Transmission of *Furcraea necrotic streak virus* (FNSV) by *Olpidium virulentus*

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ABSTRACT

Furcraea necrotic streak virus (FNSV) is the causative agent of necrotic streak disease, also known as "macana" in fique crops (*Furcraea* spp.) resulting in damage to leaf fibres and economic losses. The rhizospheric fungus *Olpidium* spp. is present in the roots of affected plants and may play a role in the disease transmission. Regarding this, FNSV infection and the role of fungi were examined in macanavirus diseased plants in Colombia.Spherical, and icosahedral (24.7 nm ± 1.98), non-enveloped virions with a granular surface were isolated from field fique plants showing compatible signs of macana disease, and the viral genome was completely sequenced. Taxonomic status was assigned through sequence analysis (*Macanavirus* genus in *Tombusviridae* family). The variability of the virus pangenome was also evaluated in diseased fique plants from ecologically different Andean regions that showed low gene flow. Furthermore, *Olpidium virulentus* zoospores were identified by internal transcribed spacer (ITS) sequencing, which were associated with viral particles and resting spores in diseased fique roots through microscopic and molecular analysis. Additionally, an *in vitro* virion-zoospore binding assay showed that FNSV and *O. virulentus* zoospores interacted. Transmission assays in lettuce (*Lactuca sativa*), a model used to study this virus, showed 100% infection when a preincubated mixture of zoospores and FNSV was added to roots, whereas 33% infection occurred when FNSV was added alone. This demonstrated *O. virulentus*'s ability to act as a vector for FNSV, potentially enhancing viral transmissibility in field fique crops. This is the first report of FNSV being transmitted by *O. virulentus*, a rhizosphere fungus.

1. INTRODUCTION

Fique (*Furcraea* spp., Asparagales: Agavaceae) is an Andean plant distributed in Colombia and northern Venezuela that has agricultural value due to the industrial use of its fibres. For this reason, fique is widely cultivated in 10 Colombian Departments with Nariño (41%) and Cauca (39%) being the largest producers (Ministerio de Agricultura y Desarrollo Rural, 2019). Fique leaves are mainly used for the extraction of long, hard, resistant fibres, accounting for 4% of their composition. The fibres constitute the raw material for various manufacturing products, such as thread, rope, sacks and crafts, including packaging for Colombian coffee and cacao exports (Kozłowski et al., 2020) Industrial residues derived from the production chain (containing short fibres that are usually discarded) are currently being revalued and proposed as new, useful products in the textile and chemical industries (Amaya Vergara, 2018; Guancha-Chalapud et al., 2020; Neves Monteiro et al., 2018) . These qualities make fique an interesting plant for cultivation in South America and other parts of the world due to its hardiness and adaptability to varied ecoregions.

Fique crop productivity is adversely affected by diseases; the most limiting one is known as “macana” which is triggered by the FSNV. This pathogen causes leaf damage leading to large-scale economic losses due to the great impact on fibre quantity and quality, evidenced by chlorotic lesions that progress to necrotic stitches and stripes (Dabek and Castaño, 1978). Disease incidence has been estimated at 26% for crops in Cauca (Flórez et al., 2013), and it usually causes the death of affected specimens. Moreover, there is no treatment to control the disease, forcing growers to replace plants, which only produce quality fibres after 4 years of planting (Ministerio de Agricultura y Desarrollo Rural, 2019). Macana disease was originally reported in Colombia’s Antioquia

Department in 1960, and FNSV was first identified by Dabek and Castano (Dabek and Castaño, 1978) .

The species FNSV was described as a member of the *Tombusviridae* family (Morales et al., 1992), a group of phytopathogens affecting many important crops. Then, it was classified as the only species of a new genus called *Macanavirus* (Adams et al., 2013) according to the coat protein's protruding domains which do not occur in viruses of the most related genus, *Necrovirus.* FNSV virions consist of about 30 nm diameter icosahedral particles containing a monopartite, single-stranded, positive-sense RNA genome (Morales et al., 1992). The only known genomic sequence (3,966 nt) reveals five open reading frames (ORFs) encoding two RNA polymerases (p25 and p78), two movement proteins (p11 and p6) and a capsid protein (p35) (Adams et al., 2013) . The existing sequence variability in the populations of this virus in its distribution area is unknown, which may limit molecular diagnosis and, therefore, hinder disease surveillance strategies.

Previous studies have shown that macana disease can be mechanical transmitted to fique plants through field tools (Dabek and Castaño, 1978), which is currently the main control measure to prevent its spread. Interestingly, lettuce plants can reproduce symptoms when infected with root sap from fique plants, thus offering a model for studying the virus and the disease (Kusunoki and Beltrán-Acosta, 2013).Some viral species belonging to the *Tombusviridae* family are known to be transmitted by root-infecting zoosporic fungi and obligate parasites on plants, such as those in the *Olpidium* genus. For example, the transmission of Cucumber necrosis virus (CNV) by *Olpidium bornovanus* has been reported (Rochon et al., 2004). Additionally, an association between *Olpidium virulentus* and Lettuce big-vein associated virus (LBVaV) and Mirafiori lettuce big-vein virus (MLBVV) has been established (Maccarone, 2010). The challenge with

these situations is that the resting spores can persist in the soil for months or years, making it difficult to control these viral diseases. Particularly, *O. virulentus* was reported in the roots of macana-diseased fique plants, suggesting that the fungus could play a role as an FNSV vector (Gonzalez et al., 2010). However, there are no reports of the relationship between *O.virulentus* and FNSV. Therefore, this study aimed to investigate FNSV infection in naturally infected Colombian fique plants, its pangenomic variability in productive areas, and the biological interaction between this virus and its putative vector, *O. virulentus*.

2. MATERIALS AND METHODS

Plant and soil sampling

Fifteen-year-old fique plants (*Furcraea* spp.) showing macana symptoms grown as a commercial crop in Colombia's Nariño department (in the El Tambo municipality, 1,260-2,300 masl, 1°24′35″N 77°23′34″O) were used to sample soil, fique roots and leaves. Composite samples of soil (approximately 500 g) and roots (50 g) were collected from at least four different points near the base of the plant stem between 30-50 cm depth. Soil samples were placed in plastic bags and root samples (including secondary roots) were washed with water to remove soil and debris and stored in paper bags. The youngest leaf that presented symptoms of the disease (chlorosis and necrotic spots) was collected from the same plant and cut into segments of approximately 30 cm in length. All samples were stored in a Styrofoam refrigerator until arrival at the laboratory. Soil samples were stored at 4 °C, while leaf and root samples were stored at -80 °C. The time elapsed between field sampling and laboratory storage did not exceed 36 hours. A larger collection of symptomatic plants' leaf and root samples from different ecological regions of Colombia was also created and stored at -80°C, covering the departments of Cundinamarca (4°51′11″N 74°5′21″W, 2,646 masl; 3 samples), Antioquia (6°37′6″N

74°58′42″W, 1,855 masl; 2 samples), Santander (6°30′18″N 72°59′42″W, 1,665 masl; 5 samples), Nariño (1°17′59″N 77°24′25″W, 2,240 masl; 7 samples) and Cauca (2°35′32″N 76°40′3″W, 1,706 masl; 1 sample). Cundinamarca, Antioquia, and Santander represent the northern Colombian Andes, while Cauca and Nariño represent the southern region. These samples were used to explore viral diversity by partial sequencing (explained later in "FNSV genome sequencing and molecular detection").

Isolating FNSV virions and proteome analysis

The procedure described by Morales *et al*., (Morales et al., 1992) was used to recover FNSV virions from fique leaves of Nariño that had chlorotic streaks (with some modifications). Briefly, leaf tissue (120 g) was homogenised in 150 mL of buffer A (0.01M EDTA, 1% β-mercaptoethanol) at 4ºC. The extract was then filtered through gauze to eliminate waste fibres and spun at 9,600xg for 15 min until clearance. The resulting sample was heated at 55ºC and ultracentrifuged at 12,000xg for 15 min; the supernatant was recovered and ultracentrifuged at 80,000xg for 90 min. The pellet was suspended in buffer A and resolved in a sucrose gradient (10-40 % w/v in phosphate buffer, pH 7.0) at 60,000xg for 4 hours. A white band was carefully recovered and suspended in buffer A (4:1 ratio). The virus stock was concentrated by ultracentrifugation at 80,000xg for 1 hour. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to estimate the quantity of viral particles at 280 nm wavelength. Viral proteins were analysed by polyacrylamide gel electrophoresis (SDS-PAGE) using a commercial molecular weight marker (XL-OptiProtein, New England-Biolabs, Ipswich, MA, USA) as a reference. Purified viruses were mixed with loading buffer (125 mM Tris-HCl, 2% SDS, pH 6.2, 10% glycerol, 0.004% bromophenol blue and 5% 2-mercaptoethanol) and heated at 100°C for 5 min. Proteins were separated in an electrophoresis chamber (Mini-PROTEAN Bio-Rad, Hercules, CA, USA) for 180 min at

80 V. The gel was stained with Coomassie brilliant blue (0.1%) for 30 min, distained for 1 h and visualised by an image analyser (Chemi-Doc BioRad, California, USA).

FNSV ultrastructural analysis

Purified virions from diseased fique plant of Nariño were observed by transmission electron microscopy (TEM) after negative staining with 1% (w/v) phosphotungstic acid (pH 7.0). In addition, infected leaves and roots were also prepared for TEM using an epoxy resin. Briefly, secondary roots of symptomatic fique plants were pre-treated with sodium hydroxide (10% w/v) at 90ºC for 10 min, followed by treatment with hydrochloric acid (1 N), and then washed with distilled water for 15 min. Approximately2 mm pieces of leaf and root were excised and fixed for 12 h in phosphate buffer (pH 7.2) containing 2% (v/v) glutaraldehyde, followed by two washes in buffer. The pieces were post-fixed in phosphate buffer containing 1% osmium tetroxide (w/v) for 4 h at 4^{o}C. The samples were washed and dehydrated in a graded series of ethanol (50%, 75%, 95% and 100%, v/v in H_2O) for 20 min each. The samples were embedded in Spurr resin (Sigma Aldrich EM 0300; San Luis, MI, USA), following the manufacturer's instructions. Thick sections were stained with toluidine blue and observed by light microscopy, while thin sections were stained with uranyl acetate and lead citrate and observed by TEM (JEOL 1400 Plus, Tokyo, Japan).

FNSV genome sequencing and molecular detection

A methodology based on RT-PCR, molecular cloning and automatic Sanger sequencing of amplicons was used to acquire the isolated FNSV virions' genome sequence (isolate from Nariño). Additionally, GeneRacer kit (Invitrogen, Carlsbad, CA, USA) was employed to determine the ends of the viral nucleic acid according to the manufacturer's instructions. Briefly, different primers were designed based on

NC_020469.1 (the only available sequence) and synthesised (Macrogen, Korea) (Table 1).

The RNAqueous™ Total RNA Isolation Kit (Ambion, Huntington, UK) was used to isolate RNA. The cDNA reactions were performed with random hexamers and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR assays were then conducted using Taq DNA polymerase (Promega, Madison, WI, USA) and the following thermal conditions: 4 min at 94ºC (1 cycle); 10s at 92ºC, 20s at 55ºC and 60s at 72ºC (35 cycles); 5 min at 72ºC (1 cycle). The PCR products were resolved by agarose gel electrophoresis, cleaned up (Wizard SV Gel and PCR Clean-Up, Promega) and molecularly cloned in pGEM-T Easy (Promega, Madison, WI, USA) or TOPO-TA vector (Thermo Fisher Scientific, Waltham, MA, USA). Then, inserts were sequenced using vector universal primers (Macrogen, Korea). In addition, Pol-F/Pol-R, PM-F/PM-R and CP-F/CP-R primers (Table 1) were used to generate PCR amplicons (genomic positions 30-501, 2,021-2,472 and 2,504-3,564, respectively) and acquire sequence for population diversity studies from the collection of 18 representative samples of the main fique-producing regions in Colombia. Moreover, CP-F/CP-R primers were used for RT-PCR assays to confirm the presence of FNSV when required. For this, samples were analysed in triplicate and results were verified by agarose electrophoresis; negative controls lacking viruses were used for each assay.

FNSV serological detection

FNSV was detected in roots and leaves using dot-blot immunoassay (DBIA) as previously reported (Toloza-Moreno et al., 2022). Briefly, polyclonal chicken-FNSV antibodies were produced in 18-week-old Babcock Brown hens and Immunoglobulin Y (IgY) was extracted from egg yolks using the EGGstract IgY Purification System Kit (Promega, Madison, WI, USA). DBIA samples were macerated with liquid nitrogen,

suspended in buffer A (1:1 w/v) and spun at 7,700xg for 15 min at 4°C. Supernatants were recovered, incubated at 55°C for 10 min, spun at 12,000xg for 15 min at 4°C and suspended in buffer A. Then, 2 µL aliquots were loaded onto nitrocellulose membrane (GE Healthcare Life Sciences, Chicago, IL, USA) and kept for 15 h at 4°C. Blocking was performed by immersing in a buffer containing 5% (w/v) skimmed milk for 1 h at 37ºC. The membranes were rinsed three times with wash buffer (Tris-HCl 0.1M, EDTA 0.2 % w/v, Tween 20 0.1 % v/v, NaCl 0.9 % w/v), incubated with chicken anti-FNSV IgY (10µg/mL) for 1 h at 37ºC with slow shaking, and then washed 3 times. Samples were blocked again for 90 min at 37ºC and rinsed. Anti-chicken-alkaline phosphatase conjugate (Sigma-Aldrich, San Luis, MI, USA) was added at a 1:5,000 dilution and incubated at 37°C for 1 h. The colour was developed by immersing membranes in a chromogenic reagent solution (SigmaFast BCIP/NBT; Sigma-Aldrich, San Luis, MI, USA). Samples were analysed in triplicate, and negative controls from specimens not infected and reagents only were used for each assay.

Maintaining and identifying *Olpidium virulentus*

Structures typical of those produced by *Olpidium* sp. in fique secondary roots were immersed in a 1% trypan blue solution for 16 h and observed by light microscopy. The spores were also observed by TEM following procedures similar to those explained above. Additionally, a soil sample associated with roots of fique crops from Cauca was used to isolate and propagate the fungus under laboratory conditions. Initially, the soil sample with fique roots was air dried for five months to eliminate the infectivity of the FNSV content (Campbell, 1985; Dias, 1970). After this period, the non-detection of viruses was confirmed by DBIA and RT-PCR. Samples were then used to inoculate lettuce (*Lactuca sativa* var. batavia) roots as bait plants, and zoospores were retrieved after exposure to dry air for another 1 month. Once again, it was confirmed that zoospores

were virus-free through DBIA and RT-PCR detection, and *Olpidium* sp. was propagated and maintained on lettuce roots using drench method, as described previously (Lot et al., 2002). Briefly, 7-day old seeds were grown in a pot containing a mixture of sterile sand and soil inoculated at a 1:10 ratio with fungi. The plants were then maintained for one month under controlled conditions at 18-26ºC, 60% relative humidity with a 12-hour photoperiod. Lettuce plants were left without watering for three days before root harvesting to retrieve zoospores, which were immersed in a glycine solution (50 mM, pH 7.6) as described by Varanda *et al*., (Varanda et al., 2011). Zoospore yield was determined by light microscopy in a Neubauer chamber. A PowerSoil DNA Isolation kit (MoBio Interactive, Toronto, Ont., Canada) was used to extract DNA for molecular characterization; PCR of the ribosomal DNA's (rDNA) internal transcribed spacer (ITS) region was used for taxonomic identification, using specific forward primers for *O. virulentus* (OLPvirF) and *O. brassicae* (OLPbraF) and a common reverse primer (OLPR), in accordance with previous reports (Herrera-Vásquez et al., 2009). A pGEM-T Easy vector system (Promega, Madison, WI, USA) was used for molecular cloning and sequencing (Macrogen, Korea). *O. virulentus* and *O. brassicae* DNAs were used as positive controls.

Bioinformatic analysis

The FNSV RNA polymerase protein sequence was compared to those of other members of *Tombusviridae* reported in GenBank (Table S1, Supporting information). Multiple alignments were performed using Clustal X and phylogeny inference was carried out using maximum likelihood with 1,000 replicates in MEGA 11 (Tamura et al., 2021) (the pairwise distances matrix was estimated using a JTT model). A similar approach was taken to study the phylogeny of different Colombian FNSV isolates, but a nucleotide concatemer derived from the PCR-generated DNA fragments corresponding

to RNA polymerase, movement protein and capsid protein ORFs was used (produced using Pol-F/Pol-R, PM-F/PM-R and CP-F/CP-R primers, respectively). In the case of *Olpidium* spp., different orthologous nucleotide sequences of the ITS region reported in GenBank (Table S2, Supporting information) were used to carry out phylogeny inference. Kimura 2-parameter (K2P) distances (Kimura, 1980) were estimated for FNSV and *Olpidium* spp. nucleotide sequences using MEGA 11 (Tamura et al., 2021).

Binding and transmission assays

FNSV virion-*O. virulentus* zoospore binding assays were conducted following a previously described method (Varanda et al., 2011), with some modifications, using DBIA and RT-PCR for viral detection. Briefly, 100 µg virions were incubated in 10 mL 50 mM glycine-NaOH (pH 7.6) solution containing 1 x 10^6 zoospore mL^{-1} for 20 min. The suspension was centrifuged at 5,600xg for 7 min and bound viruses were detected in the pellet or unbound ones in supernatant (experimental controls lacked zoospores). A transmission assay using lettuce plants (*Lactuca sativa* var. batavia) was also performed, as described by Varanda *et al*. (Varanda et al., 2011). This involved adding 5 µg purified virions to 50 mL of 50 mM glycine-NaOH (pH 7.6) solution containing 1 x 10^5 zoospores mL^{-1}. After 20 min of incubation, 1 mL of the suspension was poured into pots containing 7-day old lettuce seedlings growing in a mixture of sterile sand and peat. Whole plants were homogenised in liquid nitrogen after 15 days and suspended in 100 µL 0.01M EDTA (pH 7.8). The extracts were centrifuged at 7,700xg for 15 min and supernatants were incubated at 55ºC for 10 min. The samples were then centrifuged at 12,000xg for 15 min and evaluated by DBIA and RT-PCR. The assay consisted of three replicates (3 pots containing 5 plants each), including control treatments of zoospores lacking virus and virus lacking zoospores.

3. RESULTS

Macana disease in fique plants

Macana disease significantly affects Colombian fique crops. Less affected specimens have short chlorotic streaks on the leaves' abaxial side, especially at their bases. By contrast, severely affected plants have longer chlorotic stripes with some small necrotic spots in the middle of the leaves. Plants with generalised infection exhibit chlorosis on 90% of their leaves (observed on both abaxial and adaxial sides) and chlorotic streaks in the middle, accompanied by extensive necrotic streaks. Cross sections can reveal internal fibre damage (Figure 1).

Histological observations of the affected leaves and roots were also conducted to associate macana disease with its previously reported causative agent, FNSV (Figure 2a). Utrastructure studies of the same section that was analyzed by light microscopy revealed inclusion bodies or viroplasms containing electrodense viral particles where viral replication and assembly occur (Cook et al., 2019) (Figure 2b).

It was confirmed that sections of the fique leaves having signs of tissue damage in plants identified as sick with macana correlated with the presence of virions that could be the disease's aetiological agent. Molecular analyses were performed to confirm this assumption.

Virus isolation and phylogeny inferences

Careful viral isolation was performed to confirm fique-affecting viruses' taxonomy, previously reported as FNSV (Dabek and Castaño, 1978; Morales et al., 1992). Firstly, ultrastructural studies showed that virions have a diameter of 24.7 ± 1.98 nm and an icosahedral shape with granular surfaces, in accordance with the FNSV description (Figure 3a). SDS-PAGE structural proteome analysis (Figure 3b) revealed three detectable polypeptides (possibly the ~37 kDa capsid protein, ~25 kDa RNA polymerase

1 and ~12 kDa movement protein 1) also in accordance with the description of FNSV. This result was then validated by whole genome sequencing (WGS) which revealed a single molecule of RNA with 93.7 % identity with respect to the one reported in GenBank (FNSV-Cauca; NC_020469.1) in identical length and ORF distribution (Figure 3c). It is worth mentioning that protein sizes can vary due to possible post-translational modifications. Structural, sequence and proteomic studies corroborated the isolate's identity, consequently named FNSV-Nariño by the species to which it belongs and the geographical region where the macana diseased plant was found.

Phylogenetical inference considering the only genomic sequence available to date and other members of the *Tombusviridae* family was performed (Figure 4). This confirmed that FNSV-Nariño (this work) was closely related to FNSV-Cauca (the isolate reported in the "International Committee on Taxonomy of Viruses" ICTV), thereby belonging to the FNSV species, *Macanavirus* genus and *Tombusviridae* family (Rochon, 2012). Moreover, the tree topology revealed that macanaviruses form a clade together with gallantiviruses and alphanecroviruses, pathogens infecting tobacco, olives (Cardoso et al., 2005) and corn salad (*Valerianella locusta*) (Verdin et al., 2018) among other agricultural value plants. These viruses grouped with carmoviruses, machlomoviruses and panicoviruses defining Clade A into the Group I (Figure 4).

The variability of RNA viruses can be important as they parasitize hosts that are genetically heterogeneous or that present different states of health because of environmental conditions. Regarding this, 18 samples of fique specimens from different regions of the Colombian Andes showing evidence of macana disease were collected to explore the genetic variability of FNSV associated with its geographic distribution. RNA was extracted and three viral nucleic acid regions (encoding RNA polymerase -472 bp-, movement protein -452 bp-, and capsid protein -761 bp-), representing around 40% of the

genome, were amplified by RT-PCR and phylogenetically analysed (Figure 3c; Figure 5). The choice of these three regions was to include the variability that viral factors with different biological functions have.

The results show a division of the isolates into two genetic clusters (clade A -northern Colombian Andes region-, and B -southern Colombian Andes region-), coinciding with the geographical locations of the viruses where they were sampled (except for the only sample available from Cauca that, despite coming from the southern region, it grouped with the samples from the north). FNSV-Cauca (NC 020469.1) and FNSV-Nariño (both isolates with fully sequenced genomes) grouped in clade B and the two main Colombian productive regions show genetic isolation. In addition, the Kimura two-parameter (K2P) values were evaluated as an indicator of the distances among isolates (Kimura, 1980). Particularly, the capsid protein-related encoding region revealed the K2P values with the greatest dispersion (Figure 5b), which is to be expected for most viruses that require varying their exposed factors to sustain themselves by infecting heterogeneous populations of hosts and evading some immune responses. It should be noted that the variability among all isolates yielded K2P values ranging from 0.104 to 0.198, giving evidence that all tested samples belong to the same species. On the other hand, K2P values were significantly higher (1.090 to 1.203) when compared to one of the closest species, *Galinsoga mosaic virus* (GaMV), according to phylogeny inference (Figure 4).

Olpidium sp. and FNSV infection

Cross-sections of the macana diseased fique plants root allowed observation of epidermal cell tissue, cortical or cortical parenchyma cells, endodermis, and vascular cylinder cells with resting fungal spores of what appeared to be *Olpidium* sp., in its typical hexagonal shape. Spores were abundantly observed in the endodermis and vascular

cylinder cells close to the xylem (Figures 6a, 6b); different sized zoosporangia were detected in the vascular cylinder, presumably according to the different development stages (Figure 6c). Interestingly, ultrastructural images revealed the presence of virions inside most of the visualized zoospores (about 90 % of them) (Figure 6d). The identity of those viruses was then corroborated by RT-PCR and DBIA from isolated zoospores confirming the presence of FNSV.

To characterize the observed fungus, it was recovered from field fique roots and soil from Cauca and treated appropriately to remove FNSV (evaluated by DBIA and RT-PCR), to then be multiplied in lettuce plants under laboratory conditions by root inoculation. The ribosomal DNA's (rDNA) internal transcribed spacer (ITS) region was obtained by PCR, sequenced, and phylogenetically studied in accordance with previous reports (Herrera-Vásquez et al., 2009; Zelyüt and Ertunç, 2021). The fungus under study was closer to the isolates of *O. virulentus* (sequence similarity of 98.75 ± 0.32%) than to those of *O. brassicae* (similarity of 82.67 ± 0.27%), thus confirming its belonging to the first mentioned species as it also shows the phylogeny inference (Figure 7). The *O. virulentus*-Cauca isolate (this study) had 0.009 to 0.055 K2P distances values when compared to other reported samples from the same species, revealing very low intra-group variability. By contrast, K2P values were higher when compared to *O. brassicae* (0.246 to 0.255) or *O. bornavanus* samples (1.039 to 1.044), other *Olpidium* spp. associated with virus transmission. Interestingly, the grouping of the isolates considered in the study exhibited a phylogenetic distribution similar to that previously reported (Zelyüt and Ertunç, 2021). However, the *O. virulentus*-Cauca isolate did not align with any of the groups proposed for this species, instead forming its own distinct lineage.

Then, the fungus' role in macana disease was investigated. Firstly, zoospore binding capacity to FNSV virions was analysed through controlled mixtures of both and

subsequent evaluation of the virus fraction retained with the fungal biomass. This study showed that only 5-6% of virions were associated with the fungus when 1 x 10^6 zoospores were incubated with 100 µg of viruses. This result was similar to a previous report where Olive mild mosaic virus and *O. brassicae* were studied, and the role of the fungus as a vector of said virus was confirmed (Varanda et al., 2011). To propose a similar concept, a transmission test was then carried out on lettuce plants to explore the vector capacity of the fungus. Lettuce plants are an excellent model to multiply FNSV and *O. virulentus* under controlled conditions (Kusunoki and Beltrán-Acosta, 2013). Considering this, lettuce roots were exposed to different samples containing FNSV, zoospores, or a preincubated mixture between both by a drench method (Table 2).

A significant increase in macana disease was observed in plants (diagnosed by RT-PCR and DBIA) treated with zoospores that had previously been incubated with the viruses compared to those only exposed to the virions (100% versus 33%, respectively). Virus concentrations lower than those used without premixing with zoospores did not generate infected plants (data not shown), indicating that the availability of viruses in the soil is a limiting factor. The assay with zoospores without preincubation with viruses confirmed that they were free of viral load after undergoing treatments to eliminate any trace of infection power for FNSV, as all plants in that treatment remained uninfected.

4. DISCUSSION

FNSV is the causal agent of fique necrotic streak (generally known as macana disease), a pathology first reported in Antioquia (Colombia). Furthermore, fungal zoospores from the genus *Olpidium* have been found in the roots of diseased plants (Gonzalez et al., 2010). These fungi are considered obligate root parasites and are known to be vectors of some viruses belonging to the *Tombusviridae* family (Rochon et al., 2004;

Varanda et al., 2011). Therefore, the hypothesis that FNSV (a member of *Tombusviridae*) causes macana disease and that it is transmitted by *Olpidium* sp. was explored.

First, virions were isolated from fique leaves exhibiting symptoms associated with the disease. TEM was used to observe icosahedral shaped virions with a diameter of 25 nm, rounded margins, and granular surfaces. This feature has been attributed to the protruding (P) domain of the capsid protein in other *Tombusviridae* viruses, such as CNV, which confers a granular or rough appearance when folding out of the particle (Rochon, 2012; Sit and Lommel, 2010). Moreover, the virions have been observed within spherical bodies or viroplasms in histological studies (Dabek and Castaño, 1978), structures formed in the peroxisome membrane containing the components for RNA replication (Laliberté and Sanfaçon, 2010), which have also been observed in this study (Figure 2). Sequence analysis confirmed the close phylogenetic relationship of the samples used in this study with the only available report. The genome variability of the virus throughout the entire fique-growing area suggested a consistent genetic conservation for each evaluated region (north and south of the Colombian Andes) with a low genetic flow between them. The genetic variability of RNA viruses has been widely described in phytopathology, mainly using the capsid protein's sequence (Varanda et al., 2014). This genetic distribution can be explained by negative selection stabilizing certain gene regions (Rubio et al., 2013) and human transport effects causing similar sequences to be found in geographical distant regions. However, the results of this study suggests that the exchange of infected plant material between southern and northern production areas would be relatively infrequent or, if it exists, these strains are displaced by those that evolved as more frequent in each ecoregion. In the future, the RT-PCR and the subsequent sequencing approach presented in this study could be used as a tool to control the spread of FNSV, establish quarantine

strategies, and analyse the possible relationship between pathovariants and the progression and degree of macana disease in fique plants.

Moreover, the fungus detected in fique roots showing macana diseased symptoms caused by FNSV was visualized by TEM and genetically identified as *Olpidium virulentus* by ITS sequencing. The divergence between *O. brassicae* and *O. virulentus*, together with previously reported findings (Sasaya and Koganezawa, 2006) suggested that these two fungi belong to different species, despite having a similar morphology regarding resting spores. In fact, phylogenetic inference and the K2P distances obtained support this taxonomic separation. On the other hand, the separation between *O. virulentus* strains from Asia-Oceania and Europe-America can be attributed to the introduction of crops between these territories, as mentioned by Maccarone (Maccarone, 2010). Besides, changes in the environment possibly led to mutations enabling segregation. The clear distinction between the fique sample and other fungus isolates would indicate that it is restricted to Colombia since fique is a plant endemic to Colombia and Venezuela's Andean region (Peinado et al., 2006). Previous phylogenetic studies on this species of fungus, as well as related species, indicate that the Colombian isolate of *O. virulentus* associated with fique plants does not group with any of the proposed clades involving isolates from other regions worldwide (Zelyüt and Ertunç, 2021).

Transmission tests using lettuce plants as a model showed that FNSV can be transmitted by *O. virulentus* zoospores, as occurs with other members of *Tombusviridae* and *Olpidium* sp. (Rochon et al., 2004; Varanda et al., 2011), because it was observed that 100 % of the plants treated with a pre-incubated mixture between the zoospores and the virions became infected, unlike those treated only with the virus where 70 % remained uninfected. FNSV multiplication in plants that were only inoculated with virions can be attributed to the amount used (100 µg). In fact, smaller masses of viruses not premixed

with zoospores fail to infect lettuce plants. It is unlikely that a high concentration of free virions can be found near the roots in natural conditions. However, only a few viral particles would be necessary for the fungus to mediate transmission to the plant, as has been seen with Olive mild mosaic virus and *O. brassicae* zoospores (Varanda et al., 2011). This explains the low, but sufficient, proportion of virions that bind to zoospores.

FNSV binding to *O. virulentus* zoospores could be related to two structures associated with the viral particle, as occurs in similar cases: arms joining the RNA binding (R) and the shell (S) domains, and the P domain. According to previous reports (Kakani et al., 2003), CNV binding to *O. bornavanus* zoospore surface is due to glycoprotein receptor recognition; these factors mainly consist of mannose or fucose (sugars mainly occurring on *O. bornavanus* surface) and are recognised by the arm binding to the R and S domains (Kakani et al., 2001; Robbins et al., 1997). Mutations in these regions usually decrease binding between virions and zoospores, resulting in reduced viral transmission (Kakani, 2004). This could be caused by a necessary conformational change involving particle expansion and promoting CNV/zoospore interaction (Rochon et al., 2004). Participation of the P domain has been reported (Ohki et al., 2010) because there was lower *O. bornavanus* transmission of Melon necrotic spot virus (MNSV) mutants to susceptible plants. These assumptions should be validated in the future for the case of FNSV/zoospores (*O. virulentus*).

This work validates FNSV as the causative agent of macana disease in fique plants (*Furcraea* spp., Asparagales: Agavaceae), shows the viral genetic variability across different ecoregions of the distribution area for its main host, and reports the correlation between FNSV, macana diseased fique plants, and *Olpidium virulentus* as the viral vector that facilitates disease transmission (using the lettuce model for transmission studies). Such evidence contributes to a better understanding of FNSV infection and multiplication

in nature, providing new knowledge that can aid in the desing of control strategies to mitigate the losses associated with this disease. Based on the obtained results, it is suggested that *O. virulentus* may serve as a vector for macana disease in fique plants in Colombia.

5. ACKNOWLEDGMENTS

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6. DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the paper and its Supporting Information files. Raw data is available from the corresponding author upon reasonable request.

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8. SUPPORTING INFORMATION LEGENDS

Table S1. Sequences used in *Tombusviridae* bioinformatics analysis.

Table S2. Sequences used in *Olpidium* bioinformatics analysis.

9. TABLE LEGENDS

Table 1. Primers used for amplifying FNSV genome fragments

Table 2. FNSV transmission by *Olpidium virulentus* in lettuce plants

10. FIGURE LEGENDS

Figure 1. *Furcraea* spp. leaves affected by macana disease. Photographs showing signs of macana disease on fique leaves. **A**. Initial phase: chlorotic streaks indicated by white arrows. **B**. Disease progression with the appearance of necrotic spots in chlorotic streaks indicated by white arrows. **C**. Advanced phase where necrosis can be observed on 80% of the leaf. **D** Leaf cross-section where internal fibre necrosis can be observed.

Figure 2. Viruses in fique plants suffering macana disease. Micrographs of a macana diseased fique leaf section. **A**. Toluidine blue staining by light microscopy (10X). c: cuticle; e: epidermis; pal: palisade layer; vb: vascular bundle; x: xylem; ph: phloem. **B**. Ultrathin sections of mesophyll cells containing viral particles. White arrows show viroplasms.

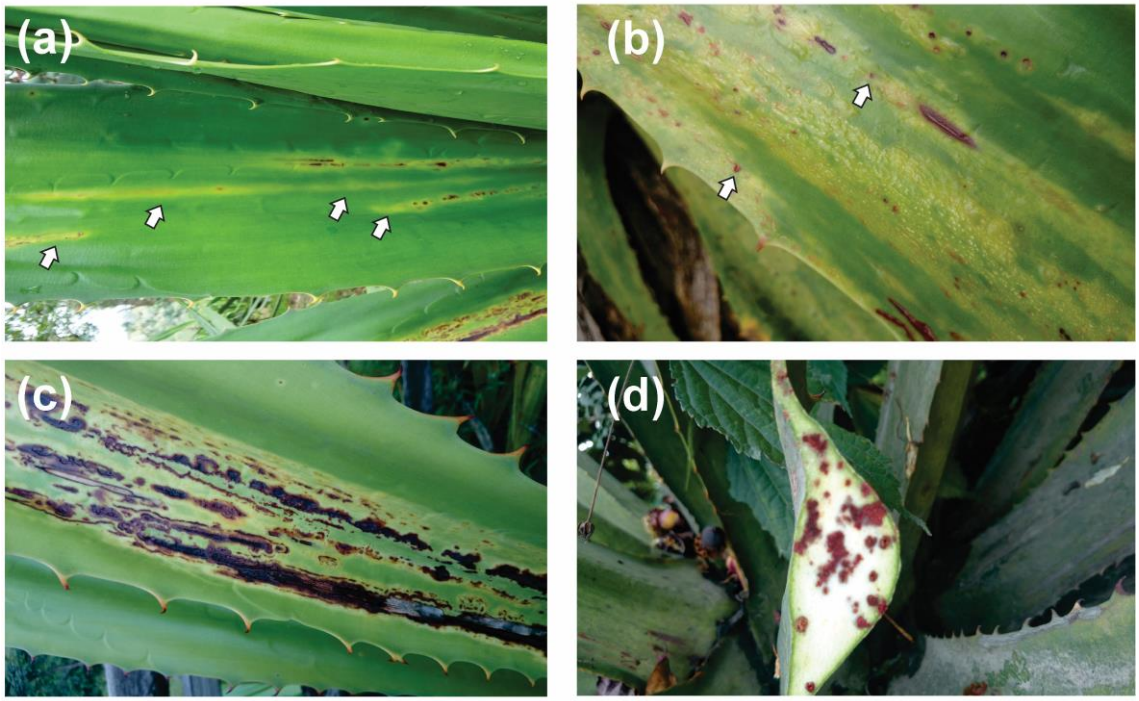
Figure 3. Characterising purified virions from diseased fique leaves. **A.** Negative staining of viral particles with phosphotungstic acid. **B**. SDS-PAGE protein analysis [some molecular weight (MW) bands are indicated in kDa]. **C**. Genome organisation map of viral nucleic acid. ORF: open reading frame; RdRp (RNA-dependent RNA polymerase); Mp: movement protein. The regions used for the Furcraea necrotic streak virus (FNSV) population studies are highlighted in grey.

Figure 4. Phylogenetic inference for FNSV. Cladogram based on the RNA polymerase protein generated by maximum likelihood method with 1,000 replicates. Numbers on nodes indicate their consistency expressed as percentages. FNSV-Nariño are highlighted in red letters. Tree scale is indicated.

Figure 5. **Phylogenetic relationships among Colombian FNSV geographic isolates.** An RT-PCR strategy was used, followed by sequencing. **A.** The maximum likelihood method (Bootstrap: 1,000 replicas) was used for constructing a phylogenetic tree based on a concatenated nucleotide sequence derived on the RT-PCR fragments corresponding to RNA polymerase, movement, and capsid encoding sequences. The previously reported FNSV-Cauca (NC_020469.1) and FNSV-Nariño (this work) were included. Numbers on nodes indicate their consistency, expressed as percentages. The geographic regions where the viruses were isolated are indicated on the map by dots, using the same colour reference as that used for the cladogram. The red star indicates the completely sequenced isolate FNSV-Nariño, while the green one indicates the region where the fungus (*Olpidium* sp.) was isolated. **B**. Average values for Kimura 2-Parameter distances (K2P) between FNSV-Cauca or FNSV-Nariño and the rest of all isolates, or those from clade A or clade B. Standard deviations are indicates as error bars.

Figure 6. Fungal spores in macana diseased fique plants. Micrographs of root cross-section of macana diseased fique plant. **A**. Vascular cylinder cells with resting spores (black arrows) (40X). X: xylem. **B**. Pericycle cells with resting spores (black arrow) (40X). **C**. Vascular cylinder cells with zoosporangia (40X). **D**. Ultrastructural image of zoospores with FNSV particles (black arrows).

Figure 7. **Phylogenetic analysis of *Olpidium* spp.** Cladogram based on the ribosomal DNA's (rDNA) internal transcribed spacer (ITS) region sequences generated by maximum likelihood method with 1,000 replicates. Numbers on nodes indicate their consistency expressed as percentages. The isolate from Cauca used on this study is indicated in red letters. The previously reported classification into species and groups is shown (Zelyüt & Ertunç, 2021). Bootstrap values and tree scale are indicated.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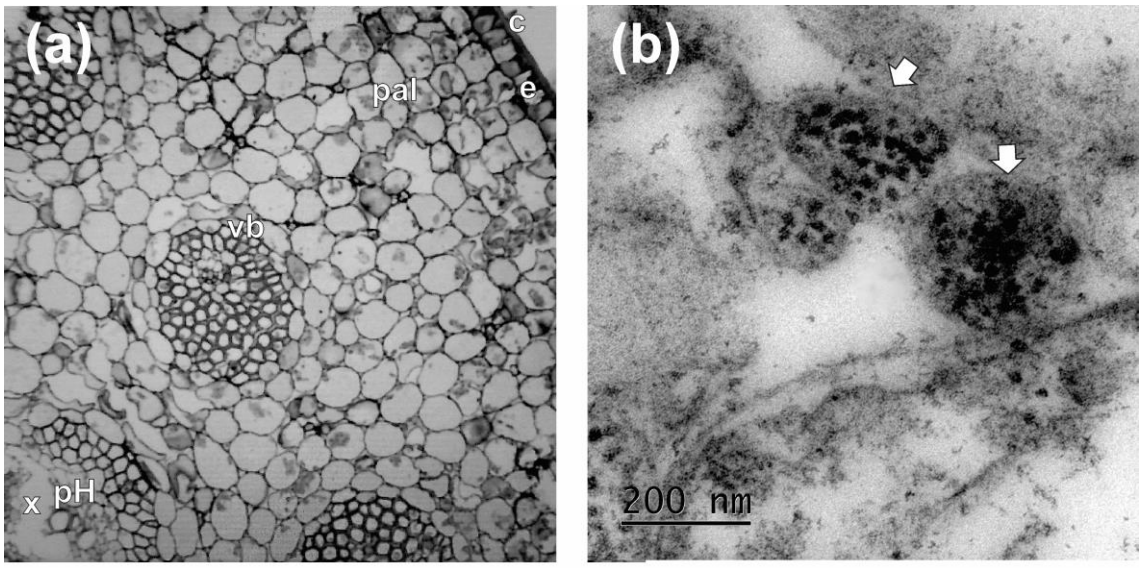
(b)
(c)
(d)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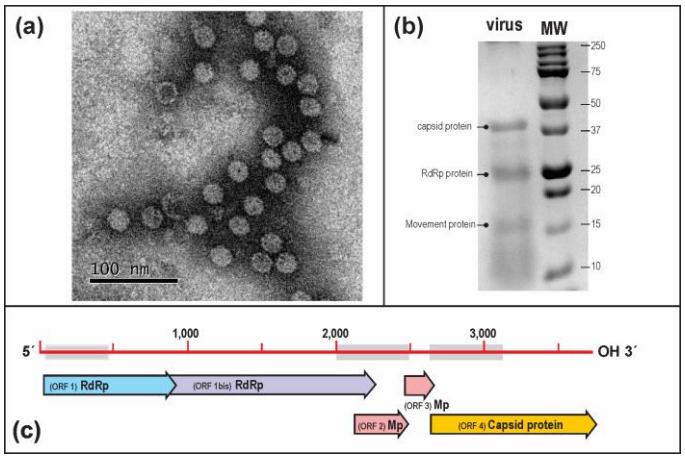
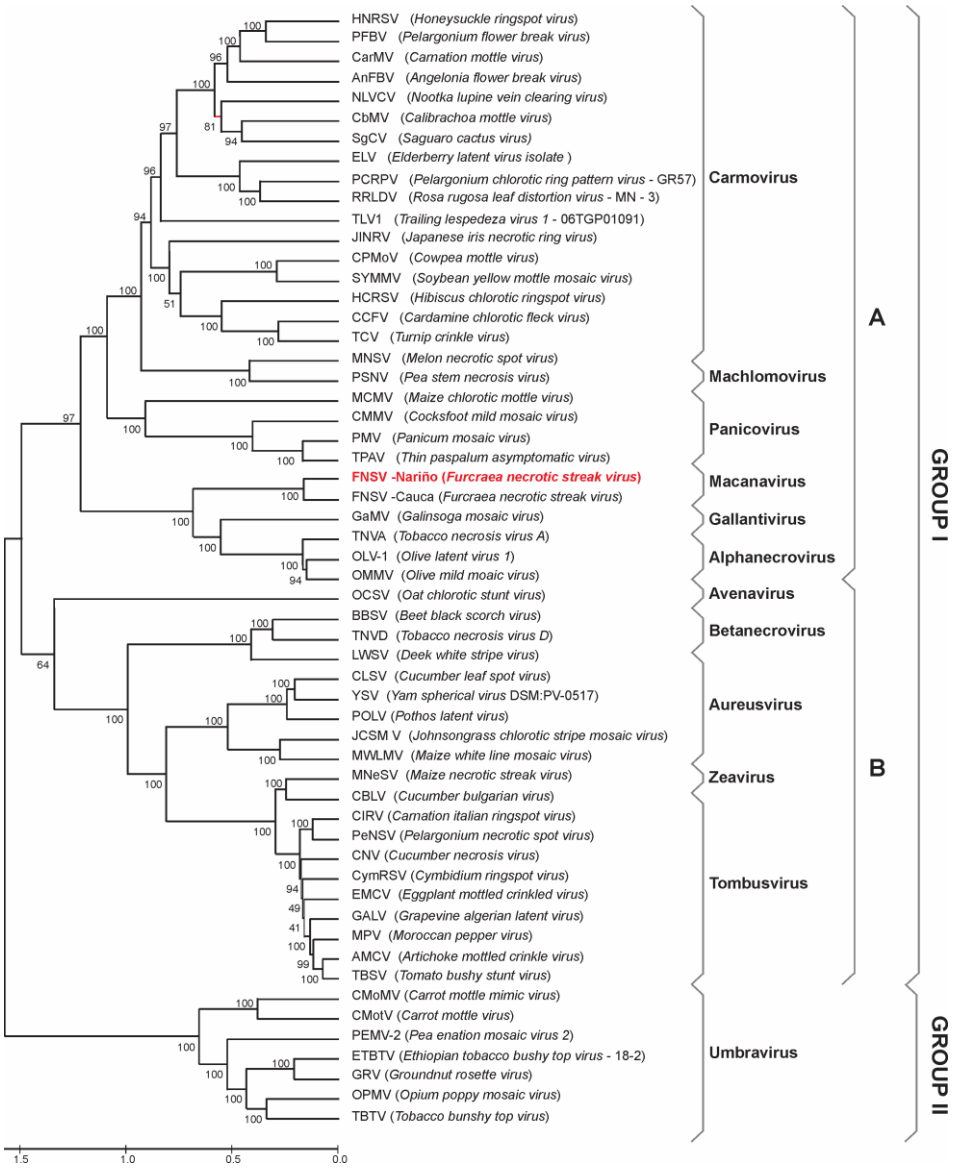
c
pal
e
vb
x pH
(b)
200 nm


(a)
100 nm
(b)
virus
MW
250
75
50
37
25
20
15
10
capsid protein
RdRp protein
Movement protein
5´
1,000
2,000
3,000
OH 3´
(ORF 1) RdRp
(ORF 1bis) RdRp
(ORF 3) Mp
(ORF 2) Mp
(ORF 4) Capsid protein
(c)


HNRSV (Honeysuckle ringspot virus)
PFBV (Pelargonium flower break virus)
CarMV (Carnation mottle virus)
AnFBV (Angelonia flower break virus)
NLVCV (Nootka lupine vein clearing virus)
CbMV (Calibrachoa mottle virus)
SgCV (Saguaro cactus virus)
ELV (Elderberry latent virus isolate)
PCRPV (Pelargonium chlorotic ring pattern virus - GR57)
RRLDV (Rosa rugosa leaf distortion virus - MN - 3)
TLV1 (Trailing lespedeza virus 1 - 06TGP01091)
JINRV (Japanese iris necrotic ring virus)
CPMoV (Cowpea mottle virus)
SYMMV (Soybean yellow mottle mosaic virus)
HCRSV (Hibiscus chlorotic ringspot virus)
CCFV (Cardamine chlorotic fleck virus)
TCV (Turnip crinkle virus)
MNSV (Melon necrotic spot virus)
PSNV (Pea stem necrosis virus)
MCMV (Maize chlorotic mottle virus)
CMMV (Cocksfoot mild mosaic virus)
PMV (Panicum mosaic virus)
TPAV (Thin paspalum asymptomatic virus)
FNSV -Nariño (Furcraea necrotic streak virus)
FNSV -Cauca (Furcraea necrotic streak virus)
GaMV (Galinsoga mosaic virus)
TNVA (Tobacco necrosis virus A)
OLV-1 (Olive latent virus 1)
OMMV (Olive mild moaic virus)
OCSV (Oat chlorotic stunt virus)
BBSV (Beet black scorch virus)
TNVD (Tobacco necrosis virus D)
LWSV (Deek white stripe virus)
CLSV (Cucumber leaf spot virus)
YSV (Yam spherical virus DSM:PV-0517)
POLV (Pothos latent virus)
JCSM V (Johnsongrass chlorotic stripe mosaic virus)
MWLMV (Maize white line mosaic virus)
MNeSV (Maize necrotic streak virus)
CBLV (Cucumber bulgarian virus)
CIRV (Carnation italian ringspot virus)
PeNSV (Pelargonium necrotic spot virus)
CNV (Cucumber necrosis virus)
CymRSV (Cymbidium ringspot virus)
EMCV (Eggplant mottled crinkled virus)
GALV (Grapevine algerian latent virus)
MPV (Moroccan pepper virus)
AMCV (Artichoke mottled crinkle virus)
TBSV (Tomato bushy stunt virus)
CMoMV (Carrot mottle mimic virus)
CMotV (Carrot mottle virus)
PEMV-2 (Pea enation mosaic virus 2)
ETBTV (Ethiopian tobacco bushy top virus - 18-2)
GRV (Groundnut rosette virus)
OPMV (Opium poppy mosaic virus)
TBTV (Tobacco bunshy top virus)
Carmovirus
Machlomovirus
Panicovirus
Macanavirus
Gallantivirus
Alphanecrovirus
Avenavirus
Betanecrovirus
Aureusvirus
Zeavirus
Tombusvirus
Umbravirus
A
B
GROUP I
GROUP II
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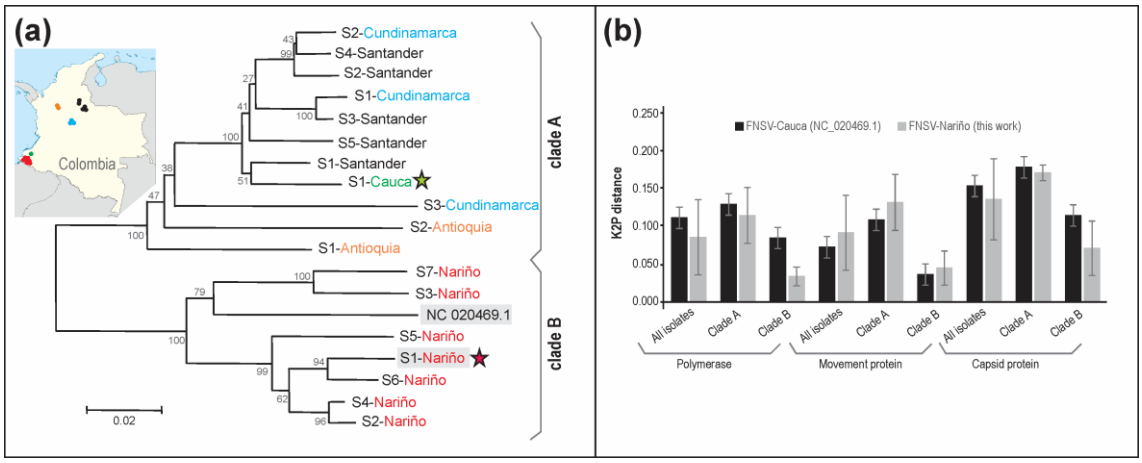
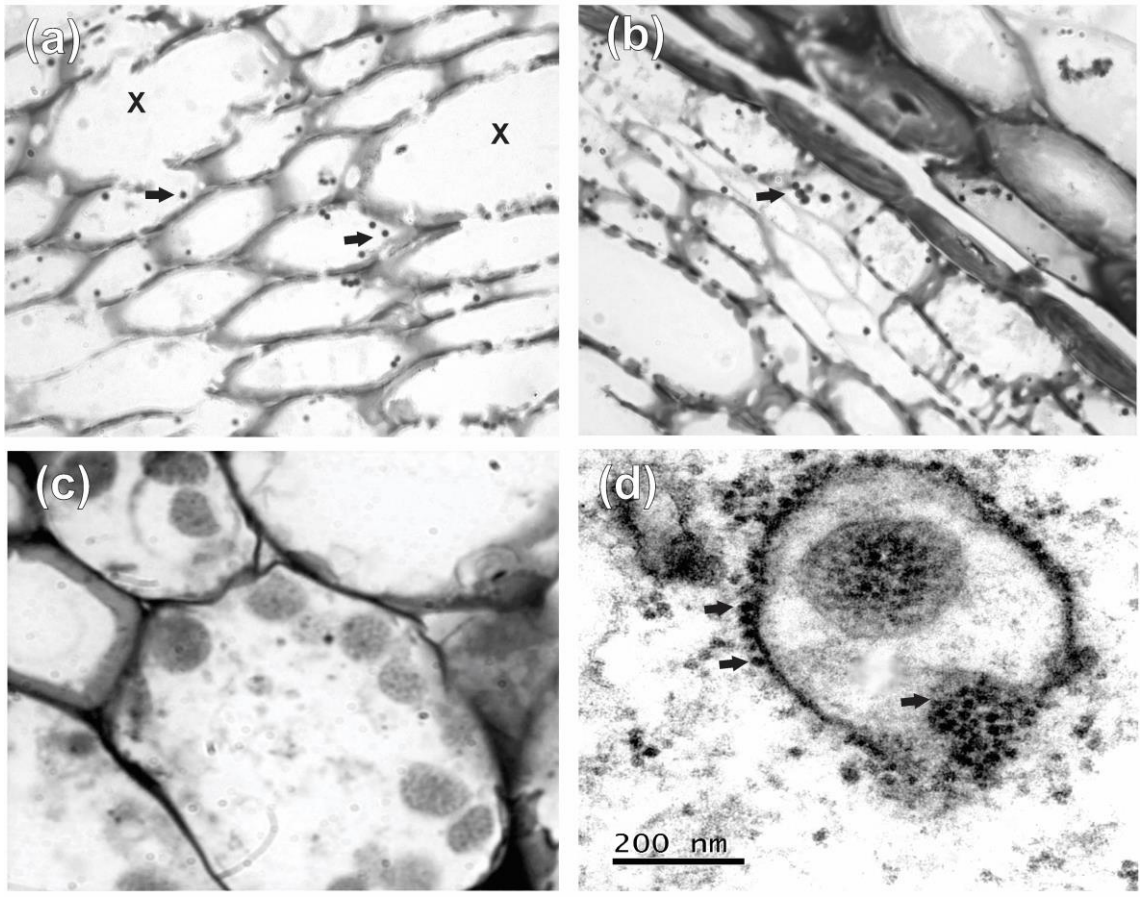
1.0
0.5
0.0


(a)
Colombia
S2-Cundinamarca
S4-Santander
S2-Santander
S1-Cundinamarca
S3-Santander
S5-Santander
S1-Santander
S1-Cauca
S3-Cundinamarca
S2-Antioquia
S1-Antioquia
S7-Nariño
S3-Nariño
NC 020469.1
S5-Nariño
S1-Nariño
S6-Nariño
S4-Nariño
S2-Nariño
0.02
clade A
clade B
(b)
FNSV-Cauca (NC_020469.1)
FNSV-Nariño (this work)
K2P distance
0.250
0.200
0.150
0.100
0.050
0.000
All isolates
Clade A
Clade B
Polymerase
Movement protein
Capsid protein


(a)
X
X
(b)
(c)
(d)
200 nm

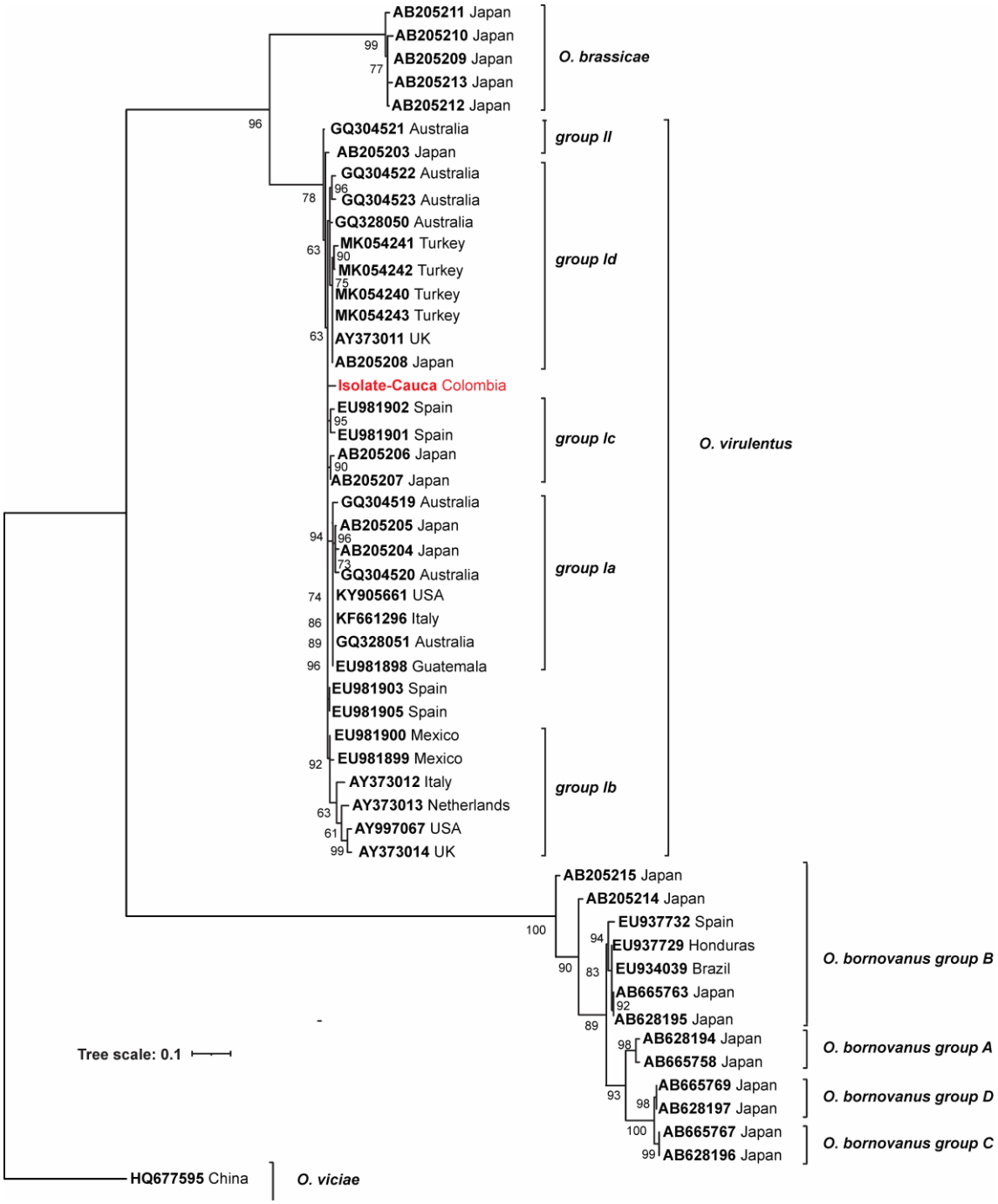

AB205211 Japan
AB205210 Japan
AB205209 Japan
AB205213 Japan
AB205212 Japan
O. brassicae
GQ304521 Australia
AB205203 Japan
group II
GQ304522 Australia
GQ304523 Australia
GQ328050 Australia
MK054241 Turkey
MK054242 Turkey
MK054240 Turkey
MK054243 Turkey
AY373011 UK
AB205208 Japan
group Id
Isolate-Cauca Colombia
EU981902 Spain
EU981901 Spain
AB205206 Japan
AB205207 Japan
group Ic
O. virulentus
GQ304519 Australia
AB205205 Japan
AB205204 Japan
GQ304520 Australia
KY905661 USA
KF661296 Italy
GQ328051 Australia
EU981898 Guatemala
group Ia
EU981903 Spain
EU981905 Spain
EU981900 Mexico
EU981899 Mexico
AY373012 Italy
AY373013 Netherlands
AY997067 USA
AY373014 UK
group Ib
AB205215 Japan
AB205214 Japan
EU937732 Spain
EU937729 Honduras
EU934039 Brazil
AB665763 Japan
AB628195 Japan
O. bornovanus group B
Tree scale: 0.1
AB628194 Japan
AB665758 Japan
O. bornovanus group A
AB665769 Japan
AB628197 Japan
O. bornovanus group D
AB665767 Japan
AB628196 Japan
O. bornovanus group C
HQ677595 China
O. viciae

Table 1. Primers used for amplifying FNSV genome fragments

Primer	***Sequence***
Pol-F	5’AGCCAGCTATACCACACAACC3’
Pol-R	5’TACCACCAAGCGGTTAGCTT3’
Pol2-F	5´GTCTTGGTGGATGCTGCGCGA3´
PM-F	5’ATATCTACATGCGGCCTTGC3’
PM-R	5’ GTTTGGGTTTCAGCGATGTT3’
PM2-R	5´CCCGTGAGAGCAAGGCCGCAT3´
CP-F	5’AACCCAAACCATTACGGTGA3’
CP-R	5’ACCCCAAACATGAATTGACC3’
CP2-F	5´TGTAGACCGCAAACTAATTGA 3´
CP2-R	5´TCCACGTGACTTGGTACACCT 3´.

Table 2. FNSV transmission by *Olpidium virulentus* in lettuce plants

Treatment	**Transmission efficiency** ***Infected plants/total plants***
	DBIA and RT-PCR
FNSV	5/15
FNSV + zoospores *O. virulentus* Cauca	15/15
Zoospores *O. virulentus* Cauca	0/15