adekiya et al., 2019). These regions are characterized by an irregular rainfall distribution and low-fertility soils (Medeiros et al., 2018; Queiroz et al., 2018). Okra is a water- and nutrient-demanding plant, despite its usual cultivation in arid and semiarid regions (Nana et al., 2019). Okra crops are grown in 43,631 rural properties in Brazil (IBGE, 2017), presenting a mean yield of 17.5 Mg/ha.

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Okra crops in Brazil are usually grown by small farmers who often have no sufficient resources to supply the proper irrigation and soil fertilization for the crop (Kamga et al., 2016). However, some alternatives have been studied and shown promising results, such as the use of plant growth-promoting rhizobacteria and water-retaining agents with high water retention capacity (Zomerfeld et al., 2021).

Rhizobacteria are bacteria associated to the root system and have symbiotic relationship with plants. They affect the soil characteristics and have an essential function in converting arid and low-quality soils into arable and fertile soils (Gouda et al., 2018). The introduction of rhizobacteria improves plant growth by increasing the number of secondary roots, thus enabling higher water and nutrient absorption (Liu et al., 2018).

Bacillus subtilis stands out among the most studied rhizobacteria (Koua et al., 2020) due to its several mechanisms of plant growth promotion and its ability to produce endospores, making it desirable to produce formulations. Lopes et al. (2019a, 2019b) found that the application of *B. subtilis* isolate 34 resulted in increases in fresh and dry weights of shoots and roots of tomato plants and improved the growth of lettuce plants.

Water retainers can be composed of several materials, including cellulose, which provides advantages such as high-water retention capacity, low cost, and biodegradability (Ranganathan et al., 2019). These polymers have significant benefits, such as enabling higher water use efficiency and cation exchange capacity and improving soil physical properties (Thombare et al., 2018). The use of water retaining polymers improves the soil water retention capacity, resulting in increased plant growth and delayed wilting point under water stress (Ahmed et al., 2016).

Water retaining provides extensions to the natural rhizosphere and maintain water supply over time, increasing the multiplication of rhizobacteria and, consequently, the colonization area of these beneficial microorganisms (Kurrey et al., 2018). Therefore, the application of rhizobacteria that favor nutrient availability for plants combined with water-retaining materials that can increase water use efficiency is an environmentally sustainable alternative (Mathes et al., 2020).

Studies about the effect of combining rhizobacteria and water-retaining materials on growth of okra plants are not yet found in the literature. In this context, the objective of this study was to evaluate the effect of the combined application of a growth-promoting rhizobacterium and a water-retaining polymer on the growth of okra plants.

MATERIALS AND METHODS

Experiment location

Laboratory and greenhouse experiments were conducted at the State University of Montes Claros (Unimontes), in Janauba, Minas Gerais, Brazil.

Viability of *Bacillus subtilis* isolate 34 grown in a culture medium with a water-retaining polymer

The growth-promoting rhizobacterium used was *B. subtilis* isolate 34 from the Bacterial Collection of the Laboratory of Phytopathology, Nematology, and Microbiology at Unimontes. The effect of the water-retaining material on bacterial growth was evaluated through an *in vitro* test. A volume of 100 µL of bacterial suspension stored in saline solution at room temperature was transferred to 200 mL Erlenmeyer flasks containing 50 mL of a rice culture medium (185 grams of rice, 185 g $C_{12}H_{22}O_{11}$, 55.5 g of NaCl, and 49.29 g KH_2PO_4 per liter) (Lopes et al., 2019b) with and without the addition (0.25 g per 50 mL) of a cellulose-based water-retaining polymer (WRP) (Polyter®) (The granules absorb 160 to 500 times their initial dry weight. Saves at least 50% of water and 30% of fertilizers. The duration of the system varies from 3 to 5 years depending on the nature of the soil.).

The flasks containing the treatments were kept under constant agitation (220 rpm) in an orbital shaker at 28°C for 32 h. Subsequently, the treatment containing WRP + rhizobacteria was subjected to an ultrasonic bath (40 kHz - Unique USC-1400) for 20 min, followed by a serial dilution from 10^{-1} to 10^{-5}. A volume of 100 µL from the 10^{-5} dilution was spread onto a Petri dish containing TSA medium (Trypic Soy and Agar) using a Drigalski spatula. The plates were incubated at 28°C for 22 h, after which the number of colony-forming units (CFU) was counted.

The experiment was conducted in a completely randomized design with two treatments and 12 replications. The means were compared by the F test at 5% probability level. The statistical analyses were performed using the software R 3.5.

Growth promotion for okra seedlings by application of *Bacillus subtilis* isolate 34 and water-retaining polymer (WRP)

Okra seeds (cultivar Santa Cruz) were sown in 60-cell plastic trays containing commercial substrate (Bioplant®; 12.5 cm^3 per cell). Seedlings were transplanted into 3 dm^3 pots containing the commercial substrate and 50 grams of hydrated WRP, 15 days after sowing. The hydration process consisted of adding 5 grams of WRP to 1.0 liter of water; the WRP absorbed the maximum amount of water after 24 hours, according to previous tests.

The culture of *B. subtilis*-34 consisted of transferring 100 µL of bacterial suspension stored in saline solution at room temperature to 200 mL Erlenmeyer flasks containing 50 mL of rice culture medium (Lopes et al., 2019a). The flasks were kept under constant agitation (220 rpm) in an orbital shaker at 28 °C for 32 hours to reach a concentration of 6.14×10^8 CFU mL^{-1} (Lopes et al., 2019b).

The okra seedlings were subjected to application of 150 mL of bacterial suspension grown in rice culture medium, which was divided into three applications of 50 mL each (at 7, 9, and 11 days after transplanting - DAT). The control treatment was subjected to application of 50 mL of distilled water at 7, 9, and 11 DAT. The plants were daily irrigated. Soil fertilizers were applied every 7 days, consisting of nitrogen ($Ca(NO_3)_2$;15% N Haifa Cal®) and potassium (K_2SO_4; 51% K Topfert®); phosphorus (simple superphosphate; 18% P_2O_5 Topfert®) was applied at the transplanting of seedlings.

The following variables were evaluated in okra plants at 60 DAT: number of leaves; leaf area; plant height; stem diameter; relative chlorophyll content (SPAD index); root volume, area, and diameter; and shoot and root dry weights. The length (L) and width (W) of third leaf of the plant was measured using a ruler (mm) to determine

Table 1. Number of colony-forming units (CFU) of *Bacillus subtilis* isolate 34 in a rice culture medium with and without cellulose-based water-retaining polymer (WRP).

Treatment	CFU (mL^{-1})	Sampling standard deviation
Rice medium with WRP	$1.97 \times 10^{9\ a}$	±8.31
Rice medium without WRP	$2.03 \times 10^{9\ a}$	±29.89
Coefficient of variation (%)	10.99	

Means followed by the same letter in the column do not differ from each other at 5% probability by the F test.

the leaf area (LA) according to the equation: $LA = (L \times W) \times 0.63$ (Oliveira et al., 2014). Plant height was measured using a ruler tape, and plant diameter was measured using a digital caliper.

Relative chlorophyll content was determined in a portable chlorophyll meter (SPAD-502 Minolta®) in three replications. Root volume, surface area, and diameter were obtained by digital image analysis (Sony® Cyber-Shot DSC-W830 HD 20.1 MP) in the software Safira (EMBRAPA, 2009). Shoot and root dry weights were obtained after drying the respective plant parts in a forced air circulation oven at 65 ºC until constant weight and, then, weighing on a precision balance.

The experiment was conducted in a randomized block design with four treatments and eight replications, totaling 32 sampling units. The treatments consisted of *B. subtilis*-34 + WRP, *B. subtilis*-34, WRP, and a control (without WRP and *B. subtilis*-34).

The data were subjected to analysis of variance by the tests of Bartlett (1937) and Shapiro and Wilk (1965) at 5% significance level. The data with normal distribution and homogeneous variances were subjected to analysis of variance. Means were compared by the Tukey's test at 5% significance level. The statistical analyses were carried out in the software R 3.5.

RESULTS

No significant difference was found for growth of *Bacillus subtilis* in the rice culture medium with or without the cellulose-based water-retaining polymer (WRP) ($p>0.05$) (Table 1).

All vegetative variables evaluated in the okra plants were affected by the treatments ($p<0.05$). The combined application of rhizobacteria + WRP increased the number of leaves by 48.87% compared to the control. All treatments increased leaf area compared to the control. The treatments with rhizobacteria + WRP, rhizobacteria, and WRP increased leaf area by 234.53%, 161.20%, and 131.06% compared to the control, respectively (Table 2).

Regarding plant height, the treatment with rhizobacteria + WRP resulted in the highest increase (107.44%) compared to the control. All treatments increased stem diameter (SD) compared to the control; rhizobacteria + WRP resulted in an increase of 86.92% (Table 2).

The application of rhizobacteria + WRP promoted increases in shoot dry weight and relative chlorophyll content of 109.41% and 35.01%, respectively, compared to the control. The treatments rhizobacteria + WRP and rhizobacteria alone significantly increased root dry weight by 154.41 and 151.02%, respectively (Table 2).

Okra plants subjected to application of rhizobacteria + WRP presented the highest root volume**,** with an increase of 418.18% compared to the control, followed by those subjected only to application of rhizobacteria (318.18%) (Table 2; Figure 1). The root surface area was larger in the plants subjected to application of rhizobacteria + WRP, presenting an increase of 357.66% compared to the control.

The percentage of fine roots was higher for plants subjected to rhizobacteria + WRP and lower for those in the control (Figure 1). The smallest variation in fine roots was found for plants subjected only to application of rhizobacteria. Fine roots contributed to 52.70% of the root system. The highest percentage of medium roots was found for plants in the treatment rhizobacteria + WRP (Table 3), whereas plants in the control treatment presented the higher percentage of thick roots (52%).

DISCUSSION

The cellulose-based water-retaining polymer (WRP) used in the culture medium did not affect the growth of *Bacillus subtilis* isolate 34, as the number of colony-forming units (CFU) was equal in the treatments with and without the addition of WRP to the culture medium. The results found in the present study for combination of a cellulose-based water-retaining polymer and *B. subtilis* are unprecedent and provide new alternatives for the field of product development.

Regarding the evaluated agronomic variables, the okra plants developed better when grown in soil with application of rhizobacteria + WRP (Tables 1, 2, 3, and Figure 1). Thus, the presence of WRP may have resulted in a favorable microclimate for the plant and rhizobacteria, as a hydrated water-retaining polymer releases water gradually, promoting increases in root volume and in release of exudates, thus contributing to bacterial multiplication. The highest plant development in the treatment with water- rhizobacteria + WRP may be due to an improved root system development, as shown by the results found for root volume and surface area.

The acquisition of soil nutrients by plants occurs

Table 2. Number of leaves (NF), leaf area (LA), plant height (AP), stem diameter (SD), shoot dry weight (SDW), relative chlorophyll content (SPAD index), and root dry weight (RDW), volume (cm^3), and surface area (cm^2) in okra plants (*Abelmoschus esculentus*) as a function of application of rhizobacteria (*Bacillus subtilis* isolate 34) and cellulose-based water-retaining polymer (WRP) to the soil.

Treatment	NF	LA (cm^2)	PH (cm)	SD (mm)
Rhizobacteria + WRP	7.25 [a]	391.11 [a]	136.12 [a]	13.01 [a]
Rhizobacteria	7.25 [a]	305.37 [a]	112.00 [ab]	9.99 [ab]
WRP	6.12 [ab]	271.25 [b]	101.37 [b]	9.93 [ab]
Control	4.87 [b]	116.96 [c]	65.62 [b]	6.96 [c]
Coefficient of variation (%)	19.14	25.60	19.31	24.43
Treatment	SDW* (g)	SPAD	RDW (g)	
Rhizobacteria + WRP	44.50 [a]	41.65 [a]	18.75 [a]	
Rhizobacteria	32.37 [ab]	35.39 [b]	18.50 [a]	
WRP	26.62 [ab]	32.84 [b]	12.87 [ab]	
Control	21.25 [b]	30.85 [b]	7.37 [b]	
Coefficient of variation (%)	24.84	10.18	33.97	
Treatment	Root volume* (cm^3)		Root surface area*(cm^2)	
Rhizobacteria + WRP	2.28 [a]		28.97 [a]	
Rhizobacteria	1.84 [a]		22.66 [ab]	
WRP	0.82 [b]		9.79 [b]	
Control	0.44 [b]		6.33 [b]	
Coefficient of variation (%)	16.18		29.20	

Means followed by the same letter in the column are not significantly different from each other by the Tukey's test at 5% probability level. Means of 8 replications. *Data transformed into $\sqrt{x+1.0}$.

through root growth and ramification, therefore, the amount of nutrients absorbed is determined by the total root surface area. Longer and fine roots, for the same mass (same metabolic consumption), present a larger surface area and, consequently, in a higher capacity to absorb nutrients, mainly those with low mobility in the soil, such as phosphorus. The rhizobacterium evaluated in the present study (*B. subtilis*-34) has been shown to be efficient in promoting the growth of vegetable, common bean, and banana plants (Lopes et al, 2019a; Lopes et al., 2019b; Santos et al., 2019; Lopes et al., 2018).

Most studies found in the literature evaluated rhizobacteria and water-retaining materials separately (Basyony and Abo-Zaid, 2018; Andrade et al., 2023), thus neglecting the potential synergistic effects of the combined use of these agents. The interaction between the growth-promoting agent and the soil conditioner significantly improved the development of okra plants under controlled conditions in a greenhouse. However, the potential for co-inoculation of both materials has not yet been investigated in the context of agricultural production in areas with low water availability in the northern Minas Gerais, Brazil. Optimizing the use of bioinoculants requires not only quantifying the isolated effect a microorganism, but also its cooperative effects (Moreira et al., 2020), as in the case of its combination with water-retaining materials.

Regarding the species of rhizobacteria used in this study (*B. subtilis*), several studies have been shown its high potential for promoting plant growth by facilitating resource acquisition and modulating hormone levels in plants (Trivedi et al., 2020). It also improves the absorption of macronutrients, such as nitrogen (Aini et al., 2019), potassium (Ramakrishna et al., 2019), and phosphorus (Kalayu, 2019), as well as micronutrients (He et al., 2019). Essentially, well-nourished plants tend to develop better than undernourished plants (Verma et al., 2019).

The results of the present study showed a significant effect of combining rhizobacteria and WRP on the root system of okra plants, as found for root volume and surface area and percentage of fine roots. Roots continuously release exudates that promote a greater activity of microorganisms, such as *B. subtilis*, acting as soil particle aggregators. Robust root systems result in better development of okra plants. Not all parts of the roots are efficient in absorbing nutrients. The root zone with the highest ion absorption is the piliferous layer, which is only present in new (thin) roots.

Cells in this zone are already expanded, but are not yet

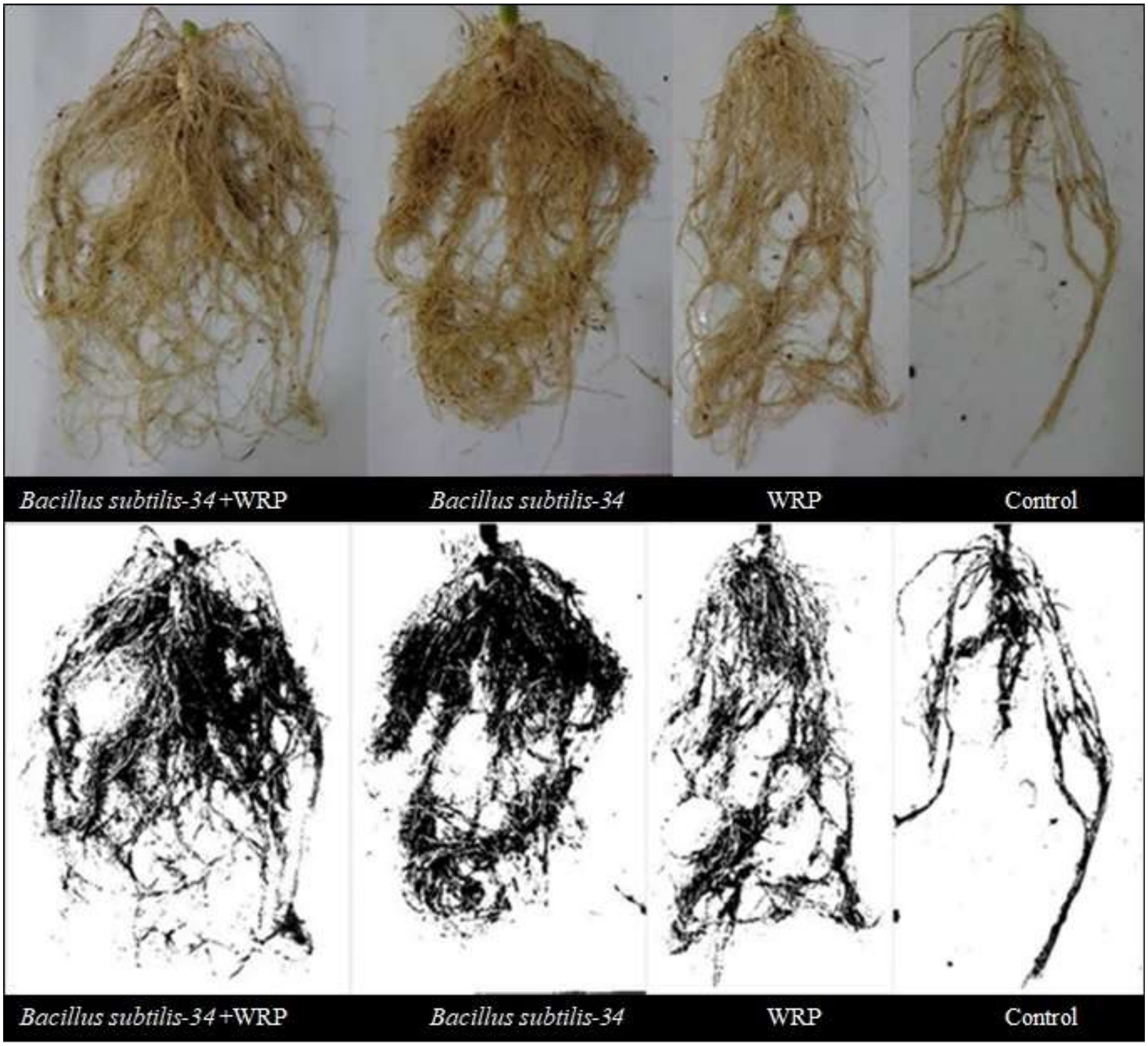

Figure 1. Roots of okra plants (Abelmoschus esculentus) subjected to application of rhizobacteria (*Bacillus subtilis* isolate 34) and cellulose-based water-retaining polymer (WRP) to the soil.

Table 3. Percentage of fine, medium, and thick roots in okra plants (*Abelmoschus esculentus*) subjected to application of rhizobacteria (*Bacillus subtilis* isolate 34) and cellulose-based water-retaining polymer (WRP) to the soil.

Treatment	% Thin roots	% Medium roots	% Thick roots
Rhizobacteria + WRP	52.71 [a]	42.73 [a]	4.56 [a]
Rhizobacteria	37.49 [ab]	48.52 [a]	13.99 [a]
WRP	26.07 [ab]	53.04 [a]	20.89 [a]
Control	21.55 [b]	26.02 [b]	52.43 [b]
Coefficient of variation (%)	20.40	19.80	21.43

Means followed by the same letter in the column are not significantly different from each other by the Tukey's test at 5% probability level. Means of 8 replications.

in secondary growth, thus presenting greater absorption of solutes. The percentage of fine roots was higher for plants in the treatments with rhizobacteria + WRP, or only rhizobacteria or WRP, which explains the greater development found for plants grown under these conditions.

Another important factor is that the diffusion rate of elements in the soil tends to decrease exponentially as the distance between them and the absorption point increases. Thus, elements close to roots diffuse towards them, but are not replenished by those that are far away, resulting in depletion. Therefore, an efficient nutrient absorption requires a continuous root growth in plants. A continuous root formation ensures the development of new roots (absorptive roots), which reach soil areas where the diffused nutrient has not been yet depleted.

Conclusions

The presence of water-retaining polymer in the culture medium did not affect the *in vitro* growth of *Bacillus subtilis* isolate 34. The combined application of water-retaining polymer and rhizobacteria promoted greater development of the okra aerial part and roots. The application of rhizobacteria with the water-retaining polymer increased the chlorophyll content (SPAD).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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