








Herbaspirillum seropedicae promotes maize growth but fails to control the maize leaf anthracnose

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Abstract *Herbaspirillum seropedicae* is an endophytic diazotrophic bacterium and a plant growth promoting bacteria. *Colletotrichum graminicola* causes the anthracnose, one of the most destructive maize diseases worldwide. The main objective of this work was to evaluate the effects of *H. seropedicae* SmR1 strain on the plant growth and leaf anthracnose of maize plants grown in substrate amended or not amended with humic substances. In the first assay, plants were pre-treated with *H. seropedicae* and inoculated with *C. graminicola* at 7, 14 and 21 days after treatment (DAT). In the second assay, plants were treated with *H. seropedicae*, grown in substrate amended with humic substances and inoculated at 3 and 7 DAT. The anthracnose severity was assessed by measurement of necrotic and chlorotic leaf area, and bacteria were quantified in leaves by quantitative PCR. *H. seropedicae* did not affect the disease severity in maize leaves, although it efficiently colonized the leaf tissues and it promoted maize leaf growth. Humic substances improved *H. seropedicae* colonization in maize.

Keywords *Colletotrichum graminicola* · Anthracnose · *Herbaspirillum seropedicae* · Plant growth promoting bacteria · Diazotrophic bacteria · Biocontrol agent

Introduction

The hemibiotrophic fungus *Colletotrichum graminicola* (Cesati) Wilson causes anthracnose in maize. It combines a transient biotrophic phase, during which the host cells remain alive, with a highly destructive necrotrophic development that kills the cells while spreading itself through the host tissue (Münch et al. 2008; Perfect and Green 2001). Anthracnose is one of the most important diseases affecting maize fields around the world (Bergstrom and Nicholson 1999; Jirak-Peterson and Esker 2011; Nicoli et al. 2016; Palaversic et al. 2009; Sukno et al. 2008), including Brazil (Cota et al. 2012). Although all maize plant tissues can be infected, the fungus attacks more frequently leaves and stalks causing the anthracnose leaf blight and anthracnose stalk rot (Bergstrom and Nicholson 1999), which represent the most economically damaging forms of the disease, resulting in severe yield losses in crops, especially under tropical conditions. The incidence of anthracnose in Brazil has increased in recent years as a result of the successive plantings, adoption of no-tillage without crop rotation and the use of susceptible genotypes. This is explained by the high ability of pathogens to survive in crop residues, leading to accumulation of inoculum in planting areas (Cota et al. 2012; Sukno et al. 2008).

There has been increasing interest in the development and use of new and environmentally safer alternatives for plant nutrition as well as protection against phytopathogens (Guerrero-Molina et al. 2015). In this scenario, the so-called plant growth promoting bacteria (PGPB) exhibit beneficial effects on plant growth and health. PGPB are known to improve plant nutrition and crop yield, while reducing negative impacts of chemical fertilizers on the environment (Babalola 2010; Patil et al. 2016; Perez-Montano et al. 2014; Vejan et al. 2016; Whipps 2001).

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Some studies have revealed that PGPB can decrease or prevent damage by plant pathogens, through mechanisms such as: (1) antagonism; (2) competition for iron; and (3) induction of systemic resistant against pathogens (Dobbelaere et al. 2003; Glick and Bashan 1997; Perez-Montano et al. 2014). Indeed, PGPB usually inhibit plant pathogens by more than one mechanism (Alabouvette et al. 1993; Patil et al. 2016).

Regarding to the beneficial effects of PGPB as biocontrol agents, several studies can be found using species of *Pseudomonas* against several pathogens, fungi, bacteria or virus (Cabanas et al. 2014; Elbadry et al. 2006; Liu et al. 1995; Nandakumar et al. 2001; Patil et al. 2016; Yasmin et al. 2016), including *Colletotrichum* spp. (Bigirimana and Hofte 2002; Gang et al. 1991; Planchamp et al. 2015; Viswanathan and Samiyappan 1999; Viswanathan and Samiyappan 2002), and even *C. graminicola* in maize (Planchamp et al. 2015). *Bacillus* has been reported to be effective against *C. acutatum* (Park et al. 2013) and other pathogens as well (Niu et al. 2011; Raj et al. 2012). *Glucanacetobacter diazotrophicus* (Arencibia et al. 2006), *Brevibacterium iodinum* (Son et al. 2014), *Azospirillum* sp. (Yasuda et al. 2009) and *Azospirillum brasilense* (Tortora et al. 2011; Tortora et al. 2012) are further examples of bacteria able to control plant pathogens at different extents under controlled conditions. Although all these studies have indicated a high potential for using PGPB as biocontrol agents against different fungal diseases on different hosts, up to now, to the best of our knowledge, there is no information if *Herbaspirillum* would be able to affect the development of plant diseases.

Herbaspirillum seropedicae is a PGPB able to colonize plant tissues endophytically. It is also diazotrophic (Chubatsu et al. 2012), that is, able to convert atmospheric nitrogen into ammonia, which can be in turn metabolized by the plant (Dobbelaere et al. 2003). *H. seropedicae*, i.e., the extensively studied SmR1 strain (Monteiro et al. 2012), has its complete genome sequenced (Pedrosa et al. 2011) and is nowadays target for application in agriculture. *Herbaspirillum* sp. was already suggested as a potential biocontrol agent by Weber et al. (2007), because its presence in plantlets of banana was associated with decrease in the severity of wilt caused by *Fusarium oxysporum* f. sp. *cubense*. *H. seropedicae* has at least 27 genes involved in iron transport and metabolism (Pedrosa et al. 2011). In fact, production of siderophores called serobactins was observed in *H. seropedicae* Z67 (Rosconi et al. 2013), and these compounds contribute to the competitive performance of *H. seropedicae* inside the host plant (Rosconi et al. 2016). Besides that, *H. seropedicae* seems to be able to modulate plant defense responses (Brusamarello-Santos et al. 2012; do Amaral et al. 2014).

Vermicompost, a renewable organic product containing humic substances (humics) with high biological activity (Martinez-Balmori et al. 2014), has been demonstrated to exert a synergistic interaction with PGPB. In this sense, vermicompost enhances the beneficial effects of PGPB agents, such as soil nutrients availability, microbial biomass and crop yield and quality. Although such effects strongly depend on the dose of vermicompost and crop species, this integrative practice has been suggested to substitute regular chemical fertilization (Song et al. 2015). Indeed, it has been shown that maize inoculation with *H. seropedicae* and humics can synergistically improve bacterial colonization in roots (Canellas et al. 2013). Combining vermicompost and *H. seropedicae* has proven to increase growth and development of tomato seedlings (Olivares et al. 2015).

Taking into account the potential use of *H. seropedicae* in maize crop, this work was the first aimed at evaluating its effect when associated or not associated with humics to control the leaf anthracnose.

Materials and methods

Microrganisms growth conditions

H. seropedicae SmR1, a spontaneous streptomycin resistant mutant of strain Z78 (ATCC 35893), was grown in an orbital shaker (120 rpm) at 30 °C in 30 mL NFbHPN medium supplemented with 5 mg/L malic acid (Klassen et al. 1997).

C. graminicola isolate MANE53 is deposited in the mycological collection (MANE) of the Laboratory of Plant Pathology, Federal University of Santa Catarina, Brazil. For fungal cultivation, pure cultures of the isolate were grown on potato dextrose agar medium (PDA) and incubated for 21 days under fluorescent light, with 12 h photoperiod at ± 25 °C.

H. seropedicae pre-treatment and growth conditions of maize plants

Maize seeds (cv. Pioneer 30F53) were surface-sterilized by immersion in 70% ethanol for 5 min, and in 2% sodium hypochlorite plus 2.5% Tween-20 solution for 30 min. Seeds were then rinsed 3 times with sterile distilled water, germinated in water moist filter paper and incubated at ± 25 °C in the dark for 3 days. In the pre-treatment, the 3-days-old seedlings remained for 30 min under constant agitation (80 rpm) (Balsanelli et al. 2010; do Amaral et al. 2014) in contact with a culture of 10^7 cells of *H. seropedicae* SmR1 mL⁻¹ diluted in sterile NFb malate broth without nitrogen source (the Optical Density of bacterial

cell cultures was measured at 600 nm using Hitachi U2910 Spectrophotometer, Tokyo, Japan). Sterile broth served as control. Seedlings were then transferred to 2 L pots containing soil.

Effect of *H. seropedicae* on leaf anthracnose

The dystrophic yellowish-red latosol, soil was collected from layer 0–20 cm in Cerro Negro, Santa Catarina, Brazil. Each kg of soil was previously supplemented with 50 mL nutrient solution (Rodríguez-Salazar et al. 2009) with reduced nitrogen (0.5 mM KNO₃). The physical composition of each kg of soil consisted of 600 g of clay, 350 g of silt and 50 g of sand. Plants were grown in greenhouse under natural light, without temperature control (average temperature about 28 °C), watered daily and weekly were added with 100 mL of nutrient solution per pot.

C. graminicola inoculation was performed at 7, 14 and 21 days after treatment (DAT) with *H. seropedicae*. First, the conidia suspension was filtered through sterile double layer cheesecloth and washed twice by centrifugation at 25 °C and 1098×g for 8 min. Whole plants were sprayed with a suspension of 5×10^5 conidia mL⁻¹ plus Tween 20 (1 µL per 10 mL inoculum) using an atomizer connected to an air compressor (58 psi), delivering about 5 mL plant⁻¹. Plants were placed in a moist chamber for 24 h (100% humidity), in the dark. Then, they were transferred to the greenhouse again. The samples were harvested at 5 days after the pathogen inoculation, when plants were 15 (7 DAT), 22 (14 DAT) and 29-day-old (21 DAT). Two leaves per plant were sampled (F1 and F2), digitally scanned and used to determine the percentages of necrotic and chlorotic leaf area, as well as the total leaf area by means of Quant[®] software (v.1.02, Federal University of Viçosa, 2003).

Effect of *H. seropedicae* on anthracnose of plants grown in humics-amended substrate

The loamy sandy fulvic neosol soil was collected in Florianópolis-SC from layer of 0–20 cm and it was composed of clay 100 kg⁻¹, silt 100 kg⁻¹ and sand 800 kg⁻¹. By the time the maize plantlets were sown, soil was treated with humics (or water). Humics were added (50 mg CL⁻¹), in a proportion of 10% v/m (or water). Humics were originated from vermicompost prepared using sugarcane filter cake as described early (Jindo et al. 2016). Plants were grown in greenhouse under natural light without temperature control (average temperature about 26.5 °C) and watered daily.

C. graminicola inoculation, now using a spore suspension of 1×10^5 conidia mL⁻¹, was performed after 3 and 7 DAT with *H. seropedicae*, as described above. Plants were placed in a moist chamber for 24 h in the dark. Then, they were transferred to the greenhouse again. Five days after

pathogen inoculation, two leaves per plant per time of treatment were detached. Harvested plants were 11 (3 DAT) and 15-days-old (7 DAT). The oldest leaf collected was digitally scanned to determine the percentage of necrotic and chlorotic leaf area and to access the leaf area using the software Quant[®], while the youngest leaf was immediately frozen in liquid nitrogen and stored – 80 °C for further analysis.

H. seropedicae quantification

H. seropedicae SmR1 was quantified by qPCR (expressed as DNA copy number and CFU) using already designed pair of primers (Pereira et al. 2014) and TaqMan probe (Dall'Asta et al. 2017). We estimated *H. seropedicae* DNA copy number/g leaf and *H. seropedicae* CFU/g leaf. Therefore, total DNA was isolated from maize tissue using DNeasy Plant Mini Kit (Qiagen) according to manufacturer instructions, and each extract was pooled of three leaves. DNA isolation from *H. seropedicae* pure culture was performed using Wizard[®] Genomic DNA purification kit (Promega[™], Madison, WI, USA) with modifications. DNA concentrations were assessed on a Thermo Scientific NanoDrop 2000 spectrophotometer (Willmington, DE, USA).

H. seropedicae DNA copy number/g leaf was established as already described, using a standard curve constructed from DNA isolated from *H. seropedicae* pure culture by plotting Cq value versus log *H. seropedicae* DNA copy number (Dall'Asta et al. 2017; Pereira et al. 2014). Furthermore, *H. seropedicae* CFU/g leaf was estimated using a standard curve constructed with DNA isolated from a serial dilution of *H. seropedicae* culture mixed with crushed plant-leaf tissues as described by Stets et al. (2015), with modifications. Maize seeds (P30F53) were surface-sterilized as described above and plants were grown under axenic conditions as in vitro experiment described previously (Pereira et al. 2014). On the 11th day, leaves were harvested and ground in liquid nitrogen using a mortar and pestle. *H. seropedicae* culture (OD₆₀₀ = 0.8; 10⁸ cells of *H. seropedicae* SmR1 mL⁻¹), was serially diluted in autoclaved 0.9% saline buffer ranging from 10⁸ to 10². A volume of 100 µL of each culture dilution was added to 100 mg of crushed leaves, and this mixture was incubated for 1 h in room temperature and stored in ultra-freezer until DNA isolation. DNA was isolated from this whole mixture using DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions and DNA concentrations were assessed by measurement of the optical density at 260 and 280 nm on a Thermo Scientific NanoDrop 2000 spectrophotometer (Willmington, DE, USA). A standard curve was generated by plotting Cq versus number of *H. seropedicae* CFU added to each reaction tube. For

both standard curves generated, amplification efficiencies were determined as described previously (Dall'Asta et al. 2017). qPCR was performed in ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) as described previously (Dall'Asta et al. 2017).

Experimental design and data analysis

Both experiments were performed in completely randomized blocks, with 4 biological replicates. In the first experiment, realized to observe the effect of *H. seropedicae* leaf anthracnose, *T* test was used to compare anthracnose extension in control and treated groups and Wilcoxon–Mann–Whitney was used to compare leaf area assessments. The experiment was repeated twice with similar results. In the second experiment, used to observe the effect of *H. seropedicae* on anthracnose of plants grown in humics-amended substrate, statistical analysis consisted of Kruskal–Wallis test to compare the groups in measurement of chlorotic and necrotic maize leaf area and also to *H. seropedicae* DNA quantification. All tests were conducted under 95% of significance using Assistat (v. 7.7 beta, Federal University of Campina Grande, Brazil, 2016).

Results

Effect of *H. seropedicae* in leaf anthracnose and maize plant growth

Two days after *C. graminicola* inoculation, leaves of control plants presented clarified areas in the site of pathogen entrance. In the third day, these sites evolved and originated oval lesions with tan centers and reddish-brown borders. At this point, some leaves already showed elongated brown lesions, which progressed through necrosis enlargement in the next 2 days, until the leaves harvesting. *H. seropedicae* pre-treatment did not affect the anthracnose disease in leaves of maize plants in this situation (Table 1). It was assessed with two leaves per plant at each analyzed period of time and no relevant differences were observed even at 7, 14 or 21 DAT. Difference was observed just at 14 DAT in one analyzed leaf in chlorotic area. The experiment was repeated twice with similar results. *H. seropedicae* promoted maize leaf growth at 21 DAT, demonstrated through the increment in maize leaf area, however, no differences were observed at 7 and 14 DAT (Table 2).

Effect of *H. seropedicae* and humics in anthracnose and *H. seropedicae* maize colonization

H. seropedicae combined with humics did not affect the maize leaf anthracnose, once disease severity assessment evaluation showed no difference in chlorotic or necrotic leaf area between the groups at 3 or 7 DAT with *H. seropedicae* (Table 3).

In order to confirm *H. seropedicae* colonization in leaves, *H. seropedicae* DNA and CFU were quantified by qPCR using standard curves. The standard curve constructed by plotting Cq x Log *H. seropedicae* DNA copy number (Fig. 1A) presented $R^2 = 0.99$ and $s = -3.4$, which correspond to an amplification *E* of 96%. The standard curve corresponding to Cq x Log CFU *H. seropedicae* (Fig. 1B), showed $R^2 = 0.99$, $s = -3.2$ and $E = 107\%$.

H. seropedicae DNA and CFU quantification presented similar results at 3 and 7 DAT (Fig. 2a, b). In view of *H. seropedicae* treated groups (+H.s – Humics and +H.s + Humics) at 3 DAT, the combined group (+H.s + Humics) tend to improve bacterial colonization in leaves. Despite that, bacterial presence was statistically higher in *H. seropedicae* treated groups (+H.s – Humics and +H.s + Humics) in comparison with *H. seropedicae* untreated groups (–H.s – Humics and –H.s + Humics) at 3 DAT. Regarding bacterial quantification at 7 DAT, *H. seropedicae* DNA and CFU found in *H. seropedicae* untreated groups (–H.s – Humics and –H.s + Humics) were statistically lower than in group with *H. seropedicae* and humics association (+H.s + Humics), suggesting that this consortium favors bacteria colonization.

Discussion

The capacity of *H. seropedicae* SmR1 associated or not to humics to control leaf anthracnose caused by *C. graminicola* was tested and colonization of *H. seropedicae* in maize leaves was assessed. Although no differences were detected in anthracnose severity in treated plants, *H. seropedicae* presence was confirmed, showing that the absence of response is not due to the failure of colonization by *H. seropedicae*.

H. seropedicae colonization in leaves was assessed through *H. seropedicae* DNA and CFU quantification based on standard curves. The reliability of these standard curves was analyzed according to previously established patterns. According to that, reliable standard curves should have R^2 values higher than 0.95 and *slope* between -3.0 and -3.9 , corresponding to PCR efficiencies of 80–115% (Zhang and Fang 2006). All qPCR parameters found in this study (Fig. 1) are in accordance to the parameters

Table 1 Chlorotic and necrotic area of maize leaves pre-treated with *H. seropedicae* and inoculated with *C. graminicola* at 7, 14 and 21 days after treatment (DAT)

<i>C. graminicola</i> inoculation DAT	Leaf ^a	Chlorotic (% area)		Necrotic (% area)	
		Control	H.s	Control	H.s
7	L1	5.0 ± 5.8	9.3 ± 7.7	91.9 ± 5.9	85.8 ± 11.2
	L2	0.4 ± 0.6	0.9 ± 1.4	33.5 ± 8.6	30.1 ± 3.2
14	L1	8.0 ± 5.1	12.7 ± 6.7	70.2 ± 12.1	62.9 ± 12.2
	L2	8.2 ^b ± 1.5	5.1 ± 1.4	13.7 ± 8.3	6.5 ± 4.4
21	L1	2.4 ± 1.7	0.8 ± 1.0	84.8 ± 7.3	89.4 ± 10.0
	L2	1.7 ± 1.2	1.5 ± 1.2	47.4 ± 14.2	64.3 ± 12.9

Mean values correspond to the average of four replicates, each one being comprised of three leaves (n = 12). Data are presented as mean ± SD. The groups represent: Control (non treated with *H. seropedicae*) and H.s (treated with *H. seropedicae*)

^aLeaves were assessed at 5 days after inoculation with *C. graminicola*

^bSignificant difference comparing *H. seropedicae* treated and untreated leaves using Student's *t* test with 95% significance level

Table 2 Maize leaves area (cm²) pre-treated with *H. seropedicae* and inoculated with *C. graminicola* at 7, 14 and 21 days after treatment (DAT)

<i>C. graminicola</i> inoculation DAT	Leaf ^a	Leaf area (cm ²)	
		Control	H.s
7	L1	28.4 ± 3.2	25.8 ± 6.5
	L2	80 ± 10.2	81.1 ± 6.6
14	L1	67 ± 22.3	67.9 ± 20.3
	L2	204.3 ± 26.6	187.7 ± 26
21	L1	38.1 ± 27.9	118.3 ± 38.4 ^b
	L2	58 ± 9.1	176.6 ± 37.4 ^b

Mean values correspond to the average of four replicates, each one being comprised of three leaves (n = 12). Data are presented as mean ± SD. The groups represent: control (non treated with *H. seropedicae*) and H.s (treated with *H. seropedicae*)

^aLeaves were assessed at 5 days after inoculation with *C. graminicola*

^bSignificant difference using Wilcoxon-Mann-Whitney with 95% significance level

established by Zhang and Fang (2006). Moreover, the present study's values represent reliable standard curves even if using the more restricted parameters (ENGL 2008), which settles minimum performance requirements for qPCR analytical methods ($R^2 \geq 0.98$, slope between - 3.1 and - 3.6, and E values from 90 to 110%). The method of using a mix of *H. seropedicae* pure culture plus maize tissue to establish the CFU/g plant tissue was used to quantify *H. seropedicae* presence in maize. This method, that combines the bacterial and plant tissue in a single extract to perform the standard curve was already used to determine *Azospirillum* sp. quantity in presence of matrix as DNA template to qPCR reactions (Couillerot et al. 2010a, b; Stets et al. 2015). However, in our study, the method was used for *H. seropedicae* CFU quantification. To the best of our knowledge, it was the first time that this method was used to estimate *H. seropedicae* CFU/g plant tissue.

Regarding maize leaf colonization (Fig. 2), results showed a tendency of increment in *H. seropedicae* DNA

Table 3 Chlorotic and necrotic area of maize leaves pre-treated with *H. seropedicae* with or without humic substances and inoculated with *C. graminicola* at 3 and 7 days after treatment (DAT)

<i>C. graminicola</i> inoculation DAT	Chlorotic (% area)				Necrotic (% area)			
	Control	-H.s +Humics	+H.s -Humics	+H.s +Humics	Control	-H.s +Humics	+H.s -Humics	+H.s +Humics
3 ^a	1.9 ± 3.7	1.3 ± 2.7	3.9 ± 2.9	1.4 ± 2.8	18.8 ± 6.9	15.5 ± 3.5	14.2 ± 7.8	15.4 ± 2.3
7	7.6 ± 2.8	11.5 ± 1.3	7.0 ± 3.8	7.8 ± 3.0	56.9 ± 11.1	50.5 ± 13.9	54.3 ± 11.6	63.9 ± 10.2

Mean values correspond to the average of four replicates, each one being comprised of three leaves (n = 12). Data are presented as mean ± SD. The groups represent: -H - HS (non-treated, without humic substances), -H + HS (non-treated, with humic substances), +H - HS (treated, without humic substances) and +H+HS (treated, with humic substances). No significant differences were obtained using Kruskal-Wallis tests ($\alpha = 0.05$)

^aAssessments were realized at 5 days after inoculation with *C. graminicola*

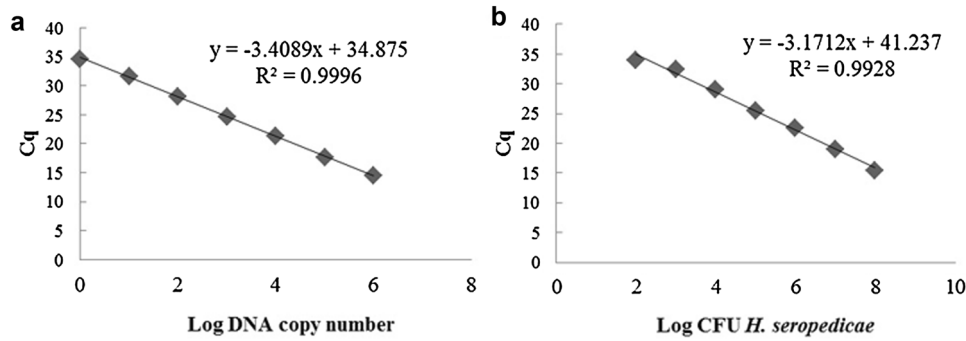
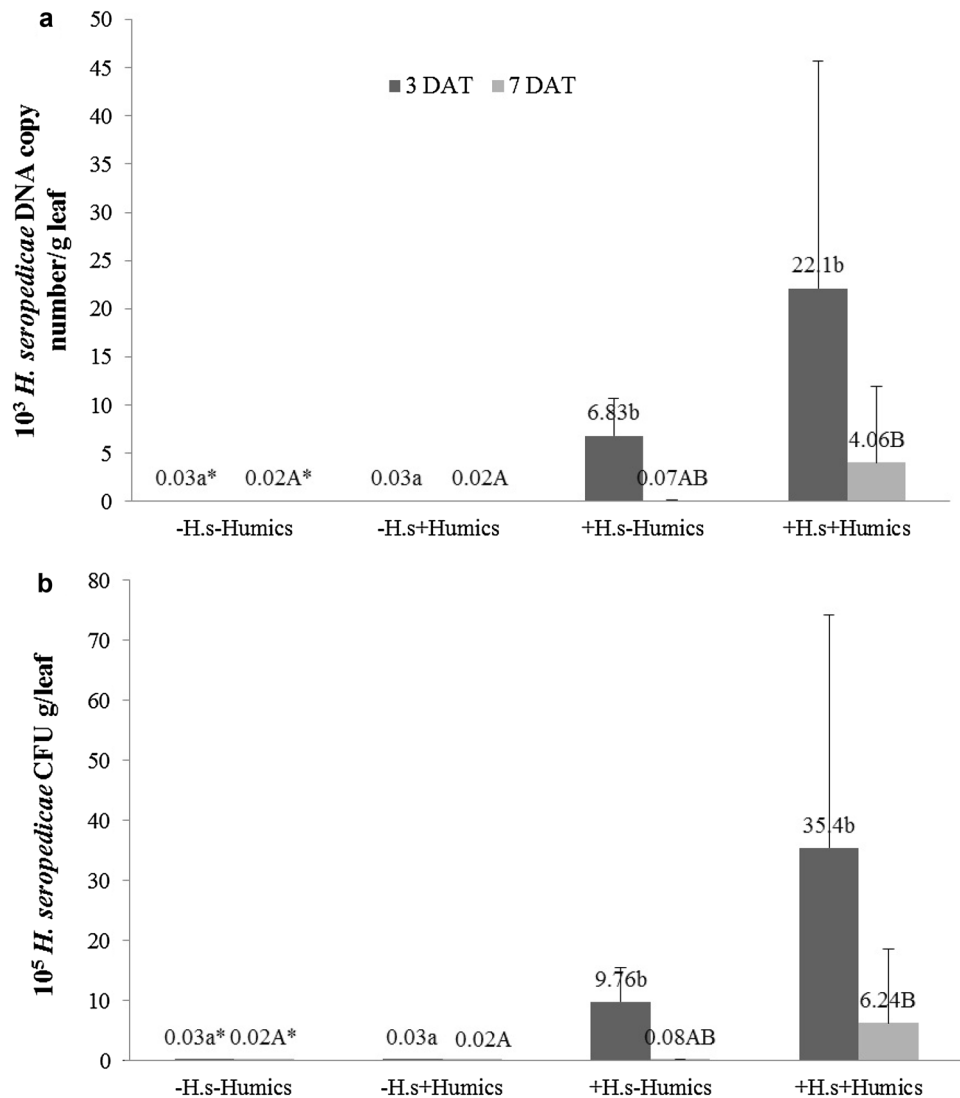


Fig. 1 qPCR standard curves for *H. seropedicae* quantification. **a** Cq versus log DNA copy number. Curve generated using DNA isolated from pure culture of *H. seropedicae* SmR1 as template. **b** Cq versus

log CFU. Curve generated using DNA isolated from 100 μ L of each serial dilution of *H. seropedicae* culture mixed with 100 mg of crushed in vitro plant-leaf tissues as template

Fig. 2 *H. seropedicae* DNA quantification in leaves of P30F53 *Zea mays*, after inoculation with *C. graminicola* 3 and 7 days after treatment (DAT) with *H. seropedicae* and with or without humic substances. The groups represent: –H.s – Humics (non treated with *H. seropedicae*, without humics), –H.s + Humics (non treated with *H. seropedicae*, with humics), +H.s – Humics (treated with *H. seropedicae*, without humics) and +H.s + Humics (treated with *H. seropedicae*, with humics): **a** *H. seropedicae* DNA copy number/g leaf (fresh weight) using standard curve generated with DNA isolated from *H. seropedicae* pure culture. **b** *H. seropedicae* CFU/g leaf (fresh weight) using standard curve generated with DNA isolated from serial dilution of *H. seropedicae* culture mixed with crushed plant-leaf tissues. Values presented as mean \pm standard deviation (n = 8). Lowercase and capital letters indicate statistical difference using Kruskal–Wallis test ($\alpha = 0.05$) at 3 and 7 DAT, respectively



copy number and CFU when *H. seropedicae* was associated with humics, if compared to *H. seropedicae* treatment isolated at 3 DAT, despite not statistically significant. At 7 DAT, it was shown that bacteria associated with humics

(+H.s + Humics) presented highest bacteria presence in comparison to *H. seropedicae* non-treated groups (–H.s – Humics and –H.s + Humics). It was already shown that humics helped the maintenance of *H.*

seropedicae population in maize roots (Canellas et al. 2013), mainly using vermicompost extracted from sugarcane cake residue (Martinez-Balmori et al. 2013). In addition, it was shown that coating maize seeds with *H. seropedicae* Z67 and humics increased the number of beneficial bacteria viable cells (da Conceicao et al. 2008). Recently it was shown that humics enhance maize root border cells survival at the same time that increase the fitness of the plant/microbial interaction. In this sense, humics are able to improve colonization of the root cap zone and influence the number and shape of border cells in maize root tips (Canellas and Olivares 2017). Despite that, our colonization results were effective, showing the accuracy of HERBAS1 pair of primers in detecting *H. seropedicae* in planta (Pereira et al. 2014), even in maize leaves inoculated with a phytopathogen and in variable experimental conditions.

At 21 DAT, *H. seropedicae* treated leaves presented an increment in its area in comparison with untreated ones, what can indicate that *H. seropedicae* promoted leaf growth. *H. seropedicae* inoculation has already shown to improve maize yield up to 34%, depending on the plant genotype (Alves et al. 2015) as well as increase maize dry weight and shoot and root lengths (Ribaudou et al. 2006). Increase in dry and fresh weight of rice plants inoculated with *H. seropedicae* was also observed at 28 (James et al. 2002) and at 30 days after inoculation (Baldani et al. 2000; Gyaneshwar et al. 2002). In fact, in view of *H. seropedicae* SmR1 genome, some genes related to plant growth promoting characteristics were identified, such as nitrogen fixation, siderophores production (and their receptors) and auxin synthesis genes, as well as a 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase gene (Monteiro et al. 2012; Pedrosa et al. 2011).

Maize plants were effectively colonized by bacteria *H. seropedicae* in this study, and the increased leaf area in treated plants observed can serve as an indicative of plant growth promotion by *H. seropedicae*. However, concerning the assessment of *H. seropedicae* control of anthracnose disease in maize plants, *H. seropedicae* SmR1 plants treatment do not exert biocontrol activity against anthracnose in maize. The association between this PGPB and humics was tested. The use of integrated disease management strategies to effectively manage diseases has been suggested as an alternative, because in general only a biocontrol agent may not be able to play this role by itself (Sahni et al. 2008). However, in our study, even the use of *H. seropedicae* SmR1 combined with humics management was not able to control the disease caused by *C. graminicola* in maize plants. In this work we tested only the SmR1 *H. seropedicae* strain and taking in account the existence of other available strains and beneficial bacteria species, there is the possibility that other strains (da Conceicao et al.

2008; Marques et al. 2008), the consortium of *H. seropedicae* strains or even with other bacteria species (da Silva et al. 2017) may be able to exert any biological activity against disease or other beneficial effect. Additionally, *C. graminicola* is considered a highly aggressive phytopathogen (Bergstrom and Nicholson 1999) and the genotype of maize contributes to the severity of symptoms resulting from infection with *C. graminicola* (Weihmann et al. 2016), it would be interesting to test its effect against other isolates and on maize plants exhibiting different levels of resistance.

Conclusion

Our data show that HERBAS1 qPCR assay efficiently quantify *H. seropedicae* plant growth promoting bacteria in maize leaves even in the presence of *C. graminicola* pathogen. Treatment with *H. seropedicae* and humics-amended substrates were not able to control anthracnose in P30F53 maize, although humics presence augmented *H. seropedicae* colonization in plants. Leaf area was increased in *H. seropedicae* colonized plants compared to control plants, *H. seropedicae* inoculation of maize leads to leaf growth promotion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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