In Vitro and *In Planta* Evaluation of *Trichoderma* spp. for the Control of *Neopestalotiopsis rosae* on Strawberry

Tom E. Schierling *, Ralf T. Voegele, Abbas El-Hasan *

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Article

In Vitro and *In Planta* Evaluation of *Trichoderma* spp. for the Control of *Neopestalotiopsis rosae* on Strawberry

Tom E. Schierling *, Ralf T. Voegele and Abbas El-Hasan *

Department of Phytopathology, Institute of Phytomedicine, Faculty of Agricultural Sciences, University of Hohenheim, Otto-Sander-Str. 5, D-70599 Stuttgart, Germany

Abstract

Neopestalotiopsis rosae is an emerging fungal pathogen causing leaf blight and fruit rot on strawberry. Due to limited fungicide availability and the small number of substances confirmed to be effective against this pathogen, alternative disease control strategies have become a focus of current research. This study aimed to assess, quantify and compare the efficacy of extracts and inocula of *Trichoderma* spp. with the conventional fungicide Switch in controlling *N. rosae*. While *T. harzianum* T16 and *T. asperellum* T23 extracts showed moderate inhibitory effects against *N. rosae*, *T. koningiopsis* T10 extracts were able to inhibit mycelial growth and conidia germination as effectively as the reference fungicide. Under greenhouse conditions, extracts from *T. koningiopsis* T10 were as effective as Switch in controlling leaf spots incited by *N. rosae*. Our findings highlight *T. koningiopsis* T10 extracts as a promising alternative to chemical fungicides in the integrated management of *N. rosae* on strawberry.

Keywords: biological control; fungal pathogen; secondary metabolites; fungicides; sustainable pest management; pesticide alternatives

1. Introduction

Strawberries are among the most economically important berry crops worldwide [1,2]. Cultivation of this crop is highly challenging, requiring significant investment of resources and labor, resulting in high production costs, particularly with regard to the persistent threat of pathogen infestation [3–5]. Multiple plant pathogens can infect strawberry plants and pose a serious threat to strawberry production [6,7]. Leaf spots, fruit, crown, and root rots as well as wilt are particularly deleterious diseases, incited by fungal pathogens such as *Ramularia* sp., *Botrytis* sp., *Phytophthora* sp., *Colletotrichum* sp., *Fusarium* sp. and *Verticillium* sp. [8–12]. Whilst it is crucial to distinguish between these different pathogens for the implementation of targeted control strategies, the emergence of new pathogens further reinforces the need for proactive monitoring systems and dynamic management responses to safeguard strawberry production [5].

Newly emerging pathogens such as *Neopestalotiopsis rosae* pose a substantial risk to strawberry production [5]. Early-stage infections by *N. rosae* may be misidentified, and even when correctly diagnosed, effective control measures are rather limited [13–16]. *N. rosae* is a fungal pathogen known to cause leaf spot, crown rot and fruit rot in strawberries [5,17–20]. Recently, multiple outbreaks of *N. rosae* have been reported in the United States, where no chemical control strategies are available [13,19], leading to significant crop losses with yield reduction of up to 70% [13,17,19,21]. In Germany, *N. rosae* has not yet been included in the national list of quarantine plant pathogens, thus no officially approved methods of control have been established yet [22].

A limited number of chemical fungicides have demonstrated partial efficacy against *N. rosae*, and options for effective management are even more restricted [13–16]. Cyprodinil and Fludioxonil either individually or in combination were able to completely inhibit mycelial growth of *N. rosae* [13]. Both fungicides are currently approved and widely utilized in Germany for the control of *Botrytis*

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cinerea and other fungal pathogens in strawberries [22]. Nevertheless, the application of chemical fungicides is associated with concerns regarding environmental and public health, particularly due to residue accumulation and the subsequent decline in biodiversity [23]. Furthermore, the emergence of fungicide resistance poses a significant risk to the long-term effectiveness of this control measure [24,25].

The sustainable management of phytopathogens can be achieved through integrated pest management (IPM) including the utilization of biocontrol agents (BCAs) [26,27]. Research on BCAs has been ongoing for several decades [28–32]. Members of the genus *Trichoderma* are among the most extensively studied, owing to their diverse mechanisms in suppressing fungal pathogens [33–37], including mycoparasitism [38–40], induction of plant resistance to pathogens [41,42], and cell wall-degrading capabilities [43,44]. Another notable mode of action is the high competition capability for space and nutrients [45]. Moreover, the production of antifungal secondary metabolites by *Trichoderma* spp. has been well characterized [46–49]. Despite numerous studies investigating secondary metabolite production by *Trichoderma* spp., their efficacy in controlling fungal pathogens remains insufficiently understood [50]. *Trichoderma* secondary metabolites have shown significant antagonistic effects against *Fusarium moniliforme* in maize and *Phakopsora pachyrihzi* on soybean [51,52]. The germination of *B. cinerea* conidia has been shown to be inhibited by gliotoxin, a metabolite produced by *Trichoderma* spp. [53]. Additionally, 6-pentyl-alpha-pyrone (6PAP) has been reported not only to reduce mycelial growth of *F. oxysporum*, *B. cinerea* and *R. solani*, but also to promote plant growth and induce systemic resistance [53,54]. There are currently limited reports describing the control of *N. rosae* using culture extracts or secondary metabolites of *Trichoderma* spp. Therefore, the present study explored the potential of *Trichoderma* spp. and their secondary metabolites as a biological control measure for *N. rosae*.

The specific objectives of this study were (1) to investigate and quantify the antifungal effects of culture extracts of *Trichoderma* spp. on *N. rosae* and to compare their efficacy with that of the conventional fungicide Switch (cyprodinil + fludioxonil), and (2) to compare their effectiveness to the BCA treatments utilizing conidial suspensions as well as to Switch in a greenhouse setting.

2. Materials and Methods

2.1. Micobial and Plant Materials

The fungal strains *Trichoderma koningiopsis* T10 (GenBank ACNO: OQ822265), *T. harzianum* T16 (GenBank ACNO: MW520837), *T. asperellum* T23 (GenBank ACNO: MW509067) and *Neopestalotiopsis rosae* AETS11 (GenBank NCBI: PQ511123) were obtained from the Institute of Phytomedicine at the University of Hohenheim. All microorganisms were cultivated on glucose-medium-7 (GM7) according to Rieker et al. 2023 [55]. Strawberry plants of the cultivated variety Herzle were provided by Hummel InVitro (Reinhold Hummel GmbH & Co. KG, Stuttgart, Germany).

2.2. Preparation of Conidial Suspensions from Trichoderma spp. and N. rosae

GM7-agar plates (90 mm ⌀) were inoculated with an agar plug (5 mm ⌀) of 14 days-old cultures of *Trichoderma* spp. or *N. rosae*. The cultures were sealed airtight with Parafilm and incubated for 14 d at 21 ± 2°C and 14/10 h (light/dark) cycle. After addition of 4 mL sterile water to each culture, fungal biomass was completely removed with a spatula. To separate conidia from mycelial fragments, the resulting suspension was filtered through two layers of sterile gauze (Hartmann, Neuhausen am Rheinfall, Switzerland) for *N. rosae* and four layers for *Trichoderma* spp. The conidial density was determined using a Fuchs-Rosenthal counting chamber (Brand, Wertheim, Germany) and adjusted to 10^7 and 10^5 conidia mL^{-1} for *Trichoderma* spp. and *N. rosae*, respectively.

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2.3. Extraction of Secondary Metabolites from Trichoderma spp.

Secondary metabolites from cultures of *Trichoderma* spp. were obtained according to the procedure described by El-Hasan et al. [52]. Briefly, 100 mL of potato dextrose broth (PDB) (Merck, Darmstadt, Germany) in 250 mL Erlenmeyer flasks were inoculated with 15 agar plugs (5 mm ⌀) from fully grown and sporulating *T. koningiopsis* T10, *T. harzianum* T16, or *T. asperellum* T23. As a negative control, agar plugs without fungal growth were used. Each treatment was performed in triplicate. Inoculated Erlenmeyer flasks were incubated at 150 rpm and 21 ± 2°C in the dark. Based on our previous studies, incubation time was set to 2 d for *T. koningiopsis* T10 (unpublished data) and 12 d for *T. harzianum* T16 and *T. asperellum* T23 [29]. Fungal biomass was filtered through sterile filter paper, and the filtrates were extracted twice with 100 mL ethyl acetate (EtOAc; Merck KGaA, Darmstadt, Germany). Organic phases were collected, dehydrated with Na_2SO_4 and subsequently evaporated in a rotary evaporator under reduced pressure (150-170 mbar) at 40°C. Dried residues were redissolved in 1 mL acetone. Extracts were subsequently sealed airtight and stored at 7°C in the dark.

2.4. Bioautography Assay

To estimate the antifungal potential of the crude extracts, thin layer chromatography (TLC) and bioautography procedures described in our previous study [46] were used with modifications. Briefly, 50 µL of the crude extract of *Trichoderma* spp. T10, T16 and T23 were applied to TLC plates (5 x 5 cm) (Machery-Nagel, Düren, Germany). GM7 extracts and acetone served as controls. Each treatment was done in triplicate. TLC plates were sprayed with a total of 30 mL GM7 at approximately 50°C, followed by a conidial suspension of *N. rosae* with a concentration of 10^5 conidia mL^{-1}, using an airbrush compressor (N026, KNF, Freiburg, Germany). Subsequently, TLC plates were incubated in a humidity chamber, at 25°C in the dark for 24 h. Each plate was photographed and fungal growth was determined using ImageJ (v. 1.54d) software (National Institute of Health, USA).

2.5. Poisoned Agar Plug Assay

To further assess the inhibitory effects of crude extracts on growth and sporulation of *N. rosae*, GM7 petri dishes (35 mm ⌀, Sardstedt, Nümbrecht, Germany) were each inoculated with one centered agar plug (5 mm ⌀) of an actively growing *N. rosae* culture. Each agar plug was then treated separately with 50 µL crude extract of *Trichoderma* spp. T10, T16, or T23, respectively. GM7 extract and acetone were used as controls. Switch (Syngenta, Basel, Switzerland) (a.i.: 375 mg g^{-1} Cyprodinil + 250 mg g^{-1} Fludioxonil) in the recommended application dosage of 2.5 µg $µL^{-1}$ was used as a positive control. Each treatment was done in triplicate. Inoculated petri dishes were incubated for 5 d at 21 ± 2°C and 14/10 h (light/dark) cycle. Fungal colonies were then photographed, and fungal growth and agar coverage rate was determined with the software ImageJ. To assess the effect on sporulation, the initial agar plugs were removed, and conidia formation was determined by adding 2 mL of sterile water to each petri dish. Fungal biomass was scraped off with a sterile spatula and filtered through two layers of sterile gauze, and conidial density was determined using a Fuchs-Rosenthal counting chamber.

2.6. Inhibition of Conidia Germination of N. rosae

To assess the inhibitory effect of crude extracts on conidial germination of *N. rosae*, a 2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole bromide (MTT) assay was utilized. *Trichoderma* spp. T10, T16, and T23 extracts, and acetone were diluted in liquid GM7 to final concentrations of 1- 5% (v/v), in 1 % increments. GM7 medium served as negative control, while Switch (2.5 µg $µL^{-1}$) in liquid GM7 served as positive control. Each treatment (100 µL) was pipetted into a flat-bottom 96 well microplate (Greiner, Frickenhausen, Germany). Subsequently, 100 µL of a conidial suspension (3.1×10^5 conidia mL^{-1}) from *N. rosae* containing 0.2 µL mL^{-1} Break-Thru S 301 (Evonik Industries, Essen, Germany) was added to each well, resulting in a final volume of 200 µL

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per well. Three wells were filled with 100 µL of liquid GM7 and 100 µL of conidia suspension from *N. rosae* without Break-Thru S 301. The remaining wells were filled with 200 µL of liquid GM7 and were used as blanks. The microplate was sealed airtight with Parafilm, placed inside a plastic box with transparent lid and incubated for 24 h at 21 ± 2°C and 14/10 h (light/dark) cycle.

Then, 50 µL MTT solution (2 mg µL $^{-1}$) prepared in salt buffer were added to each well. The salt buffer consisted of 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1,78 g L^{-1} $Na_2PO_4 \cdot 2\ H_2O$ and 0.27 g L^{-1} KH_2PO_4 in sterile deionized water. After adding the MTT to the salt buffer, the solution was filtered through a 0.2 µm microfilter.

After 1 h of incubation at 37°C with shaking in double-orbital mode at 100 rpm inside a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany), the absorbance was measured at 570 nm using the orbital (6 mm) mode with 106 reading points per well.

2.7. Greenhouse Trial

To assess the efficacy of crude extracts of *Trichoderma* spp. towards *N. rosae* on strawberry plants and compare their efficacy with the conventional-used live spores biocontrol, two months old strawberry plants cv. Herzle were planted in pots (0.34 L) containing soil (CLT; Einheitserde Werkverband, Sinntal-Altengronau, Germany). Plants were fertilized every other week with 0.2 % Wuxal® Super (Aglukon, Duesseldorf, Germany).

Crude extracts of *Trichoderma* spp. T10, T16 and T23 as well as acetone were diluted to 3% (v/v) in sterile water. Five healthy plants were placed in a tray and 2 mL of the respective treatment were pipetted onto the rhizome of each plant. Sterile water and Switch (2.5 µg µL^{-1}) served as negative and positive control, respectively. Conidial suspensions of *Trichoderma* spp. T10, T16 and T23 (10^7 conidia mL^{-1}) and *N. rosae* (10^5 conidia mL^{-1}) were prepared as described in Section 2.3. All conidial suspensions contained 0.02% Break-Thru S 301. To maintain appropriate relative humidity, trays were placed on fleece saturated with water and covered with translucent plastic boxes (l x w x h; 1.8 m x 0.6 m x 0.6 m). Trays were arranged randomly inside the greenhouse cabin. At 24 h intervals, boxes were removed for 8 h, to simulate a daily cycle of humidity. After seven days, each plant was sprayed with 2 mL of their respective BCA-treatment using an airbrush. Two days after the second BCA application, every plant was sprayed with 2 mL of the *N. rosae* conidial suspension. The experiment was done in triplicate. Plants were incubated for 37 days at 21 ± 2°C under 14/10 h (light/dark) conditions.

Leaves showing visible signs of infection were scored and the total number of infected and intact leaves of each plant was counted starting two weeks after the inoculation with *N. rosae*. Scoring was repeated at 7 day-intervals for 21 d. Disease symptoms were examined using a digital microscope (VHX-X1; Keyence Deutschland GmbH, Leinfelden-Echterdingen, Germany). The area under disease progress curve (AUDPC) for the developed leaf lesions was calculated using the formula:

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t)_1$$

where t is the time of each assessment, y is the percentage of infested plant parts in each assessment, and n is the number of readings. The variable t stands for days [56].

Subsequently, the relative AUDPC (rAUDPC) was derived from the AUDPC using the formula:

$$rAUDPC = \frac{AUDPC}{(t_{max} - t_1) \times 100}$$

2.8. Statistical Analysis

Statistical analyses were performed using the GLM procedure in SAS software (version 9.4). A one-factorial analysis of variance (ANOVA) or two-factorial analysis of variance (ANCOVA),

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followed by a least-significant difference (LSD) test were used to compare treatment means. Differences between treatments were considered significant at a probability level of $p = 0.05$.

3. Results

3.1. Mycelial Growth Inhibition of N. rosae on TLC Plates Amended with Trichoderma Extracts

The control and acetone treatments showed no fungal growth inhibition and were statistically similar, confirming that the solvent itself had no antifungal effect within this set up (Figure 1).

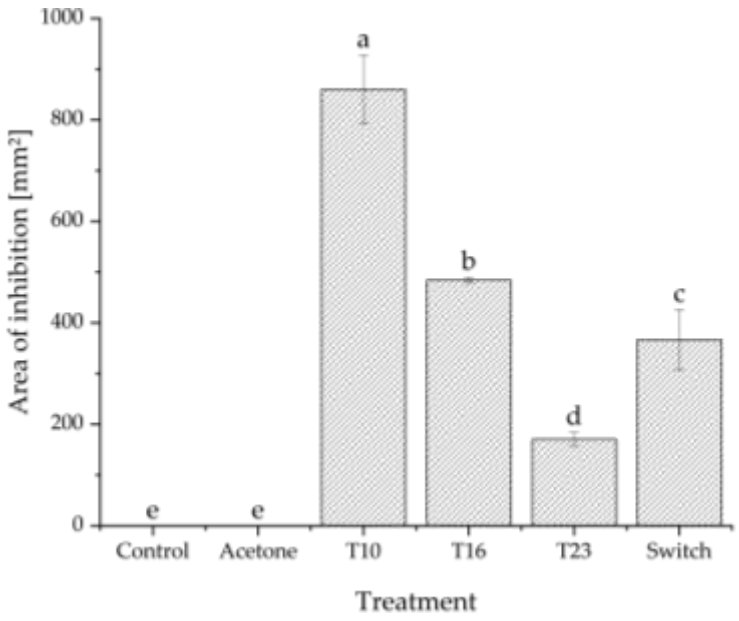

Figure 1. Mycelial growth inhibition of *N. rosae* by *Trichoderma* spp. extracts and Switch on TLC plates. Bars represent means and error bars indicate standard deviation. Treatments with the same letter are not significantly different at a probability level of $p = 0.05$.

Among the *Trichoderma* extracts, *T. asperellum* T23 exhibited a pronounced inhibitory effect, producing a small but statistically significant inhibition area ranging between 171.1 ± 13.64 mm². This was followed by the fungicide Switch, which showed a moderate inhibition of about 366.96 ± 59.67 mm². A stronger inhibitory effect was observed with *T. harzianum* T16, with an inhibition area of 484.42 ± 5.55 mm², while *T. koningiopsis* T10 produced the highest inhibition of mycelial growth reaching 860.39 ± 68.04 mm².

3.2. Poisoned Agar Plug Assay

The results show clear differences among treatments in their inhibitory effects on mycelial growth and conidia production of *N. rosae* (Figure 2, Appendix). Acetone insignificantly inhibited mycelial growth by 2.8 ± 20.9%. When treated with *T. asperellum* T23 or *T. harzianum* T16, *N. rosae* showed low growth inhibition with no significant differences compared to the acetone treatment. On the other hand, in the presence of *T. koningiopsis* T10 or Switch, *N. rosae* showed the highest growth inhibition measured by 92.3 ± 4.7% and 96.1 ± 0.9%, respectively. Both latter treatments did not differ significantly from each other.

Concentrations of conidia of *N. rosae* were highest in the GM7 control and acetone treatments at 4.19×10^6 conidia mL^{-1} and 3.87×10^6 conidia mL^{-1}, respectively. While *T. harzianum* T16 and *T. asperellum* T23 led to a moderate spore formation inhibition (45 ± 38.3% and 62.7 ± 20.6%, respectively), *N. rosae* sporulation was almost completely inhibited when treated with *T. koningiopsis* T10 (96.5 ± 4.8%) or Switch (99.9 ± 0.04 %).

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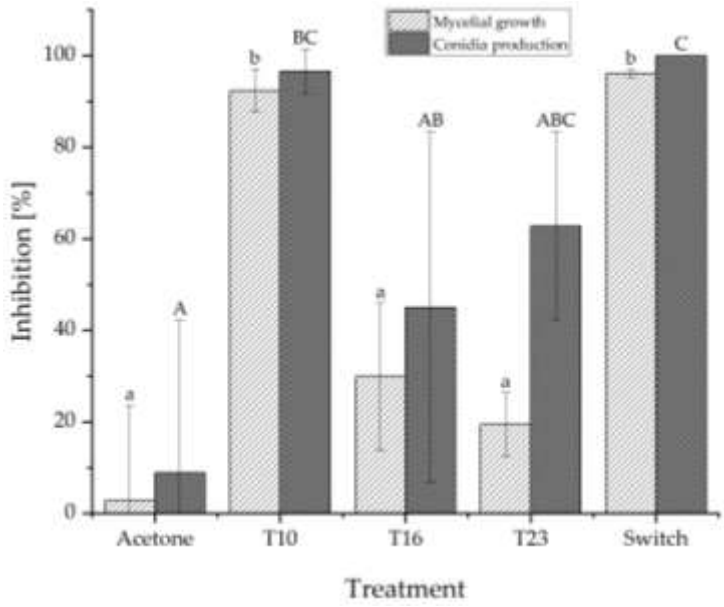

Figure 2. Inhibition of mycelial growth and conidia production of *N. rosae* following treatment with *Trichoderma* spp. extracts, or the fungicide Switch. Bars represent means and error bars indicate standard deviation. Treatments with the same letter for mean values are not significantly different from each other at a probability of $p = 0.05$.

In general, conidia production appeared to be more sensitive than mycelial growth to *T. harzianum* T16 and *T. asperellum* T23 extracts, while *T. koningiopsis* T10 extract and Switch were highly effective against both parameters.

3.3. Inhibition of Conidia Germination of N. rosae by Trichoderma Crude Extracts

The effect of *Trichoderma* spp. extracts on conidia germination of *N. rosae* in liquid culture was evaluated using the MTT assay at concentrations ranging from 1- 5% and compared with the acetone control at equivalent concentrations. Conidia germination in the acetone control remained consistently high across all tested concentrations, with values above 90% at 1 - 3% acetone and only a slight decrease observed at higher concentrations (85.1 ± 4.1% at 5%, Figure 3). This indicates that acetone, even at increasing concentrations, had minimal inhibitory effects on conidia germination of *N. rosae*. In contrast, all *Trichoderma* spp. extracts significantly reduced conidia germination relative to the acetone control in a concentration-dependent manner. Extracts of *T. asperellum* T23 caused a moderate but consistent inhibition, with germination decreasing from 85.1 ± 1.7% at 1% to 73.5 ± 3.9% at 5%. *T. harzianum* T16 extracts proved to be significantly more effective in inhibiting conidia germination, showing a progressive reduction in germination, decreasing from 83.3 ± 3.4% at 1% to 58.3 ± 2.5% at 5%. However, extracts of *T. koningiopsis* T10 exhibited the strongest inhibitory effect, ranging between 44.4 ± 0.8% and 37.5 ± 3.6% depending on their concentration.

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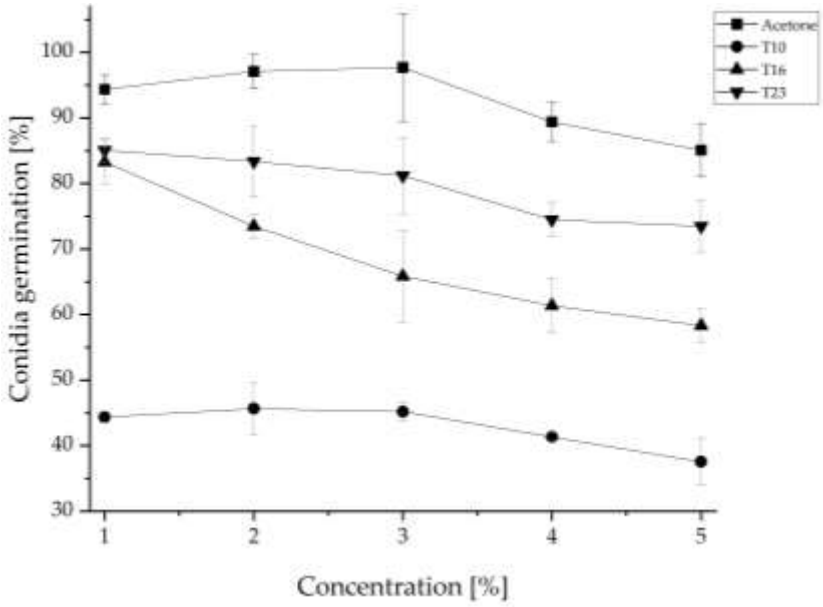

Figure 3. Effect of *Trichoderma* spp. extracts on conidia germination of *N. rosae* in liquid culture using the MTT assay. Bars represent standard deviation.

3.4. In Planta Assesment of the Effects of Crude Extracts and Conidia of Trichoderma spp. Against N. rosae

Leaves infected with *N. rosae* exhibited characteristic disease symptoms including brown leaf spots, discoloration of leaf tissue, leaf curling (Figure 4 A-C) and formation of characteristic black acervuli (Figure 4 D, E) and conidia (Figure 4 E, F), confirming the efficacy of the inoculation method and the identity of *N. rosae*.

Leaf infestation by *N. rosae*, expressed as rAUDPC (%) at 28 days post inoculation (dpi), was significantly affected by the different treatments. The infected control showed a high level of disease development (31.1 ± 22%), whereas the healthy control exhibited the lowest rAUDPC values (16.3 ± 15%; Figure 5).

Conidial (C) treatments of *Trichoderma* spp. (T10C, T16C, and T23C), as well as the extract (E) treatment T16E, produced intermediate rAUDPC values ranging between 23.8% and 27.5%. These treatments insignificantly reduced leaf infestation and remained significantly higher than the healthy control.

Among the extract treatments, clear differences were observed. Application of acetone and T23E resulted in relatively high rAUDPC values (35.8 ± 16% and 37 ± 25%, respectively) and did not differ significantly from the infected control, indicating no suppressive effect on disease development. In contrast, treatment with T10E significantly (20.5 ± 11%) reduced disease severity and was among the most effective extract treatments. The fungicide treatment (Switch) resulted in a strong reduction of disease development (17 ± 16%) and differed insignificantly from the healthy control, indicating high efficacy under the experimental conditions.

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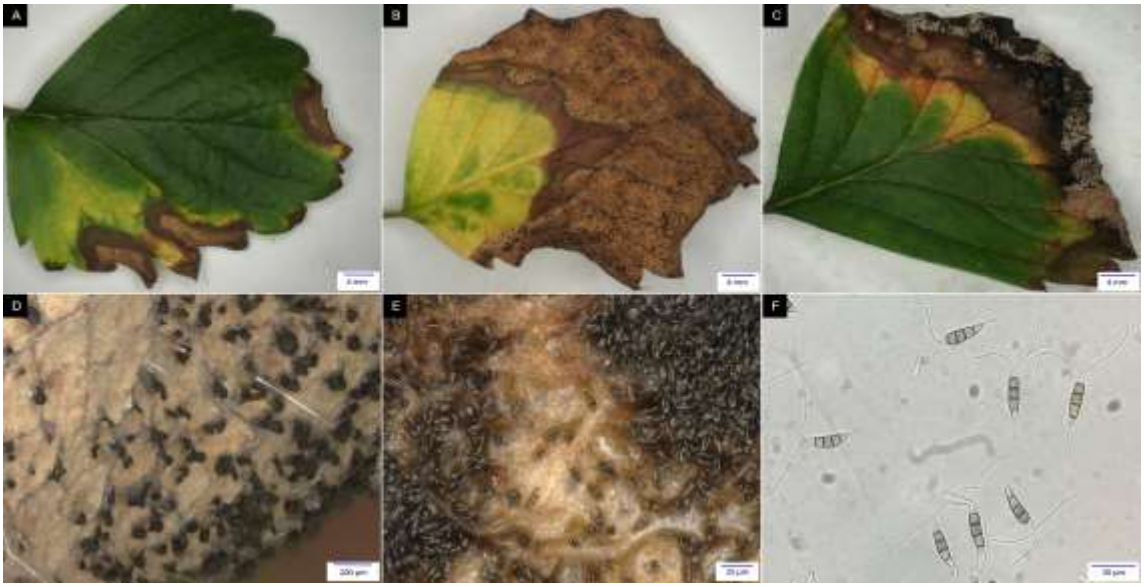

Figure 4. (A-C) Symptoms of strawberry leaves due to *N. rosae* infection. (C, D) Acervuli on strawberry leaf. (E) Conidia on strawberry leaf. (F) typical conidia under light microscope.

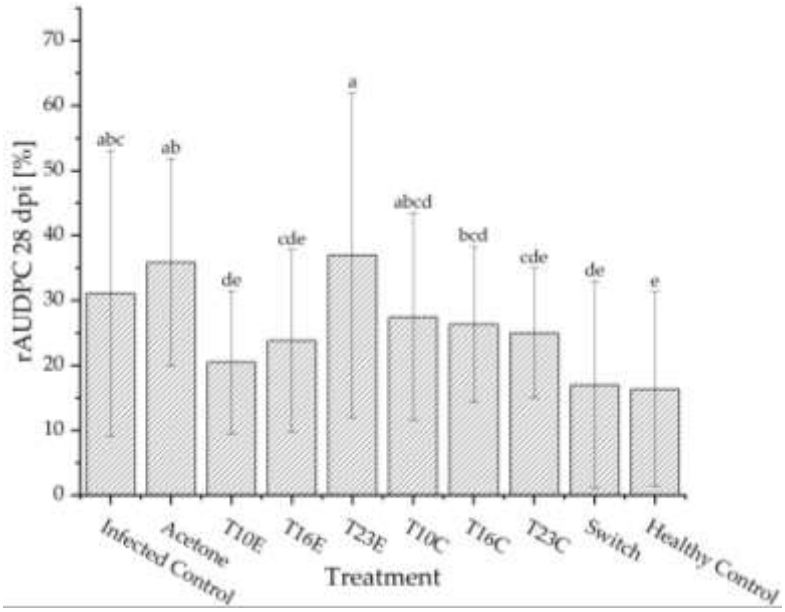

Figure 5. Effect of extracts (E) and conidia (C) of *Trichoderma* spp. on the infestation of leaves with *N. rosae*. Bars represent means and error bars indicate the standard deviation. Treatments with the same letter are not significantly different from each other at a probability of $p = 0.05$.

4. Discussion

The control of *Neopestalotiopsis* species, particularly *N. rosae* and *N. clavispora*, has become a critical priority due to their emergence as destructive pathogens causing root, crown, and fruit rot in strawberries and flower blight in ornamental plants [57]. Conventional management of these pathogens relies largely on chemical fungicides, which often fail to provide complete control and are associated with resistance development and potential health and environmental risks [58]. Consequently, research is shifting toward more effective and sustainable alternatives for integrated disease management [30,37,59–61].

In this study, we assessed the antifungal activity of *Trichoderma*-derived metabolites *in vitro* and evaluated their performance *in planta*.

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In the direct-contact TLC assay, extracts of *T. koningiopsis* T10, *T. harzianum* T16, and *T. asperellum* T23 all inhibited mycelial growth of *N. rosae*, confirming antibiosis as an important mode of action (Figure 1). Among these, *T. koningiopsis* T10 produced inhibition zones that were 2.5-fold larger than those of the reference fungicide Switch, indicating a particularly strong antifungal effect. Bacterial BCAs including *Bacillus cereus*, *B. amyloliquefaciens*, and *B. methylotrophicus* inhibited *N. clavispora* growth by about 60–79% *in vitro* [62], which is lower than the very strong inhibition observed for *T. koningiopsis* T10 extracts in our experiments. Similarly, cell-free culture filtrates of three *Bacillus* strains (two *B. velezensis* and one *B. subtilis*) significantly suppressed *N. clavispora in vitro*, with inhibition ranging from 63.3% to 73.6%. *In planta*, application of these antagonists markedly reduced root rot severity (up to 67%) and enhanced superoxide dismutase, peroxidase, and catalase activities in leaves, indicating that their cell-free metabolites act in combination with induced host resistance [63].

Comparable patterns of strong *in vitro* inhibition have been observed with botanical extracts: clove (*Syzygium aromaticum*) and turmeric (*Curcuma longa*) extracts completely suppressed mycelial growth of *Neopestalotiopsis* sp. and *Pseudopestalotiopsis* sp., whereas ginger, lemongrass, and roselle caused only partial inhibition and mangosteen extract only had a minimal effect [64].

When mycelial growth was quantified following the amendment of the agar plugs of the pathogen with *Trichoderma* extracts, *T. koningiopsis* T10 achieved 92.3% inhibition of *N. rosae* after five days, approaching the 96.2% inhibition obtained with the reference fungicide (Figure 2, Appendix). In contrast, *T. harzianum* T16 and *T. asperellum* T23 extracts caused only moderate growth suppression. *In vitro*, volatile and non-volatile metabolites produced by *T. asperellum* CMT10 exhibited strong antifungal activity against *N. clavispora*, inhibiting mycelial growth by 79.7% and 69.8%, respectively [65]. Comparable strain-dependent variability has been reported for *Trichoderma* isolates antagonizing *Fusarium* sp., *Phytophthora* sp., and *Botrytis* spp. [46,66].

Using botanicals, a high *in vitro* efficacy has been observed: cinnamon essential oil nanoemulsions inhibited *N. rosae* growth at high dilutions and completely suppressed leaf spot severity [67].

We also quantified the effect of the *Trichoderma* extracts on *N. rosae* sporulation. *T. harzianum* T16 and *T. asperellum* T23 extracts reduced conidia production by 45% and 62.7%, respectively, whereas *T. koningiopsis* T10 extracts suppressed conidiation by 96.5%, close to the 99.9% reduction achieved by the reference fungicide (Figure 2). Since conidia are the primary propagules driving *Neopestalotiopsis* epidemics [41], such strong inhibition of conidia formation is highly relevant for disease management. Similar effects on sporulation have been documented for other *Trichoderma*-based BCAs: extracts of *Trichoderma* spp., for example, markedly inhibited sporulation of *Fusarium moniliforme* and *Phakopsora pachyrhizi*, resulting in reduced lesion development and lower disease severity [51,52]. Although explicit quantification of conidia reduction is less commonly reported, *B. cereus* reduced sporulation indirectly by impairing mycelial growth [68]. Compared with botanical agents, our data are similar in magnitude to clove and turmeric extracts, which fully suppressed *Neopestalotiopsis* and *Pseudopestalotiopsis* growth at high concentrations [64].

In the conidia germination assay, *T. koningiopsis* T10 extracts inhibited *N. rosae* germination by 62.5%, whereas *T. harzianum* T16 and *T. asperellum* T23 showed a different efficacy ranking than in the sporulation assay, indicating that conidia production and germination are differentially sensitive targets. Similar discrepancies between sporulation and germination responses have been reported for other pathosystems and with other BCAs, where fungicides or extracts can strongly suppress sporulation while having more moderate effects on germination, or vice versa [52]. At the same time, we observed that acetone concentrations of 4% or higher significantly suppressed germination in the MTT assay, so *Trichoderma* extract efficacy could be reliably evaluated only up to 3% extract concentration. By comparison, Switch was the only treatment in our study that completely inhibited *N. rosae* conidial germination, which is consistent with its known multi-stage activity against several strawberry pathogens [1,13,14,56,57]. Complete inhibition of conidial germination of *Neopestalotiopsis* spp. is rarely reported. For example, cinnamon essential oil nanoemulsions markedly reduce

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germination and infection structures formation but do not achieve full suppression [67]. Similarly, *Bacillus*-based BCAs often limit germination indirectly through induced resistance rather than through direct and complete inhibition [68].

Considering the limitations of *in vitro* assays in capturing the complexity of pathogen–host interactions, both live inocula and crude extracts of *Trichoderma* spp. were evaluated *in planta* under greenhouse conditions. Our results show that conidial suspensions only moderately and not significantly reduced *N. rosae* leaf spot. This outcome suggests that under our specific conditions (*Trichoderma* strain, inoculum density, timing, environmental parameters, and host cultivar), live inocula did not establish or act sufficiently to provide robust protection. In contrast, *T. koningiopsis* T10 crude extracts significantly suppressed *N. rosae* leaf spot severity, reaching control levels comparable to the fungicide Switch (Figure 5). This finding is particularly notable because Switch is regarded as one of the most effective fungicides against *Neopestalotiopsis* and other strawberry pathogens, with proven efficacy in reducing mycelial growth, sporulation, and infection in field and greenhouse settings [15,56]. Our results show that crude extracts from *T. koningiopsis* T10 can translate their pronounced *in vitro* activity into effective *in planta* disease control. This stands in line with several reports where *Trichoderma* spp. have shown strong *in planta* effects. For example, *T. asperellum* inhibited *N. clavispora* by 66.7% *in vitro* and reduced disease severity by 75.8% on strawberry, performing similarly to chemical fungicides [69]. Acosta-González et al. [56] also demonstrated that *Trichoderma* spp. significantly suppressed *N. rosae* incidence in strawberry. Similarly, CMT10 of *T. asperellum* achieved 63.1% biocontrol efficacy against strawberry root rot caused by *N. clavispora* and concurrently promoted plant growth under greenhouse conditions [70].

Similar extract-based *in planta* efficacy has also been reported for botanicals: cinnamon essential oil nanoemulsions reduced leaf spot severity by nearly 80% without phytotoxicity [67], and clove and turmeric extracts have shown strong protection of fruits and foliage against *Pestalotiopsis*-like fungi at high concentrations [64]. Moreover, bacterial BCAs such as *B. cereus* and *B. amyloliquefaciens* also reduced disease incidence and severity e.g. *B. cereus* Bce-2 lowered *N. clavispora* leaf spot while strongly activating host defense enzymes, and *B. amyloliquefaciens* isolates reduced strawberry root rot by >57% and promoted plant growth, but their suppression levels are often slightly lower than those achieved by the synthetic fungicides [68].

Taken together, our results show that crude extract of *T. koningiopsis* T10 exhibits very strong *in vitro* activity against *N. rosae* (on mycelial growth, sporulation, and, to a lesser extent, conidial germination) and can provide *in planta* disease control equivalent to a commercial fungicide, whereas *Trichoderma* inocula were not significantly effective under the conditions tested. The effective control of *N. rosae* by fungicides such as Switch may contribute to its currently low incidence in German strawberry cultivation. However, problems could arise in the future if fungicide performance declines, or *N. rosae* develops resistance [24,25]. Nevertheless, *T. koningiopsis* T10 extracts were equally effective as Switch in suppressing *N. rosae*, suggesting its potential as a suitable alternative to conventional chemical fungicides.

5. Conclusions

The emergence of novel pathogens poses a significant threat to the cultivation of strawberries. Continuous reliance on chemical fungicides has detrimental environmental consequences and is not a sustainable practice over an extended period. The present study thus focused on the identification of biological control agents against *N. rosae*. The results of this study demonstrate that extracts from *Trichoderma* spp. can effectively inhibit growth and spore formation of *N. rosae*. Among the isolates tested, *T. koningiopsis* T10 exhibited efficacy comparable to the reference fungicide (Switch) in terms of its impact on mycelial growth, spore formation, and conidia germination of *N. rosae*. In greenhouse trials with strawberry plants, *T. koningiopsis* T10 was likewise as effective as Switch in preventing strawberry leaf infestation.

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Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
AUDPC	Area Under The Disease Progress Curve
BCA	Biological Control Agent
EtOAc	Ethyl acetate
GM7	Glucose-Medium-7
IPM	Integrated Pest Management
LSD	Least Significant Difference
MDPI	Multidisciplinary Digital Publishing Institute
MTT	2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole bromide
rAUDPC	Relative Area Under The Disease Progress Curve
6 PAP	6-pentyl-alpha-pyrone

Appendix A

Poisoned Agar Plug Assay with N. rosae

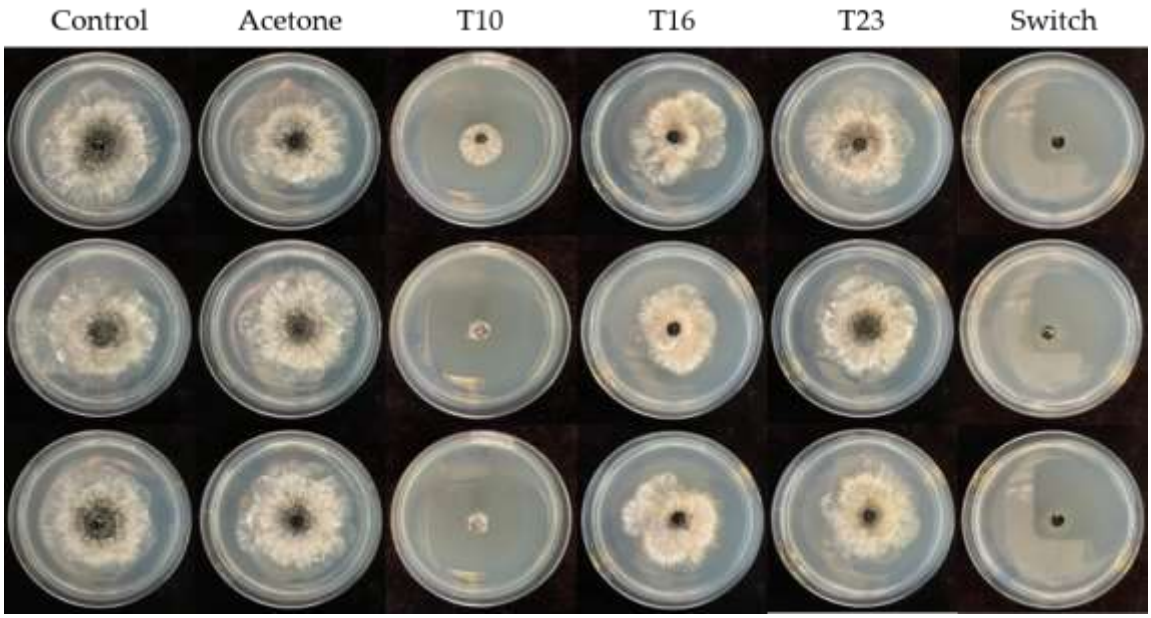

Figure A1. Mycelial growth of *N. rosae* after five days on GM7 medium amended with solvent (Acetone), extracts of *Trichoderma* spp., or Switch.

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