Research Article

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Can the banana weevil *Cosmopolites sordidus* be a vector of *Fusarium oxysporum* f.sp. *cubense* race 1? Unravelling the internal and external acquisition of effective inoculum

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Abstract

BACKGROUND: With the undergoing world outbreak of *Fusarium* wilt of bananas, it is essential to unravel all the possible process of dissemination of this disease. The host-pest interactions of the banana weevil with banana plants make this insect an important potential vector. This study, carried out in controlled conditions, explores the interaction between the banana weevil and *Fusarium oxysporum* f.sp. *cubense* race 1 (Foc), with a focus on the external and internal transport of viable fungal propagules.

RESULTS: Viable inoculum of Foc was detected very rapidly on external teguments and in the digestive tract of the insect, i.e. at the lowest time studied of 5 min after contact with infected pseudostems. Maximal inoculum acquisition occurred after 1 h contact with an inoculum source. External inoculum was higher than the inoculum present in the digestive tract, but external and internal inoculum had the same dynamics. After a contact of an infected source, external and internal inoculum decreased exponentially within 50 h, but weevils remained infested for a long time, as long as 2 or 3 days that would be enough for inoculum dispersal. Viable inoculum was also detected in feces. Foc strains isolated were pathogenic when inoculated to banana plants of the Gros Michel variety but did not provoke any symptom on Cavendish banana plants.

CONCLUSION: These results demonstrate that the infective structures of Foc remain externally viable in the digestive system and the excreta of the banana weevil. Such excreta are capable of making healthy banana plants of the Gros Michel variety.

Keywords: insect mediated dispersion; acquisition dynamics; Foc; Gros Michel cultivar; pathogenicity

1 INTRODUCTION

Insects play an important role in the spread of plant diseases when they come into contact with diseased tissues, either during feeding, oviposition, or through involuntary contact with diseased organs or viable reproductive structures of pathogens.[1–4] Insect mobility can increase the spread of pathogens, however the speed and manner in which insects disperse pathogens and their functional role in disease epidemics will depend on the type of contamination mechanism, which can be either external or internal.[5,6]

However, the transmission of fungal diseases by insects remains poorly documented as compared to insect transmission of viral diseases.[2,7–12] Among the few examples studied, the case of the role of the weevil *Sitona hispidulus* in the dispersal of Fusarium wilt of alfalfa root rot (*Fusarium oxysporum* f.sp. medicaginis) has been well documented.[13–15] It has been shown that *S. hispidulus* plays an active role in *Fusarium* wilt of alfalfa through feeding activity of larvae, the fungus been present in excised heads of the insect.[15] In the case of western corn rootworn (*Diabrotica virgifera*; Coleoptera: Chrysomelidae), it has been shown that larvae feed on corn roots, thus facilitating root colonization by the plant-pathogenic fungus *Fusarium verticillioides*.[16,17] Indeed, such interactions between root-feeding insects and plant pathogens have been reviewed recently by Willsey *et al.*[18] stating that root feeding insects might play a role in plant fungal diseases through feeding injuries that facilitate pathogen infection or being vectors of fungal pathogens. The putative role of insects as vectors of fungal

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diseases might rely on the adhesion of fungal inoculum (spores or mycelia) on their teguments during insect–plants contacts, as in the case of clover root borers.[19] Insects might also carry fungal inoculum internally in their digestive tube and then excrete viable fungal propagules.[20,21]

Fusarium wilt of bananas is one of the most important diseases of this crop worldwide, and it is caused by the fungus *Fusarium oxysporum* f.sp. *cubense* (Foc).[22–24] This pathogen belongs to the Eumicota class[25] and it has three types of asexual reproduction structures: macroconidia, microconidia and chlamydospores.[26] The Fusarium wilt of bananas is divided into four races where race 1 and race 4 are the most impactful for the banana industry worldwide.[27] The race 1 was responsible of the devastation of more than 40 000 ha of banana Gros Michel (AAA) between 1890 and 1950[24] which led to the varietal conversion to bananas from the Cavendish Subgroup (AAA) in the 1960s, the banana industry being still highly dependent on this banana Subgroup.[23,28,29] More recently, the race 4 highly pathogenic on bananas from Cavendish Subgroup emerged in Asia and had a quick and severe development in Asia over the last 10 years. First cases were recently reported in Africa and South America, which are world major production areas of Cavendish bananas.[30,31]

Understanding the dispersal of *Fusarium* wilt of bananas is a key for the development of management strategies. The first step of the dispersal is the introduction of the pathogen in a new area. Then the second step of the dispersal is the epidemics of the disease at a field or farm level. Dita *et al.*,[32] highlighted that *Fusarium* wilt of bananas can be dispersed (i) by farmers that transport infected planting material, (ii) by abiotic processes through soil adhering to footwear, vehicles, agricultural machinery, by erosion, and by contaminated water used for irrigation, and (iii) by animals, such as vertebrates, that can transport contaminated soil. All these mechanisms of fungal dispersion might explain the long distance dispersal and are taken into consideration for biosecurity measures. Recently, it has been shown that *Cosmopolites sordidus* might potentially disperse Foc spores on external teguments.[33] Then, this insect might play a role at local scale in the epidemiology of *Fusarium* wilt of bananas.

The role of this pest could potentially be very important since it is tightly associated to banana plant for feeding and for oviposition. Furthermore, the weevil is a cosmopolitan insect considered as the most limiting pest of banana cultivation in most tropical and subtropical countries.[34] *Cosmopolites sordidus* damages are caused by their larvae that mainly feed from the rhizome of banana plants. This causes a significant reduction in yield due to poor absorption of water and nutrients as well as a reduction in the longevity of the plantations. In the literature, it is mentioned that *C. sordidus* could play a role in the dissemination of several bacterial banana diseases caused by *Ralstonia solanacearum*, *Erwinia carotovora*, *Xanthomonas campestris* pv. *Musacearum*.[35] Although the dispersal capacities of *C. sordidus* are moderate, i.e. measured up to 9 m/d,[36] it has a clear potential to spread the disease within and between fields. However, the potential role of *C. sordidus* in epidemiology of *Fusarium* wilt of bananas at field level remains unclear and requires further studies. In their study, Meldrum *et al.*,[33] considered a potential external dispersal but they did not consider an internal acquisition. Furthermore, the dynamic of acquisition and permanence of the disease remains unknown while it is a key factor in the potential role of *C. sordidus* in the dispersion of this pathogen.

This study aims at a better understanding of the potential role of *C. sordidus* as an external or internal inoculum carrier. Here, the capacity of *C. sordidus* to acquire Foc race 1 externally and internally was measured in controlled experiments. We quantified and followed the dynamic of acquisition of inoculum. Importantly, we verified the viability of external and internal inoculum in order to better understand their epidemiological importance. Lastly, we discussed the implications of these fungi–insect interactions in terms of spatial epidemiology of Foc.

2 MATERIALS AND METHODS

Our study was based on three experiments (summarized in Fig. 1) that aimed at measuring (1) the external and internal acquisition of Foc by *C. sordidus* over time, (2) the external and internal survival of Foc on *C. sordidus* over time, and (3) the presence of Foc in excreta of *C. sordidus* fed from Foc-infected banana pseudostem.

2.1 Experimental design

2.1.1 Experiment 1. External and internal acquisition of Foc by C. sordidus *over time*

The objective of this experiment was to measure in controlled conditions the time required for the insects to acquire Foc both externally and internally after contact with a food source infected by the pathogen. It consisted in releasing *C. sordidus*, from a Foc-free rearing and previously isolated for 48 h without feeding before the experiment, in 252 cm^3 plastic boxes containing a 70 cm^3 pieces of pseudostems collected from Gros Michel banana plants. Each replicate included 12 boxes, ten containing a pseudostem collected from a Foc-infected plant and two containing a pseudostem collected from a healthy plant. The Foc-infested pseudostems were collected from plants showing typical external and internal symptoms of *Fusarium* wilt, while the healthy plant was brought from a Foc-free experimental area. In each box, 20 insects were released simultaneously. Then, at 0, 5, 15, 45, 90, 180, 360, 720, 1440 and 2880 min after release, two insects were removed, one to evaluate the external Foc inoculum and one to evaluate the internal Foc inoculum. In total, each replicate included 200 and 40 insects that fed from infected (100 for external and 100 for internal inoculum study) and non-infected pseudostem, respectively (Fig. 1).

For external inoculum assessment, each insect was placed in a 1 mL Eppendorf tube with 300 µL of distilled water and stirred in a vortex for 1 min at high speed. A 100 µL sample was taken, which was seeded in a Petri plate of Komada medium K2 and incubated for 5 days in constant light. The Komada medium consisted in a mixture of 900 mL of a basal medium and 100 mL of an antibiotic solution. The composition of the basal medium was: K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; FeNaEDTA, 0.01 g; L-asparagine 2 g; galactose 10 g; agar 16 g; distilled water 900 mL. After autoclaving, the basal medium was mixed with 100 mL of antibiotic solution filtered through 0,2 µm before mixing with the autoclaved basal medium containing the following agents: PCNB (pentachloronitrobenzene, 75% WP), 0.9 g; oxgall, 0.5 g; $Na_2B_4O_7{\cdot}10H_2O$ 0.5 g; streptomycin sulfate 0.3 g. The medium was adjusted to pH 3.8 with phosphoric acid 10% after being autoclaved.

For the internal inoculum analysis, each insect was disinfected twice with sodium hydroxide (NaOH) at 1000 mg/L during 5 min

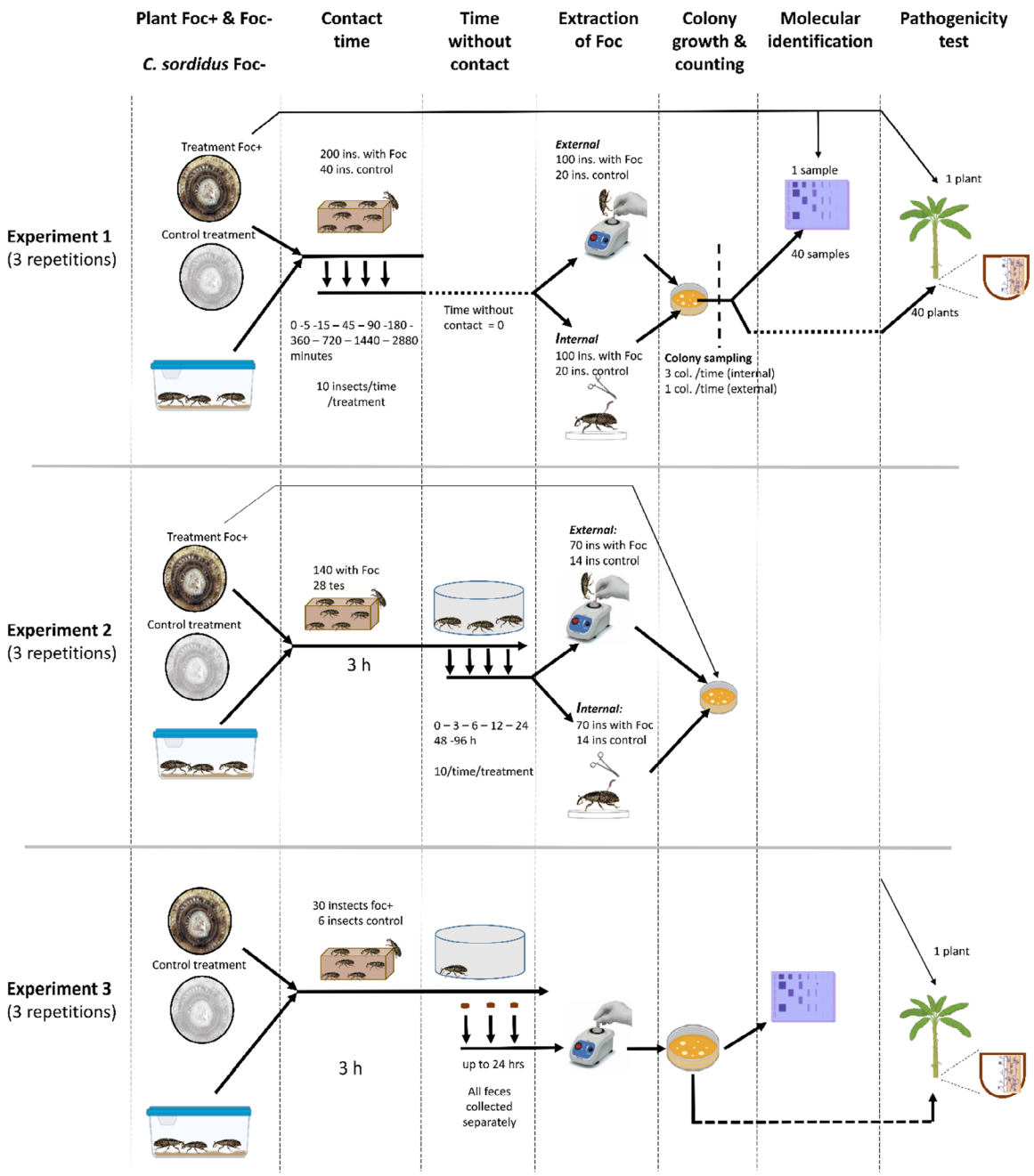

Figure 1. Description of the various experiments carried in this study.

and washed three times in sterile water. Then, with microdissection equipment, the digestive tract was extracted and placed in an Eppendorf tube with 300 µL of sterile water for its subsequent maceration with sterile glass beads in a vortex during 1 min at a high revolution. Then, 100 µL of the macerate was deposited in Petri dishes with Komada medium and incubated for 5 days at 26 °C under constant light.

On each plate, the colonies of Foc were counted on the sixth day. Each colony of Foc was characterized by its salmon pigmentation and the form of the hairy growth of the mycelium that is an important characteristic of Foc on the Komada medium.[37] The identification of *Fusarium* genus was confirmed by microscopic observation of the reproduction structures of the fungus: uni- or bi-cellular oval microconidia, presence of sickle-shaped macroconidia, globose-shaped chlamydospores.

For each repetition of the experiment, 30 *Fusarium* strains isolated from the digestive tract of the insect and ten *Fusarium* strains isolated externally from the insects were randomly selected. These strains were subjected to a molecular identification and to a pathogenicity test in order to confirm their status as Foc race 1 isolates.

2.1.2 *Experiment 2. External and internal inoculum survival of Foc on* C. sordidus *over time*

The objective of this experiment was to measure the persistence of Foc externally and internally in *C. sordidus* after a contact with Foc-infested banana pseudostems. Similar to Experiment 1, it

consisted in releasing *C. sordidus*, from a Foc-free rearing and previously isolated for 48 h without feeding before the experiment, in 252 cm^3 plastic boxes containing a 70 cm^3 piece of pseudostems collected from Gros Michel banana plants. Each replicate included 12 boxes, ten containing a Foc-infested pseudostem and two containing healthy pseudostem. After 3 h, insects were transferred to sterile boxes. Then, after 0, 180, 360, 720, 1440, 2880 and 5760 min, two insects were removed, one to evaluate the external Foc inoculum and one to evaluate the internal Foc inoculum. The assessment of external and internal Foc inoculum was performed with the same methodology as in Experiment 1. In total, each replicate included 200 and 40 insects that fed from infected and non-infected pseudostem, respectively. This experiment was completely replicated three times leading to the analysis of 720 insects.

For each repetition of the experiment, 30 *Fusarium* strains isolated from the digestive tract of the insect and ten *Fusarium* strains isolated externally from the insects were randomly selected. These strains were subjected to a molecular identification test in order to confirm their status as Foc race 1 isolates.

2.1.3 Experiment 3. Presence of Foc in excreta of C. sordidus *fed from Foc-infected banana pseudostem*

The objective of this experiment was to quantify the presence of Foc in the excreta of *C. sordidus* that fed from Foc-infested pseudostems. Thirty insects were placed in sterile plastic containers after being in contact with Foc-infected pseudostems for 3 h. Additionally, six insects were placed in the boxes with healthy pieces of pseudostem as control. After the feeding time had elapsed, the insects were moved individually (one insect per box) to sterile boxes to recover the excreta. Then, at 6, 12, 24 and 48 h, the excreta were removed (when present) in Eppendrof tubes with 200 µL of sterile water. The samples were placed in vortex for 1 min and 100 µL was taken and seeded in Petri plates with Komada medium as described in the Experiment 1. The colonies were characterized as described earlier to determine their Foc race 1 status: morphology of colonies, molecular identification, and pathogenicity test.

2.2 Weevil rearing

In a commercial Cavendish banana plantation resistant to Foc race 1, located in the canton of Siquirres, Limón province, Costa Rica, a total of 20 wedge traps were placed every 2 weeks on freshly harvested plants. One week later, the wedge traps were reviewed, and the insects trapped were collected in sterile plastic boxes. The insects were transferred to the Entomology Laboratory at the Biological Control Laboratory in Corbana located in La Rita de Pococí, Limón province, Costa Rica. In order to verify the absence of Foc, 10% of the population was sampled. An external wash of the integument was done to each insect and external Foc inoculum was detected as described in Experiment 1 (external inoculum).

2.3 Sources of pseudostem

The plants contaminated by Foc came from an experimental area located in Rita de Pococí, Limón province, Costa Rica. These plants belong to the cv Gros Michel, susceptible to Foc race 1. All selected plants showed typical symptoms of Panama disease (Fig. 2): yellow foliage, light brown to reddish-brown vascular bundles in the corm or leaf veins. In order to confirm the presence of Foc, pieces of vascular bundles were taken and placed in Komada medium. The colonies obtained were purified on potato dextrose agar (PDA) with chloramphenicol before final identification as described earlier.

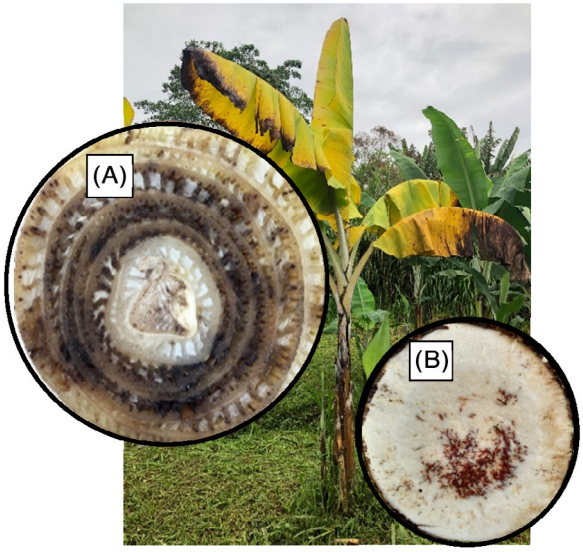

Figure 2. Typical symptoms of *Fusarium* wilt shown on banana plants selected for the provision of infected pseudostems (A) Pseudostem; (B) Corm.

2.4 Molecular analysis of strains isolated from insects

2.4.1 DNA isolation from vegetable tissue (pseudostem), animal tissue (insect), excreta of insect, and strains isolated from each sample type

The samples (50–100 mg) were placed in a 2 mL tube with per tube small steel balls and ground in a mixer (Mill MM 400 RETSCH) for 5 min before addition of 1 mL lysis buffer [1 M Tris, 0.5 M EDTA, 1% (*w/v*) SDS, pH set to 8.0 with HCl], 2 µL of proteinase K and 7 uL β-mercaptoethanol. The lysis mixture was incubated at 55 °C for 45 min, mixed by turning the tubes every 10 min and incubated again at 65 °C for 10 min. Subsequently, 300 uL chloroform-isoamyl alcohol (24:1) was added before vortex agitation carried out under cool conditions (−20 °C) for 2 min. Then the samples were centrifuged at 13 000 × *g* for 10 min. The aqueous phase was transferred to a new tube containing 400 uL polyethylene glycol (PEG 30%) and 200 ul 5 mol/L sodium chloride (NaCl), then kept at 8 °C overnight. Samples were centrifuged at 13 000 × *g* for 10 min to recover pellets, supernatants were discarded. Pellets were washed with 200 uL 70% ethanol twice, air-dried and resuspended in nuclease-free water.

2.4.2 Polymerase chain reaction conditions

Four polymerase chain reactions (PCRs) were performed with different primers in order to confirm the taxonomic position of *Fusarium* strains isolated from insects (Table 1). In the first PCR, the status of the genus *Fusarium* was confirmed through specific internal transcribed spacer (ITS) primers.[38] With the second PCR, the Foc status of strains was confirmed using specific primers designed in SIX genes.[39] In a third PCR, possible Foc R1 or Foc TR4 status of strains was tested using specific primers originally designed for Foc TR4,[40] which also match for R1 isolates from Latin America.[41] Foc strains used in all experiments collected in banana plots on experimental farms of Corbana were positive to these primers while belonging to race 1 (Conejo Barboza, personal communication). To rule out Foc TR4, a fourth PCR was

Table 1. Primer sequences used in this study in order to confirm the taxonomic status of fungal strains isolated from *Cosmopolites sordidus*

Target taxa	Primer	Target region[a]	Band size (bp)	Anneal temperature (°C)	Reference
Belongs to *Fusarium* genus	ITS-Fu-F/ITS-Fu-R	ITS	389	56.6	38
Belongs to *Fusarium oxysporum* f.sp. *cubense*	SIX9-Foc-F SIX9-Foc-R	SIX gene	260	58	39
Possible race 1 or TR4	Foc-1-F Foc-2-R	OPA02 $_{404}$ (RAPD)	242	68	40,41
Specific to tropical race 4	W2987-F W2987-R	Gene coding for an hypothetical protein	452	60	42

[a] IGS, intergenic spacer region of the rDNA; ITS, internal transcribed spacer; RAPD, random amplified polymorphic DNA; SIX, secreted in xylem.

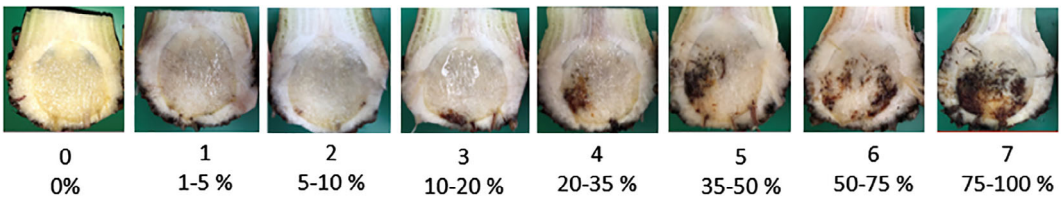

Figure 3. Disease severity scale used for evaluation of symptoms after artificial inoculation. This scale relies on internal symptoms on corms of banana plants 10 weeks after inoculation with *Fusarium oxysporum* f.sp. *cubense* race 1. The severity scale ranges from small reddish brown spots to severe rot.

performed with a specific primer from a putative virulence gene very specific for the identification of Foc TR4 strains.[42]

For all amplifications, DreamTaq PCR Master Mix (Thermo Scientific, Waltham, MA, USA) was used in 20 uL reactions according to the manufacturer's recommendations. The primer and DNA template concentration in the final PCR reaction were 0.5 mmol/L and 20 ng/uL, respectively. The following cycling conditions were used for all PCR: 94 °C for 1 min, 30 cycles of 94 °C for 30 s, and annealing temperature were primer-set dependent (see Table 1) for 30 s and 72 °C for 90 s, and finally extension 72 °C for 10 min.

2.5 Pathogenicity tests

Each *Fusarium* strain was grown in triplicate for 10 days in PDA medium with 1% chlorphenicol. For the preparation of inoculum, 20 mL of distilled water was added to Petri plates in order to remove fungal propagules. The content was filtered in a 250 mL beaker with a sterile gauze in order to separate the mycelium from the spores. The concentration of spore suspensions was adjusted to 1×10^{-6} CFU/mL with a Neubauer chamber. Inoculations of *Fusarium* strains were performed by pouring a 200 mL spore suspension directly into the plots of banana plants of 50 cm high from which laterally roots were previously cut according to the methodology described by Garcia-Batista *et al.*[31] The inoculated plants were kept in the glasshouse for 10 weeks until they were harvested for internal symptoms assessment through longitudinal cut of the corm.

Disease severity was visually assessed following a 1–7 scale where 0 = no discoloration or damage in the corm, 1 = isolated points in corm damage between 1 and 5%, 2 = damage > 5 and ≤ 10%, 3= > 10 and ≤ 20%, 4 = > 20 and ≤ 35%, 5= > 35 and 50%, 6= > 50 and ≤ 75% and 7 > 75% (Fig. 3).

2.6 Statistical analysis

In order to analyze and to represent the number of colonies measured externally and internally in relation with the time of contact between insects and Foc-infected pseudostem, we fitted a Gompertz model. The Gompertz model is particularly suitable to fit to sigmoid type growth that includes asymmetry and that follow non-linear patterns.[43]

$$y = a \exp\left(-\exp\left(\frac{\mu e}{a(\lambda - t)}\right) + 1\right) \quad (1)$$

The Gompertz equation (Eqn (1) represents the growth of y as a function of time t. It includes three parameters a, μ and λ representing population at equilibrium, the population growth rate, and the time of the inflexion of the growth, respectively. The Gompertz models were fitted as non-linear mixed effects models using the 'nlmer' function from the lme4 package,[44] including the box identification number as a random factor altering the three parameters of the model. In this analysis, the number of colonies and the time of contact were log transformed.

Similarly, to analyze and to represent the decrease of the number of colonies measured externally and internally in insects as a relation of the time after been in contact with Foc-infected pseudostem, we used exponential models (Eqn 2).

$$y = a \exp^{(b\,t)} \quad (2)$$

This equation represents the dynamic of y as a function of time t. It includes two parameters a and b, representing the initial population and the growth rate, respectively. This model was also fitted using the 'nlmer' function and included the box identification number as a random factor altering the two parameters of the model.

All these analyses only included the data from treatments with insects in contact with Foc-infected (excluding the controls). All analysis was performed using the R software 4.0.2.[45] For all fitted models, we verified graphically that the residuals were following a normal distribution.

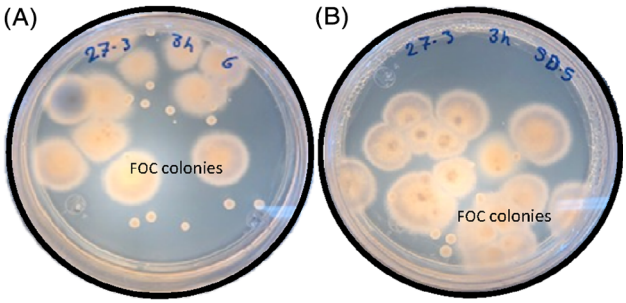

Figure 4. Example of typical fungal colonies recovered on Komada medium 7 days after (A) external or (B) internal isolements from the weevils. These pictures show typical colonies of *Fusarium oxysporum* f.sp. *cubense* (Foc race 1).

3 RESULTS

3.1 External and internal dynamics of fungal acquisition by the insect fed from infected material

From the total of colonies obtained in the Komada medium after external isolates and from the digestive system in *C. sordidus*, it was determined that more than 85% were morphologically compatible with Foc (Fig. 4). The rest of the colonies were undoubtedly contaminants. We did not observe any colonies of Foc (either externally or internally) on insects that were in contact with healthy pseudostem.

There was a good fit of the Gompertz model to the dynamics of the external inoculum as a response to the time of contact between *C. sordidus* and Foc-infected pseudostem [$a = 4.353 \pm 0.147$ (standard deviation (SD)), $\mu = 1.118 \pm 0.152$ (SD), $\lambda = 0.388 \pm 0.232$ (SD), residual degrees of freedom = 273]. As early as 5 min of contact with Foc-infected pseudostem, fungal inoculum could be detected on the external teguments of *C. sordidus*. The 90% of the maximal inoculum level was reached after an average contact time of 154 min (within the 78–301 min predicted confidence interval) with Foc-infected pseudostem (Fig. 5(A)). The three replicates showed the same trend although one replicate showed lower inoculum levels.

There was a similar trend for the dynamics of the internal inoculum in the digestive tract of *C. sordidus*, but the number of colonies was much lower as compared with those measured externally (Fig. 5(B)). The level of inoculum measured was also more variable between the different insects for a same sampling time. The Gompertz model [$a = 1.422 \pm 0.131$ (SD), $\mu = 0.678 \pm 0.229$ (SD), $\lambda = 1.705 \pm 0.412$ (SD), residual degrees of freedom = 273] highlights that 90% of the maximal inoculum was reached on average at 66 min (within the 42–166 min predicted confidence interval). Alike the external inoculum, the internal inoculum of Foc in *C. sordidus* was detected very early since 5 min after the beginning of the contact with Foc-infected pseudostem some insects already exhibited inoculum.

3.2 External and internal survival of Foc on *C. sordidus*

It was observed that the dynamics of the external inoculum of Foc on the insect tegument showed a fast exponential decrease over time after insects were removed from Foc-infected pseudostems [$a = 36.265 \pm 3.514$ (SD), $b = -0.007 \pm 0.008$ (SD), residual degrees of freedom = 205]. At 54 h the residual amount was very low and only 33% of the insects exhibited trace of external inoculum (Fig. 6(A)). The dynamics of the internal inoculum of Foc in the digestive tract of *C. sordidus* showed also a fast exponential decrease over time after insects were removed from Foc-infected pseudostems [$a = 0.311 \pm 0.051$ (SD), $b = -0.381 \pm 0.066$ (SD), residual degrees of freedom = 205]. As in Experiment 1, the amount of internal inoculum was lower than the inoculum found externally. There were always insects that did not show inoculum internally (30% at the initial time), but this proportion increased over time to reach 90% at 96 h (Fig. 6(B)).

3.3 Presence of Foc in the excreta of *C. sordidus*

The quantity and quality of the excreta recovered from *C sordidus* was low. The analysis of the 90 excreta carried out after the insects fed from the diseased pseudostem during 3 h showed that 12 excreta (13.3%) contained one or two Foc colonies and very few contaminants were observed growing on the Komada medium.

3.4 Molecular analysis of strains isolation

For each experiment developed in this investigation, molecular tools were used to confirm the identity of the Foc strains. For Experiments 1 and 2 and feces analysis, 100% of the strains isolated were confirmed as belonging to the *Fusarium* genus (Table 2). It was also confirmed that they belonged to the FOC group and that they did not match with specific race 4 (Fig. 7).

3.5 Pathogenicity test

We found that 100% of the Gros Michel banana plants inoculated with Foc strains isolated in Experiment 1 showed symptoms of Panama disease in the corm at 10 weeks after inoculation (Fig. 8 (A, B)). Most of the plants had a disease index between 20 and 35% and only a low percentage of the plants showed severe damage (Damage Index > 35%, disease severity note > 5 in the scale). No symptoms were observed in the corms of Cavendish plants inoculated as in the Gros Michel control plants (Fig. 8(C)). The 12 strains isolated from feces were also pathogenic on Gros Michel plants but not on Cavendish plants.

4 DISCUSSION

4.1 *Cosmopolites sordidus* is confirmed as an effective vector of Foc on external teguments but also in its digestive tract

Cosmopolites sordidus was previously mentioned as a potential vector in the dissemination of pathogens that affect banana plants[35] and Foc in particular.[33] To date, only an external dissemination was suggested in the literature. Here, we confirm that *C. sordidus* can be a very effective carrier of infective Foc propagules on external teguments. For the first time, we demonstrate that *C. sordidus* can also transport Foc viable propagules in its digestive tract and feces. Meldrum *et al.*[33] could not isolate Foc isolates from internal sections of *C. sordidus* from insects collected at field level. Here the insects used were starved before experiments and were fed exclusively on infected pseudostems, by another hand all the digestive tract was macerated which could explain the successful internal isolation as compared with former attempts. This new result is supported by the following facts: (i) most isolations on Komada medium were quite pure with little contaminants either on teguments, digestive tract or feces of the insect; (ii) isolates were morphologically identified as belonging to the *Fusarium* genus; (iii) molecular identifications confirmed that these isolates belonged to the Foc group; (iv) pathogenicity tests showed that these isolates were pathogenic to Gros Michel and not to Cavendish bananas confirming their race 1 status.[46]

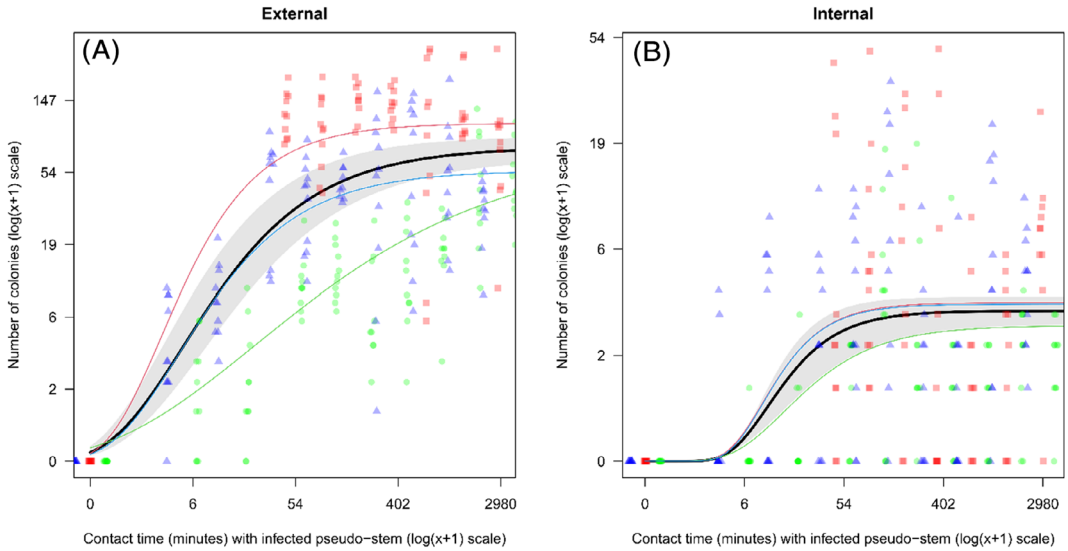

Figure 5. Dynamics of viable inoculum of *Fusarium oxysporum* f.sp. *cubense* (Foc) race 1 isolated externally (A) and internally (B) in *Cosmopolites sordidus* as a function of the time of contact between banana weevils and Foc-infected pseudostem. The experiment was replicated three times and the color of the points indicates the replicate. The black curve and the gray area show the fit and the standard error of the Gompertz model (fitted with the 'box identification number' as a random factor, see Section 2.6 for details). Colored curves show the fit for each replicate random factor. For an easier visualization, the contact time variable was slightly jittered.

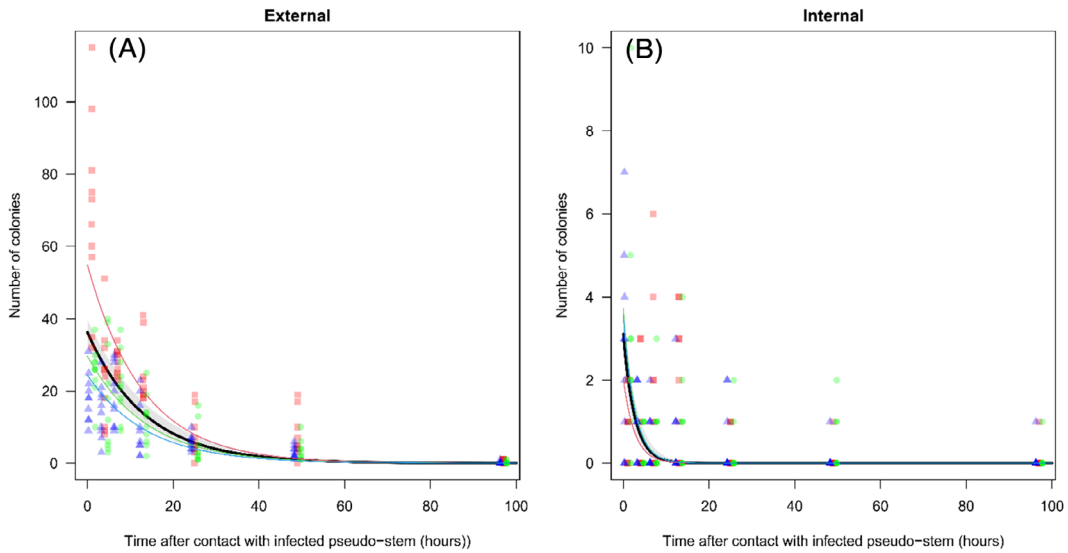

Figure 6. Decrease of viable inoculum of *Fusarium oxysporum* f.sp. *cubense* (Foc) race 1 recovered externally (A) and internally (B) in *Cosmopolites sordidus* as a function of the time after been removed from Foc-infected pseudostem. This experiment was replicated three times and the color of the points indicate the replicate. The black curve and the gray area show the fit and the standard error of the exponential models (fitted with the 'box identification number' as a random factor, see Section 2.6 for details). Colored curves show the fit for each replicate (random factor). For an easier visualization, the contact time variable was slightly jittered.

Internal inoculum was lower than external inoculum, probably because most propagules were killed in the digestive tract. Indeed, chlamydospores are more resistant than microconidia or macroconidia and might resist better through the digestive process.[47] The females visit the corms and tissues of the banana plants where they lay their eggs inside holes that they dig with their jaws and rostrum. These lesions may allow the entry of pathogens such as Foc, which is carried by the insect in its jaws or by the digestive contents that carry the eggs when they leave the female.

In our experiment 100% insects had Foc propagules on their teguments and we were also able to quantify this inoculum. During a survey on insects captured in Foc TR4-infected fields, Meldrum *et al.*[33] only detected the fungus on 10% of the trapped insects and they did not quantify the inoculum level. Overall, the quantity of propagules transported externally and internally by *C. sordidus* were not extremely high. However, Foc has the capacity of causing a disease even at low inoculum levels.[48]

4.2 Importance of transport within the digestive tract

External inoculum viability might be altered by solar radiation, washing by rain or dew from plants and leaf litter, among other factors. However, the presence of inoculum inside the insect

Table 2. Results of the four polymerase chain reaction (PCR) analyses carried out on each fungal strain isolated in the various experiments either from external teguments or digestive tract (Experiments 1 and 2); either on feces. DNA was always extracted from pure cultures

		% Positive amplification/(N° of strains)			
Experiment	Origin	Primer 1 (ITS-FU-F and -R)	Primer 2 SIX9-F and -R	Primer 3 FOC-1- and -2-F and -R	Primer 4 W2987-F and -R
1	External teguments	100 (30)	100 (30)	100 (30)	0 (30)
	Digestive tract	100 (90)	100 (90)	100 (90)	0 (90)
2	External teguments	100 (30)	100 (30)	100 (30)	0 (30)
	Digestive tract	100 (90)	100 (90)	100 (90)	0 (90)
Feces		100 (12)	100 (12)	100 (12)	0 (12)

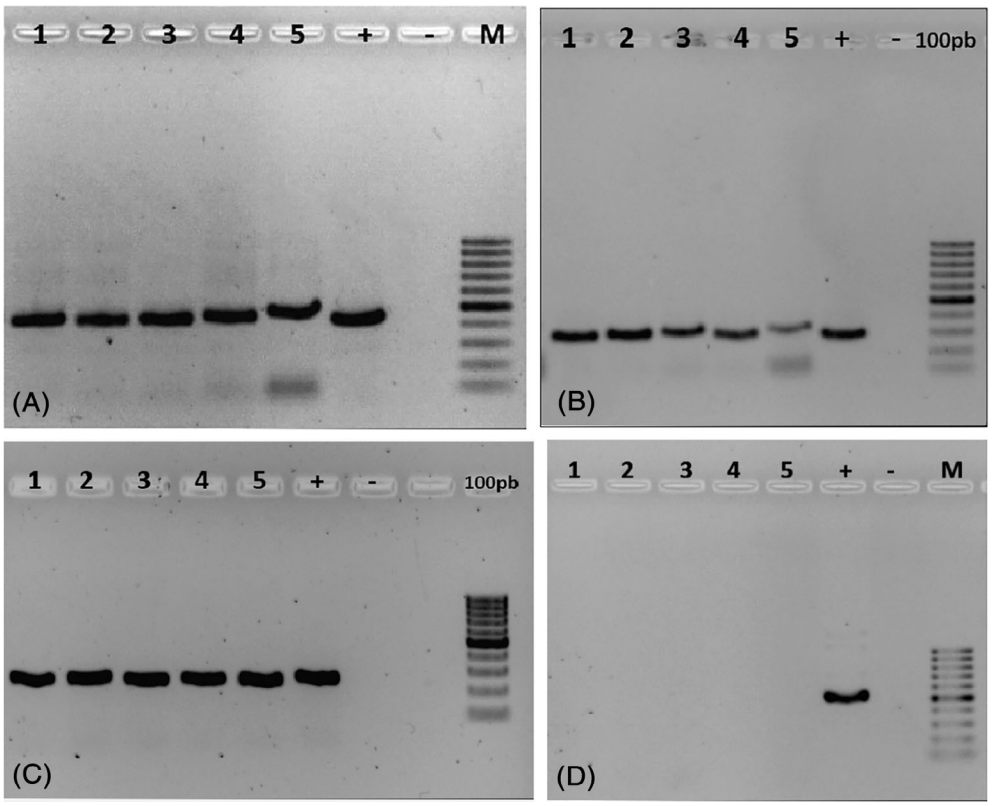

Figure 7. Agarose gel (1%) showing amplification products obtained with primers used in this study. (A) *Fusarium* spp.: ITS-Fu-F and -R, (B) *F. oxysporum* f. sp. *cubense*: FOC-1 and -2, (C) F. *oxysporum* f.sp. *cubense*: SIX9-Foc-F and -R, (D) *F. oxysporum* f.sp. *cubense* tropical race 4: W2987-F and -R. Lane 1, culture sample T2R2H; lane 2, culture sample T2R3M; lane 3, culture sample T2R3H 4, culture sample T2R4M; lane 5, culture sample T2R4H 6, DNA positive control (from (A) to (C) DNA from Foc R1 strain, and for (D) DNA from Foc TR4 strain); lane 7, DNA negative control (water); lane 8, Ladder used: GeneRuler DNA 100 bp Ready to Use Thermo Scientific.

makes it less susceptible to external environmental conditions, which might facilitate its long-distance dispersal.

Such internal presence of fungi has also been reported in other biological models. Stanghellini *et al.*[49] found chlamydospores of *Thielaviopsis basicola* in the intestinal tract of larvae and adults of shore flies (*Scatella stagnalis*) showing that these insects are involved in the dissemination of this disease to *Valerianella locusta*. Gardiner *et al.*[50] and Jarvis *et al.*[51] found that *Pythium* spp. can pass through the intestine of the fungus gnat larvae, which makes this insect play an important role as vectors of this pathogen. Likewise, Gillespie and Menzies[52] reported internal dissemination of *Fusarium oxysporum* f.sp. *radicisly copersici* by fungus gnat in tomato plants and broad beans. These examples and what were found in this investigation demonstrate that internal inoculum in the digestive tract of insects is an efficient and probably underestimated dissemination way for fungal plant pathogens. Here we have also shown that the excreta contain viable propagules of Foc but only in a smaller number of colonies in relation to what was found in the digestive tract. This may suggest that the digestive system of the weevil can destroy a great part of the viable propagules or that only a small part of fungal structures can survive in the alkaline environment of the digestive tract.

4.3 Implication of these results on the role of *C. sordidus* for Foc dispersal

Our results on the dynamics of acquisition and persistence of propagules, both externally and internally, provide a basis to

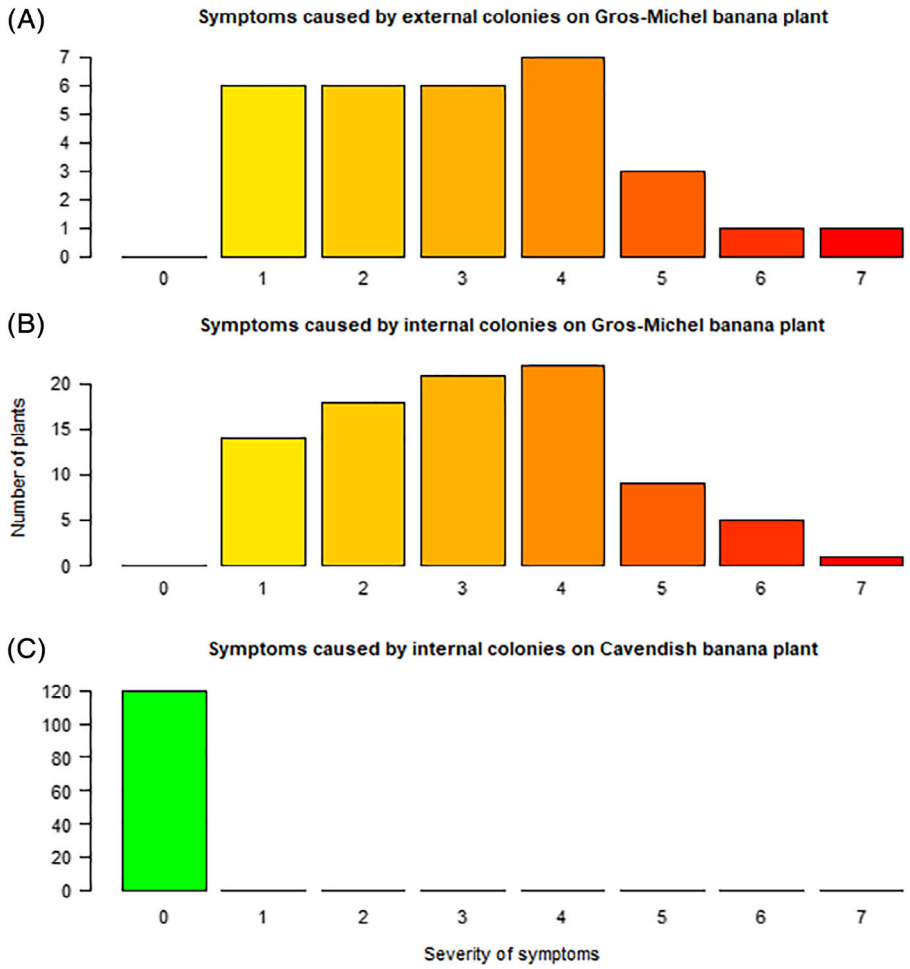

Figure 8. Disease severity on the corms of Gros Michel banana plants inoculated with different strains recovered from Experiment 1. A total of 30 strains were isolated externally (A), and 90 strains were recovered internally (B). (C) The disease severity on the corms of Cavendish banana plants isolated with these 120 isolates.

estimate the role of *C. sordidus* in spreading Foc inside and between banana fields. Here we showed that either external or internal acquisition of the fungus by *C. sordidus* is very fast since inoculum could be always detected at the shorter time of 5 min and maximal inoculum acquisition occurred after 1 h contact with an inoculum source. If internal contamination was as fast as external it might be because insects were starved before the experiments, so in natural conditions, internal contamination could depend on feeding activity of the weevils. Although the insects can get through long periods without feeding in the field, this research shows that the contamination of the insect with Foc would be rapidly effective. Weevils remain externally or internally infested for a long time, as long as 2 or 3 days that would be enough for inoculum dispersal. The rate of dissemination of the pathogen will depend on the displacement of the insect, which can be up to 9 m/d[36] and up to 35 m in 3 days,[53] so it can be expected that the dissemination of the pathogen would be of this magnitude from a single event of contamination.

Another factor that could influence the spread of the Foc in the field is the size of the insect population; larger populations are likely to spread the disease faster. The number of plants infected with Foc, the severity and external damages to the plant on which the insects feed, are fundamental in the role of *C. sordidus* on the disease spread.

All this work shows that undoubtedly *C. sordidus* is an effective vector of viable inoculum of Foc and that is a good candidate to play an important role in the epidemics of this important disease of bananas. The next step will be to prove that *C. sordidus* can efficiently transmit the disease from infected plants to healthy ones.

5 CONCLUSION

Finally, our work participates to unravel the role of *C. sordidus* as an effective vector of Foc by different ways. Inoculum acquisition is enough fast and persistent for effective disease dispersal. It is evidenced for the first time that the infective structures of Foc are capable of remaining viable in the digestive system and being expelled in the excreta, the risk of infecting healthy plants or soils is very high.

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