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Fluorescent *Pseudomonas* spp. and *Bacillus* spp. for phosphate solubilization and growth promotion of garlic[1]

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ABSTRACT

Garlic is a nutrient demanding crop which requires a high investment for fertilization and especially for phosphate (P) fertilizers, due to its high retention by the soil. It is possible to use the P solubilizing microbiota, especially rhizobacteria, to make P available and reduce fertilization. Fluorescent *Pseudomonas* spp. (CBS02) and *Bacillus* spp. (EB17) isolates were tested at triple superphosphate doses (0 % - without triple superphosphate; 50 % - 472.82 kg ha^{-1}; 100 % - 945.65 kg ha^{-1}). The experimental design was randomized blocks, in a 4 x 3 x 4 factorial scheme, with four treatments (CBS02 isolate, EB17 isolate, isolates mixture and control) and four replications. The size, fresh and dry masses, P content of the fourth leaf and yield were evaluated. The mixture of *Pseudomonas* spp. (CBS02) and *Bacillus* spp. (EB17) used in the inoculation of the garlic bulbils increased the size, dry mass and P content of the fourth leaf, as well as the yield of the crop without triple superphosphate, indicating that the P solubilization capacity of the isolates was increased when these were combined. It was possible to observe an increment in all the evaluated variables, indicating that the isolates P solubilization capacity was enhanced when they were combined.

KEYWORDS: Rhizobacteria, Alliaceae, phosphate nutrition.

RESUMO

Pseudomonas spp. fluorescente e *Bacillus* spp. para solubilização de fosfato e promoção de crescimento em alho

O alho é uma cultura exigente em nutrientes que requer alto investimento para adubação e, principalmente, fertilizantes fosfatados (P), devido à sua alta retenção pelo solo. É possível utilizar a microbiota solubilizadora de P, principalmente as rizobactérias, para disponibilizar P e reduzir a fertilização. Isolados de *Pseudomonas* spp. (CBS02) fluorescente e *Bacillus* spp. (EB17) foram testados em doses de superfosfato triplo (0 % - sem superfosfato triplo; 50 % - 472,82 kg ha^{-1}; 100 % - 945,65 kg ha^{-1}). O delineamento experimental foi em blocos ao acaso, em esquema fatorial 4 x 3 x 4, com quatro tratamentos (isolado CBS02, isolado EB17, mistura de isolados e testemunha) e quatro repetições. Avaliaram-se o porte, massas úmida e seca, teor de P da quarta folha e produtividade. A mistura de *Pseudomonas* spp. (CBS02) e *Bacillus* spp. (EB17) utilizada na inoculação de bulbilhos de alho aumentou o tamanho, a massa seca e o teor de P da quarta folha, bem como a produtividade da cultura sem superfosfato triplo, indicando que a capacidade de solubilização de P dos isolados foi aumentada quando foram combinados.

PALAVRAS-CHAVE: Rizobactérias, Alliaceae, nutrição fosfatada.

INTRODUCTION

Garlic cultivation stood out in the economic scenario by reaching 155 thousand marketable tons in 2022, in Brazil (Conab 2022). The Minas Gerais state leads the national ranking with 68.2 thousand tons, followed by Goiás and Santa Catarina (Conab 2022). The Santa Catarina state registered 19.1 thousand tons in the 2021/22 harvest (Alves 2022). Fraiburgo is one of the main producers in Santa Catarina (Epagri 2018, Gugel 2019), emphasizing the importance of the crop to the state agriculture.

Nevertheless, garlic is highly demanding in nutrients. In 2018, it was necessary to spend around US$ 552.23 to fertilize 5 ha of garlic and US$ 402.18 were spent on phosphorus (P) fertilizers (Epagri 2018). The P fertilization cost is high and aims at reducing the crop deficiency in older leaves and smaller bulbs formation (Lucini 2004).

Generally, a high amount of phosphate fertilizer is used to balance its retention in the soil due to the attachment to colloids and low mobility (Büll et al. 1998, Büll et al. 2008). However, it may cause further economic and environmental problems.

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Thus, some alternatives are studied to increase the phosphate fertilization efficiency and reduce costs and environmental problems (Chagas Junior et al. 2010). Phosphate solubilizing microorganisms are a promising alternative (Abreu et al. 2017, Oliveira-Paiva et al. 2022).

Considering the soil microbial community, phosphate solubilizing bacteria normally comprise between 1 and 50 %, while phosphate solubilizing fungi comprise between 0.1 and 0.5 % (Chen et al. 2006, Oliveira-Paiva et al. 2022). Among the rhizobacteria genera, fluorescent *Pseudomonas* spp. and *Bacillus* spp. can solubilize phosphate, make P available in the soil, help the nutrient absorption and, consequently, promote plant growth and development (Coelho et al. 2007, Marra et al. 2011, Zucareli et al. 2018).

Products containing phosphate-solubilizing bacteria are already available. They have provided significant results by increasing yield and plant quality in the field (Zucareli et al. 2018, Zamariolli et al. 2019, Oliveira-Paiva et al. 2022). Bioproducts containing *Bacillus megaterium* (BRM 119) and *Bacillus subtilis* (BRM 2084) have reduced the triple superphosphate fertilization and increased the P absorption by the roots when applied to maize seeds, thus increasing the production per hectare by 10 % (Oliveira-Paiva et al. 2020). Therefore, fluorescent *Pseudomonas* spp. (CBS02) and *Bacillus* spp. (EB17) isolated from garlic rhizosphere under laboratory conditions could stimulate the growth and yield of garlic in the field and reduce the P fertilization demand due to their capacity to solubilize phosphate *in vitro* (Botelho et al. 2019, Balbinot et al. 2020).

Thus, this study aimed to evaluate the effect of P-solubilizing isolates inoculation on garlic bulbils to reduce the triple superphosphate demand in the field.

MATERIAL AND METHODS

The experiment was carried out in Fraiburgo, Santa Catarina state, Brazil (27º05’15.9”S, 50º53’59.7”W and altitude of 1.048 m), on July 15, 2019. According to the Köppen classification, the climate in the area is Cfb (temperate climate, humid mesothermal and mild summer), and the soil is classified as a Cambisol (Santos et al. 2018), equivalent to Inceptisols (USDA 1999).

The experimental design was randomized blocks, in a 4 x 3 x 4 factorial scheme (four treatments, three triple superphosphate doses and four replications), and two bacterial isolates were evaluated: CBS02, fluorescent *Pseudomonas* spp. (Botelho et al. 2019), and EB17, *Bacillus* spp. (Balbinot et al 2020). These isolates showed significant phosphate solubilization indices for *in vitro* assays.

The treatments were CBS02 inoculation (T1), EB17 inoculation (T2), mixed isolates inoculation (T3) and control without inoculation (T4). All the treatments were combined with three doses of P_2O_5: 0, 50 and 100 % of the standard dose of 435 kg ha^{-1} of P_2O_5, whose source was triple superphosphate (TSP) (46 % of P_2O_5). The TSP amount was defined according to the CQFS-RS/SC (2016) and the soil analysis for basis fertilization as it follows: pH ($CaCl_2$) = 4.30; P = 6.97 mg dm^{-3}; K = 415.49 mg dm^{-3}; Ca = 9.53 cmol$_c$ dm^{-3}; Mg = 5.00 cmol$_c$ dm^{-3}; Al = < 0.01 cmol$_c$ dm^{-3}; H + Al = 12.74 cmol$_c$ dm^{-3}; OM = 41.00 g dm^{-3}; SB = 5.60 cmol$_c$ dm^{-3}; V = 84.98 %; CEC pH7 = 18.34 cmol$_c$ dm^{-3}.

The TSP amounts, regarding the treatments, were 100 % of the dose - 945.65 kg ha^{-1}; 50 % of the dose - 472.82 kg ha^{-1} and 0 % - without TSP. The soil had been limed at three months before planting (3.5 t ha^{-1} of dolomitic limestone).

Each of the 48 plots measured 1.0 x 1.4 m and the experimental area had 50.4 m². There were 3 double-row spaced out at 15 and 9 cm between bulbils. A Chonan virus-free cultivar with a 160-day cycle was used. Before sowing, the bulbils were vernalized in a cold chamber for 30 days, at 4 ºC, and, then, they were classified, and 5 g of bulbils (class 3), which are larger, homogeneous and have a high vigor, were selected. Peat was chosen because it is a vehicle widely used for bacteria inoculation in crops such as bean, soybean and corn, with the amount based on the inoculant manufacturers’ recommendations: 150 g of inoculant for 60 kg of soybean or bean seeds. Adjustments in the peat amounts were based on the bulbils average weight (5 g). The number of bulbils per plot and treatment was calculated and multiplied by the average weight of each bulbil (5 g), thus determining the peat amount per treatment. As they are larger seeds (bulbils), the peat amount was duplicated to ensure an adequate coverage and adhesion of the bacteria. Therefore, each inoculant received 20 g of peat.

To formulate the inoculants, each isolate, CBS02 (T1) and EB17 (T2), was inoculated into flasks containing 40 mL of liquid Luria Bertani (LB) medium. They were kept for 24 h at 28 ºC. For the mixture of isolates (T3), 20 mL of each bacterial suspension were combined in a third empty and sterilized flask and homogenized. A flask containing uninoculated liquid LB was kept under the same conditions as the control (T4). Twenty milliliters of each suspension were aseptically transferred to four flasks containing 20 g of peat. They were incubated for 72 h at 28 ºC. Subsequently, bulbils for each treatment were placed in plastic bags along with the respective inoculant, and 20 mL of a 10 % sugar solution were added to enhance adherence, following the manufacturer's recommendation for the inoculant use (300 mL of 10 % sugar solution per 60 kg of seeds). The bulbils were then shade-dried before planting. After planting, fertilization was conducted based on the aforementioned TSP doses. Irrigation was set with approximately 10 mm of water.

The assessed parameters included the size of the fourth leaf (cm), fresh and dry mass of the fourth leaf (g), leaf P content (mg g^{-1}) and yield (kg ha^{-1}). At the four-leaf phenological stage (V4), the fourth leaf from ten plants was harvested within each plot for analysis. This stage is considered the most representative for garlic development. All measurements were taken using a graduated ruler (cm). The fresh leaf mass (g) was measured using a precision scale (Mars AD3300 0.5 g) and, to determine the dry mass (g), the leaves were dried in a forced ventilation oven at 50 ºC and then weighed.

To determine the leaf phosphorus (P) content, the molybdenum blue spectrophotometry method was employed (Silva 2009). The dried leaves were crushed and passed through a 20 µm granulometric sieve. Two hundred milligrams of each sample received 1 mL of hydrogen peroxide, 2 mL of H_2SO_4 and 0.7 g of digestion mixture. The mixture was then subjected to a digester block at 180 ºC, for 30 min. Subsequently, the temperature was increased to 350 ºC and maintained for 2 hours. Following the digestion process, 50 mL of distilled water were added to the digested samples, which were then homogenized and allowed to settle. From each sample, 1 mL was taken and mixed with 2 mL of distilled water, 3 mL of ammonium molybdate (0.38 %) and three drops of ascorbic acid (20 %), until developing a blue color (Tedesco et al. 1995). The absorbance of the solution was measured at 660 ηm, using a spectrophotometer (model SP2000UV - Bel). The leaf P content was determined in mg of P per g of leaf dry mass, based on a previously defined standard curve.

The harvest was conducted at four months after sowing, and the bulbs underwent a 50-day curing process. Afterwards, they were cleaned and weighed using a precision scale.

The data were subjected to analysis of variance using the F-test with a significance level of 5 %, while the means were compared using the Skott-Knott method at a significance level of 5 %, employing the Sisvar software version 5.8 (Build 92).

RESULTS AND DISCUSSION

The analysis of variance, along with its unfolding, indicated significance for the mean square of the bacterial inoculations (bacteria) and TSP doses (D), individually or for their interactions, except for the fourth leave fresh mass, which showed no significant difference (Table 1).

The statistical unfolding analysis revealed significant results, particularly for the combination of the two isolates.

A correlation between the masses of the fourth leaf and the bulbs was described by Biasi (1986), and,

Table 1. Analysis of variance (mean square) of fluorescent *Pseudomonas* spp. and *Bacillus* spp. inoculated on garlic associated to triple superphosphate doses.

Variance factors	DF	FLS	FLFM	FLDM	P content	Yield
Bacteria (B)	3	4.29*	0.14^{ns}	0.01^{ns}	2.08*	0.06*
Doses (D)	2	3.25^{ns}	0.41^{ns}	0.02*	0.39^{ns}	0.00^{ns}
B * D	6	2.08*	0.15^{ns}	0.00^{ns}	0.80*	0.05*
Block	3	4.61*	0.31^{ns}	0.01^{ns}	0.89^{ns}	0.03^{ns}
Residue	33	1.62	0.15	0.00	0.53	0.01
Average		46.72	3.88	0.47	4.54	1.39
CV (%)		2.72	10.05	16.69	16.10	7.49

* Significant at the F test ($p < 0.05$); ^{ns} not significant; DF: degree of freedom; FLS: fourth leaf size; FLFM: fourth leaf fresh mass; FLDM: fourth leaf dry mass.

later, it was also observed for the leaf size. Hence, it can be assumed that the leaf characteristics can reflect the plant nutritional status, especially regarding the P content. Therefore, the size, mass and P content of the fourth leaf were used as parameters to evaluate the isolates inoculation effects.

There were no significant differences observed among the treatments, including the control, *Pseudomonas* (CBS02) and *Bacillus* (EB17), as well as the TSP doses, regarding the size of the fourth leaf (Table 2). However, a significant difference was observed when comparing the treatments with the mixture of isolates, without the addition of TSP (Table 2). A similar effect was observed for the different TSP doses, with the mixture showing a significant increase without and with the full TSP dose (Table 2).

The largest leaf sizes were observed with the mixture inoculation without TSP fertilization and at the full TSP dose (48.47 and 48.29 cm, respectively). Considering the absence of exogenous P sources (0 % of TSP), such increase suggested that the phosphate solubilization was improved by the bacterial mixture, leading to enhanced absorption by the plants. The P content in the soil was classified as low (Santos et al. 2018) for the soil type and crop. The P extraction was performed with Mehlich 1, which removes the P that is not available to plants. Cambisols have a clay content of around 30 % (Santos et al. 2018), so the P amount was considered low. This indicates that the bacteria were able to solubilize and/or make available the P fractions that were initially unavailable to the plants due to the low P content in the unfertilized soil. This is supported by the higher leaf P content observed with the inoculation of two bacteria without phosphate fertilization (Table 2).

In vitro analyses of both isolates have shown their ability to solubilize phosphate (Botelho et al. 2019, Balbinot et al. 2020), suggesting this mechanism as a leaf size promoter. It is important to note that there are differences in phosphate solubilization mechanisms among microorganisms (Silva Filho & Vidor 2001). Possibly, the solubilization mechanisms of each isolate complemented each other, increasing the P availability to the plants. These results are similar to those by Hartmann et al. (2009 and 2010), who analyzed *Pseudomonas* spp. (W6), *Bacillus megaterium* (W19), *Bacillus cereus* (UFV40) and P solubilizers after transplanting onion seedlings. For number of leaves, they observed a positive interaction of bacterial inoculation and onion growth promotion after transplanting. The isolates also contributed to an increase in plant height. Similar effects have been observed in different plants (Ibarra-Galeana et al. 2017, Mattos et al. 2020).

Considering the full P dose (100 % of TSP), possibly, the isolates mixture effect relates to other plant growth promoting-mechanisms, since P was available because TSP is a soluble source (Table 2). It seems that the phosphate solubilization in the treatment without TSP was complemented between the isolates. Moreover, there might be additional mechanisms that enhance the inoculation effect on plant growth at 100 % of TSP. Considering the IAA production, which is a phytohormone (Taiz & Zeiger 2004), these isolates were able to produce it (Botelho et al. 2019, Balbinot et al. 2020), what could enhance their plant growth promoting ability. They produced approximately 20 µg mL^{-1} of IAA, which has been shown to stimulate plant growth (Florentino et al. 2017, Balbinot et al. 2020). It may have increased the root development and nutrient absorption reflected in the fourth leaf size. Increases in leaf size affect the photosynthetic rate, contributing to bulb development and harvest increase (Lucini 2004). All plant growth mechanisms, including P solubilization, can stimulate such effect on plant growth and production. Multiple effects of plant growth promoting rhizobacteria

Table 2. Inoculation effect of isolates on garlic growth and yield at triple superphosphate doses.

Treatment	FLS (g)			P content (mg P g^{-1} DM)			Yield (t ha^{-1})			FLDM (g)
	0 %	50 %	100 %	0 %	50 %	100 %	0 %	50 %	100 %	
Control	46.30 bB*	45.95 bB	46.30 bB	2.97 bB	2.99 bB	3.86 bA	9.07 bB	9.36 bB	9.86 bB	0.49 b
CBS02	46.30 bB	46.30 bB	46.30 bB	2.66 bB	2.76 bB	2.85 bB	9.64 bB	9.78 bB	10.64 bB	0.52 b
EB17	46.39 bB	46.30 bB	47.44 bB	2.84 bB	2.97 bB	2.97 bB	9.65 bB	9.50 bB	9.78 bB	0.45 b
CBS02 + EB17	48.47 aA	46.32 bB	48.29 bA	3.87 aB	3.25 bB	3.24 bB	11.93 aA	10.28 bB	9.57 bB	0.59 a
CV (%)		2.72			17.88			7.49		16.69

* Means followed by the same letter do not differ statistically by the Scott-Knott method, at a significance level of 5 %. Lowercase letters indicate comparisons among inoculations, while capital letters indicate comparisons among triple superphosphate doses. CV: coefficient of variation; FLS: fourth leaf size; FLDM: fourth leaf dry mass.

mechanisms were observed for different plants (López-Ortega et al. 2013, Mideksa et al. 2016, Silva et al. 2022, Vocciante et al. 2022). Suryanto et al. (2017) observed a positive inoculation effect of *Bacillus*, *Lactobacillus* and actinomycete isolates, single or combined, on pepper growth. Isolates have showed an ability to solubilize phosphate and/or produce IAA.

There was no significant difference among the treatments and doses, with the fourth leaf fresh mass reaching approximately 3.88 g. However, it was possible to observe the isolates mixture influence on the leaf dry mass (table 2), reinforcing the possibility of multiple actions of other mechanism(s). This treatment showed the highest leaf dry mass (0.59 g). It is likely that other mechanisms were acting on the leaf parameters, since the accumulation of leaf dry matter was higher with the two isolates mixture.

The P availability influenced the bacterial mixture for the leaf size increment (Table 2). Significant differences were observed for the CBS02 and EB17 mixtures without TSP and the control with 100 % of TSP (Table 2), concerning the fourth leaf P content analysis, with averages of 3.87 and 3.86 mg P g^{-1} of dry mass, respectively. The leaf P content increase for the isolates mixture without TSP suggested their ability to solubilize P pre-existing in the soil, pointing out a significant effect on the P availability for garlic. The leaf P content in the isolates mixture (3.87 mg P g^{-1} of dry mass) reached the recommended range of 3-5 mg P g^{-1} of dry mass (Resende & Cecílio Filho 2009). This result is particularly significant, since no P source was applied, suggesting the isolates action. This result was higher than that found by Jacon (2013), who evaluated P sources for garlic crops and obtained around 1.9 mg P g^{-1} of dry mass, even using simple superphosphate. This value was lower than those obtained in the treatments of the present experiment, even in the control without TSP (Table 2). It may indicate the presence of a phosphate-solubilizing microbial community in the soil, since the leaf P content was relatively high and like all the treatments without P fertilization, except for the isolates mixture (Table 2). Mideksa et al. (2016) evaluated the effect of phosphate solubilizers on chickpea and observed that the inoculation of some bacterial isolates resulted in a P shoot content increase, when compared to the treatment with soluble P fertilizer. It also stimulated the nodulation and, consequently, the shoot N content. These findings support the hypothesis of integrated mechanisms at play, as observed in some of the results from this study.

The higher fourth leaf P content observed in the control with the full TSP dose could be attributed to the isolates absence. Probably, they absorbed part of the available P, thereby reducing the plant uptake in the inoculated treatments, suggesting that the introduced bacterial populations could establish and colonize the environment. Despite the P content reduction by inoculations, there was no competition for the nutrient, since the P values remained relatively high and there were no deficiency symptoms in the plants. Although there was no statistical difference, the highest leaf P content among the inoculated treatments was observed in the isolates mixture (Table 2). Similar results were obtained by Büll et al. (2008), who detected an increase in the P content of garlic leaf at 100 % of TSP without the bacterial isolate inoculation.

Regarding yield, no significant differences were observed for the control, single inoculations and TSP doses. However, the CBS02 and EB17 mixture without TSP was superior to the other treatments (Table 2), reaching a value (11.93 t ha^{-1}) higher than the average yield of the Santa Catarina state, estimated at 10.5 t ha^{-1} (Alves 2022). It increased the yield by 2.86 t ha^{-1}, when compared to the control without TSP, and by 2.07 t ha^{-1}, to the same treatment, with a full fertilizer dose (Table 2). This result corroborates the CBS02 and EB17 mixture effect determined in other parameters. They indicate that the combined bacteria could solubilize P from soil and, without fertilization, supply its demand for plant development, reflecting on yield.

The P-solubilizing bacteria effect, capable of supplying plants and reflecting on yield, without P fertilizers, has been reported. Hartmann et al. (2009 and 2010) inoculated onion seeds with rhizobacteria isolates and observed that *Pseudomonas* sp. (W6), *Bacillus megaterium* (W19) and *Bacillus cereus* (UFV40) increased by 48, 46 and 45 % the production of bulbs per hectare, respectively, when compared to the control, which received standard crop fertilization. Chaves et al. (2013) observed that *P. fluorescens* inoculation on corn produced 87 % of relative agronomic efficiency, when comparing TSP fertilization and control without P.

There was a correlation among yield, fourth leaf size and leaf P content (Table 2), suggesting that solubilizing phosphate was one of the growth-promoting mechanisms for garlic, especially in the isolates mixture. For the isolates combination, it was possible to find out that the exogenous P source did not increase yield, despite improving the fourth leaf size and dry mass (Table 2). It also suggested that other mechanisms induced these accumulations, but not directly the P solubilization/absorption, since TSP is a P soluble source, and its P leaf content was not significant at 100 % of TSP for the inoculated treatments (Table 2).

The control yield at 100 % of TSP was below the average, in the range of 10.5 t ha^{-1} (Table 2). However, this treatment and the isolates mixture without TSP showed the highest leaf P contents (Table 2), suggesting that P was absorbed, but not translocated to the bulbs, reflecting on yield.

The lack of effect for 50 % of TSP on the parameters (Table 2) was not expected. However, this may suggest that the amount of available P was insufficient to increase the plants P uptake, or even stimulate endogenous or introduced microbiota action. Therefore, more studies are needed.

CONCLUSIONS

1. The isolates mixture of CBS02 *Pseudomonas* spp. and EB17 *Bacillus* spp., used for garlic bulbs inoculation, increased the size, dry mass, fourth leaf P content and yield without P fertilization. Thus, it is suggested that the P solubilization was a plant growth-promoting mechanism improved by bacterial combination. However, other plant growth-promoting mechanisms may be involved, because some parameters, such as size of fourth leaf, were increased at the full triple superphosphate dose;
2. The isolates mixture demonstrated efficacy and potential to reduce the P fertilization, requiring additional analyses to deepen the isolates effect and understand the P-solubilizing bacterial community in regional soils.

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