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In Vitro Evaluation of the Development of *Fusarium* in Vanilla Accessions

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Abstract: Vanilla is an economically important crop for low-lying humid tropical regions. World demand for natural vanilla is increasing, but cultivated plants face serious phytosanitary problems. The disease known as Fusarium wilt is mainly related to the fungus *Fusarium oxysporum* f. sp. *vanillae*, and for its management, the pathogen–host relationship must be understood. Four in vitro multiplied vanilla accessions were evaluated: two *Vanilla planifolia* from Colombia and Mexico, one from *V. odorata*, and one (1) F1 hybrid (*V. rivasii* × *V. trigonocarpa*). In addition, three isolates of *Fusarium* from different symptomatic plants present in small-scale agroforestry systems: (1Fov) *F. oxysporum* f. sp. *vanillae* from leaf, (2Fov) *F. oxysporum* f. sp. *vanillae* from root and (3Fs) *F. solani* also from root. Plants with two months of growth were inoculated in vitro by immersion of roots, and the development of Fusarium wilt was recorded for 15 days, using a severity scale to describe symptoms and to calculate the area under the disease progress curve (AUDPC). No statistical differences were found when analyzing the interaction between *Fusarium* isolates and vanilla accessions. However, when independently analyzing the design factor *Fusarium* isolates, there were significant differences; the 1Fov isolate of *F. oxysporum* f. sp. *vanillae* induced the highest symptoms as well as death in some plants of all accessions, while *F. solani* was considered a secondary pathogen. There were no statistical differences for the vanilla accessions factor, but the values of AUDPC and symptoms observed suggest a slight resistance in all the accessions. Therefore, it is suggested to explore the vanilla gene pool to generate multiplication material with resistance genes and to contribute with genetic improvement to successfully integrate the management of Fusarium wilt in commercial systems.

Keywords: *Fusarium* spp.; vanilla plant pathology; *Vanilla* spp.

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1. Introduction

The genus *Vanilla* (Family Orchidaceae) has approximately 120 species [1,2], of which *V. planifolia* is cultivated due to the aromatic properties of its fruits [3]. Vanilla is the most widely used flavoring in the food industry, and natural vanilla is the second most expensive spice in the world market after saffron [1,4]. Vanilla fragrance is valued in both the food and cosmetic industries [5].

Despite its economic importance, the vanilla crop is attacked by pathogens due to its low genetic variability, as a consequence of continuous vegetative propagation [6–10]. Among the various diseases that affect Vanilla crops, those caused by *Fusarium* species are considered the most limiting [11,12].

The symptomatology of Fusarium wilt in vanilla begins with brown spots on the roots and chlorosis on the stem; they advance as dry rot in terrestrial and aerial roots and chlorotic lesions and necrotic points in fruits and leaves; subsequently, generalized wilting occurs, followed by the absence of new shoots and finally the death of the plant [11,13–15]. The cause of the disease has been associated with *Fusarium* species, including *F. solani* [12,13,16], *F. oxysporum* f. sp. *vanillae* [11,14,17], and *F. oxysporum* f. sp. *radicis-vanillae* [3,13].

Fusarium attack limits the productivity and growth of *Vanilla* spp. crops [6,12] but also presents a high risk to human health due to the fruit's potential contamination with mycotoxins, including: (a) fumonisins B1 (FB1; PubChem CID: 2733487) and B2 (FB2; PubChem CID: 2733489), which have nephrotoxic, hepatotoxic, cardiotoxic, immunosuppressive, neurotoxic, teratogenic, embryotoxic, pulmotoxic and cytotoxic effects; (b) thrichothecenes such as DON (PubChem CID: 40024), NIV (PubChem CID: 5284433), T−2 toxin (PubChem CID: 5284461) and HT−2 toxin (PubChem CID: 10093830) registered to have genotoxic, mutagenic, teratogenic, immunosuppressive, hepatotoxic, neurotoxic and hemotoxic effects; and (c) zearalenone as ZEA (PubChem CID: 5933650) with estrogenic, genotoxic, mutagenic, teratogenic, immunosuppressive and hemotoxic effects [18]. Therefore, it is necessary to generate strategies to control the development of Fusarium wilt in the plant. Among the different strategies for managing the disease, the use of resistant genotypes is considered one of the most viable alternatives [1,6,19].

For this reason, detailed information on *Vanilla* spp.–*Fusarium* spp. interactions are required [3]. Therefore, studies should be carried out on the response of said interaction in the vanilla crop's primary and secondary genetic pools, to find potential material that contributes to the genetic resistance to the attack of *Fusarium* spp. and initiates crop improvement. This approach is viable since the existence of *Vanilla* species expressing different degrees of disease severity has been shown in previous studies [3,13].

Likewise, it must be recognized that some endophytic fungi are reported as latent pathogens or saprophytic, potentiating one of the two roles in response to environmental factors or to the decrease in the expression of resistance genes of their hosts [20–22]. Studies show the same *Fusarium* species acting as a phytopathogen or bio controller depending on the host plant [23]; however, in *Vanilla* spp., it has been possible to isolate *Fusarium* spp. from asymptomatic tissue, naming it as an endophyte, and from symptomatic tissue without confirming its pathogenic role based on Koch's postulates [24].

Therefore, for the present investigation, the following hypotheses were raised: (1) In different accessions of vanilla acclimatized in a greenhouse and subsequently multiplied in vitro, the presence of fungal endophytes will be low or non-existent due to the conditions provided in a balanced environment, guaranteeing the availability of healthy plant material for fulfilment of Koch's third postulate [25,26]. (2) *Fusarium* species obtained from symptomatic tissue and inoculated into healthy vanilla accessions multiplied in vitro will all induce symptoms in the hosts tested. However, the highest values of the disease, according to the standardized severity scale of Koyyappurath et al. [13], will be associated with *V. planifolia* (NSF092) that comes from Mexico, since this material is propagated clonally to establish commercial crops around the world, which influences its low genetic variability and therefore greater affection by Fusarium wilt [8,11,12,14].

In this study, the objectives were: (1) To characterize the community of cultivable fungal endophytes present in vanilla accessions multiplied in vitro. (2) To evaluate the pathogenicity of three *Fusarium* isolates in vanilla accessions multiplied in vitro, including material from *V. planifolia* crop´s primary and secondary genetic pool, implementing the standardized disease severity scale of Koyyappurath et al. [13]. (3) To evaluate the development of Fusarium wilt in vanilla accessions multiplied in vitro, by calculating the area under the disease progress curve (AUDPC).

2. Materials and Methods

2.1. Vanilla Material Multiplied In Vitro

Two (2) accessions of *Vanilla planifolia* with different provenance, one from Colombia (NSF021) and one from Mexico (NSF092—*V. planifolia* variety "Mansa" [27]; in addition, one (1) accession of *V. odorata* (NSF023) and one (1) of a hybrid F_1 (*V. rivasii* NSF059 × *V. trigonocarpa* NSF073) were propagated and maintained in vitro in 25 × 150 mm Fisherbrand sterile glass tubes, with 15 mL of the artificial culture medium called VAI−002−1. That medium consists of ORCHIMAX salts (Duchefa Biochemie B.V.), 25.3 g, adding Benzyl Amino Purine (BAP), 2.0 mg, Naphthalene–Acetic Acid (ANA), 1.0 mg, Sucrose, 20 g; Duchefa Agar, 7.0 g, 1000 mL distilled water. This methodology is implemented by the Gene Editing Platform of the Alliance between Bioversity International and the International Center for Tropical Agriculture (CIAT-Palmira).

The in vitro propagated plants were kept in a growth room at 28 ± 2 °C, with a photoperiod of 16/8 h, luminosity of 120–150 µmol m^{-2} s^{-1}, and without regulation of environmental humidity, until reaching two months old.

2.2. Characterization of Fungal Endophytes in Vanilla Accessions Multiplied In Vitro

To characterize the community of endophytic fungi of the vanilla accessions, multiplied in vitro, stem, leaf, and root sections were made from two plants taken at random from each accession. These tissue sections were disinfected with 1% hypochlorite and 70% alcohol, going through three washes with sterile distilled water (SDW); each process was performed for one minute. Once the tissues were disinfected, they were allowed to dry on sterile absorbent paper to remove excess moisture. Once dry, they were transferred to Potato Dextrose Agar acidified at 2.5% PDAa and incubated at 26 °C [28]. The material was reviewed during the following 10 days to determine the expression of endophytic fungi.

2.3. Reactivation, Obtaining of Monosporic Cultures, and Identification of Fungal Isolates

Three *Fusarium* isolates were obtained from symptomatic *V. planifolia* tissue from different plants growing in small-scale agroforestry systems in Valle del Cauca, Colombia (Table 1) [24]. Two *F. oxysporum* isolates, one from the leaf (1Fov) and another from the root (2Fov), with a purple tonality of the colony, although with different mycelial development, and an *F. solani* isolate also from the root (3Fs), with a white and yellow colony tonality were recovered.

Table 1. *Fusarium* isolates were used to evaluate the development of *Fusarium* wilt in vanilla accessions in vitro.

Code	Fungus Species	Characteristics of Colony	Tissue	Accession Number in GenBank
1Fov	*F. oxysporum* f. sp. *vanillae*	Purple tonality, cottony mycelium	Leaf	OP035624
2Fov	*F. oxysporum* f. sp. *vanillae*	Purple-violaceous tonality, creeping mycelium	Root	OP035625
3Fs	*F. solani*	White hue with yellow areas, cottony mycelium	Root	OP035626

These isolates were reactivated in a PDAa medium and kept for eight days in incubation at 26 °C [28]. Monosporic cultures were obtained from these isolates, implementing the methodology of Gilchrist-Saavedra et al. [29]. A conidial suspension was prepared by scraping the mycelium and conidia, from which an aliquot of 500 µL of the suspension was taken, which was dispensed in a water agar (AA) medium. Observations were made at intervals of 24, 48, and 72 h to determine germination of individual conidia, to be transferred into individual Petri dishes with PDAa and to guarantee their monosporic origin.

The isolates were identified as species, based on: (1) morphological characteristics. Macroscopic description (color and texture of the mycelium) and microscopic description (aspect of micro and macroconidia) were recorded in the colonies grown in PDAa. (2) molecular analysis. DNA was extracted from the colonies, following the protocol of Lee and Taylor [30]. Subsequently, the ITS region of the rRNA was amplified by PCR using the universal primers ITS4 (5′-TCCTCCGCTTATTGATATGC−3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG−3′) [31]. The PCR reactions were prepared in a final volume of 50 µL, adding 5 µL of DNA + 45 µL of Master Mix (5 µL of Taq Buffer (KCl-MgCl) + 5 µL of $MgCl_2$ + 5 µL DNTPs + 5 µL of primer Forward + 5 µL of primer Reverse + 19.5 µL of water + 0.5 µL of Taq polymerase). Amplification was performed in a thermocycler using the following program for ITS-rRNA with an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min [32]. The PCR products were purified and sent to Macrogen for sequencing. The obtained sequences were edited using BioEdit [32] and identified by BLAST searches in the NCBI database (http://blast.ncbi.nlm.nih.gov/) and FUSARIUM-ID, taking into account the highest percentages of similarity shown in those molecular database [33]. (3) Phylogenetic analysis. The consensus sequences were aligned and analyzed manually with the help of the MEGA6 program [34]. Based on the matrix obtained, it was possible to determine the nucleotide substitution model, taking into account the Bayesian Information Criterion (BIC) using the ModelGenerator version0.851 software of Keane and collaborators (Maynooth, Ireland) [35]. The phylogenetic analysis was determined using the maximum likelihood (ML) method using the 2-parameter Kimura model, and the internal reliability of the nodes was determined using the bootstrap method with 1000 iterations. As an external group of the phylogenetic tree, the species *Rhizoctonia solani* (HQ263348) was used.

2.4. Inoculation of Fusarium Isolates in In Vitro Multiplied Vanilla Accessions

The methodology of Koyyappurath et al. [13] was modified by allowing monosporic *Fusarium* cultures to grow in a PDAa medium for 10 days. For inoculation, a conidial suspension adjusted to a concentration of 1×10^6 [36] was prepared with the aid of a Neubauer chamber.

For inoculation, 20 plants per accession were used, which were removed from the growth medium, washed with sterile distilled water (SDW), and gently cleaned with sterile absorbent paper to remove residual activated carbon residues from the medium. The root immersion in the conidial suspensions for five minutes was implemented. For negative controls, roots were submerged for the same time using SDW [13,22].

Once the inoculation was carried out, the seedlings were individualized in 25 × 150 mm Fisherbrand sterile glass tubes, with 2 mL of SDW and incubated at a constant temperature of 26 °C, relative humidity of 80%, and a photoperiod of 12 h in a chamber of growth brand Tecnal model TE−381. The plants were evaluated every two days during the 15 days after inoculation (DAI) to observe the development of symptoms in roots and aerial organs.

To comply with Koch's fourth postulate, to re-isolate the inoculated microorganism in the different treatments, 15 DAI or earlier, depending on the development of symptoms, cuts of diseased tissue of the plants treated with isolates of *Fusarium* spp. were transferred to PDAa using three repetitions.

2.5. Evaluation of the Development of Symptoms in Vanilla Accessions Multiplied In Vitro

To evaluate the symptom induction capacity of the three *Fusarium* isolates inoculated on seedlings of different vanilla accessions, the standardized scale of Koyyappurath et al. [13] was implemented, which categorizes and describes the disease into four levels of expressed symptomatology. Severity scale: 0 = no symptom (0% of the affected plant); 1 = dull leaves (10% of the plant affected); 2 = visible localized browning in affected tissue (20% of affected plant); 3 = brown areas and visible mycelium (60% of the affected plant); 4 = totally decomposed or dead plant (100% of the affected plant).

For this research, the development of Fusarium wilt (terrestrial stem and root rot) was evaluated on the affected organs after the roots were inoculated, to subsequently calculate the area under the progress curve of the disease (AUDPC). Symptomatic tissue measurements were made such as aerial roots, roots, and root collars that penetrated the medium, stem, and leaves throughout the 15 DAI of evaluation. These measurements were made using photographs analyzed in the IMAGE J program with java 1.8.0_112 for greater precision of the values obtained.

2.6. Statistical Data Analysis

To perform the statistical analysis of the data, a completely randomized experimental design—CA—was followed, consisting of 16 treatments and five repetitions per treatment. All analyses were performed in the statistical software R (version 3.6.3) of R core Team (Vienna, Austria). If the data did not meet the assumptions of normality and homogeneity of variance, a bifactorial ANOVA model of fixed effects was applied, using the ANOVA function 2way. R, with 10,000 Legendre permutations [37]. This procedure recognizes statistical differences, at a 95% confidence level, independently concerning the design factors, which for the present investigation, corresponded to *Fusarium* isolates, vanilla accessions and the interaction among both factors.

To determine statistically significant differences within each design factor, multiple comparisons of the possible pairs of means were performed with the pairwise Permutation Test function with Bonferroni correction. Additionally, to statistically interpret the development of the Fusarium wilt, the area under the disease progress curve (AUDPC) was calculated, using the AGRICOLAE package and the AUDPC function.

3. Results

3.1. Community of Fungal Endophytes in Vanilla Accessions Multiplied In Vitro

There was no expression of fungal microorganisms in Petri dishes with PDAa for seedings of disinfected vanilla tissue multiplied in vitro (Figure 1). Therefore, the hypothesis raised about the low presence or absence of cultivable fungal endophytes in the plant material was accepted.

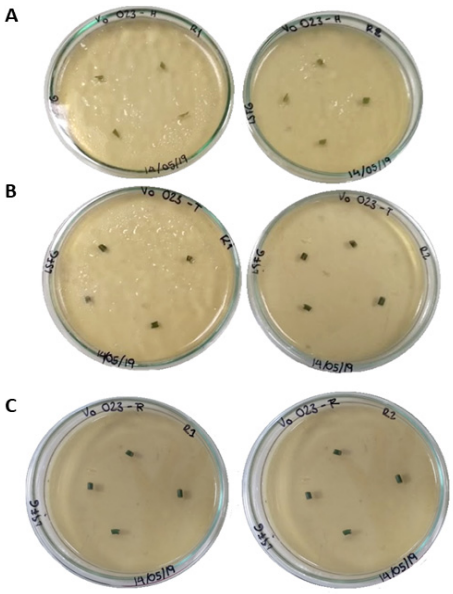

Figure 1. Tissue seeding of vanilla accessions multiplied in vitro 10 days later, for isolation of endophytic fungi on PDAa: (**A**) leaf cuts; (**B**) stem sections; (**C**) main terrestrial root cuttings.

3.2. Identification of Fungal Isolates

The isolates with codes 1Fov and 2Fov from the leaf and root, respectively, were identified as *F. oxysporum* f. sp. *vanillae*, while the isolate coded as 3Fs, isolated from the root, was identified as *F. solani*. The morphological characteristics of the identified colonies are presented in Table 1 and Figure 2. At the molecular level, the sequences obtained for the ITS region of the rRNA, corresponding to the 1Fov and 2Fov isolates, presented a similarity of 100% with reference sequences of the *F. oxysporum* species (ON740952, MW789025, ON181985), while the sequence obtained for the 3Fs isolate presented 100% similarity with sequences of the *F. solani* species (KY978584, MK333991, ON514027).

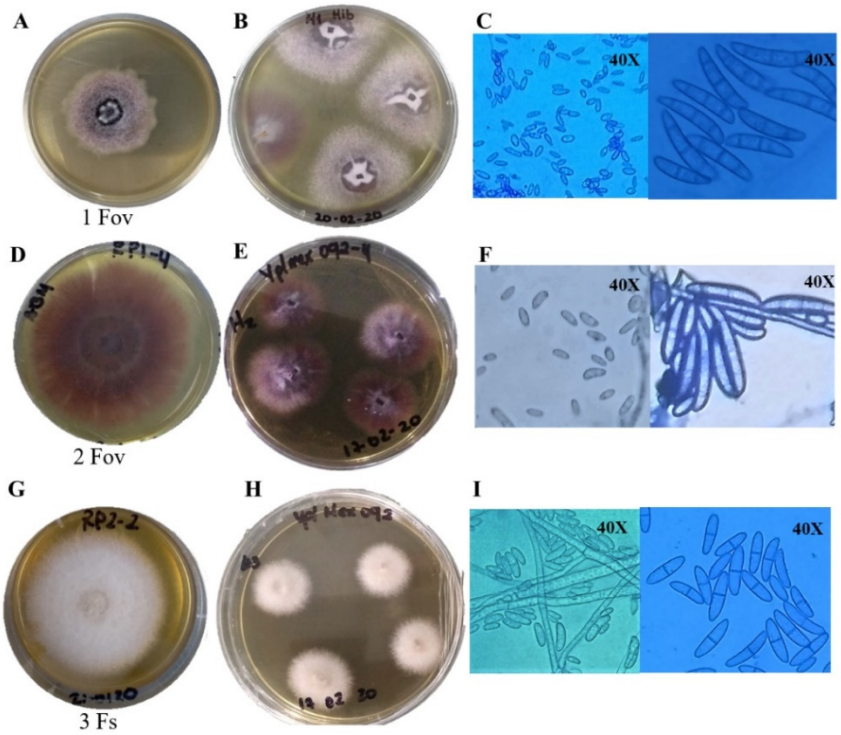

Figure 2. Identified colonies, re-isolation of *Fusarium* spp. inoculated to evaluate the development of symptoms in vanilla accessions. (**A**,**D**,**G**) Colonies on the obverse of monosporic origin of the two isolates *F. oxysporum* f. sp. *vanillae* (1Fov and 2Fov) and the *F. solani* isolate (3Fs); (**B**,**E**,**H**). Obverse colonies re-isolated from symptomatic tissue of inoculated plants; (**C**,**F**,**I**) microconidia (left) and macroconidia (right) characteristics of the genus *Fusarium* obtained from each re-isolation.

According to the constructed phylogenetic tree (Figure 3), the sequences corresponding to the 1Fov and 2Fov isolates identified as *F. oxysporum* f. sp. *vanillae* were grouped in the same clade with reference sequences of *F. oxysporum* of the same and other special forms (JQ975403, MG905829, MG905422, AY380575, KM005080 and KT261749 of vanilla; KR071144 and MG136705 of tomato; EU022522 of Banana), with Bootstrap support of 92%. On the other hand, the 3Fs sequence identified as *F. solani* were grouped in the same clade with reference sequences from the same species (KY978584 from an undefined host, ON514027 from the medicinal plant *Hygrophila auriculata*), with Bootstrap support of 100%. The sequences of these species, including those generated in the present study, are grouped into clades apart from other *Fusarium* species reported in vanilla crops such as *F. concentricum*, *F. verticillioides*, *F. mangiferae*, *F. nygamai*, and *F. proliferatum*.

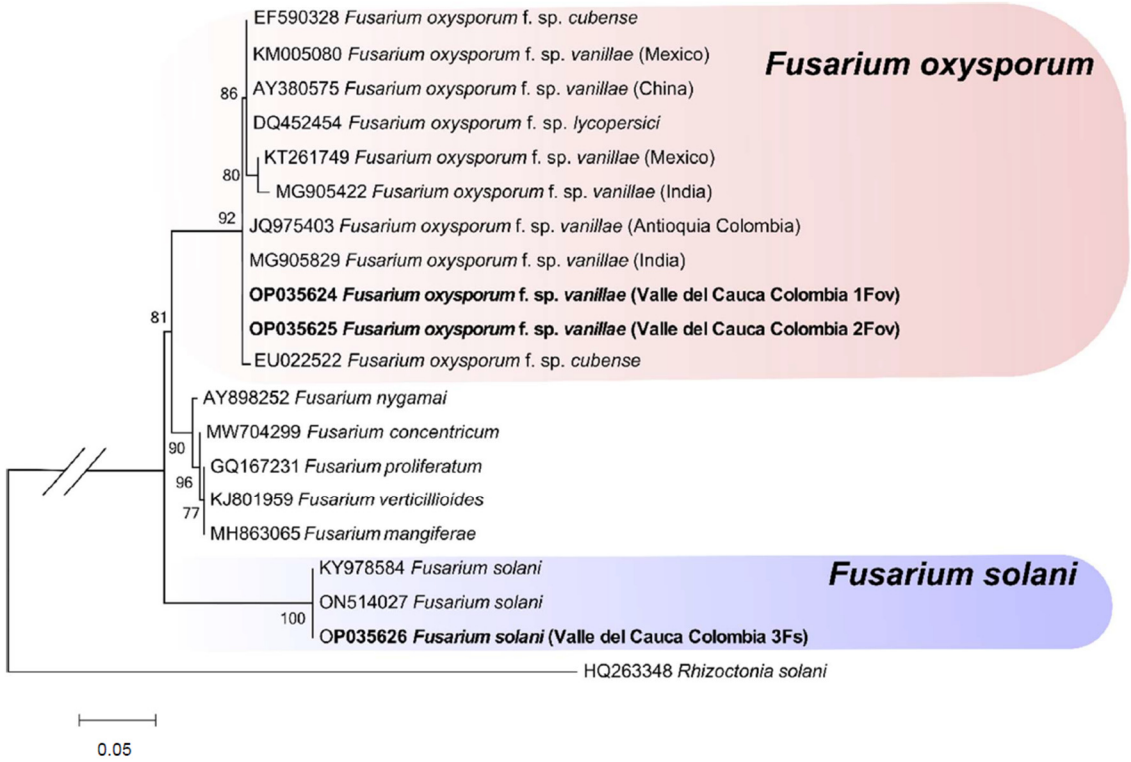

Figure 3. Phylogenetic tree obtained by the Maximum Likelihood method based on the Kimura 2-parameter model of consensus sequences of the partial Internal Transcribed spacer-ITS region of the rRNA. The sequences obtained in this study are indicated in bold. The numbers in the branches indicate a Bootstrap value ≥70%. The species *Rhizoctonia solani* was included as an outgroup.

3.3. Evaluation of Fusarium Wilt in Vanilla Accessions Multiplied In Vitro

The three *Fusarium* isolates evaluated induced symptoms in all vanilla accessions, with different expressions of development. The two isolates of *F. oxysporum* f. sp. *vanillae* (1Fov and 2Fov) were the first to induce symptoms in the host, which occurred at the fourth DAI; the *F. solani* isolate (3Fs) induced symptoms at six DAI.

When analyzing the symptomatology evaluated based on the severity scale of the disease, of the three inoculated isolates, 1Fov, *F. oxysporum* f. sp. *vanillae*, was the only one capable of inducing seedling death, level 4 (plant decomposed or dead, 100% of the plant affected), during the 15 DAI. The infective process induced death to plants at 10 DAI in the two accessions of *V. planifolia* (NSF092 and NSF021) and *V. odorata* (NSF023), while in the hybrid material F_1 (NSF059 × NSF07), death occurred at 12 DAI. The 2Fov isolate, *F. oxysporum* f. sp. *vanillae*, induced symptom expression up to level 3 (brown areas and visible mycelium; 60% of the plant affected) at 15 DAI, in all the plant material evaluated.

On the other hand, the 3Fs, *F. solani* isolate, at 15 DAI, induced symptoms at level 2 (visible localized browning in affected tissue; 20% of the affected plant). However, of the three isolates the most severe symptoms were induced by 1Fov, *F. oxysporum* f. sp. *vanillae*, and observed in the two accessions of *V. planifolia* (NSF092 and NSF021).

Concerning the results obtained from the statistical analysis, the bifactorial ANOVA showed significant differences when considering the design factors independently. For the *Fusarium* isolates factor, highly significant differences were found ($F_{3, 624} = 150.990$, $p < 0.00009$); meanwhile, for the vanilla accessions factor, the differences were significant ($F_{3, 624} = 2.95$, $p = 0.032$). For both cases, the analysis was based on the response factor, which was the development of symptoms in seedlings after two months of development. However, no significant differences were found when analyzing the interaction of both variables; that is, there was no statistical difference between treatments ($F_{9, 624} = 1.321$, $p = 0.219$).

When performing the statistical analysis based on the multiple comparisons of means with the Bonferroni correction test, which is more sensitive to detecting statistical differences, the data showed that there were differences for the *Fusarium* isolates factor (Table 2), but no longer highly significant, with the 1Fov isolate being the one that induced the greatest symptomatology in the vanilla accessions (Figure 4A). However, the same statistical test this time did not detect differences within the vanilla accessions factor (Table 2, Figure 4B).

Table 2. Multiple comparisons of means with Bonferroni correction to detect statistical differences within each design factor.

***Fusarium* Isolates**				***Vanilla* Accessions**			
Comparison	**Stat**	***p* Value**	***p* Adjust**	**Comparison**	**Stat**	***p* Value**	***p* Adjust**
Control−1Fov	0–12.85	8.82×10^{-38}	2.646×10^{-37}	NSF021-NSF023	0–0.5231	0.6009	0.7172
Control−2Fov	0–12.35	4.89×10^{-35}	9.784×10^{-35}	NSF021-NSF059 × NSF073	0–0.9097	0.363	0.5445
Control−3Fs	0–10.4	2.542×10^{-22}	3.813×10^{-25}	NSF021-NSF092	0–2.185	0.02889	0.1733
1Fov−2Fov	0–3.316	0.0009141	9.141×10^{-4}	NSF023-NSF059 × NSF073	0–0.3623	0.7172	0.7172
1Fov−3Fs	0.958	0	0.000 × 10e	NSF023-NSF092	0–1.608	0.1078	0.3234
2Fov−3Fs	0 8.264	2.22×10^{-16}	2.664×10^{-16}	NSF059 × NSF073-NSF092	0–1.278	0.2013	0.4026

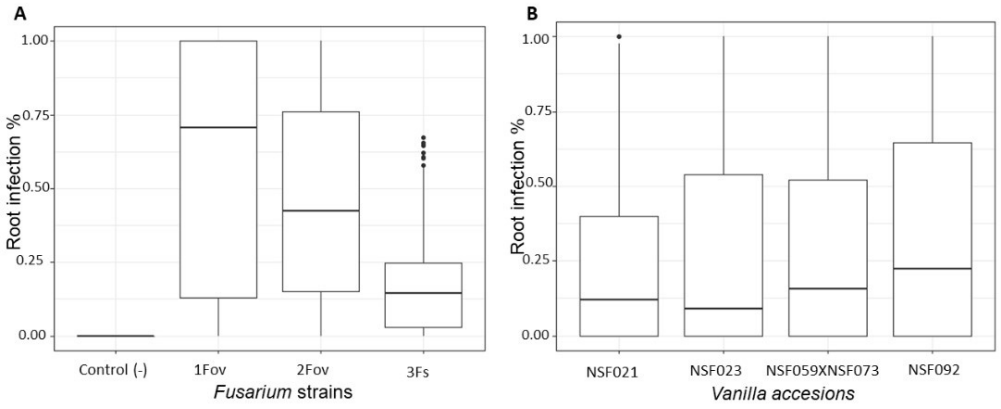

Figure 4. (**A**) Plot of design factor designated as *Fusarium* isolates; The atypical points of the 3Fs isolate belongs to the percentage of infected root in the accessions NSF092 (Points 1,2, 4 and 6 from top to bottom), NSF023 (point 3) and NSF056XNSF073 (point 5), and (**B**) design factor graphic designated as vanilla accessions; The atypical point illustrated in the image belongs to a repetition of the NSF021 accession with the 1fov isolate.

3.4. Description of Symptoms Induced by Isolates of Fusarium spp. In Vitro Vanilla Accessions

The two isolates of *F. oxysporum* f. sp. *vanillae* (1Fov and 2Fov) that caused the most severe symptoms initially induced stem chlorosis in seedlings, followed by brown spots, and finally root rot in contact with the culture medium. It was observed that the symptoms of rotting began with a wet appearance and later dry, followed by strangulation of the root neck and stem, as well as necrosis in some of the aerial root tips (Figure 5). In the two accessions of *V. planifolia* (NSF092 and NSF021), the most severe root throttling was recorded (Figure 5E–G), and in *V. odorata* (NSF023) in particular, stem rot was found to be limited to the internodes (Figure 5F). In the F1 hybrid (NSF059 × NSF07), the symptoms were mild (Figure 5C), with mycelial development and wet rot in roots in contact with the culture medium.

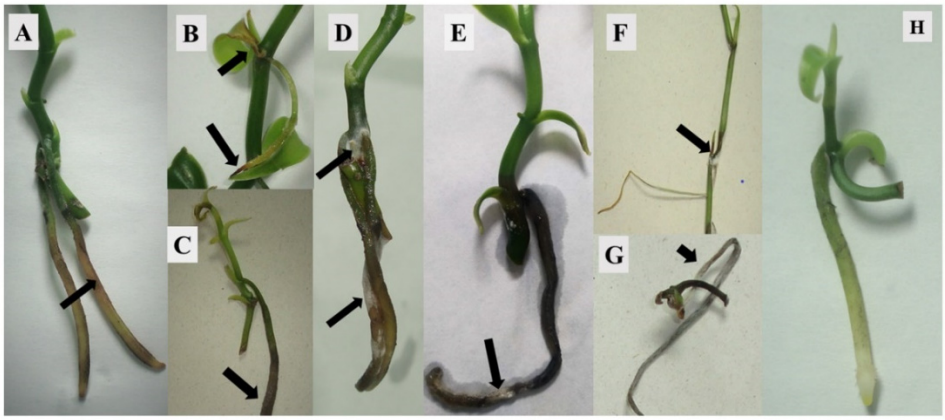

Figure 5. Development of symptoms caused by isolates of *Fusarium oxysporum* f. sp. *vanillae* in vanilla accessions. (**A**) Root browning in contact with the culture medium, *V. planifolia* NSF092; (**B**) aerial root with necrotic tip and wilted leaf, *V. planifolia* NSF021; (**C**) development of mycelium and wet root rot in contact with the culture medium of the F1 hybrid (NSF059 × NSF07); (**D**) wet root rot in contact with the culture medium, *V. planifolia* NSF092; (**E**) root dry rot in contact with the culture medium, *V. planifolia* NSF092; (**F**) stem strangulation, *V. odorata* (NSF023); (**G**) root strangulation in contact with the culture medium, *V. planifolia* NSF092; (**H**) control plant not inoculated with the pathogen. Symptoms and signs of the disease are shown with black arrows.

Plants inoculated with *F. solani* showed mild symptoms of browning, necrosis, and some cases of soft rot at the root tip in contact with the culture medium (Figure 6); however, the greatest symptoms were observed in *V. planifolia* NSF092. The seedlings used with the negative controls (inoculation only with SDW) did not present symptom development, as observed in Figure 5H.

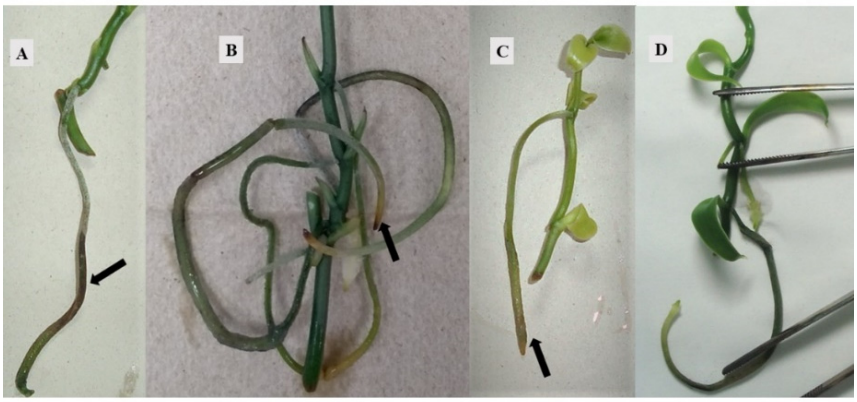

Figure 6. Development of symptoms caused by *Fusarium solani* in vanilla accessions. (**A**) Root browning in contact with the culture medium, *V. planifolia* NSF092. (**B**) necrotic tips on roots in contact with the culture medium, *V. odorata* (NSF023); (**C**) mild root rot in contact with the culture medium of the F_1 hybrid (NSF059 × NSF07). (**D**) control plant not inoculated with the pathogen. Black arrows shown symptoms and signs of the disease.

3.5. Re-Isolation of Fusarium spp. Isolates Following Koch's Postulates

The re-isolation in the PDAa medium of the fungi was achieved from the symptomatic tissue of the inoculated plants in the three repetitions. It was possible to obtain only colonies with similar characteristics to those that originated in monosporic cultures, information that was complemented with the descriptions of reproductive structures under the microscope at 10X and 40X. Typical microconidia and macroconidia of the *Fusarium* genus were visualized according to Agrios [35] (Figure 2). The septa of macroconidia in *F. oxysporum* f. sp. *vanillae*

ranged between 3 and 4 (Figure 2C–F), while in *F. solani*, they were between 2 and 3 (Figure 2I).

3.6. Evaluation of the Development of Fusarium Wilt in Vanilla Accessions In Vitro

The values of the calculation of the area under the disease progress curve, AUDPC, in all the evaluated accessions, were located in a wide general range, between 0 and 10.0 (Figure 7), without statistical differences. However, plants inoculated with *F. oxysporum* f. sp. *vanillae* (1Fov) presented the highest AUDPC values that were between 8.6 and 10.0, which when interpreted according to the levels of the severity scale of Koyyappurath et al. [13], would be plants slightly resistant to the development of Fusarium wilt. Meanwhile, the lowest values were in a range between 0.94 and 3.9, and mostly correspond to inoculations with *F. solani* (3Fs).

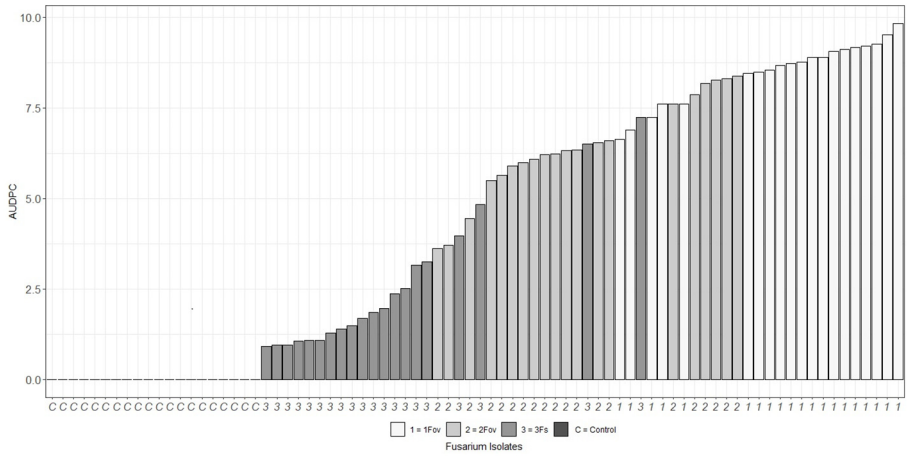

Figure 7. Fusarium wilt development gradient in vanilla accessions, calculated using the area under the disease progress curve—AUDPC. 1Fov = *F. oxysporum* f. sp. *vanillae* from leaf, 2Fov = *F. oxysporum* f. sp. *vanillae* from root and 3Fs = *F. solani* from root.

When performing the AUDPC analysis for each accession, it was found that the highest values were obtained when inoculating *F. oxysporum* f. sp. *vanillae* (1Fov): in *V. planifolia* NSF092, the value was 9.84, followed by *V. planifolia* NSF021 with 9.52, the hybrid F_1 (NSF059 × NSF07) with 9.26 and finally *V. odorata* (NSF023) with 9.20. In contrast, the lowest values of AUDPC were recorded with the isolate *F. solani* (3Fs) which were values of 7.23 in *V. planifolia* NSF092, 3.97 in *V. odorata* (NSF023), 3.24 in *V. planifolia* NSF021 and 3.15 in the hybrid F_1 (NSF059 × NSF07).

4. Discussion

4.1. Community of Fungal Endophytes in Vanilla Accessions Multiplied In Vitro

As cultivable microorganisms included fungi were not expressed in PDAa from the tissue of the vanilla accessions cultivated in vitro, it is interpreted that propagated plants via meristems can be free from the expression of endophytes due to the absence of differentiated conductive tissue (xylem and phloem) in this type of explant [38], but also because of the different disinfection and asepsis procedures due to the methodologies implemented for plant tissue culture [39]. This suggests that the in vitro propagation methodology of *Vanilla* spp. developed in the Gene Editing Platform of the Alliance between Bioversity International and the International Center for Tropical Agriculture (CIAT-Palmira) is effective in eliminating microorganisms, which guaranteed in this study healthy plant material for the fulfilment of Koch's third postulate. Therefore, it is confirmed that the symptomatology observed in the *Vanilla* spp. plant's was caused by the inoculated *Fusarium* isolates in the present study, complying with Koch's third postulate [36].

Likewise, since no cultivable endophytic microorganisms were found in the processed samples, the possibility of the presence of pathogens or biocontrollers latent in the in vitro vanilla material as mentioned in other studies is ruled out, which is positive due to the elimination of pathogens transmissible in the planting material such as *F. oxysporum*, *F. solani*, and *Phoma* spp., but unfavorable by eliminating microorganisms with a biocontrol role in vanilla [9,19,20]. There is research that mentions fungal endophytes in *V. planifolia* with bioprotective properties [21]. Similarly, the biocontrolling role of fungal endophytes on pathogenic *Fusarium* species has been reported in different plants, both under in vitro and in situ conditions [22,40,41].

4.2. Identification of Fungal Isolates

The morphological characteristics and the DNA sequences recorded for the *Fusarium* colonies analyzed in this study confirmed the presence of *F. oxysporum* f. sp. *vanillae* and *F. solani* in symptomatic vanilla plants in small-scale cultivation in agroforestry systems in Valle del Cauca, Colombia. These *Fusarium* species have been reported to cause symptoms in vanilla crops from different countries, including India, Mexico, Indonesia, China, Puerto Rico, and Colombia [4,12,14,33,42]. Phylogenetically, these species were separated from other species of the same genus reported in vanilla crops, including *F. concentricum*, *F. verticillioides*, *F. mangiferae*, *F. nygamai*, *F. semitectum*, *F. proliferatum*, and *F. sporotrichioides*, which coincide with other studies [33].

4.3. Symptoms Induced by Fusarium spp. Isolates in Vanilla Accessions In Vitro

The two isolates of *F. oxysporum* f. sp. *vanillae* (1Fov and 2Fov) induced rotting symptoms in stem and root, as reported by Pinaria et al. [12] in investigations with *Vanilla* spp. in India. Similarly, this coincides with the greenhouse reports obtained by Cardona et al. [4], and in the field by Hernández- Hernández [11], as well as by Tombe and Liew [43] and Bhai and Dhanesh [17].

During the 15 DAI, the two isolates of *Fusarium oxysporum* f. sp. *vanillae* induced the most severe symptoms, isolated from plants other than symptomatic *V. planifolia*; 1 Fov was obtained from the leaf and 2 Fov from the root. Research studies in Reunion Island, Madagascar, and Indonesia by Koyyapurath et al. [13] and Pinaria et al. [12] found *F. oxysporum* f. sp. *vanillae* (Fov) as the causal agent of stem and root rot, likewise, with *F. oxysporum* f. sp. *radicis-vanillae* (Fov) causing terrestrial root and root collar rot. The results obtained show that there are differences between the two isolates of the species *Fusarium oxysporum* f. sp. *vanillae* in the ability to induce symptoms, even when both come from the same botanical species (*V. planifolia*) but develop in different production systems. This indicates that studies on the microbiome and its functionality in vanilla should be expanded in natural and commercial systems [1,8].

Meanwhile, the isolate of *F. solani* (3Fs) obtained from the symptomatic root of *V. planifolia* was the one that induced the mildest symptoms. These results coincide with reports in India, Indonesia, and Puerto Rico causing rot and necrosis in the roots of *V. planifolia* in commercial vanilla systems, and indicated as a secondary pathogen [12,13,16].

4.4. Re-Isolation of Isolates of Fusarium spp. Inoculated in Vanilla Accessions In Vitro

The fungal colonies re-isolated from the plant material inoculated in the present investigation expressed similar characteristics to those observed in the monosporic cultures used.

The characteristics of mycelium of purple tonality with white and violet mycelium in the colonies of *F. oxysporum* f. sp. *vanillae* coincide with the registered ones in other studies. From symptomatic tissue of watermelon (*Citrullus lanatus*) and asymptomatic pine (*Pinnus* sp.) plants, colonies of *Fusarium oxysporum* with purple and purple-violaceous pigmentation were re-isolated, in addition to macroconids with 3 and 4 septa, characteristic of this fungal species [44,45]. Likewise, in studies with *Vanilla* plants, colonies of *F. oxysporum* f. sp. *vanillae* showed colony development and shades similar to those observed in the present

investigation [42]. In the case of the re-isolations of *F. solani*, the colonies presented cottony mycelium with a yellowish-white tonality, descriptions similar to those reported by Robles et al. [45]; and macroconidia with 3 septa, as described by Martínez-Fernández et al. [46].

Therefore, based on the macroscopic and microscopic characteristics of both fungal species, Koch's fourth postulate is met by obtaining the same microorganism inoculated to induce symptoms in healthy hosts of vanilla accessions in vitro [36].

4.5. Evaluation of Symptoms Development and Description of Fusarium Wilt in Vanilla Accessions In Vitro

When analyzed together, the AUDPC values for the development of Fusarium wilt and the records obtained from the symptoms based on the standardized severity scale of Koyyappurath et al. [13] reveal that the two accessions of *V. planifolia* (NSF092 and NSF021), without presenting statistical differences and with low values, were the ones that expressed the greatest development of the disease.

Concerning AUDPC, for *V. planifolia* NSF092, its value was 9.84 and *V. planifolia* NSF021 presented 9.52; on the severity scale, the levels were 4 and 3, respectively. However, the greatest symptoms were observed in the *V. planifolia* (NSF092) material from Mexico. However, these results do not allow for accepting the second hypothesis proposed in this investigation, because both accessions of *V. planifolia* (NSF021 and NSF092) developed symptoms induced by the isolates of *F. oxysporum* f. sp. *vanillae* (1Fov and 2Fvo) without finding statistical differences. Other studies reveal that there may be accessions of *V. planifolia* with different degrees of response to pathogenic species of *Fusarium* spp., that is, material that is resistant, slightly resistant, moderately susceptible, susceptible, and highly susceptible [3,13]. Likewise, among *V. planifolia* plants of a same accession that are in vitro propagated, there is variation in disease severity after inoculation with *F. oxysporum*, which has been attributed to the somaclonal variation in different investigations [6]. These authors found that for 60 days (after two cycles of selection), 35.7% (40) of the buds were resistant to *F. oxysporum* strain H3 filtrates in a concentration of 50%, and that of the total buds selected, the 26% expressed systemic resistance to the disease under in vivo conditions.

The development of Fusarium wilt in plants of different vanilla accessions throughout the Indian Ocean reached AUDPC values above 30 and was categorized as susceptible. However, those with values below 30 were classified as slightly resistant [3,13]. When analyzing the results of AUDPC obtained in the present study, in general, the values were low, as they did not exceed the record of 10.0; therefore, it can be suggested that the evaluated material would be classified as slightly resistant. However, in the cited research, vanilla accessions and isolates of *Fusarium* spp. were evaluated differently from those used in the present study.

In relation only to the *Fusarium* isolates, 1Fov, *F. oxysporum* f. sp. *vanillae*, was the only one that induced seedling death in the two accessions of *V. planifolia* (NSF021 and NSF092), with a record of level 4 of the severity scale at 10 DAI. These results under in vitro conditions agree with investigations in India and others under greenhouse conditions [4] and in the field [11,42] by indicating this species of *Fusarium* as the causal agent of stem and root rot in *Vanilla* spp. [12].

5. Conclusions

Without statistical differences, all the accessions evaluated in vitro developed Fusarium wilt, but the AUDPC values were not higher than 10.0, which postulates the plants as slightly resistant, according to the severity scale of Koyyappurath et al. (2016). However, the two accessions of *V. planifolia* (NSF092 and NSF021) were the ones that expressed the highest development of the disease (9.84 and 9.52) and symptomatology (on the severity scale levels 4 and 3). Meanwhile, the material corresponding to the *V. planifolia* crop's primary and secondary genetic pool, the hybrid F_1 (NSF059 × NSF07) and *V. odorata* (NSF023), expressed milder symptoms and lower AUDPC, in which genetic expression analysis and prospection of resistance genes are required.

There is a need to reduce the pressure of phytopathogenic microorganisms in the production systems of *Vanilla* spp., mainly *Fusarium* spp., which is achieved by integrating agronomic practices that are environmentally friendly and with greater emphasis in the use of resistant materials. Therefore, interaction studies among *V. planifolia* crop's primary and secondary genetic pools and *Fusarium*, as in this investigation, could identify plant material with resistance genes that accurately contribute to the integrated management of phytosanitary problems, mainly Fusarium wilt. Identifying slightly resistant vanilla accessions partially mitigates the problem, but efforts for the obtention of durable and effective plant material with polygenic resistance against the different genetic variants of the pathogen are necessary for an effective solution to a systemic and limiting pathogen such as some *Fusarium* species.

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