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Sporormiella isomera - Solanum lycopersicum - Botrytis cinerea: ESCAPE ROOM FOR FRIEND AND FOE BEHAVIOUR

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Abstract

The endophytic fungus Sporormiella isomera strain HTF58 shows potential as a biological control agent against the tomato pathogen Botrytis cinerea. The antagonistic capacity of HTF58 against B. cinerea was evaluated through in vitro and in vivo tripartite bioassays. Establishing sufficient endophyte biomass before pathogen introduction resulted in the strongest antagonistic effect, significantly reducing symptoms and improving seedling growth. However, in vivo trials indicated that simultaneous inoculation of HTF58 and B. cinerea did not confer effective protection but did promote young plants roots growth (fresh weight: HTF58 = 22 ± 10.4 g vs. negative control = 7.6 ± 6.7 g; dry weight: HTF58 = 5 ± 2.45 g vs. negative control = 1.8 ± 1.79 g, $p<0.05$). Colony morphology and expansion of the endophytic S. isomera were influenced by the type of carbon and nitrogen nutrient sources provided. Organic nitrogen sources like D-alanine promoted regular lobular growth (36% increase compared to the minimal synthetic medium, $p<0.05$) and enhanced pigment production. S. isomera HTF58 exhibits plant growth-promoting traits, with pre-inoculation being a key factor for successful pathogen suppression.

Key words*: biocontrol, biologicals, fungal ecology, plant protection, tomato.*

INTRODUCTION

Nearly all plants rely on symbiotic relationships with fungi, which provide numerous benefits, such as improved nutrient acquisition, increased tolerance to abiotic stress, and enhanced resistance to pathogens (Saikkonen et al., 1998; Rodriguez et al., 2009; Boiu-Sicuia & Cornea, 2020; Zhou et al., 2022). These interactions can significantly influence plant health, productivity, diversity, and interactions with phytopathogens, which has drawn considerable interest in ecological research (Van Bael et al., 2012). The complexity of plant-endophyte interactions, along with the evolutionary and functional aspects of the communities involved, suggests that using endophytes to enhance plant defence mechanisms is a promising strategy for pest and disease management. This approach has potential applications in biological control and sustainable agriculture (Giménez et al., 2007). Endophytes isolated from medicinal plants are of particular interest due to their ability to synthesize secondary metabolites like those of their host plants (Strobel, 2003; Kusari et al., 2008). Additionally, their biosynthetic potential to produce antibiotic, antiparasitic, antifungal, and insecticidal compounds, which effectively inhibit the growth of other microorganisms, including plant pathogens, highlights their biotechnological importance (Schulz et al., 2002; Saikia et al., 2022). Following the rationale for the collection of plants for endophyte isolation and natural product discovery proposed by Strobel et al. (2004), *Artemisia thuscula* is an endemic species of Canary Islands, with an ethnobotanical history (Benjumea et al., 2005), therefore highly interesting in the search for endophytes displaying bioactivity (Coşoveanu et al., 2012; Coşoveanu et al., 2018). Derived from the rationale of Strobel et al. (2004) where plants with unconventional biology may be considered as promising source of compounds, fungi isolated from unconventional setting may be subject of interest. *Sporormiella* is a genus of ascomycete fungi in the family Sporormiaceae (Pleosporales), encompassing over 80 species distributed in subboreal and temperate regions worldwide. While most species are coprophilous (found obligatory living on dung of livestock and wild herbivores (González-Menéndez et al.,

2017; Kruys & Wedin, 2009), some have been identified as saprobic or, in rare cases, as plant endophytes (Arenal et al., 2007; Doveri, 2004; Gauchan et al., 2020; González-Menéndez et al., 2017; Kumaresan et al., 2013; Leyte-Lugo et al., 2013; Li et al., 2007; Suryanarayanan et al., 2018; Yang et al., 2016). This converts at least some of the species of the genus as fimiculous (optionally live on dung but that are also able to develop on other substrates as well). Coprophilous fungi can produce a variety of bioactive specific metabolites (Bills et al., 2013; Sarrocco, 2016), involved in defence mechanisms against other competing microorganisms (Bills et al., 2013). Specifically, the *Sporormiella* genus is known to produce a variety of secondary metabolites, including xanthones, chromones, macrolide lactones, organic acids, triterpenoids, steroids, and nitrogen-containing compounds (Chen et al., 2021; Gonzalez-Menendez et al., 2017; Leyte-Lugo et al., 2013). Previous works have underlined the ability of the coprophilous and fimiculous groups may have to exploit and compete for nutrients, hence the antagonistic interactions (Sarrocco, 2016). *Botrytis* genus currently includes 35 species (Bi et al., 2023), with most members acting as necrotrophic pathogens. These fungi induce the death and lysis of host cells to access cellular nutrients (Cheung et al., 2020). *Botrytis cinerea*, one of the most notorious phytopathogenic species, has over 500 host species, mainly dicotyledonous plants, though it also affects some monocots. Due to its scientific and economic significance, *B. cinerea* has been ranked as the second most important plant pathogen worldwide (Pérez-Hernández, 2017; Yusoff et al., 2020; Cheung et al., 2020). Currently, the control of *B. cinerea* relies primarily on chemical methods, with approximately 8% of the global fungicide market dedicated to managing this pathogen (Cheung et al., 2020). However, this approach faces significant challenges due to the increasing resistance of the pathogen, which reduces the effectiveness of fungicides, and the risks associated with chemical residues on food safety and environmental sustainability (Wang et al., 2019). As a result, new strategies for the biological control of *B. cinerea* are being developed, including the use of antifungal biological agents such as oils, phytohormones, antifungal proteins, and peptides (Aumer et al., 2020; Kou et al., 2023). These alternatives aim not only to improve the efficacy of control but also to minimize the negative impacts on food safety and the environment. Among the economically important crops impacted by *B. cinerea* are the tomatoes (Bikdeloo et al., 2021). In addition to its agricultural importance, *Solanum lycopersicum* serve as model plants for studying fruit growth, development, ripening, and plant-microbe interactions (Delian et al., 2021).

Within the present study we propose i) to gain insights on the nutrient modulation of fungal culture morphology and expansion of *Sporormiella isomera* strain HTF58 and ii) to evaluate the beneficial potential this strain may have for tomato seedlings in absence/presence of the phytopathogen *Botrytis cinerea* using scenarios designed to simulate real-life conditions. The hypothesis of this study was that if the endophyte would colonize the seed during the germination process, before the pathogen arrival, seedling development would be enhanced, and pathogen-induced symptoms would be reduced.

MATERIALS AND METHODS

Endophytic fungal isolation

The endophytic fungus strain HTF58 was isolated from the stem of an apparently healthy individual of *Artemisia thuscula* Cav. from Tenerife, Canary Islands (Coşoveanu, 2018). Surface sterilization of plant fragments and incubation for fungal emergence was carried out as previously detailed (Coşoveanu, 2018) in the laboratory of the Unit of Phytopathology (CIPEV group - Integrated Control of Plant Pest and Diseases) at the Universidad de La Laguna.

DNA extraction, amplification, and sequencing

DNA extraction, amplification, and sequencing of HTF58 were performed at the SEGAI Sequencing Service (La Laguna, Spain) using the E.Z.N.A. Fungal DNA Kit (OMEGA Bio-Tek, Norcross, Georgia, USA). PCR conditions followed those previously described by Coşoveanu et al. (2018). Molecular identification was performed with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4

(5′-TCCTCCGCTTATTGATATGC-3′) primers to amplify the 5.8S ribosomal DNA and the internal transcribed spacer (ITS1 and ITS2) regions (White et al., 1990). Also, the nu-LSU-362-fw (5′-GCGCACAAGTAGAGTGATC-3′) and LR5 rev (5′-TCCTGAGGGAAACTTCG-3′) primers were used to amplify the nuclear large ribosomal subunit (LSU) of rDNA (Galloway, 2000; Vilgalys, 2005). Sequences were analysed using the BLASTN search tool with the Megablast algorithm on the NCBI platform and the MycoID tool in BioloMICS software with reference databases FunCBS and MIRRI available through Mycobank where the most similar hits were obtained.

Dual culture assay

An antagonism assay was performed to evaluate the inhibitory activity of the endophytic fungus HTF58 against *Botrytis cinerea* according to the previously detailed procedure (Coşoveanu et al., 2016). The *B. cinerea* strain B05.10 (Pérez-Hernández, 2017; Quidde et al., 1999), was kindly provided by the Department of Biochemistry, Microbiology, Cellular Biology, and Genetics of the University of La Laguna, Tenerife.

Optimization of growth and morphological characterization

Endophytic fungal strain HTF58 was cultured on minimal synthetic vitamin agar medium (SM) supplemented with various carbon and nitrogen sources, substituting glucose and KNO_3, respectively (Asina et al., 1977). The base composition of synthetic minimal medium included (per litre): KNO_3 (1 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $CaCl_2$ (0.1 g), trace elements (1 mL), biotin (5 µg), thiamine (100 µg), glucose (2 g), and agar (10 g). The trace element solution was prepared by dissolving 100 mg $CuCl_2$, 200 mg $FeSO_4$, 20 mg $MnCl_2$, 20 mg $NaMoO \cdot 2H_2O$, and 10 mg H_3BO_3 in acidified hydrochloric acid until a clear solution was obtained. The used carbon sources were D-xylose, D-mannose, D-cellobiose, maltose, sorbitol, L-rhamnose, malt extract, L-arabinose, D-galactose, and starch. Nitrogen sources included D-alanine, ammonium nitrate, sodium nitrate, calcium nitrate, urea, and L-glutamic acid. The two vitamins, biotin and thiamine, were substituted in between or eliminated. Colony morphology and average diameter were assessed after two weeks of incubation at 25°C, in darkness.

***In Vitro* Tripartite Assay**

A preliminary assay was conducted to evaluate the capacity of the fungal strain HTF58 to endophytically colonize the new tomato host. The inoculation was carried out by i) simultaneously introducing the seeds with the endophyte and ii) 7 days post-seed introduction of the endophyte on Hoagland medium (further used for the *in vitro* tripartite assay). Root, stem and leaves fragments were used after three weeks of incubation to test the detection of the endophyte. For the isolation process 10 seedlings per treatment were used with four fragments per PGA plate. The plates were incubated in darkness, at 25 °C for one week and emerged colonies were further purified and identified.

The tripartite *in vitro* assay was conducted to evaluate the interaction between tomato seedlings, the endophytic fungus HTF58, and the pathogen *Botrytis cinerea* to test the biological control potential of HTF58, using previously detailed procedure (Coşoveanu et al., 2021) with the following modifications. Briefly, sterilized commercial tomato seeds of the DELOS F1 variety (Fitó, 2021) were used as plant host. The endophytic strain HTF58 inoculum was obtained from a 7 days-old colony, and the pathogenic strain B05.10 inoculum from a 4 days-old colony cultivated on the PGA medium. The pathogen was inoculated at a 2 cm distance from the seed, and the endophyte was placed at 1 cm distance due to its slower growth rate, aiming to synchronize the time of contact between the fungi and the seeds. The order of inoculation for *S. isomera*, *B. cinerea* and seeds was performed in five different time scenarios (Figure 1). In the first strategy (T1), the endophytic fungus was given an advantage by being inoculated first. In T1A, after the endophyte, the pathogen was inoculated, followed by the seed. In T1B, the order between the pathogen and the seed was reversed. In the 2nd strategy (T2) the endophyte, pathogen, and seed were inoculated at the same time. In the 3rd strategy (T3), the seed was inoculated first. In T3A, the seed was inoculated first to allow its development, followed by the

endophyte and subsequently the pathogen. In T3B, after the seed, the pathogen was inoculated, and the endophyte was inoculated last. For each scenario, three controls were maintained: seed and endophyte (C1), seed and pathogen (C2), and a negative control with the seed alone (C3).

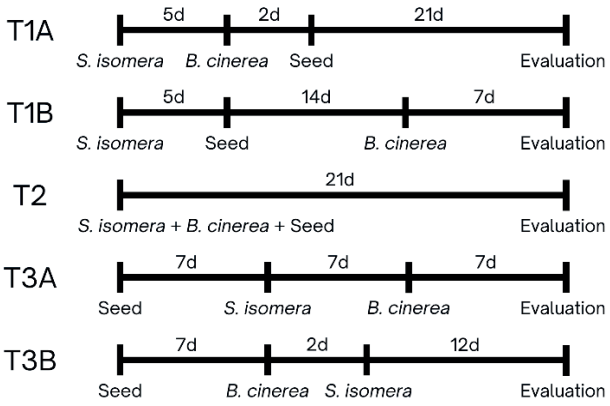

Figure 1. *In vitro* tripartite assay: Inoculation order of *S. isomera*, *B. cinerea* and tomato seeds in each time scenario

Each scenario was conducted in triplicate, with two biological repetitions with randomized configuration. Petri dishes were incubated in a plant growth chamber (Sanyo MLR-351) under a 12:12 hour light/dark regime of 18:25ºC, with 55-60% relative humidity, and 70 µmol m^{-2} s^{-1} light intensity. Seedlings were assessed and scanned 21 days after the introduction of seeds in the tripartite interaction (Figure 1). The assessments on seedlings were carried to evaluate the growth and visible disease and potential phytotoxicity symptoms using scales of two indices. The development index (ID) reflects the developmental stage of the seedlings being: 0 - not germinated, 1 - radicle emergence, 2 - plumule emergence, 3 - complete development with presence of cotyledons or leaves. The symptoms severity incidence was calculated using necrosis index (IN) based on Iacomi et al. (2004) with modifications, being: 0 – healthy; 1 - 1/3 of the radicle damaged; 2 - 2/3 of the radicle damaged; 3 - radicle completely damaged; 4 - seed/seedling completely covered with mycelium/spores (Rodriguez-Sabina et al., 2022). Fresh and dry weights (70°C for 48 h) of radicle and plumule were performed and length of both plant parts was assessed using ImageJ 1.53e (Wayne Rasband, NIH).

***In vivo* tripartite assay**

To determine the optimal natural solid medium for growth, *Sporormiella isomera* and *Botrytis cinerea* were cultured on three different substrates and their combinations, under darkness, at 25°C, for four weeks. The media included oatmeal, wheat, rice, and combinations of oatmeal-wheat, oatmeal-rice, and wheat-rice. For the single-ingredient medium, 10 g of oatmeal or rice flakes and 5 g of wheat bran were used. For the combined media, 5 g of oatmeal or rice and 2.5 g of wheat were used. To each medium, 5 mL of H_2Od water was added, and the mixture was autoclaved (121 °C, 20 min). Each growth substrate was tested in triplicate and assessments were made visually on the colonization capacity of the fungal strains.

The *in vivo* tripartite assay was based on the methodology described by Aydoğdu (2022). Commercial seeds DELOS F1 variety (Fitó, 2021) were sterilized as previously mentioned and were planted in 7 x 7 x 14 cm trays filled with a universal substrate mix containing peat, perlite, and Agrosil® root enhancer (COMPO brand), previously autoclaved. The trays were placed in a plant growth chamber (Sanyo MLR-351) under same environmental condition as the seedlings *in vitro* assay, previously detailed. After 30 days, the substrate of each pot, up to 3 cm deep was removed and replaced with 5 g of each fungal inoculum surrounding the stem of each plant, finally covered by 1 cm of the initial substrate. The inocula of both fungi (colonized natural substrate) was prepared in autoclavable bags with 100 g of oatmeal and 50 g of wheat, which were autoclaved, and 100 mL of sterile H_2Od water was added. Each bag was inoculated with 15 mycelial plugs of 4 mm in diameter and incubated at 25 °C for 21 days. The experimental treatments were as follows: i) HTF58, ii) HTF58 + *Botrytis*, iii) oat-wheat (negative control) and iv) *Botrytis* (positive control). Twenty-five plants were used per treatment. The trays were rotated every three days to homogenize growth conditions. Twenty-five days post-inoculation, plants were evaluated for growth and health indicators. Measurements included plant height, fresh and dry biomass of both shoot and root systems (15 random plants were used for the weigh; values are expressed as weight/plant sample). A visual assessment of overall plant vigour, stem collar

symptoms, root condition (root rot or discoloration), and whether plants maintained an upright posture, indicating structural health.
To confirm the presence of the inoculated fungi, classical isolation methods were performed using stem and root samples from five random plants per treatment, with three fragments plated per dish with PGA. Each organ was sampled in triplicate.

Statistical analysis
For the characterization of colony morphology based on carbon and nitrogen sources, ANOVA was conducted using JASP v.0.15 to assess the differences among nutrient complex groups and their effects on fungal growth. Significant differences between groups were further examined with Tukey post-hoc multiple comparisons test. Additionally, correspondence analysis (CA) was performed using PAST v.3.18 to explore associations and evaluate the strength of relationships between nutrient complexes and colony morphology.
For the *in vitro* and *in vivo* tripartite assays, treatment groups were compared based on fresh and dry biomass, as well as root and plumule length, and Development Index (ID) and Necrosis Index (IN), using ANOVA when the assumptions of homogeneity of variance and normality were met, followed by Tukey post-hoc test, to identify significant differences; otherwise, the Kruskal-Wallis test was applied, followed by Dunn test with Bonferroni correction for multiple comparisons. All statistical analysis were conducted at a significance level of 0.05.

RESULTS AND DISCUSSIONS

Molecular identification of *S. isomera* strain HTF58
Both ITS and LSU regions sequences obtained from the endophytic fungus strain HTF58 (NCBI library under references PP886655 and PP886654, respectively) showed 100% similarity with the reference strain CBS 166.73, identified as *Sporormiella isomera* S.I. Ahmed & Cain (Vu et al., 2019).

Dual culture assay
The endophytic fungus HTF58 showed high antagonistic activity against *Botrytis cinerea*, inhibiting mycelial growth at 0.74 cm indicating significant potential for biological control of this pathogen (Figure 2).

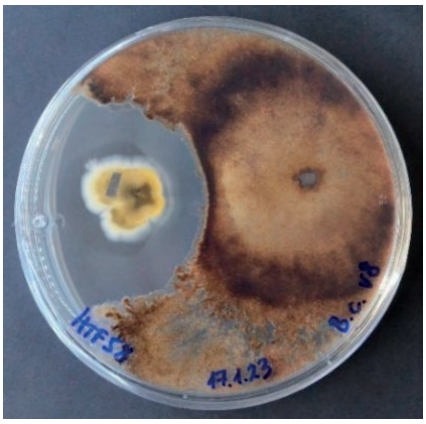

Figure 2. Dual culture assay with *S. isomera* strain HTF58 (left) and *B. cinerea* B05.10 (right)

Effect of carbon and nitrogen sources on colony morphology and diameter
Three nutrient source combinations were selected to assess their effects on the morphology and diameter of the colony of the endophytic fungal strain HTF58: i) multiple carbon and organic nitrogen sources, ii) multiple carbon and inorganic nitrogen sources, and iii) combinations of glucose with organic/inorganic nitrogen sources (Rodriguez-Sabina et al., 2019). Colony morphology was classified into six distinct categories: circular shape, lax growth, lobular growth, irregular edge growth, pigment production, and arbuscular growth (Figure 3). Irregular edge growth of colony was not observed in medium with mannose or malt extract (which contains mannose). Only irregular edge with lax growth of colony was observed under a single source of carbon and none of inorganic nitrogen. Also, in presence of inorganic nitrogen and glucose, no arbuscular growth and lax mycelium were observed. In the correspondence analysis of carbon-nitrogen sources and colony morphology, the first two axes explained 60.57% of the variance (eigenvalues: axis 1 = 0.40, axis 2 = 0.24) (Figure 4). Pigment production was strongly associated with Axis 1 (loading = 0.94), showing a positive correlation with D-mannose (2), D-galactose (10), starch (11), D-alanine (12), and nitrogen sources like sodium nitrate (14) and calcium nitrate (15). Circular growth was positively correlated with Axis 2 (loading = 2.86), reflecting distinct growth patterns under

specific treatments. It was primarily associated with malt extract (8), D-alanine (12), sodium nitrate (14), and no carbon or nitrogen source (24). Lax growth was mostly driven by malt extract (8), urea (16), and the combination of glucose and potassium nitrate (18). Both lax and arbuscular growth were positioned far from Axis 1 and Axis 3 (loadings = -2.06 and -1.66), respectively, indicating that these traits are less frequent in the dataset. Compounds like L-rhamnose and malt extract were distributed across both Axis 1 and Axis 2 clustering near the origin. This suggests these treatments induce intermediate growth patterns or do not dominate a particular morphological trait. Approximately 80% of the fungal colonies produced pigments when organic nitrogen was present, suggesting that organic nitrogen may regulate pigment biosynthesis, potentially linked to secondary metabolite production with antifungal properties or stress resistance (Lomax & Learner, 2006). Strain HTF58 colonies produced both lobular regular growth and pigments when i) basal medium, ii) basal medium without one or both vitamins, iii) nitrates (calcium or sodium), iv) starch or v) D-alanine sources were used. Changes in morphology may be due to altered gene expression related to morphogenesis and secondary metabolite production (Brakhage & Schroeckh, 2011; Bulgakov, 2008).

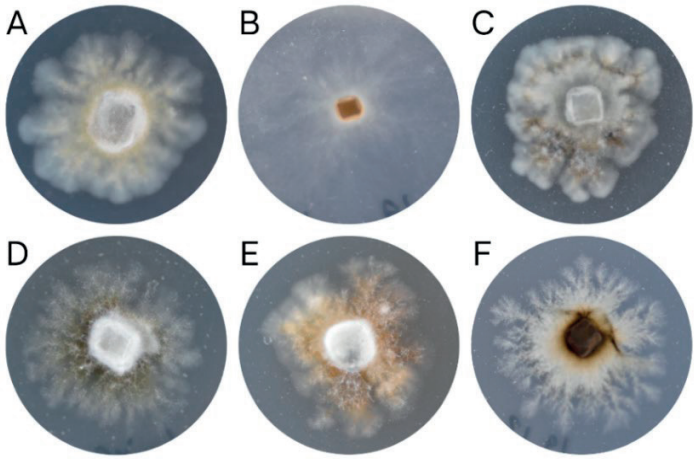

Figure 3. Morphological groups of endophytic fungal strain HTF58, considering all combinations of nutrient elements: A) circular; B) lax; C) lobular; D) irregular border; E) pigment production; F) arbuscular growth

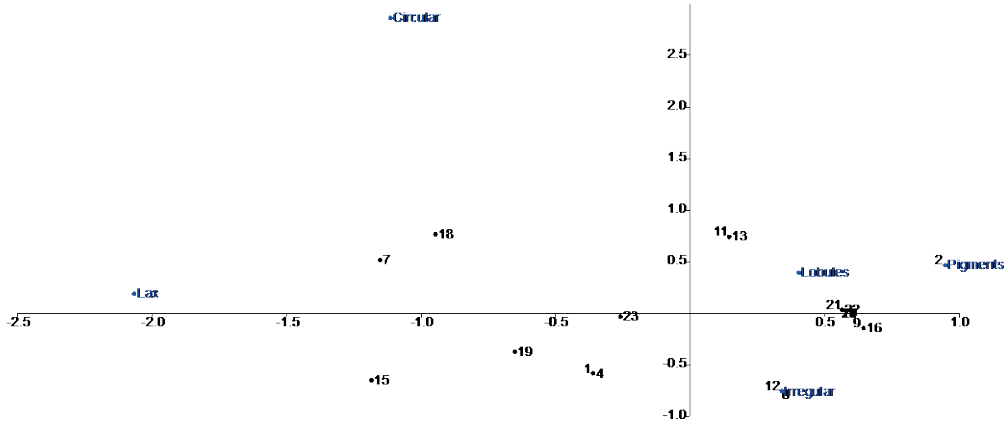

Figure 4. Correspondence analysis of colony morphological characteristics (circular shape, lax growth, lobular growth, irregular edge growth, pigment production) in presence of: 1: D-xylose; 2: D-mannose; 3: D-cellobiose; 4: Maltose; 6: Sorbitol; 7: L-rhamnose; 8: Malt extract; 9: L-arabinose; 10: D-galactose; 11: Starch; 12: D-alanine; 13: Ammonium nitrate; 14: Sodium nitrate; 15: Calcium nitrate; 16: Urea; 17: L-glutamic acid; 18: Glucose + KNO_3; 19: no Glucose + KNO_3; 20: Glucose no KNO_3; 21: no vitamins; 22: Thiamine no Biotin; 23: no Thiamine + Biotin; 24: no C or N source)

In the "multiple carbon sources + inorganic nitrogen" group, the combination of malt extract and L-rhamnose led to the greatest increase in colony diameter (Table 1, Tukey post-hoc test, $p < 0.05$). Similarly, in the "multiple carbon sources + organic nitrogen" group, D-alanine addition increased colony diameter by 36% compared to the basal medium (Tukey post-hoc test, $p < 0.05$). When glucose was the sole carbon source, adding urea, enhanced growth by 46% compared to the basal medium (Tukey post-hoc test, $p < 0.05$). The variability in colony diameter shows that medium composition significantly influences fungal growth. Malt extract and L-rhamnose, within the "multiple carbon sources + inorganic nitrogen" group, produced the largest colonies (Tukey post-hoc test, $p < 0.05$), potentially acting as metabolic enhancers (Fleck et al., 2011). Nitrogen sources such as D-alanine and urea also boosted biomass production. D-alanine increased colony diameter by 36%, indicating that specific amino acids stimulate fungal growth. Similarly, urea, when combined with glucose, enhanced growth by 46% (Tukey post-hoc test, $p < 0.05$), possibly due to easily assimilable nitrogen form promoting protein synthesis and essential cellular functions (Walker et al., 2017). The addition of D-alanine and urea, suggest a potential link between nutrition, growth, and antifungal compound production. These results highlight the importance of nutritional conditions in modulating the morphology and growth of endophytic fungi like *S. isomera*.

Table 1. Average diameter and standard deviation (cm) of the endophytic fungal strain HTF58 *S. isomera* under different nutrient sources (SM - minimal synthetic vitamin agar medium substituting C/N compounds)

Carbon + Organic Nitrogen	Diameter ± sd	Carbon + Inorganic Nitrogen	Diameter ± sd
D-alanine	2.37 ± 0.05^{b}	D-xylose	1.73 ± 0.08^{ab}
Urea	2.56 ± 0.18^{b}	D-mannose	2.00 ± 0.03^{ab}
L-glutamic acid	1.74 ± 0.11^{a}	D-cellobiose	1.61 ± 0.06^{a}
SM - KNO_3	2.27 ± 0.07^{b}	Maltose	2.04 ± 0.23^{ab}
SM - Glucose & KNO_3	1.75 ± 0.13^{a}	Sorbitol	1.74 ± 0.11^{ab}
		L-rhamnose	2.40 ± 0.13^{c}
		Malt extract	2.57 ± 0.14^{c}
		L-arabinose	2.36 ± 0.35^{c}
		D-galactose	1.88 ± 0.03^{ab}
		Starch	1.70 ± 0.12^{ab}
		Ammonium nitrate	2.10 ± 0.11^{b}
		Sodium nitrate	1.63 ± 0.08^{a}
		Calcium nitrate	1.61 ± 0.10^{a}
		Complete SM	1.80 ± 0.11^{ab}
		SM - Vitamins	1.78 ± 0.06^{ab}
		SM - Biotin	2.14 ± 0.30^{b}
		SM - Thiamine	1.60 ± 0.15^{a}
		SM - Glucose & KNO_3	1.75 ± 0.13^{ab}

*Different letters indicate significant differences using Tukey post-hoc test, $p < 0.05$.

Organic and inorganic nitrogen sources differentially influenced pigment production and colony expansion, with compounds like D-alanine and urea notably promoting growth. The trial shows that the choice of nitrogen source significantly influenced colony expansion, morphology, and pigmentation. When KNO_3 was substituted with D-alanine, colonies exhibited greater size, lobular morphology, and pigmentation. This suggests that the nature of the nitrogen source plays a crucial role in fungal growth dynamics. KNO_3, as an inorganic nitrogen source, provides nitrate that the fungus must reduce and assimilate, requiring energy to convert it into amino acids. In contrast, D-alanine is an organic nitrogen source that can be directly incorporated into cellular metabolism, bypassing the need for nitrate reduction, and potentially facilitating more efficient growth and secondary metabolite production, such as pigments. The elimination of both or one of the two vitamins (biotin or thiamine) from the basal medium, did not interfere with lobular regular growth or pigments production. Yet, when eliminating thiamine, colony size decreased (with thiamine and no biotin = 2.14 ± 0.30b versus no thiamine with biotin = 1.60 ± 0.15, Tukey test $p < 0.05$),

indicating that thiamine serves as a cofactor that may enhance metabolic pathways linked to colony expansion. The ability to adjust morphology and secondary metabolite production in response to different carbon and nitrogen sources may be an adaptive mechanism that allows endophytic fungi to colonize various ecological niches and compete with other microorganisms within the plant host environment.

In Vitro Tripartite Assay

A preliminary assay testing the capacity of the endophyte to colonize the new tomato host revealed successful establishment of strain HTF58 in multiple plant tissues. When seeds and the endophyte were introduced simultaneously, the fungal strain was successfully isolated from 92.5% of root samples, 10% of stem samples and 12.5% of leaf samples. A similar colonization pattern was observed in a second trial where the endophyte was introduced after seed germination, showing colonization frequencies of 95% in the roots, 20% in the stems, and absence in the leaf fragments).

The hypothesis of the tripartite *in vitro* study was that if the endophyte would colonize the seed during the germination process, before the pathogen introduction, seedling development would be enhanced, and pathogen-induced symptoms would be reduced. The tripartite interaction was evaluated across different scenarios, with the outcomes depending on two main factors i) which arrives before (seed, endophyte, or pathogen) and ii) the duration of their interaction (Coşoveanu et al., 2021). Across all scenarios, the control groups of the endophyte + seeds (C1) were similar to negative control groups of seeds (C3), suggesting that the endophyte is harmless to the seedlings. In the positive control groups consisting of pathogen + seeds (C2), symptom severity ranged from mild to severe, ultimately, in some cases, leading to the death of seeds or seedlings. These findings indicate that the selected *B. cinerea* strain B05.10 is capable of successfully colonizing tomato seeds and seedlings, thereby inducing disease symptoms.

In the T1 strategy, where the endophyte was given a five-day head start before either pathogen or seed introduction, two scenarios were evaluated based on the order of pathogen and seed introduction: T1A - endophyte was inoculated first, followed by seed and then pathogen introduction, T1B - the order was reversed between seeds and pathogen.

In T1A, significant decrease in fresh and dry weight was observed in the positive control group of pathogen (C2) compared to negative control group of seeds (C3) ($p < 0.05$; Table 2). The treatment group exhibited increased values of fresh weight compared to the positive control with pathogen (T = 45.7 ± 5.51 mg versus C2 = 14.7 ± 1.53 mg), although no significant differences were noted in dry weight. The average root length was lower in the positive control group with pathogen compared to the treatment group and the seed-only or endophyte control groups (C2 = 0.22 ± 0.03 cm; T = 0.58 ± 0.1 cm; C3 = 0.4 ± 0.10 cm; C1 = 0.54 ± 0.04 cm; Tukey post-hoc test, $p < 0.05$). The differences in plumule size were more pronounced, with no growth observed in the positive control group with pathogen. In the treatment group radicle size increased by 45% (0.58 ± 0.10 cm) and plumule size by 18% (1.93 ± 0.14 cm), compared to the seed-only group (radicle: 0.4 ± 0.10 cm and plumule: 1.64 ± 0.81 cm). The treatment group supported normal seedling development with an ID scale increase of 30% compared to seed-only group, although not statistically different (T = 2.44 ± 0.53 versus C3 = 1.89 ± 0.93). In contrast, the positive control group with pathogen allowed only radicle emergence (ID = 1 ± 0; Dunn test, $p < 0.05$). The protective role of the endophyte was evident avoiding death of seedlings observed in the positive control group with pathogen (IN: T = 0.11 ± 0.33 vs. C2 = 4 ± 0; Dunn test, $p < 0.05$).

In T1B scenario, no significant differences were found in weight (fresh and dry) or length (radicle and plumule) between the treatment and control groups. The development index showed no variation, likely because the pathogen was inoculated after the seedlings had already developed for 14 days, limiting its impact on the scale values. However, differences in the symptom index were evident between the positive control with the pathogen and the other groups (Table 2), suggesting that the endophyte provided protection to the radicle. Previous study using a similar procedure with tomato

seedlings and various fungal endophytes concluded that one of the most effective time strategies was when the endophyte preceded the pathogen, with seeds introduced last (Coşoveanu et al., 2021).

The tomato seed coat is a rich source of organic nitrogen and carbon, making it a suitable target for fungal degradation using enzymes such as pectinases, cellulases, and ligninases, among others. While cellulases facilitate the loosening of cell walls, aiding in nutrient accessibility and root growth enhancement (Libertini et al., 2004), pectin degradation generates oligosaccharides that act as damage-associated molecular patterns (DAMPs), triggering growth-related pathways alongside defence mechanisms (Benoit et al., 2012; van den Brink & Vries, 2011). Additionally, lignin degradation releases compounds that may stimulate root elongation through growth-associated hormonal pathways (Rehman et al., 2022; Cha et al., 2017). In the present scenarios, the fimiculous endophytic fungus strain HTF58 was cultured in Hoagland medium, which provides essential minerals but lacks organic carbon and nitrogen sources beyond inorganic nitrogen forms (e.g., nitrates and ammonium) (Gamborg & Wetter, 1975). The endophytic fungus managed to colonize the substrate under these nutrient-limited conditions. However, once the seed was introduced, the presence of the seed coat likely provided richer sources of organic nitrogen and carbon (e.g., proteins, polysaccharides). This shift in nutrient availability may have triggered the fungus to upregulate the production of cell wall-degrading enzymes, allowing it to access these additional resources. Consequently, this enzymatic activity could have indirectly promoted seedling growth, as observed in the increased values in fresh weight, radicle length, plumule size, and development index, in both T1A and T1B scenarios, compared to the control group of seedlings only (Table 2).

In the T2 strategy, where the seed, endophyte, and pathogen were introduced simultaneously, the treatment group showed similar values for fresh weight, plumule size and development index compared to seed-only and endophyte control groups. In contrast, the presence of the pathogen (C2), led to a significant decrease in fresh weight, plumule length and both the development (ID) and symptom (IN) scales, compared to seed-only group. This indicates that the pathogen adversely affected seed germination, ultimately to the seeds or seedlings being entirely covered with mycelium and spores (IN: C2 = 4 ± 0).

In treatment group compared to the positive control group, only plumule size showed a significant increase (T = 2.19 ± 0.36 cm vs. C2 = 0 ± 0 cm), the other biometric variables, including the symptom index, remained similar. This suggests that any potential protective effect of the endophyte was not observed in this scenario. In a previous study involving the direct simultaneous confrontation of endophyte, seedling and pathogen, a reduction in the negative effects of pathogen was reported. The authors proposed competition for resources and growth stimulation of seedlings by presence of both pathogen and endophyte as potential factors (Coşoveanu et al., 2021). *S. isomera* is a low-growing fungus *in vitro*, regardless of the nutrient medium, indicating that the capacity it may have to produce antifungal compounds may be limited by the biomass it can achieve within a given time. In contrast, *B. cinerea*, is a relatively fast-growing fungus, enabling it to reach the seed in a shorter time frame.

In the T3 strategy, the results varied between two scenarios: i) T3A - the seed was placed first, followed by endophyte and then pathogen introduction and ii) T3B - the order was reversed between the endophyte and pathogen.

In T3A, where the pathogen was introduced after partial seedlings development, biometric variables were similar among the seed-only control, endophyte control and treatment groups. However, all these groups showed a significant increase in fresh weight compared to the positive control group with the pathogen, with the highest value observed in the treatment group (T = 43 ± 2.65 mg vs. C2 = 19.7 ± 4.62 mg; Tukey post-hoc test, $p < 0.05$). Despite the pathogen being inoculated 14 days after seed introduction, it still caused complete damage to the radicle (IN: C2 = 3 ± 1.5; Dunn test, $p < 0.05$).

In T3B scenario, where the endophyte was introduced last, following seed germination and two days after the pathogen inoculation, no potential beneficial effect of the endophyte was observed. Fresh weight values in the treatment group were similar to those in the positive

control with the pathogen (T = 16.7 ± 10.5 mg and C2 = 6.67 ± 2.31 mg) and no protective effect on the seedlings protection was detected (IN: T = 3.33 ± 1.32 and C2 = 4 ± 0). These results suggest that the absence of the endophyte for two days after the introduction of the pathogen, despite the seedlings already being developed, contributed to the complete death of the seedlings.

Table 2. *In vitro* tripartite assay "*S. isomera* - tomato seedlings - *B. cinerea*" - average values and standard deviation of weight (dry and fresh - mg) and length of radicle and plumule (cm) and index of development (ID scale) and index of symptoms (IN scale) for the scenarios T1A, T1B, T2, T3A and T3B

		Dry weight	Fresh weight	Radicle	Plumule	ID	IN
T1A	C1	4.33 ± 0.58^{a}	52.3 ± 4.04^{a}	0.54 ± 0.04^{a}	2.26 ± 0.10^{a}	2.56 ± 0.53^{a}	0 ± 0^{a}
	C2	2.67 ± 0.58^{a}	14.7 ± 1.53^{b}	0.22 ± 0.03^{bc}	0 ± 0^{b}	1 ± 0^{b}	4 ± 0^{b}
	C3	4.67 ± 0.58^{a}	37 ± 9.85^{a}	0.4 ± 0.10^{ab}	1.64 ± 0.81^{ab}	1.89 ± 0.93^{a}	0 ± 0^{a}
	T	3 ± 1^{a}	45.7 ± 5.51^{a}	0.58 ± 0.10^{a}	1.93 ± 0.14^{ab}	2.44 ± 0.53^{a}	0.11 ± 0.33^{a}
T1B	C1	6.67 ± 1.53^{a}	49.7 ± 6.81^{a}	0.50 ± 0.02^{a}	2.28 ± 0.07^{a}	2.44 ± 0.73^{a}	0 ± 0^{a}
	C2	5.33 ± 0.58^{a}	36.3 ± 0.58^{a}	0.50 ± 0.03^{a}	1.77 ± 0.32^{a}	2 ± 0^{a}	1.78 ± 1.79^{b}
	C3	6 ± 1^{a}	42 ± 1.73^{a}	0.46 ± 0.13^{a}	2.10 ± 0.06^{a}	2.33 ± 0.71^{a}	0 ± 0^{a}
	T	3.67 ± 1.53^{a}	44 ± 9.54^{a}	0.46 ± 0.07^{a}	1.93 ± 0.39^{a}	1.89 ± 0.78^{a}	0 ± 0^{a}
T2	C1	4 ± 1^{a}	45 ± 7.55^{a}	0.46 ± 0.13^{a}	2.35 ± 0.64^{a}	2.33 ± 1^{a}	0 ± 0^{a}
	C2	4 ± 1^{a}	16 ± 4^{bc}	0.56 ± 0.39^{a}	0 ± 0^{b}	0.67 ± 0.5^{b}	4 ± 0^{b}
	C3	6.33 ± 0.58^{a}	54.7 ± 7.57^{a}	0.52 ± 0.12^{a}	2.8 ± 0.21^{a}	2.44 ± 0.53^{a}	0 ± 0^{a}
	T	2.67 ± 1.53^{a}	28 ± 18.7^{ab}	0.63 ± 0.09^{a}	2.19 ± 0.36^{a}	2.22 ± 0.67^{a}	2 ± 1.94^{b}
T3A	C1	5 ± 1.73^{a}	35.7 ± 5.13^{a}	0.40 ± 0.03^{a}	2.28 ± 0.30^{a}	2 ± 0^{a}	0 ± 0^{a}
	C2	3.33 ± 0.58^{a}	19.7 ± 4.62^{b}	0.60 ± 0.13^{a}	2.28 ± 0.14^{a}	2.11 ± 0.33^{a}	3 ± 1.5^{b}
	C3	5.33 ± 0.58^{a}	40.3 ± 7.23^{a}	0.56 ± 0.07^{a}	2.51 ± 0.53^{a}	2 ± 0^{a}	0 ± 0^{a}
	T	5.67 ± 0.58^{a}	43 ± 2.65^{a}	0.44 ± 0.04^{a}	2.39 ± 0.06^{a}	2.11 ± 0.33^{a}	0 ± 0^{a}
T3B	C1	6.33 ± 0.58^{a}	39 ± 4.58^{a}	0.47 ± 0.04^{a}	2.51 ± 0.18^{a}	2.33 ± 0.5^{a}	0 ± 0^{a}
	C2	4.67 ± 1.15^{a}	6.67 ± 2.31^{b}	0.48 ± 0.10^{a}	1.73 ± 0.56^{a}	1.89 ± 0.33^{a}	4 ± 0^{b}
	C3	6 ± 1^{a}	33.7 ± 6.43^{a}	0.54 ± 0.13^{a}	2.36 ± 0.46^{a}	2 ± 0.5^{a}	0 ± 0^{a}
	T	5.67 ± 0.58^{a}	16.7 ± 10.5^{ab}	0.52 ± 0.16^{a}	1.75 ± 0.51^{a}	2.11 ± 0.33^{a}	3.33 ± 1.32^{b}

In analysing the time scenarios, we expected the index of necrosis to increase in positive control group with pathogen based on i) the duration of interaction, ii) the seedling developmental stage (with seeds stage as most sensitive), and iii) nutrient stress experienced by the pathogen (synthetic medium supporting minimal nutrient conditions). The predicted ranking was T1A > T2 > T3B > T1B and T3A. Results showed that for T1A and T2, where seeds first encountered the pathogen at 0 days, necrosis was highest (IN = 4) after 21 days of interaction. In T3B, where seeds had 7 days to germinate before pathogen exposure, necrosis was still at the maximum after 14 days of interaction. However, in T1B and T3A (14 days pre-pathogen seedling development), necrosis was reduced (IN: T1B = 1.78 ± 1.8; T3A = 3 ± 1.5), also having a shorter interaction duration (7 days). These results indicate that the time of interaction and seedling developmental stage significantly reduced necrosis, with older seedlings displaying greater resistance.

Conversely, we expected that the index of development in the endophyte-seeds group treatment would increase with i) the number of days of interaction and ii) the duration of nutrient stress experienced by the endophyte prior to seeds exposure. The expected ranking was T1A > T1B > T2 > T3A > T3B. Across all

scenarios, whether the endophyte interacted with the seed from day 1 (T2), experienced presumed nutrient limitation before seed introduction (T1A and T1B = 5 and 7 days pre-seeds, respectively) or encountered a seedling (T3A and T3B = 7 and 9 days post-seeds, respectively), the index values remained similar, ranging from 2 to 2.56 ± 0.5. This suggests that neither the duration of interaction nor the nutrient conditions significantly affected the developmental index obtained by seedlings in presence of the endophyte.

The protective effect of the endophyte assessed in the treatment groups among scenarios based on the symptom index, was expected to be influenced by two main factors: i) the number of days the endophyte interacted with the seeds before the pathogen introduction and ii) the duration the endophyte remained alone before either the seed or pathogen introduction. It was expected that the protective effect (i.e. lower IN values) would be inversely proportional to the number of days. The predicted ranking was T1B < T1A < T3A < T2 < T3B. Similar symptom index values were observed for the first three scenarios (T1B = 0 ± 0; T1A = 0.11 ± 0.33; T3A = 0 ± 0), indicating that the protective effect of the endophyte was consistent across different conditions. This was true regardless of i) the age of the endophyte at the time of the seed introduction - 5 days old in T1B, 7 days old in T1A or after seed germination in T3A, and ii) whether the pathogen was present or absent prior to seeds introduction. For instance, in T1A the endophyte was cultivated solely for 5 days followed by co-culture with the pathogen for 2 days and then the seeds were introduced. These results suggest that the endophyte can fully protect seedlings, with minimal symptom incidence, when the pathogen is introduced in between the two other actors. The last two scenarios were in accordance with the expected ranking, the endophyte only slightly protecting when simultaneous introduction was performed, and almost total damage of seedlings was observed when the endophyte was introduced at the end (IN: T2 = 2 ± 1.94 and T3B = 3.33 ± 1.32).

The results from the *in vitro* assay support the hypothesis that prior introduction of the endophyte before the pathogen enhances seedling development and reduces symptoms caused by *Botrytis cinerea*. These findings suggest that the timing of endophyte colonization is a key factor for its efficacy in protecting seedlings against pathogens (Molina-Montenegro et al., 2023). Significant effect of *Sarocladium strictum*, *Anthracocystis floculossa* and *Penicillium olsonii* on reducing *Fusarium* head blight was observed when endophytes were applied at least two days before pathogen (Rojas et al., 2020). In tomato plants, *Verticillium dahlie* was excluded from roots when the endophytic strain was inoculated before or simultaneously with the pathogen (Shittu et al., 2009). Endophytic *Aspergillus terreus* was isolated from a halophytic plant and inoculated in tomato plants few days before artificial contamination with *Fusarium oxysporum.* Authors concluded that the endophyte is a promising strain with both plant growth promotion and antagonistic traits, emerging as a potential biological control agent (Alhaddad et al., 2024). The favourable coexistence between endophyte and host plant revealed that fungal-induced resistance remains an important mechanism used by endophytic microorganisms in disease suppression. Specific endophytic fungal compounds with hormonal responses are usually recognized by the host plant (Adeleke et al., 2022).

Our findings indicate that achieving sufficient endophyte biomass before the arrival of the pathogen leads to the most effective antagonistic performance, significantly impeding pathogen-induced symptoms and enhancing seedling growth. Specifically, the scenarios T1A (5 days of endophyte cultivation, followed by 2 days in co-culture with the pathogen, and finally seeds introduction), T3A (7 days of seeds cultivation, followed by 7 days in co-culture with the endophyte, and lastly pathogen introduction) and T1B (5 days of endophyte cultivation, followed by 14 days of co-culture with the endophyte and lastly pathogen introduction) exhibited significant improvements and have in common that the endophyte has time to establish itself either alone or in co-culture with the seedlings.

In Vivo Tripartite Assay

Multiple substrate colonization trials were performed using organic substrates like oatmeal, wheat, rice, and combinations of oatmeal-wheat,

oatmeal-rice, wheat-rice to select the optimal growth medium for both *Sporormiella isomera* and *Botrytis cinerea*. The oatmeal-wheat mixture (1:1) was selected as suitable substrate for both fungi, further used to inoculate 30 days-old plants.

The proposed tripartite *in vivo* assay aimed to closely simulate a real-life scenario encountered during transplanting, where the substrate may already be contaminated with *B. cinerea* either living saprophytically or present as sclerotia in soil to mitigate potential disease outbreaks and to ease the application process, the introduction of *S. isomera* was proposed at this stage. After 25 days post-inoculation, significant differences in root development were observed between the control group with the endophyte and the negative control group (containing only oatmeal-wheat mixture). The endophyte control group exhibited higher fresh (Figure 5A) and dry weights (Figure 5B) of roots, indicating that the endophyte positively influenced root growth (fresh weight: endophyte control group = 22 ± 10.4 mg vs. negative control = 7.6 ± 6.7 mg; dry weight: treatment endophyte = 5 ± 2.45 mg vs. negative control = 1.8 ± 1.79 mg; $p < 0.05$). In contrast, similar values were observed for the treatment group with endophyte and pathogen compared to positive control group with *B. cinerea*, suggesting that under the test conditions, the endophyte did not stimulate root growth in the presence of the pathogen.

For shoot fresh weight (Figure 5C), a similar pattern was observed as with root weight, between the control group with endophyte and the negative control group (fresh weight: endophyte control group = 1362 ± 342 mg vs. negative control = 991 ± 303 mg), suggesting that the endophyte promotes shoot growth in tomato plants. However, for dry weight while there was a trend toward higher values in the treatment group compared to positive control with pathogen, differences were not statistically significant (Figure 5D).

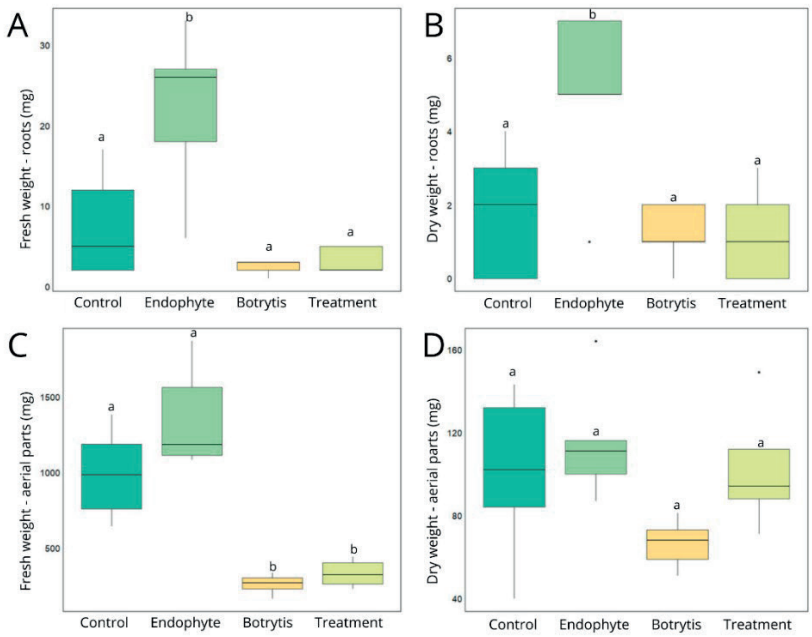

Figure 5. Assay *in vivo Sporormiella isomera* HTF58 - tomato plants - *Botrytis cinerea*: A) Fresh weight of roots; B) Dry weight of roots; C) Fresh weight of aerial parts; D) Dry weight of aerial parts. Average values are shown with letters indicating differences between treatments (Tukey post-hoc test/Dunn test, $p < 0.05$)

In both the positive control with pathogen and the treatment group, the pathogen was successfully isolated from both the roots and shoots (colonization frequency: treatment roots = 55% and stem = 35%; positive control roots = 44% and stem = 40%). Together with the observed symptoms of necrotic roots and collar necrosis and colonization of mycelium and spores, results indicate that the strain B05.10 was able to establish itself even in the presence of the endophyte. The endophyte was scarcely isolated only from root samples in samples of control with endophyte (colonization frequency = 2.2%). This emphasizes that in scenarios where the endophyte is inoculated simulta-neously with the pathogen, the potentially

beneficial effects are lost, reinforcing the importance of inoculation timing for successful biocontrol. Previous studies have shown that endophytes require time to colonize plant tissues and activate defence mechanisms before the introduction of the pathogen (Adeleke et al., 2022). In the *in vivo* tripartite assay, the positive effect of *S. isomera* in promoting the development of tomato plants was evident in the fresh and dry weights of the roots and shoots. The strain HTF58 was successfully isolated from tomato roots at the end of the trial, although with low values. These results indicate that *S. isomera* strain HTF58 has an endophytic behaviour in the new host, and it can promote growth under non-pathogenic conditions. In a similar study procedure, on the patovar "*Sclerotinia rolfsii* - groundnut" the effect of native strains *T. harzianum* and *T. aggressivum* was evaluated positively indicating plant growth promoting features (Aydoğdu, 2022). This finding aligns with previous research demonstrating the ability of endophytes to enhance plant growth under normal cultivation conditions (Alhaddad et al., 2024; Sinno et al., 2020). However, the lack of significant differences in the treatment groups treated with the endophyte in the presence of the pathogen suggests that under the conditions tested, *S. isomera* was unable to effectively protect the plants from *Botrytis cinerea* infection. This contrasts with some studies that have demonstrated the ability of endophytes to protect plants against pathogens by inducing defence responses or producing antifungal metabolites (Aydoğdu, 2022). Factors such as the plant substrate, load or speed of the pathogen might have been critical determinants in the lack of observed protection. When both the pathogen and the endophyte are introduced simultaneously, their interaction often depends on the relative growth rates of each organism. In some cases, this can lead to antagonism, with the outcome determined by nutrient competition or the release of secondary metabolites. However, pre-emptive inoculation with endophytes tends to provide a stronger defence mechanism against pathogens (Coşoveanu et al., 2021). Besides our *in vitro* results where simultaneous co-inoculation was one of the less desirable scenarios, and the posterior confirmation with young plants (*in vivo*), other studies have also pronounced in favour of pre-inoculation time of the endophyte as critical for successful biocontrol (Muhammad et al., 2024). The enhancement of root development by the endophytic fungus observed both in the *in vitro* as well as in the *in vivo* trials, should be further explored using techniques such as cellular microscopy and transcriptomics. These methods would enable a fine-scale examination of root architecture changes and gene expression patterns associated with fungal colonization, shedding light on the cellular and molecular mechanisms through which strain HTF58 influences root growth dynamics. Also, a comparative study on the enzymatic activity on plant-derived substrate versus dung could reveal the presence of enzymes capable of degrading plant cell walls and utilizing organic nitrogen sources. The study would provide insights into how the strain adapts the metabolic processes based on available nutrient sources and could uncover pathways involved in nitrogen cycling and nutrient acquisition.

CONCLUSIONS

Nutrient sources had a significant impact on pigment production, colony expansion and the development of regular lobular morphology. Compounds like i) D-alanine or ii) thiamine combined with inorganic nitrogen, led to all three effects, indicating a connection between nutrient availability, morphological changes, and the antagonistic potential of strain HTF58. This adaptability may support its ecological success within host plants. Bioassays with *Sporomiella isomera* strain HTF58 showed it is harmless to tomato seedlings and young plants, with potential to promote growth metrics. Effective protection against *Botrytis cinerea* depends on pre-inoculation timing, allowing the endophyte to establish.

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