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Potential of *Pseudomonas* and *Trichoderma* from the Brazilian Amazon as Biocontrol Agents against the Wheat Blast Disease

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Abstract: Blast is one of the most significant wheat diseases, causing high yield losses in susceptible varieties under favorable conditions in Latin America, Southeastern Asia and Eastern Africa. The disease is caused by the ascomycetous fungal pathogen *Pyricularia oryzae Triticum* lineage (*PoTl*). Chemical control with fungicides has been used as a management strategy; however, the effectiveness of the major classes of high-risk site-specific systemic fungicides has been reduced due to the widespread prevalence of resistance, especially in Brazil. Biological control is seen as a highly important and sustainable strategy to minimize the impact of yield losses associated with wheat blast in areas where fungicides are ineffective. In our study, we specifically aimed to determine the biological control potential of the three isolates of fluorescent *Pseudomonas* and three of *Trichoderma* as the antagonists of *PoTl*, both in in vitro and under greenhouse conditions. Additionally, we aimed to describe the ultrastructural interactions among the biocontrol agents and the pathogen in vitro by means of scanning electron microscopy (SEM). Fluorescent *P. wayambapalatensis* 'Amana' or *Pseudomonas* sp. nov. 'Yara', both from the *P. putida* group, and *Trichoderma koningiopsis* 'Cachara' significantly reduced *PoTl* in vitro mycelial growth and the blast disease severity on wheat plants. The SEM analyses revealed ultrastructural antagonistic mechanisms: biofilm formation, direct antagonism and mycoparasitism. Further research on the topic should include the development of stable formulations of the *Pseudomonas*- and *Trichoderma*-based biocontrol agents selected in our study for managing the wheat blast disease and the field tests of the biofungicide formulations obtained thereafter.

Keywords: biocontrol; antagonism; *Pseudomonas*; *Trichoderma*; *Pyricularia oryzae Triticum* lineage

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1. Introduction

Wheat blast is one of the most significant cereal diseases in countries from Latin America (Brazil, Bolivia, Paraguay and Argentina), Southeast Asia (Bangladesh) and Eastern Africa (Zambia), causing high yield losses on susceptible varieties under favorable weather conditions [1,2]. In certain Northern America and the European Union countries, wheat blast has been designated to be a major quarantine disease [3]. The pathogen associated with wheat blast is the ascomycetous fungus *Pyricularia oryzae Triticum* lineage (*PoTl*) [4,5]. *PoTl* mainly attacks the heads and spikelets of wheat plants. Initial symptoms on heads begin as bleached-centered elliptical lesions on glumes. The fungus may infect the rachis, resulting in the partial or total sterility of the heads and empty grains. The spikelets above the infection point in the rachis die and become white bleached. Sporadically, under highly favorable conditions, leaf spots may also be detected on infected wheat plants [6].

The integrated disease management (IDM) of wheat blast precludes the adoption of several strategies, including crop rotation to minimize fungal infection from primary inoculum derived from perithecia formed on crop residues; the use of certified pathogen-free seeds; changing the sowing dates to avoid coincidence of the plant's flowering stage with disease-favorable weather conditions; regional diversification of cultivars based on the pathogen's predominant virulence groups; and fungicide sprays [7]. However, due to the pathogen's high genetic and virulence diversity, resistance to wheat blast is not durable, rendering IDM fully dependent on chemical control based on spraying systemic fungicides on the plant ears [7]. However, the effectiveness of the major site-specific systemic fungicide classes labeled for wheat diseases management (such as strobilurins, triazoles and succinate dehydrogenase inhibitors) is considered very low due to the widespread distribution of resistance in the country [8–11]. Therefore, considering the serious scenario of the lack of durable resistance combined with the ineffectiveness of systemic fungicides, biological control emerges as an important sustainable management strategy against wheat blast and its resulting high yield losses. So far, there have been no biofungicides labeled by the Ministry of Agriculture, Livestock and Supply (MAPA) [12] for managing wheat blast in Brazil.

Fungal and bacterial antagonists play an important role as microbial biocontrol agents (BCAs) in managing plant pathogens and diseases and can be delivered as biofungicides [13–15]. Biofungicides used for the biological control of plant pathogens are microorganisms-based formulations, which include antagonistic fungi [16,17] and bacteria [18–22]. Among fungi-based formulation, species from the genus *Trichoderma* are the most common biocontrol agents [23–26]. In comparison, fluorescent species from the genus *Pseudomonas* are the most common antagonists among bacteria-based formulations, with emphasis on the *P. fluorescens* and *P. putida* groups, which are also reported as plant growth promoting bacteria [27–30]. The development of biofungicides for managing wheat blast aims to meet the growing demand of modern society for more sustainable, less environmentally impactful agriculture and higher food safety derived from agricultural produce with lower pesticide residues [22,31].

Considering the pressing need for a sustainable management strategy to control wheat blast in Brazil, the present study aimed to determine the potential of antagonistic bacteria and fungi for the biocontrol of the disease caused by *PoTl*. Three fluorescent *Pseudomonas* species [*P. wayambapalatensis* and two *Pseudomonas* sp. nov. (one from the *P. putida* group and another from the *P. asplenii* group] and three *Trichoderma* species (*Trichoderma koningiopsis*, *T. lentiforme* and *T. virens*), all obtained from naturally suppressive Amazon soils from Brazil, were selected for this study. Their role as biocontrol agents of another foliar disease on a Poaceae host have been previously characterized by Nunes [32] and Vicentini et al. [30] using foliar sprays of formulations. Our intent was to explore ways to expand their scope as biocontrol agents against wheat blast. We hypothesize that these bacteria and fungi have extended biocontrol capabilities, which include the wheat blast disease. If this hypothesis holds true, follow up developments on formulations could result in the labelling of the first biofungicide for wheat blast control in South America.

2. Materials and Methods

For this study, fluorescent *Pseudomonas* bacteria and fungal antagonists from the genus *Trichoderma* were bio-prospected from naturally suppressive Brazilian Amazon soils in Paranaita County, Mato Grosso State. They were previously characterized as effective biocontrol agents against the leaf blight and sudden death diseases of the forage grass pasture *Urochloa brizantha*, caused by the basidiomyceteous fungus *Rhizoctonia solani* AG-1 IA [30,32,33] (Table 1).

Table 1. Fluorescent *Pseudomonas* bacteria and fungal antagonists from the genus *Trichoderma* used in this study.

Isolates	Species	References
Amana	*Pseudomonas wayambapalatensis* (*P. putida* group)	Vicentini et al. [30]
Poti	*Pseudomonas* sp. nov. (*P. asplenii* group)	Vicentini et al. [30]
Yara	*Pseudomonas* sp. nov. (*P. putida* group)	Vicentini et al. [30]
Cachara	*Trichoderma koningiopsis*	Nunes [32]
Jaú	*Trichoderma virens*	Nunes [32]
Jurupoca	*Trichoderma lentiforme*	Nunes [32]

2.1. In Vitro Antagonism of Fluorescent Pseudomonas against the Wheat Blast Pathogen

The *PoTl* colonies were grown on potato dextrose agar medium (PDA: potato dextrose, 20.8 g L^{-1}; agar, 15 g L^{-1}) supplemented with chloramphenicol and streptomycin (50 µg mL^{-1} of each) and incubated at 25 ± 0.2 °C for 15 days and 12-h photoperiod. Three fluorescent *Pseudomonas* spp. strains (Amana, Poti and Yara) were grown in a liquid Luria-Bertani culture medium (LB, 20 g L^{-1}) in a shaker for 12 h at 28 °C and 200 rpm, when the final optical density at 620 nm (OD_{620}) of the culture was measured and adjusted to ≈0.8.

The in vitro antagonism experiment was established in a completely randomized design, with 4 repetitions, by pairing 7-mm-diameter mycelial colony disks from 3 individual *PoTl* isolates available in our fungal collection (12.1.146, 12.1.207, and 12.1.047, obtained in 2012 from infected wheat plants sampled in Mato Grosso do Sul, Rio Grande do Sul and Brasilia, respectively) with 3 strains of the antagonistic bacteria from fluorescent *Pseudomonas* species. The antagonist bacteria inoculum consisted of 1 mL of LB liquid medium containing the antagonist at $OD_{620} \approx 0.8$). The pairings were set on Petri dishes containing King B medium by positioning the individual *PoTl* isolate in the center of the plate and the three bacterial strains on a triangle shape with each edge at 0.5 cm from the plate's margin. A negative control was included (LB medium only). The pairings between *PoTl* and the antagonists were incubated for 7 days at 25 °C.

The fungal pathogen mycelial growth (C) was measured 7 days after the pairings, using the methodology of Camporota [34] adapted by Vicentini et al. [30], in which C = DT/DE*100, where DT is the growth radius of the *PoTl* colony towards the antagonistic *Pseudomonas* bacteria and DE, the distance separating the two colonies. The data were analyzed using the *F* test to detect the significance of the treatment effect and the Tukey test at 5% for comparison between means. The experiment was repeated once.

2.2. In Vitro Antagonism of Trichoderma against the Wheat Blast Pathogen

The *PoTl* colonies were grown on PDA culture medium with chloramphenicol and streptomycin, as described in Section 2.1, and incubated at 25 ± 0.2 °C for 15 days for a 12-h photoperiod. Antagonistic *Trichoderma* spp. isolates [32] were initially reactivated and then also cultivated for inoculum production in PDA medium with chloramphenicol and streptomycin and incubated at 25 °C for a 12-h photoperiod for 5 days.

The experiment was set up in a completely randomized design, with 4 repetitions, by pairing 4-mm-diameter mycelial colony disks from 3 individual isolates of *PoTl* (12.1.146, 12.1.207, 12.1.047) with *T. koningiopsis* Cachara, *T. virens* Jaú and *T. lentiforme* Jurupoca. A negative control without the antagonistic fungi was also included. The pairings were set on Petri dishes containing PDA medium by positioning the individual *PoTl* isolate in the center of the plate and a mycelium disc from a single antagonistic *Trichoderma* isolate positioned on the opposite side at 0.5 cm from the plate's margin.

The fungal pathogen mycelial growth (C) was measured 7 days after the pairings, using the methodology of Camporota [34], as described in Section 2.1. The data were analyzed similarly to the method described in Section 2.1. The experiment was repeated once.

2.3. Scanning Electron Microscopy Analyses of In Vitro Pathogen–Biocontrol Agents Interactions

The samples chosen for the ultrastructural studies of the interactions between biocontrol agents and *PoTl* comprised the treatments from the in vitro experiments described in the Sections 2.1 and 2.2: (1) Amana vs. *PoTl*; (2) Poti vs. *PoTl*; (3) Yara vs. *PoTl* and (4) *T. koningiopsis* Cachara vs. *PoTl*. Colony disks from these antagonism experiments were sampled at 7 days after the pairings, fixed in 70% formalin acetic alcohol (FAA) [35] and stored under refrigeration. The fixed samples were dehydrated in ethanol series treatment (at 70, 80, 90 and 99.5%), dried at critical point and metallized with gold. The images were acquired using a Zeiss EVO/LS15 Scanning Electronic Microscope at the Chemistry–Physics Department (at Unesp Ilha Solteira Campus). The scanning electron microscopy analyses were conducted to characterize the biocontrol agents antagonist action against *PoTl*.

2.4. Potential of Pseudomonas and Trichoderma as Biocontrol Agents Controlling Wheat Blast

Seeds of wheat plants cv. TBIO Sossego (Biotrigo Genética) with no fungicide treatment were sown in 700 mL pots containing the plant substrate Topstrato HT Vegetables and kept at greenhouse conditions at 26 ± 2 °C and 70% relative humidity. The pots were irrigated daily and fertilized every 20 days with 0.7 g N-P-K (10-10-10) per pot. Thinning was carried out at 15 days after emergence (DAE) leaving only three plants per pot.

The inoculum of the antagonistic fluorescent *Pseudomonas* isolates [30] (Table 1) were prepared in Erlenmeyer containing 20 mL of liquid LB culture medium, kept at 28 °C under agitation at 190 rpm for 16 h, until the bacterial suspension reached $OD_{620} \approx 0.8$ (equivalent to 6.2×10^8 cfu mL^{-1}). The bacterial suspensions were then centrifuged for 15 min at 5000 rpm, the supernatant LB medium drained and the resulting bacterial pellet resuspended in similar volume of sterile distilled water and finally adjusted to a final suspension with $OD_{620} \approx 0.8$.

The inoculum of the antagonistic *Trichoderma* spp. isolates [32] (Table 1) were grown on PDA with chloramphenicol and streptomycin and incubated for 7 days at 25 °C and for a 12-h photoperiod. Fungal spores were harvested using distilled water and 0.01% tween 20 and the final conidia suspension was adjusted at 10^9 conidia mL^{-1} based on counts from a Neubauer chamber.

The inoculum of the fungal pathogen was obtained by harvesting spores from 50 Petri dishes for each isolate, half of which (N = 25 plates) containing oat medium (60 g L^{-1} of oat flour, 15 g L^{-1} agar) and another half containing rice–bran–oat–meal medium (15 g L^{-1} of rice bran, 15 g L^{-1} of oat flakes, 5 g L^{-1} of dextrose and 20 g L^{-1} of agar), both with chloramphenicol and streptomycin (50 mg mL^{-1} of each). The plates were incubated for 15 days at 25 °C and 12 h photoperiod. The conidia of the pathogen produced on both culture media were harvested and a mixed inoculum suspension that included *PoTl* isolates 12.1.146, 12.1.207 and 12.1.047 was prepared. The conidia suspension was prepared in sterile distilled water plus 0.01% Tween 20 and adjusted to $\approx 10^4$ conidia mL^{-1} using a Neubauer chamber for the subsequent spraying of the leaves and ears of wheat plants.

The biocontrol agents were sprayed on the entire wheat plant at 60 days after emergence at the Feeks' head stage 10.5 [36], 7 days before the inoculation of the pathogen. The following treatments were applied: (1) Amana; (2) Poti; (3) Yara; (4) Cachara; (5) Jau; (6) Jurupoca; (7) Amana + *PoTl*; (8) Poti + *PoTl*; (9) Yara + *PoTl*; (10) Cachara + *PoTl*; (11) Jau + *PoTl*; (12) Jurupoca + *PoTl*; (13) negative control (no *PoTl*) and (14) positive control (+*PoTl*).

Soon after the application of the biocontrol agents, the plants were transferred to a growth chamber set at 25 °C, adjusting the relative humidity to 90% with nebulization, for 24 h, under complete darkness. Subsequently, the 12 h photoperiod was reestablished. Seven days later, the pathogen was inoculated, and the plants were kept in the same growth chamber for another 14 days under the same incubation conditions of 25 °C and for a 12-h photoperiod, until the evaluation of the treatments effect was performed.

The evaluation of the biocontrol treatments effect was carried out 14 days after inoculation by determining the severity of blast symptoms on wheat ears, which were

digitally photographed. The heads infected area was measured with the aid of the image analysis software Assess from APS (ASSESS: Image Analysis Software for Plant Disease Quantification, Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada) [37]. Data were analyzed using the *F* test to detect the significance of the treatment effect and the 5% Scott–Knott test to compare between means. The experiment was repeated once.

3. Results

Initially, *Pseudomonas* and *Trichoderma* isolates were evaluated for antagonism to the causal agent *PoTl* through in vitro experiments and for the biocontrol potential of wheat blast in vivo.

3.1. In Vitro Antagonism of Fluorescent Pseudomonas against the Wheat Blast Pathogen

While the mycelial growth of *PoTl* isolates was significantly reduced ($p \leq 0.05$) by the three strains of fluorescent *Pseudomonas* species tested (Table 2, Figures 1 and 2), the *P. wayambapalatensis* strain 'Amana' resulted in the highest in vitro inhibition of the fungus relative mycelial growth, ranging from 33 to 52%.

Table 2. Analysis of variance of the in vitro antagonism effect of fluorescent *Pseudomonas* strains against *Pyricularia oryzae Triticum* lineage.

Source of Variation	df	SS	MS	*F*	*p*
Treatments	3	8381.53	2793.84	180.60	0.0000 ***
Error	28	433.16	15.47		
Total	31	8814.69			
CV(%): 5.09					

*** Significance by the *F* test at $p \leq 0.001$.

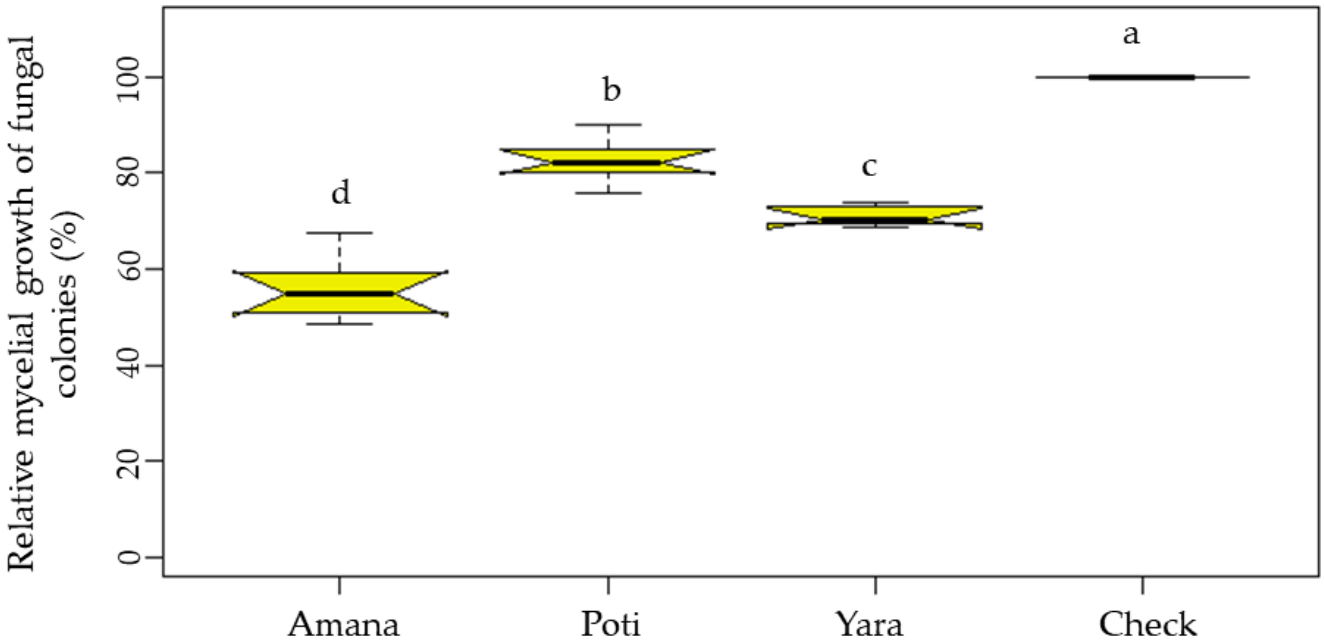

Figure 1. Boxplot distribution of the relative mycelial growth of *Pyricularia oryzae Triticum* lineage (*PoTl*) under in vitro antagonism by three strains of fluorescent *Pseudomonas* species. Each boxplot represents the distribution of values from 3 *PoTl* isolates (12.1.047, 12.1.146, and 12.1.207). Means followed by the same letters (a–d) are not significantly different using the Tukey test at $p \leq 0.05$.

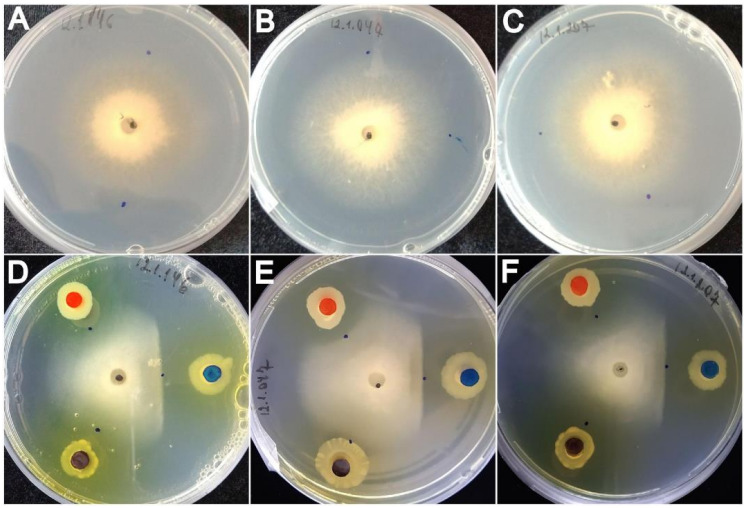

Figure 2. In vitro antagonism by strains of fluorescent *Pseudomonas* species against *Pyricularia oryzae Triticum* lineage (*PoTl*). (**A**–**C**): *PoTl* isolates only (12.1.146, 12.1.047 and 12.1.207). (**D**–**F**): *P. wayamba-palatensis* 'Amana' (blue mark; *P. putida* group); *Pseudomonas* sp. nov. 'Poti' (black mark; *P. asplenii* group) and *Pseudomonas* sp. nov. 'Yara' (red mark; *P. putida* group) paired with *PoTl* (colony in the center).

3.2. In Vitro Antagonism of Trichoderma against the Wheat Blast Pathogen

The three antagonistic *Trichoderma* species significantly reduced *PoTl* mycelial growth (at $p \leq 0.05$) (Table 3, Figures 3 and 4). While *T. koningiopsis* 'Cachara' and *T. lentiforme* 'Jurupoca' caused the highest inhibition of the pathogen's relative mycelial growth, their inhibitory effects were significantly different from *T. virens* 'Jaú'. The general inhibitory effect by *Trichoderma* species ranged from 67 to 81%.

Table 3. Analysis of variance of the in vitro antagonism effect of *Trichoderma* species against *Pyricularia oryzae Triticum* lineage.

Source of Variation	df	SS	MS	*F*	*p*
Treatments	3	33,858.42	11,286.14	1287.57	0.0000 ***
Error	28	245.43	8.77		
Total	31				
CV(%): 6.20					

*** Significance by the *F* test at $p \leq 0.001$.

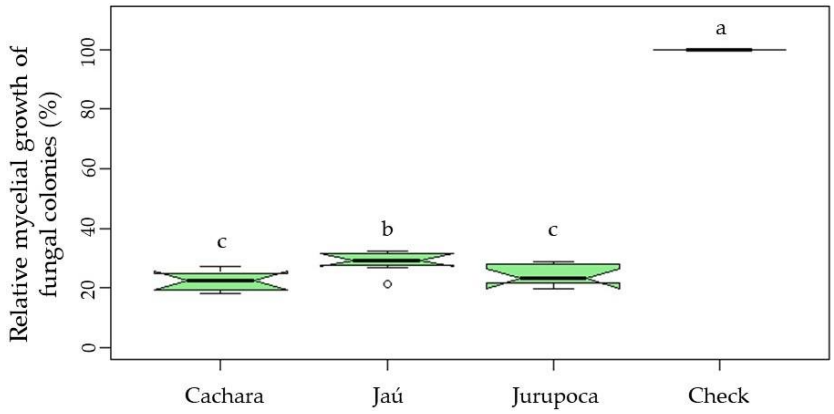

Figure 3. Boxplot distribution of relative mycelial growth of *Pyricularia oryzae Triticum* lineage (*PoTl*) under in vitro antagonism of three *Trichoderma* species. Each boxplot represents the distribution of values from three *PoTl* isolates (12.1.047, 12.1.146 and 12.1.207). Means followed by the same letters (a–c) are not significantly different using the Tukey test at $p \leq 0.05$.

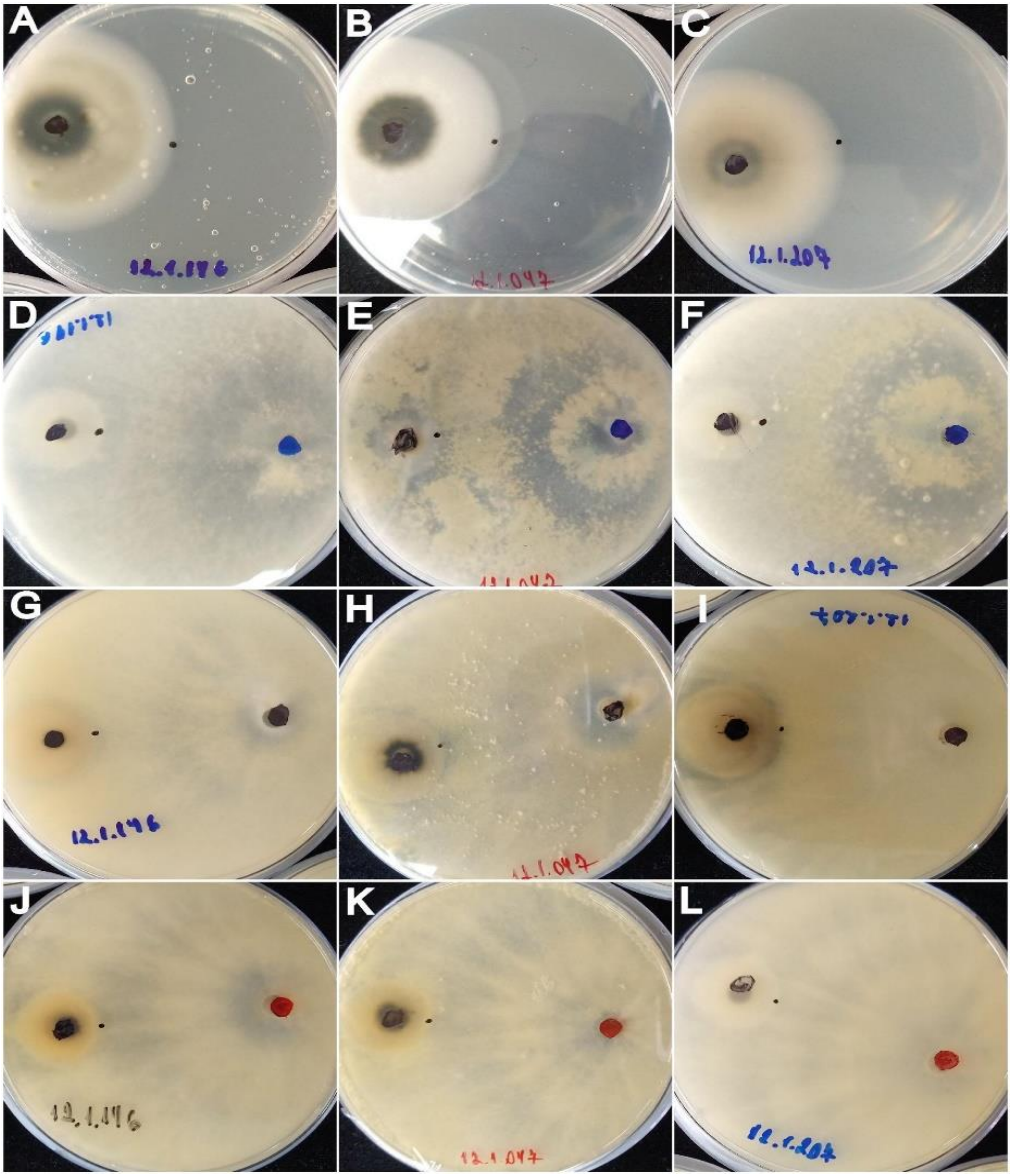

Figure 4. In vitro antagonism of *Trichoderma* species against *Pyricularia oryzae Triticum* lineage (*PoTl*). (**A**–**C**): *PoTl* isolates only (12.1.146, 12.1.047 and 12.1.207); (**D**–**F**): *PoTl* isolates paired with *T. koningiopsis* 'Cachara'; (**G**–**I**): *PoTl* isolates paired with *T. virens* 'Jau'; (**J**–**L**): *PoTl* isolates paired with *T. lentiforme* 'Jurupoca'.

3.3. Scanning Electron Microscopy Analyses of In Vitro Pathogen–Biocontrol Agents Interactions

The scanning electron micrographs (SEM) from the antagonistic in vitro tests allowed the observation of bacterial cells' (from the Amana, Poti and Yara *Pseudomonas* strains) colonization on the *PoTl* hyphae surface, with biofilm development (Figure 5). The Amana strain aggressively grew over the *PoTl* hyphae, with extensive biofilm formation (Figure 5A,B). Both Poti and Yara strains also developed biofilm over the *PoTl* hyphae surface (Figure 5C–F). In addition, the interaction with the Yara strain led to *PoTl* hyphae damage (Figure 5F, arrow).

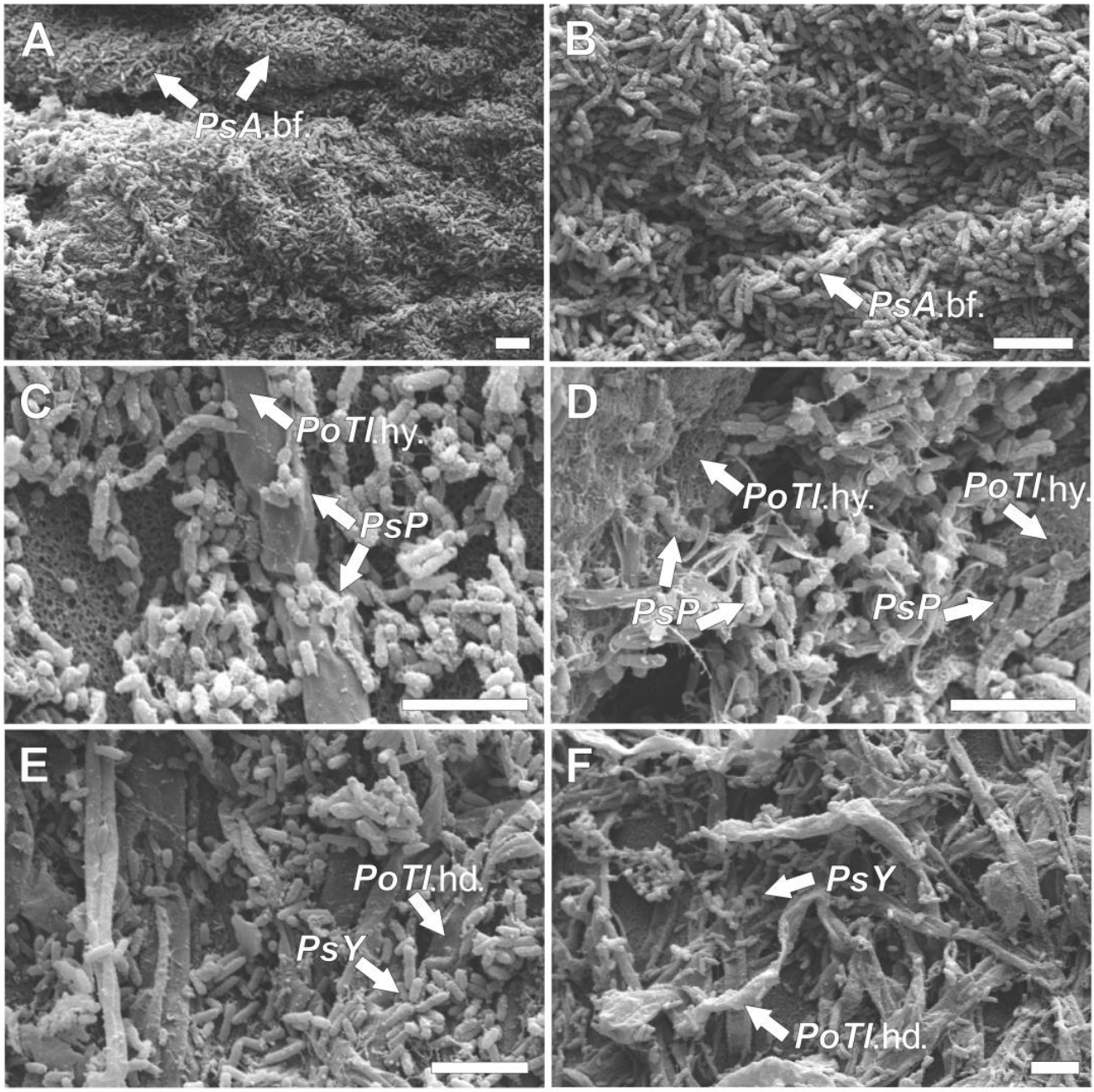

Figure 5. In vitro antagonism by strains of *Pseudomonas* species against *Pyricularia oryzae Triticum* lineage (*PoTl*) on PDA medium. (**A**,**B**): Bacterial biofilm formation by *Pseudomonas wayambapalatensis* 'Amana' (*PsA*.bf.) completely covering the *PoTl* hyphae. (**C**,**D**): the bacterial cells of *Pseudomonas* sp. nov. 'Poti' (*PsP*) growing and colonizing *PoTl* hyphae (*PoTl*.hy.). (**E**,**F**): Colonization of *Pseudomonas* sp. nov. 'Yara' (*PsY*) above *PoTl* pathogen, with hyphae damage (*PoTl*.hd.) (**F**). Scale bars: 5 µm.

In comparison, we also performed SEM analyses of the in vitro interaction of the *T. koningiopsis* Cachara isolate against *PoTl*. Extensive hyphae growth and abundant sporulation of *Trichoderma* over the *PoTl* aerial mycelium were observed (Figure 6A,B,D–F). Mycoparasitism was also detected, which was characterized by *Trichoderma* forming a hyphal coiled structure to parasitize *PoTl* (Figure 6C).

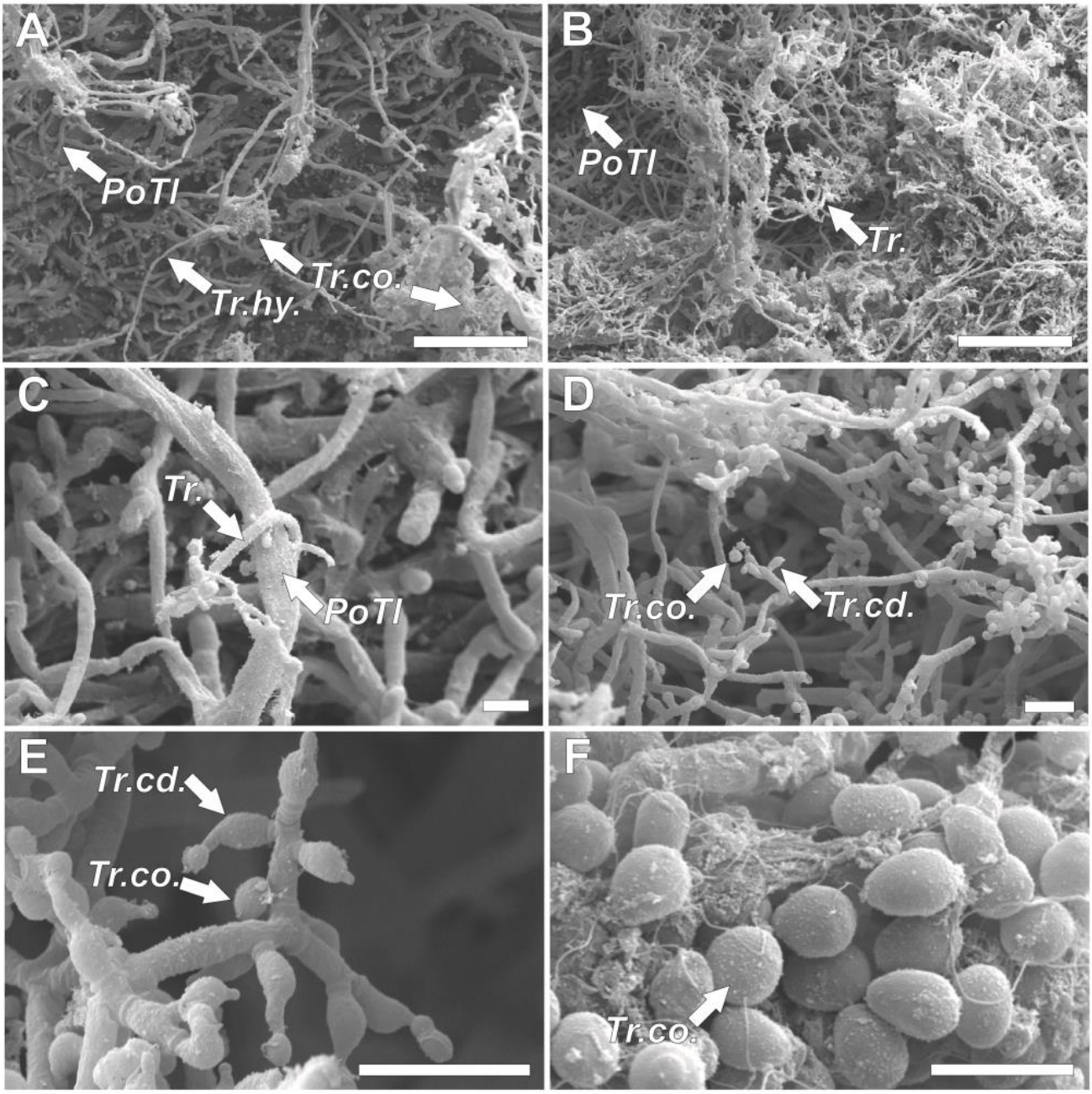

Figure 6. In vitro antagonism by the antagonistic fungus *Trichoderma koningiopsis* 'Cachara' (*Tr.*) against *Pyricularia oryzae Triticum* lineage (*PoTl*) on PDA medium. (**A**,**B**): *Trichoderma* hyphae (*Tr.hy.*) growth above *PoTl* hyphae. (**C**): *Trichoderma* parasitizing *PoTl* hyphae. (**D**–**E**): *Trichoderma* conidia (*Tr.co.*) produced from conidiophores (*Tr.cd.*). (**F**) Abundant *Trichoderma* conidia (*Tr.co.*) in detailed close-up. Scale bars: 100 µm (**A**,**B**), 10 µm (**C**–**E**), 5 µm (**F**).

3.4. Potential of Pseudomonas and Trichoderma as Biocontrol Agents Controlling Wheat Blast In Vivo

The two in vivo experiments of wheat blast biocontrol were analyzed together because there were no significant differences between replicates and the interaction between treatments and experiments was not significant, indicating the complete reproducibility of the observations, regardless of the experiment (Table 4). The joint analysis of the experiments indicated significant differences among biocontrol treatments ($p \leq 0.05$) in reducing blast severity (Table 4).

Table 4. Analysis of variance of the biocontrol potential of *Pseudomonas* and *Trichoderma* species in reducing blast severity in wheat cv. Sossego.

Source of Variation	df	MS	*F*	*p*
Treatments	13	7087.59	29.01	0.0000 ***
Experiments (1 and 2)	1	0.62	0.003	0.9606 ^{NS}
Blocks	2	145.24	0.59	0.5636 ^{NS}
Treatments*experiments	13	562.36	2.30	0.0582 ^{NS}
Treatments*blocks	25	70.30	0.29	0.9974 ^{NS}
Error	16	244.34		
Total	70	105.407.43		
CV(%): 52.51				

*** Significant by the *F* test at $p \leq 0.05$ and not significant (^{NS}). The experiment was repeated once.

A significant reduction in head blast severity was observed in wheat plants treated with the fluorescent *P. wayambapalatensis* 'Amana' or *Pseudomonas* sp. nov. 'Yara', both from the *P. putida* group, or with the antagonist *T. koningiopsis* 'Cachara'. These treatments did not even differ significantly from the non-inoculated check (Figure 7). For the remaining *Pseudomonas* (*Pseudomonas* sp. nov. 'Poti' + *PoTl*) or *Trichoderma* treatments (*T. virens* 'Jau' + *PoTl* and *T. lentiforme* 'Jurupoca' + *PoTl*), the average head blast severity was above 60%. These two *Trichoderma* isolates did not differ from the non-treated positive check inoculated only with *PoTl*.

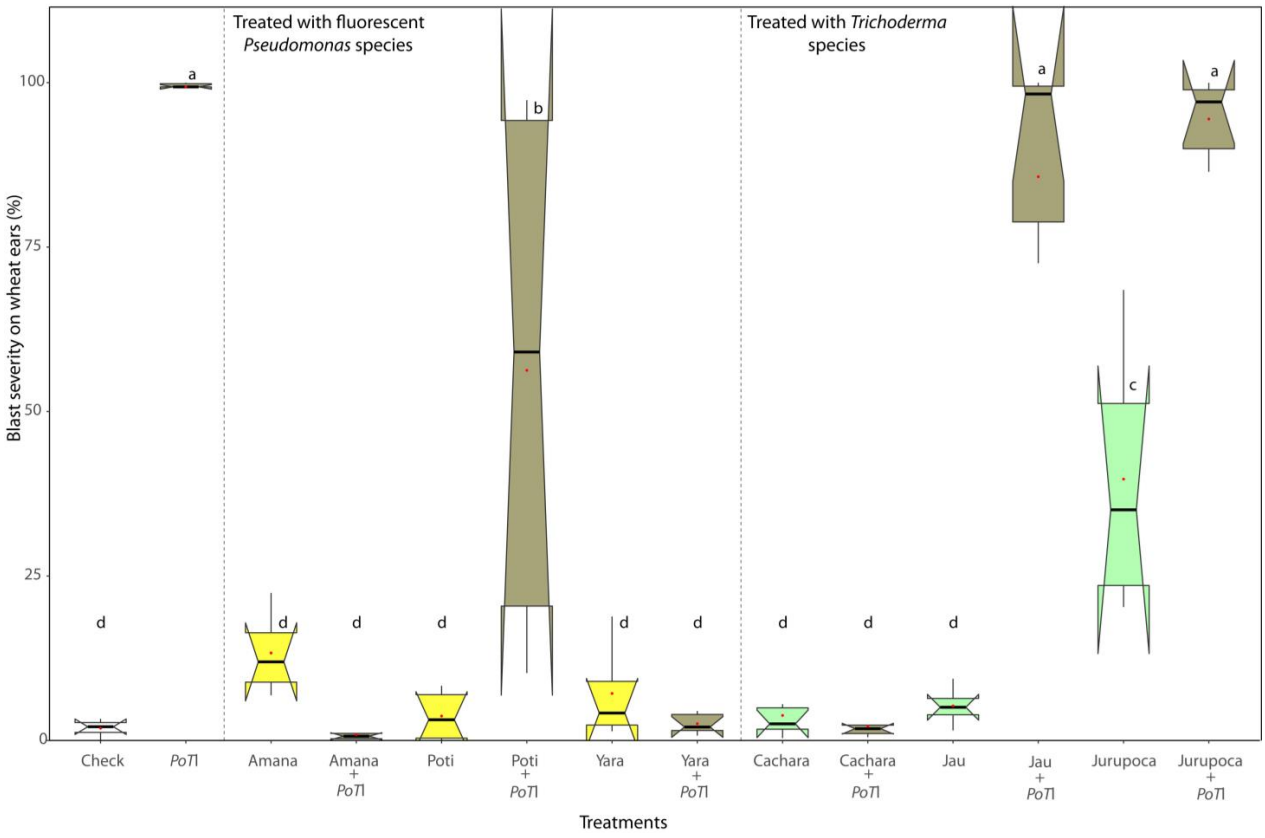

Figure 7. Severity of head blast on wheat cv. Sossego inoculated or not with *Pyricularia oryzae Triticum* lineage (*PoTl*), individually treated with three strains of fluorescent *Pseudomonas* species (strains 'Amana', 'Poti' and 'Yara') or three strains of *Trichoderma* species (strains *T. koningiopsis* 'Cachara', *T. virens* 'Jau' and *T. lentiforme* 'Jurupoca') as potential biocontrol agents. The plants were inoculated with a mixed inoculum composed of three *PoTl* isolates (12.1.047, 12.1.146 and 12.1.207) at $\approx 10^4$ conidia mL^{-1}. Means followed by the same letters (a–d) are not significantly different according to the Scott–Knott test at $p \leq 0.05$.

All non-inoculated plots (represented by yellow or light green boxplots) treated only with the potential bacterial or fungal biocontrol agents, had significantly lower severity values, also not significantly distinct from the non-inoculated negative check. The only exception was the treatment with *T. lentiforme* 'Jurupoca', which showed a slightly higher disease severity, though significantly different from the positive check (Figures 7 and 8). The incidence of head blast in this particular treatment may be associated with seedborne inoculum, since we opted for not treating the seed lot with fungicides.

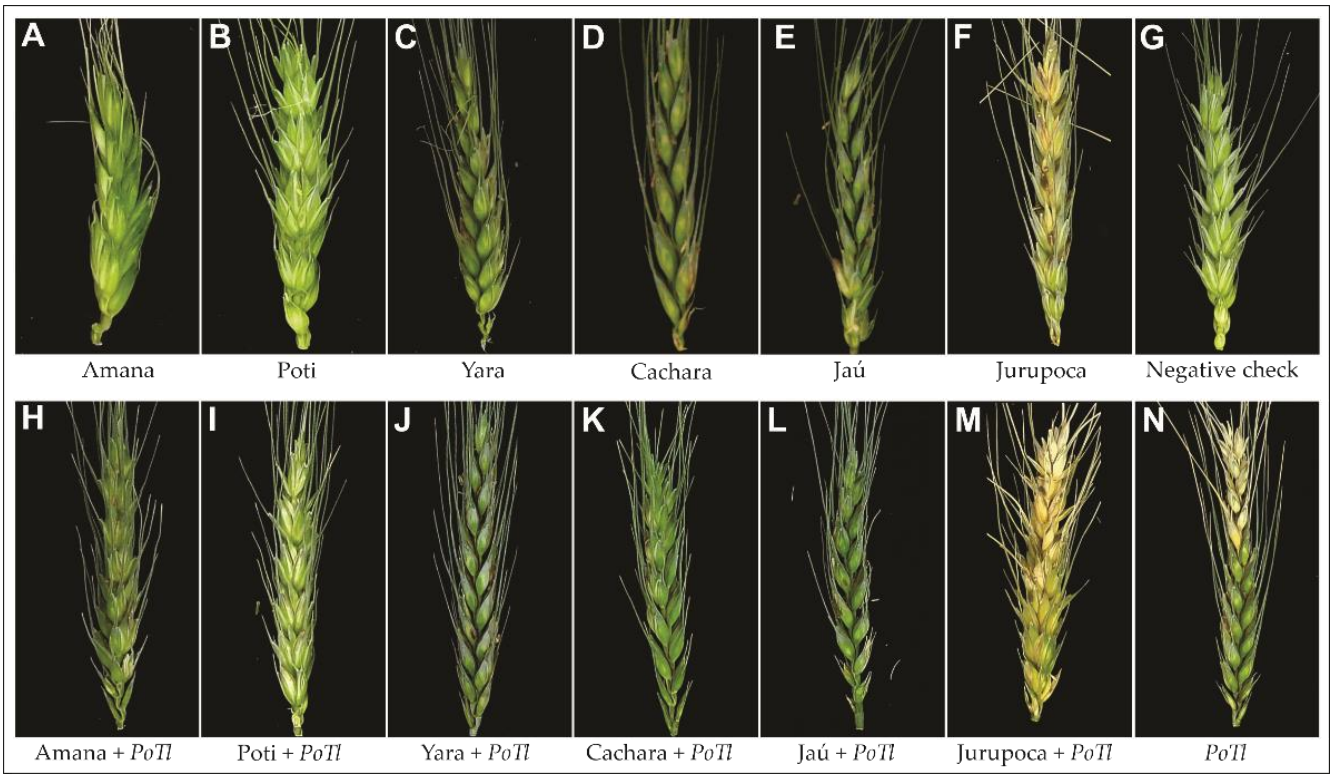

Figure 8. Heads of wheat cv. Sossego inoculated or not with *Pyricularia oryzae Triticum* lineage *(PoTl)*, and treated with bacterial and fungal antagonists, which included fluorescent *Pseudomonas* species (strains 'Amana', 'Poti' and 'Yara') (**A**–**C**,**H**–**J**) or *Trichoderma* species (strains 'Cachara', 'Jaú' and 'Jurupoca') (**D**–**F**,**K**–**M**). (**G**): Negative check. (**N**): Positive check inoculated only with *PoTl*. Bleached ears depicted in M and N had partial or total sterile spikelets from the infection point in the rachis with empty grains.

4. Discussion

In this study, three strains of fluorescent *Pseudomonas* (Amana, Poti and Yara) and three strains of *Trichoderma* spp. (*T. koningiopsis* 'Cachara', *T. lentiforme* 'Jurupoca' and *T. virens* 'Jau') obtained from naturally suppressive soils from the Amazon biome were bio-prospected for their role as biocontrol agents of the wheat blast disease caused by *P. oryzae Triticum* lineage.

The aerial spraying of *P. wayambapalatensis* 'Amana' or *Pseudomonas* sp. nov. 'Yara', both from the *P. putida* group on the leaves and heads of wheat plants resulted in significant disease control, causing a high reduction in the severity of the wheat head blast disease (from 100% of diseased area in the positive check to a maximum of 5% in plots treated with the biocontrol agents). These two strains of fluorescent *Pseudomonas* inhibited 33 to 52% of fungal mycelial growth (Figures 1 and 2) and grew aggressively, with extensive biofilm formation, over the *PoTl* hyphae, resulting in hyphae damage, as detected by the SEM analyses (Figure 5).

Under field conditions, ears of winter wheat were found to be consistently colonized at a high density by *Pseudomonas* species at the late milk dough stage. These *Pseudomonas*

were able to reduce the production of *Alternaria* and *Fusarium* mycotoxins in wheat grains. However, these naturally occurring bacterial antagonists were found unevenly distributed in the wheat field [15]. The delivery of the antagonistic fluorescent *Pseudomonas*, such as *P. wayambapalatensis* 'Amana' or *Pseudomonas* sp. nov. 'Yara', could also have the potential as biocontrol agents against the production of mycotoxins and other wheat head fungal pathogens.

With respect to the general mechanisms of biocontrol, there have been several reports of biofilm formation by *Pseudomonas* species, by which bacterial microcolonies attach to surfaces suitable for growth, including the fungal mycelial mat [38–41]. Biofilm is defined as a multicellular aggregation of bacteria established on biotic or abiotic surfaces that can improve their survival under adverse environmental conditions [42]. Bacteria growing in biofilms are known to have considerable advantages in natural environments, so bacteria living in biofilms or microcolonies are significantly more tolerant of antibiotics, biocides, and other forms of environmental stress [43–45]. In addition to cells, the extracellular matrix, which contains exopolysaccharides, proteins, nucleic acids and lipids, is the main ingredient for biofilm establishment [46].

Besides the evidence of direct bacterial antagonism against *PoTl* by parasitism (Figure 5), it is also probable that it occurred by antibiosis from the secretion of metabolites that cause fungal hyphae damage [30,47]. Vicentini et al. [30] reported that Amana and Yara strains of fluorescent *Pseudomonas* produced siderophores, while only Amana showed protease and chitinase in vitro activity and none had cellulase activity. As a matter of fact, other siderophore-producing fluorescent *Pseudomonas* inhibited the mycelial growth of *P. oryzae Oryza* lineage, which causes the rice blast disease, as well as *R. solani* AG-1 IA, which is associated with the rice sheath blight disease [48,49]. Anti-fungal metabolites produced by fluorescent *Pseudomonas* antagonists of plant pathogens include phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (DAPG), pyroluteorin, and pyrrolenitrine, which are among the known metabolites [47]. Beneficial *Pseudomonas* species from the *P. koreensis* and *P. putida* groups with biocontrol abilities produce an array of antimicrobial secondary metabolites, such as cyclic lipopeptides (CLPs), that can control the rice blast disease-induced resistance and by direct antagonism. These CLPs included lokisin, the white line-inducing principle (WLIP), entolysin and N3 [49]. Fluorescent *Pseudomonas* also can promote plant growth [28,29].

Considering the role of *Trichoderma* species as fungal antagonists against the wheat blast pathogen, despite the significant in vitro inhibition of *PoTl* mycelial growth (varying from 63 to 71% overall) by *T. koningiopsis* 'Cachara', *T. virens* 'Jau' and *T. lentiforme* 'Jurupoca' (Figures 3 and 4), only *T. koningiopsis* 'Cachara' reduced blast severity on wheat cv. Sossego under greenhouse conditions. In fact, the aerial spraying of *T. koningiopsis* 'Cachara' was so extremely successful in reducing the blast disease severity that this treatment did not differ significantly from the non-inoculated check (Figure 7). The efficacy of *T. koningiopsis* 'Cachara' as biocontrol agent is unique for the wheat blast pathosystem and for other *Pyricularia*-associated pathosystems as well, such as the rice blast disease (*P. oryzae Oryza* lineage). However, other *Trichoderma* species have been reported as efficacious biocontrol agents against rice blast. For example, *T. asperellum* reduced the severity of rice leaf blast by 85% with curative spraying, utilizing mycoparasitism and antibiosis mechanisms [50]. *Trichoderma harzianum* was also reported to be effective in controlling rice blast disease by hyperparasitism [51]. Seed-coating with *T. atroviridae* induced resistance against *P. oryzae* in *Lolium multiflorum* [52].

In terms of mechanisms, the ability of *T. koningiopsis* 'Cachara' to directly antagonize *PoTl* was demonstrated by the hyphae of the fungal antagonist engaging the hyphae of the pathogen (Figure 6). In fact, the SEM analyses of the in vitro interaction between *T. koningiopsis* 'Cachara' and *PoTl* indicated extensive hyphae growth, abundant sporulation and the development of hypha coiled structures (Figure 6), which supported mycoparasitism [51,53,54]. By definition, mycoparasitism is the ability of organisms to actively parasitize fungi [54]. This ability to feed on fungi, dead or alive, has been shown to be an

ancestral form of nutrition in all species of *Trichoderma* [55]. Mycoparasitism by *Trichoderma* involves a sequence of events, including host location, recognition, contact, coiling, the formation of hook-shaped structures with appressoria function, direct penetration, folding and the development of parallel hyphae. All these steps can be detected by scanning electron microscopy (SEM) [50,56,57].

In addition to parasitism, other direct mechanisms are usually involved in the antagonism of *Trichoderma* against other plant pathogenic fungi by the direct interaction with plant roots or other organs, such as niche competition, antibiosis, resistance to diseases, tolerance to abiotic stresses and plant growth promotion [53,58–60]. The antagonistic activity may result from the production of metabolites, such as harzianic acid, alamethicins and tricolines, in addition to the activity of lytic enzymes, such as chitinases, glucanases and proteases [50,61–63].

Further research on the topic should include the development of stable formulations of the *Pseudomonas*- and *Trichoderma*-based biocontrol agents selected in our study for managing the wheat blast disease and field tests of the biofungicides formulations obtained thereafter.

The pressing demands for sustainable farming with reduced chemical pesticide (fungicides) input, lower level of residues on pre- and postharvest and a lesser impact on the environment and food safety [64,65] has led to a substantial increase in biopesticide development in Brazil [52]. There are already 65 commercial biofungicides currently labeled by the Ministry of Agriculture, Livestock and Supply (MAPA) for the biological control of crop diseases in Brazil [12]. The majority of these biofungicides are *Trichoderma*-based actives, including *T. afroharzianum*, *T. asperelloides*, *T. asperellum*, *T. atroviride*, *T. endophyticum*, *T. harzianum*, *T. koningiopsis*, *T. reesei*, *T. stromaticum* and *T. viride*, totaling 34 commercial products. Based on efficacy data, these biofungicides were labeled mostly for managing diseases caused by soilborne pathogens, such as *Fusarium oxysporum*, *F. oxysporum* f. sp. *lycopersici*, *F. solani* f.sp. *glycines*, *F. solani* f.sp. *phaseoli*, *R. solani*, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum* and *Thielaviopsis paradoxa* and a single foliar disease (common bean antrachnosis caused by *Colletotrichum lindemuthianum*). Considering biopesticides with fluorescent *Pseudomonas* as active ingredients, there are only two formulations with *P. chlororaphis* or *P. fluorescens* labeled for controlling the insect pests *Bemisia tabaci* race B, *Dalbulus maidis* and *Euschistus heros* [12].

Thus far, no *Trichoderma*- or fluorescent *Pseudomonas*-based biofungicides have been labeled in Brazil for the management of wheat foliar and head diseases, which include wheat blast [12]. Considering that a sustainable management strategy to control wheat blast is warranted, we foresee that the opportunities for the development, labeling and marketing of biofungicides for the biocontrol of wheat blast are promising in Latin America, Southeast Asia and East Africa.

5. Conclusions

Fluorescent *P. wayambapalatensis* 'Amana' or *Pseudomonas* sp. nov. 'Yara', both from the *P. putida* group, and *Trichoderma koningiopsis* Cachara significantly reduced both *PoTl* in vitro mycelial growth and blast disease severity in wheat plants. The SEM analyses revealed ultrastructural antagonistic mechanisms: biofilm formation, direct antagonism and mycoparasitism.

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