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Biocontrol of *Xanthomonas axonopodis pv. passiflorae* With Bacteria Isolated From Passion Fruit

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Abstract

The yellow passion fruit *Passiflora edulis Sims f. flavicarpa Deg* is affected by the bacterium *Xanthomonas axonopodis pv. passiflorae* (Pereira) Dye, causing the bacterial blight disease, for which no effective bactericide is available. The use of live microorganisms in the treatment of pests have shown positive results in the control. This research sought to select bacteria residing in the passion fruit phyllosphere to function as a biocontrol agent of *X. axonopodis pv. passiflorae*. The selected bacteria were isolated from the passion fruit tree leaves collected in four municipalities of the state of Roraima, and thermostable compounds test and greenhouse test were performed. In total, 28 bacterial isolates were obtained. In the test with thermostable compounds, the isolates RR02, RR04, RR06, RR14, RR16, RR17, RR19, RR20, RR21, RR22, RR23, RR25, and RR27 stood out. In the greenhouse tests, the isolates RR03, RR07, RR09, RR13, RR16, RR21, and RR23 stood out. The results showed potential for the control of *X. a. pv. passiflorae* in *in vitro* and *in vivo* tests.

Keywords: antibiosis, passion fruit, bacterial blight, severity, thermostable compounds

1. Introduction

The yellow passion fruit (*Passiflora edulis Sims f. flavicarpa Deg.*) belongs to the Passifloraceae family composed of 18 genera and 630 species, from which the genus *Passiflora* stands out due to its economic importance. The passion fruit tree is characterized as a tropical climate plant with wide geographical distribution of its product. This fact contributes to the increase in the production of the fruit, with promising results for the Brazilian economy. The fruit can be consumed *in natura* or in pulp form, and it can also be used in the pharmaceutical and pharmacological industries. (Wagner Júnior et al., 2021).

Despite being an important crop for the country's economy, the cultivation of passion fruit presents many challenges caused by fungi, viruses, and bacteria. The most recurrent issue is the passion fruit bacterial blight, caused by the bacterium *Xanthomonas axonopodis pv. passiflorae* (Pereira) Dye (Ereno, 2011).

The first occurrence of the bacterium in Brazil was observed in 1967, in the state of São Paulo. In Roraima, the first record of the disease occurred in July 2005, affecting the yellow passion fruit in a cultivation in the Monte Cristo region (Halfeld-Vieira & Nechet, 2006).

The symptoms of bacterial blight can be observed by a soaking aspect of the tissue; the presence of dark green spots on the leaves; and, in its initial stage, by lesions present in the leaf margins, which advance to severe burning and yellowish halo around the necrotic tissue, leading to the defoliation of the plant (Halfeld-Vieira & Nechet, 2006).

This bacterium is resistant to any existing chemical controls (Junqueira, 2011). Manica (2022) states that the passion fruit culture has few registered treatment products, most of which are formulated with copper hydroxide, copper oxychloride, and kasugamycin. Agrolink (2023) indicates the following products Auge®, Fegatex®, Kasumin®, Supera/Swat®, and Timorex Gold®.

To obtain a product that is effective and less aggressive to the environment, researchers study the control of pests and diseases with the application of microorganisms, a practice known as biocontrol. These works use the population of microorganisms that live in the phyllosphere of plants given the great diversity of biotic and abiotic events that determine their dynamics and the existence of more resistant microorganisms in antagonistic activity (Macagnan et al., 2021). The study by Wang et al. (2008) verified that biological controls cause minimal environmental impact, which can lead to the improvement of cultivation techniques, thus avoiding great losses caused by diseases in the crop.

2. Method

2.1 Collection of Plant Material and Isolation of Bacteria

The leaves of the passion fruit tree were collected in the state of Roraima, in the municipalities of Boa Vista, Cantá, Mucajaí, and Pacaraima; leaves of plants without application of chemical pesticides were favored due to their greater variety of bacteria with antagonistic potential.

The samples were processed at the Plant Pathology Laboratory of EMBRAPA (Brazilian Agricultural Research Corporation) Roraima. Half of a healthy passion fruit leaf was deposited in a 250 mL Elenmeyer flask containing 100 mL of sterile sodium chloride solution (0.85% NaCl) and was stirred for 50 min.

For each extract, a serial dilution was performed up to 10^{-8}, and 100 µL of the solutions obtained were added to Petri dishes containing solid culture medium 523 (Kado & Heskett, 1970), being spread on the surface with a Drigalski spatula for sowing and, subsequently, the plates were kept in a BOD incubator at 27 °C for 24 h (Halfeld-Vieira et al., 2015).

2.2 Pathogenicity Test

For the pathogenicity test, an isolate of *X. a. pv. passiflorae* was taken from the collection of bacteria culture of the Plant Pathology Laboratory of EMBRAPA Roraima, which was lyophilized and stored in a refrigerator. The bacteria were activated in liquid culture medium 523.

After 24 h, with the aid of a metal handle, the bacteria were sown in Petri dishes containing solid medium 523. Once its growth was observed, the bacteria isolated in 0.85% sodium chloride solution were added and sprayed on seedlings; after which they were kept in a moist chamber for 24 h. The seedlings were evaluated daily, a plate was then prepared with the diseased part for analysis under a microscope (Silva et al., 2020).

2.3 Testing With Thermostable Compounds

The bacteria selected for thermoresistant antagonistic activity were activated in liquid culture medium 523; after being stirred for 72 h, they were taken to the autoclave for 20 min, at a temperature of 121 °C, to obtain only the compounds resistant to the submitted temperature.

From the material, 5 mL was pipetted into 50 mL of molten solid culture medium 523 and poured into 9 cm Petri dishes.

With *X. a. pv. passiflorae* activated in liquid medium, serial dilution, up to 10^{-8}, was performed in 0.85% sodium chloride solution, and 100 µL were transferred in Petri dishes for each repetition. The evaluation was performed after 24 h, by counting colonies with the aid of a magnifying glass.

2.4 Greenhouse Testing

For the greenhouse test, yellow passion fruit (*P. edulis Sims f. f. Degs*) seeds were obtained from the crops at BRS Gigante Amarelo and were planted in tubes with substrate sand, soil, and manure (1:1:1), prepared at EMBRAPA Roraima; the growth of the seedlings was monitored until they had the ideal size for the *in vivo* control test, which was defined as having eight true leaves.

The bacteria selected for antagonistic activity were replicated in test tubes containing solid culture medium 523 and maintained in a BOD incubator at 27 °C, for 24 h. After this period, 15 mL of 0.85% NaCl solution was added and stirred for 30 seconds to obtain a homogeneous solution of each bacterium.

In the greenhouse, with the aid of sprinklers, each plant was colonized with an isolated antagonist candidate by spraying the bacterial suspension, maintaining them in a moist chamber for 24 h.

After 24 h of colonization, the plants were sprayed with a suspension of *X. a. pv. passiflorae* cells, which were taken to a moist chamber for 24 h with the aid of a plastic bag.

The experimental design adopted was completely randomized, with 5 repetitions. After 14 days of inoculation, the plants were evaluated according to the severity of the disease, compared to the diagrammatic scale elaborated by Miranda (2004).

2.5 Statistical Analyses

The data for the variables 'Colony Formation' and 'Isolate Severity' were submitted to the Shapiro-Wilk normality test. Once the distribution normality was observed, analysis of variance was applied, followed by the Skott-Knott test ($p > 0.05$). When normality was not reached, the Kruskall-Wallis test (KW) was used, followed by the Dunn post-test ($p > 0.05$). All analyses were performed with the aid of the Prisma GraphPad software, version 9.1.4.

3. Results

In total, 28 bacteria were obtained from the phyllosphere of the passion fruit tree leaves collected in Boa Vista, Cantá, Mucajaí, and Pacaraima, all with different physical characteristics, namely color, border shape, and surface.

The bacterial isolate of *X. a. pv. passiflorae* (RRXAP01), conserved and lyophilized, presented growth in culture medium 523. Fourteen days after the inoculation of the plants for the pathogenicity test, the symptoms of the disease were identified: lesions at the borders of the leaf; soaking aspect of the tissue, with dark green coloration; yellowish halo around the necrotic tissue; and the defoliation of the plant. These symptoms are described in a study by Halfeld-Vieira; Nechet (2006). Bacterial exudation in the diseased tissues was also verified via light microscopy. Tissue fragments were macerated and sown in culture medium 523 to confirm pathogenicity.

3.1 Testing With Thermostable Compounds

Figure 1 shows the values of the Colony Forming Units (CFU.mL-1.10^8) of *X. a. pv. passiflorae* for each bacterial isolate tested, against the antagonistic activity by means of direct inhibition by thermostable compounds.

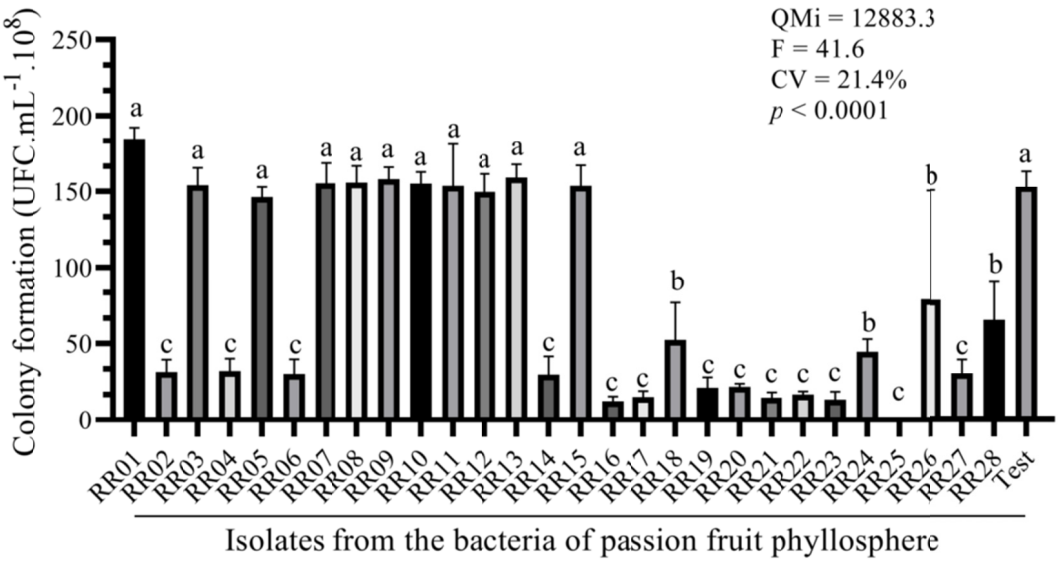

Figure 1. Values of the Colony Forming Units (CFU) of *X. a. pv. Passiflorae* when submitted to direct contact with thermostable compounds produced by bacteria isolated from the passion fruit phylloshpere. Isolates with the same letter on the bars do not differ from each other, according to the Skott-Knott test ($p > 0.05$). Mean chi-square of isolates; F: F-test in ANOVA; CV: coefficient of variation

3.2 Greenhouse Testing

Regarding the results of the greenhouse test (Figure 2), the percentage of disease severity was evaluated after 14 days of inoculation. In this evaluation, the diagrammatic scale was used for leaves in the seedling phase, available in the work of Miranda (2004), with the following percentages: 2%, 5%, 11%, 26%, and 59%.

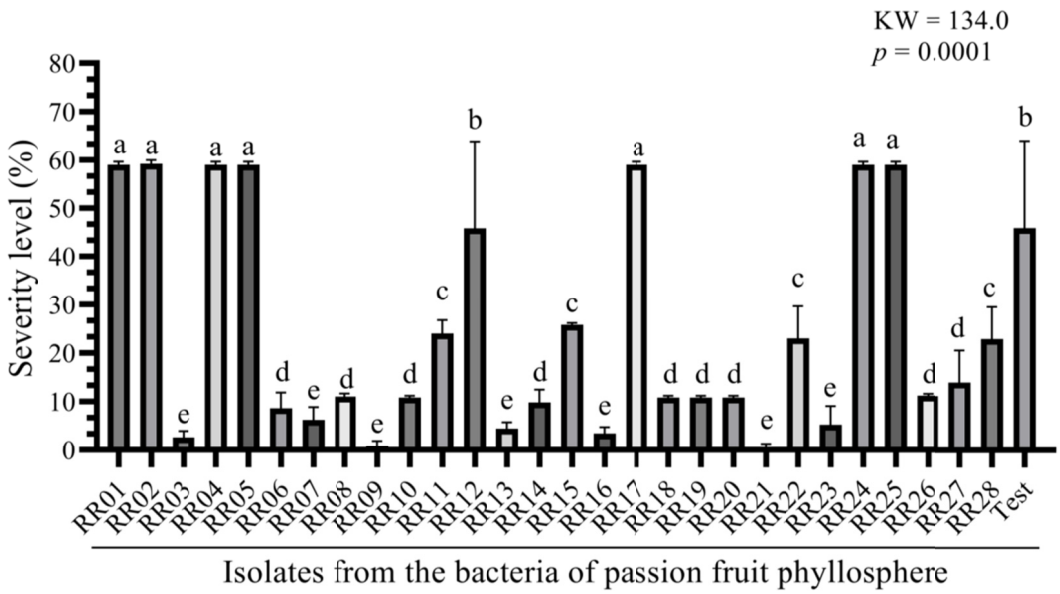

Figure 2. Severity levels of *X. a. pv. Passiflorae* in passion fruit seedlings, presented after 14 days of exposure in leaves previously treated with the bacteria isolated from the passion fruit tree phyllospheres. Isolates with the same letter are in the same group in the ranking formed by the Dunn post-test ($p > 0.05$). KW: Kruskall-Wallis test value

In Figure 3A, C, E and G, one can observe the difference between the samples used as controls and the three isolates with better results.

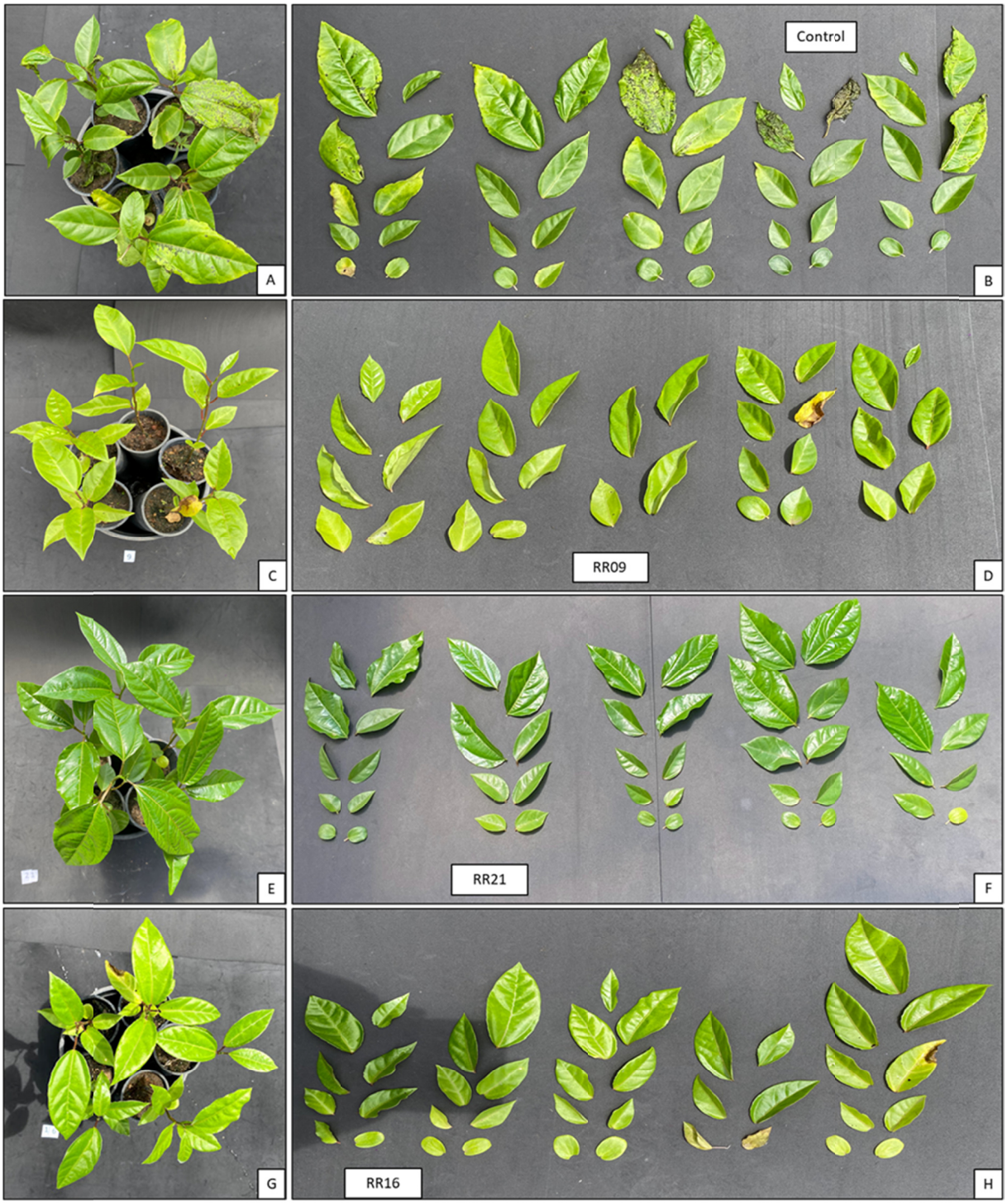

Figure 3. Seedlings after 14 days: A) Control; B) Leaves of the control seedlings arranged for better visualization of the disease; c) RR09 isolate; D) Leaves of the isolate RR09 seedlings arranged for better visualization of the disease; e) RR21 isolate; F) Leaves of the isolate RR21 seedlings arranged for better visualization of the disease; G) RR16 isolate; H) Leaves of the isolate RR16 seedlings arranged for better visualization of the disease

4. Discussion

4.1 Pathogenicity Test

Based on the results herein presented, we were able to isolate the bacteria from the phyllosphere of the passion fruit of four municipalities of the state of Roraima. In the study by Silva (2013), 102 bacteria were isolated from the phyllosphere of the passion fruit from the state of Roraima.

As mentioned in the results, there are a variety of isolates, which can be classified by groups based on their physical characteristics. Notably, it is not possible, with this analysis alone, to identify the bacterial strains.

4.2 Testing With Thermostable Compounds

Observing Figure 1, we can verified that the CFU values of the isolates RR01, RR03, RR05, RR07, RR08, RR09, RR10, RR11, RR12, RR13, and RR15, did not present statistical difference when compared with the control; thus, they did not present potential in the treatment of *X. a. pv. Passiflorae.*

The lowest CFU values are presented in ascending order: RR25, RR16, RR17, RR23, RR21, RR22, RR19, RR20, RR27, RR14, RR06, RR02, and RR04; all of which showed a reduction of more than 70% in the formation of colonies in the Petri dish.

Fernandes (2019), in their study, considered as significant the result with percentages higher than 50% of inhibition of the pathogen. This fact enhances the efficacy of the isolates of our study, since only reductions greater than 70% were adopted as a significant result.

Additionally, these tested compounds can be considered thermostable since no bacterial growth of the selected isolates for antagonistic activity was observed after the evaluation period.

Fernandes (2019) highlights that these thermostable compounds are highly applicable in *in vivo* conditions since they can tolerate heat from the environment. Especially for the passion fruit crops that are predominantly grown in tropical climates, such as the one in Roraima that presents high temperatures.

4.3 Greenhouse Testing

Figure 2 shows that the isolates RR01, RR02, RR04, RR05, RR12, RR17, RR24, and RR25 presented severity levels equal to or higher than 59%. Not inhibiting symptoms of the disease in the leaves of passion fruit plants.

The isolates RR06, RR08, RR10, RR14, RR18, RR19, RR20, RR26, and RR27 presented 5% of the symptoms of the disease (Figure 3).

The seedlings that were treated with the isolates RR03, RR07, RR09, RR13, RR16, RR21, and RR23 presented the lowest percentages of disease severity levels (less than or equal to 2%).

In Figure 4B, the control, which presented 59% of signs of the disease, shows leaf falls and intense yellowing, in addition to the complete wilting of some leaves.

Figure 4D shows the seedlings that were sprayed with isolate 9, here the disease manifested itself in only one of the leaves of the sample. When compared with the control, isolates 9, 21, and 3 reduced the symptoms of the disease by 98.61%, 98.61%, and 95.83%, respectively. In Figure 4F, referring to isolate 21, we can observe that only one leaf presented a slight sign of the disease.

It is also worth mentioning that isolate 21 presented a low severity of the disease, in addition to a greater growth of the seedlings and a greater intensification of their green coloration.

Another important fact to be highlighted is that isolate 21 also showed efficient results in the *in vitro* test with thermostable compounds, confirming its effectiveness against bacterial blight. Isolate 16 also showed control potential in both tests but did not indicate promotion of seedling growth.

In Silva's (2013) research, the severity levels of *X. a. pv. Passiflorae* ranged from 1.1% to 27.5% for bacteria isolated from the state of Roraima, Pará, and São Paulo, and in the state of Roraima their isolates presented a maximum 15.3% severity. In our study, 17 isolates presented similar inhibition percentages. Silva et al. (2008), when the evaluation of the level of severity, observed a 39.1% reduction regarding the symptoms of the disease caused by *Xanthomonas axonopodis pv. phaseoli* (Xap) in beans (cv. BRS Valente). In Rocha & Moura (2013), they significantly reduced fusarium wilt, ranging from 22.5% to 76% in tomato, caused by *Ralstonia solanacearum* and *Fusarium oxysporum f. sp. lycopersici* (FOL).

The study of Silva (2013) draws attention to the fact that, despite the wide variety of bacteria present in the phyllosphere of plants, only a small part showed antagonistic activity. This observation is in agreement with our research, in which only isolates 16 and 21 showed positive results for both pathogen control tests.

In our study, of the 28 tested isolates, seven presented a percentage of inhibition of up to 2%, corresponding to 25% of the total number of isolates. When compared with the studies of Silva (2013), from the total of 224 bacteria tested, only 4.4% (which corresponds to 10 isolates) showed potential for the inhibition of *X. a. pv. Passiflorae.*

5. Conclusion

The results of the present study revealed that some of the bacteria isolated from the phylloplane of passion fruit leaves have antibacterial properties for the control of *X. axonopodis pv. passiflorae* in *in vitro* and *in vivo* tests in a greenhouse, being an alternative and less aggressive option for the environment.

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