A multidisciplinary characterization of *Lysinibacillus fusiformis* strain S4C11: *in planta* and *in silico* analyses reveal a plant-beneficial microbe

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ABSTRACT:

Despite sharing many of the traits that have allowed the genus *Bacillus* to gain recognition for its agricultural relevance, the genus *Lysinibacillus* is not as well known and studied. The present study employs *in vitro*, *in vivo*, *in planta*, and *in silico* approaches to characterize *Lysinibacillus fusiformis* strain S4C11, isolated from the roots of an apple tree in northern Italy.
The *in vitro* and *in vivo* assays demonstrated that strain S4C11 possesses an antifungal activity against different fungal pathogens, and is capable of interfering with the germination of *Botrytis cinerea* conidia, as well as inhibit its growth through the production of volatile organic molecules. *In planta* assays showed that the strain possesses the ability to promote plant growth, that is not host-specific, both in controlled conditions and in a commercial nursery. Biocontrol assays carried out against phytopathogenic viruses gave contrasting results, suggesting that the strain does not activate the host's defense pathways.
The *in silico* analyses were carried out by sequencing the genome of the strain through an innovative approach that combines Illumina and High-Definition Mapping methods, allowing the reconstruction of a main chromosome and two plasmids from strain S4C11. The analysis of the genes encoded by the genome contributed to the characterization of the strain, detecting genes related to the biocontrol effect detected in the experimental trials.

KEYWORDS (Maximum 6): *Lysinibacillus fusiformis*; Plant-growth promotion; Biocontrol; Volatile Organic Compounds; Genome sequencing; HD-Mapping;

ABBREVIATIONS:

AN: *Aspergillus* sez. *nigri*
BC: *Botrytis cinerea*
CMV: *Cucumber mosaic virus*
CymRSV: *Cymbidium ringspot virus*
DVB/CAR/PDMS: Divinylbenzene/Carboxen/Polydimethylsiloxane
FC: *Fusarium culmorum*
FV: *Fusarium verticillioides*
GIP: Growth inhibition percentage

GIPv: Growth inhibition percentage by volatile compounds
GR: Conidial germination rate
HD-Mapping: High-Definition Mapping
I%I: percentage infection index
LB: Lysogeny broth
PDA: Potato dextrose agar
PV: *Phomopsis viticola*
PVY: *Potato virus Y*
RS: *Rhizoctonia solani*
SPME-GC-MS: Solid Phase Micro Extraction- Gas Chromatography- Mass Spectrometry
TGY: Tryptone glucose yeast broth
TGYA: Tryptone glucose yeast agar
VOCs: Volatile organic compounds

INTRODUCTION

The genus *Lysinibacillus* has been described as a reclassification of some bacteria that were previously included in the *Bacillus* genus RNA group 2. The advance in bacteria taxonomy studies brought to this change in classification due to the differences at genomic level and in the composition of the peptidoglycan in their cell walls, which included lysine (Ahmed *et al.*, 2007). Among the bacteria that were re-classified in this study, was the species *Lysinibacillus fusiformis*, a soil bacterium known to be rod-shaped, motile, aerobic, Gram-positive, and spore forming.

Many strains belonging to *L. fusiformis* have received interest in recent years for their biotechnological potential, in particular for their ability to produce relevant molecules and enzymes (especially esterases and peptidases) with potential industrial application (Zhao *et al.*, 2015; Divakar *et al.*, 2017; Mechri *et al.*, 2017; Jabeur *et al.*, 2020). Also, this species is well-known for its potential in the antagonism towards plant pathogens in laboratory trials, in particular fungi (Ahmad *et al.*, 2014; Liu *et al.*, 2016; Pudova *et al.*, 2018), and for its high tolerance and detoxifying ability towards several pollutants, including heavy metals and petroleum, making it suited for bioremediation techniques (He *et al.*, 2011; Mohamed and Farag, 2015; Huang *et al.*, 2016; Gholami-Shiri *et al.*, 2017; Mathivanan *et al.*, 2018; Jinal *et al.*, 2019).

Despite not having been mainly investigated as a plant-associated bacterium, several strains of *L. fusiformis* have been isolated from plant material and were found to have good plant-growth promotion and biocontrol activities (Vendan *et al.*, 2010; Trivedi *et al.*, 2011; Rahmoune *et al.*, 2016; De Mandal *et al.*, 2018; Damodaran *et al.*, 2019).

The plant-growth promotion features of *L. fusiformis* strains have some of their mechanisms elucidated by these works, reporting typical plant-growth promoting traits such as production of auxins and siderophores, and solubilization of phosphate. The same cannot be said for the mechanisms underlying the biocontrol and antagonistic abilities of this species. The study from Pudova and colleagues (2018) identified *in silico* the genes for the production of several bacteriocins but no single antibiotic molecule produced by *L. fusiformis* has been identified *in vivo*. Also, Trivedi and colleagues (2011) report that *L. fusiformis* is detected exclusively in the roots of healthy citrus trees, and not in those infected by '*Candidatus* Liberibacter asiaticus'.

This study suggests that *L. fusiformis*, together with other bacteria that are likewise associated to healthy plants, can exert direct antagonism effect and induce the plant defense responses. Despite these evidences, the molecular basis of the biocontrol activity of *L. fusiformis* remains unknown.

This study aims to expand the knowledge on the interaction between plant and *L. fusiformis* by employing strain S4C11, isolated from the roots of healthy apple trees in a study investigating the differences in bacterial communities in healthy or '*Candidatus* Phytoplasma mali'-infected apple trees (Bulgari *et al.*, 2012). The biocontrol and plant growth-promoting ability of strain S4C11 were investigated using *in vitro*, *in vivo*, and *in planta* experiments. In parallel to functional experiments, we also analyzed the genome of the strain, since none of the *L. fusiformis* genomes presently available in public repositories belong, to the best of our knowledge, to strains characterized to be plant-beneficial.

The analysis of genomes from plant-beneficial microorganisms is considered very important to shed light into the genetic basis of this phenotype. However, despite the introduction of second-generation sequencing technologies consistently increased the availability of bacterial genomes, these did not always allow to unravel the genetic basis behind complex phenotypes, such as biocontrol (Shiebani-Tezerji *et al.*, 2015). The de-novo assembly of such data (short reads, 100–300 base pairs) results indeed in fragmented assemblies, because repetitive sequences in bacterial genomes are frequently longer than the read length. Still, determining the complete genome sequence is essential, for example to properly study functional islands, often containing information such as resistance or plant-microbe interaction genes, which are frequently flanked by repetitive elements, and to determine whether they are localized in chromosomes or plasmids (Liao *et al.*, 2019). To overcome these limits, we previously employed long-read based nanopore sequencing to assemble the genome of *P. pasadenensis,* that unraveled the presence of plant-associated traits (Passera *et al.*, 2018).

In the present study, in order to generate a contiguous assembly of *L. fusiformis* genome, a hybrid approach was used, by integrating the short-read sequencing data obtained with the Illumina technology and the whole genome mapping data generated using the novel approach of High-Definition (HDM) genome mapping provided by Nabsys. In Nabsys HD-Mapping, high-molecular-weight DNA is nicked in a sequence-specific manner with nicking endonucleases and each nick site is labelled with a proprietary tag protein. The tagged molecules are then translocated through a solid-state nanochannel and the relative position of tags is identified by measuring the electrical resistance change inside the channel. Determination of labelling pattern allows to reconstruct a physical map of the DNA under study, thus allowing to identify genome structure and/or structural variants (Kaiser *et al.*, 2017; Oliver *et al.*, 2017). To our knowledge, the genome of *L. fusiformis* strain S4C11 reported in the present work represents the first bacterial genome generated with the combination of Nabsys mapping technology and Illumina data.

In summary, these genomic and functional studies determined *L. fusiformis* strain S4C11 to be a plant-beneficial bacterium with ability i) to antagonize the growth of phytopathogenic fungi in *in vitro* and *in vivo* conditions, ii) to greatly promote plant growth in several plant species both in experimental greenhouse (controlled conditions) and in a commercial nursery (working conditions), iii) to stimulate plant defenses, causing the up-regulation of a regulator gene of Induced Systemic Resistance. Furthermore, the genome of this strain encodes for several functions that can explain the observed phenotype and suggest further experiments to study if

this strain can offer protection from abiotic stresses, as well as promote growth and mitigate damage caused by biotic stresses.

MATERIALS AND METHODS

Bacterial and fungal strains

Lysinibacillus fusiformis strain S4C11 was isolated from the roots of an apple plant in a previous study: in particular, the apple plants were sampled in two consecutive years, March 2011 and March 2012, as part of a survey on the phytoplasma-associated disease apple proliferation carried out in the orchard of the Minoprio Foundation, in the Vertemate con Minoprio town (CO). Strain S4C11 was among those that were recovered exclusively from healthy plants (Bulgari *et al.*, 2012). This strain was cultivated on LB High Salt Agar plates (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, agar 15 g/L) at 25 °C and was stored in a 20% glycerol solution at -80 °C for long conservation periods.
The fungal phytopathogens used in this study all come from the mycological collection of the DiSAA's phytopathology lab. The strains used in this study are: *Botrytis cinerea* Pers. Strain MG53 (which will be identified as BC in the rest of the study), isolated from wheat kernels in 2014; *Aspergillus* sez. *nigri* strain AsN1 (which will be identified as AN for the rest of the study) isolated from rotting grape berries in 2015; *Fusarium verticillioides* (Sacc.) Nirenberg strain GV2245 (which will be identified as FV for the rest of the study) isolated from a corn ear showing pink rot symptoms in 2011; *Fusarium culmorum* Sacc. strain GV2144 (which will be identified as FC for the rest of the study) isolated from maize in 2011; *Phomopsis viticola* Sacc. strain PV1 (which will be identified as PV for the rest of the study) isolated from grape berries in 2012; and *Rhizoctonia solani* (Cooke) Wint strain RS1 (which will be identified as RS for the rest of the study) isolated from millet in 2012. These fungal isolates were cultivated on potato dextrose agar (PDA, Difco™) at 20 °C and stored at 4°C.

Biochemical assays for plant-growth promotion traits

The following biochemical *in vitro* assays were carried out to determine the presence of some common plant-growth promotion or plant-associated traits: (i) siderophore production, using CAS agar medium as described by Alexander and Zuberer (1991); (ii) catalase activity, tested by dripping 3% hydrogen peroxide directly on the bacterial colonies; (iii) production of indole acetic acid, using the colorimetric method described by Pilet and Chollet (1970); (iv) chitinase production, using the medium reported by Sridevi *et al.* (2008).

In vitro antifungal assays

The ability of strain S4C11 to inhibit the growth of phytopathogenic fungi was assayed *in vitro* using different techniques.

A dual-culture assay was carried out as described in Passera *et al.*, 2017, using strain S4C11 and the fungal strains AN, BC, FC, FV, PV, and RS. Briefly: droplets from an overnight liquid culture of strain S4C11 (approximately 2 x10^6 CFUs) were placed on four sterilized cellulose disks around the inside edge of a Petri dish containing a Tryptone Glucose Yeast Extract Agar medium (TGYA - 5 g/L tryptone, 1 g/L glucose, 3 g/L yeast extract, 15 g/L agar). After two days of incubation at 24 °C, a plug (0.5 cm in diameter) was taken from actively growing mycelium of the target fungus and was placed in the middle of the plate. As negative controls, plates containing (i) the fungus alone, (ii) the fungus and blank sterilized filter paper discs, and (iii) the fungus and discs inoculated with 20 µL of sterilized LB broth were used.

Fungal growth, as mycelial growth diameter, was measured 5, 7, and 14 days post inoculation (dpi). Each test was carried out with plates in triplicate and three independent measures were made for each plate at each measuring time. The Growth inhibition percentage (GIP) was calculated as [1-(D1/D2)]x100, where D1 is the radial colony growth on the bacteria-treated plate, D2 is the radial colony growth on the control plate (Passera *et al.*, 2017).

Having given a good result in the antagonism assays and being a most widespread pathogen growing on hundreds of different plant species, all further *in vitro* and *in vivo* assays focused on BC as the fungal pathogen to employ.

A dual-plate assay was carried out as described by Chaurasia *et al*., 2004, using strain S4C11 and the fungal strain BC. Briefly, 100 µl of an overnight culture of strain S4C11 in LB broth (approximately 10^6 CFU), were diffused on the surface of a TGYA plate and incubated at 25 °C. After two days, a fungal mycelial plug (0.5 cm in diameter) was taken from a plate containing actively growing mycelium and was inoculated onto another TGYA plate. Under sterile conditions, the lid of the plate bearing the bacteria was replaced by the upturned plate containing the fungal inoculum, and the plates were sealed together with Parafilm. After fungal inoculation, all the plates were kept at 25 °C in the dark, and the fungal growth was measured 14 dpi. Each test was made with plates in triplicate, three independent measures being made for each plate. The growth inhibition percentage, determined by volatile compounds (GIPv), was calculated as previously described.

Lastly, an assay was carried out as described in Passera *et al*., 2017 to determine the ability of strain S4C11 to inhibit the germination of conidia produced by BC, using the spectrophotometry method reported by Raposo and colleagues in 1995. In this assay, the samples were composed of 50% conidia suspension in TGY broth (10^4 conidia/ml) and 50% either (i) TGY, (ii) cell suspension of S4C11, at a final concentration of approximately 10^6 CFU/ml (iii) S4C11 overnight culture broth, sterilized by centrifugation and filtering (S4C11 CF), (iv) S4C11 overnight culture broth, sterilized by centrifugation and filtering, and autoclaving at 121 °C for 15 minutes (S4C11 121), or (v) S4C11 overnight culture broth, sterilized by centrifugation and filtering, and treated with proteinase K (200 µg/ml) for 30 minutes at 37 °C (S4C11 PK). These last treatments were tested to determine if the components of the culture broth relevant to the inhibition of conidia germination could be degraded by a proteinase or by a heat treatment. Each sample was prepared in a 96-wells optical plate for immunological assays (8 replicates per treatment) and the plate was incubated at 24 °C for 48 hours. Germination of conidia was determined by absorbance at a wavelength of 492 nm at 12, 18, 24, 36, and 48 hours post inoculation.

At 48 hours from the start of the experiment, germination was also evaluated by direct observation under an optical microscope (20X; Easylab CX40, Olimpus) using a Kova counting grid, considering each spore to have germinated if the length of germination tube was twice as

long as the conidium diameter (Chen *et al*. 2008). For each observation, 100 spores were visually analyzed and determined to be either germinated or non-germinated, and three observations were carried out for each treatment. Conidial germination rate (GR) was calculated as (G/C)x100, where G is the number of germinated conidia detected, and C is the total number of conidia counted.

Characterization of Volatile Organic Compounds

The VOCs produced by strain S4C11 were analyzed by means of Solid Phase Micro Extraction-Gas Chromatography- Mass Spectrometry (SPME-GC-MS) using the method described by Passera *et al.*, 2019. Briefly, a layer of LBA (as described before, but with 3% agar) was prepared inside a 20 mL headspace glass vial put in horizontal position. Strain S4C11 was then inoculated on the LBA layer so that, during extraction step, the fiber would be in contact only with the volatile compounds present in the headspace over the LBA layer, avoiding any contact with the solid media or the microorganisms. The vials were prepared in triplicate with the aim of following up the volatile production at different time points (1, 2, 3, 4, and 5 days of incubation). For the middle time point (3 days of incubation), an additional set of three replicates were set up. In order to distinguish the volatiles produced by the bacterial strain from the volatiles produced by the growth medium, both vials with only TGYA medium and vials in which strain S4C11 was growing were prepared, and left in incubation at 24 °C in the dark until analysis (36 vials in total). The volatiles were adsorbed at 50 °C on a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber and desorbed at 260 °C in the injection port of an Agilent Technologies 6890N/5973N gas chromatograph-mass spectrometer equipped with a 60 m × 0.25 mm × 0.25 µm 100% polyethylene glycol column (Zebron ZB-WAX plus, Phenomenex). The analytical conditions were the same described before (Passera *et al.*, 2019).

In vivo antifungal assay

The ability of strain S4C11 to reduce infection from BC was evaluated in an experiment carried out on (i) detached tomatoes in postharvest conditions (Passera *et al*., 2019) and (ii) detached grapevine leaves. For this assay, ripe and healthy cherry tomatoes, grown organically in Italy and purchased in a local grocery, and healthy grapevine leaves obtained from the vines present in the University's experimental greenhouse were used. Detached tomatoes of uniform size, free of visible blemishes, or grapevine leaves were surface sterilized in a 70% ethanol solution in water for 5 minutes, rinsed in water three times, and dried on filter paper under a laminar flow hood. After drying, the equatorial area of each tomato was pierced 4 times by a needle. Bacterial strain S4C11 was inoculated singly by a 5 minutes soaking of each tomato or grapevine leaf in a bacterial suspension (approximately 10^6 CFUs/ml in Ringers solution, Sigma Aldrich), and then left to dry on filter paper under a laminar flow hood. The BC conidia were inoculated by applying a 20 µl drop of conidial suspension (5 x10^5 conidia/ml) into each puncture wound for tomatoes, and by applying three drops of conidial suspension on the surface of each grapevine leaf. For each treatment (non-treated, BC alone, bacterial strain S4C11 and BC) 10 tomatoes were put on a sterile ceramic tray in a glass chamber, containing a wet piece of filter paper to maintain a relative humidity of 95% inside the chamber, or three grapevine leaves were put in a Petri dish containing 1% Agar-Water substrate to maintain 95%

relative humidity, and incubated at 20 °C in the dark. All the aforementioned procedures were carried out under sterile conditions. Each treatment was carried out in triplicate. The tomatoes were evaluated visually to determine fungal colonization at 7 days after inoculation, while the grapevine leaves were evaluated at 3, 5, and 7 days after inoculation.

The results were expressed as visual classes ranging from 0 for healthy tissues to 7 for completely infected tissues, according to the scale presented in a previous work (Vercesi *et al.*, 2014). Visual classes were transformed into a percentage infection index (I%I) according to the formula proposed by Townsend and Heuberger (1943).

In planta growth-promotion assays

The ability of strain S4C11 to promote growth of *Capsicum annuum* L. var Zebo F1 (referred to as Zebo in the rest of the study) plants was assayed under experimental greenhouse conditions. Fifteen days-old seedlings of Zebo pepper were inoculated by root dipping with a PBS solution containing 10^6 CFU/mL of strain S4C11 or mock-inoculated using only PBS. The height of these plants, 7 per treatment, was monitored weekly over a period of two months and compared between treatments. During growth, these plants received no additional treatments and were simply watered to avoid drought stress.

Furthermore, a similar assay was conducted in a commercial nursery greenhouse, to examine the performance of strain S4C11 on more host plants and under realistic operative conditions. These assays were carried out in a dedicated area, kept separated from plants destined to commercialization, in a nursery located in the Monza-Brianza province of Lombardy, Italy.

In this assay, different plant species were used: leek (*Allium ampeloprasum*) var. Matejko RZ F1 (which will be indicated as leek in the rest of the study), chicory (*Cichorium intybus*) var Uranus (which will be indicated as chicory in the rest of the study), green beans (*Phaseolus vulgaris*) var. Pike (which will be indicated as bean in the rest of the study), zucchini (*Cucurbita pepo*) var Tarmino F1 (which will be indicated as zucchini in the rest of the study), hot pepper (*Capsicum frutescens*) var. A Mazzetti (which will be indicated as pepper in the rest of the study). These plants were chosen to test the performance of strain S4C11 on a variety of plants belonging to different families, among the varieties most requested by the customers of the nursery. The inoculum of strain S4C11 was carried out by pouring 50 mL of a suspension of strain S4C11 diluted in tap water (final concentration of 2.5×10^6 CFU/ml) in each pot right after transplant, without altering any of the other working conditions of the nursery.

Among the standards employed by the nursery, it is important to mention that they regularly employ a biological plant-growth promotion product in the sowing soil, composed of arbuscular mycorrhiza belonging to the *Glomus* genus and a combination of non-mycorrhizal filamentous fungi (*Trichoderma* sp.), bacteria (*Bacillus subtilis* and *Streptomyces* spp.) and yeasts (*Pichia pastoris*). The plants were fertilized through fertirrigation, supplying nitrogen, potassium, calcium, iron, phosphorus, magnesium, sulfur, boron, copper, manganese, molybdenum, and zinc. Lastly, insecticide treatments were carried out in the nursery against *Frankliniella occidentalis*, *Trialeurodes vaporariorum*, and *Aphis* spp.

Inoculum was carried out on the 25th of May 2018, and the initial height of plants was measured on the same day. Height was measured again on the 1st of June, 8th of June, and 22nd of June. On the 13th of July, the fresh weight of the aerial part of the plants was measured. In particular,

for the beans this fresh weight includes the weight of the fruits produced, while for zucchini the fruits were removed before determining fresh weight and weighed separately.

In planta biocontrol assays

The ability of strain S4C11 to induce plant defense responses was assayed using *Nicotiana benthamiana* as a host plant and challenging it by mechanically inoculating different viruses. Two-weeks old seedlings of *N. benthamiana* were inoculated by pouring 20 mL of a bacterial suspension in Ringer's solution (final concentration 10^6 CFU/mL), or mock-inoculated with 20 mL of sterile Ringer's solution. A day after this inoculation, 21 of these plants (3 biological replicates, 7 plants each) for both treatments were collected for RNA extraction and quantification of the expression pattern of some defense-related genes (as detailed in subsection "RNA extraction and relative quantification of virus concentration and gene expression"). After one week, 10 plants per treatment were mechanically inoculated with one among *Cymbidium ringspot virus* (CymRSV) strain DSMZ PV-0272, *Cucumber mosaic virus* (CMV) strain DSMZ PV-0504, or *Potato virus Y* (PVY) strain DSMZ PV-1036. Inoculum of the virus was obtained by grinding leaves of infected *N. benthamiana* plants in a 0.05 M phosphate buffer (pH 7, containing DIECA at 5 mM and EDTA at 1 mM). Ten mock-inoculated plants were kept as healthy control plants, without virus inoculation. The development of symptoms was monitored on all plants after 5, 7, and 10 days from inoculation, after which the plants were sampled for RNA extraction and virus quantification. Symptoms were quantified by assign to each plant a class (from 0 to 5), and then converting the class to a percentage of symptom severity using the formula of Townsend and Heuberger (1953).

At the tenth day after inoculation, leaf samples were collected to carry out extraction of RNA and quantification of the virus (as detailed in subsection "RNA extraction and relative quantification of virus concentration and gene expression")

RNA extraction and relative quantification of virus concentration and gene expression

RNA was extracted from samples of *N. benthamiana* leaves following the protocol detailed by Gambino *et al*., 2015.

Relative quantification was carried out through a two-step Real-Time PCR, starting from 1 µg of RNA per sample and using a M-MLV reverse transcriptase (Thermo Fisher, USA) for the synthesis of cDNA, and using the Sybr Green chemistry in a StepOnePlus Real-Time PCR thermocycler (Thermo Fisher Scientific, USA).

The reaction mix had the following composition for all the primer pairs used in the study: 1x Power Sybr Green Master Mix (Thermo Fisher Scientific, USA), 300 nM of forward and reverse primer, 2 µL of cDNA, water to reach the volume of 10 µL.

The relative quantification was carried out using the protein phosphatase 2 (*PP2A*) as housekeeping gene for normalization, and analyzing the expression of the following targets: (i) enhanced disease susceptibility 1 (*EDS1*) gene, an upstream gene in the signaling pathway of salicylic acid-mediated plant defense responses (Wiermer *et al.*, 2005), from *N. benthamiana*; (ii) non-expressor of pathogenesis related genes 1 (NPR1) gene, a master regulator gene in

plant defense responses (Spoel *et al.*, 2003), from *N. benthamiana*; (iii) pathogenesis-related protein 2b (*PR2b*), a molecular marker of systemic acquired resistance (van Loon *et al.*, 2006), from *N. benthamiana*; (iv) RNA polymerase from CymRSV; (v) movement protein from CMV; (vi) coat protein from PVY. All the primer pairs used in the study are reported in Table 1. Each amplification reaction was carried out in triplicate, and each reaction plate included six negative controls containing only reaction mix and sterile water.

The primer pair for CymRSV, designed in this study, has been tested for its specificity against nucleic acids from healthy host plant *N. benthamiana* and several plant pathogenic viruses (CMV, Potato Virus A, Potato Virus X, PVY, Tobacco Mosaic Virus, Tobacco Rattle Virus, Tomato Aspermy Virus) and was found to give amplification only for the intended target virus.

After amplification, relative gene expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001), and expressed as $\ln 2^{-\Delta\Delta Ct}$.

Table 1. Primers used in this study

Target Gene	Primer sequence (5'-3')	Reference
PP2A – *N. benthamiana*	GAC CCT GAT GTT GAT GTT CGC T	Liu *et al.*, 2012
	GAG GGA TTT GAA GAG AGA TTT C	
EDS1 – *N. benthamiana*	GGA CAA TGG GAG AAG CAG AA	Zhang *et al.*, 2012
	GAA CGC ATC ATA ATA CCC GA	
NPR1 – *N. benthamiana*	GGC CTT GCC TCA TGA TAT TG	Zhang *et al.*, 2012
	GCT ACA GCA TAA TGG AGA GC	
PR2b – *N. benthamiana*	CTA AAG AGG GTA GCC CAA GA	Zhang *et al.*, 2012
	GTC CCA AAC TCC ACC AGA GA	
RNA polymerase – CymRSV	GTA CAT GCG TCA CTT GGG GA	This study
	TCT GAC CAT CTT CCA ACC GC	
Movement protein – CMV	CTG ATC TGG GCG ACA AGG GA	Feng *et al.*, 2006
	CGA TAA CGA CAG CAA AAC AC	
Coat protein – PVY	AGC GGT ACA ACT TGC ATA CGG	Yang *et al.*, 2014
	GAT GTT TGG CGA GGT TCC A	

Statistical Analyses

The data obtained from the dual-culture antagonism assays, *in vivo* biocontrol assays, plant growth promotion assays (weight), *in planta* biocontrol assays, virus quantification and gene expression were analyzed through an independent samples Student's t-Test to identify significant differences between the non-treated and S4C11-treated samples ($p < 0.05$).

The data obtained from the conidia germination assay and the plant growth promotion assays (plant height) throughout the whole duration of those experiments were compared by performing a general linearized model test, optimized for repeated measures, followed by Tukey's exact post-hoc test ($p < 0.05$)

Illumina sequencing

Strain S4C11 was cultivated in 200 mL of LB broth at 24 °C overnight and the genomic DNA was extracted using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldich), following the manufacturer's instruction. Genomic DNA was quantified with the Qubit dsDNA HS Assay kit (Life Technologies), purity and integrity were assessed with Nanodrop 1000 spectrophotometer (Thermo Scientific) and by capillary electrophoresis on a 2200 TapeStation (Agilent Technologies), respectively. Illumina libraries were produced starting from 1 µg of genomic DNA, which was sheared using the Covaris S220 instrument (Covaris Inc. Woburn, MA). Size selection of fragments 500bp in length was conducted on agarose gel at 1.8% and libraries were produced using TruSeq DNA Sample Prep Kit (Illumina, San Diego, CA) according to manufacturer instructions. Sequencing was performed on a HiSeq1000 instrument with 100 x 2nt Pair end protocol using the TruSeq PE Cluster v3 kit (Illumina, San Diego, CA) according to manufacturer instructions. Sequenced reads underwent the following quality filtering procedure: reads with a number of N >10% of the total read length or with > 50 bases with a quality score < 7 (Base call accuracy < 80%) were removed using a custom script, adapters were clipped using Scythe v0.980 (https://github.com/ChrisJohnRiley/Scythe), bases on both 3' ends with a quality < 20 (Base call accuracy < 99%) were trimmed using Sickle v0.940 (https://github.com/najoshi/sickle), fragments with one of the mate of length < 20 bp were removed.

Nabsys High-Definition Mapping

High molecular weight genomic DNA for HD-Mapping was extracted from Strain S4C11 grown in LB broth at 25 °C overnight (OD~3) using the Macherey-Nagel NucleoBond AXG 20 column system (Bethlehem, PA) in conjunction with Macherey-Nagel NucleoBond Buffer Set III with minor revisions. Briefly, cell pellet was lysed at 37°C for 30 min in NucleoBond G3 Buffer supplemented with lysozyme and proteinase K. NucleoBond G4 Buffer was added to the lysate and incubated at 60˚C for 1 h with gentle mixing every 30 min, and afterwards the lysate was transferred onto a AXG20 column (previously equilibrated with buffer N2) and eluted by gravity flow. Column-bound DNA was washed 3 times with buffer N3 and subsequently eluted with 1ml buffer N5. Eluted DNA was precipitated with 0.7 volumes of isopropanol and the DNA pellet was washed with 70% ethanol, resuspended in 100 µL of TE buffer mixing 10x with a wide bore tip and incubated at 50˚C for 2 hours and subsequently at room temperature overnight with gentle mixing.

For the Nt.BspQI/Nb.BbvCI map, the purified DNA sample was nicked with Nt.BspQI (8.8 U/µg) in 1X NEBuffer 3 at 50°C for 1 hour followed by the addition of Nb.BbvCI (7 U/µg). The sample was then incubated at 37°C for 1 hour followed by 20 min at 80°C. For the Nb.BssSI map, the purified DNA sample was nicked with Nb.BssSI (20 U/µg) in 1X NEB Buffer 3 and incubated at 37°C for 1 hour followed by 20 min at 80°C. Nicking enzymes and NEB Buffer 3 were purchased from New England Biolabs, Ipswich, MA. Nabsys proprietary tags were attached by incubating the nicked DNA with the tag at room temperature for 30 min. The samples were then coated with RecA protein (Enzymatics, Beverly, MA) in the presence of ATPγS (Sigma Aldrich, St. Louis, MO) at 37°C for 2h.

For the Nt.BspQI/Nb.BbvCI map, mapping data (average coverage of 308x, reads ≥ 60kb) were collected utilizing the Nabsys HD-Mapping platform and assembled using Nabsys software

Asm1 (parameters: microbial, read length ≥ 60kb). For the Nb.BssSI map, mapping data (average coverage of 388x, reads ≥ 40kb) were collected utilizing the Nabsys HD-Mapping platform and assembled using Nabsys software Asm1 (parameters: microbial, read length ≥ 40kb).

Genome Assembly and Annotation

Filtered reads were de novo assembled using SOAPdevo2 vr223 (Luo *et al.*, 2012) and Velvet v1.1.06 (Zerbino and Birney, 2008) [2] using numerous kmer length. SOAPdenovo2 assemblies were performed using modified parameters "-M 2 -e 1 - F" while Velvet assemblies were performed using default parameters. Assembly generated using SOAPdenovo2 with kmer length ranging from 87 to 95 presented the best contiguity metrics and were selected for the further analysis. Scaffolding of NGS contigs (Illumina) with Nabsys HD maps was performed using Nabsys software CompareAssemblyToReference, v1.12.0.3 (parameters: interval size match = ±(300bp + 0.03*IntervalSize), small interval threshold = 500bp). The assembled sequences were investigated for putative assembled plasmid genomes by BLAST search, against the NCBI plasmid genomes database.

Annotation was carried out with RASTtk pipeline (Brettin *et al.*, 2015) (setting build metabolic model option) on the hybrid genome assembly including the HDE mapping generated with Nt.BspQI/Nb.BbvCI. Redundant rRNA and tRNA features were filtered in the final annotation. 13 Genes with Open Reading Frames spanning gap regions were manually inspected and 12 of these were identified as chimeric genes and thus removed in the final annotation. Gene clusters related to the production of secondary metabolites was predicted using the antiSMASH 3.0 online tool (Weber *et al.* 2015) with the following parameters: strictness relaxed, mode all.

Annotation of plasmids was manually curated by comparing the predicted proteins with the Uniprot Swissprot Bacteria database and the available annotation of *L. sphaericus* (NCBI ID1582) and *L. fusiformis* genomes (NCBI ID2727) using BLASTp version 2.2.28+. Hits with more than 50% coverage were picked. Additionally, plasmid protein sequences were compared against the Pfam-A database, version 32 using the hmmscan command (the HMMER package, version 3.1b1) (Eddy, 1998). Hits with Pfam-A database (El-Gebali *et al.*, 2019) were filtered using a custom script. Functional annotation was also performed using Egg-NOGmapper version 2 (Huerta-Cepas *et al.*, 2017) selecting the bacteria taxa and non-electronic gene ontology evidence terms. Diamond mode was chosen for mapping.

The origin of replication was identified using a homology-based approach. Origin of replication of bacterial and plasmid species deposited in DoriC10.0 database (Luo and Gao, 2018) were aligned on the genome using BLAST tool v.2.6.0+ (Camacho *et al.*, 2009). Only matches showing at least 80% of query coverage were retained. The origin of replication on the main chromosome was confirmed using the web-based tool Ori-Finder (Gao and Zhang, 2008). Default *E. coli* DNaA box (TTATCCACA) was set as species-specific DnaA box. Predicted origin of replication was inspected in its proximity for the presence replication-related genes according to Rahman *et al.*, 2015.

RESULTS

Biochemical assays for plant-growth promotion traits

The assays carried out allowed to determine the presence of some typical plant-growth promotion or plant-associated traits in strain S4C11.

In particular, S4C11 showed ability to produce siderophores, detoxify hydrogen peroxide, and produced indole-acetic acid at a concentration of 68.168 $\pm$ 2.998 µg/mL, starting from an initial concentration of 1 mg/mL of tryptophan. The assay to determine the degradation of chitin gave negative results.

In vitro antifungal assays

Lysinibacillus fusiformis Strain S4C11 demonstrated to have an antifungal effect against four of the six phytopathogenic fungal strains it was tested against in dual-culture assays (Fig 1). In particular, it is capable of significantly reducing the growth of the utilized strains of *Aspergillus nigri* (AN), *Botrytis cinerea* (BC), *Phomopsis viticola* (PV), and *Rhizoctonia solani* (RS) for the whole duration of the experiment. The growth inhibition percentage (GIP) is lower for PV (GIP = 54%) but has values above 80% for the other three fungal strains (Fig 1C). Against *Fusarium culmorum* and *F. verticillioides*, there is a significant difference in the growth of the fungi in the presence of S4C11, but only at 5 dpi and with low GIP (GIP = 32% and GIP = 3% for FC and FV, respectively) (Fig 1A). This effect has a short duration, as there is no difference between the control and treated condition for subsequent timepoints.

The assay carried out in dual-plate against BC resulted in a significant reduction in growth of the fungus, although less pronounced than when growing in dual-culture conditions (GIPV = 65%).

The assay regarding the germination of BC conidia showed that strain S4C11 can inhibit the germination of conidia in all the tested conditions (Fig 2A). The best result was obtained using the cell suspension of strain S4C11 (S4C11 CS), in which no germination was detected for the whole duration of the experiment. The use of the cell-free culture broth (S4C11 CF) had still a strong inhibitory effect on germination of BC, but this condition was less effective than in the presence of bacterial cells. The treatment with proteinase K (S4C11 PK) or with heat (S4C11 121) reduced the inhibitory effect against BC conidia germination, although this difference (vs CS and CF) was not statistically significant.

Microscopy observation of the conidia at 48 hours from the start of the experiment allowed to determine the conidia germination percentage (CG%) to be 77% for NT, 19% for S4C11PK and S4C11 121, 15% for S4C11 CF, and 0% for S4C11 CS. Moreover, the microscopy observation revealed that, while in the non-treated control the conidia could be either non-germinated (Fig 2B) or germinated (Fig 2C) as normal, in the presence of S4C11 cells there was evidence of degraded conidia surrounded by bacterial cells (Fig 2D) which could occasionally be found in large clusters including several degraded conidia (Fig 2E).

Characterization of VOCs produced

The GC-MS analysis carried out identified a total of 13 relevant volatile molecules (Table 2). Two clearly distinguishable close peaks were both identified as methylthiol isovalerate (both identification probabilities > 90 %). This finding was interpreted as two different isomers of the same compound being produced, and the results of abundance presented in the table are the sum of the two peaks.

A PCA analysis on the abundance of these 13 molecules throughout the 5 considered time points detected 2 principal components that explain 85% of the total variation among samples (Fig 3A): PC2 (Y-axis) account for 23% of variation and mostly separates the different time points, indicating that strain S4C11 modulates the release of VOCs through time. PC1 (X-axis), which contributes to almost 62% of the variation, clearly separates the vials containing strain S4C11 from those with only the growth media. While at the first two days of incubation the profiles of control vials and S4C11-inoculated vials are more similar, the 3-days timepoint is a turning point in the volatile profile of S4C11: during this day a large increase in the production of signature volatiles can be noticed. The loading plot of the PCA (Fig 3B) identifies 11 molecules that drive the first principal component, that can be divided in two groups: 3-methylbutanale, 2-methylbutanale, benzaldehyde, and furfural are a group on the left side of the graph and are molecules typical of the growth media without bacterial inoculation, while the group on the right, which includes acetone, 2-butanone, 5-methyl 3-hexanone and multiple sulfur compounds, are the molecules that characterize S4C11.

Comparison in the abundance between control vials and S4C11-inoculated vials for each compound can be seen in Figure 3C. Acetophenone is the only molecule for which there is no significant difference between control and S4C11. The abundance of 2-methylbutanale, 3-methylbutanale, furfural, and benzaldehyde is significantly lower in S4C11-inoculated vials compared to control ones, while for all other compounds the abundance in the S4C11-inoculated vials is significantly higher.

The data obtained at different timepoints allowed to describe a trend in volatile emission by strain S4C11: the production of volatiles steadily increases in the first days, reaching a peak at 3 days of incubation, and then progressively decreases. The abundance of methylthiol isovalerate, the most abundantly produced molecule by strain S4C11, is presented as an example of this trend (Fig 4D), but the trend is the same for all other relevant molecules produced by the strain. These findings are in accordance with a trial previously carried out in which, after 14 d of incubation no significant amounts of the characterizing molecules were found (data not shown) in the headspace of the vials inoculated with S4C11.

504 Table 2. Results of the VOCs analysis through GC-MS.

Molecule	RT	QI		Abundance 1 dpi	Abundance 2 dpi	Abundance 3 dpi	Abundance 4 dpi	Abundance 5 dpi
Acetone [67-64-1]	5.29	43	LBA	2'235'802 ± 34'328	2'392'223 ± 49'999	3'110'734 ± 90'393	3'038'877 ± 45'493	2'907'969 ± 7'302
			S4C11	2'181'394 ± 77'143	3'866'594 ± 103'866	7'894'853 ± 1'334'721	7'519'125 ± 904'463	9'841'102 ± 842'177
2-butanone [78-93-3]	6.76	43	LBA	395'301 ± 24'349	446'166 ± 2'871	537'844 ± 24'997	483'861 ± 21'179	455'077 ± 87
			S4C11	511'157 ± 56'436	762'270 ± 23'789	1'153'123 ± 195'626	874'835 ± 8'967	1'084'310 ± 86'566
2-pentanone [107-87-9]	9.27	43	LBA	686'957 ± 6'319	712'762 + 7'190	522'690 ± 307'614	194'674 ± 5'501	173'081 ± 4'406
			S4C11	773'779 ± 87'892	798'578 ± 18'038	642'563 ± 146'103	403'805 ± 23'630	744'817 ± 32'012
2-methylbutanal [1730-97-8]	7.07	57	LBA	250'066 ± 7'455	250'799 ± 3'692	462'423 ± 216'849	644'188 ± 26'053	619'892 ± 25'815

			S4C11	1'500 ± 0	1'500 ± 0	1'500 ± 0	1'500 ± 0	1'500 ± 0
5-methyl 3-hexanone [623-56-3]	13.94	57	LBA	1'500 ± 0	1'500 ± 0	1'500 ± 0	1'500 ± 0	1'500 ± 0
			S4C11	1'500 ± 0	63'227 ± 8'307	198'406 ± 91'090	179'952 ± 28'017	205'407 ± 29'675
methylthiol isovalerate (I & II)[a]	19.90 & 20.23	57	LBA	1'500 ± 0	1'500 ± 0	1'500 ± 0	1'500 ± 0	1'500 ± 0
			S4C11	95'368 ± 52'529	1'171'814 ± 155'616	3'875'116 ± 1'242'707	3'435'676 ± 567'462	2'204'725 ± 166'472
3-methylbutanal [590-86-3]	7.18	58	LBA	641'091 ± 1'531	542'340 ± 42'334	996'709 ± 373'532	1'397'137 ± 25'238	1'330'302 ± 11'462
			S4C11	14'486 ± 1'738	8'744 ± 2'845	29'257 ± 12'795	12'329 ± 1'189	2'921 ± 947
methylthiol butyrate [2432-51-1]	16.47	71	LBA	1'500 ± 0	2'062 ± 219	1'525 ± 33	1'500 ± 0	1'500 ± 0
			S4C11	17'732 ± 1'403	94'919 ± 59'964	530'898 ± 291'948	777'792 ± 65'192	778'706 ± 4'295
Dimethyldisulfide [624-92-0]	13.65	94	LBA	171'829 ± 9'598	165'959 ± 467	452'284 ± 265'831	735'935 ± 20'791	738'778 ± 49'425
			S4C1	166'964 ± 19'462	338'529 ±	1'02	1'347'112 ± 146'340	1'453'560 ± 173'152

			1		26'779	1'057 ± 423'106		
Furfural [98-01-1]	27.83	96	LBA	637'928 ± 4'716	633'725 ± 7'767	729'926 ± 31'733	692'130 + 6'592	647'546 + 8'919
			S4C11	25'266 ± 15'844	5'787 ± 4'469	1'500 ± 0	1'500 ± 0	1'500 ± 0
Benzaldehyde [100-52-7]	29.58	105	LBA	6'859'666 ± 49'960	7'727'183 ± 427'114	10'220'381 ± 135'198	11'478'049 ± 140'196	9'954'094 ± 268'096
			S4C11	165'484 ± 2'477	46'879 ± 35'846	129'500 ± 75'246	92'914 ± 56'052	93'861 ± 61'362
Acetophenone [98-86-2]	32.74	105	LBA	194'712 ± 10'234	179'056 ± 18'095	233'408 ± 75'410	245'287 ± 60'721	307'943 + 4'237
			S4C11	201'072 ± 7'060	211'766 ± 7'733	269'762 ± 60'931	201'346 ± 14'281	188'056 ± 18'908
Dimethyltrisulfide [3658-80-8]	25.53	126	LBA	36'116 ± 4'115	28'792 ± 923	79'918 ± 41'337	106'120 ± 2'307	123'983 ± 30'234
			S4C11	21'824 ± 5'505	163'641 ± 41'160	557'102 ± 351'924	658'366 ± 449'190	197'029 ± 21'897

First column indicates molecule name and CAS number, second column indicates Retention Time, third column indicates the Quant Ion, columns from fifth to ninth indicate the average abundance ± standard deviation for the molecule at 1, 2, 3, 4, and 5 dpi, in control and S4C11-inoculated samples. In the table, an abundance level of 1500 indicates that the quant ion area is below the noise level (1550).

[a]: the CAS number of methylthiol isovalerate is 23747-45-7, but the two detected isomers may have different identifiers.

In vivo antifungal assays

In order to assess the capability of fungal growth inhibition also *in vivo*, tomato berries and grapevine leaves were inoculated with BC alone or in the presence of S4C11. Strain S4C11 significantly reduced the growth of BC on both the tested plant material. (Fig 4). On tomato berry, the reduction in BC-induced symptoms was very sharp, causing a reduction of almost 40% in the severity of symptoms (Fig 4A), as evident from berry appearance, on which the presence of mycelium is greatly reduced (Fig 4B, 4C). Another difference is in the firmness of the berry, which is preserved in treated berries, while the fruits became excessively soft when no treatment was applied.

For grapevine, the reduction of symptom severity was less intense, but became greater over time: no significant difference in symptom severity was observed at 3 dpi; while a statistically significant reduction in symptom severity, of approximately 10% and 20%, was observed at 5 dpi and 7 dpi, respectively (Fig 4D). Already at 3 dpi the non-treated leaves showed large portions affected by *B. cinerea* (Fig 4E), while the affected areas on S4C11-treated leaves were smaller (Fig 4F).

In planta growth promotion assays

In order to assess the ability of strain S4C11 to promote the growth of different plant species in different conditions, two separate greenhouse trials were set up, in an experimental and in a commercial greenhouse, using six different plant species belonging to five different families. Strain S4C11 significantly increased the height of all tested plants, both in experimental greenhouse (Fig 5A) and in the nursery (Fig 5B-F). This increase in height had a stronger statistical significance for the Zebo and zucchini plants, followed by the pepper and bean plants, and the least significant effect on chicory and leek. The increase in height ranges from 14% in chicory to 70% in Zebo, with an average value of 36%. It is interesting to point out that the major increase in height was registered in the experimental greenhouse test, in which the plants received no fertilization, suggesting that the effects of strain S4C11 are more evident when available nutrients are limited. The growth promotion effect was not as widely registered when measuring the fresh weight of plants: significant differences were registered only in the weight of fruits produced by zucchini and in the fresh weight of pepper plants (Fig 5G), while for the other plants the increase in height was not accompanied by a significant increase in biomass. The effects of the treatment with strain S4C11 on the different plant species are visually reported in Figure 6.

In planta biocontrol assays

Inoculation of the three different viruses (CMV, CymRSV, and PVY), a type of pathogen with completely different interaction mechanisms with the host compared to fungi, on *N. benthamiana* plants, either non-treated or inoculated with strain S4C11, showed different effects on the development of symptoms depending on the virus (Fig 7A-C). CMV gave mild symptoms on non-treated plants at 5 and 7 dpi, but developed stronger symptoms at 10 dpi, while the

symptoms remained mild on plants treated with strain S4C11. A similar trend was observed for CymRSV, with non-treated plants showing mild symptoms until the tenth day from inoculation: at this timepoint non-treated plants developed severe symptoms, while the symptoms on S4C11-treated plants remained milder. The situation was different for plants inoculated with PVY, as no significant difference in symptoms developed on non-treated vs S4C11-treated plants were observed until 10 dpi, when S4C11-treated plants showed significantly stronger symptoms than those registered on non-treated plants.

The symptoms registered were not correlated with the relative abundance of virus detected in the infected plants (Fig 7D): in CMV-infected plants, the virus load was significantly higher in plants treated with strain S4C11, even though the symptom severity was significantly lower. For plants infected by CymRSV and PVY no significant difference in the virus abundance was detected, even though the symptoms were significantly lower or higher, respectively, in S4C11-treated plants.

The gene expression analysis, carried out on genes related to plant resistance pathways, showed that, of the three analyzed genes (EDS1, NPR1, PR2b), only NPR1 is affected by the inoculation of strain S4C11 (Fig 7E). The gene EDS1 has expression rates similar to the internal reference PP2A, having an average abundance level close to 0 in both non-treated and S4C11-treated plants; gene NPR1 is expressed less than the reference gene in S4C11-treated plants, but has significantly higher expression in NT-treated plants; gene PR2b has lower average expression than the other two genes both in non-treated and S4C11-treated plants, but there are no significant differences between the two.

Features of *L. fusiformis* S4C11 genome

In order to gain insight into the molecular mechanisms underlying the plant-beneficial properties of L. fusiformis, we sequence its genome and analyzed the gene content. De novo genome assembly of *Lysinibacillus fusiformis* strain S4C11 was obtained from short-read sequencing data generated by Illumina technology comprising 37,784,883 fragments for a total of 7.6Gb and 1575X expected coverage (currently being deposited in NCBI: SRA, AN will soon be available). The Illumina-based assembly, consisting of 46 sequences with N50 of ~365Kb and GC 36.9% (Table 3), was subsequently scaffolded using the physical mapping data generated with Nabsys HD maps on the basis of Nt.BspQI/Nb.BbvCI nickases (coverage 308X, Table 3). The final genome of *Lysinibacillus fusiformis* strain S4C11 consisted of 3 main sequences of 5,038,130bp, 192,921bp and 137,487bp (Table 3 and Supplementary Table 1). The same genome structure was confirmed using mapping data generated with a different enzyme (Nb.BssSI, coverage 388X), that also allowed to demonstrate the circularity of the largest scaffold (Supplementary Figure 1), namely the main chromosome. The same analysis revealed also the circularity of the two minor sequences (Supplementary Figure 2), that can be therefore considered large plasmids, when taking into account also their size and the presence of typical plasmid-related genes, as demonstrated in the subsequent analysis.

Table 3. *Lysinibacillus fusiformis* S4C11 Genome Assembly statistics; complete Assembly statics are reported in Supplementary Table X.

	NGS assembly	HD Map	Hybrid

	(Illumina)	assembly (Nabsys)	assembly
Number of contigs/scaffolds	46	7	3
Genome size (bp)	5,069,506	4,970,266	5,038,130
Average length (bp)	153,621	927,833	1,679,376
N50 (bp)	365,524	634,146	4,707,722
# Gap	4	/	18
Gap size (bp)	4	/	87,480
CG (%)	36.9	/	35.2
Chromosome (bp)	/	/	4,707,722
Plasmid 1 (bp)	/	/	137,487
Plasmid 2 (bp)	/	/	192,921

589

590 The bacterial origin of replication was estimated with high-confidence to be located in the region
591 between 4,535,768-4,537,882 bp in the chromosome sequence, based on homology search in
592 the DoriC database, as well as using Ori-Finder. Subsequent annotation using RAST predicted
593 the chromosomal replication initiator protein DnaA at location 4,536,363-4,537,712 and several
594 other replication-related genes in the near vicinity, such as DNA gyrase subunit A (4,542,527-
595 4,544,992 bp) and subunit B (4,540,576-4,542,501 bp), as well as the DNA recombination and
596 repair protein RecF (4,539,359-4,540,474 bp) (Supplementary Figure 3).

597 The genome of strain S4C11, annotated with the RAST software, was predicted to have 5,306
598 genes. Of these, 97 are non-coding RNAs (rRNA, tRNA), 3,571 encode for non-hypothetical
599 proteins, and 1,638 encode for hypothetical proteins (Table 4).

600 **Table 4.** *Lysinibacillus fusiformis* S4C11 Genome Annotation statistics

Genome Annotation	
Number of Coding genes	5,306
Cumulative Gene lenght (bp)	4,362,647
Protein Coding genes	5,209
Protein Coding genes without function prediction	1,638
tRNA genes	82
rRNA genes	15
Gene Statistics	
Max length (bp)	8,079
Min length (bp)	90
Average (bp)	835.2
Median (bp)	738

601

602 Integration of RAST annotation with manual curation allowed to identify gene sets with a
603 predicted function not related to primary metabolism, which are reported in Table 5, grouped
604 together by function. Also, the AntiSMASH analysis identified 8 regions dedicated to secondary
605 metabolism on the chromosome of strain S4C11, while none was identified on the plasmids
606 (Table 6).

Table 5. Categories of non-primary metabolic genes identified in strain S4C11

Category	Chromosome	Plasmid 1	Plasmid 2
Biocontrol	19	0	0
Conjugation	19	1	1
Detoxification and Stress-related genes	130	14	3
Antibiotic resistance	24	1	1
DNA damage	15	2	2
Heavy metal resistance	12	3	0
Mono- / Dioxygenases	18	3	0
Oxidative stress	18	3	0
Temperature stress	10	0	0
Motility and Chemotaxis	95	7	3
Chemotaxis	47	7	2
Flagella and Motility	48	0	1
Plant growth-promotion	5	0	0
Quorum quenching	2	0	0
Siderophores	33	1	0
Synthesis	10	1	0
Utilization	23	0	0
Sporulation	104	5	2
Transcription Regulation	199	14	6
AcrR	25	3	0
AraC	15	3	0
ArsR	6	0	0
GntR	19	0	0
HxlR	5	0	0
IclR	7	0	0
LuxR	12	0	1
LysR	21	2	0
MarR	15	1	0
MerR	13	2	0
OmpR	6	0	0
PadR	5	1	0
TetR	3	0	0
Xre	3	0	0
YafY	3	0	0
Transport systems	258	23	8
ABC transporters	207	19	6
DMT transporters	10	0	1

First column indicates the categories of genes (underlined) and, if relevant, subcategories (in italics). Second, third, and fourth column indicate the number of genes present in the chromosome, plasmid 1, and plasmid 2, respectively. Numbers of any category include those of subcategories, when present.

Table 6. Results of the AntiSMASH analysis of S4C11 genome

Region	Type	Closest match	Similarity	Position
Region 12.1	Betalactone	Fengycin	46%	180'195 - 204'419
Region 12.2	Terpene	N/A		1'428'869 - 1'449'690
Region 12.3	Siderophore	Petrobactin	33%	2'215'421 - 2'228'967
Region 12.4	Bacteriocin	N/A		2'330'257 - 2'339'149
Region 12.5	NRPS	Molybdenum cofactor	29%	2'524'141 - 2'586'067

Region 12.6	Bacteriocin	N/A		2'916'028 - 2'926'264
Region 12.7	NRPS-like	Kijanimicin	4%	3'999'797 - 4'042'964
Region 12.8	T3PKS	N/A		4'179'019 - 4'220'101

First column reports the genomic region identified by the software. The second the type of secondary metabolite predicted to be produced by the region. The third and fourth column indicate the closest known region and the similarity with this match, respectively. The fifth column indicate the position of the region on the genome, giving starting and ending position in bp.

Annotation results showed that the most represented category of genes are those encoding for elements of transport systems, with a total of 258 genes, most of which belong to the ABC-type transporters, 10 belong to the DMT-type transporters, and the remaining 41 belong to less-represented types of transporters in the genome.

The second most-abundant category is represented by transcriptional regulators, with 199 genes predicted to have this function; of these 199 genes, 158 belong to 15 different families of transcription regulators (reported in table 5) for which at least three genes were found in the genome, while the remaining 42 belong to 15 families of which only one or two genes were found. Of the former 15 families, the most represented ones are AcrR, LysR, GntR, AraC, and MarR.

The following category by abundance is represented by genes related to detoxification of toxic compounds and resistance to stress, with 130 genes. The genome of strain S4C11 is predicted to encode proteins used to counteract oxidative stress (e.g. catalase, super-oxide dismutase), temperature stress (e.g. heat-shock and cold-shock proteins), and DNA damage such as that caused by UV light (e.g. UvrX). The presence of several mono- and dioxygenases predicts the ability to detoxify several cyclic/aromatic compounds. The genome of S4C11 furthermore presents genes related to resistance against several antibiotics (bacitracin, beta-lactams, blasticidin, chloramphenicol, fosfomycin, fosmidomycin, nitroimidazole, streptothricin, tetracycline) and metals (aluminum, arsenic, cadmium, chrome, cobalt, copper, lead, tellurium, zinc).

The fourth most abundant category is represented by genes related to sporulation, with 104 genes. This category was expected of a spore-forming, Gram + bacterium.

The fifth most abundant category is related to motility and chemotaxis, with a total of 95 genes. These genes are split almost evenly between the genes related to the regulation, synthesis, and use of flagella (48 genes), and those related to chemotaxis (47 genes).

The last categories include (i) the genes related to synthesis and use of siderophores (33) genes, among which were identified the anthrachelin, bacillibactin, staphylobactin and, through AntiSMASH, petrobactin; (ii) genes related to conjugation and natural competence; (iii) plant growth-promotion, which include six genes related to synthesis of the plant hormone auxin; (iv) biocontrol, which include genes related to the synthesis of phenazines, bacteriocins, degradation or utilization of chitin, and non-ribosomal peptide synthases, two of which were predicted by AntiSMASH to be similar to clusters related to the production of the antibiotics fengycin and kijanimicin (low similarity); and (v) quorum quenching, including two genes encoding for enzymes that can degrade N-acyl homoserine lactones, a major quorum sensing molecule of bacteria.

Furthermore, the analysis of the genome allowed the detection of genes encoding the full pathway for synthesis of methylthiol isovalerate and methylthiol butyrate (consisting of 8 enzymes) starting from leucine, as proposed by Sourabié and colleagues (2012) in the genus *Brevibacterium*.

The annotation of plasmids allowed to assign a putative function to many genes present on these genetic elements: 154 out of 219 genes on plasmid 1 (70%) and 78 out of 122 genes on plasmid 2 (64%). Most of these genes are related to replication and partition and methylation of the plasmids, which are base functions for the conservation of the plasmids in the cell. The other functions identified comprise: conjugation, detoxification and stress-related, chemotaxis, siderophore production, sporulation, transcription regulation, and transport systems for plasmid 1; conjugation, detoxification and stress-related, motility and chemotaxis, sporulation, transcription regulation, and transport systems for plasmid 2 (Table 5). The most interesting genes on plasmid 1 are those related to the resistance against oxidative stress, as well as the detoxification of cyclic compounds and heavy metal, possibly giving an advantage to strain S4C11. In addition to the genes already reported in Table 5, plasmid 2 carries genes encoding for discoidin proteins with adhesive properties, possibly being involved in the adhesion to the host tissues.

DISCUSSION

The genus *Lysinibacillus*, obtained from a re-categorization of some species from the genus *Bacillus* (Ahmed *et al.*, 2007), does not share the fame for agricultural studies of the latter, despite sharing many of the traits that made many *Bacillus* species relevant for agriculture.

The species *Lysinibacillus fusiformis*, in particular, has been identified as a plant-beneficial microbe living in association with the roots of apple trees (Bulgari *et al.*, 2012), cereals (Damodaran *et al.*, 2018), citrus (Trivedi *et al.*, 2011), ginseng (Vendan *et al.*, 2010), and tomato (Rahmoune *et al.*, 2017), but little information is available on the possibility to use this species as a plant growth-promoting agent in agriculture, outside of these studies in which strains of this species were isolated.

The results obtained in this study demonstrate the ability of *L. fusiformis* strain S4C11 to act as an antifungal agent against isolates of different phytopathogenic species, such as *Aspergillus nigri*, *Botrytis cinerea*, *Phomopsis viticola*, and *Rhizoctonia solani*, inhibiting the growth of these fungi from 54% to 100% after an incubation of 14 days. The bacterial strain proved ineffective in reducing the growth of two isolates of different *Fusarium* species: *Fusarium culmorum* and *Fusarium verticillioides*, obtaining only minimal inhibition of the fungal growth and losing effectiveness after one week from inoculum. Other strains of *L. fusiformis* were already reported to have an antifungal effect and being effective against isolates of *Fusarium* (Damodaran *et al.*, 2018; De Mandal *et al.*, 2018), suggesting that different strains can have a specialized antagonistic effect against different fungi. In this study, the antifungal effect was examined more in-depth against *B. cinerea* (BC), determining that strain S4C11 is capable of inhibiting the growth of the fungal colony also through the production of volatile organic compounds (VOCs), as well as of inhibiting the germination of BC conidia both with cell suspension and cell-free culture broth, suggesting the production of extracellular diffusible compounds that can inhibit the germination of the spores. Furthermore, the cell-free culture broth treated with either proteinase

K or heat maintained most of the inhibitory effect on conidia, revealing that the main active compounds released are not degraded by proteinase activity and are heat-stable. Since Bacillaceae are known to produce antibiotic lipopeptides that are resistant to degradation and heat (Romero *et al.*, 2007; Ongena and Jacques, 2009), and the genome of strain S4C11 showed genes that could be involved in the biosynthesis of such molecules, in particular with a cluster identified as similar to that needed for the production of fengicyn, it is reasonable to hypothesize that strain S4C11 could use such molecules to act against fungi. Further studies will be carried out to describe the non-volatile, antifungal molecules produced by strain S4C11.

The characterization of the volatile molecules produced by strain S4C11 highlighted the presence of ketones and compounds that contain sulfur. While the effect of these ketones on fungal growth is unknown, dimethyl disulfide and dimethyl trisulfide are well-documented as antifungal molecules (Rosskopf *et al.*, 2006; Wang *et al.*, 2009; Ossowicki *et al.*, 2017). Two other sulfur-containing compounds that are produced, and at a much higher abundance, are methylthiol isovalerate and methylthiol butyrate: while no literature is available on the biocidal effect of these volatiles, it has been previously reported that other compounds with a methylthio group produced by bacteria can have a biocidal effect (Groenhagen *et al.*, 2013), suggesting that these highly-abundant VOCs can contribute to the antifungal effect of S4C11.

The antifungal effect seen *in vitro* against BC was also confirmed by *in vivo* assays, in which plant organs treated with strain S4C11 showed significantly milder grey mold symptoms compared to untreated controls, inoculated only with the pathogen. Even if the application of the bacterial strain on produce that can be consumed raw, such as tomato berries, would not be advisable and could raise safety concern, the data suggest that single molecules produced by strain S4C11 could have a potent antifungal effect, being useful as treatments to preserve perishable crops from spoilage. The effect of antimicrobic molecules extracted from the culture broth of *L. fusiformis* against foodborne pathogens were already reported by Ahmad and colleagues in 2014, showing them to be effective not only against toxigenic fungi, but also against several bacteria, such as *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Vibrio cholera*, making this an interesting avenue to investigate.

The most promising ability displayed by strain S4C11 is the plant growth-promoting effect that it exerted on several different plant species, both in an experimental greenhouse and in a nursery environment, in which the plants were already treated with a commercial plant-growth promoting product and received fertilization. Strain S4C11 managed to consistently promote the growth of plants belonging to five different families (Alliaceae, Asteraceae, Cucurbitaceae, Fabaceae, Solanaceae), showing a broad-range effect that does not seem to be host-specific. While the effect on the height of the plants is consistent for all examined species, a statistically significant increase in biomass (fresh weight) was not detected in all the examined species. The most outstanding result obtained in this experiment regards the increase in fruit weight produced by zucchini, which was on average more than twice that of non-treated control. This is, to the best of our knowledge, the first report of a similar result from the *L. fusiformis* species. Considerations that can be made from this experiment are that i) strain S4C11 worked as a plant-growth promoter when inoculated as a single strain in the experimental greenhouse experiment, but also enhanced and contributed to the effect of the already in-use plant growth-promoter products used in the nursery, showing a good aptitude for being employed in the production of formulations for agriculture; ii) apart from the increased yield, other parameters of the fruits produced by zucchini treated with strain S4C11 might be important to consider. The application of the bacteria might have positive or negative effects on the nutritive content of the

edible part of the treated plants but, at the moment, the only reported effect of this kind is a positive one: an increase of available iron in wheat kernels and soy beans after a treatment with siderophore-producing *L. fusiformis* (Sharma *et al.*, 2019).

The *in planta* biocontrol assays were carried out against viruses, pathogens that cause particularly high damage in greenhouses and nurseries in which the controlled conditions promote the survival and proliferation of several vectors. The results obtained against three different viruses (Cymbidium Ringspot Virus, CymRSV; Cucumber Mosaic Virus, CMV; and Potato Virus Y, PVY), all of which have a genome composed of a single-stranded positive RNA (ssRNA+), are not consistent: a significant reduction of symptom severity was registered for CymRSV and CMV, while for PVY the symptom severity was significantly higher in the S4C11-treated plants than in the non-treated controls. Also, these results do not match the ones obtained from virus quantification, in which no significant differences were registered in the S4C11-treated and non-treated plants. It is important to point out that while there are reports of both symptoms caused by virus and virus concentration being reduced by a bacterial treatment (Kumar *et al.*, 2016), the two parameters are not necessarily correlated (Raupach *et al.*, 1996). These observations suggest that, rather than having a specific effect that counteracts the replication of ssRNA+ viruses in the host, the treatment with strain S4C11 generally promotes the health of the plant host, possibly allowing it to react in a more positive way to the infection against some viruses, but is not effective against others. This can also be seen from the gene expression of three defense-related plant genes. The genes EDS1 and PR2b show no difference in expression between non-treated and S4C11-treated plants, while the gene NPR1, which is a master regulator gene in both SAR and ISR (Pieterse *et al.*, 2014), is down-regulated in S4C11-treated plants, indicating that the defenses of inoculated plants are not strengthened by the application of strain S4C11, even though the symptoms developed on those plants were milder for two out of three viruses.

Integration of Illumina data with HD mapping based on Nabsys technology allowed the reconstruction of the complete and contiguous genome of S4C11 in three closed circular contigs, subsequently identified as a main chromosome and two large plasmids. Annotation of S4C11 unraveled important features of this bacteria as a highly-adaptative biocontrol agent, including the massive presence of transport and regulatory genes. These genes, coupled with those encoding for different defense and detoxification pathways can indicate a flexible genome, able to reshape its functions to deal with several stresses. This is in accordance with previous studies that demonstrated that strains of *L. fusiformis* can be applied for bioremediation from arsenic (Mohamed and Farag, 2015), chromate (He *et al.*, 2011; Huang *et al.*, 2016), lead (Mathivanan *et al.*, 2018), and pollutants derived from oil refineries (Gholami-Shiri *et al.*, 2017). Strain S4C11 showed genomic traits related to the detoxification of all these heavy metals and compounds, as well as several genes related to the detoxification of cadmium, cobalt, and tellurium, which might need further investigation for a possible application in bioremediation or phytoremediation.

The only known plant growth-promoting genes detected in the genome of strain S4C11 are those related to the synthesis of indole-acetic acid (IAA), an auxin phytohormone, namely a typical trait of plant growth-promoting microorganisms (Hayat *et al.*, 2010). The effective production of auxin by strain S4C11 was verified *in vitro* and beside being present, it was also detected to be very high, in accordance with other works on different *L. fusiformis* strains (Vendan *et al.*, 2010; Damodaran *et al.*, 2019). Still, it cannot be excluded that other mechanisms, currently not known and identifiable by genome annotation, are involved.

Another relevant feature of this strain is its biocontrol potential, that was demonstrated by the several assays carried out in this study. The genetic base of these features is not fully elucidated, but some elements were unraveled by genome annotation. In particular i) the presence of genes related to the production of bacteriocins and non-ribosomal peptides assembly pathways, which can produce molecules that can directly antagonize the growth of other microorganisms (Ongena *et al.*, 2008); ii) quorum-quenching genes, which may be relevant to biocontrol, as the virulence of many phytopathogenic bacteria can be activated only after a certain quorum is achieved (von Bodman *et al.*, 2003; Simionato *et al.*, 2007); also relevant to this particular topic could be the ability, reported for other strains of *L. fusiformis*, to disturb the formation of biofilm formation by other bacteria (Pradhan *et al.*, 2014); iii) the production of chitinase, an important protein for the antagonism towards fungi, which will need further investigation. Genes related to this function have been found, but not the whole pathway that is needed to use chitin as a nitrogen and carbon source, comprising tens of genes (Passera *et al.*, 2018). Strain S4C11 demonstrated no chitinase activity in the dedicated functional assay but was able to degrade spores of BC during the *in vitro* germination assay, which suggests that the chitinase activity for this strain is not related to starvation and some other signal is involved in its activation.

Assembly of S4C11 genome by employing HD mapping allowed the recognition of two independent genomic portions that represent large plasmids, a feature that was not identified by simply comparing the nucleotide sequence with NCBI plasmid database. These plasmids may be relevant for S4C11 activity as they encode for useful traits that may assist strain S4C11 in surviving stresses (plasmid 1) and in colonizing a host plant (plasmid 2). Despite not encoding for functions/metabolic pathways directly linked to plant-growth promotion or biocontrol effect of the strain, these features may be useful for the possible employment of such a strain in the environment. In addition, the absence of coding regions for antibiotic molecules or other active substances keep the risk associated with their spreading in the ecosystem low.

A last positive trait identified in the genome is the spore-formation mechanism present in strain S4C11, as expected of a *Lysinibacillus*. The formation of endospores is a highly desirable trait for the production of commercial formulates, as it allows the bacteria to be easily stored for long periods of time with a minimal loss of vitality (Emmert and Handelsman, 1999; Kokalis-Burelle *et al.*, 2005).

CONCLUSIONS

The present study is, to the best of our knowledge, the first to analyze in depth and with a multidisciplinary approach the potential for agricultural application of a strain of *Lysinibacillus fusiformis*, a bacterial species that is often found associated with crops but not sufficiently studied. The results obtained highlight the versatile effects of strain S4C11 regarding biocontrol and, especially, plant growth-promotion, as well as providing insights on the mechanisms and genetic elements underlying the observed effects, paving the way for further studies in the characterization of this species.

The deep integration of innovative genomic approaches (HD mapping) with multidisciplinary *in vivo*/*in vitro* functional experiments led to the full characterization of *Lysinibacillus fusiformis* strain S4C11. These scientific and technological advancements highlighted the versatile effects of strain S4C11 regarding biocontrol and, especially, plant growth-promotion as well as provided

insights on the mechanisms and genetic elements underlying the observed effects, thus paving the way for further studies in the characterization of this species for agricultural application.

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Data Statement

All data obtained as part of this study is available as part of the manuscript or found at (currently being deposited in NCBI: SRA, AN will soon be available).

Figure Captions:

Figure 1. Graphs reporting the antifungal effect registered in dual-culture assays. The box-plot graphs describe the growth of the different phytopathogenic fungal strains when cultured in absence (NT) or presence (S4C11) of strain S4C11 at A) 5 dpi, B) 7 dpi, C) 14 dpi. The top of each graph reports the species to which each fungal strain belongs: *Aspergillus nigri* (AN), *Botrytis cinerea* (BC), *Fusarium culmorum* (FC), *Fusarium verticillioides* (FC), *Phomopsis viticola* (PV), or *Rhizoctonia solani* (RS). The Y-axis reports the radial growth of the fungal colony expressed in centimeters. The number reported on each section of the graph indicates the average Growth Inhibition Percentage (GIP) calculated for each fungus at that time point. Stars between two bars indicate statistically significant differences in the values of radial growth according to a Student's T-test (* = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$).

Figure 2. Results of the conidia germination *in vitro* assay. A) Graph reporting the absorbance measured at 492 nm (Y-axis) at different hours from the start of the experiment (X-axis). Different lines indicate the results obtained in the different treatments: 50% conidia suspension in TGY broth (10^4 conidia/mL) and 50% either TGY (BC); cell suspension of S4C11, at a final concentration of approximately 10^6 CFU/mL (S4C11 CS); S4C11 overnight culture broth, sterilized by centrifugation and filtering (S4C11 CF); S4C11 overnight culture broth, sterilized by centrifugation and filtering, and autoclaving at 121 °C for 15 minutes (S4C11 121); S4C11 overnight culture broth, sterilized by centrifugation and filtering, and treated with proteinase K (S4C11 PK). Different letters (a,b,c,d) on the right side of the lines indicate

statistically significant differences in the results throughout the duration of the experiment, determined by a general linear model, optimized for repeated measures, followed by the Tukey's exact post-hoc test ($P < 0.05$).
Pictures of optical microscopy taken at 48 hours from the start of the experiment showing B) non-germinated condia from the BC condition; C) germinated conidia from the BC condition; D) detail of non-germinated conidia with visible bacterial growth surrounding them from S4C11 CS condition; E) detail of a cluster of non-germinated and degraded conidia from S4C11 CS condition. The white bar in the lower-right corner of each picture corresponds to 10 µm.

Figure 3. Results of VOCs analysis. A) PCA based on abundance of compounds. Each marker represents a single sample that was analyzed. Different shapes of the markers indicate different time points, while different colors indicate blank controls with only the growth medium (LBA) or samples containing the bacteria (S4C11). The X-axis reports Principal component 1 (61.8%) while the Y-axis reports Principal component 2 (23.2%) B) Loading plot of PCA, indicating how each single molecule contributes to the determination of Principal components 1 and 2 C) Box-plot showing comparison of abundance between blank controls (LBA) and bacteria-inoculated samples (S4C11), regardless of the time point of the analysis. Stars between two bars indicate statistically significant differences in the values of radial growth according to a Student's T-test (* = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$). D) abundance at different time points for the prevalent molecule produced: methylthiol isovalerate.

Figure 4. Results of the *in vivo* antifungal assays. A) Graph reporting the Infection percentage index (I%I) registered on tomato berries at 7 days after infection with *B. cinerea* alone (NT) or also treated with strain S4C11 (S4C11). Stars between two bars indicate statistically significant differences in the values of radial growth according to a Student's T-test (*** = $P < 0.001$).
Pictures showing the symptoms registered on berries infected B) with *B. cinerea* alone or C) also treated with strain S4C11 7 days post inoculation.
D) Graph reporting the Infection percentage index (I%) registered on grapevine leaves at 3, 5, or 7 days after infection with *B. cinerea* alone (NT, in white) or also treated with strain S4C11 (S4C11, in green). Stars between two bars indicate statistically significant differences in the results according to a Student's T-test (* = $P < 0.05$).
Pictures showing the development of symptoms at 3 dpi on grapevine leaves inoculated E) with *B. cinerea* mycelium alone or F) also treated with strain S4C11.

Figure 5. Results of the plant growth-promotion assay, graphs. Graphs reporting on the Y-axis the height of the plants in centimeters and on the X-axis the progression of height in three weeks from the inoculation. Separate lines indicate the non-treated plants (NT, in black) or S4C11-treated plants (S4C11, in green). The graphs report the height of plants of A) bell pepper Zebo, grown in experimental greenhouse; B) bean, C) zucchini, D) pepper, E) chicory, or F) leek, grown in nursery. Stars between the lines indicate statistically significant differences in the results throughout the three weeks of observation, determined by a general linear model, optimized for repeated measures, followed by the Tukey's exact post-hoc test (* = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$).
G) Graph reporting the fresh weight of plants in grams on the Y-axis, and the different plants on the X-axis, divided among non-treated (NT, in white) and S4C11-treated (in green). The weight reported corresponds to the whole aerial part of the plant for all the tested species, except for zucchini: the weight reported for zucchini is that of the fruits that were produced by the plants.

Stars between two bars indicate statistically significant differences in the values according to a Student's T-test (* = P < 0.05).

Figure 6. Results of the plant growth-promotion assay, pictures of the plants. Pictures of the plants at 2 weeks post inoculation: A) bell pepper Zebo, grown in experimental greenhouse; B) bean, C) zucchini, D) pepper, E) chicory, or F) leek, grown in nursery. For each picture in the panel, the plants shown on the left-hand side are the non-treated controls, while those on the right-hand side of the dashed line are the S4C11-treated plants.

Figure 7. Results of the *in planta* biocontrol assay against viruses and gene quantification. The graphs report A) the infection percentage index (I%I) determined on the *N. benthamiana* plants at 5 days , B) 7 days, or C) 10 days after being inoculated with either CMV, CymRSV, or PVY. The graphs compare the I%I of non-treated plants (NT, in white) and S4C11-treated plants (S4C11, in green). Stars between two bars indicate statistically significant differences in the results according to a Student's T-test (* = P < 0.05; ** = P < 0.01, *** = P < 0.001). D) The graph reports the relative virus quantification expressed as ln2^(-ΔΔCT) in comparison to the PP2A housekeeping gene of *N. benthamiana*. The X-axis divides the values among different viruses (CMV, CymRSV, PVY) in the non-treated plants (NT, in white) and S4C11-treated plants (S4C11, in green). No significant differences among results were detected by a Student's T-test (P > 0.05).
E) The graph reports the relative gene expression, expressed as ln2^(-ΔΔCT), in comparison to the PP2A housekeeping gene of *N. benthamiana*. The X-axis divides the values among different genes (EDS1, NPR2, PR2b) in the non-treated plants (NT, in white) and S4C11-treated plants (S4C11, in green). Stars between two bars indicate statistically significant differences in the results according to a Student's T-test (*** = P < 0.001).

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