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Isolation and characterization of a new *Leptobacillium* species promoting tomato plant growth

Luisa Liu-Xu[1]✉, Begonya Vicedo[1], Kalliope K. Papadopoulou[2], Gemma Camañes[1] & Eugenio Llorens[1]✉

Endophytes can be a promising alternative for sustainable agronomic practices. In this study, we report for the first time a root-colonizing fungal strain (Sl27) of the genus *Leptobacillium* as a tomato (*Solanum lycopersicum*) endophyte, with no clear homology to any known species. Performed analyses and assays, including morphological and physiological characterization of the fungal isolate, provided insights into the ecological niche and potential agronomical and industrial applications of the fungal isolate. The ability of Sl27 to establish a symbiotic relationship with the host plant was assessed through experiments under controlled conditions in the growth chamber and greenhouse. Seed-inoculation showed no detrimental effects in the three tomato genotypes studied (TH-30, ADX2, MO-10). The influence of Sl27 on growth parameters of the host plant was dependent on the tomato genotype, with TH-30 showing the most prominent improved phenotype. Moreover, chlorophyll and lycopene content in fruits were enhanced. These findings provide a basis for further studies on the potential application of this new isolate for improving crop performance.

Keywords Fungal endophyte, Beneficial microorganisms, *Leptobacillium* sp., *S. lycopersicum*, Fungal characterization

The microbiome has recently become a fundamental component of plant research. Microorganisms interact closely with plants, influencing their development and forming potentially lifelong associations as endophytes[1,2]. These interactions are studied to understand their influence on plant behavior and the potential applications[3–6]. Endophytes have been reported to confer resistance to plants against abiotic and biotic stress, enhance nutrient acquisition and growth, and serve as potential sources for compounds of agronomic or industrial interest[7–9]. These benefits present a sustainable alternative to traditional agricultural techniques. The role of plant endophytes is crucial for reducing reliance on agrochemicals and mitigating their environmental and biological impacts[10]. However, there remains limited knowledge about the functions of most fungal communities in plant development and behavior[11,12]. Numerous efforts have been made in studying beneficial endophytic fungi. The most studied endophytic fungi are ascomycetes, with nearly 75% of studies focusing on species from the genera *Epichloe*, *Serendipita*, *Penicillium*, *Fusarium* and *Trichoderma*[13]. These fungal endophytes have been shown to help plants overcome several challenges[14–16].

Plant-microorganism interactions vary among plant species. Many studies have used tomato plants (*Solanum lycopersicum*) to examine the effects of endophytes from different origins[17–19]. Tomato is not only a model dicotyledonous plant for research but also one of the most relevant crops wordwide[20]. Studies on the isolation and application of fungal endophytes from tomato plants have explored their roles under conditions such as organic nitrogen availability[21], water stress[22,23] and nematode infections[24]. These findings highlight the potential of in-depth studies on the tomato microbiota. For instance, previous research showed that certain traditional genotypes demonstrated better heat stress resistance than common commercial varieties[25], which could be potentially attributable to differences in the microbiome structure and composition between traditional and commercial genotypes[26].

In this work we report the discovery of a novel endophyte from a traditional tomato genotype. This study includes the isolation of the fungal strain from healthy tomato roots, characterization of the fungal isolate, bioassays to assess its potential applications, and evaluation of its effect on tomato plant development under controlled conditions to demonstrate their beneficial interaction.

[1]Department of Biology, Biochemistry and Natural Sciences, Universitat Jaume I, Castellón de la Plana, Spain. [2]Department of Biochemistry and Biotechnology, University of Thessaly, Larissa, Greece. ✉email: lliu@uji.es; ellorens@uji.es

Material and methods

Fungal isolation

Several fungal endophytic strains were isolated from apparently healthy tomato plants of several traditional genotypes. These genotypes are also known as landraces, which are locally produced through traditional management and open pollination. These landraces included red tomato from Thessaloniki (TH-30) and Montfavet (MO-10), hanging tomato from Alcalà de Xivert (ADX2), Cor de Bou and VI.

The study was conducted by acquiring seeds of the tomato genotypes to minimize influence of specific characteristics of soil. These seeds were obtained from the Institute for the Conservation and Improvement of the Valencian Agrodiversity (COMAV) at the Polytechnic University of Valencia, Spain. The seeds were surface-sterilized using 75% ethanol for 3 min, followed by a 4% sodium hypochlorite solution for 1 min and rinsed three times with sterile distilled water to remove residual ethanol and sodium hypochlorite.

All the plants from which the fungal isolates were obtained were managed uniformly. Sterilized seeds were sown in vermiculite substrate in appropriately sized pots under growth chamber conditions (light/dark cycle of 16/8 h, temperature of 26/18 °C, and 75% relative humidity) and watered with 10% Hoagland solution for 4 weeks. Plant material was then sampled, with leaves, stems and roots surface-sterilized by immersion following this sequence: 70% ethanol for 1 min, 4% sodium hypochlorite solution for 3 min and rinsed three times with sterile distilled water to remove residual ethanol and sodium hypochlorite. The plant tissues were further cut into segments using sterilized scalpels to fit into 90 cm petri plates.

The segments of leaves, stems and roots parts were placed separately on 10% Potato Dextrose Agar (PDA) (Scharlab, Germany) plates, ensuring contact between the tissue and the media, and cultivated at 26 °C in darkness. Daily checks were performed to detect and purify fungal colonies by transferring them to fresh plates. The purified fungal isolates were later preserved by cryopreservation with glycerol, following the method of Ofek-Lalzar et al.[27].

Molecular identification

Fungal mycelium from pure cultures was harvested, and 1 g of fungal tissue was dried for DNA extraction using the CTAB method[28]. PCR was performed using universal primers for the internal transcribed spacer (ITS) region (Sup. Table 1) and GoTaq® DNA Polymerase (Promega Biotech Ibérica S.L.). The PCR protocol was programmed for 35 cycles as follows: Denaturation 15 s at 95 °C, annealing 15 s at 55 °C, extension 30 s at 72 °C. The integrity of the DNA amplicons was verified by agarose gel electrophoresis, followed by PCR purification. DNA samples were then sent to the sequencing service of the *Instituto de Biología Molecular y Celular de plantas (IBMCP)* in Valencia, Spain, for amplicon sequencing. Molecular identification was conducted using the Basic Local Alignment Search Tool (BLAST) from the USA National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi/). Identification was further studied using the UNITE database, including BOLD and INSD datasets as recommended by Refs.[29,30]. In addition, sequences were compared to the potential matches in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) at a threshold value of 0.001. This process was repeated targeting the large ribosomal subunit (LSU) and beta-tubulin (TUB) regions (Sup. Table 1).

A phylogenic tree was constructed using the ITS sequence of the isolate in MEGA ver.11. The analysis aimed to determine the isolate's phylogenetic position among its closest relatives as identified by the aforementioned databases. ITS sequence data from GenBank were used for *Simplicillium* sp., *Leptobacillium* sp. strains, and *Cladosporium herbarum* type as outgroup. The Tamura-Nei model and neighbor-joining (maximum composite likelihood) statistical methods were applied, along with a bootstrap test of 500 replicates.

Morphological characterization

The fungal isolate was maintained in axenic cultures on PDA at 26 °C for 4 weeks and transferred to fresh PDA for subcultures under the same conditions.

The morphological analysis followed the guidelines provided by Watanabe[31], including macroscopic observations of colony morphology (color, texture) and microscopic examination of hyphal structure, spore morphology and germination using a biological microscope.

For the microscopic observations, tissue from the mycelium edges was mounded on glass slides using the Scotch tape method as described by Harris[32]. Additionally, colonies grown in liquid medium (Potato Dextrose Broth, PDB) were examined by placing a small drop on a glass slide. Staining with 0.1% Lacto-fuchsine 1:1 (*Sigma-Aldrich, Germany*) was performed for color contrast when necessary[33].

A conidia solution was obtained by filtering a PDB fungal culture through a sterilized cheesecloth, followed by centrifugation for 5 min at 5500 rpm and 16 °C and resuspension in 0.85% NaCl[22]. Subsequently, 20 µL of the conidia solution was spread on glass slides, with addition of PDB as a nutrition source. These slides were then placed into petri dishes containing 5 mL of sterile 10 mM $MgSO_4$ solution to maintain high humidity. The petri dishes were sealed and incubated at 26 °C for 12 and 24 h before using the glass slides for microscopical observation.

Physiological characterization

Evaluation of growth conditions

Optimal fungal growth conditions were assessed varying key environmental parameters such as light, temperature, pH and nutrient source. Growth rate measurements were taken at different temperatures (ranging from 10 °C to 35 °C) and pH levels (from 3.0 to 8.0). The optimal temperature was calculated based on a non-lineal regression model:

$$Growth\,rate = k \times (T - 9.5)^a \times (35.5 - T)^b$$

where T is the temperature, and a, b, k are the model constants.

Nutrient requirements were assessed between PDA (*Scharlab, Spain*) and maltose extract agar (MEA) (*Scharlab, Spain*). In addition, the industrial PDA (1x, 2x) was compared with homemade PDA, which was prepared by boiling 400 g of white potato in 1L of distilled water for 1 h, filtering the broth through cheesecloth and supplementing it with 20 g/L of sucrose and 15 g/L of agar–agar (*Scharlab, Spain*).

All tests were performed in triplicate (3 replicates for each condition). Media assay was conducted for 21 days, and temperature and pH for 14 days, using fungal plugs of 7 mm from the mycelia margins of 2-week-old solid cultures.

Production of compounds of interest

Indole-3-Acetic Acid (IAA) production was evaluated using the Salkowski colorimetric assay[34]. The fungal isolate was cultured with and without the presence of tryptophan (1% L-tryptophan) in PDB medium for 14-day-old. Salkowski reagent (*Sigma-Aldrich, Germany*) was added to the culture filtrates, and samples were incubated on a shaker in the dark for 20 min for color stabilization. Absorbance was measured at 530 nm, and hormone concentrations were determined using a standard curve prepared with IAA (*Sigma-Aldrich, Germany*) stock dilutions.

The effects on phosphorus macronutrient were assessed on media plates. Insoluble tricalcium phosphates (TCP) were used as the sole source of phosphorus in slightly modified Pikovskaya medium (PVK)[35], commonly used for detecting phosphate-solubilizing microorganisms. After incubation for 2 weeks at 26 °C, the appearance of a light halo around the colonies indicated phosphate solubilization.

Siderophore production was examined using the Fe (III)—chrome azurol (CAS) complex (*Santa Cruz Biotechnology*) colorimetric assay. Layered CAS-media agar plates were prepared according to Andrews et al.[36] to prevent media toxicity. The plates consisted of a CAS layer topped with a layer of PDA, with a fungal plug placed on the PDA layer. Incubation at 26 °C for 2 weeks allowed for observation of color changes in the CAS layer, indicating reduction of Fe (III) to Fe (II).

Effect of fungal isolate in plant development

The effects of the endophytic isolate were evaluated using the original host plant, *S. lycopersicum*. Initially, a test was conducted to assess the viability of the interaction between tomato seedlings and the fungal strain, monitoring the plantlets over week. Afterwards, two experiments were performed under controlled conditions, one in a growth chamber and another in a greenhouse. Each experiment was conducted three times independently. Tomato plants were then evaluated to detect any physiological changes between inoculated and non-inoculated plants.

Plant experiment in growth chamber

The experiment involved three tomato genotypes: ADX2, TH-30 and MO-10. The selected tomato genotypes were based on results found in Fernández-Crespo et al.[25]. A susceptible (TH-30), a neutral (ADX2) and a resistant (MO-10) genotype against heat stress were selected to evaluate its development in interaction with Sl27.

Seeds were surface sterilized with 75% ethanol for 3 min, followed by a 4% sodium hypochlorite solution for 1 min and rinsed three times with sterile distilled water[27]. Clean seeds were then inoculated by immersion in a fungal conidia solution for 6 h at 27 °C on a rotation shaker. The conidia solution, obtained from liquid culture in PDB, was adjusted to a concentration of 10^5 spores/mL, as determined by Neubauer chamber counting. Control plants underwent the same procedure but were treated with sterile distilled H_2O instead of conidia solution. After the inoculation period, seeds were dried on filter paper before being sown in pots containing vermiculite substrate. After a germination period of 7 days, plantlets of similar size were selected, ensuring a minimum of 10 biological replicates for each condition and genotype. The plants were grown in the growth chamber and watered with controlled amounts of 10% Hoagland solution for 4 weeks. At the end of the experiment, the following growth parameters were measured: Root length, root fresh and dry weight, basal diameter, stem length, stem fresh and dry weight, and chlorophyll content.

Plant experiment under greenhouse conditions

This experiment was conducted with two tomato genotypes, TH-30 and MO-10. These genotypes were chosen based on their distinct growth responses observed in the growth chamber experiment, with ADX2 genotype exhibiting intermediate results.

The seeds underwent the same procedure as in the previous experiment. Following the germination period, selected plantlets were transferred to individual pots containing a mixture of peat and perlite (4:1) and placed in the greenhouse, ensuring 10 plants for each genotype and condition. After one day of acclimation, 1 mL of conidia solution was re-inoculated onto the root surface of the plantlets through irrigation. Periodic measurements were taken for aerial growth parameters: length, number of main leaves, flowers and fruits. The plants were allowed to grow for 3 months. Fully ripe fruits were collected weekly, quickly frozen and stored at − 20 °C until further analysis. Root length and weight measurement were taken on the last day of the experiment.

For lycopene quantification, fruits were analyzed using spectrophotometry at 503 nm, following the steps in Anthon & Barrett[37]. Tomatoes were diced and 200 g of each genotype was homogenized using a blender (1500W) at low speed and rounds of 30 s to prevent heating. Tomato juice was obtained by straining through a fine sieve (0.1 mm) and stored in the dark at 4 °C until analysis. 10 replicates were prepared using 100 µL of juice for each sample. A solvent solution with a 2:1:1 ratio of hexane, ethanol, and acetone was prepared. Samples were

sealed and incubated in the dark for 1 h at 150 rpm on an orbital shaker. Distilled water (1 mL) was added to facilitate phase separation in the samples before measurement.

Inoculation and colonization success rate

To verify the presence of the endophyte in the inoculated plants, several roots and leaves from both control and inoculated plants were sampled. DNA extraction and PCR amplification were carried out as stated previously in this work, using specific designed primers for the fungal isolate (Sup. Table 1). Sample DNA extracted from plant tissue was compared to purified fungal strain DNA to determine the presence of the endophyte in inoculated plant tissue and its absent in control plant tissue. In addition, the ability to colonize another Solanaceae species such as *Nicotiana benthamiana* without showing pathogenic behavior was examined.

Results

Endophyte characterization

Molecular identification

The fungal isolates Sl27 and Sl33, isolated from traditional Spanish tomato genotypes (Cor de Bou and ADX-2), returned the same molecular identification. Following work with isolate Sl27 confirmed it to belong to the genus *Leptobacillium* (Table 1), formerly classified in *Simplicillium*, with *Leptobacillium leptobactrum* as the closest match. However, phylogenetic analysis using the maximum likelihood method (Tamura-Nei model) revealed that the isolate occupies a distinct position from both *Leptobacillium leptobactrum* and *Leptobacillium chinense* (Fig. 1).

Morphological characterization

Fungal colonies grown on PDA plates exhibited a white or cream color mycelium on both the upper and reverse sides (Fig. 2a). After 14 days, the mycelium reached 3 to 4 cm in diameter. The texture was dense and difficult to break down, with a velvety appearance and entire margins. In liquid culture, the mycelium formed creamy, smooth spheres (Fig. 2b). A layer of mycelium was formed on the medium's surface suggesting an affinity for aerobic metabolism. Prolonged incubation resulted in the dissipation of mycelium margins or the darkening on the reverse side (Fig. 2c).

The microscopy examination revealed the presence of coenocytic hyphal threads with few ramifications (Fig. 3). Spreading a conidia suspension on glass slides revealed erect, unbranched conidiophores. The conidia were fusiform to cylindrical, measuring 5-8um, with the initial conidium having a more ovate-round shape.

Physiological characterization

Fungal growth of isolate Sl27 was unaffected by light/dark regime, showed a preference for homemade PDA, and was restricted to a temperature range of 15 °C to 30 °C (Fig. 4). Optimal growth was calculated to occur at 24.05 °C ($R^2 = 0.9855$), with a pH range of 3.5 to 5. The isolate could recover after returning to the optimal temperature of 25 °C but did not survive incubation at 35 °C.

Sl27 produced a small amount of IAA (2ug/mL) after 1 week of culture, with production increasing (4ug/mL) when the medium was supplemented with 1% L-tryptophan (Sup. Figure 1). The isolate produced siderophores, reducing Fe (III) in CAS-agar plates, with a visible reduction halo after 2 days and complete reduction after 10 days**.** Sl27 displayed uncertain phosphate-solubilizing ability in the presence of TCP. Additionally, it produced orange pigmentation in both solid and liquid media and exhibited guttation after one week under optimal conditions (Sup. Fig. 1).

Characterization summary

*Taxonomy***:** *Leptobacillium sp.*

Systematic position: Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Cordycipitaceae

Source: *Solanum lycopersicum var.* Cor de Bou, Spain, March 2019

GenBank Accession: SUB14746316 ITS PQ363272

Culture traits: Growth rate of 2 cm/week (slow), mycelium is white to cream (above) and cream to brown (reverse). Mycelial structure is dense. Single conidiophores with fusiform 5-8µm diameter spores.

Optimal growth conditions: PDA medium. Optimal temperature 24 °C and pH 3.5–5. No influence of light/dark regimen.

Characteristics assessment: Low production of IAA, unclear phosphate solubilizing ability, high siderophore production.

Effects on plant growth

In a preliminary short-term assay with tomato plantlets, the fungal isolate Sl27 did not have any negative impact on the plantlets (Sup. Fig. 2), allowing for subsequent growth chamber and greenhouse experiments.

Growth chamber experiment

The influence of isolate Sl27 on seed-inoculated plants in the growth chamber generally led to growth enhancement to varying degrees, depending on the genotype (Fig. 5, Sup. raw data). TH-30 showed significant improvement in shoot and root fresh and dry weight, root length, shoot basal diameter, and chlorophyll content for $p < 0.05$. However, the improvement in these parameters was not always significant in ADX2 and MO-10.

Top 10 matches in NCBI (Genbank database)								
	Scientific Name	**Max Score**	**Total Score**	**Query Cover**	**E value**	**Per. Ident**	**Acc. Len**	**Accession**
ITS	*Gloeotinia sp.*	911	911	98%	0	99.8	599	MK267767.1
	Hypocreales sp.	911	911	98%	0	99.8	517	MG543967.1
	uncultured fungus	911	911	98%	0	99.8	547	KX430917.1
	Ascomycota sp.	911	911	98%	0	99.8	528	KX953318.1
	Hypocreales sp.	911	911	98%	0	99.8	562	KY471669.1
	Hypocreales sp.	911	911	98%	0	99.8	574	KY471668.1
	Leptobacillium leptobactrum var. calidius	911	911	98%	0	99.8	518	KU382173.1
	uncultured fungus	911	911	98%	0	99.8	584	KU164758.1
	uncultured fungus	911	911	98%	0	99.8	585	KU164747.1
	uncultured fungus	911	911	98%	0	99.8	583	KU164733.1
LSU (fw)	*Leptobacillium leptobactrum*	1109	1109	100%	0	100	896	MW493117.1
	Leptobacillium chinense	1109	1109	100%	0	100	927	NG_069745.1
	Leptobacillium chinense	1109	1109	100%	0	100	861	MH871193.1
	[Verticillium] insectorum	1109	1109	100%	0	100	826	KX425621.1
	Leptobacillium chinense	1109	1109	100%	0	100	841	MH871535.1
	Leptobacillium chinense	1109	1109	100%	0	100	825	MT974414.1
	Simplicillium sp.	1109	1109	100%	0	100	901	OL871271.1
	Leptobacillium chinense	1109	1109	100%	0	100	901	MH870810.1
	Leptobacillium leptobactrum	1103	1103	100%	0	99.83	897	KJ130983.1
	Leptobacillium leptobactrum	1103	1103	100%	0	99.83	931	ON834394.1
β-TUB (fw)	*Hypocreales sp.*	523	523	100%	5E-144	99.65	360	KY488510.1
	Gloeotinia sp. LJC-2011	523	523	100%	5E-144	99.65	617	JN393557.1
	Aquanectria filiformis	298	298	100%	3E-76	85.57	604	KX611499.1
	Sarocladium dejongiae	287	287	100%	7E-73	84.75	680	MK069406.1
	Mariannaea elegans	287	287	100%	7E-73	85.47	516	KX986145.1
	Mariannaea chlamydospora	281	281	100%	3E-71	85.12	539	KX986147.1
	Sarocladium sasijaorum	278	278	95%	4E-70	85.05	577	MW890144.1
	Calonectria clavata	268	268	100%	3E-67	84.08	522	DQ190548.1
	Calonectria clavata	268	268	100%	3E-67	84.08	515	DQ190547.1
	Calonectria clavata	268	268	100%	3E-67	84.08	523	AF333396.1

TOP 10 matches in UNITE (BOLD + INSD databases)								
	Scientific name	Score	Prcnt	E-value	Rstart	Rend	MisM	Reference
ITS	*Leptobacillium leptobactrum*	847.9	99.8%	0.0	54	549	1	OW987708 (https://app.plutof.ut.ee/sequence/view/13129106)
	Leptobacillium leptobactrum	847.9	99.8%	0.0	58	553	1	OW985233 (https://app.plutof.ut.ee/sequence/view/13105597)
	Leptobacillium leptobactrum	847.9	99.8%	0.0	68	563	1	OW983854 (https://app.plutof.ut.ee/sequence/view/13106974)
	Hypocreales	847.9	99.8%	0.0	32	527	1	OP874684 (https://app.plutof.ut.ee/sequence/view/16300425)
	Gloeotinia	847.9	99.8%	0.0	45	540	1	OK663505 (https://app.plutof.ut.ee/sequence/view/13087538)
	Gloeotinia	847.9	99.8%	0.0	54	549	1	MZ422981 (https://app.plutof.ut.ee/sequence/view/12178013)
	Verticillium	847.9	99.8%	0.0	18	513	1	MW776224 (https://app.plutof.ut.ee/sequence/view/6077398)
	Leptobacillium leptobactrum	847.9	99.8%	0.0	18	513	1	MW776222 (https://app.plutof.ut.ee/sequence/view/6077400)
	Verticillium	847.9	99.8%	0.0	32	527	1	MW760836 (https://app.plutof.ut.ee/sequence/view/6132590)
	Verticillium	847.9	99.8%	0.0	43	538	1	MW760823 (https://app.plutof.ut.ee/sequence/view/6132603)

Table 1. Top 10 matches for ITS, LSU, and β-TUB region sequences from the NCBI (GenBank) database, along with the top 10 matches for ITS region sequences from the UNITE (BOLD + INSD) databases. The matches are ranked based on sequence similarity and alignment scores. The table includes the accession numbers, species names, percentage identity, and alignment lengths.

Greenhouse experiment

Growth parameters in the greenhouse experiment focused on plant height and the number of leaves, flowers and fruits (Fig. 6, Sup. raw data). The number of leaves (counted as new apical sprouts) and flowers (counted as flowering buds) increased in 7-week-old tomato plants inoculated with Sl27, especially in TH-30. Height improvement was consistent over time and not restricted to a particular growth stage, while a notable sprout spurt was observed in the second week for inoculated plants. The fructification stage was not significantly altered by the fungal endophyte, but fruits from inoculated plants had higher levels of lycopene in both tomato varieties.

Inoculation success rate

The presence of the fungal endophyte was confirmed in all inoculated roots through PCR (Sup. Fig. 3). Sl27 was also detected in some leaf tissues of inoculated plants and was absent in the roots and leaves of non-inoculated plants. In addition, Sl27 successfully colonized *Nicotiana benthamiana* roots without adversely affecting plant growth and health.

Discussion

Isolate Sl27: an endophytic strain of *Leptobacillium sp.*

Most of the research on the efficacy of endophytic fungi on plants focuses on certain species, with about half of the studies in the last 25 years focusing on Poaceae[13]. These circumstances leave many endophytic fungi understudied, presenting a promising research area for sustainable agriculture.

Among the less studied genera, *Leptobacillium* has gained interest recently. Initially, this genus was described including only the single species *Leptobacillium leptobactrum*[38], previously known as *Verticillium leptobactrum*. Subsequently, several species have been described in various environments, such as *L. cavernicola* from paleolithic cave walls[39] and *L. symbioticum* from decayed plant tissue[40]. Some species previously assigned to the related genus *Simplicillium* were reclassified as *Leptobacillium*, including *L. coffeanum*[41] and *L. filiforme*[42], increasing the genus's species count. Notably, some of these species, isolated from healthy plant tissues, have demonstrated beneficial effects such as nematode resistance[43] and pathogen growth inhibition via volatile emissions[41].

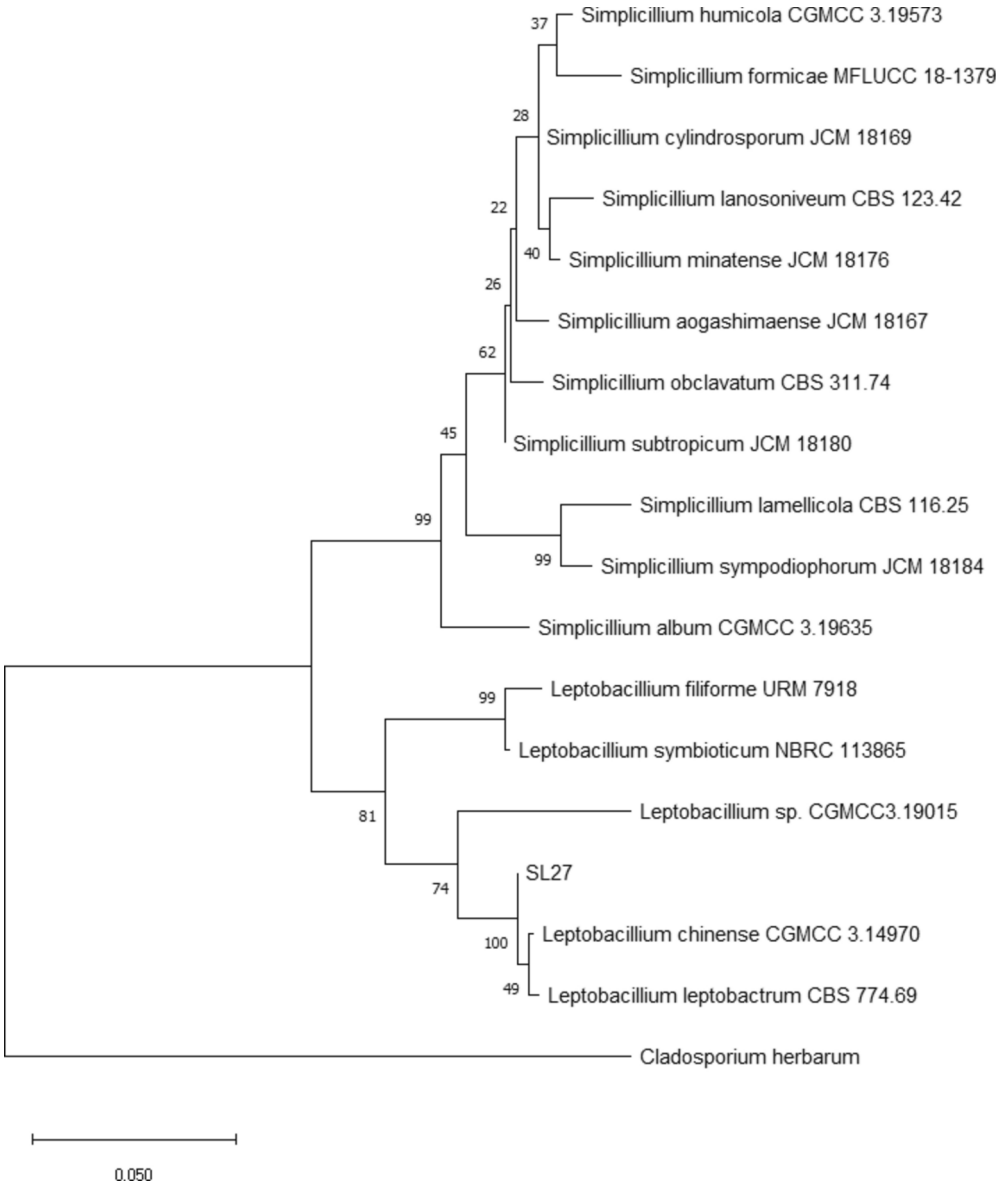


Fig. 1. Evolutionary analysis of isolate Sl27 seen as phylogenetic position calculated by Maximum Likelihood method (Tamura-Nei model). Taxa clustered together is shown in percentage and branch length is measured in number of substitutions per site.

In this study, the BLAST analysis of the fungal strain Sl27, obtained from healthy tomato roots of the Cor de Bou genotype, indicated close resemblance to species in the *Simplicillium* and *Leptobacillium* genera. Given that *Simplicillium* species are mostly entomogenous[39], Sl27 may belong to *Leptobacillium*. The mycelia of Sl27, in terms of color, texture, and slow growth rate (1–2 cm/week), closely matched those in the *Leptobacillium* genus. Microscopic structures, such as solitary conidiophores and microconidia, resembled *L. leptobactrum,* further supported by molecular identification and phylogenetic analysis. Like Sl27, *L. leptobactrum* has been reported from plant tissues[38]. However, Sl27 differed in its ability to grow at 30 °C, optimal conditions and spore characteristics from previously described species[40,41,44,45]. Further analysis is being conducted to clearly identify isolate Sl27, potentially representing a new strain of *Leptobacillium* sp.

Endophytic fungi are highly diverse and influenced by environmental factors[2,46,47]. Thus, experimental conditions such as light, temperature and pH are crucial in assessing fungal behavior and host plant interaction[48]. Sl27 grew best at 25 °C on nutrient-rich media (homemade PDA or double industrial PDA) to show the same growth of other faster-growing fungal species, and had optimal growth at pH 3.5–5. These conditions align with other fungi, and the growth characteristics of Sl27 are similar to previously reported *Leptobacillium* strains, indicating that Sl27's slow growth is not due to suboptimal culture conditions, and may be a trade-off for the ability to produce some antimicrobial compounds[49]. In addition, most plants grow in slightly acidic to neutral pH, which could be relevant for a plant-microorganism interaction to occur[50,51].

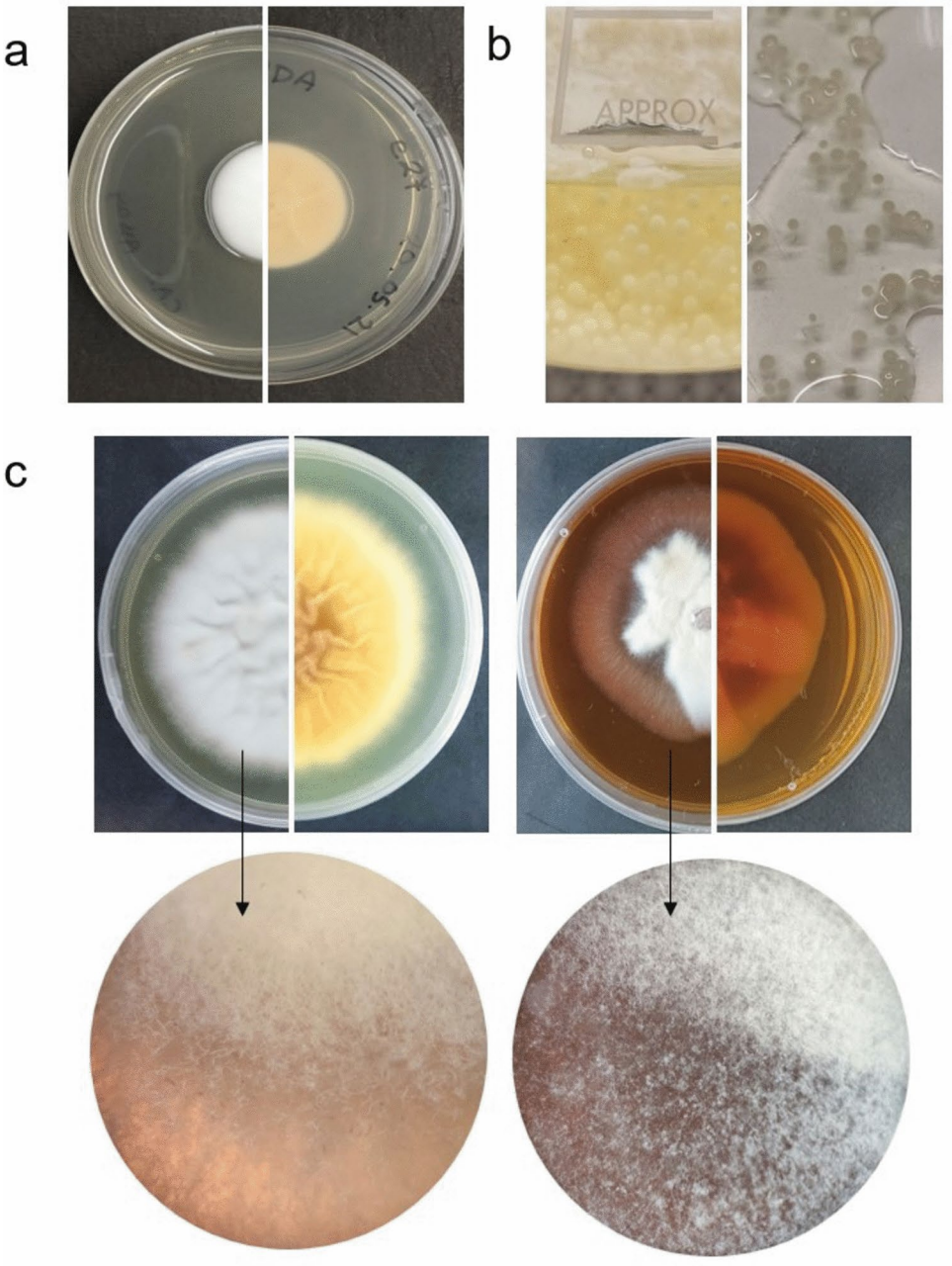


Fig. 2. Macroscopic features of isolate Sl27 in different growth conditions. (**a**) Front and reverse view of culture grown in normal conditions on a PDA plate. (**b**) Mycelia spheres produced in liquid culture under constant agitation (150 rpm). (**c**) Front and reverse view of cultures grown for over 2-week periods with magnifying glass observation of mycelial margins.

Sl27 synthesizes indole acetic acid (IAA) when supplemented with L-tryptophan, indicating potential for plant growth promotion, though production is low. This trait, combined with its ability to produce siderophores for binding insoluble iron (III), could benefit the host plant under nutrient stress[52]. Despite its slow growth, siderophore production was observed as early as day 2. While many soil microorganisms possess this ability, beneficial effects are primarily reported for bacteria[53,54] rather than fungal endophytes.

Inoculation with Sl27 can improve plant development

The study of plant growth-promoting fungi is significant due to their ability to enhance plant health and resistance to pathogens. One of the most well-studied growth-promoting endophytes is *Serendipita indica*, a basidiomycete isolated from soil that confers various beneficial effects on the host plant including growth promotion and stress resistance[55]. Similarly, dark septate endophytes are frequently reported as growth-promoting species in plants like cabbage, chili and tomato[21,56,57].

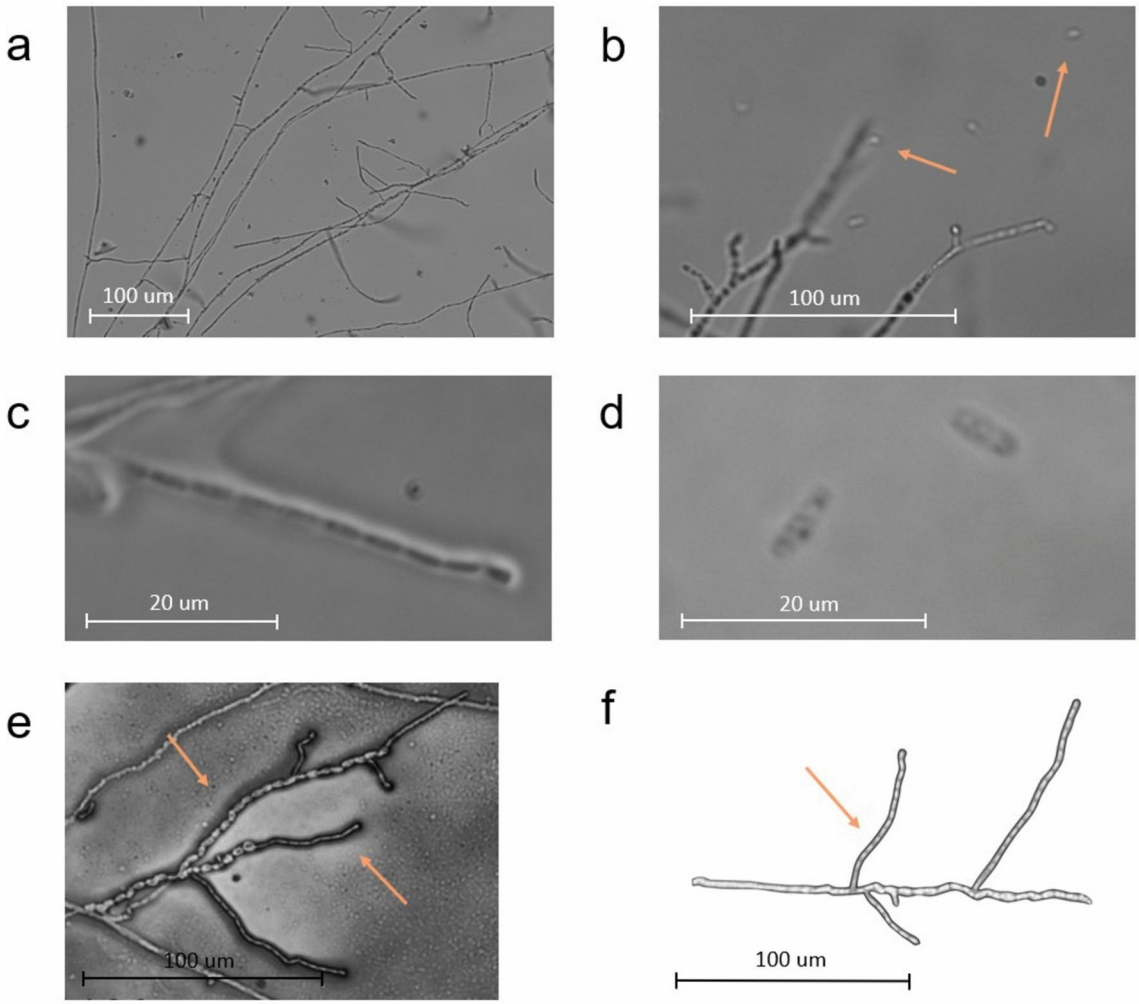
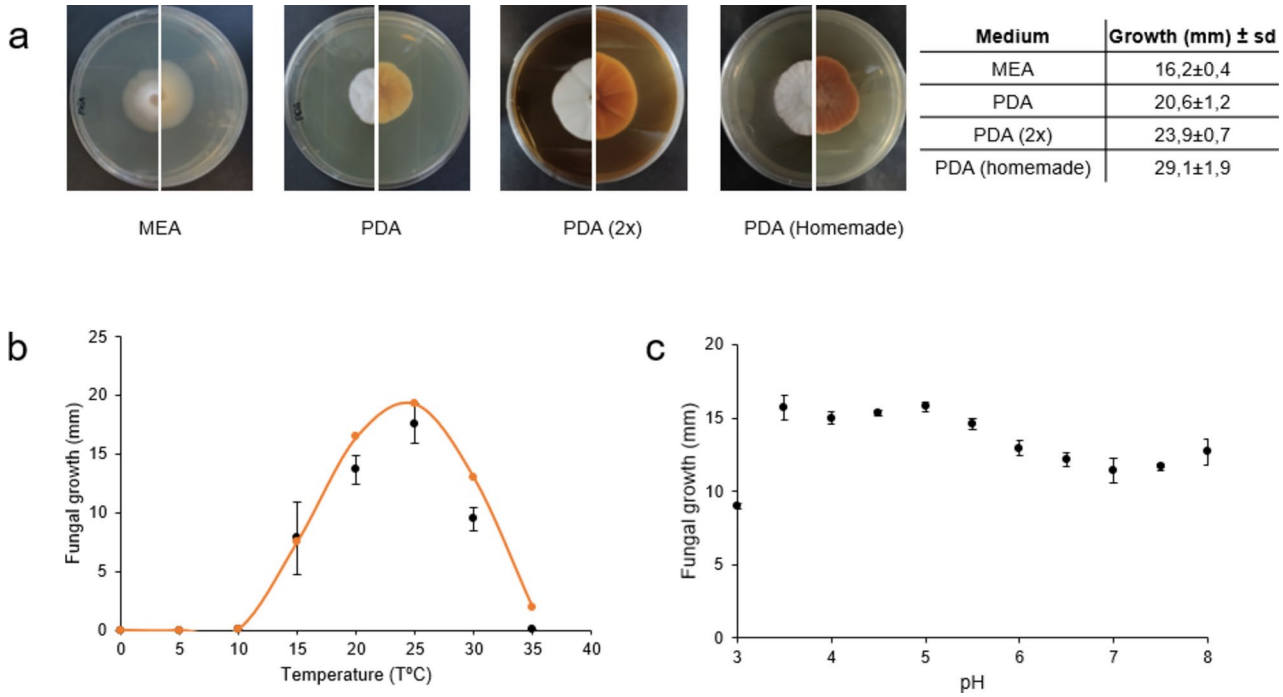


Fig. 3. Morphological characterization of isolate Sl27 at the microscopic level, with images converted to black and white for enhanced contrast: (**a**) Normal mycelial structure; (**b**) Colony edge detail with presence of conidia; (**c**) Conidiophore detail; (**d**) Conidia; (**e**) Conidiophore structure at double contrast with distinction between phialide and conidial chain; (**f**) Conidiophore representation with long conidial chain.

To study the interaction between the endophytic fungal strain Sl27 and tomato plants, and to evaluate its potential for promoting plant growth, we assessed how Sl27 affects host plants under controlled conditions. It was crucial to confirm that Sl27 behaves as an endophyte and does not exert pathogenic effects on plant development. We hypothesized that Sl27 would exhibit neutral or positive effects on the host plant, based on previous reports of endophytes isolated and inoculated in the same host species[58]. The preliminary assay showed no notable effects on plants in the presence of Sl27. While the effects of fungal endophytes might not be visible in the early stages of plant development, differences may arise at later stages or when plants are under stress[22].

The development of seed-inoculated tomato plants was either positive or neutral, suggesting a mutualistic relationship between the isolated fungus and its host plant species. The growth parameters of the TH-30 genotype were significantly improved in the growth chamber experiment. Previously categorized as heat-sensitive[25], this genotype exhibited enhanced growth following inoculation with Sl27. The specificity of the effects of the fungal isolate on different tomato genotypes may be related to the greater phenotypic sensitivity of TH-30, particularly to heat stress. Inoculation with Sl27 also improved parameters such as chlorophyll content in the leaves and lycopene content in the fruits. Filamentous fungi are known sources of carotenoid pigments, which are often related to secondary metabolites with biological activity[59]. The use of microbial inocula to enhance quality traits in tomato fruit production has not been well-explored, although a significant increase of this carotenoid compound is of interest for industrial applications and crop management. The promotion of morphological parameters such as plant and root length varied across the genotypes and experiments. However, in greenhouse experiments, a trend toward improved was noted for both TH-30 and MO-10 genotypes. Improvements in root structure, fructification and tomato lycopene content suggest that the endophyte plays a role in enhancing root structure to facilitate improved nutrient acquisition, possibly through mechanisms related to the production of siderophores and other bioactive compounds. This enhanced nutrient uptake may increase, at least, the TH-30 genotype's resistance to high temperatures and other abiotic stresses associated with climate change.



Medium	Growth (mm) ± sd
MEA	16,2±0,4
PDA	20,6±1,2
PDA (2x)	23,9±0,7
PDA (homemade)	29,1±1,9

Fig. 4. Growth pattern of 2-week-old cultures of isolate Sl27 under different conditions: (**a**) Several media: MEA, PDA, 2 × PDA, homemade PDA; (**b**) Several temperatures (10 °C–35 °C) with growth model curve; (**c**) Media pH ranging from acidic (3) to slightly alcaline (8).

The variability found in the experimental results may be actually common in many endophytes. For instance, Hoyos et al.[60] studied several isolates of *Trichoderma* sp., known as biocontrol agents and growth promoters, and found variation in their ability to produce growth-promoting compounds like auxins, with no clear correlation to growth promotion in plants. Similarly, Attia et al.[61] studied several plant growth-promoting fungi isolated from rhizosphere soil and their effect on conferring resistance to tomato against Fusarium wilt, finding improved photosynthetic pigments but variable effects on plant morphological indicators.

In addition, experimental factors, such as differences between growth chamber and greenhouse conditions and seasonal variations, may contribute to the variability of plant responses[58]. Factors like substrate pH can influence plant response and interaction with endophytes. Thus, in this study we demonstrated the endophytic behavior of this novel fungal isolate Sl27 by confirming it does not negatively affect plant development and observing its potential beneficial influence on growth. Further studies are needed to determine specific beneficial functions this endophyte could confer on tomato plants, which could lead to new agronomically relevant practices.

Conclusion

The novel endophytic fungal strain Sl27 has been confirmed to act as an endophyte in tomato roots, where it was originally discovered. While phylogenetic analysis and morphological characterization did not lead to a definitive identification, most results suggest that it is closely related to *Leptobacillium leptobactrum*. To the best of our knowledge, this is the first time this species has been studied as an endophyte in an agronomically important crop. Inoculating tomato seeds with Sl27 resulted in beneficial effects under controlled conditions, including increased plant height, enhanced chlorophyll content, and elevated lycopene levels in the fruits. These advantages were particularly prominent in the genotype TH-30, and no detrimental effects were observed over the course of 8 weeks. Given that TH-30 is sensitive to heat stress, further investigation is required to determine whether the growth enhancement associated with Sl27 contributes to improved tolerance to abiotic stress. This discovery opens new opportunities for exploration in the research field of beneficial microorganisms and is significant for both the biological study of plant-microbial interactions and sustainable agricultural management, as it has the potential to improve plant health and reduce the need for chemical inputs in the context of climate change.

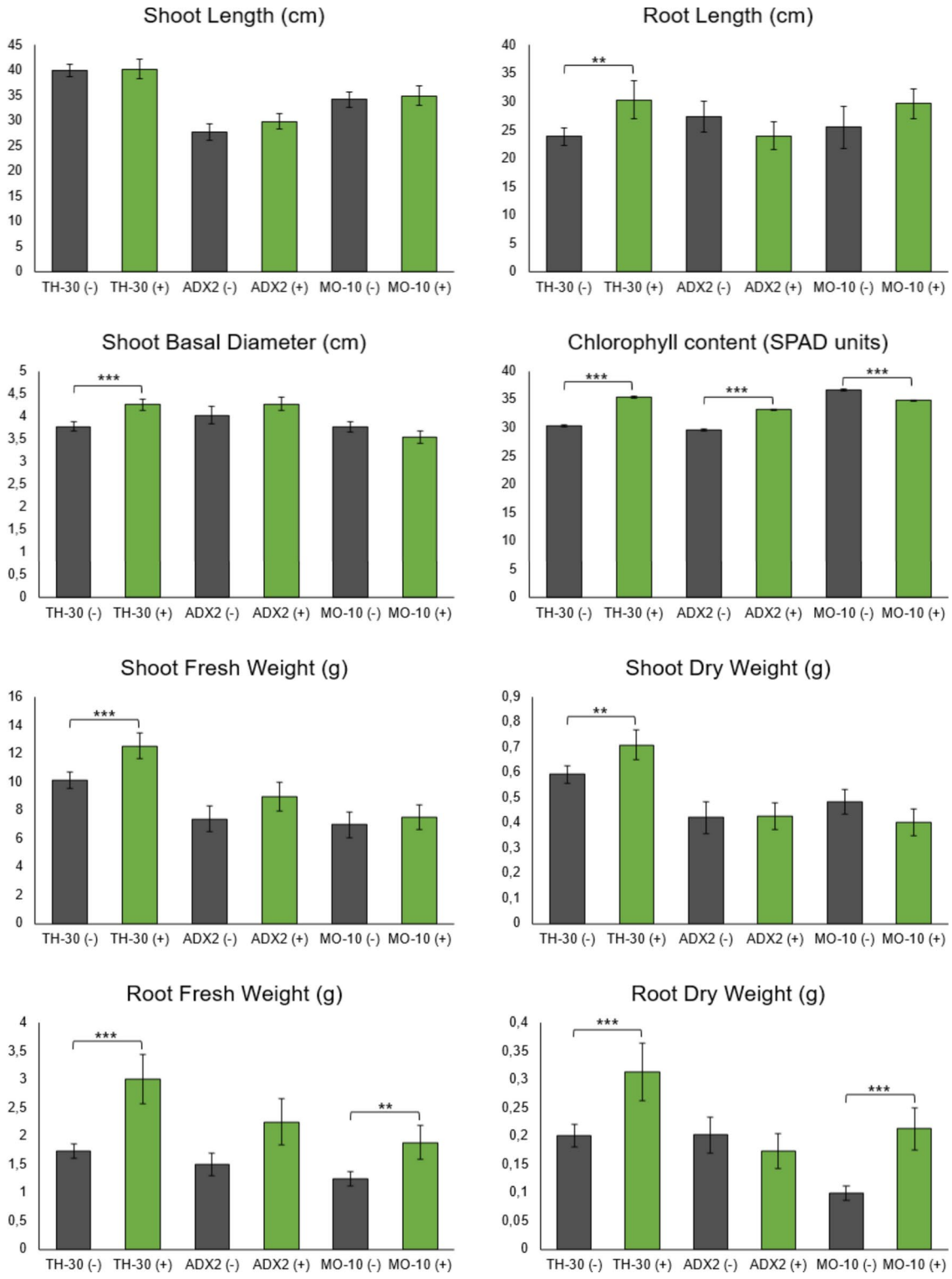


Fig. 5. Growth parameters for non-inoculated (−) and inoculated with Sl27 (+) tomato plants of 4 weeks under normal conditions in the growth chamber. Study parameters are as follows: Shoot Length (cm); Root Length (cm); Shoot basal diameter (cm); Chlorophyll content of leaves (SPAD units); Shoot fresh weight (g); Shoot dry weight (g); Root fresh weight (g); Root dry weight (g). Statistical significance of differences between groups was determined using ANOVA: (*) $p < 0.1$, (**) $p < 0.05$, (***) $p < 0.01$.

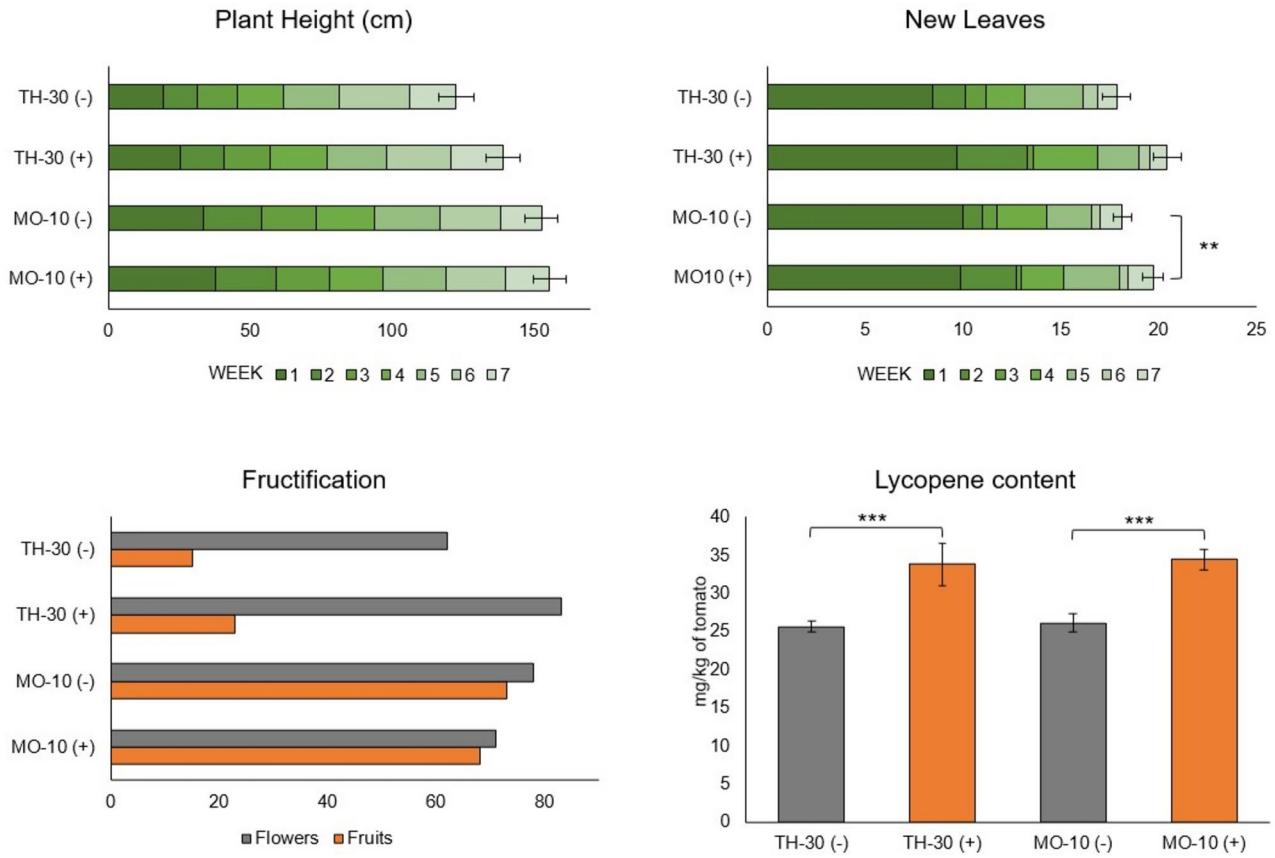


Fig. 6. Growth parameters for non-inoculated (−) and inoculated with Sl27 (+) tomato plants of 7 weeks under normal conditions in the greenhouse: Plant height increase for each week; Number of main leaves counted as new apical leaves for each time point; Number of identified flowers and fruits; Lycopene quantification from tomato juice. Statistical significance of differences between groups was determined using ANOVA: (*) $p < 0.1$, (**) $p < 0.05$, (***) $p < 0.01$.

Data availability

The data generated or analysed during this study are included within the manuscript and its supplementary information files. The sequence data that support the findings of this study is available in the GenBank repository with the accession number SUB14746316.

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Author contributions

LL-X performed the experiments, interpreted the data, wrote the main manuscript and prepared the figures and tables. BV analyzed and interpreted the data and contributed to the writing of the manuscript. KP supervised the experiments and contributed to the writing of the manuscript. GC contributed to the writing of the manuscript. EL designed the research, supervised the experiments, analyzed the data and contributed to the writing of the manuscript. All authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.L.-X. or E.L.