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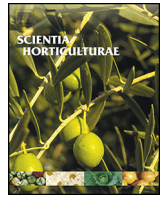
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Protective effect of organic substrates against soil-borne pathogens in soilless cucumber crops



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ABSTRACT

The development of sustainable crop protection is expected by vegetable producers and highly encouraged by authorities. For crops grown in soilless systems, vegetable fibers are relevant for both agronomical and plant protection purposes. This work examines their potential against the soil-borne pathogen *Fusarium oxysporum* f. sp. *radicis-cucumerinum*.

Wood fiber, coir fiber and peat were tested over two cucumber cropping periods. *Fusarium* blight symptoms were monitored on cucumber, and fungal community structure (PCR-TTGE) in substrates. Substrate sterilization and bio-augmentation with antagonistic strains were also studied; they did not modify protection. Compared to the other substrates, wood fiber increased protection at the end of the first assay, but did not during the second assay. Differences in crop season and plant density may have impacted on cucumber physiology and may have indirectly modified rhizosphere fungal community structure.

The sole determination of microbial activity in substrates is not sufficient to predict protection. Growth conditions, substrate type and the microbiome altogether impacted on the protection of cucumber.

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1. Introduction

Consumers are more and more mindful of the quality of the vegetables they consume. In parallel, the impact of agricultural practices on the environment is more and more under focus. New agricultural systems, such as soilless culture, can increase productivity whatever the climate conditions, but also optimize the management of inputs (fertilizers, pesticides), within a given economic and environmental framework. They also aim at controlling diseases more efficiently (Gullino et al., 2015). Mineral soilless culture substrates, which hardly contain any microorganisms, have been widely used. Organic substrates are also in use because they appear more natural, they host microorganisms likely to be useful for plant health and thus they can supply part of plants'

nutritional needs. Peat is the most commonly used material in organic substrates because it exhibits unequalled stability and physico-chemical properties (Al Naddaf et al., 2011). Composts are added to the growing media to improve microbial activity. They can substantially reduce disease severity (Khalil, 2013). However, peat bogs represent a slowly renewable pool and sanitary issues remain lingering despite the use of pesticides. Other materials (coir or wood fibers) are now being used instead of peat (Olle et al., 2012; Robin, 1997). Their physical resilience is considered as sufficient under cropping conditions, but little is currently known about their physico-chemical and microbiological properties.

Soilless culture of cucumber represents a substantial economic stake round the world (2,114,000 tons produced every year in Europe). Knowing i) how severe some pathogens can be (especially the formae speciales forms of *Fusarium oxysporum* on cucumber; Abeyasinghe, 2012), ii) the restricted use of several active molecules, and iii) the producers' wish to adopt agro-ecological production practices, other means of protection need to be developed.

Phytopathogenicity is related to the microbial populations present in the culture substrates. Yet, organic matter type appears as a determining factor for the development of both

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Table 1
Physico-chemical and microbial characteristics of the substrates (\pm : standard deviation, $n=3$).

Substrate	Substrate origin	Process	C:N ratio	pH ^a (water)	Organic matter ^b (g dw kg ⁻¹)	Dry bulk density ^c (kg dw m ⁻³)	Water storage capacity ^c (ml l ⁻¹)	Microbial organic C ^d (mg dw kg ⁻¹)	Bacterial CFUs ^e (substrate g ⁻¹)	Fungal CFUs (substrate g ⁻¹)
PiF1s	Pine 1	Screw grinding	612	4.7	996	72.4	101	457.5 (± 216.8)	2.1×10^6 ($\pm 5.2 \times 10^5$)	5.7×10^7 ($\pm 2.6 \times 10^7$)
CoF	Coconut	Unknown	130	6.4	962	59.4	116.8	388.6 (± 103.3)	9×10^6 ($\pm 9 \times 10^5$)	2.19×10^5 ($\pm 1.04 \times 10^4$)
SpPn	Sphagnum	Extracted	48	6.9	867	110.2	284.9	875.9 (± 30.8)	9.3×10^6 ($\pm 3.16 \times 10^6$)	1.23×10^6 ($\pm 4.06 \times 10^5$)

^a NF EN 13037 (2000) standard method.

^b NF EN 13039 (2011) standard method.

^c NF EN 13041 (2000) standard method.

^d NF ISO 14240-2 (2011) standard method.

^e Estimation of the number of culturable bacteria (TSA + cycloheximide) and fungi (PDA + streptomycin and tetracycline).

phytopathogenic microorganisms and their antagonists (Domeño et al., 2011; Kleiber et al., 2012; Pérez et al., 2002)

Environment-friendly control strategies, among which microbiological control, are of growing interest for the sector. Many studies have addressed the role of microorganisms in plant protection against pathogens, including soil-borne ones (Mercier and Manker, 2005; Shanmugam and Kanoujia, 2011). The specific physico-chemical properties of organic substrates are believed to drive microbial development, and sometimes lead to a protective effect against opportunistic pathogens (Clematis et al., 2009; Martínez et al., 2013). Competition for nutrients and/or space and antibiosis phenomena occur among microorganisms (Benítez et al., 2004), but our understanding of these mechanisms still remains to be further examined. In this context, we previously compared the biochemical composition of different organic substrates, the related microbial activities, and the microbial community structures (Montagne et al., 2015). Our results confirmed that specific microbial activities and microbial population structures are related to material type (wood fiber, coir fiber, peat) and therefore to specific organic compositions. Hence it appeared interesting to investigate the involvement of specific microbial communities in the control of soil-borne pathogens of soilless crops.

The present study aims at testing the responsiveness of pine wood fiber, coir fiber and peat to the host/pathogen pair *Cucumis sativus* L.-*Fusarium oxysporum*, f. sp. *radicis-cucumerinum* (FORC), a pathogen of cucumber. Two bioassays were performed: one in summer 2014, and the other in spring 2015, under greenhouse production conditions. Plant growth, pathogen attack symptoms, and the evolution of the rhizospheric fungal community structure were monitored throughout the two bioassays.

2. Materials and methods

2.1. Organic substrates

The following substrates were studied: pine wood fiber (PiF1s), coir fiber (CoF), and peat (SpPn) (Florentaise company, France). Their physico-chemical and microbiological properties are presented in Table 1.

2.2. Plant: cropping and management

Cucumber (*Cucumis sativus* L. Galaxy F1, ENZA Zaden, Enkhuizen, The Netherlands) non-treated seeds were placed in vermiculite shelves (25 °C, 12 h photoperiod). After germination, plantlets were transferred to pots filled with 1.1 l of substrate. A drop-irrigation-fertilization system was used. The nutrient solution (pH 5.6, electrical conductivity 1.5 dS m⁻¹) was prepared from mineral fertilizers (Plant Prod 229 and 216, Fertel, Boulogne-Billancourt,

France) to reach the equivalent of a 10-2-8 nitrogen-phosphorus-potassium ratio.

Crops were grown under the greenhouse, minimum temperature was 18 °C, and openings allowed for aeration when temperatures reached 24 °C. During the cultivation period, plants were propped, fruit were collected at the end of the assays, and biological protection was applied to control the development of pest insects by using the auxiliary insects *Euseius gallicus*, *Phytoseiulus persimilis*, and *Steiner nemafeltiae* (Biobest, Belgium). Climatic parameters are summarized in Table 3.

2.3. Microorganisms: growth conditions and inoculum preparation

Two antagonistic strains and one pathogenic strain were studied in the bioassays:

- the antagonistic strain *Fusarium oxysporum* (MIAE 00047, UMR1347 Agroécologie, INRA DIJON; Alabouvette et al., 1987) isolated from soil from Châteaurenard (France) in 1976 (Fo47);
- the *Trichoderma atroviridae* strain (MUCL45632, NIXE Laboratoire, Sophia Antipolis, France) (Tricho);
- the pathogenic strain *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (FORC), first isolated from diseased plants at a producer's farm, and then identified by PCR using specific FORC primers (Lievens et al., 2008).

Microbial culture and substrate inoculation were performed as follows:

- the Fo47 strain was grown on Potato Dextrose Broth (PDB, Laboratorios CONDA, Spain) under shaking at 100 rpm at 26 °C. It was inoculated when seeds were sown (mixed with vermiculite) and when plantlets were potted, at a concentration of 10³ conidia per ml of non-tyndallized substrate. Conidia were collected after filtration on a 48- μ m nylon filter (Buisine, France), and were enumerated on a Malassez counting chamber;
- the *Trichoderma* strain MUCL45632 was first grown on wood fiber for 3 weeks to reach a high concentration, and then the inoculum was mixed with the PiF1s substrate to reach a final concentration of approximately 10⁵ cells g⁻¹ of non-tyndallized substrate;
- the FORC strain was grown in the same conditions as the Fo47 strain. Conidia were collected and enumerated as described above. FORC was inoculated at the surface of the pots at a concentration of 5 \times 10³ conidia per ml of substrate (tyndallized or not), when plantlets were at the 3–4 leaf stage.

To prevent microorganisms leaching from the substrates, in the week following inoculation plants were irrigated with the same amount of water as in the following weeks, but it was provided in smaller quantities and more frequently.

Table 2
Conditions of the two bioassays.

Treatment	Bioassay 1		Bioassay 2	
	FORC-free	FORCadded	FORC-free	FORCadded
With prior tyndallization	PiF1s/FORC-free/Tynd	PiF1s/FORC/Tynd	PiF1s/FORC-free/Tynd	PiF1s/FORC/Tynd
	CoF/FORC-free/Tynd	CoF/FORC/Tynd	–	–
	SpPn/FORC-free/Tynd	SpPn/FORC/Tynd	–	–
Without prior tyndallization	PiF1s/FORC-free/No Tynd	PiF1s/FORC/No Tynd	PiF1s/FORC-free/No Tynd	PiF1s/FORC/No Tynd
	CoF/FORC-free/No Tynd	CoF/FORC/No Tynd	CoF/FORC-free/No Tynd	CoF/FORC/No Tynd
	SpPn/FORC-free/No Tynd	SpPn/FORC/No Tynd	SpPn/FORC-free/No Tynd	SpPn/FORC/No Tynd
Without prior tyndallization, with bio-augmentation	PiF1s/FORC-free/Fo47	PiF1s/FORC/Fo47	PiF1s/FORC-free/No Tynd/Tricho	PiF1s/FORC/No Tynd/Tricho
	CoF/FORC-free/Fo47	CoF/FORC/Fo47	–	–
	SpPn/FORC-free/Fo47	SpPn/FORC/Fo47	–	–

Table 3
Temperature and light intensity in the greenhouse during the bioassays.

Bioassay	Temperature (°C)		Light intensity (J cm ² day ⁻¹)	
	Bioassay 1	Bioassay 2	Bioassay 1	Bioassay 2
Min	9.5	10.7	482	530
Max	36.7	42.7	2644	3064
Mean	21.4	22.1	1775	2017

2.4. Microbial analysis

Different microbiological analyses were performed at each sampling time and at the end of each assay:

– *F. oxysporum* was enumerated in the rhizosphere and the roots of 5 plants per condition, in bioassay 1 only: the rhizospheres from three plants from one block were used, and from one plant from the other two blocks. Suspensions/dilutions were prepared, using 1 g of vortexed substrate in 45 ml of sterile water. Then the appropriate dilutions were enumerated in Petri dishes by spreading 1 ml of microbial suspension mixed with Komada medium (Komada, 1975). The *F. oxysporum* (Fo47 or FORC) density was expressed as colony-forming units (CFU)/g of substrate.

F. oxysporum was also enumerated in the plants' roots. Roots were first disinfected by dipping 30 s in 70% ethanol, and then rinsed by bathing 15 s in two successive sterile water baths. At least five root fragments from a same plant were laid on Komada agar medium. Petri dishes containing microbial suspensions or root fragments were all read after five days of incubation at 25 °C in the dark, followed by 3 days under light to reveal the purple pink *F. oxysporum* colonies (Davet and Rouxel, 1997);

– the structure of rhizospheric fungal communities was determined: Rhizospheric substrate was kept at –20 °C and then analyzed by PCR-TTGE (Polymerase Chain Reaction–Temporal Temperature Gel Electrophoresis; Jaffrès et al., 2009; Montagne et al., 2015). Briefly, microbial DNA extracted from the substrate was amplified according to Joly's protocol (Joly et al., 2012). This step amplifies part of fungal ITS 1, based on the fact that certain ITS 1 zones vary according to microorganisms. The amplicons migrated through an acrylamide gel under a temporal temperature gradient to reveal sequence differences among the variable regions of fungal DNA. The gels were stained with GelRed (Biotium, United States), and then fungal profiles were developed under UV light. Genetic fingerprints (number, position, and intensity of the bands) were compared using FPQuest Software (Bio-Rad, United States).

2.5. Experimental design

Two assays were conducted at two different seasons. The first crop was grown in late summer 2014, and the second one in spring 2015. The conditions studied in the two assays are detailed

in Table 2. Some substrates were first sterilized by tyndallization (Marchal, 1976) while others were bio-augmented by adding pathogenic or antagonistic microorganisms (FORC, *F. oxysporum* Fo47, and *T. atroviridae* MUCL45632).

A light/temperature gradient was applied in the greenhouse. That is why a 3-block system was used, each block containing one replicate of each of the conditions. In each block, 3 plants were grown per condition and per sampling time-point, i.e. 9 plants in total. Two samplings (S1 and S2) were performed during the bioassays, and a third one (S3) at the end. First samples (S1) were taken seven days after inoculation with FORC, i.e. after 32 days of cultivation in bioassay 1, and 34 in bioassay 2. Samples S2 were taken 21 days after inoculation with FORC, i.e. after 46 days of cultivation in bioassay 1, and 44 in bioassay 2. Samples S3 were taken at the end of the bioassays, i.e. after 69 days of cultivation in bioassay 1, and 78 in bioassay 2. Substrate pH, electrical conductivity (following the standard NF EN 13038, 2000) and microbial analysis were recorded at the beginning of the cropping periods, during samplings, and at the end of the cropping periods.

The different conditions were randomly distributed in each block. Crop density was 45 pots (1.1-l) per m² in bioassay 1, and 12.5 pots per m² in bioassay 2. Crop density was reduced in the second assay because plants were too difficult to handle at the end of first assay.

Plant size, leaf and fruit numbers were evaluated over the two cropping periods. In parallel, the outbreak of symptoms related to FORC attack was monitored by evaluating i) the number of healthy plants, ii) the number of diseased plants exhibiting a yellowish, narrower stem base and pronounced wilting of the aerial parts, and iii) the number of dead plants in which more than half of the aerial part was dry and yellow.

2.6. Data analysis

Analyses of variance (ANOVA) were performed at the end of the 2 bioassays, using Tukey's test with a 5% significance threshold. Only FORC-free conditions were analyzed to test the effects of the "substrate type", "tyndallization", "Fo47" and "Trichoderma" factors on plant growth. For plant health data, ANOVA analyses were supplemented by a Newman-Keuls multiple comparison test to compare the numbers of dead plants among conditions at the end of the two bioassays. Variance analyses and multiple comparison tests were generated using R 3.1.1 (Free Software Foundation) and the SNK.test function of the agricolae package.

Fungal community structure analysis was performed based on genetic fingerprints. Pearson's test was used to compare them, and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to create a dendrogram based on similarity coefficients (Ibekwe et al., 2010).

3. Results

3.1. Initial substrate characteristics and plant growth

Whatever the substrate, organic matter contents were high, but C/N significantly differed between substrates (Table 1). Because peat had a higher water storage capacity than the other two substrates, water regimes were adapted accordingly (Paul and Lee, 1976).

Initial microbial carbon was twice higher in peat than in wood fiber and coir fiber.

The mean concentration of culturable bacteria in the substrates was 10^6 – 10^7 CFUs g^{-1} , and the mean concentration of culturable fungi varied from 10^5 to 10^7 CFUs g^{-1} . PiF1s contained 100 times more fungi than CoF and 10 times more than SpPn, but half as many bacteria as the other substrates.

Climatic conditions were monitored throughout the two cropping periods (Table 3). On average temperature was similar for the two crops, but mean light intensity was higher during bioassay 2. As for substrate pH and electrical conductivity, they remained close to 7.5 and 200 $\mu S\ cm^{-1}$ throughout the 2 bioassays, respectively.

The height of control (FORC-free) plants was measured during the bioassays. Mean final size was 225 cm in bioassay 1, and 170 cm in bioassay 2. Control plants developed in a homogeneous manner over time in the two bioassays: there was no significant difference among conditions or among blocks; *p*-values were 0.1185 in bioassay 1, and 0.4616 in bioassay 2. Moreover, at the end of the bioassays, leaf and fruit numbers were similar among conditions, but the number of fruit was higher in bioassay 2, with 4 fruits per plant in bioassay 2 vs. 2 in bioassay 1. Pot density differed too: it was lower in bioassay 2, with 12.5 pots m^{-2} vs. 45 pots m^{-2} in bioassay 1.

3.2. Disease suppression in the different treatments

The pathogen dose was inoculated at the 3–4 true leaf stage to allow for gradual and sufficient expression of the disease. Whatever the bioassay, FORC-related symptoms came out one month after inoculation, i.e. after 55 days of cultivation. Statistical data analysis did not reveal any block effect. Mean percentages of healthy, diseased and dead plants were determined at the end of each bioassay. In the first bioassay (Fig. 1), no plant was found diseased in the FORC-free conditions.

Significant differences in the number of dead plants among conditions were determined by one-way ANOVA (*P*-value: 7.3×10^{-10}). Then a multiple comparison was performed to form homogeneous groups based on the numbers of dead plants. No dead plant was observed in the FORC-free controls, which clustered in group c. In the 9 FORC-treated conditions, wood fiber-grown plants displayed average mortality rates ranging between 22 and 33% depending on the condition, coir fiber-grown plants between 55 and 77%, and peat-grown plants between 55 and 77% too. The mortality rates of non-tyndallized wood fiber-grown plants (PiF1s/FORC/No Tynd.) and bioaugmented wood fiber-grown plants (PiF1s/FORC/Fo47) did not significantly differ from the controls, with 33 and 22%, respectively. However, bioaugmented wood fiber-grown plants also displayed a significantly lower number of dead plants than the plants of 2 out of the 3 peat treatments (SpPn/FORC/Tynd and SpPn/FORC/Fo47, with 77% mortality rate each), and 2 out of the 3 coir fiber treatments (CoF/FORC/No Tynd and CoF/FORC/Tynd, with 77% mortality rate each).

A two-way ANOVA solely applied to the FORC conditions showed an effect of the substrate (*P*-value: 0.005204). This confirmed the existence of a significant difference between the mean highest mortality rates and the mean lowest mortality rates. Consequently, the plants grown on wood fiber were less severely attacked by the pathogen than those grown on coir fiber and peat. By con-

trast, treatments (tyndallization or bioaugmentation with Fo47) altogether had no effect on mortality rates.

In the second bioassay (Fig. 2), among control conditions (i.e. FORC-free substrates), only one plant exhibited *Fusarium* blight symptoms although it had not been inoculated with FORC. As compared to the first bioassay, the percentage of healthy plants was lower, and so was the percentage of dead plants, but the percentage of diseased plants was higher.

A one-way ANOVA was applied whose variable was the number of dead plants and whose factor was the 10 conditions, and then a multiple comparison test was performed (Fig. 2). The homogeneous groups corresponding to dead plants are indicated by letters. On this basis, the FORC-inoculated raw substrates (with or without prior tyndallization) shared the same group as the control substrates.

Further in detail, considering the non-tyndallized conditions and FORC-inoculated substrates, no dead plant was observed among CoF-treated plants, and PiF1s and SpPn treatments did not show significant differences as compared to previous treatments.

The percentage of diseased plants was similar among the three substrates, with 66%. As regards the tyndallization factor, we noted the same tendency as in bioassay 1 for substrate PiF1s only, namely that prior tyndallization of the substrate (PiF1s/FORC/Tynd) appeared to increase the percentage of dead plants, with 33% vs. 11% for the same non-tyndallized substrate (PiF1s/FORC/No Tynd). Nevertheless, these differences were not significant, as shown by ANOVA results (groups ab and b, respectively). By contrast, bioaugmentation with MUCL45632 (PiF1s/No Tynd/Tricho) had a significant negative effect on plant mortality (66%) as compared to FORC/PiF1s/No Tynd (11%).

The percentages of diseased and/or dead plants differed from those obtained in bioassay 1. In other words, the time-course of the disease appeared faster in bioassay 1, with a higher percentage of dead plants, while in bioassay 2 plants were only diseased, with a similar percentage of healthy plants.

3.3. *F. oxysporum* plate count

F. oxysporum populations were enumerated in the first bioassay (Table 4). Fo47 and FORC were not phenotypically discriminated on the Komada medium used to assess the CFU count.

At S1, the Fo47-free, FORC-inoculated conditions, and the Fo47-bioaugmented conditions (both FORC-free and FORC-inoculated) displayed a high *F. oxysporum* density (ca. 3 log (CFU) g^{-1}). By contrast, the FORC- and Fo47-free conditions did not reveal the presence of *F. oxysporum*. Therefore the presence of *F. oxysporum* corresponded to the FORC-inoculated or Fo47-bioaugmented conditions.

At S2, populations were at a balance at around 3 log (CFU) g^{-1} for the FORC and Fo47 conditions.

As compared to peat and coir fiber, in the presence of FORC wood fiber appeared to be more responsive to the *F. oxysporum* strains, whether pathogenic or not (PiF1s FORC/No Tynd and PiF1s/FORC/Tynd).

At S3 (the end of the assay), *F. oxysporum* was found in the "FORC-free/No Tynd." and "FORC-free/Tynd" conditions, suggesting contamination from the air or from spores present in the substrates. However, the same was noted with tyndallized substrates, and tyndallization proved efficient since fungal amplification at the beginning of the assays was not possible (data not shown). Contamination from the air therefore appears as a relevant hypothesis.

To monitor the development of *F. oxysporum* strains, detection tests were carried out in plant roots (data not shown). At least 20% of the root systems, all conditions included, contained *F. oxysporum*. *F. oxysporum* distribution according to the different conditions was similar in the roots and in the rhizosphere. A maximum of 100%

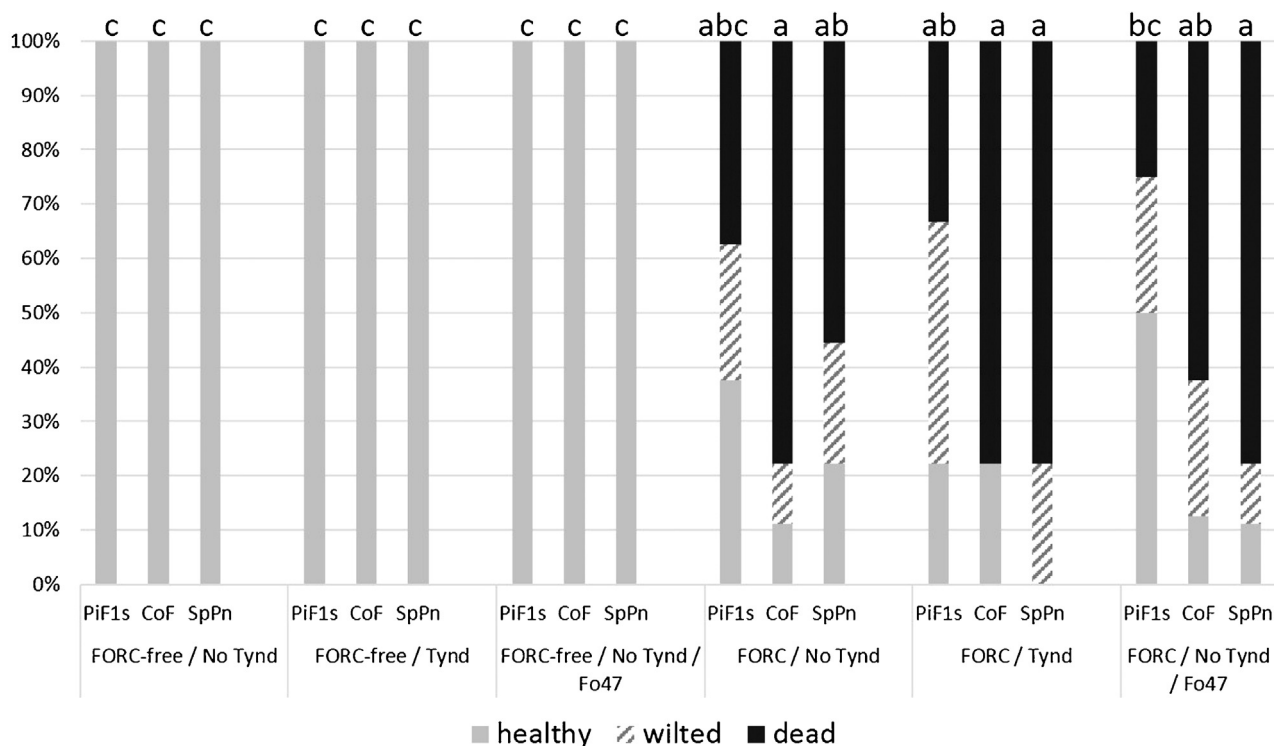


Fig. 1. Percentages of healthy, wilted and dead plants at the end of bioassay 1 (n = 3, 9 plants). Different letters of the homogenous groups (a, c, ab, bc and abc) indicate significant differences among treatments for the number of dead plants (black bars only) according to Newman-Keuls test (P = 0.05)

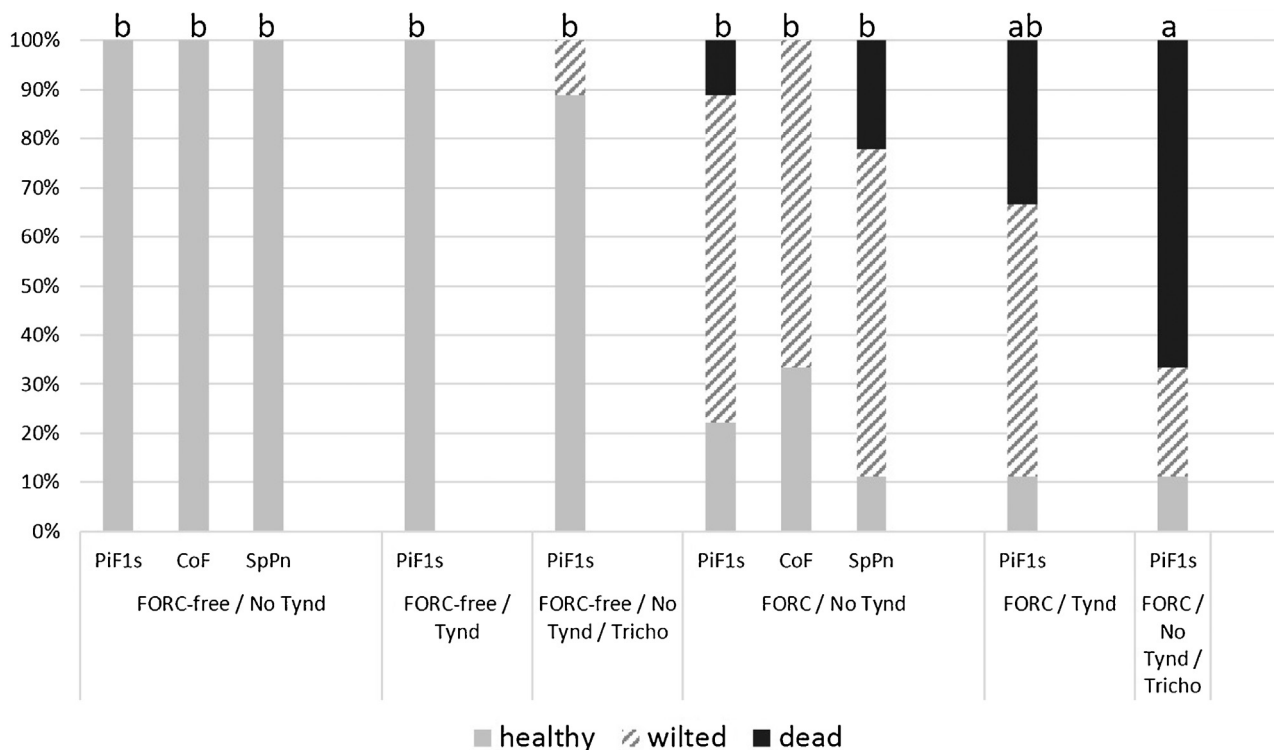


Fig. 2. Percentages of healthy, wilted and dead plants at the end of bioassay 2 (n = 3, 9 plants). Different letters of the homogenous groups (a, b and ab) indicate significant differences among treatments for the number of dead plants (black bars only) according to Newman-Keuls test (P = 0.05)

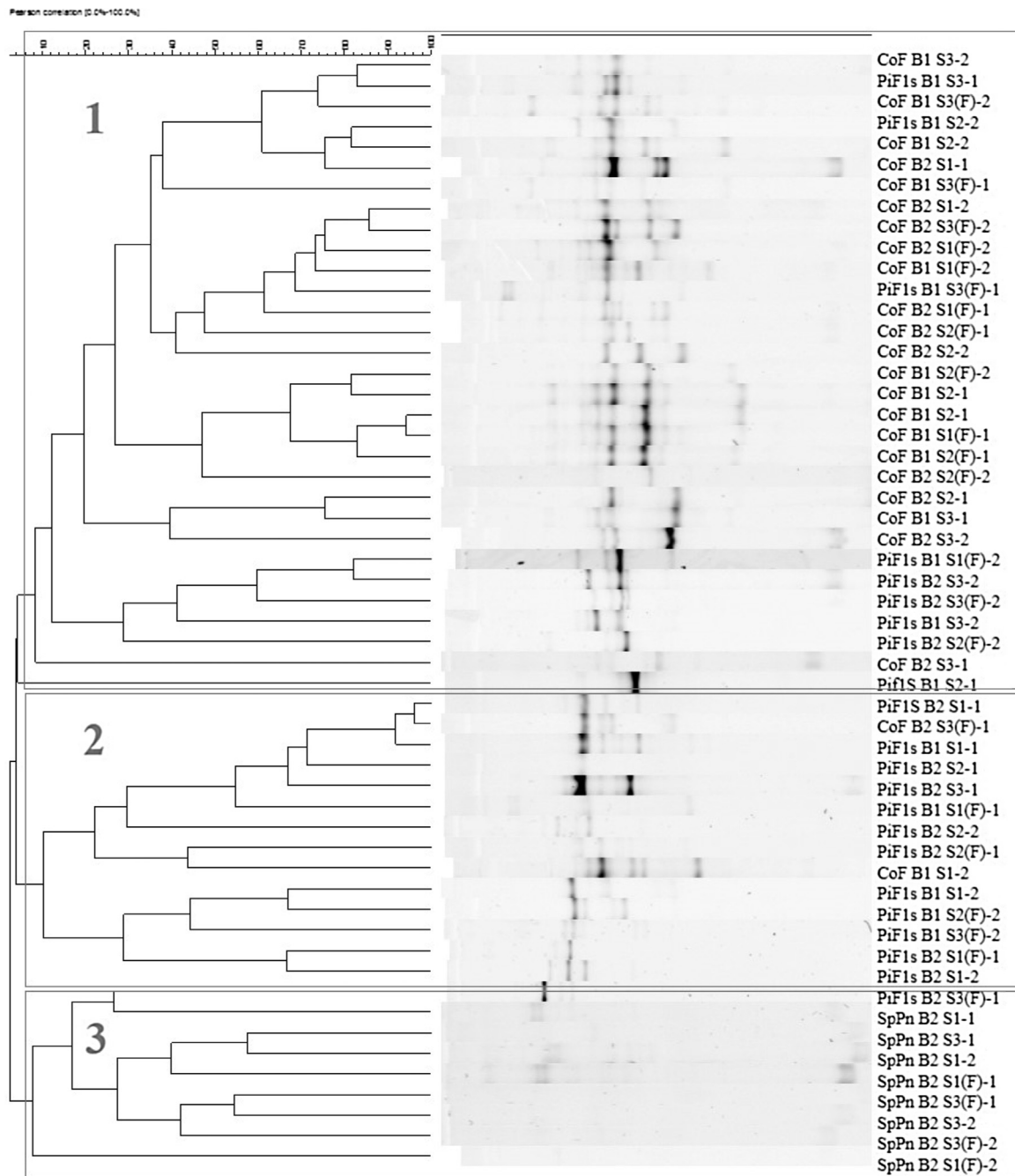


Fig. 3. Fungal community structures of PiF1s, CoF and SpPn throughout the two bioassays B1 (in 2014) and B2 (in 2015). Grey rectangles symbolize the three clusters named 1, 2 and 3 on Fig. S1 FigS. S1, S2 and S3 are samples. (F) indicates the samples that received the phytopathogen FORC. –1 and –2 are replicates. Technical difficulties did not allow us to obtain the SpPn B1 (2014) profile.

infected plants was found in the FORC/Tynd, FORC/Fo47 and FORC-free/Fo47 substrates.

3.4. Characterization of the fungal population structure

The fungal structures of the 3 FORC-free or FORC-inoculated substrates (NoTynd, FORC or FORC-free) were sampled at S1, S2 and S3 during the two bioassays (Fig. 3). For a given condition, replicates noted –1 and –2 displayed rather low similarity percentages. Nevertheless, comparisons between the fungal structures of the two bioassays can be made, all conditions included. Above all, fungal structure depended on substrate nature (CoF represented by rectangle 1, PiF1s represented by rectangle 2, and SpPn represented by

rectangle 3), whatever the year or the presence/absence of FORC. More precisely, in rectangle 1 which mainly represents CoF substrates, a PiF1s cluster was present and differed again from the CoF cluster.

As regards CoF (rectangle 1), similarity between bioassay 1 (B1) and bioassay 2 (B2) was low. Therefore the fungal structure of CoF was related to the year. The modification of the fungal community structure of CoF in bioassay 1 as compared to bioassay 2 could explain the different protection levels supplied by this kind of substrate.

If we specifically analyze PiF1s (Fig. 4) at the first (S1) and last (S3) sampling time-points of bioassays 1 and 2, sample structures rank according to sampling date, not according to the year.

Table 4
CFUs of *Fusarium oxysporum* per g of rhizospheric substrate after 32 days (S1), 46 days (S2), and at the end of the cropping season (after 69 days, S3) in bioassay 1 (means \pm standard deviations; n = 5). Data are expressed in log-transformed values.

	PiF1s			CoF			SpPn		
	32 d	46 d	69 d	32 d	46 d	69 d	32 d	46 d	69 d
FORC-free/No Tynd.	0.00 ± 0.00	0.00 ± 0.00	2.83 ± 2.58	0.00 ± 0.00	0.00 ± 0.00	2.37 ± 2.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
FORC/No Tynd.	3.88 ± 0.72	1.98 ± 2.87	4.63 ± 0.93	2.59 ± 2.41	0.53 ± 1.19	4.51 ± 0.48	0.79 ± 1.77	2.21 ± 2.04	4.02 ± 0.49
FORC-free/Tynd.	0.00 ± 0.00	0.73 ± 1.63	0.53 ± 1.19	0.00 ± 0.00	0.00 ± 0.00	1.71 ± 1.58	0.00 ± 0.00	0.00 ± 0.00	0.59 ± 1.32
FORC/Tynd.	3.72 ± 0.85	2.65 ± 2.66	4.46 ± 2.53	3.58 ± 0.56	1.40 ± 1.92	4.89 ± 0.75	2.71 ± 1.61	2.80 ± 1.72	4.45 ± 0.32
FORC-free/Fo47	2.96 ± 1.80	1.12 ± 1.54	3.22 ± 2.04	1.24 ± 1.70	1.85 ± 1.73	3.68 ± 0.50	1.85 ± 1.73	0.73 ± 1.63	3.33 ± 0.41
FORC/Fo47	2.98 ± 0.32	2.11 ± 1.96	2.17 ± 2.04	3.50 ± 2.04	0.53 ± 1.19	2.21 ± 2.05	2.63 ± 1.52	2.88 ± 1.71	4.12 ± 0.89

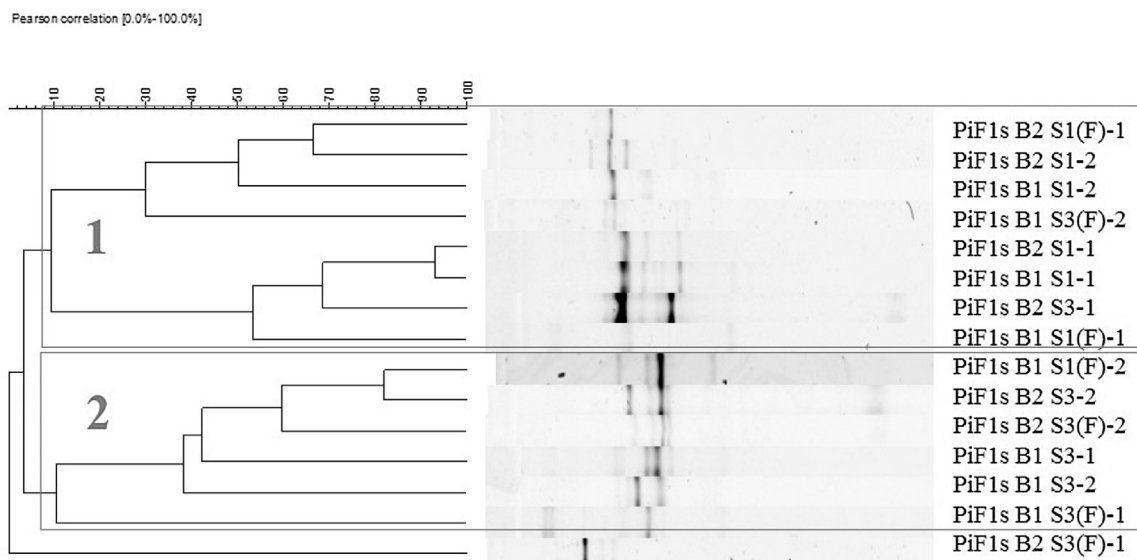


Fig. 4. Fungal community structure of PiF1s throughout the two bioassays B1 (in 2014) and B2 (in 2015). Grey rectangles symbolize the two clusters named 1 and 2 on Fig. S1 and S3 are samples. (F) indicates the samples that received the phytopathogen FORC. –1 and –2 are replicates.

4. Discussion

This study underlines the implication of organic substrates in crop bioprotection against a pathogen of cucumber (FORC), and on the reproducibility of that protection under conditions nearing large-scale production conditions.

Plants grew well, whatever the substrate type. Our substrates are suited for soilless culture, although some physico-chemical and microbiological parameters (Table 1: water retention capacity, biochemical composition, granulometry, special microbial populations, etc.) differed among the three substrates.

Apart from one plant affected by FORC in the second bioassay, control (FORC-free) plants did not display any *Fusarium* blight symptoms, whereas FORC-inoculated plants did (the disease came out after 55 days). The only difference between these two conditions was the addition of FORC, so the symptoms were indeed related to the presence of FORC, and were not related to possible indigenous pathogenic strains. *F. oxysporum* density on Komada medium during the first bioassay actually showed that FORC and/or Fo47 had settled in the substrates (Table 4).

In the first bioassay, tyndallization had a stronger impact when applied to PiF1s, with a lower number of healthy plants as a result of wood fiber tyndallization. That is why that factor only was tested again on PiF1s in the second bioassay. The Fo47 factor did not yield

homogeneous results across all substrates, so it was replaced by *Trichoderma* (only tested on PiF1s), a strain known for its growth-stimulating effects, which seems to settle well in wood fiber. Peat initially contained the greatest amount of microorganisms (it had the highest rate of microbial carbon). The culturable fungi/bacteria ratio was approximately 1, and was the highest (> 1) in wood fiber. These initial differences implied different plant protection potentials. *Fusarium* blight differed in its expression depending on the substrate throughout the two bioassays. Plant fiber processing (screw grinding for wood fiber, unknown for coir fiber) could explain this lesser microbial colonization, while peat was simply extracted mechanically.

Looking at the number of dead plants at the end of the bioassays, it appeared that substrate type had a significant influence on mortality. Mortality was lower in plants grown on wood fiber (PiF1s), particularly in the first assay, despite the high number of *F. oxysporum* CFUs. This may have resulted from the presence of different *F. oxysporum* populations, with predominance of non-pathogenic ones. Competition among them may explain this low mortality rate (Dhingra et al., 2006). Moreover, these populations possibly triggered plant resistance before FORC attack. Besides, distinguishing between diseased and dead plants allowed us to assess the time-course of the disease in the two bioassays. Mortality occurred later in bioassay 2, but adding *Trichoderma* caused

it to occur earlier. FORC attack occurs under plant weakness conditions; it is all the more virulent as plants are weakened or their inner resources are depleted. In our bioassays, symptoms probably first appeared concomitantly with combined stresses, such as plant fruit load – for example, at the end of bioassay 2, plant biomass (vegetative parts + fruit) reached 2250 g on average – or increases in temperature and light intensity (Table 3). In bioassay 1, the disease symptoms burst out all of a sudden, and plants rapidly died. Conversely, symptoms came out more progressively in bioassay 2. Temperature increased more gradually, so plants probably got acclimated more easily. As a result, fewer plants died, more plants became diseased (22–66% depending on the conditions, vs. 0–44% in bioassay 1). Moreover, the higher plant density in bioassay 1 probably intensified these stresses.

Tyndallization drastically reduces initial microbial population densities in the substrates, while maintaining their physico-chemical and structural properties. It did not significantly modify plant protection as compared to non-tyndallized substrates. This means that the microbial populations naturally present in the substrates have little influence on plant protection, which is surprising and contradicts previously published results (Clematis et al., 2009). The analysis of the fungal community structure over time did not evidence any noticeable difference between tyndallized and non-tyndallized samples. This confirms that in the case of tyndallized substrates, re-colonization is under the control of the substrate, and therefore of its physico-chemical characteristics (Table 1). As crops grew, CoF fungal community structure diverged from PiF1s fungal community structure (Fig. 3). Competition for space and/or access to substrates probably explain why protection was modulated.

If we specifically analyze PiF1s (Fig. 4) at the first (S1) and last (S3) sampling points of the 2 B1 and B2 bioassays, we can note that the sampling date influenced fungal community structure independently of the year or the addition (or not) of the phytopathogen FORC. The evolution of the fungal community structure according to cultivation stage could be explained by quantitative and/or qualitative variations of rhizodeposits in the course of the cropping period (Zhang et al., 2014). Plants grew under different growth conditions in the 2 bioassays, so we can assume that root exudate composition was different too. When associated to other factors such as substrate temperature and humidity, these parameters may explain that the rhizospheric microbial community structure was modified in the course of the 2 bioassays, and in turn the response to the pathogen. We can indeed note that, especially for CoF, the fungal populations of the two assays evolved (Fig. 3). Different populations in CoF B1 and CoF B2 could explain why they provided different levels of protection in the 2 bioassays. Gao et al. (2015) worked on cucumber crops; they showed that microbial communities differed depending on the season and the cultural practices (e.g. grafting).

Fo47 was only studied in the first bioassay, on the 3 substrates. *F. oxysporum* Fo47 antagonizes the causal agents of *Fusarium* blight on tomato and melon (Fuchs et al., 1997; Migheli et al., 2000). Besides, other non-pathogenic *F. oxysporum* strains compete for nutrients with FORC (Alabouvette et al., 2009; Mandeel and Baker, 1991). In bioassay 1, Fo47 did not influence on mortality. In the same way, Fo47 had no effect in CoF or SpPn, probably due to a too low Fo47/FORC ratio (Olivain et al., 2004).

MUCL45632 was also studied as an antagonistic agent, only in PiF1s in bioassay 2. Numerous *Trichoderma* species can protect crops by producing chitinases (antibiotic effect) or cellulases (competition for nutrients) and allow for plants to develop properly by improving mineral bio-availability (Benítez et al., 2004; Boehm et al., 1993; John et al., 2010; Lemanceau et al., 2012). Yet, under our experimental conditions the *Trichoderma* strain we used (MUCL45632) had no positive effect on cucumber growth. On average, the number of dead plants in PiF1s was even higher in the presence of MUCL45632 (Fig. 2). The MUCL45632 patent presents

it as a stimulant of plant growth (Nixe, 2008). We did not specifically monitor this strain in this work, so we cannot conclude about its ability to survive and to be active in PiF1s.

5. Conclusions

Plant sensitivity to pathogens remains greatly dependent on cropping conditions that are in turn dependent on the season, even though the organic composition of substrates can reduce pathogen virulence. Microbiological control based on the use of organic substrates therefore appears to be efficient up to a certain phytopathogen threshold. Our 2 bioassays also showed how important it is to study bio-control methods case by case, and how difficult it is to reproduce suppressiveness effects.

Conflict of interest

No conflict of interest declared.

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