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Evaluation of *Tulasnella* and *Ceratobasidium* as Biocontrol Agents of Fusarium Wilt on *Vanilla planifolia*

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Abstract: Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *vanillae* (Fov), is a disease that results in significant losses in commercial vanilla production. The genera *Ceratobasidium* (Ceratobasidiaceae) and *Tulasnella* (Tulasnellaceae), which are often reported as mutualistic symbionts in orchids, belong to the form genus *Rhizoctonia*, a paraphyletic group of fungi with potential for pathogen biocontrol. We assayed the antagonistic properties of the form genus *Rhizoctonia* from the roots of neotropical orchids: two *Tulasnella* spp. isolates (Bv3 and Er1) and one *Ceratobasidium* sp. (Er19). In a dual culture, we found that form genus *Rhizoctonia* isolates can generate a biocontrol effect against *Fusarium* through the mechanisms of antibiosis and competition for space and nutrients. On histological observations, orchid root endophytes also demonstrated potential for mutualistic symbiosis development by establishing themselves on the surface and within the root tissue of *Vanilla planifolia* accessions multiplied in vitro (NSF021 and NSF092). However, in plant assays, the form genus *Rhizoctonia* isolates did not reduce symptom expression or disease development due to infection by Fov in the host. These results contribute to the knowledge of the interactions between tropical orchids and their microbiota and demonstrate the need for multidisciplinary studies for the implementation of integrated management strategies for Fusarium disease in commercial systems.

Keywords: *Fusarium*; pathogenicity; severity; biological control; fungal disease; colonization; orchid mycorrhizae; root endophytes; form genus *Rhizoctonia*

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1. Introduction

Within the Orchideaceae family, the genus *Vanilla* has approximately 120 species [1], of which about 35 produce aromatic fruits from which vanilla is obtained [2], a natural flavoring with a wide demand in the global market for its use in the food, cosmetic and pharmaceutical industries [3]. Colombia is one of the countries with the greatest orchid diversity in the world, with approximately 4300 species recorded and represents a center of *Vanilla* diversity [4,5]. Here, 24 species of *Vanilla* are reported, of which 18 are aromatic and 3 are endemic to the country (*Vanilla espondae*, *V. javieri* and *V. rivasii*) [5]. *Vanilla planifolia*, which has natural and locally adapted populations in Colombia [2,5], is the principal cultivated species worldwide and the main source of natural vanilla commercially [6]. However, the continuous vegetative propagation of the plants has generated a low genetic variability, which favors the attack of pathogens in the vanilla crop [2,7]. Among the various plant pathogens affecting the crops, the fungus *Fusarium oxysporum* f. sp. *vanillae*. (Fov), the causal agent of Fusarium wilt [8–10], is the main threat in vanilla production systems worldwide [7,9,11]. Meanwhile, species such as *F. solani* or *F. proliferatum* are reported in the vanilla crop as saprophytes or latent pathogens, which can become part of the pathogenic complex in response to changes in environmental conditions [9,10].

Currently, the application of chemical fungicides is the most widely used strategy for the management of diseases caused by *Fusarium* [12]. However, these agrochemicals represent a risk to human health [13,14]. Moreover, in the vanilla crop, chemical

fungicides can generate problems in plant nutrition and seed germination due to the harmful effect on orchid mycorrhizal fungi (OMF) and other beneficial microorganisms in the rhizosphere [15,16]. Orchid mycosymbionts are often found in a paraphyletic group, encompassing different clades within the phylum Basidiomycota, called form genus *Rhizoctonia* or "rhizoctonias" [17,18]. This fungal group is characterized by the lack of reproductive structures in the asexual stage (anamorph), by the presence of bifurcations that generate right angles with respect to the main hypha and a constriction at the point of bifurcation with an adjacent septum [17,19,20]. The form genus *Rhizoctonia* can present a variety of ecological strategies, based on the genotype of the fungus and the host plant species [21,22]. *Thanatephorus cucumeris* (anamorph: *Rhizoctonia solani*, Ceratobasidiaceae) that causes diseases in various crops is considered the most important pathogen within this group [13,17,21]. On the other hand, isolates expressing their teleomorph (sexual stage) in the genera *Ceratobasidium* (Ceratobasidiaceae) and *Tulasnella* (Tulasnellaceae) are frequently reported as mycorrhizal orchid, endophytes, saprophytes and antagonists of soil pathogens [19–22].

The potential of form genus *Rhizoctonia* for biocontrol of plant pathogens in different host plant species is mainly related to its ability to establish itself in the tissue and to activate systemic resistance genes in the plant [21,22]. However, mycoparasitism and competition for space and nutrients have been reported as mechanisms of action that could contribute to the protective effect on plants [22–24]. The mycorrhizal association of *V. planifolia* with OMF of form genus *Rhizoctonia* is facultative during the adult photosynthetically active stage [20]. The presence of mycorrhizal symbiosis improves the nutritional status of plants through carbon exchange, which occurs when the plant degrades hyphal fungal coils, called pelotons, in the cortex cells [17,19,20]. It should be noted that not all the form genus *Rhizoctonia* isolates that grow endophytically in orchid roots can be considered mycorrhizal, due to the little or no potential of some isolates for seedling nutrition [15,20]. Nonetheless, inside the orchid tissue, there is a large diversity of fungi with beneficial potential [21,22,25] and the natural populations of *Vanilla* in Colombia show a very low incidence of diseases compared to commercial crops [5].

Research on plant–antagonist–pathogen interactions is minimal, especially in tropical ecosystems [2,5,21]. Therefore, the hypotheses addressed in this study were as follows: (1) The form genus *Rhizoctonia* endophytes in orchid roots can exert biocontrol against *Fusarium* in plant assays. This is supported by previous findings where form genus *Rhizoctonia* isolates identified in the *Ceratobasidium* and *Tulasnella* genera have shown potential as biocontrol agents against *F. culmorum* in wheat (*Triticum aestivum*) [22], as well as against *Rhizoctonia solani* in rice (*Oryza sativa*) [21]. (2) If a biocontrol effect exists from the form genus *Rhizoctonia*, it will be greater in the *Vanilla* plants of the Colombian accession (NSF021) compared to the commercial accession from Mexico (NSF092). This is because the plant material NSF021 comes from natural populations exposed and adapted to higher microbial diversity, which favors the selection of genotypes resistant to pathogens [26,27]. In the present study, the objectives were (1) to identify the in vitro biocontrol mechanism of the form genus *Rhizoctonia* endophyte of orchid roots against *Fusarium* spp. using dual culture tests, (2) to characterize the colonization of form genus *Rhizoctonia* in the in vitro multiplied material of *V. planifolia*, and (3) to evaluate the development of Fusarium wilt in plants of two accessions of *V. planifolia* in the interaction with the form genus *Rhizoctonia* and *F. oxysporum* f. sp. *vanillae*.

2. Materials and Methods

2.1. Microorganisms

One fungal isolate (Bv3) corresponding to the genus *Tulasnella* was obtained from the roots of *Vanilla rivasii* growing in a small agroforestry system under *circa situm* conditions in Valle del Cauca, Colombia (Table 1). The other two form genus *Rhizoctonia* isolates (Er1 and Er19) were isolated and characterized in a previous study of potential OMF associated with the threatened species *Cattleya quadricolor* [28]. Isolates of *Fusarium* spp. were obtained

from symptomatic tissue of different accessions of *V. planifolia,* and their pathogenicity was evaluated on in vitro multiplied *Vanilla* plants [10]. All fungal microorganisms were transferred to a Potato Dextrose Agar Merck Sigma-Aldrich, Germany (PDA) medium at 50% of the commercial concentration of the product (39 g/L) to maintain the active mycelium [29].

Table 1. Isolates of form genus *Rhizoctonia* and *Fusarium* spp. used in this study.

Code	Fungus Species	Host	References
Bv3	*Tulasnella* sp.	*Vanilla rivasii*	Present study
Er1	*Tulasnella* sp.	*Cattleya quadricolor*	Ramírez-Bejarano [28]
Er19	*Ceratobasidium* sp.	*Cattleya quadricolor*	Ramírez-Bejarano [28]
1Fov	*F. oxysporum* f. sp. *vanillae*	*Vanilla planifolia* (NSF021)	Mosquera-Espinosa et al. [10]
2Fov	*F. oxysporum* f. sp. *vanillae*	*Vanilla planifolia* (NSF092)	Mosquera-Espinosa et al. [10]
3Fs	*F. solani*	*Vanilla planifolia* (NSF092)	Mosquera-Espinosa et al. [10]

2.2. Identification of Bv3 Isolate

Molecular identification: DNA extraction of the Bv3 isolate was performed according to the protocol of Lee and Taylor [30]. Subsequently, amplification of the ITS region of the rRNA was performed using the protocol of Mosquera-Espinosa et al. [21], with the universal primers ITS4 and ITS5 [31]. To prepare the PCR reaction, 5 µL of purified DNA was mixed with 45 µL of a master mix (5 µL of Taq buffer (KCl-MgCl), 5 µL of $MgCl_2$, 5 µL of DNTPs, 5 µL of forward primer, 5 µL of reverse primer, 19.5 µL of water and 0.5 µL of Taq polymerase) in a final volume of 50 µL. Amplification was carried out in a thermal cycler with the following program: an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 45 s, 53 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. Finally, an additional cycle at 10 °C for 5 min was performed. The PCR product was purified and sequenced in both directions. The consensus sequence was edited using BioEdit and subsequently compared with the most similar sequences through BLAST searches in the NCBI database (http://blast.ncbi.nlm.nih.gov/ accessed on 10 August 2023).

2.3. Dual Culture Tests between Form Genus Rhizoctonia and Fusarium spp.

The biocontrol potential of form genus *Rhizoctonia* isolates was evaluated in vitro using the dual culture technique [23], which allows for the evaluation of several mechanisms. The assays were carried out in 9 cm diameter Petri dishes with 50% PDA. Three form genus *Rhizoctonia* isolates were confronted against three *Fusarium* isolates for a total of 15 treatments, including controls and six repetitions per treatment. From 10 days' pure cultures of form genus *Rhizoctonia* and *Fusarium*, 0.5 mm diameter discs were obtained as inocula and were each placed at opposite sides of the Petri dish, 1 cm from the margin.

To evaluate the mechanism of biocontrol by antibiosis in the dual test, the growth of both fungi was measured and the percentage of radial growth inhibition (PRGI) was calculated. The experiment readings were taken every 48 h for 10 days.

$$\text{PRGI} = \frac{R1 - R2}{R1} \times 100 \quad (1)$$

$R1$ = radial growth of the pathogen evaluated in pure culture (mm);
$R2$ = radial growth of the pathogen in the dual culture (mm).

Two adjusted indicators were used to evaluate competition for space and nutrients: the Ortiz and Orduz scale [32] and the location by class with the Bell et al. scale [33]. The readings were taken 10 days after inoculation (DAI). The scale of Ortiz and Orduz [32] classifies the invasiveness of the biocontrol agent on the pathogen colony: level 0 = no invasion of the *Fusarium* colony; level 1 = invasion of 25% of the *Fusarium* colony surface; level 2 = invasion of 50% of the *Fusarium* colony surface; level 3 = invasion of 100% of the

Fusarium colony surface; and level 4 = invasion of 100% of the *Fusarium* colony surface and sclerotia production.

Location by class with the scale of Bell et al. [33] categorizes the degree of antagonism into five levels: Class 1 = biocontrol agent overgrowth, covering the entire surface of the medium and reducing the *Fusarium* colony; Class 2 = biocontrol agent overgrowth covering at least 2/3 of the medium surface; Class 3 = biocontrol agent and *Fusarium* each colonizing half of the medium surface (more than 1/3 and less than 2/3), with one of the fungi not overlapping the other; Class 4 = *Fusarium* colonizing at least 2/3 of the surface of the medium and resisting invasion by the biocontrol agent; and Class 5 = overgrowth of *Fusarium* colonizing the entire surface of the medium and invading the biocontrol agent colony.

To evaluate mycoparasitism, slides were made from the contact zone of the confronted colonies. The slides were observed under the light microscope at 40× and 100×, in order to characterize different somatic or sexual structures (appressoria, papilla-like structures or hyphal coils) reported to be associated with mycoparasitism or antibiosis [23,24,34].

2.4. In Vitro Multiplication of Vanilla planifolia

In vitro multiplication of plant material was carried out following the methodology of Mosquera-Espinosa et al. [10] Two accessions of *V. planifolia* of different origins were multiplied in vitro, one from natural populations in Colombia (NSF021) and the other from a commercial accession from Mexico (NSF092-*V. planifolia* variety "Mansa" [35]). The accessions were propagated and maintained in vitro in sterile Fisherbrand 25 × 150 mm glass tubes with 15 mL of VAI 002 1 culture medium. The in vitro propagated plants were kept in a germination chamber (Tecnal, Ourinhos, Brazil, model TE-381) for two months at a constant temperature of 26 °C, with 80% relative humidity and a photoperiod of 12 h under continuous artificial illumination. After this time, the plant material was used to evaluate the microbial interaction and to determine the possible biocontrol of Fusarium wilt.

2.5. Inoculation of Fungal Microorganisms on In Vitro Multiplied Material of V. planifolia

The isolate 1Fov of *Fusarium oxysporum* f. sp. *vanillae* was selected because in previous work [10], this fungus presented the highest symptom induction capacity on in vitro multiplied material of *Vanilla*. The three isolates of the form genus *Rhizoctonia* evaluated in dual culture tests were used as potential biocontrol agents. Six plants with two months of growth of two accessions of *V. planifolia* were used for each treatment. The plants were removed from the culture medium, and the roots were washed with sterile distilled water (SDW) and wiped with sterile absorbent paper to remove activated charcoal residues from the medium. Subsequently, they were individualized in test tubes with 2 mL of SDW [9,10].

To inoculate endophytes of orchid roots from the form genus *Rhizoctonia*, cultures that had been grown for 10 days in PDA 50% and incubated at 26 °C were used. The inoculation was performed 30 days prior to the inoculation of 1Fov to ensure the establishment of potential biocontrol agents in the host tissue [21,22]. Disks with a diameter of 5 mm containing only mycelium were taken and placed in contact with the roots immersed in water. This method of inoculation was chosen because water's physicochemical properties enable biochemical communication between plants and microorganisms under natural conditions [36]. During the 30 DAI, the plants were incubated in a germination chamber under the conditions described above during the plant multiplication process. After this time, the description of symptoms in plants inoculated with the form genus *Rhizoctonia* was carried out [21,26].

For the inoculation of 1Fov, the roots were immersed in conidial suspensions adjusted to a concentration of 1×10^6 conidia using a Neubauer chamber [10]. Each treatment, consisting of six plants, was inoculated by immersing the roots in 25 mL of the conidial suspensions for five minutes under constant agitation. Absolute controls (plants inoculated only with SDW) and positive controls for the biocontrol agent (plants inoculated only with the form genus *Rhizoctonia*) were also immersed in SDW for the same duration. After

inoculation with 1Fov, the plants were returned to the germination chamber to allow for host infection and the expression of Fusarium wilt symptoms. The plants were evaluated every 48 h during the 15 DAI [9].

2.6. Evaluation of Symptom Development in Plant Biocontrol Assays

The standardized scale of Koyyappurath et al. [9] was used, which categorizes and describes the severity of the disease in four levels according to the expressed symptomatology: level 0 = no symptom (0% of the affected plant); level 1 = dull leaves (10% of the affected plant); level 2 = localized browning visible in affected tissue (20% of the affected plant); level 3 = brown areas and visible mycelium (60% of the affected plant); and level 4 = fully decomposed or dead plant (100% of the affected plant). To complement the analysis and statistically interpret the development of Fusarium wilt, the area under the disease progress curve (AUDPC) was calculated for each treatment [9,10].

2.7. Characterization of Form Genus Rhizoctonia Colonization in V. planifolia Accessions In Vitro

To characterize the colonization of the form genus *Rhizoctonia* inside the plant tissue, the methodology of Bleša et al. was used [22]. In the present study, the hyphae of the fungi belonging to the form genus *Rhizoctonia* were followed up to fungal penetration in root tissues (velamen and cortex). The percentage of colonization and formation of pelotons in cortex cells was not evaluated, since the biological analysis was focused on the biocontrol potential on *Fusarium* as a plant pathogen and not on the nutritional function in the host plant. Samples were taken from aerial roots and those in contact with the culture medium, which were fixed in formaldehyde, alcohol and acetic acid (FAA) [37]. Transverse cuts were made to obtain three portions of approximately 0.5 cm from the root of each plant [19]. The sections were washed with 3% KOH for 3 min and 1% HCl for 1 min and mounted on slides with a drop of 0.05% methylene blue [22]. Subsequently, the sections were observed under a light microscope at 40× and 100× to verify the presence of the fungus in the cortex cells. On the surface of the tissue, observations were made with the naked eye and using stereoscopy at 10× and 50× magnification [13].

2.8. Statistical Data Analysis

For the statistical analysis of the dual culture tests, a completely randomized experimental design with 15 treatments including controls and six repetitions per treatment was used. The analyses were carried out in R software (version 4.2.1). To evaluate the mechanism of biocontrol by antibiosis, the response factor was the percentage inhibition of radial growth (PRGI) of *Fusarium*. Because the data did not meet the assumptions of normality and homoscedasticity, a two-factor ANOVA fixed effects model was performed, using the ANOVA 2way. R package, with 10,000 permutations ($p \leq 0.05$) [38].

This analysis recognizes statistical differences independently in each of the design factors at this stage: form genus *Rhizoctonia* isolates, *Fusarium* isolates and the interaction between both factors. Once significant differences between the design factors were identified, a multiple comparison test of means was run with the pairwise function test with Bonferroni correction ($p \leq 0.05$) [10].

For the statistical analysis of the biocontrol effect of the form genus *Rhizoctonia* on the *Fusarium* isolate 1Fov in *V. planifolia* accessions, a completely randomized experimental design with 16 treatments including controls and six repetitions per treatment was used. Subsequently, the area under the disease progress curve (AUDPC) was calculated using the AUDPC function of the AGRICOLAE library. Once the AUDPC values were calculated, a three-factor ANOVA was performed using the ANOVA 3way. R package, with 10,000 permutations ($p \leq 0.05$) [38], to determine the effect of the design factors at this stage: form genus *Rhizoctonia* isolate, *Fusarium* isolate, *V. planifolia* accession and the interaction between these on the response factor that was Fusarium wilt development. Subsequently, a Tukey's rank test was run with the Tukey HSD function for pairwise comparisons and analysis of the interaction between factors ($p \leq 0.05$).

3. Results

3.1. Molecular Characterization of Bv3 Isolate

The sequence corresponding to the ITS region of the rRNA, obtained from isolate Bv3 (OR536424), showed the highest percentage of similarity (96.07%) with a reference sequence from an isolate of *Tulasnella* sp. (OQ678403), obtained from the roots of the orchid *Paphiopedilum purpuratum* [39].

3.2. Identification of the Biocontrol Mechanism in Dual Culture Tests

During macroscopic observation of the interaction zone between fungi of the form genus *Rhizoctonia*. And *Fusarium* spp., no inhibition halos were observed in any of the treatments. However, different patterns of interaction between fungal isolates were evident (Figure 1).

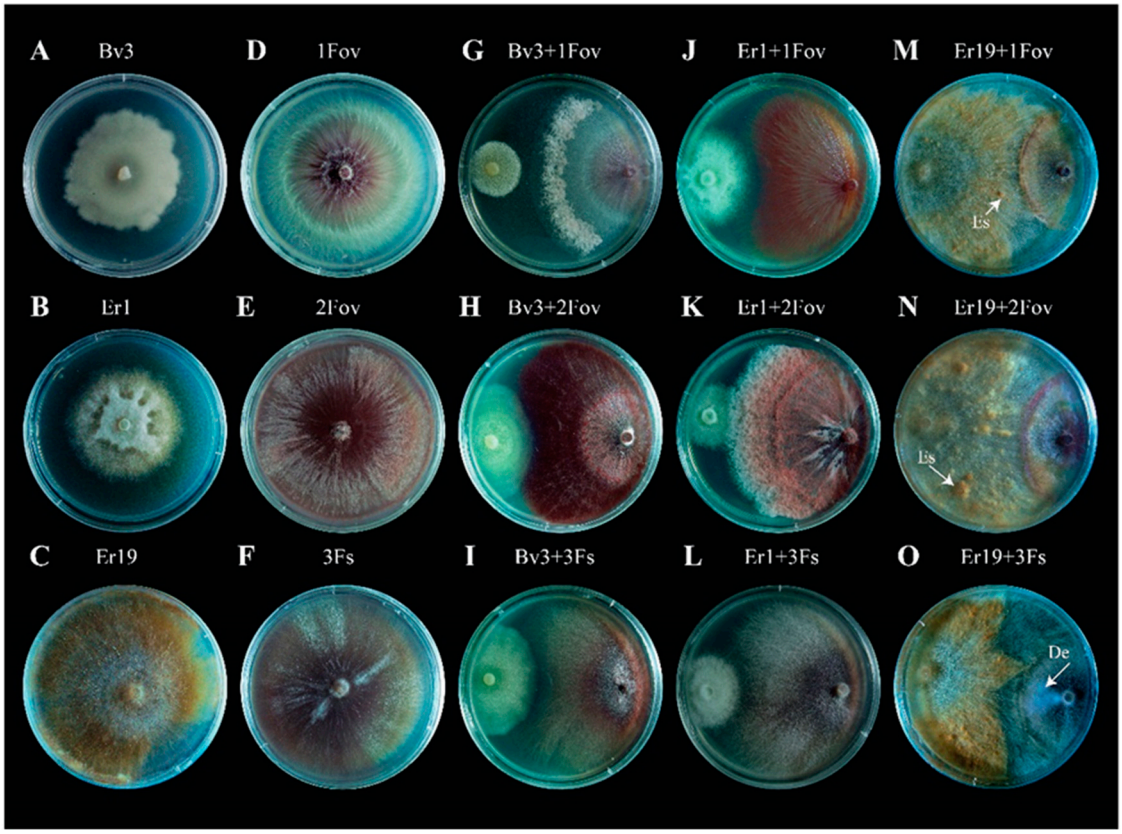


Figure 1. Dual culture tests to identify the in vitro biocontrol mechanism of three form genus *Rhizoctonia* isolates on three isolates of *Fusarium* spp. (**A**–**C**) Controls for form genus *Rhizoctonia* (**D**–**F**) Controls for *Fusarium* spp. (**G**–**O**) Dual cultures (form genus *Rhizoctonia* + *Fusarium*) De: development of 3Fs sclerotia. Es: sclerotia Er19 (Petri dishes of 9 cm diameter). The controls correspond to the microorganism growing in pure culture.

In the contact zones of some of the pairwise interactions between *Tulasnella* spp. (Bv3 and Er1 with the three isolates of *Fusarium* spp., there was a loss of pigmentation and a decrease in the concentration of *Fusarium* spp. hyphae (Figure 1H,J,L). In addition, during microscopic observation, cell lysis was evidenced in the hyphae of *Fusarium* spp. (Figure 2B,D) and less frequently in the hyphae of the form genus *Rhizoctonia* (Figure 2C). In the dual cultures, isolates 1Fov and 2Fov generated abundant growth of aerial hyphae on the periphery of the colony, forming a "barrier" (Figure 1G,K); sometimes, this interaction with the form genus *Rhizoctonia* also produced a change in the tonality of the colonies of 1Fov and 2Fov from purple or violet to intense red. On the other hand, morphological

alterations in *Fusarium* spp. conidia or a decrease in their number were not observed in any of the treatments (Figure 2C,E).

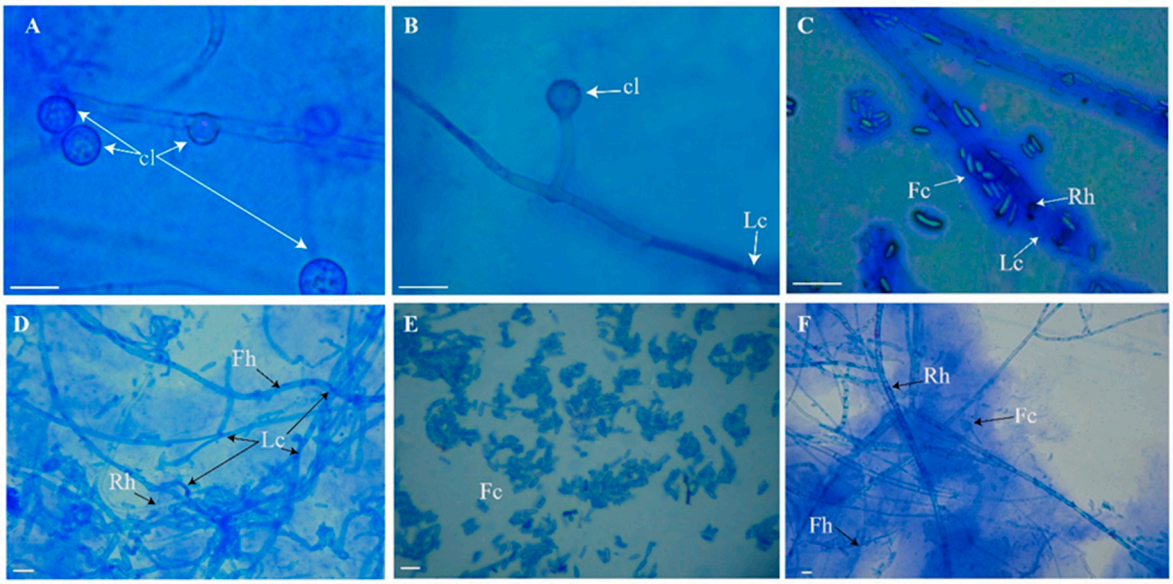


Figure 2. Interaction between the form genus *Rhizoctonia* as biocontrol agent and *Fusarium* spp. as pathogen in the contact zones of colonies confronted in dual culture. (**A**,**B**) Chlamydospores of 3Fs in interaction with Er19. (**C**) Hyphae of Bv3 and conidia of 2Fov. (**D**) Cell lysis in hyphae of 1Fov in interaction with Bv3. (**E**) Conidia of 1Fov in interaction with Bv3. (**F**) Hyphae of Er1 and clusters of microconidia of 2Fov. cl: chlamydospore, Lc: cell lysis, Fh: *Fusarium* hyphae, Fc: *Fusarium* sp. conidia, Rh: form genus *Rhizoctonia* hyphae (Scale bar = 20 µm).

The growth of the three isolates of *Fusarium* spp. was inhibited only in the presence of *Ceratobasidium* sp. (Er19), which also caused degradation of the pathogen's mycelium expressed in the darkening of the colonies (Figure 1M,N). In addition, the 3Fs isolate in interaction with Er19 generated chlamydospores (Figure 2A,B) and initiated the development of sclerotia (Figure 1O), somatic structures of resistance that were not evidenced in other treatments.

Regarding the results obtained from the statistical analysis of the PRGI, the bifactorial ANOVA showed significant differences when considering design factors independently. For the isolate factor of the form genus *Rhizoctonia*, highly significant differences were found ($F = 313.26$; $p = 0.00009$), while no significant differences were found when analyzing the *Fusarium* isolate factor ($F = 0.5045267$; $p = 0.60213979$). Likewise, the interaction between both groups of microorganisms did not show significant differences ($F = 0.9430736$; $p = 0.44075592$). Therefore, there were no statistical differences between treatments. When performing the multiple comparison of means test with the Bonferroni correction (Table 2), which is more sensitive in detecting statistical differences, highly significant differences were confirmed for the form genus *Rhizoctonia* isolates factor, with Er19 being the isolate that generated the highest values of radial growth inhibition of *Fusarium* spp. (Table 2, Figure 3A).

The in vitro invasiveness and degree of antagonism exerted by the form genus *Rhizoctonia* on *Fusarium* spp. was measured (Table 3). Isolate Er19 (*Ceratobasidium* sp.) invaded the total surface of the pathogen's colony and reduced the growth of all three isolates of *Fusarium* spp. In addition, it was able to produce sclerotia (Figure 1M,N). Therefore, the mean values for treatments 13, 14 and 15 were grouped in level 3 of the Ortiz and Orduz [32] scale and in class 1 of the Bell et al. [33] scale.

Table 2. Multiple comparison of means with Bonferroni correction to detect statistical differences within each factor evaluated in the dual culture tests.

Form Genus *Rhizoctonia* Isolates				*Fusarium* Isolates			
Comparison	**Stat**	***p* Value**	***p* Adjust**	**Comparison**	**Stat**	***p* Value**	***p* Adjust**
Bv3–Er1	0–0.08359	0.9334	1	1Fov–2Fov	0–0.0739	0.9411	1
Bv3–Er19	−5.775	7.697×10^{-9}	2.309×10^{-8}	1Fov–3Fs	0–0.2706	0.7867	1
Er1–Er19	−5.736	9.675×10^{-9}	2.903×10^{-8}	2Fov–3Fs	0–0.1979	0.8431	1

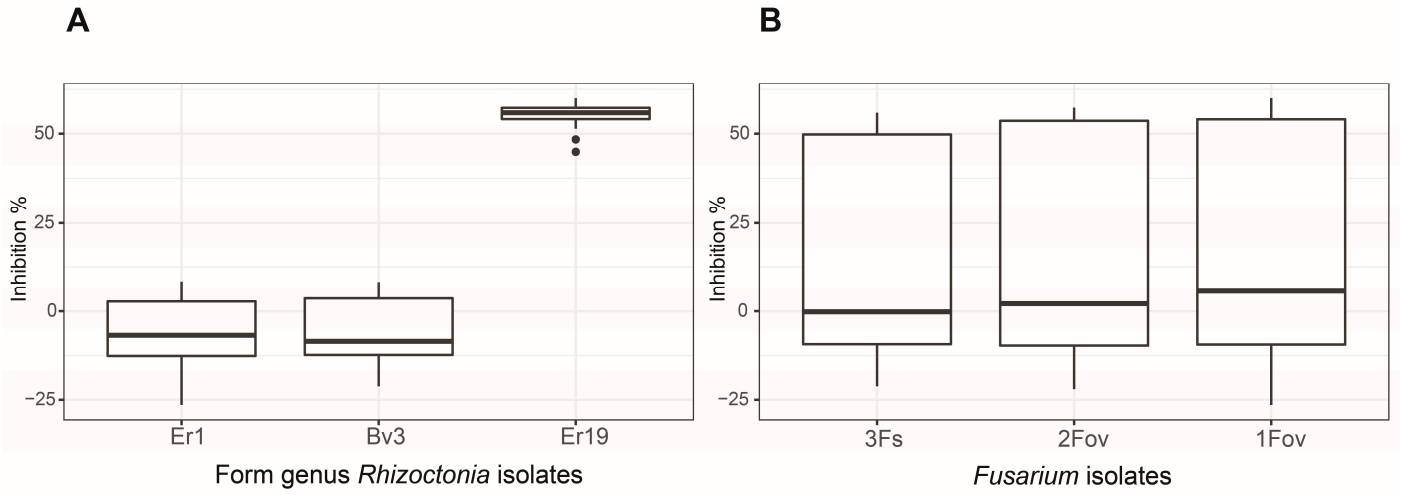


Figure 3. (**A**) Graph for the design factor isolates from the form genus *Rhizoctonia*; atypical values for the isolate Er19 correspond to the PRGI obtained for a repetition of the dual test with 3Fs (point 1 from the bottom up) and another with 1Fov (point 2 from bottom to top). (**B**) *Fusarium* isolate design factor graph. Negative PRGI values indicate that radial growth of *Fusarium* spp. was higher in dual testing, with respect to control.

Table 3. Degree of antagonism with the location by class Bell et al. [33] and in vitro invasiveness of the biocontrol agent with the scale of Ortiz and Orduz [32] for three isolates of the form genus *Rhizoctonia* on three isolates of *Fusarium* spp. (Average SD of 6 repetitions).

Treatment	Form Genus *Rhizoctonia* + *Fusarium*	In Vitro Invasiveness	Degree of Antagonism
7	Bv3 + 1Fov	0.2 ± 0.4	4.0 ± 0.0
8	Bv3 + 2Fov	0.3 ± 0.5	3.5 ± 0.5
9	Bv3 + 3Fs	0.3 ± 0.5	3.7 ± 0.5
10	Er1 + 1Fov	0.2 ± 0.4	3.7 ± 0.5
11	Er1 + 2Fov	0.2 ± 0.4	4.3 ± 0.8
12	Er1 + 3Fs	0.2 ± 0.4	3.8 ± 0.4
13	Er19 + 1Fov	3.2 ± 0.4	1.0 ± 0.0
14	Er19 + 2Fov	3.5 ± 0.5	1.0 ± 0.0
15	Er19 + 3Fs	2.8 ± 0.8	1.0 ± 0.0

Treatments 1 to 3 correspond to controls for the form genus *Rhizoctonia* and treatments 4 to 6 correspond to controls for *Fusarium* spp.

In contrast, the two isolates of *Tulasnella* spp. (Bv3 and Er1) showed slow growth in the pure and dual cultures, in vitro invasiveness on *Fusarium* spp. for these isolates was less than 25%, and they did not stop the growth of the pathogen. Therefore, the mean values for their respective treatments (7, 8 and 9, and 10, 11 and 12) were in class 4 of the Bell et al. [33] scale and did not exceed level 1 of the Ortiz and Orduz [32] scale. However, both Bv3 and Er1 presented the ability to protect their colony from *Fusarium* spp. invasion (Figure 1H–J). It is noteworthy that, during macroscopic observation, no alteration in morphology or pigmentation was found in the colonies of the form genus *Rhizoctonia*

isolates. This fact suggests that the *Fusarium* spp. isolates did not produce any inhibitory effect on the *Rhizoctonia* form genus, since their mycelium did not undergo changes in the dual culture.

3.3. Description of Symptoms Induced by the Form Genus Rhizoctonia in V. planifolia Accessions

The *Ceratobasidium* sp. isolate (Er19) was the only form genus *Rhizoctonia* isolate to induce symptoms on in vitro multiplied material of *V. planifolia* (NSF021 and NSF092). Initially, dry rotting of aerial roots occurred (Figure 4G,I2), followed by corky-looking sunken lesions on the stem (Figure 4F); sometimes reddish-brown spots (Figure 4I1); and later, wet rotting of some roots in contact with the culture medium (Figure 4J). The most severe symptoms occurred in two plants of the Colombian accession (NSF021), where chlorotic spots were observed on leaves that later turned into necrotic lesions; stem drying; generalized wilting; and finally, death of the plants before 30 DAI (Figure 4H).

In contrast, some plants inoculated with the *Tulasnella* spp. isolates (Bv3 and Er1) generated new leaves, shoots and aerial roots (Figure 4A,C). While plants used as absolute controls (inoculated only with SDW) did not show symptom expression or the development of new tissues (Figure 4K,L).

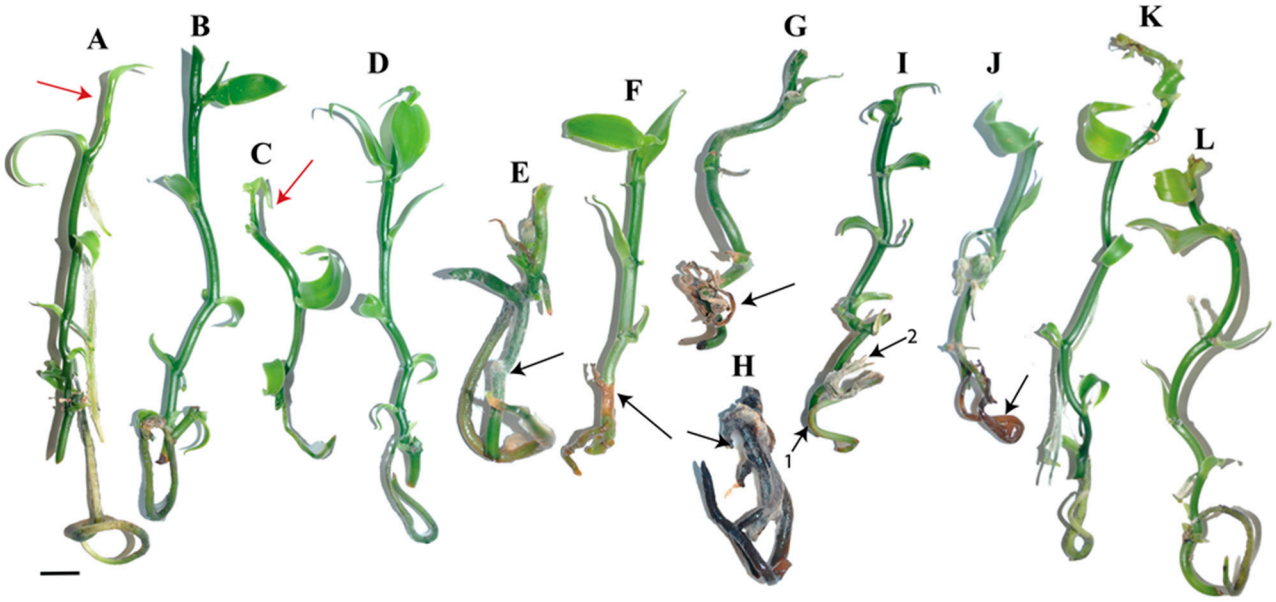


Figure 4. Examples of symptom development caused by form genus *Rhizoctonia* isolates on accessions of *Vanilla planifolia*. (**A**) New shoot on plant without symptoms, NSF021 inoculated with Bv3. (**B**) Plant without symptoms, NSF092 inoculated with Bv3. (**C**) New leaf on plant without symptoms, NSF021 inoculated with Er1. (**D**) Plant without symptoms, NSF092 inoculated with Er1. (**E**) Plant without symptoms with mycelium development, NSF021 inoculated with Er19. (**F**) Corky-looking sunken lesion on stem, NSF092 inoculated with Er19. (**G**) Dry rot on aerial roots, NSF092 inoculated with Er19. (**H**) Completely collapsed plant, NSF021 inoculated with Er19. (**I**) 1. Reddish-brown spots on root in contact with growing medium; 2. dry rot of aerial roots, NSF092 inoculated with Er19. (**J**) Wet rot of roots in contact with growing medium, NSF092 inoculated with Er19. (**K**) Absolute control, NSF021. (**L**) Absolute control, NSF092. Symptoms and signs are indicated by black arrows; new tissue development by red arrows (scale bar = 1 cm).

3.4. Colonization of the Form Genus Rhizoctonia on the Surface of the Tissue

Three days after inoculation (DAI) of the plant material, hyphal growth of Bv3 and Er1 became visible on the surface of the root in contact with the culture medium, and after 20 DAI, the mycelium extended to the root collar, where a hyphal network with a mucous appearance and whitish pigmentation was observed (Figure 5A). Isolate Er19 presented a faster growth, with the hyphae on the root in contact with the culture medium becoming

visible at 2 DAI and extending over the entire surface in less than 10 DAI. The hyphal network also presented a mucous appearance. However, the pigmentation was brown or brownish (Figure 5B).

Er19 generated abundant mycelium until it reached the aerial organs of the plant, sometimes inducing lesions where white sclerotia subsequently developed (Figure 5F,H). In contrast, no growth of Bv3 or Er1 was observed above the root collar penetrating the culture medium.

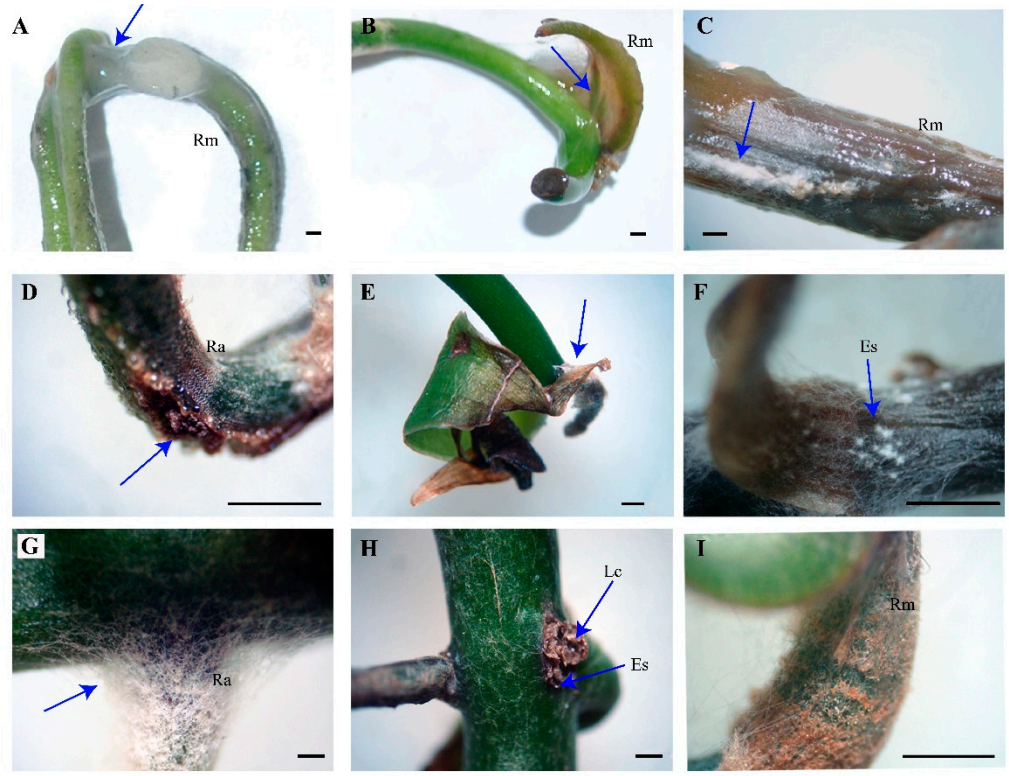


Figure 5. Colonization and/or symptom development of form genus *Rhizoctonia* on the surface of *V. planifolia* tissue. (**A**) Hyphal network of Bv3. (**B**) Hyphal network of Er19. (**C**) Signs of Er19 in root with wet rot. (**D**) Corky lesion and signs of Er19. (**E**) Mycelium of Er19 on leaf with necrotic spots. (**F**) Sclerotia of Er19 in necrotic tissue. (**G**) Mycelium of Er19 on healthy root. (**H**) Mycelium of Er19 on stem. (**I**) Mycelium of Er19 on root collar. Symptoms and signs are indicated by blue arrows. Lc: corky lesion, Ra: aerial root, Rm: root in contact with culture medium, Es: sclerotia (scale bar = 1.0 mm).

3.5. Colonization of the Form Genus Rhizoctonia within the Tissue

The growth of form genus *Rhizoctonia* isolates was more abundant on roots in contact with the culture medium, specifically in areas with the presence of root hairs. The hyphae were intertwined with the root hairs (Figure 6A) and were observed in the intercellular spaces of the velamen cells or as directly crossing them (Figure 6B,C). They then penetrated the hypodermis through the passage cells and reached the cortex without forming pelotons (Figure 6D,F); no hyphae reached the endodermis or vascular cylinder.

Up to the time of observation (45 DAI), no evidence of pathogenic activity by form genus *Rhizoctonia* isolates inside plant tissue, such as plasmolysis or degradation of cell structure and shape, was observed. However, it was not possible to process the roots inoculated with Er19 that presented rotting due to their high state of degradation.

Different colonization patterns of the form genus *Rhizoctonia* were observed depending on the accession of *V. planifolia*. No hyphae of Er1 were found penetrating the tissue of accession NSF021. Isolates Er19 and Bv3 penetrated the rhizodermis cells and reached the cortex in both accessions. However, Er19 was the only isolate found in aerial roots,

where it only penetrated to the velamen (Figure 6G,H). On the other hand, no anatomical differences were observed in the roots of both accessions that could influence colonization (Figure 6I).

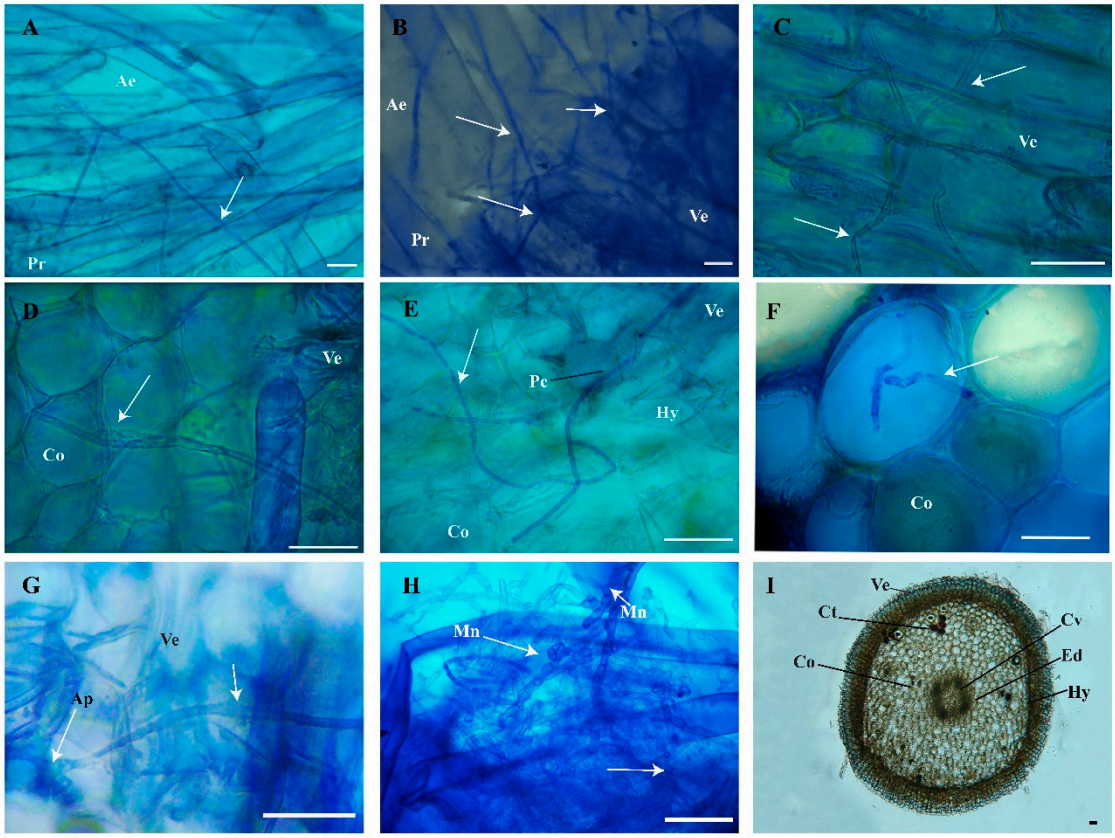


Figure 6. Colonization of form genus *Rhizoctonia* inside the tissue of *V*. *planifolia*. (**A**) Hyphae of Bv3 in the zone with presence of root hairs. (**B**) Penetration of Er1 through the rhizodermis. (**C**) Bv3 in velamen cells. (**D**) Bv3 crossing the velamen into the cortex. (**E**) Penetration of Er19 through the hypodermis into the cortex. (**F**) Bv3 in cortical cell. (**G**,**H**) Penetration of Er19 into aerial roots. (**I**) Anatomy of the root (NSF021) in contact with the culture medium. Ae: extra-radical area, Pr: root hair, Ve: velamen, Co: cortex, Pc: passage cell, Hy: hypodermis, Ed: endodermis Ct: tannifer cells, Ap: appressorium, Mn: monilioid cells, Cv: vascular cylinder (scale bar = 30 µm). Hyphae of the form genus *Rhizoctonia* are indicated by white arrows.

3.6. Effect of Plant–Antagonist-Pathogen Interaction on Symptom Expression

The plants of the two *Vanilla planifolia* accessions in interaction with each of the three form genus *Rhizoctonia* isolates and *F*. *oxysporum* f. sp. *vanillae* (1Fov) showed characteristic Fusarium wilt symptoms, with different developmental expressions (Figure 7).

When evaluating plant symptomatology with the disease severity scale, 1Fov induced symptoms in the positive controls for the pathogen (plants inoculated only with 1Fov) at 4 DAI, level 2 of the standardized scale of Koyyappurath et al. [9] (localized browning visible in affected tissue—20% of the affected plant). Death of plants of the Colombian (NSF021) and Mexican commercial (NSF092) accessions occurred at 12 and 15 DAI, respectively, level 4 (totally decayed or dead plant—100% of the plant affected).

Plants of both *V*. *planifolia* accessions previously treated with Bv3 and Er1 showed no differences in relation to symptom induction by 1Fov, considering the positive controls for the pathogen. However, plants inoculated previously with Er19 and then 1Fov presented symptoms at level 2 at 2 DAI and plant death; level 4 occurred at 10 DAI for NSF021 and at 12 DAI for NSF092.

It is important to highlight that the *Ceratobasidium* (Er19) isolate can induce some symptoms like 1Fov in the root, such as browning and brown areas (Figures 4I1 and 7B). However, Er19 does not generate stem or root strangulation (Figure 4E); moreover, the *Fusarium* isolate 1Fov induces wet rot and subsequent dry rot in roots in contact with the culture medium (Figure 7A), while Er19 only caused wet rot (Figures 4J and 5C). Therefore, Er19 is not considered to induce the expression of Fusarium-wilt-associated symptoms in the host.

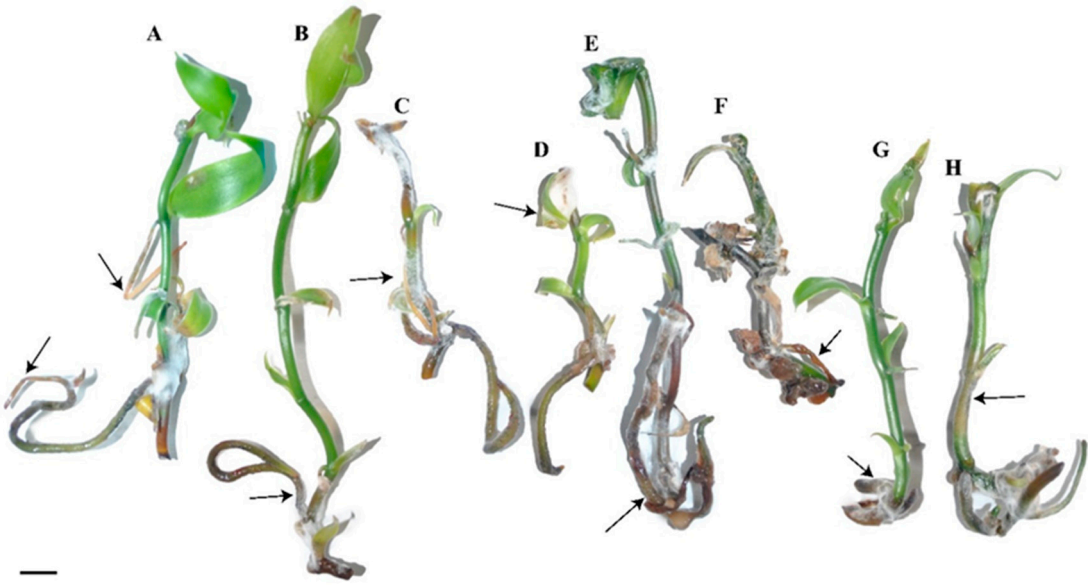


Figure 7. Symptomatology in *V*. *planifolia* accessions in interaction with form genus *Rhizoctonia* and *Fusarium oxysporum* f. sp. *vanillae*, 1fov. (**A**) Strangulation and dry rotting of roots, NSF021 inoculated only with 1Fov. (**B**) Root browning on contact with culture medium and mycelium development, NSF092 inoculated only with 1Fov. (**C**) Stem wilt, NSF021 inoculated with Bv3 and 1Fov. (**D**) Leaf wilt, NSF092 inoculated with Bv3 and 1Fov. (**E**) Wet rot of roots, NSF021 inoculated with Er19 and 1Fov. (**F**) Root strangulation in contact with the growing medium and generalized wilt, NS092 inoculated with Er19 and 1Fov. (**G**) Dry rot of root in contact with growing medium, NSF021 inoculated with Er1 and 1Fov. (**H**) Stem strangulation and mycelium development, NSF092 inoculated with Er1 and 1Fov. Plants had 10 DAI of 1Fov and 40 DAI of each form genus *Rhizoctonia* isolate. Symptoms and signs are indicated by black arrows (scale bar = 1 cm).

3.7. Effect of Plant–Antagonist–Pathogen Interaction on Fusarium wilt Development

The area under the disease progress curve (AUPDC) was calculated from the percentage severity measurements at 15 DAI. A continuous gradient of AUPDC values from 16 treatments was recorded to interpret disease development and possible biocontrol of the pathogen by endophyte form genus *Rhizoctonia* (Figure 8). AUPDC values ranged from 0.0 to 11.28. Absolute controls (treatments 1 and 2, plants inoculated only with SDW) and positive controls for the biocontrol agent (treatments 3 to 8, plants inoculated only with the form genus *Rhizoctonia*) showed no expression of Fusarium wilt. Therefore, the AUDPC values were zero. Treatments 14 and 16 presented the highest values (11.28–10.87), and the lowest values belonged to treatments 10 and 12 (2.11–2.09).

Regarding the statistical analysis of the AUDPC calculation results, the multifactorial ANOVA showed significant differences when considering the design factors independently. Significant differences were found for the factors form genus *Rhizoctonia* isolates ($F = 11.577230$; $p < 0.00009999$), *Fusarium* isolates ($F = 573.569442$; $p < 0.00009999$) and the interaction between both groups of microorganisms ($F = 573.569442$; $p < 0.00009999$). On the other hand, the *V*. *planifolia* accession factor showed no statistical differences ($F = 1.030388$; $p = 0.38446155$). Similarly, the interaction between form genus *Rhizoctonia* isolates, *Fusarium*

and plant accessions did not show statistical differences in the analysis (F = 1.945267; p = 0.38636136). Therefore, statistical differences are considered to exist in at least one of the eight treatments generated by excluding the *V*. *planifolia* accession factor from the statistical test.

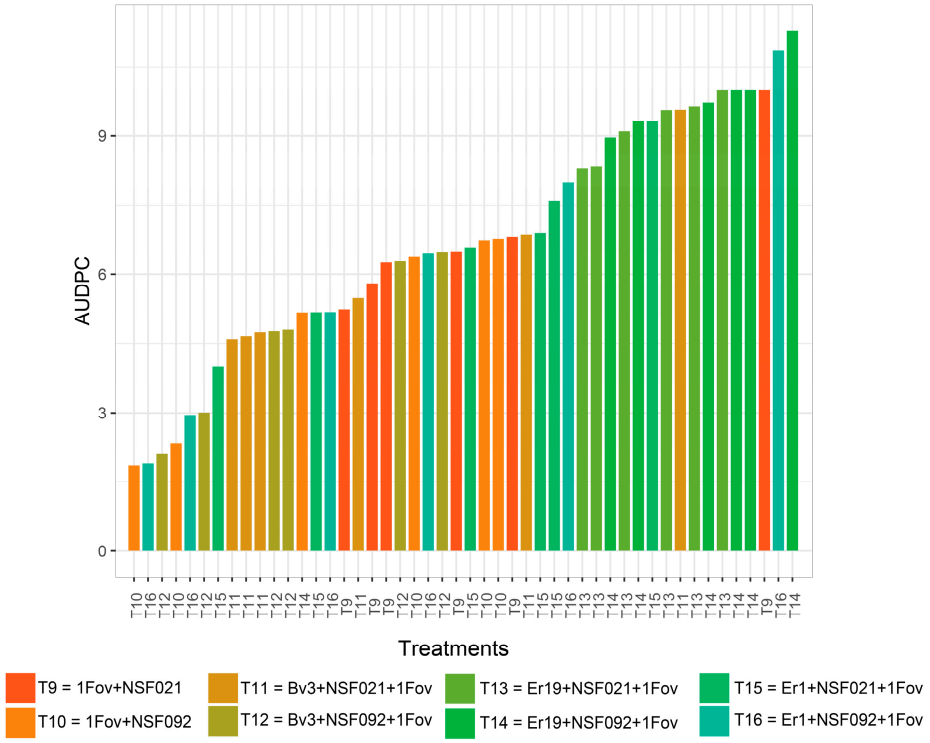


Figure 8. Gradient of Fusarium wilt development using the area under the disease progress curve-AUDPC in the conformal treatments.

When pairwise comparisons were performed with Tukey's test to detect statistical differences in the interaction between factors, no significant differences were found in the AUDPC means between plants previously treated with *Tulasnella* spp. (Bv3 and Er1) and plants used as positive control for the pathogen ($p > 0.05$) (inoculated only with 1Fov, Table 4). In contrast, plants inoculated with Er19 and 1Fov were significantly different from the rest of the treatments according to Tukey's test ($p < 0.05$), presenting the highest mean AUDPC (Table 4) and the highest expression of symptomatology (Figure 7E,F). It should be noted that the *V*. *planifolia* accession factor was not included in the analysis because there were no statistical differences in the ANOVA.

Table 4. Pairwise comparison of means with Tukey's test for statistical differences in the interaction of design factors: form genus *Rhizoctonia* isolates and *Fusarium* sp. isolate (mean ± SD of 6 replicates per treatment). Values of AUDPC means followed by different letters are significantly different (Tukey, $p < 0.05$).

Treatments	Form Genus *Rhizoctonia* + *Fusarium*	AUDPC
13, 14	Er19 + 1Fov	9.5 ^{a} ± 0.8
15, 16	Er1 + 1Fov	6.2 ^{b} ± 2.6
9, 10	1Fov	5.8 ^{b} ± 2.1
11, 12	Bv3 + 1Fov	5.3 ^{b} ± 1.9
3, 4	Bv3	0.0 ^{c} ± 0.0
7, 8	Er1	0.0 ^{c} ± 0.0
5, 6	Er19	0.0 ^{c} ± 0.0
1, 2	Absolute controls	0.0 ^{c}± 0.0

4. Discussion

4.1. Dual Culture Tests

The different interaction patterns observed in the dual tests between the form genus *Rhizoctonia* and *Fusarium* spp. may be associated with the simultaneous action of different biocontrol mechanisms reported in the literature such as antibiosis and competition for space and nutrients [23,34]. In the present study, two isolates of *Tulasnella* spp. (Bv3 and Er1) and one of *Ceratobasidium* sp. (Er19) caused loss of pigmentation, decreased hyphal concentration and cell lysis in *Fusarium* spp. in the contact zone. Additionally, Er19 caused darkening of the pathogen colonies. This may be due to the action of metabolites or extracellular enzymes, which can degrade the cell wall of the pathogen hyphae [25,32,33]. It is important to note that very few studies report *Ceratobasidium* causing these morphological and physiological alterations in phytopathogenic fungi [23]. There are no previous reports of the fungal genus *Tulasnella* acting through the antibiosis mechanism. On the other hand, changes in the tonality of *Fusarium* spp. colonies and the development of aerial hyphae forming "barriers" are characteristics that could be related to the defense against the antagonistic activity of the form genus *Rhizoctonia* [34].

In the present study, the form genus *Rhizoctonia* isolates did not generate structures that could be associated with mycoparasitism. However, Siwek et al. [24] reported two binucleate isolates of *Rhizoctonia* sp. generating appressoria and hyphal coils on the surface of *Pythium ultimum* var. *sporangiiferum*, the causal agent of bell pepper wilt (*Capsicum* sp.); nonetheless, the molecular processes and ecological factors involved in the expression of the different biocontrol mechanisms exerted by this group of fungi are still unknown [22].

Regarding the evaluation of the ability of form genus *Rhizoctonia* isolates to compete for space and nutrients, the *Ceratobasidium* sp. (Er19) was the only efficient isolate to rapidly colonize the culture medium, invading the colony of the three *Fusarium* spp. isolates and inhibiting their growth. This coincides with the results of Gonzáles et al. [23], when evaluating an isolate of *Ceratobasidium* sp. obtained as an endophyte on watermelon (*Citrullus lanatus*) against several pathogens of the same plant, including *F. solani*, *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *niveum*. Our results of the PRGI evaluation and competition for space and nutrients suggest that *Tulasnella* spp. isolates have a lower potential as biocontrol agents in vitro compared to *Ceratobasidium* sp. due to the slow growth of the former, which is typical of potential orchid mycorrhizal fungi [26].

4.2. Symptoms Induced by Form Genus Rhizoctonia

In this research, the isolate Er19 of *Ceratobasidium* sp., isolated from *C. quadricolor*, an epiphytic orchid endemic to the geographic valley of the Cauca River [28], was the only one to induce symptoms in *V. planifolia* plants (NSF021 and NSF092). Some of the symptoms observed were dry rotting of aerial roots, stem drying, and generalized wilting. Bhai and Dhanesh [11] report isolates of *Rhizoctonia* sp. and *Rhizoctonia solani* associated with this symptomatology in plantations of *V. planifolia* in India. Although there are studies of the pathogenicity of *Ceratobasidium* spp. in various crops [13,21,40], this is not the case for the genus *Tulasnella*. This taxon is most frequently reported as an endophyte, saprophyte and mutualistic symbiont in orchids, with no reports to date of this group of fungi acting as plant pathogens [17,20,22], as we also show here.

On the other hand, the development of new tissues in plants inoculated with the form genus *Rhizoctonia* coincides with that reported by Hossain et al. [41] and Zhao et al. [42]. However, no root pelotons were observed in the present study. Therefore, the effects on plant growth could be related to the production of phytohormones (auxins and cytokinins) in response to the interaction with this group of microorganisms [43].

4.3. Colonization of V. planifolia by Form Genus Rhizoctonia

To date, there are only two published investigations describing the penetration process and colonization pattern of a potentially pathogenic fungus in the root tissue of *Vanilla* spp. plants [44,45], neither of these related to the form genus *Rhizoctonia* and its simultaneous

potential as a mutualistic symbiont and biocontrol agent. Under the experimental conditions of the present study, hyphae of *Tulasnella* spp. (Bv3 and Er1) and *Ceratobasidium* sp. (Er19) were observed to enter the root through the velamen and subsequently penetrate the passage cells of the hypodermis to reach specific regions of the cortex. This is consistent with what has been described for the colonization of *Tulasnella* sp. in orchids of the genus *Paphiopedilum* [46]. The passage cells have thin walls and a large size and are inserted in the root hypodermis; their function is to regulate the apoplastic entry of water and other solutes into the cortex [44]. In orchids, the passage cells have been studied as the entry point to the cortex for pathogenic fungi such as *F. oxysporum* f. sp. *vanillae* [45] and mycorrhizal fungi such as *Tulasnella* spp. [46]. Based on the above and considering the hyphal network observed on the root surface of the *V. planifolia* accessions, it could be considered that the form genus *Rhizoctonia* can compete for space with *Fusarium* spp. not only by establishing itself in the host tissue but also by obstructing the pathogen's access points. During the histological observation, hyphae of *Tulasnella* sp. isolated from *C. quadricolor* (Er1) were recorded in the commercial Mexican vanilla accession root tissues (NSF092) but not in the Colombian accession (NSF021). These results suggest that in vitro vanilla accessions from different production systems respond differently to interaction with microorganisms, despite being the same botanical species (*V. planifolia*), which is consistent with previous studies [10].

4.4. Plant–Microorganism Interaction in Symptom Expression

The isolate 1Fov of *F. oxysporum* f. sp. *vanillae* induced stem and root rot symptoms on the two *V. planifolia* accessions inoculated with each of the three form genus *Rhizoctonia* isolates. The most severe symptoms were observed in plants inoculated 30 days earlier with *Ceratobasidium* (Er19). There were no biological or statistical differences in symptom expression between plants inoculated only with 1Fov (positive control for the pathogen) and those treated previously with *Tulasnella* spp. These results differ with previous investigations that reported the form genus *Rhizoctonia* endophyte of orchid roots decreasing the severity of the symptoms induced by the pathogen [21,22]. However, in the present study, the experimental conditions, host and isolates evaluated were different. When the AUDPC values and severity scale records were analyzed together, the results indicate that the accession from Colombia (NSF021) showed the highest disease development, although the statistical analysis did not find significant differences for the *V. planifolia* accession design factor. In contrast, Mosquera-Espinosa et al. [21] reported that the commercial accession from Mexico (NSF092) expressed the most severe symptoms and the highest development of Fusarium wilt among four different accessions of *Vanilla* multiplied in vitro, including NSF021. It should be noted that in the present study, as in Mosquera-Espinosa et al. [21], the 1Fov isolate and the same in vitro mother material of *V. planifolia* (NSF021 and NSF092) were used. This difference observed between the studies could be associated with the somaclonal variation that has been reported in different investigations [47,48]. In the case of *V. planifolia*, Ramírez-Mosqueda et al. [49] found that during 60 days under in vitro conditions (after two selection cycles), 35.7% (40) of the shoots were resistant to *F. oxysporum* f. sp. *vanillae* filtered at a concentration of 50%, while 26% of the total shoots expressed systemic resistance to the disease under greenhouse conditions.

4.5. Plant–Microorganism Interaction in the Development of Fusarium Wilt

For the analysis of AUDPC means, the *V. planifolia* accession factor was not considered. Likewise, the highest AUDPC mean value was 9.5, which is interpreted as moderate disease development according to Koyyappurath et al. [9] and was seen in plants of both accessions previously treated with *Ceratobasidium* sp. On the other hand, the means of AUDPC for plants treated with *Tulasnella* sp. from *C. quadricolor* (6.25) and *Tulasnella* sp. from *V. rivasii* (5.3) are interpreted as mild disease development. However, the mean of plants inoculated only with *F. oxysporum* f. sp. *vanillae* (5.8) (positive control for the pathogen) is also interpreted as a slight development of the disease.

Therefore, the AUDPC values and the biological response of the host do not allow the acceptance of the hypotheses put forward in this study because there is no evidence of a biocontrol effect that can be attributed to the isolates of the *Rhizoctonia* form genus, inducing resistance in plants. Furthermore, this biocontrol effect was not greater in the Colombian accession (NSF021); on the contrary, this accession presented the greatest expression of symptoms and the most rapid development of the disease. The absence of a biocontrol effect by the form genus *Rhizoctonia* could also be associated with somaclonal variation. This is because several studies have documented the loss of adaptive characteristics in plants that have undergone micropropagation [47,47,50]. The incidence and effect of genetic and epigenetic modifications that occur during continuous multiplication of the plant material are highly unpredictable [47]. Therefore, in the present study, it is possible to consider a negative effect on genes involved in the induction of systemic resistance in *V. planifolia* accessions in vitro.

Even though the form genus *Rhizoctonia* isolates evaluated did not generate a reduction in Fusarium wilt development in the host, it should be noted that the mean AUDPC values for plants inoculated only with isolate 1Fov (5.8) were lower than those reported by Mosquera-Espinosa et al. [10], who obtained values close to 10 when evaluating the accessions used in this study under similar in vitro experimental conditions. These differences, although statistically comparable, may have important practical implications for plant recovery in commercial systems [51]. However, changes in AUDPC as a response factor cannot be explained only with plant essays, since other factors such as the genetics of the host and of the microorganisms (antagonist and pathogen) and the interaction at the molecular level between the parties must be considered [52]. In the present study, the AUDPC values for control plants inoculated only with the pathogen suggest a lower severity of the disease induced by the 1Fov isolate, with respect to that reported by Mosquera-Espinosa et al. [10]. The possible decrease in virulence of 1Fov could be related to the changes in nuclear dynamics reported during the development of *Fusarium oxysporum* mycelium, from a uninucleate to a multinucleate state, after completion of the colony initiation [53]. This genetic rearrangement increases genotypic plasticity [53,54]. In addition, it is potentially related to the pathogenicity and virulence of the microorganism, which is why it is considered an important source of genetic variability in fungi [54,55].

5. Conclusions

In the present study, the potential of three isolates of the form genus *Rhizoctonia* as biocontrol agents of Fusarium wilt on *V. planifolia* was evaluated. In the dual tests, morphological alterations in the somatic structures of *Fusarium* spp. were presented, which are attributed to the in vitro antagonistic activity of *Tulasnella* spp. (Er1 and Bv3) and *Ceratobasidium* sp. (Er19) through the mechanisms of antibiosis and competition for space and nutrients. However, these results do not correspond to those obtained when evaluating the microorganisms in vitro–in vivo on a host, where no biocontrol effect against Fusarium wilt by orchid root endophytes was evidenced in the accessions of *V. planifolia* (NSF021 and NSF092), even though these fungi exhibited the ability to colonize the root surface and to penetrate the cortex. These findings demonstrate the need to continue studying the simultaneous potential of the form genus *Rhizoctonia* and other endophytes as a mutualistic symbiont in *Vanilla* and possible biocontrol agents of pathogens under field and greenhouse conditions. In addition, it is important to evaluate in future research other factors (genetic, environmental and agronomic) that may affect the activity of the biocontrol microorganism and its relationship with the host plant.

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A.T.M.-E.; drafting—revision and editing, S.M.-B., N.S.F. and A.T.M.-E. All authors have read and agreed to the published version of the manuscript.

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