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Eelgrass Slabs, a Soilless Culture Substrate That Inhibits Adhesion of Fungi and Oomycetes and Enhances Antioxidant Activity in Tomato

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ABSTRACT: Composed of a marine plant, *Zostera* sp., eelgrass slabs are a novel organic substrate for soilless cultures used in tomato production. The benefit of using eelgrass slabs for growing tomatoes was assessed by comparing it with coconut fiber slabs in regard to contamination by *Pythium* spp. and to the antioxidant properties of tomato fruits. First, tomato root contamination by *Pythium* spp. was studied by direct plate counting, and a molecular comparison of fungal and oomycete communities was conducted using PCR-DHPLC. Second, the antioxidant properties of tomato fruits were analyzed by measuring total phenol and carotenoid contents and by evaluating radical scavenging activity. Compared to plants grown on coconut fiber slabs, those on eelgrass slabs presented a lower rate of *Pythium* spp. root contamination. Moreover, culture on eelgrass slabs produced fruits with better radical scavenging activity and higher total phenol content compared to controls. Carotenoid content was not affected by the type of substrate. This study highlights the value of detrital leaves of *Zostera* sp. as a substrate for soilless culture that reduces root contamination and also promotes the production of tomato fruits with better nutritional value.

KEYWORDS: eelgrass (*Zostera* sp.), tomato (*Lycopersicon esculentum* cv. Plaisance), soilless culture, antiadhesive substance, antioxidant activity

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is a widely consumed fruit, rich in healthy dietary components [1] and generally produced in soilless systems. Environmental control is the main advantage of soilless cultures, which provide ideal conditions for plants and, consequently, frequently result in greater yields than traditional cultural methods. In Europe, 95% of tomatoes cultivated in greenhouses are grown in soilless culture conditions.[2] Among the various types of substrates available, rockwool (an inorganic substrate) and coconut fibers are the most widely used in soilless culture substrates in Brittany, which is the leading area of tomato production in France. However, recent decades have shown an increase in industrial byproduct recycling due to consumer and regulatory pressure. In addition, some organic substrates, such as coconut fibers, are sometimes difficult to obtain. Depending on the type of substrate chosen, cultural practices should be adapted: in particular, irrigation, nutritional amendments, pH regulation, and management of energy in the greenhouse.[3]

Originally, soilless systems were developed as open systems where excess nutrient solutions were discarded. However, this caused eutrophication outside the greenhouse. As a result, plants were immune to most soilborne pathogens. In recent years, closed hydroponic systems have been developed to minimize pollution. However, in a closed system with recycled nutrient solutions, root disease still can be a major problem, if proper cultural practices are not followed.[4] Microbial contamination of the root system in these soilless systems can arise from many sources, that is, plant material, growing medium, and water.[5] In tomato, *Pythium* spp. is one of the most commonly found pathogens in the rhizosphere, where it can cause root rot diseases.[6] Prevention of these infections has become a major challenge in the past decade,[5,7−9] fostering the development of methods to disinfect nutrient solutions or to introduce biocontrol agents into the rhizosphere.

In addition to microbial contamination due to nutrient solution recycling, one of the main problems with soilless cultures is the disposal of spent culture substrates such as rockwool, the recycling of which is still an environmental concern in many countries.[10] Thus, there is currently a strong demand for organic substrates that are readily available, affordable, and suitable as growing media.[11] Recently, Floury et al.[12] suggested using eelgrass leaves as an organic substrate for soilless culture.

Eelgrass (*Zostera* sp.) is a marine plant that is widely distributed in the northern hemisphere.[13] Eelgrass beds are important ecosystems, providing protection and breeding grounds for many marine organisms. They also produce large amounts of detritus that washes ashore on beaches and that has become a costly nuisance requiring regular removal and disposal.[14] The French Atlantic coast is particularly affected by eelgrass detritus because the most extensive intertidal meadows of *Zostera noltii* in western Europe are located in the Arcachon Bay (southwestern France).[15] The use and development of this important natural biomass are therefore of interest. Nevertheless, few studies have investigated this material and its potential. Davies et al.[14] showed that eelgrass fibers were useful for reinforcing composite materials

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and could be employed in biodegradable structures. Recently, Achambale et al.[15,16] indicated that eelgrass leaves can be sources of zosteric and rosmarinic acids, two secondary metabolites. Both are bioactive compounds: zosteric acid is known as an antiadhesive molecule,[17] and rosmarinic acid shows various biological properties such as antiviral, antibacterial, antioxidant, and anti-inflammatory activities.[18,19]

In this work, we studied the use of detrital leaves of *Zostera* sp. as an organic substrate for soilless tomato production. To do so, we compared two organic substrates: eelgrass slabs and coconut fiber slabs. Two biological aspects were investigated. First, we studied tomato root contamination by *Pythium* spp., to determine if the culture on eelgrass slabs reduced the attack of this oomycete. Quantification was done by directly plating roots on selective media and by performing a molecular comparison of fungal and oomycete communities (PCR-DHPLC). Second, we estimated the antioxidant activity of tomato fruits, to determine whether eelgrass slabs induced higher antioxidant activity than coconut fiber slabs. This was carried out by measuring total phenol and carotenoid contents and by evaluating radical scavenging activity.

MATERIALS AND METHODS

Substrates. Eelgrass slabs (60 cm × 25 cm × 10 cm) provided by AlgiePlus (Pleudaniel, France) were constituted of dry, compact *Zostera* sp. leaves. Commercial coconut fiber slabs (75 cm × 4 cm × 20 cm) purchased from Palmeco were used as controls.

Fruit and Root Sampling. Tomato (*L. esculentum* cv. Plaisance, Thomas Seeds, France) were sown in October 2009. About 3 months after sowing, tomato plants were transplanted into eelgrass or coconut fiber slabs in a heated greenhouse on the northern coast of Brittany (Ploubazlanec, France). Plant nutrition and chemical pest and disease control followed standard commercial growing practices. Roots and fruits of six tomato plants in each condition were randomly collected at the end of production (November 2010).

Roots were used for directly measuring *Pythium* spp. contamination. After harvesting, fruits were cleaned with deionized water, rapidly rinsed, and cut in small pieces. For each experimental condition, one subsample was stored at −25 °C and then freeze-dried. The freeze-dried material was ground to a fine powder. The second subsample was stored at −25 °C before the extraction of carotenoids.

Root Colonization by Fungi and Oomycetes. *Plate Counting To Assess Root Colonization by Pythium spp.* Root colonization by *Pythium* spp. was quantified by direct plating, as described by Vallance et al.[20] Briefly, 200 nondisinfected root fragments (for each substrate, 10 root segments of 10 mm per plate) were put in contact with selective medium (CMA-PARP) and incubated at 25 °C. After 48 h, *Pythium* spp. thalli were counted, and the ratio of the number of colonized fragments to the number of analyzed fragments in each condition was determined.

DNA Extraction. Genomic DNA was extracted from the rhizosphere of plants growing on eelgrass and coconut fiber slabs using the FastDNA SPIN Kit for soil (MP Biomedicals, Illkirch, France) with slight modifications to the manufacturer's instructions.[8] Briefly, 200 mg of finely ground environmental material was homogenized with 122 μL of lysis MT buffer and 978 μL of sodium phosphate in a multimix two-tissue matrix tube using a FastPrep instrument for 40 s at a speed setting of 4.0. The total DNA extract was resuspended in 60 μL of sterile distilled water during the final step of extraction and was quantified using a Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, MA). Samples were standardized to 20 ng μL^{-1}.

PCR Amplification of Fungal and Oomycete DNA. PCR amplifications were performed using the ITS1 and ITS2 primer set. These primers amplify fungal nuclear rDNA (ITS1 region).[21] PCR was carried out in a 50 μL reaction volume using 1 μL of genomic DNA (final concentration roughly 20 ng μL^{-1}), 5 U of Taq polymerase (Promega, France), 10 μL of 5× PCR buffer, 4 μL of 25 mM $MgCl_2$, 10 μM of each primer (1 μL), and 2.5 mM dNTPs (4 μL). PCR amplification conditions included an initial denaturation step at 95 °C (3 min), followed by 25 cycles at 95 °C (1 min), 60 °C (1 min), and 72 °C (1 min) and a final 10 min elongation step at 72 °C. PCR products were generated using both PTC-100 and PTC-200 DNA thermocyclers (MJ Research Inc., Waltham, MA). PCR amplicons were verified by loading 5 μL of PCR product onto 1% analytical grade agarose gels (Promega) in 1% Tris-borate-EDTA (TBE, Promega) and followed by subsequent staining with ethidium bromide (0.625 μg mL^{-1}) for 20 min. DNA bands were visualized under UV light and analyzed using Quantity One 1-D Analysis software version 4.4 (Bio-Rad). The Bench Top 100bp (Promega) was used as the DNA molecular weight ladder.

Table 1. Optimal DHPLC Conditions for the Analysis of Fungal Communities in the Tomato Rhizosphere at 54.5 °C

gradient name	time (min)	% buffer A[a]	% buffer B[b]
loading	0	55	45
start gradient	0.1	48	52
stop gradient	18.1	39	61
start clean	18.2	55	45
stop clean	18.7	55	45
start equilibrate	18.8	55	45
stop equilibrate	20.3	55	45

[a] Buffer A, 0.1 M triethylammonium acetate (TEAA). [b] Buffer B, 0.1 M TEAA with 25% acetonitrile at an eluent flow rate of 0.5 mL min^{-1}.

Analysis of Fungal and Oomycete Communities Using Denaturing High-Pressure Liquid Chromatography (DHPLC). In this study, a DHPLC assay was developed to separate the PCR-amplified ITS1 rDNA gene fragments.[22] For each PCR amplicon, 5 μL was injected into the autosampler of the system, without prior purification. Separation of PCR products was analyzed via the elution of partially melted DNA molecules, which is achieved through interaction with an ion-pairing reagent, triethylammonium acetate (TEAA), and the DNAsep HT cartridge matrix of the WAVE microbial analysis system (Transgenomic, Ohama, NE). The buffers used for the DHPLC were buffer A, 0.1 M triethylammonium acetate, and buffer B, 0.1 M TEAA with 25% acetonitrile. Separation conditions were optimized by adjusting optimal temperature, elution gradients, and column flow rate. Our overall best running conditions for analysis of PCR products are shown in Table 1. The total running time for each sample was 20.3 min, including the cleaning and equilibration steps. Separated PCR products were analyzed using a UV detector (L-7485) and a fluorescence detector (L-2480). Analysis of chromatographic data was facilitated using Navigator software version 1.6.2 (Transgenomic). Peaks with the same retention time in DHPLC analysis represent the same operational taxonomic unit (OTU). The area of each OTU peak was used as quantitative data for the multivariate analysis (principal components analysis, PCA).

Peak areas obtained by DHPLC were used in a covariance matrix (substrate type in rows and OTU in columns) and analyzed using a PCA, which provided an ordination of fungal communities on a factorial map based on the scores of the first two principal components. To study the genetic structure of the rhizosphere fungal communities, one of the main advantages of DHPLC fingerprinting is that it detects rapid changes in microbial communities in the absence of prior knowledge on their composition. This method also avoids the biases introduced by cultural methods. PCA was performed using R software.

Biochemical Analysis. *Total Phenol Content.* Phenolic compounds were extracted from 200 mg of the freeze-dried subsample ground to a powder and homogenized with 5 mL of water/ethanol (1:1)

under magnetic stirring at 4 °C for 20 min. After centrifugation for 15 min (4 °C, 4000*g*), the resulting pellet was extracted twice following the same protocol. The supernatants were collected, pooled, and filtered on glass wool. The obtained extract was concentrated by rotary evaporation at 30 °C. The residue was dissolved in deionized water.

Total phenol content was determined using the Folin−Ciocalteu reagent following the colorimetric method given by Sanoner et al.[23] Measurements were carried out in triplicate, and calculations were based on a calibration curve obtained with gallic acid. Total phenol content was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

Carotenoids. Carotenoids were extracted following the method of Georgé et al.,[24] using the acetone/petroleum ether mixture. Lycopene and β-carotene were quantified spectrophotometrically using the equations[25]

$$C_{\beta\text{-carotene}} = 4.624A_{450} - 3.091A_{503} \quad (1)$$

$$C_{\text{lycopene}} = 3.956A_{450} - 0.806A_{503} \quad (2)$$

where C is the concentration of carotenoids expressed in μg mL^{-1} and A_{450} and A_{503} are the absorbances at 405 and 503 nm, respectively

Table 2. Root Colonization by *Pythium* spp. and Antioxidant Compounds and Activity in Tomatoes Cultivated on Eelgrass Slabs or Coconut Fiber Slabs

	coconut fiber slabs	eelgrass slabs
root colonization[a] by *Pythium* spp. (%)	82 (±5.6) a	61.5 (±9.6) b
antioxidant properties[b]		
total phenol content (mg g^{-1} DW)	3.16 (±0.09) a	3.37 (±0.11) b
β-carotene (mg 100 g^{-1} FW)	0.62 (±0.02) a	0.58 (±0.07) a
lycopene (mg 100 g^{-1} FW)	1.21 (±0.19) a	1.24 (±0.21) a
radical scavenging activity (IC_{50}, mg mL^{-1})	1.95 (±0.07) a	1.69 (±0.11) b

[a] Root colonization: values (± standard deviation) reported are the means of 20 measurements. [b] Antioxidant compounds and activity: each value (± standard deviation) is the mean of 9 replicates. For each tested variable (one per row), means with different letters indicate significant differences ($P < 0.05$).

Antioxidant Activity. The scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined using the Marwah et al. method.[26] Briefly, the reaction medium contained 2 mL of 100 μM DPPH violet solution in ethanol and 2 mL of plant extract (or water for the control). The reaction mixture was incubated in the dark at room temperature for 15 min, and absorbance was recorded at 517 nm. The decrease in absorbance upon addition of test samples was used to calculate the antiradical activity, as expressed by the inhibition percentage (% IP) of DPPH radicals, using the equation

$$\%\ \text{IP} = [(A_c - A_s)/A_c] \times 100$$

where A_c and A_s are the absorbances of the control and of the test sample after 15 min, respectively.

From a plot of concentration against % IP, a linear regression analysis was performed to determine the IC_{50} (extract concentration resulting in 50% inhibition) value for each sample.

Statistical Analysis. To compare fungal fingerprints in coconut fiber and eelgrass slabs, data obtained from the DHPLC profiles were compared using PCA implemented in R software. This method provided an ordination of fungal communities, which were plotted in two dimensions based on the scores in the first two principal components.

For the analysis of the antioxidant compounds and the antioxidant activity of tomato fruits, results were expressed as the mean ± SD of nine independent determinations. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered to be significant at $P < 0.05$. All statistical analyses were performed with Statgraphics Plus 2.1 (Statistical Graphics Corp., Inc., Rockville, MD).

RESULTS

Root Colonization by *Pythium* spp. Assessed by Plate Counting. Results for tomato root contamination by *Pythium* spp. according to type of slabs used for soilless culture are given in Table 2. Tomato plants grown on eelgrass slabs showed a relatively low contamination rate (about 61.5%) compared to those cultivated on coconut fiber slabs (82%).

Effect of Substrate on the Genetic Composition of the Rhizosphere Fungal Community. DHPLC uses the length polymorphism of the ITS1 rDNA gene of the nuclear ribosomal region. Chromatograms obtained were straightforward, with

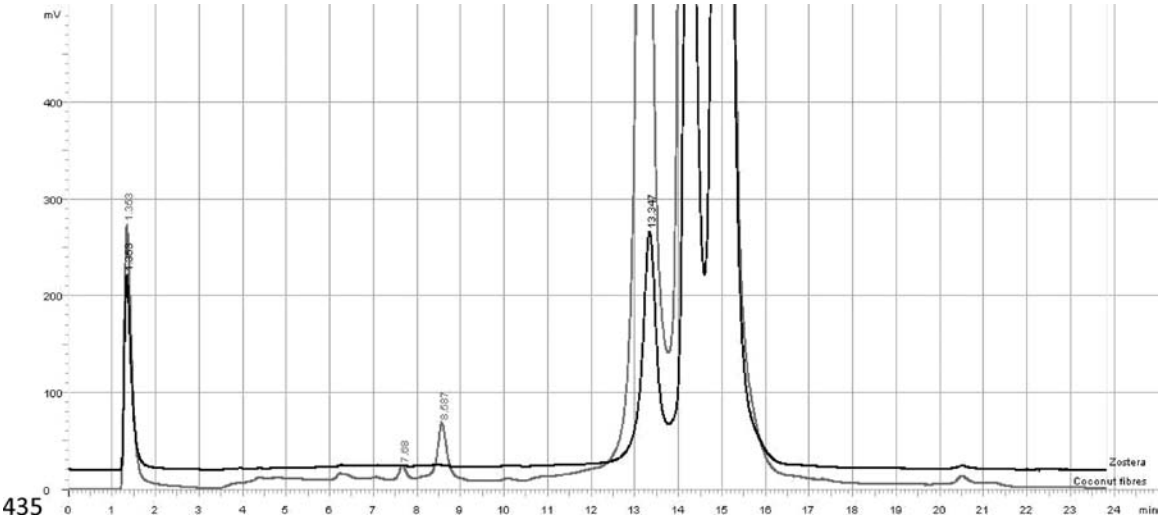

Figure 1. Fingerprints of fungal and oomycete communities obtained using DHPLC with a fluorescence detector: black line, example of a profile obtained from the rhizosphere of a tomato plant grown on an eelgrass slab; gray line, example of profile from the rhizophere of a tomato plant grown on a coconut fiber slab.

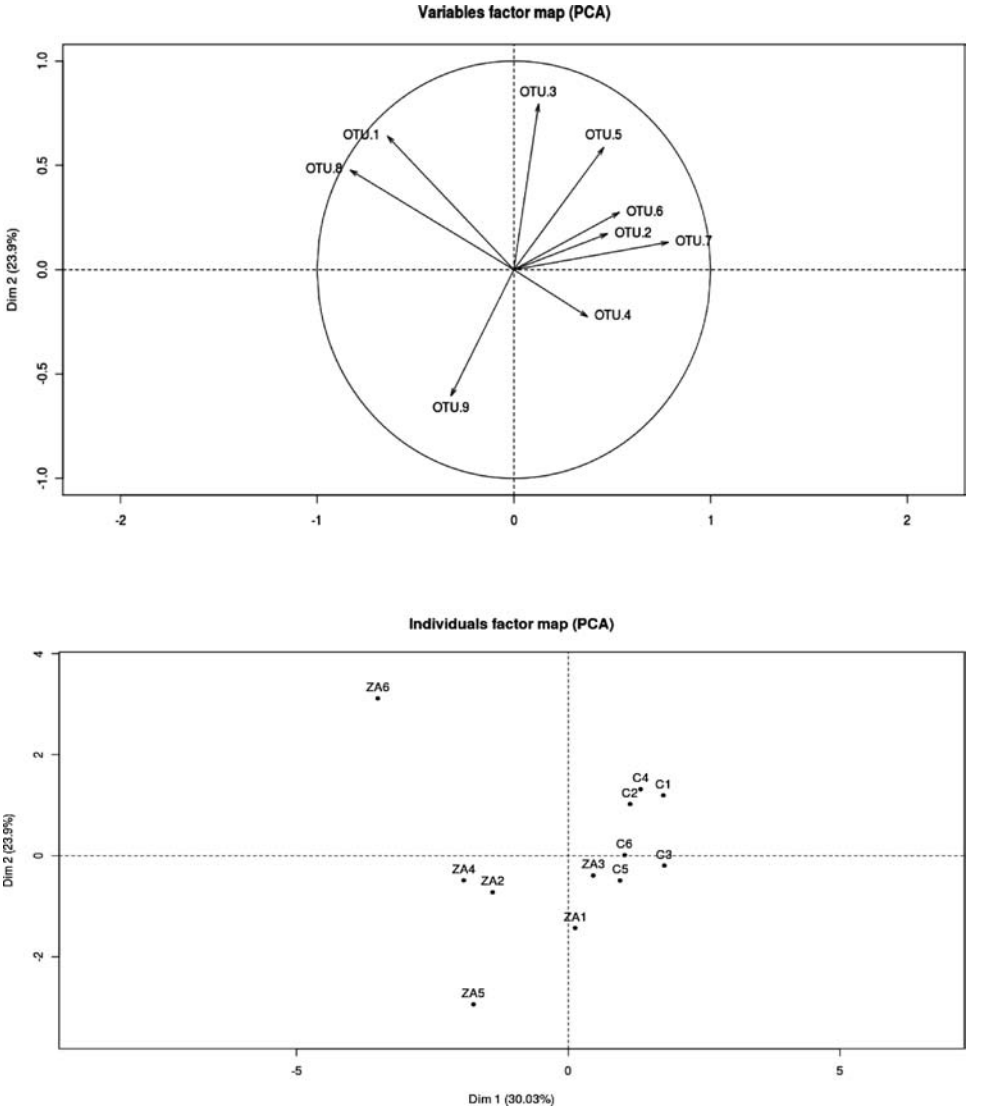

Figure 2. Principal component (PC1 vs PC2) plots generated from the DHPLC profiles obtained from fungal and oomycete DNA extracted from the rhizosphere of tomato plantes grown on eelgrass slabs (ZS) or coconut fiber slabs (CF). Peaks with the same retention time during DHPLC analysis represent the same operational taxonomic unit (OTU). The peak area of each OTU was used as quantitative data for the multivariate analysis (PCA).

peaks ranging from 4 to 14 min of retention time, demonstrating that the fungal communities were not highly diverse whatever the substrate (Figure 1). For eelgrass and coconut fiber substrates, fungal fingerprints were determined for each of the six slabs. PCA on the obtained fingerprints significantly discriminated between substrate type along the first PCA axis, which explained 30.03% of the variability (Figure 2). These results demonstrate a persistent effect of substrate composition on fungal diversity. Axis PC2 (23.9% of the total variability) discriminated among the fungal communities associated with each specific eelgrass slab (i.e., among the repetitions).

Effect of Substrate on the Antioxidant Compounds of Tomato Fruits. Results on antioxidant compound contents and radical scavenging activity in tomato fruits, according to the substrate used in soilless culture, are presented in Table 2. Carotenoid contents, either lycopene or β-carotene, did not differ by type of culture substrate: there were no significant differences between plants grown on eelgrass slabs and plants cultured on coconut fiber slabs. However, tomato fruits from plants grown on eelgrass slabs showed the highest total phenol content, with 6% more than tomatoes grown in coconut fiber slabs (Table 2). These tomato fruits also had low IC_{50} values compared to those produced on coconut fiber slabs (Table 2), indicating that plants grown in eelgrass slabs produced fruits with a better radical scavenging activity than controls grown on coconut fiber slabs.

DISCUSSION

Impact of Substrate on Fungal Communities. Growing tomatoes on eelgrass slabs showed a decrease in root colonization by *Pythium* spp. This result can potentially be explained by the release of phenolic compounds from eelgrass leaves in the tomato root environment and particularly those of zosteric acid (ZA), a secondary compound synthesized by *Zostera* sp. ZA (*p*-(sulfoxy)cinnamic acid) is a phenolic acid composed of a sulfate phenolic group at one end and a carboxylic acid group at the other. Achambale et al.[15] recently reported the presence of

this molecule in dead leaves of *Z. noltii* and *Z. marina*, with contents ranging from 65 to 456 μg g^{-1} DW and from 51 to 692 μg g^{-1} DW, respectively. ZA is a substance known for its antiadhesive properties. For example, this compound can inhibit the attachment of marine bacteria, barnacle larvae, and zoospores from the green alga *Enteromorpha* at nontoxic concentrations on artificial surfaces.[27,28] Stanley et al.[29] also showed that ZA can inhibit the adhesion of fungal spores (*Magnaporthe grisea* and *Colletrotrichum lindemuthianum*) on polystyrene and leaf surfaces. ZA can be used as an alternative natural antifoulant product, because it is much less toxic than chemical antifoulants such as tributylin (TBT).[17] Barrios et al.[30] suggested that ZA should be incorporated into silicone coatings with a slow-release system to prevent bacterial attachment. At 500 ppm ZA in solution, the bacterial cover is reduced by 93 and 96% for Lake Erie bacteria and *Pseudomonas putida*, respectively. Thus, the difference in the *Pythium* root contamination rate in our study, in favor of tomatoes cultured on eelgrass slabs, may be attributable to the release of zosteric acid near the roots resulting from the degradation of *Zostera* leaves. ZA may prevent adhesion of *Pythium* spp. zoospores on tomato roots by interfering with their ability to attach to the surface.[17]

The molecular comparison of fungal and oomycete communities using a PCR-DHPLC approach showed that substrate composition had a persistent impact on fungal diversity. Vallance et al.[5] demonstrated an increase in the complexity of the fungal fingerprints at the end of the cultural season compared to the beginning, whatever the experimental condition. This observation suggests that the complexity and size of microflora increases over time. According to the literature, biological processes in the rhizosphere are strongly affected by plant root exudates, which consist of easily degradable organic carbon compounds that attract specific microbial populations and stimulate their growth. Therefore, in this study, the differences in fungal populations between substrates may result from differences in nutrient availability. The differences in rhizosphere microflora may also be the result of pathogenic attacks by *Pythium* spp. and secondary colonization due to subsequent nutrient leakage on coconut fiber slabs.[31]

Impact of Substrate on Antioxidant Properties. Our results on antioxidant compounds and radical scavenging activity in tomato fruits were similar to those found by other authors. Thus, the carotenoid contents quantified in this study (about 0.6 mg 100 g^{-1} FW for β-carotene and 1.2 mg 100 g^{-1} FW for lycopene) were similar to those given in the literature, ranging from 0.3 to 1.07 mg 100 g^{-1} FW and from 0.4 to 10.4 mg 100 g^{-1} FW, respectively.[32−34] Similarly, phenolic compound contents are often reported at values of between 1.8 and 6.0 mg g^{-1} DW in tomato fruits, and antioxidant activity ranges from 1.3 to 1.8 mg mL^{-1}.[33−36]

The difference in total phenol content between the two studied substrates could be attributed to the physicochemical properties of the slab. Although both substrates are organic in nature, slabs composed of eelgrass leaves are not inert like those composed of coconut fibers. Continuous watering with nutrient solution on eelgrass slabs may therefore result in the release of molecules in the root environment. Toor et al.[36] demonstrated that the type of fertilizer plays a major role in determining antioxidant compound levels in tomato. The release of nutrients from chicken manure and grass-clover mulch can be used for the synthesis of C-based secondary compounds, such as phenolic compounds. Thus, eelgrass slabs may behave like these organic fertilizers:[36] its slow degradation releases nutrients used for total phenol synthesis.

The difference in radical scavenging activity according to the type of soilless culture substrate may be related to total phenol content in tomato fruits. In particular, fruits harvested on eelgrass slabs had the lowest IC_{50} (meaning the highest antioxidant activity) and the highest total phenol content, compared to those produced on coconut fiber slabs. Phenolic compounds are secondary metabolites known for their antioxidant activity,[37] particularly their ability to scavenge free radicals, to donate hydrogen atoms or electrons, or to chelate metal cations.[38] Furthermore, they contribute to a great extent to the color and sensory characteristics of fruits and vegetables.[38] At the cellular level, phenolic compounds participate in protecting the cell against the harmful action of reactive oxygen species (ROS), mainly free oxygen radicals, produced in response to environmental stresses, such as drought, high light intensity, or mineral nutrient deficiency.[39] Plants containing high concentrations of antioxidants show considerable resistance to oxidative damage caused by ROS.[39] It is well-known that the intake of fruits and vegetables is positively correlated to the prevention of diseases such as atherosclerosis, cancer, diabetes, and arthritis and can also retard aging.[40] Thus, the possibility of producing tomatoes with strong antioxidant activity may be advantageous for human health.

In summary, growing tomatoes on eelgrass slabs resulted in fruits with better antioxidant activity compared to fruits produced on coconut fiber slabs. This stronger antioxidant activity was accompanied by a higher total phenol content. However, this type of substrate did not influence carotenoid content, because β-carotene and lycopene levels did not vary with substrate used in soilless culture.

This study also demonstrated that culture on eelgrass slabs reduced root contamination by *Pythium* spp. by 25%. This may be related to the release of zosteric acid, a compound known for its antiadhesive properties, during the decomposition of eelgrass. This molecule may thus prevent the adhesion of *Pythium* spp. zoospores to roots.

In conclusion, this study showed that eelgrass can be used as a substrate for growing fruits and vegetables in hydroponic conditions: not only does it reduce root contamination, but it also leads to the production of fruits with better nutritional value.

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ABBREVIATIONS USED

ZA, zosteric acid; DHPLC, denaturing high-pressure liquid chromatography; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GAE, gallic acid equivalents.

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