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Understanding the Influence of Applying Two Culture Filtrates to Control Gray Mold Disease (*Botrytis cinerea*) in Tomato

Lobna Hajji-Hedfi [1,*], Abdelhak Rhouma [1], Hichem Hajlaoui [1], Fedi Hajlaoui [1] and Nazih Y. Rebouh [2,*]

[1] Regional Centre for Agricultural Research of Sidi Bouzid, CRRA, B.P. 357 Gafsa Road Km 6 Sidi Bouzid, Sidi Bouzid 9100, Tunisia; hajlaoui2001@yahoo.fr (H.H.); fedi.hajlaoui7@gmail.com (F.H.)
[2] Department of Environmental Management, Institute of Environmental Engineering, RUDN University, 6 Miklukho-Maklaya St., 117198 Moscow, Russia
* Correspondence: elhajjilobna@yahoo.fr (L.H.-H.); rebukh_nya@pfur.ru (N.Y.R.)

Abstract: *Botrytis cinerea*, a causal agent of gray mold disease, is one of the most destructive fungal pathogens that leads to substantial global economic crop losses, especially for tomato plants. The present study aims to investigate the inhibitory effect of two microbial culture filtrates (BCA filtrate alone and combined with salicylic acid) of *Trichoderma longibrachiatum* and *Pseudomonas* sp. against the phytopathogenic fungus *B. cinerea* on tomato plants. The biochemical modifications, gray mold disease incidence, and fruit quality parameters of the tomatoes were determined according to tested treatments. The results showed that both fungi and bacteria were able to solubilize phosphate and produce IAA and HCN. *T. longibrachiatum* could produce hydrolytic enzymes (chitinase, protease, and glucanase). Otherwise, *Pseudomonas* sp. showed the capacity to produce catalase and amylase enzymes. Both microbial culture filtrates inhibited the hyphae growth of *B. cinerea*. The biocontrol efficacy, in vitro, was significant: up to 50% in terms of the growth inhibition rate at a concentration of 40%. The tomato seedlings' growth was promoted by the separate preventive treatments of each micro-organism culture filtrate. In addition, disease severity in the tomato seedlings and fruit was significantly reduced. Furthermore, the combined treatment of tomato fruit with culture filtrates and salicylic acid induced significant biochemical and physiological changes in fruit firmness, juice yield, total protein, and ROS enzyme activities. The culture filtrates of *T. longibrachiatum* and *Pseudomonas* sp. can be recommended as an effective microbial biofungicide to control gray mold disease under storage conditions.

Keywords: antifungal activity; biocontrol; *Trichoderma longibrachiatum*; *Pseudomonas* sp.; plant growth-promoting

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1. Introduction

Tomato is one of the most consumed crops worldwide due to its nutritional value expressed by its high vitamin C and lycopene content [1]. However, gray mold generates significant economic losses during growing seasons, storage, and transport [2]. This disease is essentially caused by the pathogenic agent *Botrytis cinerea*, which is a ubiquitous fungal pathogen that is widely distributed and infects a broad range of plant species. This fungal pathogen causes serious pre- as well as postharvest diseases and has a disastrous economic impact on tomatoes [3,4].

The pathogenic mechanism of *B. cinerea* is variable within different attack modes and is able to survive in favorable or unfavorable conditions. Indeed, this fungus' pathogenesis (in particular, growth development and virulence), was found to be related to reactive oxygen species and extracellular proteins [5]. Furthermore, *B. cinerea* is able to invade almost all plant parts at both preharvest and postharvest, which leads to this fungus being difficult to control [5,6].

Over the previous decades, chemical fungicides remained the most common method for controlling *B. cinerea*. However, this pathogenic fungus develops resistance to several

fungicides [7,8]. In addition, the toxic residues in tomato fruit and soil are threatening human health, soil, and the environment [9]. Biological control can be used as an alternative to reduce the disease incidence of gray mold disease caused by *B. cinerea* [10].

Microbial biological control agents (MBCAs) have emerged as a viable alternative to chemical fungicides, and they are considered key players in modern sustainable agriculture [11–14]. To date, many fungi and bacteria have been isolated from the rhizosphere of cultivated crops and have been selected as biocontrol agents against *B. cinerea*. Moreover, numerous studies reported that the *Trichoderma* species is able to inhibit tomato gray mold and other tomato fungal diseases [15]. In addition, numerous bacteria, mainly the *Pseudomonas* and *Bacillus* genera, showed significant antagonistic potential [13,16].

At present, the successful development and commercialization of biofungicide products require several investigations into any well-adapted and efficient antagonistic microorganisms in order to be used (as singular or in microbial consortia) as an effective mode of action [13,17]. In fact, various modes of action can be involved in biological disease control [17,18].

The objectives of the present study were (1) to explore (in vitro and in vivo) the efficacy of microbial antagonists, *Trichoderma longibrachiatum* and *Pseudomonas* sp., to reduce gray mold severity on tomato seedlings and fruits, and (2) to assess the morphometric, physiological, and biochemical effect of two BCA inoculations formulated based on these micro-organisms on tomato fruit.

2. Materials and Methods

2.1. Fungal Strains

The *Botrytis cinerea*, *Trichoderma longibrachiatum*, and *Pseudomonas* sp. used in the present study were obtained from the laboratory of Plant Protection and Biological Sciences, Regional Centre for Agricultural Research (Sidi Bouzid, Tunisia). This phytopathogen was isolated from infested tomato fruits (cv. Firenze) cultivated in a greenhouse (Regueb, Sidi Bouzid, Tunisia). However, *T. longibrachiatum* and *Pseudomonas* sp. were isolated from the rhizosphere of the same greenhouse.

2.2. In Vitro Plant-Growth-Promoting Activities

T. longibrachiatum and *Pseudomonas* sp. were evaluated in vitro for properties that are known to be essential for plant growth-promoting activities, such as IAA production (IAA), hydrocyanic acid production (HCN), atmospheric nitrogen fixation (N), and phosphate solubilization (P) and for their capacity to produce extracellular enzymes such as catalase production (Cat), pectinase production (Pec), proteolytic activity (Pro), amylolytic activity (Amy), β-1,3-glucanase activity (Glu), and chitinase (Chit).

IAA: the production of IAA is determined according to the standard method. The isolated colony is spread on Luria-Bertani (LB) agar culture medium, supplemented with a concentration of 5 mL of tryptophan, 0.06% of SDS, and 1% of glycerol. A disc of Whatman paper is placed on the surface of the agar culture medium, incubated at a temperature of 28 °C for 48 h, recovered, and treated with Salkowski's reagent (2% of 0.5 M $FeCl_3$ in 35% perchloric acid). The discs are saturated in a Petri dish during the impregnation in the reagent after 10 to 30 min. A color change in the filter paper from yellow to orange-red (weak production), brown (moderate production), or pinkish-brown (strong production) confirms IAA production.

HCN: Petri plates were filled separately with 15 mL of LB and PDA agar amended with glycine 4.4 g L^{-1}; then, a one-disc plug (0.5 cm diameter) of fungi and bacteria colony was placed onto PDA and LB media after solidification. A Whatman No. 1 paper soaked in an alkaline picrate solution (2.5 g picric acid, 12.5 g Na_2CO_3 in 1 L of distilled water; pH 13) was glued to the lids of the Petri plates containing *T. longibrachiatum* and *Pseudomonas* sp. cultures. The Petri plates were incubated at 28 ± 2 °C for 4 days (*T. longibrachiatum*) and 3 days (*Pseudomonas* sp.). A color change in the filter paper from yellow to orange-red

(weak production), brown (moderate production), or reddish-brown (strong production) confirms cyanogenic potential [19,20].

N: *T. longibrachiatum* and *Pseudomonas* sp. were inoculated into an N-free medium and incubated at 30 °C for 5 days. The *T. longibrachiatum* and *Pseudomonas* sp. that were able to grow formed a visible film on the surface of the medium and were considered nitrogen fixers [19].

P: phosphate solubilization was determined based on the cultivation of *T. longibrachiatum* and *Pseudomonas* sp. in Pikivoskaya medium. Phosphate-solubilizing fungi and bacteria were detected by the formation of a clear halo [19,21].

Cat: a part of the colony of *Pseudomonas* sp. and *T. longibrachiatum* was transferred into a microscopic slide and mixed with a drop of H_2O_2. The production of air bubbles indicates positive catalase activity [20].

Pec: *T. longibrachiatum* and *Pseudomonas* sp. were inoculated into a Pectino-Congo Red agar medium and incubated at 30 °C for 5 days. Pectinase production of fungi and bacteria was detected by the formation of a clear halo [21].

Pro: Petri plates were filled separately with 15 mL of LB and PDA agar amended with gelatine 10 g L^{-1}; then, a one-disc plug (0.5 cm diameter) of fungi and bacteria colony was placed onto PDA and LB media after solidification. After 5 days of incubation at 30 °C, the Petri dishes were flooding with a solution containing 10 mL of distilled water, 2 mL of HCL at 1.5 g $HgCl_2$. Proteolytic production of fungi and bacteria was detected by the formation of a clear halo [20,21].

Amy: the demonstration of this activity is carried out on nutrient agar (*Pseudomonas* sp.) and a PDA medium (*T. longibrachiatum*) containing 1% soluble starch; then, a one-disc plug (0.5 cm diameter) of fungi and bacteria colony was placed onto PDA and LB media after solidification. The Petri plates were incubated at 30 °C for 7 days. After incubation, the Petri dishes were sprayed with a solution containing Lugol and rinsed in distilled water. Starch hydrolysis is manifested by the appearance of a clear halo around the colony; on the other hand, a negative result reveals a brown color around the culture.

Glu: *T. longibrachiatum* and *Pseudomonas sp.* were inoculated on an enriched medium with β-1,3-glucanase (barley flour). After 5 days of incubation at 30 °C, the Petri dishes were flooded with Congo solution (0.1%). β-1,3-glucanase activity is reflected by the presence of clear areas around the bacterial colonies and fungal disc plug.

Chit: *T. longibrachiatum* and *Pseudomonas sp.* were inoculated on an enriched medium with colloidal chitin. Colloidal chitin was obtained from 40 g of powdered chitin, which was added to 600 mL of concentrated HCL; this mixture must be stirred for 60 min. The colloidal chitin precipitates, following the addition of 2 L of cold water, were recovered by filtration. Chitinolytic activity is reflected by the presence of clear and creamy areas around bacterial colonies and fungal disc plugs.

2.3. In Vitro Evaluation of T. longibrachiatum and Pseudomonas sp. Culture Filtrates against Botrytis cinerea

The antifungal activity of *T. longibrachiatum* and *Pseudomonas* sp. filtrates on the mycelial growth inhibition of *B. cinerea* was evaluated in vitro using dual culture techniques. A total of 25 mL of each filtrate concentration was incorporated separately under aseptic conditions into 200 mL of molten potato dextrose agar (PDA) medium. The bacterial cultural filtrate was obtained by transferring a loop full of bacterial colony, cultivated previously in King's B medium, into nutrient broth conical flasks and incubated in an electric shaker for 48 h at 200 rpm at 32 °C. The fungal cultural filtrate was obtained by transferring mycelia plugs into flasks containing sterile potato dextrose broth (PDB) and incubated with agitation at 150 rpm for 10 days [22]. Then, each fungal cultrate was filtered through 0.45 µm pore size filters to remove the mycelial pellets and then refiltered via a 0.22 µm membrane filter. Culture filtrates were kept in the refrigerator until the bioassay. In this experiment, four concentrations (C1: 20%; C2: 40%; C3: 60%; C4: 80%) of *T. longibrachiatum* and *Pseudomonas* sp. filtrates were used separately for their efficacy.

The percentage of mycelial growth inhibition (MGI) was evaluated 8 days after inoculation according to Formula (1), as demonstrated by [23].

$$\text{MGI (\%)} = (1 - \text{Ce}/\text{Ct}) \times 100; \quad (1)$$

where Ce is the radial growth diameter of *B. cinerea* in the presence of filtrate. Ct is the radial growth diameter of *B. cinerea* in the absence of filtrate.

The mycelial growth rate (MGR) was measured according to Formula (2), as reported by [24].

$$\text{MGR (mm/h)} = [\text{D1}/\text{Te1}] + [(\text{D2} - \text{D1})/\text{Te2}] + [(\text{D3} - \text{D2})/\text{Te3}] + \ldots\ [(\text{Dn} - \text{Dn} - 1)/\text{Ten}] \quad (2)$$

where D: radial growth diameter of *B. cinerea* per day (1, 2, 3, 5, 6, and 8 days after inoculation); Te: incubation time.

2.4. In Vivo Evaluation of T. longibrachiatum, Pseudomonas sp. and/or Salicylic Acid on Tomato Seedlings in the Presence of Botrytis cinerea

Tomato seeds (cv. Firenze) were sterilized via treatment with ethanol, washed with sterile distilled water two times, and dried at room temperature. After thorough drying, the tomato seeds were treated separately with *T. longibrachiatum* and *Pseudomonas* sp. filtrates (80%), salicylic acid ($C_6H_4(OH)CO_2H$), *T. longibrachiatum* filtrate (80%) + salicylic acid, and *Pseudomonas* sp. filtrate (80%) + salicylic acid. This assay was carried out by dipping seeds into a flask containing each treatment mentioned above for 30 min. After 24 h, the seeds were inoculated with *B. cinerea* (10^6 spores/mL). Two controls were performed; one by dipping the tomato seeds only in distilled water (negative control) and the second with the pathogen only (positive control). The treated seeds were placed in Fahraeus medium with an average of seven seeds per Petri plate and were subsequently incubated for 21 days in a growth chamber with a 12 h/12 h day/night photoperiod. The experimental design was a randomized complete block, arranged in three blocks of 10 Petri plates each, and the entire experiment was repeated twice.

2.4.1. Effect of Culture Filtrate on Tomato Seedling Gray Mold Disease Severity

At the end of the experiment (21 days of incubation), the tomato seedlings were carefully removed from the Petri dishes. Symptoms of gray mold were scored using a scale from 0 to 4, according to [25]. The disease severity index (DSI) was processed by McKinney's Formula (3):

$$\text{DSI (\%)} = (\Sigma \text{vn})/(\text{NV}) \times 100 \quad (3)$$

where v represents the numeric value of the disease index scale, n is the number of seedlings assigned to the disease index scale, N is the total number of seedlings, and V is the numeric value of the largest disease index scale.

The resistance level of each treatment to gray mold was determined according to its DSI; EE: extremely effective (DSI = 0%), HE: highly effective (DSI = 0.1 to 5%), E: effective (DSI = 5.1 to 25%), I: ineffective (DSI = 25.1 to 50%) and HI: highly ineffective (DSI = 50.1 to 100%) [26].

2.4.2. Determination of Phenotypic and Plant Growth Parameters

Chlorophyll content was also measured using the method described by [27]. Chlorophyll content data were processed by using Formulas (4)–(6):

$$\text{Chlorophyll a (Chl a)} = 12.41 \times \text{absorbance } 663 - 2.59 \times \text{absorbance } 645 \quad (4)$$

$$\text{chlorophyll b (Chl b)} = 22.90 \times \text{absorbance } 645 - 4.68 \times \text{absorbance } 663 \quad (5)$$

$$\text{total chlorophyll (Chl T)} = \text{Chl a} + \text{Chl b} \quad (6)$$

Chl a, Chl b, and Chl T were expressed in mg/g fresh weight.

Other agronomic measurements of fresh weight (FWS; g) and length (SL; cm) of tomato seedlings were assessed. DSI, Chl a, Chl b, Chl T, SL, and FWS were evaluated on 30 tomato seedlings per treatment and per block (3 blocks).

2.5. In Vivo Evaluation of T. longibrachiatum, Pseudomonas sp. and/or Salicylic Acid on Tomato Fruits Inoculated with Botrytis cinerea

Healthy tomato fruits (*cv*. Firenze) at the ripening stage with uniform size were harvested from the biological field (Sidi Bouzid, Tunisia) and immediately transported to the laboratory. The selected fruits were rinsed with sterile distilled water, immersed in NaClO (2.5%) for 3 min, washed two times with sterile distilled water, and dried under a sterile flow cabinet. Fruits were wounded (5 mm deep) on the blossom end with a sterile needle. A total of 20 µL of each treatment was pipetted onto each wound site. After 2 h, the tomato fruits were inoculated with 20 µL of spore suspension of *B. cinerea* (10^6 spores/mL). Seven treatments were tested; T1: negative control (fruits treated only with 20 µL of distilled water), T2: positive control (fruits treated only with 20 µL of *B. cinerea* 10^6 spores suspension/mL), T3: *T. longibrachiatum* filtrate (80%), T4: *Pseudomonas* sp. filtrate (80%), T5: salicylic acid, T6: *T. longibrachiatum* filtrate (80%) + salicylic acid, and T7: *Pseudomonas* sp. filtrate (80%) + salicylic acid. Treated fruits were placed in plastic containers on sterile wet paper with an average of six.

Containers were enclosed in a plastic bag to maintain high humidity (>90%) and were subsequently incubated for 7 days in a growth chamber with an 8 h/16 h day/night photoperiod at 21 °C. The experimental design was a randomized complete block, arranged in three blocks of 10 containers each, and the entire experiment was repeated twice [28].

After 7 days, the pathological, morphometric, physicochemical, and biochemical attributes were investigated to determine the antifungal activity of the above-mentioned treatments on tomato fruits.

2.5.1. Effect on Gray Mold Severity

The percentage of fruit area covered by gray mold (PFA) was estimated by using Formula (7), as described by [29]:

$$\text{PFA (\%)} = (\text{LAP}/\text{TLA}) \times 100 \quad (7)$$

where LAP is the lesion diameter covered by gray mold; TLA is the total diameter of the tomato fruit. The disease severity index (DSI) and resistance level of each treatment were also evaluated.

2.5.2. Determination of Morphometric Fruit Quality

The morphometric quality of the fruits was studied by analyzing two parameters; the firmness and the color density of the tomato fruits. The firmness was determined according to [30]. The fruits' color density (L and a/b) was evaluated using a Konica Minolta CR10 colorimeter (Tokyo, Japan) [31,32].

2.5.3. Determination of Physiochemical Fruit Parameters

Some physicochemical measurements were evaluated regarding the tomato fruits, such as water content, juice yield, pH, titratable acidity, sugar content, nitrate content, and electrical conductivity.

Water content is determined by using Formula (8):

$$\text{WC (\%)} = (\text{FF} - \text{FS})/(\text{FF}) \times 100 \quad (8)$$

where WC = water content; FF = fresh weight of the fruit; FS = dry weight of the fruit.

The juice content is determined by using Formula (9):

$$\text{JC (\%)} = (\text{weight of juice})/(\text{fresh weight of fruit}) \times 100 \quad (9)$$

where JC = juice content; FF = fresh weight of the fruit; FS = dry weight of the fruit.

A pH meter electrode was inserted into the tomato juice and the pH was recorded [33].

The sugar content was measured and expressed in Brix degree by pouring one to two drops of tomato juice on the prism of a digital pocket refractometer (type: Atago pal-, Japan) [34].

The nitrate content and electrical conductivity of the juice were respectively determined using a nitrashek device (type LAQUA-twin Germany) and a portable conductivity meter (type HI 99301-Hanna-Virginia—USA).

The titration technique was used to evaluate the titratable acidity (TA) of the juice by using Formula (10), as described by [32]:

$$\text{TA} = \text{k} \times \text{a} \times \text{f}. \quad (10)$$

where TA: titratable acidity, defined as [g AC/10 mL juice]; K: conversion factor for citric acid conversion = 0.64; f: sodium hydroxide solution 0.1 M factor = 1, and a: volume of 0.1 M sodium hydroxide solution used (in mL).

2.5.4. Determination of Antioxidant Enzymatic Activities

To understand the biochemical changes of tomato fruits treated preventively with salicylic acid and aqueous seed extract (separately or in combination), a set of biochemical parameters were assessed. For the enzyme activities, peroxidase activity was carried out, according to [35]. Catalase activity was evaluated according to the method in [36]. The method in [37] was employed to determine polyphenol-oxidase. Ascorbate peroxidase was evaluated according to the method in [38].

2.5.5. Determination of Stress Markers

Total phenolic content was assessed according to the method of Folin-Ciocalteu [38]. Malondialdehyde (MDA) was determined according to [39]. Total protein content was calculated according to the method described by [40].

2.6. Statistical Analysis

Statistical analysis was performed using the mean values of the replicates. The data were analyzed by ANOVA using SPSS version 20.0 statistical software (SPSS, SAS Institute, Cary, NC, USA). The homogeneity of the variances and normality was checked by applying Duncan's multiple range test. The differences between the treatments were determined by Duncan's multiple range test. All statistical tests were performed with a significance level of 5% ($p \leq 0.05$).

3. Results

3.1. In Vitro Plant-Growth-Promoting Activities

T. longibrachiatum and *Pseudomonas* sp. exhibit plant growth-promoting (PGP) traits, as they are both able to produce IAA and HCN and solubilized phosphate. *Trichoderma* was shown to be able to produce protease, glucanase, and chitinase enzymes. Moreover, *Pseudomonas* sp. displays the potential to produce amylase and catalase enzymes (Table 1).

Table 1. Extracellular enzyme activities and plant-growth-promoting traits of *T. longibrachiatum* and *Pseudomonas* sp.

Microorganism	Cat	Pec	Amy	Pro	Chit	Glu	AIA	HCN	N	P
T. longibrachiatum	+	-	-	+	+	+	+	+	-	+
Pseudomonas sp.	+	-	+	-	-	-	+	+	-	+

Cat: catalase production; Pec: pectinase production; Amy: amylolytic activity; Pro: proteolytic activity; Chit: chitinase enzyme activity; Glu: β-1,3-glucanase activity; I IAA: AA production; HCN: hydrocyanic acid production; N: atmospheric nitrogen fixation; P: phosphate solubilization; +: presence; -: absence.

3.2. In Vitro Evaluation of T. longibrachiatum and Pseudomonas sp. Culture Filtrates against Botrytis cinerea

Pseudomonas sp. and *T. longibrachiatum* were assessed for their antagonism effect on the causal agent of tomato gray mold (*Botrytis cinerea*) through in vitro experiments (Figure 1; Table 2). The mycelial growth of *B. cinerea* was affected significantly ($p < 0.01$) by the concentrations of the two bio-agents (C1: 20%; C2: 40%; C3: 60%; 80%) and the sampling moments (1, 2, 3, 5, 6, and 8 days after incubation). The maximum values for *B. cinerea* diameter were observed on the eighth day of incubation after being treated with four concentrations of *T. longibrachiatum* and *Pseudomonas* sp. A significant decrease in mycelial growth was noted as the concentration of *T. longibrachiatum* and *Pseudomonas* sp. increased, with the highest reduction occurring at 80%. In contrast, the lowest growth was recorded at a concentration of 20%. It can be concluded that *T. longibrachiatum* and *Pseudomonas* sp., at 80% concentration, seemed to be the most effective treatment, with *B. cinerea* seeing mycelial growth from 1.2 mm (control = 5.32 mm) and 1 mm (control = 5.12 mm) 8 days after incubation (Figure 1). The effects of different concentrations of the culture filtrate of *T. longibrachiatum* and *Pseudomonas* sp. on the mycelial growth rate were tested (Table 2), and the lowest radial growth was shown by *T. longibrachiatum* at concentrations of 80% (0.83 mm h^{-1}) and 60% (0.99 mm h^{-1}) (control = 2.14 mm h^{-1}). The minimum mycelial growth rate was observed in the culture filtrate of *Pseudomonas* sp. (0.80 mm h^{-1}) at a concentration of 80%, followed by concentrations of 60% (0.97 mm h^{-1}) and 40% (0.85 mm h^{-1}) (control = 1.81 mm h^{-1}) (Table 2). The culture filtrate of *T. longibrachiatum* and *Pseudomonas* sp. at a concentration of 80% increased the mycelial growth inhibition of *B. cinerea*, obtaining data ranging from 79.63 to 80.47%, respectively. However, *B. cinerea* showed good resistance against *T. longibrachiatum* at a concentration of 20%, with an inhibition rate below 50% (Table 2).

Table 2. Effect of *T. longibrachiatum* and *Pseudomonas* sp. filtrates at various concentrations on the mycelial growth rate and inhibition of *Botrytis cinerea* after 8 days of incubation at 28 ± 2 °C under laboratory conditions.

Treatments	Mycelial Growth Rate (mm h^{-1})		Mycelial Growth Inhibition (%)	
	T. longibrachiatum	***Pseudomonas* sp.**	***T. longibrachiatum***	***Pseudomonas* sp.**
C1	1.37 ± 0.03 b [a]	1.01 ± 0.07 b	41.80 ± 0.62 d	67.58 ± 0.24 b
C2	1.15 ± 0.01 c	0.85 ± 0.12 bc	57.67 ± 0.32 c	75.78 ± 0.57 ab
C3	0.99 ± 0.04 d	0.97 ± 0.14 bc	68.52 ± 0.54 b	69.53 ± 0.39 ab
C4	0.83 ± 0.004 e	0.80 ± 0.03 c	79.63 ± 0.81 a	80.47 ± 0.90 a
Control	2.14 ± 0.04 a	1.81 ± 0.03 a	nd	nd
p-value [b]	<0.01	<0.01	<0.01	<0.01

[a] Duncan's multiple range test; the values followed by the various superscripts differ significantly at $p \leq 0.05$. [b] probabilities associated with individual F tests. The data are the average of nine Petri dishes per replicate (with three replicates). C1: 20%; C2: 40%; C3: 60%; C4: 80%; nd: not determined. Means ± standard error.

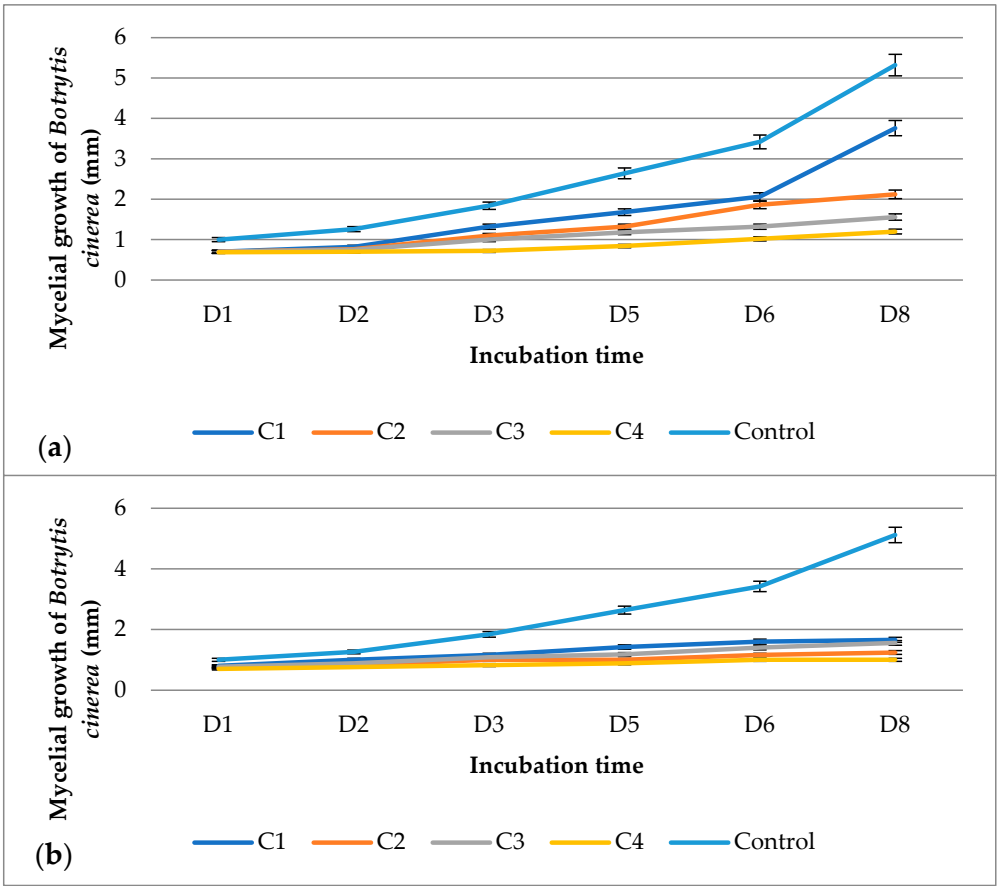

Figure 1. Effect of *T. longibrachiatum* (**a**) and *Pseudomonas* sp. (**b**) filtrates at various concentrations (C1: 20%; C2: 40%; C3: 60%; C4: 80%) on the mycelial growth (mm) of *Botrytis cinerea* at six incubation times (1 [D1], 2 [D2], 3 [D3], 5 [D5], 6 [D6], and 8 [D8] days) under laboratory conditions. The letters above the bars indicate significant differences between treatments within the experiments ($p \leq 0.5$) according to Duncan's multiple range tests. Data are the average of nine Petri dishes per replicate (with three replicates).

3.3. In Vivo Evaluation of T. longibrachiatum, Pseudomonas sp., and/or Salicylic Acid on Tomato Seedlings in the Presence of Botrytis cinerea

3.3.1. Effect of Culture Filtrates on Gray Mold Disease Incidence

The ANOVA tests showed significant differences ($p < 0.01$) in the disease severity index (DSI) observed between the treatments. All applications significantly decreased the gray mold disease severity index. *T. longibrachiatum* (9.40%) and *Pseudomonas* sp. (10.80%) induced the best results, with a decrease in DSI (positive control = 92.60%) (Table 3). The effectiveness of the treatment using *Trichoderma* sp., *Pseudomonas* sp., and/or salicylic acid was variable. The treatment with *T. longibrachiatum* and *Pseudomonas* sp. applied separately or in combination with salicylic acid revealed its ability to protect tomato seedlings against gray mold (resistance level = R). However, all treatments using only salicylic acid have shown their ineffectiveness (resistance level = S) in decreasing gray mold attacks. Thus, the positive control was highly sensitive to gray mold (resistance level = HS) (Table 3).

Table 3. Effect of preventive treatments using *T. longibrachiatum* filtrate, *Pseudomonas* sp. filtrate, and/or salicylic acid on disease severity index, chlorophyll a, chlorophyll b, total chlorophyll content, fresh weight, and the length of tomato seedlings in the presence of *Botrytis cinerea* under laboratory conditions.

Treatments	DSI	Resistance Level	Chl a (mg g^{-1} Fresh Weight)	Chl b (mg g^{-1} Fresh Weight)	Chl T (mg g^{-1} Fresh Weight)	Fresh Weight (g)	Seedling Length (cm)
Negative control	0 ± 0 f [a]	EE	6.13 ± 0.66 a	0.85 ± 0.24 cd	6.98 ± 0.41 a	0.61 ± 0.09 a	13.18 ± 0.85 a
Positive control	92.60 ± 1.71 a	HI	1.66 ± 0.11 de	0.68 ± 0.04 de	2.34 ± 0.09 g	0.17 ± 0.06 c	7.08 ± 0.81 e
T. longibrachiatum filtrate	9.40 ± 1.52 e	E	4.08 ± 0.02 b	2.43 ± 0.02 b	6.52 ± 0.02 b	0.69 ± 0.09 a	11.5 ± 0.61 b
Pseudomonas sp. filtrate	10.80 ± 1.38 e	E	2.13 ± 0.01 c	0.92 ± 0.01 c	3.05 ± 0.01 e	0.44 ± 0.07 b	11.6 ± 1.14 b
Salicylic acid	37.80 ± 1.59 b	I	2.10 ± 0.02 cd	0.63 ± 0.009 e	2.73 ± 0.02 f	0.38 ± 0.08 b	11.38 ± 0.44 bc
T. longibrachiatum filtrate + Salicylic acid	15.60 ± 1.05 d	E	1.36 ± 0.03 e	2.34 ± 0.02 b	3.70 ± 0.01 d	0.26 ± 0.04 c	9.54 ± 0.79 d
Pseudomonas sp. filtrate + Salicylic acid	22.20 ± 1.48 c	E	2.56 ± 0.02 c	2.72 ± 0.03 a	5.28 ± 0.01 c	0.43 ± 0.08 b	10.26 ± 1.21 cd
p-value [b]	<0.01	nd	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Duncan's multiple range test; the values followed by the various superscripts differ significantly at $p \leq 0.05$. [b] Probabilities associated with individual F tests. DSI: disease severity index; Chl a: chlorophyll a; Chl b: chlorophyll b; Chl T: total chlorophyll content; EE: extremely effective; HE: highly effective; E: effective; I: ineffective; HI: highly ineffective. Data are the average of 30 tomato seedlings per treatment and per block (three blocks). Means ± standard error.

3.3.2. Effect of Culture Filtrate on Tomato Seedlings' Phenotype and Growth

The results showed that chlorophyll a (Chl a) content increased significantly ($p < 0.01$) after treatments with *T. longibrachiatum* (4.08 mg g^{-1} fresh weight), *Pseudomonas* sp. (2.13 mg g^{-1} fresh weight), and *Pseudomonas* sp. + salicylic acid (2.56 mg g^{-1} fresh weight) in the presence of *B. cinerea* (Table 3). The highest content of Chl band Chl T was recorded for the tomato seeds treated preventively with *T. longibrachiatum* (2.43 and 6.52 mg g^{-1} fresh weight, respectively), *T. longibrachiatum* + salicylic acid (2.34 and 3.70 mg g^{-1} fresh weight, respectively), and *Pseudomonas* sp. + salicylic acid (2.72 and 5.28 mg g^{-1} fresh weight, respectively) against *B. cinerea* (Table 3).

Table 3 shows a significant variation ($p < 0.01$) in the fresh weight (FWS) and length (SL) of the seedlings in the different seed treatments when compared to the positive (0.17 g and 7.08 cm, respectively) and negative (0.61 g and 13.18 cm, respectively) controls. Tomato seeds treated separately with *T. longibrachiatum* and *Pseudomonas* sp. in the presence of *B. cinerea* improved regarding the fresh weight (0.69 and 0.44 g, respectively) and the length (11.5 and 11.6 cm, respectively) of the seedlings (Table 3).

Disease severity index (DSI) was negatively and significantly correlated with Chl a (r = −0.521), Chl T (r = −0.661), SL (r = −0.822), and FWS (r = −0.796). Chl a/Chl T and FWS/SL were highly correlated, with r values of 0.868 and 0.773, respectively (Table 4).

Table 4. Correlation coefficients between the disease severity index, chlorophyll a, chlorophyll b, total chlorophyll content, fresh weight, and the length of tomato seedlings.

	Chl a	Chl b	Chl T	SL	FWS
Chl b	−0.068				
Chl T	0.868 **	0.436 *			
SL	0.679 **	0.022	0.624 **		
FWS	0.585 **	0.327	0.690 **	0.773 **	
DSI	−0.521 *	−0.385	−0.661 **	−0.822 **	−0.796 **

* Significant at a level of 5%. ** Significant at a level of 1%. DSI: disease severity index; Chl a: chlorophyll a; Chl b: chlorophyll b; Chl T: total chlorophyll content; FWS: fresh weight of seedlings; SL: seedling length.

3.4. In Vivo Evaluation of T. longibrachiatum, Pseudomonas sp., and/or Salicylic Acid on Tomato Fruits Inoculated with Botrytis cinerea

3.4.1. Effect of Culture Filtrates on Fruit Gray Mold Disease Severity

The effectiveness evaluation of *T. longibrachiatum*, *Pseudomonas* sp., and/or salicylic acid in controlling gray mold on tomato fruits showed that the percentage of fruit area covered by gray mold (PFA) and disease severity index (DSI) was significantly ($p < 0.01$) lower compared to the positive controls (92.99 and 94.37%, respectively). Thus, (*T. longibrachiatum* + salicylic acid) and (*Pseudomonas* sp. + salicylic acid) induced the best results with a decrease in PFA (13.97 and 23.29%, respectively) and DSI (13.01 and 17.51%, respectively) (Table 5). The results of the resistance level to gray mold indicated that only the tomato fruits treated with *T. longibrachiatum*, *T. longibrachiatum* + salicylic acid, and *Pseudomonas* sp. + salicylic acid against *B. cinerea* seemed to be the most effective (resistance level = R) (Table 5).

Table 5. Effect of preventive treatments using *T. longibrachiatum* filtrate, *Pseudomonas* sp. Filtrate, and/or salicylic acid after 7 days of application on the percentage of fruit area covered by gray mold and the disease severity index on tomato fruits inoculated with *Botrytis cinerea* under laboratory conditions.

Treatments	PFA	DSI	Resistance Level
Negative control	0 ± 0 e [a]	0 ± 0 e	EE
Positive control	92.99 ± 0.88 a	94.37 ± 0.12 a	HE
T. longibrachiatum filtrate	24.52 ± 0.30 c	23.1 ± 0.29 c	E
Pseudomonas sp. filtrate	33.21 ± 0.78 b	27.31 ± 0.25 bc	I
Salicylic acid	40 ± 0.51 b	31.97 ± 0.08 b	I
T. longibrachiatum filtrate + Salicylic acid	13.97 ± 0.33 d	13.01 ± 0.56 d	E
Pseudomonas sp. filtrate + Salicylic acid	23.29 ± 0.07 c	17.51 ± 0.15 d	E
p-value [b]	<0.01	<0.01	Nd

[a] Duncan's multiple range test; the values followed by the various superscripts differ significantly at $p \leq 0.05$. [b] Probabilities associated with individual F tests. PFA: percentage of fruit area covered by gray mold; DSI: disease severity index; EE: extremely effective; HE: highly effective; E: effective; I: ineffective; HI: highly ineffective; Nd: not determined. Data are the average of 30 tomato fruits per treatment and per block (three blocks). Means ± standard error.

3.4.2. Effect of Culture Filtrates on Tomato Fruit Morphometric and Quality

The ANOVA tests showed significant differences in the effectiveness of *T. longibrachiatum*, *Pseudomonas* sp., and/or salicylic acid against *B. cinerea* ($p < 0.01$) in terms of fruit firmness (FF), pH, electrical conductivity (EC), water content (WC), juice yield (JY), titratable acidity (TA), sugar content (Brix), and color density (L and a/b) (Table 6). Generally, the application of the biological agents and/or salicylic acid produced excellent results against *B. cinerea* with respect to the evaluated morphometric parameters of tomato fruits, with improvements, at times, exceeding 50% when compared with the controls (Table 6). The ANOVA classified the two treatments (negative control (3.07) and *Pseudomonas* sp. + salicylic acid (3.03)) into the same homogeneous group, exhibiting the highest fruit firmness. In the same sense, the tomato fruits treated with *Pseudomonas* sp. and *T. longibrachiatum* + salicylic acid against *B. cinerea* saw enhanced fruit firmness, and the values ranged between 2.5 and 2.33, respectively (Table 6).

All treatments significantly decreased the pH of the fruits in comparison to the positive controls (4.37), as the values varied from 3.55 (fruits treated with *Pseudomonas* sp.) and 3.74 (fruits treated with *T. longibrachiatum* separately or in combination with salicylic acid) (Table 6). Salicylic acid, applied separately or in combination with *T. longibrachiatum* in the presence of *B. cinerea*, exhibited the lowest electrical conductivity, which ranged from 1.76 to 1.92 mS cm^{-1} (negative control = 2.14 mS cm^{-1}; positive control = 2.63 mS cm^{-1}) (Table 6). The tomato fruits treated separately with *T. longibrachiatum* (98.43%) or in combination with salicylic acid (97.65%) decreased significantly in terms of water content (negative control = 98.15%; positive control = 99.59%) (Table 6). *T. longibrachiatum* (69.22%), *Pseudomonas* sp.

(56.56%), and *Pseudomonas* sp. + salicylic acid (56.02%) exhibited the highest juice yield (negative control = 43.47%; positive control = 30.66%) (Table 6).

The application of salicylic acid separately (1.18 g/10 mL juice) or in combination with *Pseudomonas* sp. (1.08 g/10 mL juice) enhanced titratable acidity. The same results were obtained for the tomato fruits treated only with distilled water (1.18 g/10 mL juice). However, the fruits treated only with *Pseudomonas* sp. (0.83 g/10 mL juice) and *T. longibrachiatum* (0.83 g/10 mL juice) showed their ineffectiveness at reducing titratable acidity. The highest increase in sugar content was recorded for the tomato fruits treated separately with *Pseudomonas* sp. or in combination with salicylic acid (Table 6). The different measurements carried out on the color density revealed that treatment with *T. longibrachiatum*, *Pseudomonas* sp., and/or salicylic acid decreased the value of L and a/b (Table 6).

Table 6. Effect of preventive treatments using *T. longibrachiatum* filtrate, *Pseudomonas* sp. Filtrate, and/or salicylic acid after 7 days of application on the morphometric parameters of tomato fruits inoculated with *Botrytis cinerea* under laboratory conditions.

Treatments	Fruit Firmness	pH	Electrical Conductivity (mS cm^{-1})	Water Content (%)	Juice Yield (%)	Titratable Acidity (g/10 mL Juice)	Sugar Content (° Brix)	Color Density	
								L	a/b
Negative control	3.07 ± 0.15 a [a]	3.59 ± 0.01 b	2.14 ± 0.23 d	98.15 ± 0.10 f	43.47 ± 0.05 e	1.18 ± 0.02 a	6.27 ± 0.15 a	39.47 ± 0.75 a	1.09 ± 0.07 a
Positive control	0 ± 0 e	4.37 ± 0.55 a	2.63 ± 0.19 a	99.59 ± 0.5 a	30.66 ± 0.15 g	0.89 ± 0.01 d	4.93 ± 0.11 c	41.80 ± 0.60 a	1.23 ± 0.05 a
T. longibrachiatum filtrate	2.1 ± 0.17 c	3.74 ± 0.01 b	2.57 ± 0.02 b	98.43 ± 0.01 e	69.22 ± 0.05 a	0.83 ± 0.01 e	5.30 ± 0.43 c	33.87 ± 0.49 b	1.16 ± 0.13 a
Pseudomonas sp. filtrate	2.5 ± 0.28 b	3.55 ± 0.01 b	2.34 ± 0.11 c	98.96 ± 0.05 d	56.56 ± 0.05 b	0.83 ± 0.01 e	5.87 ± 0.05 b	36.33 ± 0.53 b	0.87 ± 0.10 b
Salicylic acid	1.7 ± 0.05 d	3.73 ± 0.02 b	1.76 ± 0.01 g	99.42 ± 0.02 b	53.92 ± 0.06 d	1.18 ± 0.02 a	5.07 ± 0.11 c	34.87 ± 0.23 b	1.14 ± 0.12 a
T. longibrachiatum filtrate + Salicylic acid	2.33 ± 0.10 b	3.74 ± 0.05 b	1.92 ± 0.01 f	97.65 ± 0.04 g	39.68 ± 0.11 f	1.02 ± 0.01 c	4.97 ± 0.06 c	35.33 ± 0.36 b	0.89 ± 0.08 b
Pseudomonas sp. filtrate + Salicylic acid	3.03 ± 0.06 a	3.56 ± 0.06 b	2.12 ± 0.05 e	99.13 ± 0.04 c	56.02 ± 0.05 c	1.08 ± 0.05 b	5.97 ± 0.21 ab	36.40 ± 0.29 b	1.19 ± 0.17 a
p-value [b]	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Duncan's multiple range test; values followed by various superscripts differ significantly at $p \leq 0.05$. [b] Probabilities associated with individual F tests. Data are the average of 30 tomato fruits per treatment and per block (three blocks). Means ± standard error.

3.4.3. Effect of Culture Filtrates on Antioxidant Enzymatic Activities

The applications of *T. longibrachiatum* + salicylic acid and *Pseudomonas* sp. + salicylic acid increased the peroxidase (4.17 and 4.16 units mg^{-1} min^{-1}, respectively) and catalase (43.84 and 42.56 µmol H_2O_2 mg protein^{-1}, respectively) activities, while the lowest POX (1.96 units mg^{-1} min^{-1}) and CAT (32.12 µmol H_2O_2 mg protein^{-1}) were registered for the tomato fruits inoculated only with *B. cinerea* (negative control: POX = 4.06 units mg^{-1} min^{-1}; CAT = 44.58 µmol H_2O_2 mg protein^{-1}) (Table 7). Table 7 shows significant PPO activity in the different tomato fruit treatments when compared to the positive (19.03 units mg^{-1} min^{-1}) and negative (10.92 units mg^{-1} min^{-1}) controls. The most significant increase was detected for salicylic acid (19.93 units mg^{-1} min^{-1}), whereas *Pseudomonas* sp. + salicylic acid (7.22 units mg^{-1} min^{-1}) recorded the lowest activity of PPO (Table 7). The treatments increased the APX activity when compared to the positive control (15.78 µmol mg^{-1} min^{-1}). The increase in APX was greater for those tomato fruits treated with salicylic acid (44.17 µmol mg^{-1} min^{-1}) and the untreated control (39.39 µmol mg^{-1} min^{-1}) (Table 7). Salicylic acid applied separately (4.82 µg g^{-1}) or in combination with *Pseudomonas* sp. (4.94 µg g^{-1}) against *B. cinerea* on tomato fruits showed the greatest TPC (positive control = 2.88 µg g^{-1}; negative control = 5.48 µg g^{-1}) (Table 7).

Table 7. Effect of preventive treatments of *T. longibrachiatum* filtrate, *Pseudomonas sp.* Filtrate, and/or salicylic acid on catalase and peroxidase activities, polyphenol-oxidase, ascorbate peroxidase, total phenolic content, total protein content, and malondialdehyde in tomato fruits inoculated with *Botrytis cinerea* under laboratory conditions.

Treatments	POX (Units mg^{-1} min^{-1})	PPO (Units mg^{-1} min^{-1})	CAT (µmol H_2O_2 mg $protein^{-1}$)	APX (µmol mg^{-1} min^{-1})	TPC (µg g^{-1})	TP (mg g^{-1})	MDA (µmol g^{-1})
Negative control	4.06 ± 0.08 ab [a]	10.92 ± 0.03 e	44.58 ± 0.23 a	39.39 ± 0.92 b	5.48 ± 0.27 a	8.45 ± 0.47 c	1.75 ± 0.02 b
Positive control	1.96 ± 0.24 b	19.03 ± 0.15 b	32.12 ± 0.15 e	15.78 ± 0.25 f	2.88 ± 0.06 d	37.13 ± 0.13 a	5.73 ± 0.04 a
T. longibrachiatum filtrate	3.79 ± 0.07 b	15.15 ± 0.21 c	38.36 ± 0.09 c	32.61 ± 0.57 c	4.58 ± 0.08 bc	12.64 ± 0.93 b	2.28 ± 0.98 b
Pseudomonas sp. filtrate	3.76 ± 0.07 b	13.74 ± 0.52 d	36.32 ± 0.10 d	25.68 ± 0.63 d	4.60 ± 0.34 bc	12.33 ± 0.39 b	2.79 ± 0.16 b
Salicylic acid	3.78 ± 0.28 b	19.93 ± 0.11 a	36.28 ± 0.24 d	44.17 ± 0.45 a	4.82 ± 0.42 bc	11.94 ± 0.26 b	2.65 ± 0.01 b
T. longibrachiatum filtrate + Salicylic acid	4.17 ± 0.13 a	14.75 ± 0.09 c	43.84 ± 0.18 ab	30.37 ± 0.73 c	4.35 ± 0.29 c	12.64 ± 0.38 b	2.62 ± 0.71 b
Pseudomonas sp. filtrate + Salicylic acid	4.16 ± 0.11 a	7.22 ± 0.57 f	42.56 ± 0.09 b	22.82 ± 0.75 e	4.94 ± 0.05 b	8.99 ± 0.35 c	1.90 ± 0.04 b
p-value [b]	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Duncan's multiple range test; values followed by various superscripts differ significantly at $p \leq 0.05$. [b] Probabilities associated with individual F tests. POX: peroxidase; PPO: polyphenol oxidase; CAT: catalase; APX: ascorbate peroxidase; TPC: total phenolic content; TP: total protein content; MDA: malondialdehyde. Data are the average of 30 tomato fruits per treatment and per block (three blocks). Means ± standard error.

3.4.4. Effect of Culture Filtrates on Protein, Total Phenols, and MDA Contents

Fruits treated with *Pseudomonas* sp. + salicylic acid in the presence of *B. cinerea* exhibited the lowest total protein content. The TP values ranged from 8.99 mg g^{-1} (*Pseudomonas* sp. + salicylic acid) to 12.64 mg g^{-1} (*T. longibrachiatum* applied separately or in combination with salicylic acid) (positive control = 37.13 mg g^{-1}; negative control = 8.45 mg g^{-1}) (Table 7). Tomato fruits treated with *T. longibrachiatum*, *Pseudomonas* sp., and/or salicylic acid in the presence of *B. cinerea* reduced significantly in terms of malondialdehyde when compared to the positive control (5.73 µmol g^{-1}), as the values ranged from 1.75 µmol g^{-1} (negative control) to 2.79 µmol g^{-1} (*Pseudomonas* sp.) (Table 7).

DSI/MDA, DSI/TP, DSI/pH, DSI/PFA, PFA/TP, and PFA/WC were highly correlated, with r values ranging from 0.748 and 0.982. DSI and PFA have a significant negative correlation with CAT (−0.848 and −0.877, respectively), APX (−0.610 and −0.589, respectively), POX (−0.960 and −0.921, respectively), TPC (−0.886 and −0.868, respectively), and FF (−0.945 and −0.928, respectively). FF and POX showed a high positive correlation, with r values of 0.911. TP has a significant negative correlation with POX (−0.965) and FF (−0.925) (Table 8).

Table 8. Correlation coefficients between fruit firmness, the potential of hydrogen, electrical conductivity, water content, juice yield, titratable acidity, sugar content, color density (L and a/b), nitrate content, percentage of fruit area covered by gray mold, disease severity index, catalase and peroxidase activities, polyphenol oxidase, ascorbate peroxidase, total phenolic content, total protein content, and malondialdehyde.

	APX	POX	PPO	TPC	MDA	TP	FF	pH	EC	WC	JY	TA	Brix	L	a/b	PFA	DSI
CAT	0.360	0.793 **	−0.712	0.675 **	−0.629 **	−0.725 **	0.817 **	−0.556 **	−0.481 *	−0.757 **	0.076	0.520 *	0.461 *	−0.258	−0.202	−0.877 **	−0.848 **
APX		0.573 **	0.141	0.659 **	−0.500 *	−0.626 **	0.407	−0.428	−0.639 **	−0.311	0.324	0.604 **	0.104	−0.441 *	−0.062	−0.589 **	−0.610 **
POX			−0.577 **	0.815 **	−0.753 **	−0.965 **	0.911 **	−0.751 **	−0.614 **	−0.589 **	0.522 *	0.401	0.455 *	−0.627 **	−0.298	−0.921 **	−0.960 **
PPO				−0.547 *	0.509 *	0.569 **	−0.795 **	0.538 *	0.056	0.328	−0.241	−0.179	−0.765 **	0.043	0.039	0.613 **	0.611 **
TPC					−0.785 **	−0.895 **	0.873 **	−0.730 **	−0.529 *	−0.396	0.505 *	0.538 *	0.635 **	−0.408	−0.247	−0.868 **	−0.886 **
MDA						0.791 **	−0.754 **	0.639 **	0.414	0.431	−0.505 *	−0.348	−0.567 **	0.468 *	0.332	0.755 **	0.775 **
TP							−0.925 **	0.812 **	0.581 **	0.492 *	−0.612 **	−0.415	−0.537 *	0.620 **	0.286	0.910 **	0.954 **
FF								−0.796 **	−0.433 *	−0.542 *	0.477 *	0.355	0.699 **	−0.427	−0.293	−0.928 **	−0.945 **
pH									0.415	0.347	−0.522 *	−0.249	−0.517 *	0.497 *	0.285	0.714 **	0.781 **
EC										0.212	0.027	−0.795 **	−0.014	0.363	0.242	0.485 *	0.538 *
WC											−0.015	−0.084	−0.136	0.269	0.443 *	0.748 **	0.685 **
JY												−0.207	0.252	−0.721 **	0.006	−0.410	−0.465 *
TA													0.222	0.022	0.135	−0.397	−0.419
Brix														0.147	−0.127	−0.539 *	−0.547 *
L															0.273	0.465 *	0.527 *
a/b																0.321	0.327
PFA																	0.982 **

* Significant at a level of 5%. ** Significant at a level of 1%. FF: fruit firmness; pH: the potential of hydrogen; EC: electrical conductivity; WC: water content; JY: juice yield; TA: titratable acidity; Brix: sugar content; L: color density; L) a/b: color density a/b; NC: nitrate content; PFA: percentage of fruit area covered by gray mold; DSI: disease severity index; POX: peroxidase; PPO: polyphenol oxidase; CAT: catalase; APX: ascorbate peroxidase; TPC: total phenolic content; TP: total protein content; MDA: malondialdehyde.

4. Discussion

Botrytis cinerea is one of the major tomato diseases. Several bioassays were conducted to reduce the damage and the disease incidence of this pathogen. Therefore, during the screening of new BCA programs, understanding the potential employed mechanism is an essential step [13,17]. In addition, plant-growth-promoting (PGP) bacteria and fungi are known to impart induced systemic resistance to fungal plant diseases. They have been shown to elicit plant defense systemically against *B. cinerea* [41,42]. Previous investigations revealed that different PGP species protect the plants from various pathogens by activating plant defense genes and other enzymes, many of which act as primary reactive oxygen species scavengers [19–21].

During the current study, the microbial culture filtrate of soil fungus and bacteria at different concentrations inhibited the development of the phytopathogenic fungus *B. cinerea*. The inhibitory effect of culture filtrate of both of the tested BCAs suggested that the potential antagonistic mechanism is possible due to the secondary metabolites. These results are in accordance with previous studies, which have shown the promising antifungal potential of micro-organisms against the postharvest fungus *B. cinerea* by using secondary metabolites [15,16,41,42].

Different *Trichoderma* species were confirmed for suppressing *B. cinerea* growth, particularly by culture filtrates or by directly applying their secondary metabolites [15,43]. Microbial volatile compounds (VOCs) have recently been reported as antifungal sources against several phytopathogenic fungi, and they have been shown to use numerous mechanisms, like the disruption of the cell wall and membrane structures, leading to intracellular lysate leakage and oxidative stress induction [42,44]. Similarly, in the current study, the results demonstrated that *T. longibrachiatum* exhibits plant-growth-promoting (PGP) traits by producing IAA, HCN, and solubilized phosphate. Furthermore, *Trichoderma* has been shown to be able to produce protease, glucanase, and chitinase enzymes, allowing for the inhibition process of *B. cinerea*.

The disease severity index in plants is a key parameter to assess the efficacy of the tested treatments [45]. In the current investigation, the gray mold disease severity index was significantly reduced by a single application of each micro-organism on tomato seedlings. In addition, the preventive treatment of tomato seeds with the culture filtrate of *T. longibrachiatum* showed the highest chlorophyll content, fresh weight, and length of seedlings, which may be due to the influence of *T. longibrachiatum* on improving plant-growth-related functions like PSII efficiency, CO_2 plant assimilation, stomatal conductance, leaf water potential, and relative water content. This finding agrees with others reported in [46]. The authors demonstrated that *T. longibrachiatum* allows for the maintenance of an optimal hydration cell level by accumulating osmolytes, which improve water uptake. Similarly, ref. [47] reported that *T. longibrachiatum* helps to enhance photosynthetic efficiency and chlorophyll fluorescence, which enables plants to mitigate stress.

The use of beneficial micro-organisms in protecting plant health and promoting growth has been shown in several *Trichoderma* [15,48] and *Pseudomonas* [16,49,50] species on different agricultural crops. In the present study, the preventive treatments of *T. longibrachiatum*, *Pseudomonas* sp., and those associated with salicylic acid significantly reduced the gray mold and disease severity index on the tomato fruits inoculated with *Botrytis cinerea*. This is probably due to the ability of this micro-organism to inhibit the enzymatic activity of plants, which decreases the negative effect of the pathogen, as demonstrated in various studies [51,52].

Recently *T. longibrachiatum* has been reported to inhibit the mycelial growth of *F. solani* on common bean (*Phaseolus vulgaris* L.) and to induce defense responses by showing the significant upregulation of the defense-related genes PR1, PR2, PR3, and PR4 [53]. Furthermore, in a previous study [54], a significant nematicide effect of *T. longibrachiatum* against root-knot nematode, plant-growth-promoting attributes, and inducing tomato defense responses was reported.

The fruits treated separately with salicylic acid + *T. longibrachiatum* and salicylic acid + *Pseudomonas* sp. as biological control agents against *B. cinerea* not only decreased the severity of the disease but also improved the biochemical, physicochemical, and morphometrical reactions. These results are close to those obtained by Colla et al. [55] and Ruiz-Cisneros et al. [56], who showed that *Trichoderma* spp. and *Pseudomonas* spp. could be effective against the infection of tomato fruits by *B. cinerea*, *Alternaria solani*, *Fusarium oxysporum*, and *Phytophthora infestans*, as measured by the levels of decay, firmness, ethylene production, total soluble solids content, color, weight, size, titratable acidity, and bromatological composition. As stated by Singh et al. [57] and Vukelić et al. [58], the quality of tomato fruits treated separately with *Trichoderma* spp. was altered in the sense of increased total flavonoids content, decreased starch, and increased bioaccumulation index for Fe and Cr. Borrero et al. [59] and Silva-Beltrán et al. [60] recorded reduced levels of total phenolic content, total antioxidant capacity, total and individual anthocyanins, and antioxidant proteins, which appear to generally indicate a ROS-enriched environment in tomato fruits treated with *Trichoderma* against *Fusarium* spp. Seo et al. [61], Konappa et al. [62], and Oszust et al. [63] documented that the combination of *Trichoderma* spp. with *Pseudomonas* spp. offered impressive protection against postharvest diseases on tomato fruits. The innate immunity elicited by the treatment of tomato fruits with *Trichoderma* spp. and *Pseudomonas* spp. was analyzed by the expression of defense-related enzymes (polyphenol oxidase, phenylalanine ammonia-lyase, and peroxidase) [62,63]. The activity of defense-related enzymes might, then, indicate the resistance of tomato fruit against *B. cinerea*, with an increase in their activity and accumulation depending on the treatments and the physiological condition of the fruit. A series of morphological and biochemical changes trigger the synthesis of defense chemicals against plant phytopathogens that suppress or retard their development [62,64].

5. Conclusions

The use of beneficial micro-organisms (bacteria and fungi) can include biological control agents and/or biostimulants for crop growth and yield. The microbial culture filtrates of *T. longibrachiatum* and *Pseudomonas* sp. showed the inhibition of *B. cinerea* growth that infects tomato plants. The antifungal activity towards the causal agent of gray mold disease due to the cultures filtrates was attributed mainly to the antibiotic activity of microbial secondary metabolites. This research gives an insight into the mechanisms of action of microbial biocontrol agents and biostimulants.

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