ORLANDO MACIEL RODRIGUES JR.

TOMATO EARLY BLIGHT BIOCONTROL AND PLANT GROWTH PROMOTION BY *Clonostachys* spp.

Dissertation presented to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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BIOGRAPHY

Orlando Maciel Rodrigues Junior, filho de Ana Nizete Fontes Vieira Rodrigues e Orlando Maciel Rodrigues, nasceu em Belém-PA em 20 de abril de 1988.

Em 2006 ingressou no curso de Medicina na Universidade Estadual do Pará e tardou três anos até ir em busca da carreira que realmente gostaria de seguir.

Em 2010, ingressou no curso de Agronomia da Universidade Federal Rural da Amazônia, Belém-PA, cursou o ano letivo de 2013 na CAH University of Applied Sciences, Holanda, e finalmente graduou-se em janeiro de 2017.

Em março de 2017, iniciou o mestrado em Fitopatologia na Universidade Federal de Viçosa.

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ABSTRACT

RODRIGUES Jr., Orlando Maciel, M.Sc., Universidade Federal de Viçosa, February, 2019. **Tomato early blight biocontrol and plant growth promotion by *Clonostachys* spp**. Advisor: Lucas Magalhães de Abreu.

Given that fungal biocontrol agents not only suppress diseases, but also confer benefits for plants, this work aimed to evaluate the effect of different *Clonostachys* species against tomato early blight and on plant growth and biomass accumulation. Regarding biocontrol, we hypothesized that non-pathogenic, epiphytic isolates of *Cladosporium* added to leaf surfaces could act as a living support for the mycoparasitic *Clonostachys* spp. in a combined application scheme, enhancing the biocontrol effects. We also hypothesized that extending the *Clonostachys* application after the *Alternaria linariae* inoculation might provide a 'curative' effect on diseased plants. First, isolates of five *Clonostachys* species were sprayed alone or in combination with a mix of four *Cladosporium* spp. isolates twice before pathogen inoculation. No marked evidence was found to support the benefit of combined application of these BCA's. In the second assay, preventive spraying with single isolates of three *Clonostachys* species resulted in in more than 50% reduction in disease intensity, reaching a maximum of 72% with C. *chloroleuca* isolate UCBV 12. Additional post-inoculation spray did not result in enhanced biocontrol efficacy or detectable curative effects. In a distinct set of experiments, 21 isolates from nine different *Clonostachys* species, plus two isolates of *Beauveria* and *Trichoderma*, were tested for growth promotion of tomato seedlings after either single-step seed microbiolization or seed microbiolization followed by biodrenching of saturated conidial suspensions after seedling emergence. Tomato seedlings were evaluated for length and dry matter. Single-step seed microbiolization was inefficient in providing benefits. Contrastingly, clear plant growth promotion effects were detected after biodrenching. Seven isolates from five *Clonostachys* species consistently resulted in significant plant mass increases ranging from 21% to 33%, the latter result obtained with the isolate UCBV 35 of C. *byssicola*. *Clonostachys* isolates probably were able to colonize rhizosphere due to more efficient form of conidial delivery through drenching, a practice that can be easily adopted in tomato seedling production. Plant growth promotion seems to be another common feature of *Clonostachys* genus, just as the mycoparasitism ability.

RESUMO

RODRIGUES Jr., Orlando Maciel, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Biocontrole da pinta preta e promoção de crescimento em tomate por *Clonostachys* spp**. Orientador: Lucas Magalhães de Abreu.

Dado que agentes de controle biológico não apenas suprimem doenças, mas também podem conferir benefícios a plantas, este trabalho objetivou avaliar o efeito de diferentes espécies de *Clonostachys* contra a pinta-preta do tomateiro e sobre o crescimento vegetal e acúmulo de biomassa. Em relação ao biocontrole, nossa hipótese era que isolados não patogênicos do epífito *Cladosporium* aplicados à superfície foliar poderiam atuar como suporte vivo para os micoparasitas *Clonostachys* spp. em um esquema combinado de aplicação, melhorando os efeitos de biocontrole. Nossa outra hipótese era que estender a aplicação de *Clonostachys* após inoculação com *Alternaria linariae* proveria um efeito "curativo" sobre a doença. Inicialmente, isolados de cinco espécies de *Clonostachys* foram aspergidos, isoladamente ou em combinação com mistura de quatro isolados de *Cladosporium* spp., duas vezes antes da inoculação do patógeno. Nenhuma evidência marcante sustentou a existência de benefício na aplicação combinada desses ACB's. No segundo ensaio, aspersão preventiva com isolados de três espécies de *Clonostachys* resultou em mais de 50% de redução na intensidade da doença, atingindo um máximo de 72% com *C. chloroleuca* UCBV 12. A aspersão adicional pós-inoculação não resultou em maior eficácia de biocontrole ou detecção de efeitos curativos. Em um conjunto distinto de experimentos, 21 isolados de nove espécies de *Clonostachys*, mais dois isolados de *Beauveria* e *Trichoderma*, foram testados para promoção de crescimento de mudas de tomate. As sementes foram tratadas ou com em uma única etapa de microbiolização ou com microbiolização seguida de *biodrenching* de suspensão saturada de conídios após a emergência das plântulas. As mudas de tomate foram avaliadas quanto ao comprimento e peso da seca. A microbiolização de sementes em única etapa foi ineficiente em oferecer benefícios. Entretanto, efeitos claros de promoção de crescimento foram detectados após o *biodrenching*. Sete isolados de cinco espécies de *Clonostachys* resultaram de maneira constante em aumento significativo de biomassa com amplitude de 21% a 33%, sendo este último resultado obtido pelo isolado UCBV 35 – C. *byssicola*. Isolados de *Clonostachys* provavelmente foram capazes de colonizar a rizosfera devido à forma mais eficiente de aplicação de conídios pela suspensão no substrato, uma prática que pode facilmente ser adotada na produção de mudas de tomate. Promoção de crescimento de plantas mostra-se como mais uma característica comum ao gênero *Clonostachys*, tal qual a habilidade micoparasítica.

1. General introduction

Being one of the most cultivated horticultural crops in Brazil and worldwide (Camargo Filho; Camargo, 2017), it is no surprise that tomato cultivation faces challenges regarding phytopathogens and thus has always required intensive use of chemical defensives for successful mass production. Among the tomato diseases, early blight, caused by *Alternaria linariae* (Ascomycota: Pleosporaceae), is of great concern, especially due to its destructive potential and the lack of full genetic resistance to it so far (Lima; Sales; Caixeta, 2017).

Alternaria linariae initially damages older leaves, causing the characteristic target-shaped dark spots, which then enlarge and cause early leaf abscission. The disease progresses from the lower to the upper aerial parts of the plant and may cause its death. Losses in fields or greenhouses are intense and can compromise production, varying from 20% to almost 80% (Chaerani; Voorrips, 2006).

The most common approach to early blight control is the frequent use of fungicides in order to keep infection rates low. However, apart from the financial costs, the heavy reliance on these agrochemicals may be a risk for the laborers and consumers health, and for the environment (Aktar; Sengupta; Chowdhury, 2009; Runkle et al., 2017; Santovito et al., 2018). Additionally, if synthetic fungicide use would be the sole answer for this phytosanitary issue, all tomato producers would be automatically excluded from the highly profitable organic market. Field populations of *Alternaria* under pressure by the constant use of fungicides are being selected and the arising resistant genotypes may pose a threat for the intensive production systems if other control solutions remain unavailable (Pasche; Wharam; Gudmestad, 2004; Avenot; Morgan; Michailides, 2008).

One possible solution is the use of biological control agents (BCA's), which are generally considered harmless to humans and to the environment (Ocampo-Suarez et al., 2017). BCAs act against phytopathogens by resource competition, antibiosis and/or parasitism and can reduce disease rates in many crops. Many BCAs are studied and tested for protection against soil-borne pathogens and are able to effectively colonize roots (Montesinos, 2003; Fravel, 2005). However, plant aerial parts present a very different habitat when compared to the rhizosphere and successful epiphytic organisms must be able to live despite the harshness of above-ground conditions, such as direct solar irradiance, UV light exposure, scarce free nutrients, the washing effect of heavy rainfall and wider temperature changes throughout the day (Vorholt, 2012; Moreira; May De Mio, 2015). BCA candidates selected from this kind of habitat increase the likelihood of a reliable biocontrol strategy. Antagonistic soilborne fungi and bacteria are commonly sprayed on aerial plant parts by inundation to control plant diseases, but their resilience on the phyllosphere may be low, requiring repeated applications.

One of the most studied fungal BCAs outside the ubiquitous genus *Trichoderma* is *Clonostachys rosea*, whose isolates are well-known mycoparasites, with abundant published reports regarding *in vitro* parasitism assays and biocontrol of diseases in crops like strawberry, roses, tomato and wheat (Borges; Saraiva; Maffia, 2015; Xue et al., 2009; Cota; Maffia; Mizubuti, 2008; Jensen et al., 2007; Morandi et al., 2003; Morandi; Sutton; Maffia, 2000). Phylogenetically close species of *Clonostachys* also present similar antagonistic activities towards other fungi but have not been studied with similar intensity. The genus *Clonostachys* is species diverse, with a simple search in the Mycobank database (2018) returning 76 records. Brazil accounts for a portion of this diversity and several species have been found in the country, such as *C. rosea, C. byssicola, C. candelabrum, C. pseudochroleuca, C. rhizophaga, C.*

rogersoniana and C. chloroleuca (Abreu et al., 2014; Moreira et al., 2016). With that in mind, our Biological Control Lab team at UFV is currently testing the biocontrol effect of different *Clonostachys* species, whose isolates came from different Brazilian habitats and locations, against tomato and potato early blight.

Clonostachys spp. are commonly found in soil and in recently dead plant tissues as saprotrophs (Karlsson et al., 2015), but their survival and activities may be compromised on the inhospitable aerial parts of living plants, as shown by the sharp decline in viable cell counts of *C. rosea*, even as soon as 3 days after application (Morandi; Mattos; Santos, 2006). The successful biocontrol gray mold diseases by *C. rosea* depends on successive weekly applications, due to the limited survival span of the BCA on the phyllosphere (Cota et al., 2008).

Some groups of melanized fungi are ubiquitous colonizers of plant leaf surfaces and commonly regarded as non-pathogenic saprotrophs, including *Aureobasidium pullulans* (Vero et al., 2009) and several *Cladosporium* species belonging to the *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* species groups. In fact, the application of a *Cladosporium cladosporioides* alone has shown to be effective in the biocontrol of apple scab (Köhl et al., 2015). These fungi, however, are not aggressive mycoparasites/predators of other fungi, a feature shared by successful biocontrol agents belonging to *Clonostachys*, *Coniothyrium* and *Trichoderma* species (Hermosa et al., 2012; Jensen et al., 2007; Verkley et al., 2004).

Considering the aim of prolonging the survival and mycoparasitic activities of *Clonostachys* species applied to the phyllosphere of tomato plants, we hypothesized that efficient epiphytic non-pathogenic fungi belonging to the genus *Cladosporium*, sprayed together with *Clonostachys* isolates, could co-colonize the tomato leaf surfaces and act as nutrient sources for the mycoparasites. Therefore, combined use of two fungi as BCA's could further enhance biocontrol effect against tomato early blight. Additionally, we wanted to assess if an application of *Clonostachys* spp. isolates after inoculating *A. linariae*, might present a 'curative' effect on tomato leaves.

In addition to direct antagonism towards plant pathogens, BCA's may also positively interact with the host plants, priming them for induced resistance against diseases and insects, increasing their performance under abiotic stress and promoting plant growth (Fischer et al., 2005; Jetiyanon; Kloepper, 2002; Shoresh; Harman; Mastouri, 2010). The latter effect is commonly observed with the application *Trichoderma* spp. and many rhizobacteria species to the rhizosphere (Contreras-Cornejo et al., 2009; Liu et al., 2018; Sharma; Sindhu; Sindhu, 2018; Tucci et al., 2011). Due to the common detection of *Clonostachys* spp. in soil and their ability to colonize the rhizosphere (Chatterton; Jayaraman; Punja, 2008; Xue, 2003), a soil spore inundation could provide a positive plant growth effect on tomato in a similar manner as obtain for other crops (Johansen et al., 2005).

The benefits of soil drenching would be the ease of adopting this measure in commercial seedling production and the advantageous boost in plant growth and dry matter accumulation. In this sense, our other aim was to explore the Brazilian *Clonostachys* diversity in terms of testing different isolates from different species in order to assess whether they could offer enhanced tomato seedling development.

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2. Article I - Biocontrol of tomato leaf blight with *Clonostachys* and *Cladosporium*

2.1 Abstract

With the aim of evaluating the effect of different *Clonostachys* species against tomato early blight, we hypothesized that non-pathogenic, epiphytic isolates of *Cladosporium* added to leaf surfaces could act as a living support for the mycoparasitic *Clonostachys* spp. in a combined application scheme, enhancing the biocontrol effects. We also hypothesized that extending the *Clonostachys* application after the *Alternaria linariae* inoculation might provide a 'curative' effect on diseased plants. First, isolates of five *Clonostachys* species were sprayed alone or in combination with a mix of four *Cladosporium* spp. isolates twice before pathogen inoculation. No marked evidence was found to support the benefit of combined application of these BCA's. In the second assay, preventive spraying with single isolates of three *Clonostachys* species resulted in in more than 50% reduction in disease intensity, reaching a maximum of 72% with C. *chloroleuca* isolate UCBV 12. Additional post-inoculation spray did not result in enhanced biocontrol efficacy or detectable curative effects. *Clonostachys* spp. and *Cladosporium* spp. do not seem to act synergistically in terms of enhancing tomato early blight biocontrol. The biocontrol effects of *Clonostachys* spp. application are mainly preventive. *Clonostachys chloroleuca* is able to reduce tomato early blight.

Keywords: Mycoparasitism, biological control, Solanaceae.

2.2 Introduction

Clonostachys rosea and other *Clonostachys* species are common fungal antagonists which can be used in biocontrol of plant diseases. These fungi are soil-dwellers and, apart from their use against soil-borne diseases, including those not caused by fungi (Lahlali and Peng 2014; Allen G. Xue 2003; Tian, Li, and Sun 2014; Iqbal et al. 2018), *Clonostachys* species have been investigated for the biocontrol of above-ground plant diseases. Well documented results include controlling gray mold disease of shoots, flowers and/or fruits of strawberry, roses, fuchsia and tomatoes (Morandi, Sutton, and Maffia 2000; Silvera-Pérez et al. 2010; Gong et al. 2017; Borges, Saraiva, and Maffia 2015; Saraiva et al. 2015; Cota et al. 2008), as well as fusarium head blight on wheat spikes(Xue et al. 2009).

Effectiveness of *Clonostachys* biocontrol depends on the number and intervals of aerial spraying, such as two weekly sprays for robust gray mold control on strawberry (Cota et al. 2008) and consecutive sprays during wheat anthesis for fusarium head blight (Xue et al. 2009). Given the harsh environmental conditions that phylloplane are subjected to (Costa et al. 2013), survival and development of *Clonostachys* on the aerial plant parts might be influenced by pre-existing, abiotic stress-tolerant fungi that could be preyed on and used as a living nutrient source.

Clonostachys are unspecific mycoparasites and are able to survive on different fungal species, including *Trichoderma* isolates used in combined applications on plant surfaces for possible enhanced biocontrol activity(Krauss et al., 2013). These may include well adapted colonizers of the phylloplane, such as the ubiquitous *Cladosporium* species belonging to the *C. cladosporioides* and *C. sphaerospermum* groups, which by themselves might present some biocontrol activity (Torres et al. 2017; Köhl et al. 2015). Therefore, we lifted the question that a combined foliar application of *Cladosporium* and *Clonostachys* could enhance survival chance of the biological control agent at the expense of the adapted phylloplane colonizer. Moreover, the sole application of *Cladosporium* was effective in apple leaf scab biocontrol in field trials (Köhl et al. 2015).

To test this living-support hypothesis, we sprayed conidial suspensions of *Clonostachys* isolates belonging to five species on tomato plants, either alone or in combination with a mixture of epiphytic *Cladosporium* isolates and evaluated their biocontrol effect on tomato early blight. *Clonostachys* can parasitize *Alternaria* species and have been used in the biocontrol of seed decay and damping-off on carrot caused by seedborne *Alternaria* pathogens (Jensen et al., 2004; Lübeck; Knudsen, 2002; Turhan, 1993).

The sprayings were all conducted before inoculation of tomato plants with *Alternaria linariae*, in alignment with the most commonly studied practice in biocontrol for plant aerial parts, i.e. the preventive application of fungal mycoparasites. Given that antagonistic interactions are thought to occur mostly before pathogen infection (Jensen, Lübeck, and Jørgensen 2016), ‘curative’ application is not widely considered as a viable option.

However, biological control agents may still have indirect beneficial effects in different plant species, such as plant immune response elicitation and pathogens sporulation inhibition (Sutton et al. 2002; Roberti et al. 2008; Gong et al. 2017). Thus, we have also evaluated the effect of spraying tomato plants with five *Clonostachys* species isolates before *Alternaria* inoculation and with an additional spraying thereafter, right on the early blight onset, in order to assess a putative curative biocontrol effect.

2.3 Material and methods

2.3.1. *In vitro* mycoparasitism of *Clonostachys rosea* on *Cladosporium* sp. isolates

Nine *Cladosporium* isolates, previously obtained as tomato and potato epiphytes, and as culture contaminants, were selected from the collection and inoculated in the center of 60 mm petri dishes containing OA medium. After four days of growth, the *Cladosporium* cultures were challenged with *Clonostachys rosea* isolate UCBV 133 (=NCR61/F) (Nobre et al., 2005) by placing a 100µL-drop of saturated conidial suspension in the dish center. In order to produce the saturated conidial suspension, a 60 mm petri dish with fully sporulated *C. rosea* isolate UCBV 133 received 5 mL of distilled sterilized water and was scraped. Seven days after inoculation, a visual inspection was carried out to assess which *Cladosporium* isolates were most parasitized, presenting their dark mycelium taken over by the C. *rosea* pinkish-white mycelium. Four highly parasitized *Cladosporium* isolates were chosen for the further experiments.

2.3.2 Combined use of *Cladosporium* and *Clonostachys* against tomato early blight

Five *Clonostachys* spp. isolates were chosen from the UCBV (*Unidade de Controle Biológico de Viçosa)* fungal collection, each representing a species, namely isolates UCBV 06 (*C. rhizophaga*), UCBV 08 (*C. rogersoniana*), UCBV 12 (*C. chloroleuca*), UCBV 109 (*C. byssicola*) and UCBV 133 (*C. rosea*). These *Clonostachys* isolates as well as the *Cladosporium* ones were grown in 20 grams of cooked sterilized rice for 10 days, regularly shaken and kept under 16h photoperiod provided by fluorescent lights.

The colonized rice was blended by an immersion blender in 100 mL of tap water. The final suspensions were adjusted to 1g/L and consisted of: each one of the five *Clonostachys* isolates, separately; all four *Cladosporium* isolates mixed together; and each *Clonostachys* isolate with the *Cladosporium* mixture. The other treatments were the control using only water (from now on simply named *Alternaria* treatment) and the fungicide suspension (Chlorothalonil, 2g/L). Treatments were sprayed on cherry-tomato plants (Carolina cultivar, ‘Feltrin Sementes’) at 36 and 43 days after sowing. Forty-eight hours after the second spraying, plants were inoculated with a suspension adjusted to 10^3 conidia/mL containing three *Alternaria* spp. isolates. All plants were kept under high humidity in plastic tunnels inside the greenhouse and temperature remained 25ºC ±3º.

Disease severity was evaluated 5 and 7 days after inoculation by taking images of four leaves per plant, from the middle third to the lower third, and by posterior diseased leaf area estimation in the Fiji software. This assay was planned as complete randomized design, with 13 treatments and 8 replicates, summing 108 potted tomato plants.

2.3.3 Pre and Post-inoculation *Clonostachys* spraying for putative curative effect on tomato leaf blight

The same five *Clonostachys* isolates used in item 2.2.2 were again grown in 20g of sterilized rice for 10 days and separately blended in 100 mL of tap water with an immersion blender. Final suspensions were adjusted to 10g/L and used as follows: spraying twice only before *Alternaria* inoculation; spraying twice before *Alternaria*, followed by a third *Clonostachys* spraying four days post inoculation. The control treatments were water spraying (used as baseline and termed simply *Alternaria*) and fungicide suspension spraying (Chlorothalonil, 2g/L). All treatments were applied at 28 and 35 days after germination and inoculation was performed at 42 days after germination. Then, only the post-inoculation treatments were sprayed 46 days after germination. All plants were kept in plastic tunnels inside the greenhouse for high humidity and temperature range of 25ºC ±3º . All 12 treatments had 8 replicates, in a total of 96 plants. Disease severity was evaluated 7 and 11 days post inoculation, with the same method aforementioned in item 1.2. This assay was planned as complete randomized design, with 12 treatments and 8 replicates, summing 96 potted tomato plants.

2.3.4 Image and Data analysis

Leaf images were taken against white background. The area of three apical leaflets per leaf was selected and the background was removed in Photoshop CC 2015. Then, in software Fiji, the severity percentage was calculated.

Data were recorded in Microsoft Excel spreadsheets and then analyzed in RStudio and the available packages. Data organization was done with package ‘tidyverse’, data normalization with ‘BestNormalize’, analysis of variance and comparison of means with ‘easyanova’, ‘car’ and ‘agricolae’, bootstrapped confidence intervals with ‘Rcompanion’ and plots with ‘ggplot2’ and ‘ggpubr’. The ‘agricolae’ package was also used to calculate the area under the disease progress curve (AUDPC).

Whenever anova normality and homoskedasticity assumptions were met, either with untransformed or transformed data, post-hoc significant differences between means were grouped by Scott-Knott test.

Bootstrapped confidence intervals of the means were set to 100,000 iterations and 95% confidence. The confidence interval lower limit of *Alternaria* treatment was used as visual reference in plots.

Simple effect size percentages of severity and AUDPC were calculated by:

$$Simple\ Effect\ Size = 100\ \times \frac{Treatment - Control}{Control}$$

The simple effect size represents the percentage of gain or loss relative to a baseline, i.e., the *Alternaria* treatment value.

2.4 Results

2.4.1 Combined use of *Cladosporium* and *Clonostachys* against tomato early blight

Five and seven days after inoculating *Alternaria*, only the fungicide treatment significantly differed from other treatments, as shown in bootstrapped confidence intervals for severity percentages and the area under the disease progress curve (AUDPC) plot (Figures 1-3).

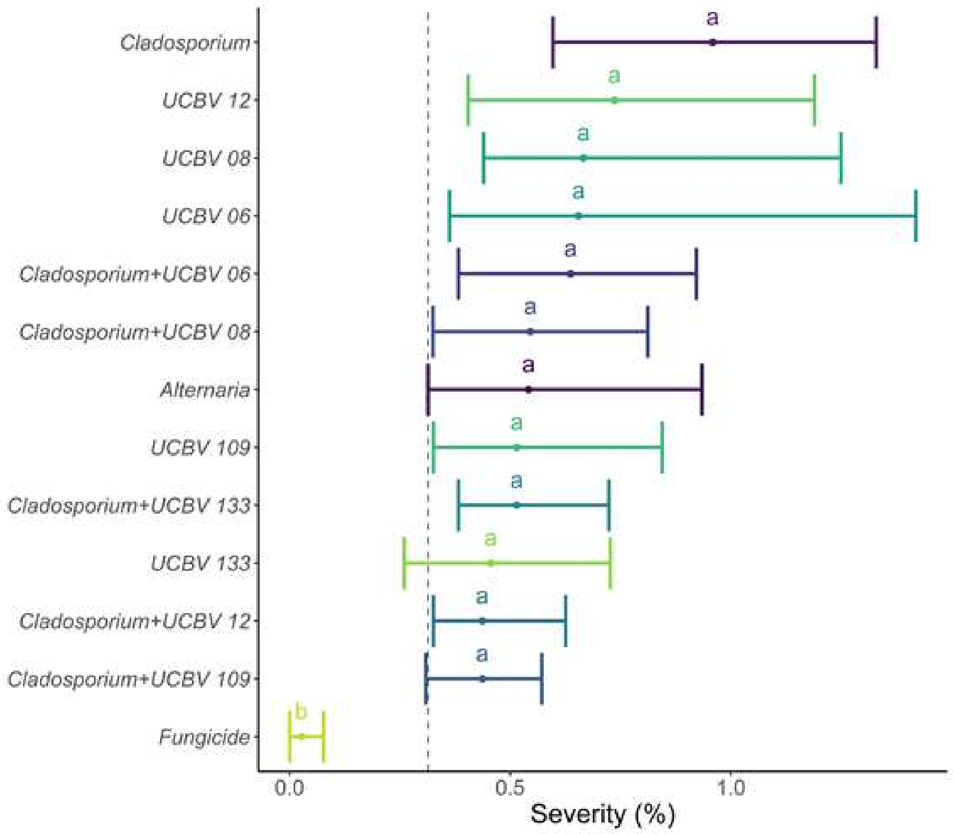

Figure 1: Tomato early blight severity (%), 5 dai. Plants were sprayed twice with *Clonostachys* and/or *Cladosporium* isolates prior to pathogen inoculation. Bootstrapped confidence intervals of the means. Dashed line represents the *Alternaria* treatment lower limit as visual reference.

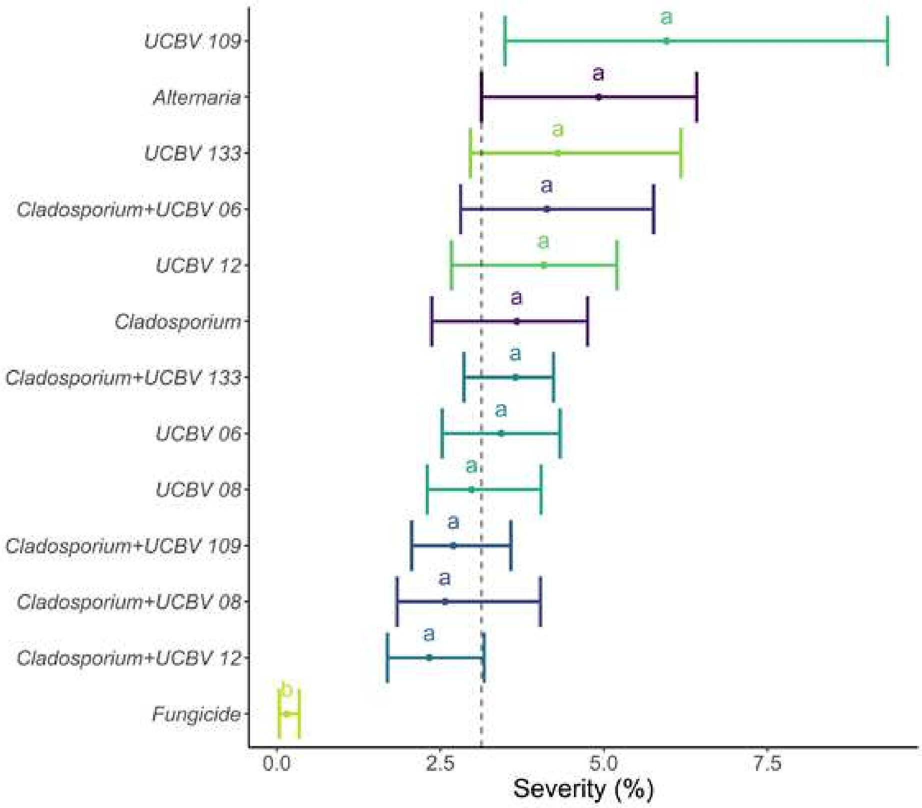

Figure 2: Tomato early blight severity(%), 7 dai. Plants were sprayed twice with *Clonostachys* and/or *Cladosporium* isolates prior to pathogen inoculation. Bootstrapped confidence intervals of the means. Dashed line represents the *Alternaria* treatment lower limit as visual reference.

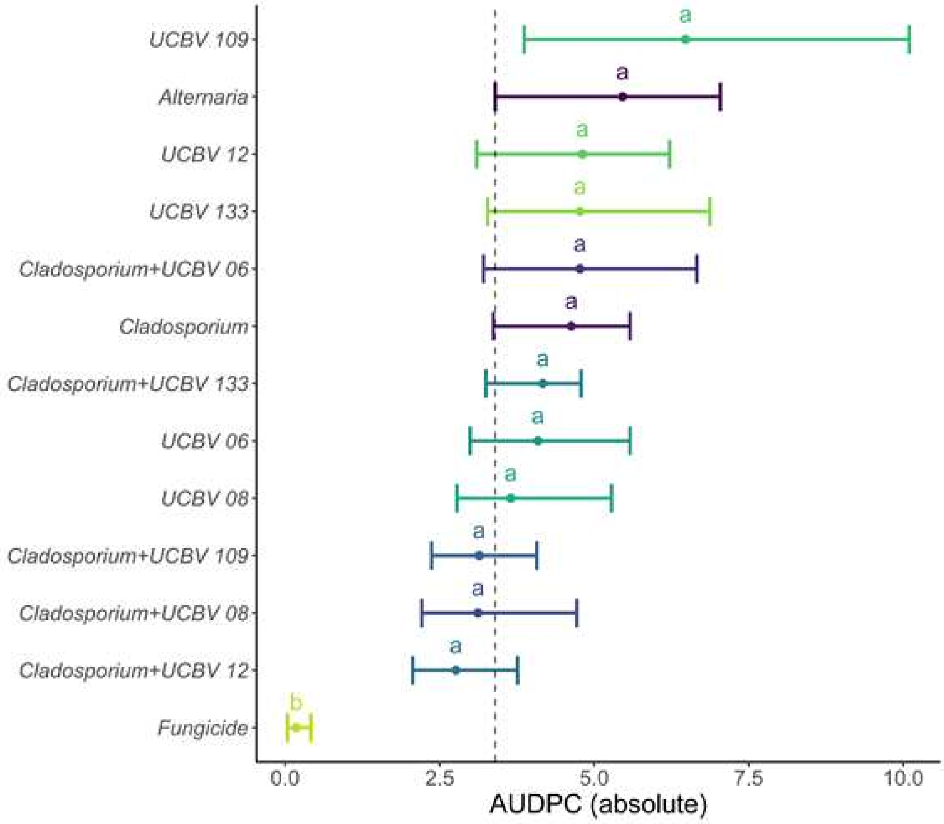

Figure 3: Tomato early blight absolute AUDPC. Plants were sprayed twice with *Clonostachys* and/or *Cladosporium* isolates prior to pathogen inoculation. Bootstrapped confidence intervals of the means. Dashed line represents the *Alternaria* treatment lower limit as visual reference.

Application of *Clonostachys* spp. Isolates UCBV 08, UCBV 12, UCBV 109 and UCBV 133 combined with *Cladosporium* apparently tended towards lower early blight severity compared to their individual use. Simple effect size of biocontrol treatments on AUDPC varied from a 49.3% reduction for isolate 12+*Cladosporium* to a 18.6% increase for isolate 109.

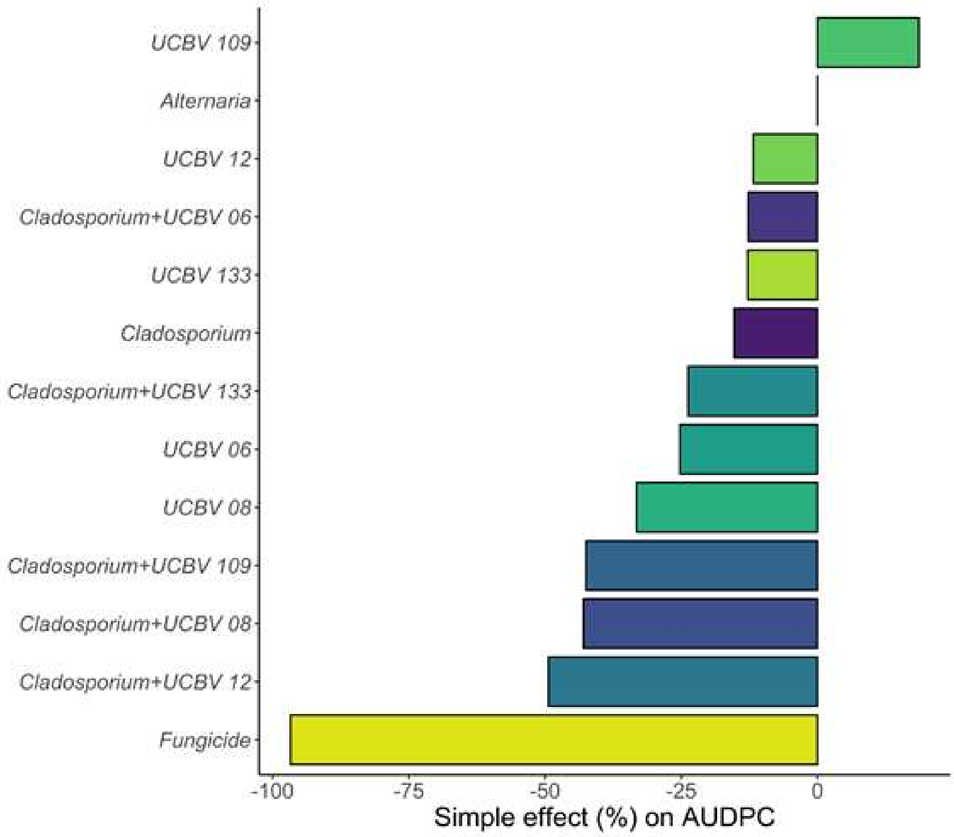

Figure 4: Simple effect size (%) of tomato early blight absolute AUDPC. Plants were sprayed twice with *Clonostachys* and/or *Cladosporium* isolates prior to pathogen inoculation.

Nonetheless, since the biological control agents performance was statistically similar to the *Alternaria* treatment, the aforementioned tendencies of combined use of organisms for more suppressive effects were not enough evidence to include *Cladosporium* in our subsequent extended-spraying experiment.

2.3.2 Pre and Post-inoculation *Clonostachys* spraying for putative curative effect on tomato leaf blight

Severity assessment carried out at 7th and 11th day post inoculation and the calculated AUDPC are summarized in bootstrapped confidence intervals (Figures 5-7).

Significant differences ($p<0.001$) were detected in the first evaluation between the groups formed by fungicide treatment and UCBV 12; UCBV 109, UCBV 133 extended , UCBV 06 extended and

UCBV 08; and the remaining treatments that grouped with *Alternaria* .

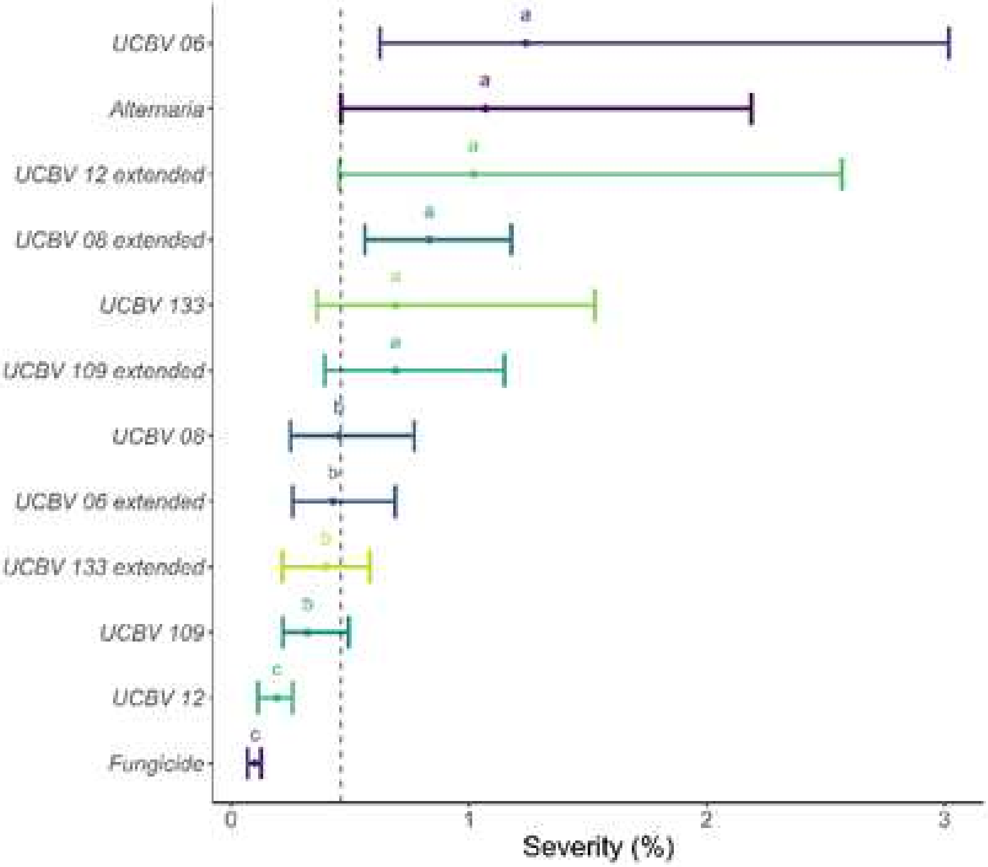

Figure 5: Tomato early blight severity(%), 7 dai. Plants received protective only or protective plus curative (extended) sprayings with *Clonostachys* isolates. Bootstrapped confidence intervals of the means. Dashed line represents the *Alternaria* treatment lower limit as visual reference.

In the second evaluation, the significant differences were detected only between fungicide, UCBV 12 and the remaining treatments (Figure 6). The absolute AUDPC followed this same pattern (Figure 7).

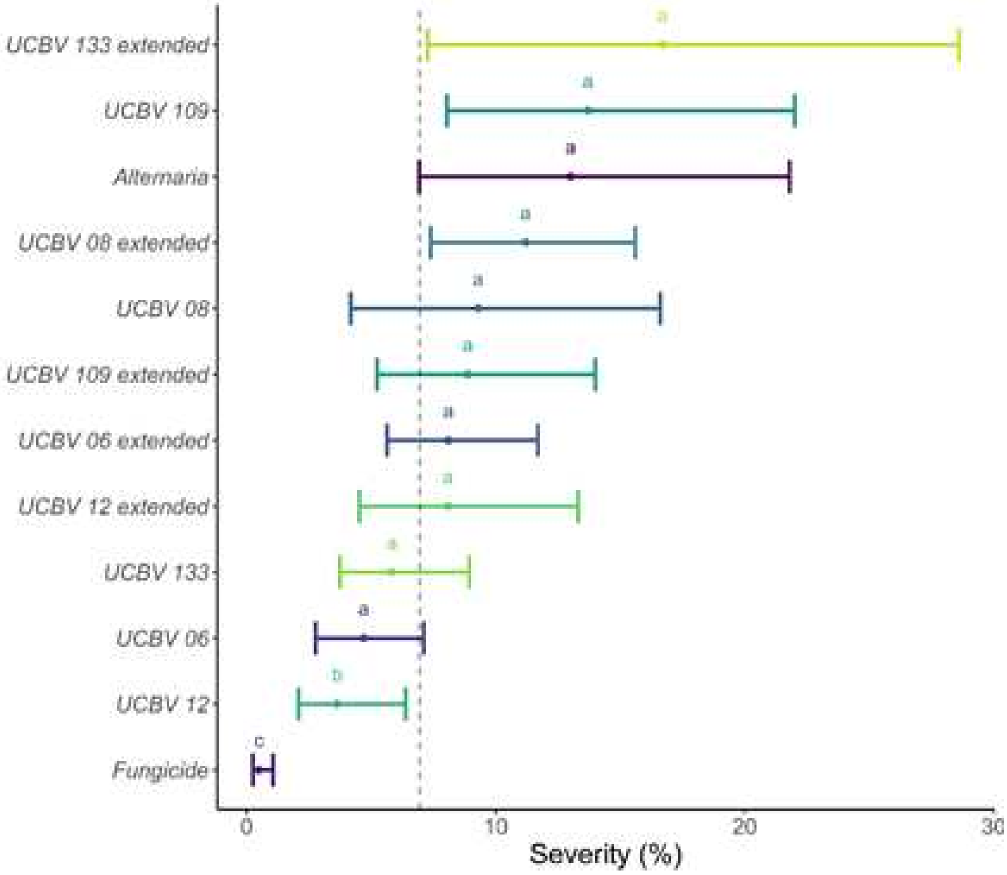

Figure 6: Tomato early blight severity(%), 11 dai. Plants received protective only or protective plus curative (extended) sprayings with *Clonostachys* isolates. Bootstrapped confidence intervals of the means. Dashed line represents the *Alternaria* treatment lower limit as visual reference.

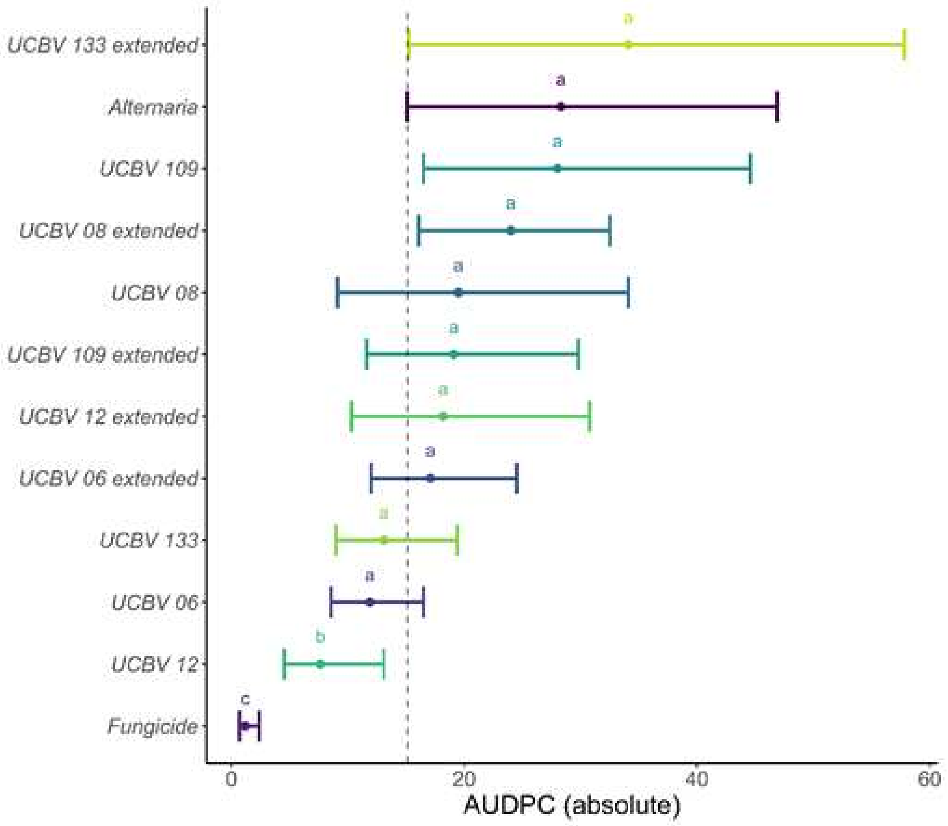

Figure 7: Tomato early blight absolute AUDPC. Plants received protective only or protective plus curative (extended) sprayings with *Clonostachys* isolates. Bootstrapped confidence intervals of the means. Dashed line represents the *Alternaria* treatment lower limit as visual reference.

Simple effect size showed reduction of almost 73% on AUDPC for UCBV 12, while the extended application of UCBV 133 increased it by nearly 20% (Figure 8).

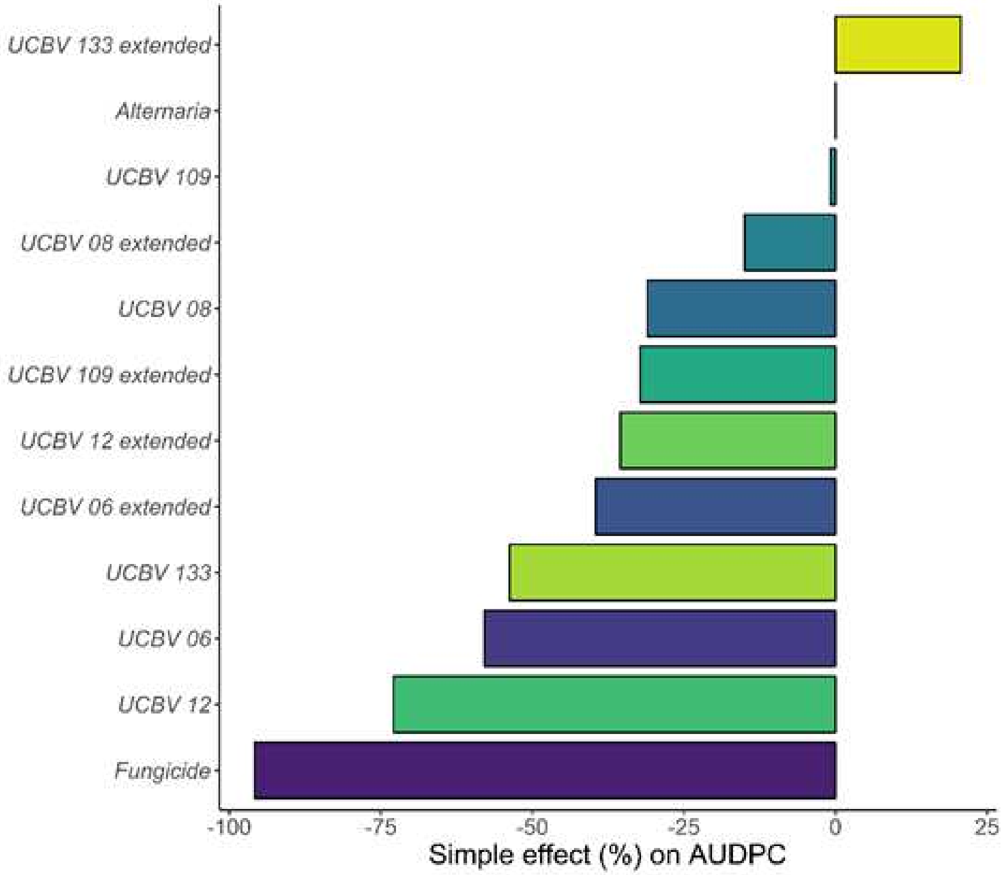

Figure 8: Simple effect size(%) on tomato early blight absolute AUDPC. Plants received protective only or protective plus curative (extended) sprayings with *Clonostachys* isolates.

Taken together, these findings suggest that the BCA's should be used individually, in a preventive way, since curative action was not detectable against tomato early blight.

2.5 Discussion

The combined application of *Cladosporium* and *Clonostachys* tended towards lower tomato early blight severity and, consequently, lower AUDPC values. However, this observed tendency lacked statistical support to indicate whether some *Clonostachys* isolates, namely UCBV 12, 08, 109 and 133, may have reduced the disease impact due to their combined use with the *Cladosporium* mix.

This approach deserves further investigation, possibly with prolonged evaluation times, since the maximum disease severity observed at 7 dai was low (< 10%), making it difficult to detect effects due to BCA treatments. In more progressed disease conditions, the response of treatments might differ markedly, with potential evidence for sporulating *Alternaria* lesions invaded by antagonists and reduced lesion expansion rates caused by enhanced plant immune response induced by the the applied BCA's (Perazzolli et al., 2008). Such an invasion type of established lesions on leaves was reported in studies showing that *Cladosporium* spp. were capable of controlling white rust (*Puccinia horiana*) in chrysanthemum without penetrating teliospores (García-Velasco et al., 2005; Torres et al., 2017).

Cladosporium cladosporioides was shown as an effective antagonist against *Venturia inaequalis*, significantly reducing scab incidence on apple leaves and fruits in field trials, with relative reductions ranging from approximately 50 to 52% in leaves and from 45 to 46% in fruits (Köhl et al., 2015). In contrast, decrease in tomato early blight severity ascribed to the application of the *Cladosporium* mix alone only reached 12.5% in our study. Time from *V. inaequalis* arrival to disease evaluation was long enough to detect these significant changes, especially given required period for fruit development. Spraying *C. cladosporioides* after infection also resulted in significant disease reduction. In contrast, our study focused in a much shorter evaluation period, since our previous trials indicated higher disease levels by the evaluation time (data not shown). So, regardless of any tendency of combined use of BCA's, disease severity was low in both fifth and seventh days after *Alternaria* inoculation and no strong suppressive effects could be detected.

Under more realistic conditions, such as the ones described in apple scab trials (Köhl et al., 2015), plants are constantly subjected to different pathogens and plant aerial parts act as landing zones for a myriad of fungal spores. Biocontrol studies tend to focus on preventive strategies, although plant pathogens may arrive at any moment. Repeated applications of BCA's during the cropping cycle also result in direct contact of the antagonists with infected tissues and lesioned areas, and thus, a direct or indirect curative effect on established lesions could take place. Our second study aimed to evaluate if a putative 'curative' effect might play an important role, i.e., mimicking a scenario in which the biological control agent acts after the pathogen arrival and yet mitigates damages.

Curative effect has been reported for *Clonostachys* rosea with striking disease reduction: tomato plants infected with *Botrytis cinerea* showed nearly no disease after two 10-day spaced applications after pathogen inoculation in stems. (Utkhede; Mathur, 2006). In our study, however, the post-inoculation application of *Clonostachys* conidial suspensions followed by prolonged incubation of plants inside plastic tunnels – under very high humidity and elevated temperatures – had more negative impact on plants regarding their pathogen susceptibility than any detectable curative effect by the applied *Clonostachys*.

Thus, the artificial conditions necessary to induce disease in a more homogenous and reproducible form in the experiment may prevent observations of curative effects that occur in real conditions, in which BCA's are applied at regular shorter intervals (e.g. twice a week), even after the disease onset (Cota et al., 2008; Elad, 2000).

After two preventive applications, 3 out of 5 tested isolates resulted in more than 50% reduction in tomato early blight severity when compared to the *Alternaria* treatment. These *Clonostachys* isolates belong to three species, evidencing that potential for biocontrol is a common feature in the genus. Disease severity decreased nearly 72% when plants were treated with UCBV 12 (*C.*

chloroleuca), the highest-performing biological treatment in our experiments.

Interestingly, UCBV 12 was the lowest-performing treatment in a single-step seed microbiolization assay for plant growth promotion (Rodrigues Jr., 2019), suggesting that it may be more amenable to the use on phylloplane and other aerial parts, rather than survival on a seed coat.

C. chloroleuca was recently described from a collection in Brazil (Moreira et al., 2016) and no reports of biocontrol activities by isolates of this species would be expected. However, one green isolate of *Clonostachys* used as biocontrol agent against *Fusarium*- and *Sclerotinia*-incited diseases in China (Sun; Sun; Li, 2015; Tian; Li; Sun, 2014), commonly referred to as '*C. rosea* 67-1', was found to belong to *C. chloroleuca* by multiple gene phylogenetic studies (Moreira et al., 2016). So, this new species has already been showing interesting biocontrol results.

Our results showed that preventive applications of selected *Clonostachys* isolates alone resulted in high levels of disease suppression. We could only asses the effects of spraying *Clonostachys* isolates on the monocycle of the disease. But it can be assumed that repeated sprayings during the crop cycle may protect plants against infections by primary and secondary inocula continuously brought by air in variable quantities. Weekly sprays with BCA's are commonly suggested for efficient protection, although two weekly applications may necessary in some real situations (Cota et al. 2008). Considering the feasibility of weekly sprayings with *Clonostachys* on commercial tomato plantations and the large suppressive effect obtained in this study, where artificially high loads of mixed pathogen inoculum was used, it is highly probable that this approach may work properly under field and greenhouse conditions.

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3. Article II - Growth promotion of tomato plants by *Clonostachys* spp.

3.1 Abstract

Apart from their mycoparasitic ability, biological control agents may also show direct benefits to the host plants instead of acting solely on pathogens, such as improved nutrient assimilation, plant defenses priming and growth promotion. Few *Clonostachys* species have been studied for the latter benefit and thus, this work aimed to investigate the plant growth promotion in tomato by these fungi. Twenty-one isolates from nine different *Clonostachys* species, plus two isolates of *Beauveria* and *Trichoderma*, were tested for growth promotion of tomato seedlings after either single-step seed microbiolization or seed microbiolization followed by biodrenching of saturated conidial suspensions after seedling emergence. Tomato seedlings were evaluated for length and dry matter. Single-step seed microbiolization was inefficient in providing benefits. Contrastingly, clear plant growth promotion effects were detected after biodrenching. Seven isolates from five *Clonostachys* species consistently resulted in significant plant mass increases ranging from 21% to 33%, the latter result obtained with the isolate UCBV 35 of C. *byssicola*. *Clonostachys* isolates probably were able to colonize rhizosphere due to more efficient form of conidial delivery through drenching, a practice that can be easily adopted in tomato seedling production. Plant growth promotion seems to be another common feature of *Clonostachys* genus, just as the mycoparasitism ability.

Keywords: Seedling enhancement, seed imbibition, biodrenching.

3.2 Introduction

Traditionally, fungal biocontrol control agents (BCA's) were investigated due to their antagonistic abilities against plant pathogens and thought to exert disease suppression through inoculum eradication and protective action on plant surfaces. Later studies showed that direct interaction between BCA's and plants were largely responsible for their desired outcome of disease suppression. These interactions result not only in induced disease resistance, but also in abiotic stress tolerance, enhanced nutrition and growth promotion (Bae et al., 2009; Contreras-Cornejo et al., 2009; Harman et al., 2004; Li et al., 2015).

Studies involving direct plant gains in growth and accumulated matter by the use of *Trichoderma* species are more advanced and include some already commercially available isolates. These isolates promote plant growth and health and plant hosts seem to benefit particularly well with early contact, i.e., from germination of coated seeds onwards (Harman et al., 2004). Rhizocompetent isolates of *Trichoderma* spp. and *Piriformospora indica* effectively colonize the surface and interior of roots in many plant species and can promote lateral growth and proliferation of the root system in several cases. These effects are caused by different modes of interaction, including production of auxin-like and other secondary metabolites by the fungi, efficient solubilization of phosphates and micronutrients from soil, making them more available to the roots, and direct influence on plant metabolism through molecular communication between symbionts, e.g. leading to increased rates of nitrogen assimilation and photosynthetic activities (Altomare et al., 1999; Fischer et al., 2005; Vargas; Mandawe; Kenerley, 2009).

Clonostachys rosea and other less commonly known species within this genus are unspecific mycoparasites and fungal antagonists used in biocontrol of plant diseases, such as gray mold, fusarium head blight of wheat and pea root rot complex (Cota et al., 2008; Xue, 2003; Xue et al., 2009). *Clonostachys* spp. are common soil-dwellers and inhabitants of rhizosphere, also capable of endophytic colonization of different hosts. Rhizosphere colonization by some isolates of C. rosea may lead to enhanced growth and increased plant dry mass in e.g. barley and tomato plants (Johansen et al., 2005; Ravnskov et al., 2006). Nevertheless, the plant beneficial effects of these fungi are commonly described as secondary to the biocontrol abilities and no systematic evaluation of plant growth promotion by *Clonostachys* strains has been described.

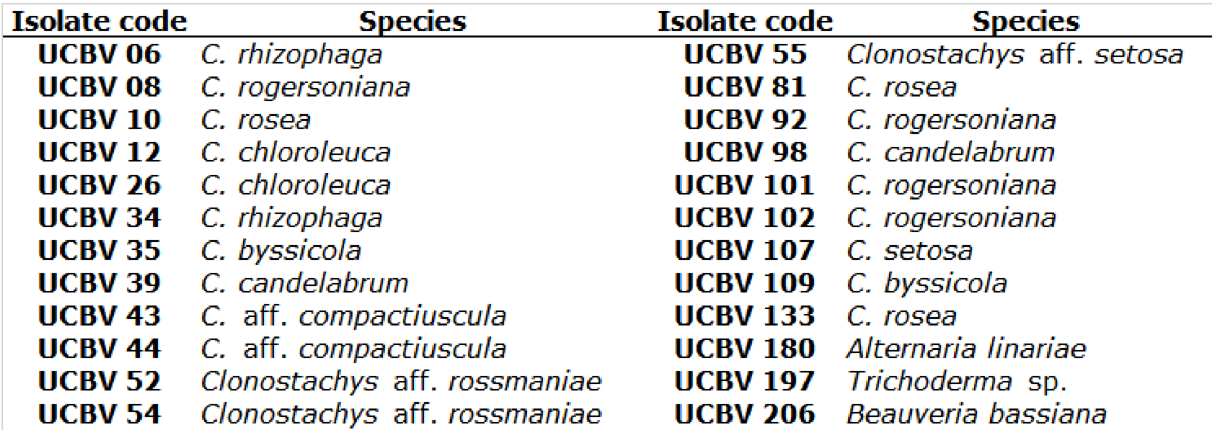
In this study we hypothesized that different *Clonostachys* species may offer benefits for plant hosts through early contact with emerging plants. Thus, we tested the growth promotion effect of isolates from nine *Clonostachys* species on potted tomato seedlings, which came into contact with these BCA’s prior to sowing either by sole seed microbiolization or by seed microbiolization followed by soil drenching with spore suspension.

3.3 Material and methods

3.3.1 Single-step Seed microbiolization

Twenty-one isolates from nine *Clonostachys* species were selected from the UCBV collection, as well as three other isolates – *Beauveria bassiana*, *Trichoderma* sp. and *Alternaria linariae* (Table 1).

Table 1: List of fungal species and isolates



Isolate code	Species	Isolate code	Species
UCBV 06	*C. rhizophaga*	**UCBV 55**	*Clonostachys* aff. *setosa*
UCBV 08	*C. rogersoniana*	**UCBV 81**	*C. rosea*
UCBV 10	*C. rosea*	**UCBV 92**	*C. rogersoniana*
UCBV 12	*C. chloroleuca*	**UCBV 98**	*C. candelabrum*
UCBV 26	*C. chloroleuca*	**UCBV 101**	*C. rogersoniana*
UCBV 34	*C. rhizophaga*	**UCBV 102**	*C. rogersoniana*
UCBV 35	*C. byssicola*	**UCBV 107**	*C. setosa*
UCBV 39	*C. candelabrum*	**UCBV 109**	*C. byssicola*
UCBV 43	*C.* aff. *compactiuscula*	**UCBV 133**	*C. rosea*
UCBV 44	*C.* aff. *compactiuscula*	**UCBV 180**	*Alternaria linariae*
UCBV 52	*Clonostachys* aff. *rossmaniae*	**UCBV 197**	*Trichoderma* sp.
UCBV 54	*Clonostachys* aff. *rossmaniae*	**UCBV 206**	*Beauveria bassiana*

All fungi were cultured for 10 days in 60 mm petri dishes containing oatmeal agar medium (OA). Conidia were scraped in 5 mL of sterile distilled water and approximately 100 disinfested cherry-tomato seeds (*Carolina* cultivar from *Feltrin Sementes*) per treatment were imbibed in 1mL of conidial suspension for 18 h inside 2 mL microtubes. That seed-disinfection process was carried out by 30 seconds immersion in 70% ethanol, then a 20-minute soaking in diluted commercial bleach (10% bleach in distilled water) and rinsing for five times in sterile distilled water. Seed Imbibition in water was conducted as a control.

Seeds were sown in 500-mL plastic cups containing an isovolumetric mixture of commercial substrate and soil. After germination, only one plant per plastic cup was maintained. This experiment was planned as complete randomized design, with 25 treatments and 16 replicates. Each replicate consisted in one plant, randomly arranged in two elevated benches inside a greenhouse. Plants were watered daily until saturation of the substrate. The evaluation for shoot and root length and dry matter weight was conducted 28 days after sowing. Plants were carefully washed for thorough substrate removal and aligned in a bench for digital image capture beside a ruler for later software-assisted measurements. Root and shoot of each replicate were separated in identified paper bags and placed in a 60ºC-oven for complete drying for at least 3 days until constant weight.

Additionally, seeds were also inoculated in tubes containing MS medium (Murashige; Skoog, 1962) after microbiolization to check the viability of isolates, since these commercial seeds contain thiram on their coating.

3.3.2 Seed microbiolization followed by biodrenching

The same procedures mentioned in item 2.1 were carried out until sowing. Seven days after sowing, all seedlings received 1 mL of saturated conidial suspension directly on the substrate, next to the stem base. The saturated conidial suspension was prepared by scraping two 90 mm petri dishes containing fully-grown isolates, each dish with 15 mL of sterilized distilled water. Plants were grown on four elevated benches, each bench containing all types of treatments. This experiment was planned in a complete randomized block design, with four blocks and 25 treatments. Each experimental unit consisted of four plants, whose individual values were pooled into a mean per block. Plants were watered daily until saturation of the substrate. The evaluation for shoot and root length and dry matter weight was conducted 26 days after sowing. The processing of samples was the same as the methods in item 3.2.1.

3.3.3 Image processing and data analysis

Plant images were taken against a black background beside a 30-cm ruler. Plant parts were measured by the freehand line tool in Fiji after setting a scale in cm. Roots were measured from stem collar to longest root tip and aerial parts from stem collar to apical meristem.

Raw data were recorded in Microsoft Excel spreadsheets and then analyzed in RStudio. Data organization was done with package ‘tidyverse’, data normalization with ‘BestNormalize’, analysis of variance and comparison of means with ‘easyanova’, ‘car’ and ‘agricolae’, bootstrapped confidence intervals with ‘Rcompanion’ and plots with ‘ggplot2’ and ‘ggpubr’. The ‘agricolae’ package was also used to calculate the area under the disease progress curve (AUDPC).

Whenever anova’s normality and homoskedasticity assumptions were met, either with untransformed or transformed data, post-hoc significant differences between means were grouped by Scott-Knott test. Otherwise, the non-parametric medians test of ‘agricolae’ package was used.

Bootstrapped confidence intervals of the means or medians were set to 100,000 iterations and 95% confidence. The confidence interval lower limit of control treatment was used as visual reference in plots.

Simple effect size percentages of plant dry matter and length were calculated by:

$$Simple\ Effect\ Size = 100\ \times \frac{Treatment - Control}{Control}$$

The simple effect size represents the percentage of gain or loss relative to a baseline, i.e., the control treatment value.

3.4 Results

3.4.1 Seed microbiolization alone

The observed values of root and shoot dry mass and of plant length are summarized in the following bootstrapped confidence intervals plots containing a central point estimate: mean plant dry matter (Figures 1-2) and median plant length (Figures 3-4). In the latter case, median values were used in non-parametric test for plant length since data could not be normalized.

Microbiolization treatments with 10 isolates yielded root dry mass values statistically equivalent to the control treatment. Treatments with the remaining isolates actually resulted in decreased root dry mass of plants in comparison to the control.

The same pattern occurred with the aerial dry mass and the combined data for the whole plants, where treatments with eleven isolates were equivalent to the control, and the rest of treatments resulted in decreased mass values.

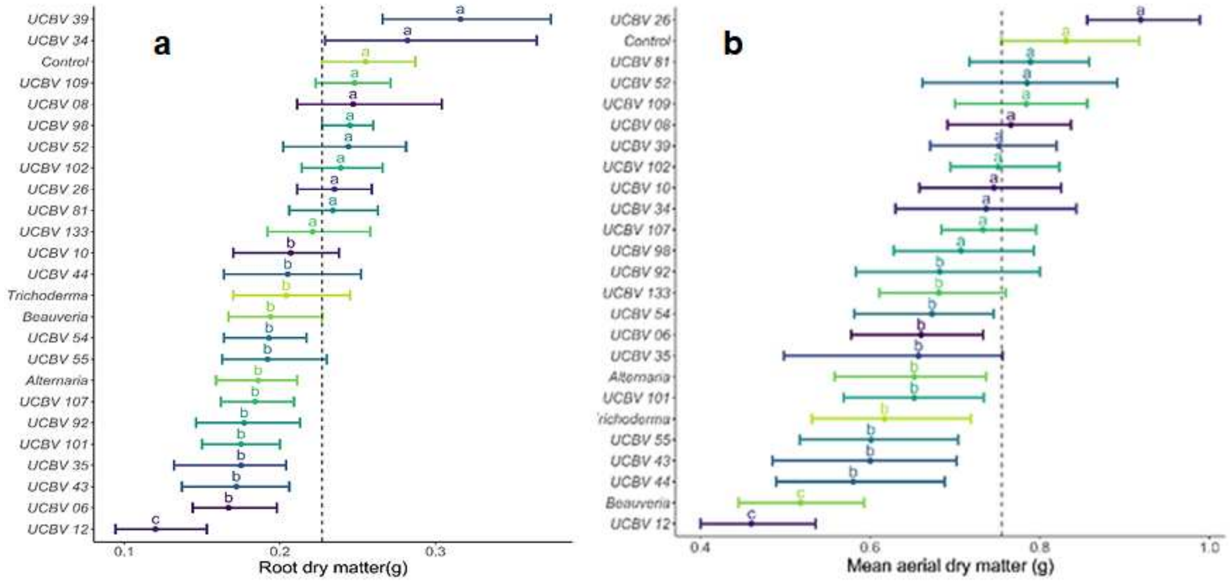


Figure 1: Root (**a**) and aerial dry matter (**b**) (in grams) of tomato plants seed-treated with different fungal isolates. Bootstrapped confidence intervals of the means. Dashed line represents the control treatment lower limit as visual reference. Means grouped by Scott-Knott test, after significant differences ($p<0.001$) in anova.

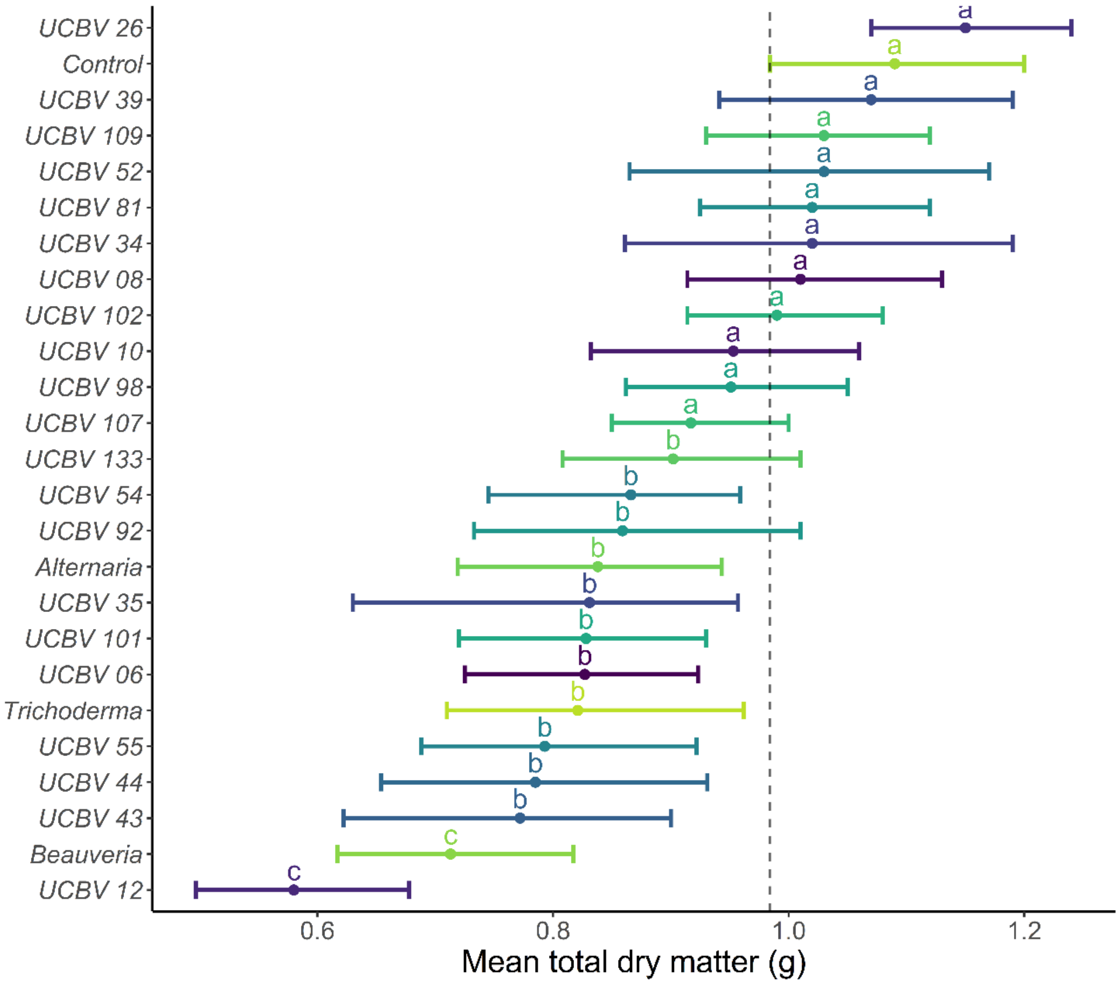


Figure 2: Total dry matter (in grams) of tomato plants seed-treated with different fungal isolates. Bootstrapped confidence intervals of the means. Dashed line represents the control treatment lower limit as visual reference. Means grouped by Scott-Knott test, after significant differences (p<0.001) in anova.

Regarding plant length, only UCBV 98 (*C. candelabrum)* yielded significantly higher root and total length medians when compared to control. There was no significant difference among treatments for the aerial part length (p> 0.17).

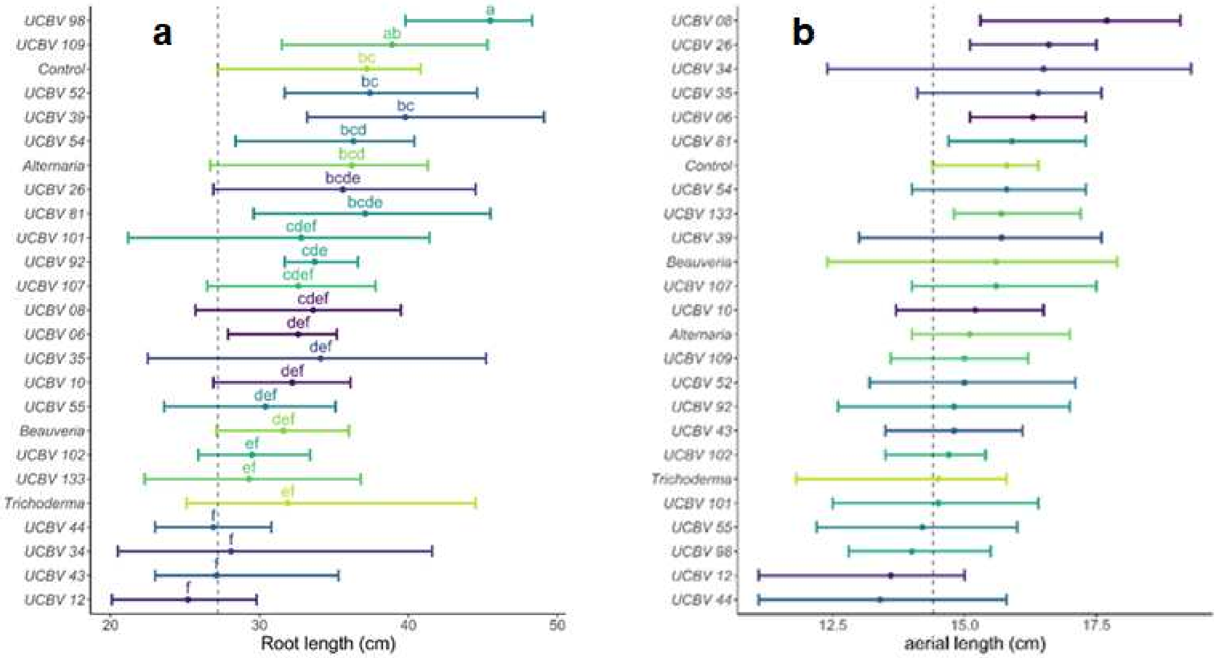


Figure 3: Root (**a**) and aerial part (**b**) length (in cm) of tomato plants seed-treated with different fungal isolates. Bootstrapped confidence intervals of the medians. Dashed line represents the control treatment lower limit as visual reference. Medians grouped by medians test, after detection of significant differences only for aerial length (p<0.005).

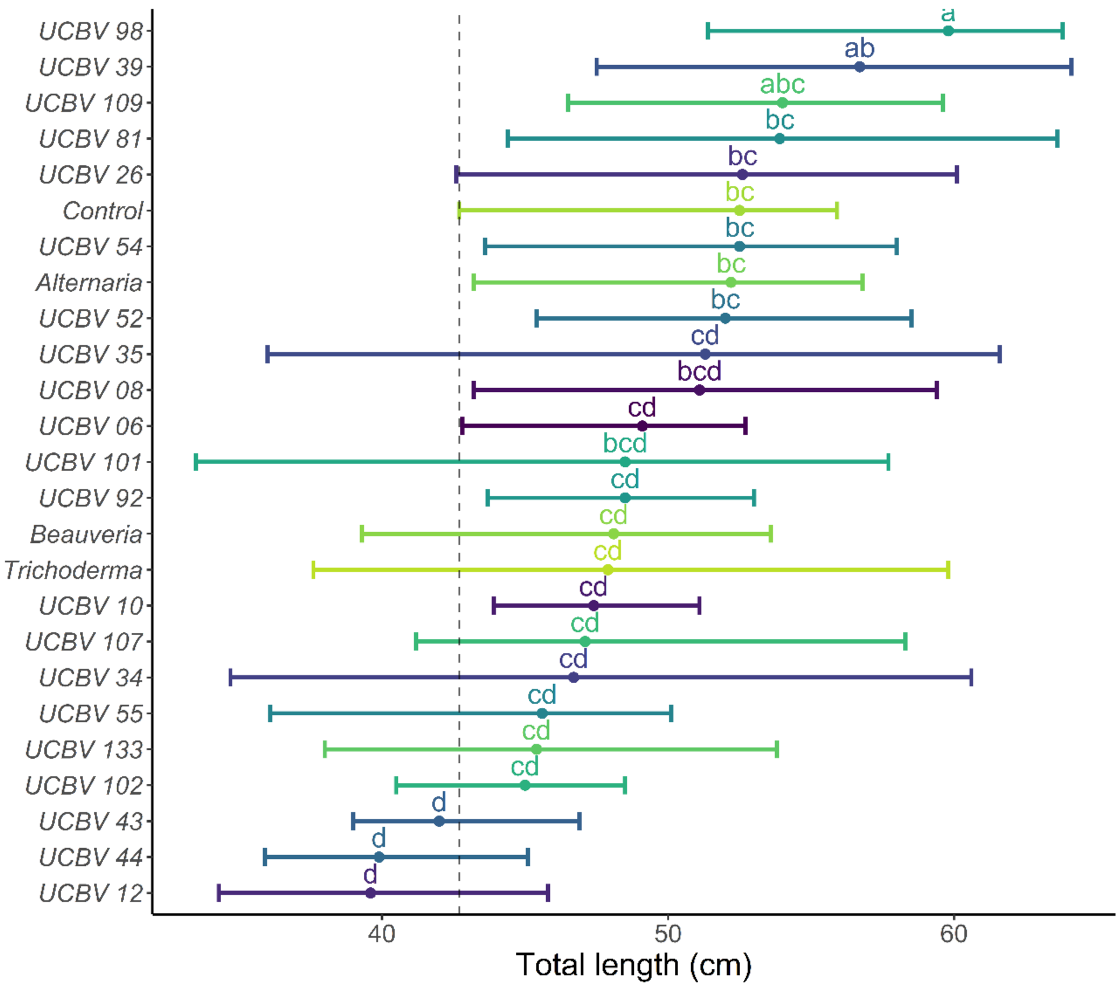


Figure 4: Total length (in cm) of tomato plants seed-treated with different fungal isolates. Bootstrapped confidence intervals of the medians. Dashed line represents the control treatment lower limit as visual reference. Meadians grouped by medians test, after detection of significant differences (p<0.01).

The simple effect size for total dry matter and total length are presented in Figure 7. UCBV 26 (*C. chloroleuca)* provided around 6.2% gain in total dry matter, whereas all other treatments ranked lower than the control treatments with decreases ranging from 1.7% to 46.5%. As for total length, treatments with seven isolates from four species yielded gains ranging from 0.75% to 22.4%, while reductions ranged from 1.2% to 23.4%.

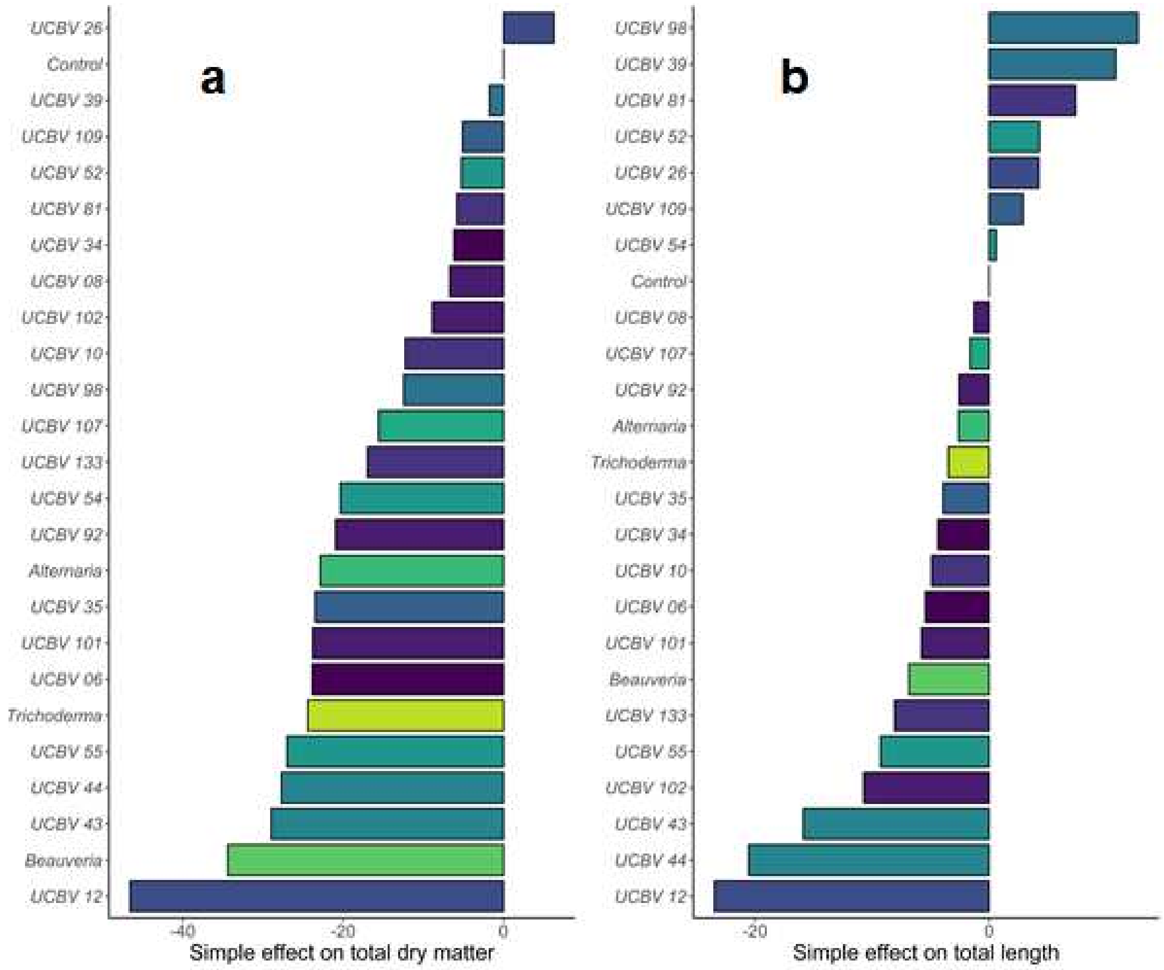


Figure 5: Simple effect size of total plant dry matter (**a**) and length (**b**), in relation to control treatment, of tomato plants seed-treated with different fungal isolates.

In non-parametric Kendall's correlation, total dry mass and total length were positively associated and weakly correlated, with only 33% of variation in total length given by changes in total dry matter ($p<0.001$).

A fungal viability test showed that all isolates grew from treated seeds after incubation on MS medium (data not shown), demonstrating that coating of commercial seeds containing thiram was not able to eradicate the applied fungal treatments before sowing.

3.4.2 Seed microbiolization and biodrenching

In this experiment, each seedling received a 1-mL drenching of concentrate conidial suspension near the stem collar seven days after sowing, in addition to seed microbiolization. This time, only three out of 16 plants in the *Alternaria* treatment survived when drenched with the pathogen. Their measurements were also compromised since the fungus induced lesions in the collar region and resulted in poor growth. There were no deaths among other treatments.

Nine treatments had statistically more of dry root matter than the control, ten treatments had more aerial part matter (Figure 6) and twelve had more total dry matter than the control (Figure 7). Treatments with *Clonostachys* isolates UCBV 35, 92, 102, 55, 81, 44 and 133 were consistently superior in comparison to control values in all three measurements. Treatment with isolate UCBV 107 (*C. setosa*) resulted in total dry matter similar to *Alternaria*, although no disease symptoms or plant deaths were observed.

In the combined analysis of root and shoot, the twelve treatments that resulted in significant higher dry matter when compared with the control were divided in two groups, with the best treatments composed by four isolates of *C. byssicola* (UCBV 35), *C.* aff. *compactiuscula* (UCBV 44) and *C. rogersoniana* (UCBV 92 and 102) (Figure 7).

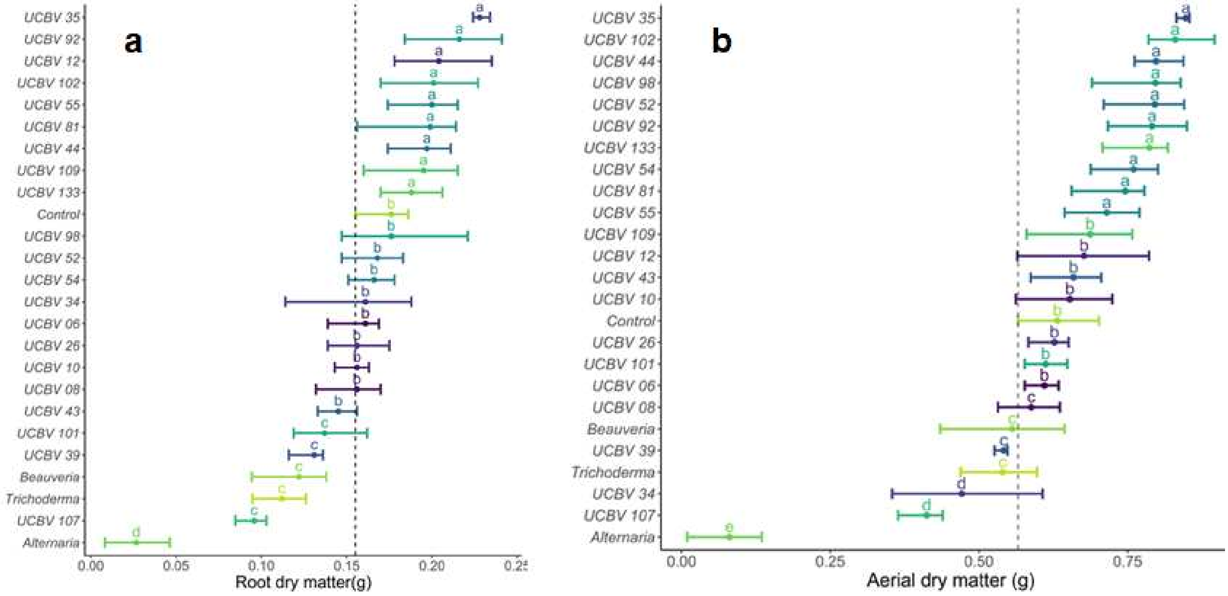


Figure 6: Root (**a**) and aerial part (**b**) dry matter (in grams) of tomato plants seed-treated followed by drenching with different fungal isolates. Bootstrapped confidence intervals of the means. Dashed line represents the control treatment lower limit as visual reference. Means grouped by Scott-Knott test, after significant differences (p<0.001) in anova.

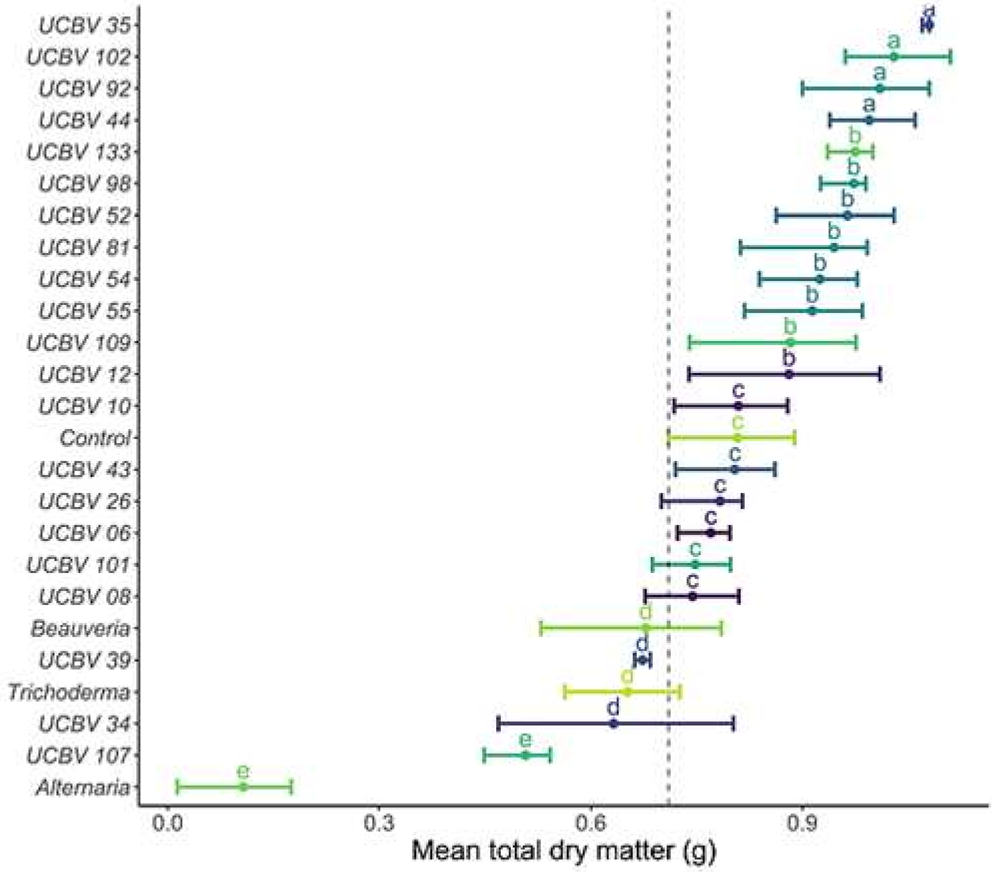


Figure 7: Total plant dry matter (in grams) of tomato plants seed-treated followed by drenching with different fungal isolates. Bootstrapped confidence intervals of the means. Dashed line represents the control treatment lower limit as visual

reference. Means grouped by Scott-Knott test, after significant differences (p<0.001) in anova.

The analysis of plant length showed that four treatments were superior to the control in the aerial part, however there was no significant difference in comparison to the control in root and total plant length.

In non-parametric Kendall's correlation, total dry mass and total length were also positively associated and weakly correlated, with only 15% of variation in total length given by changes in total dry matter (p<0.006). In practical terms, measuring plant size is not a direct inference for its dry matter.

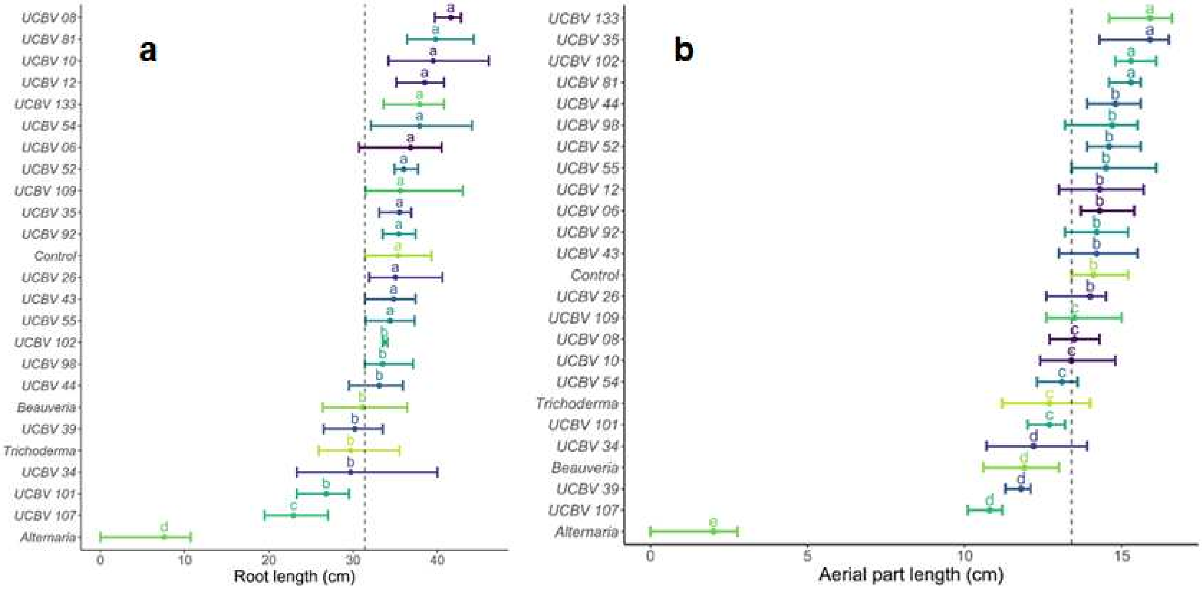


Figure 8: Root (**a**) and aerial part (**b**) length (in cm) of tomato plants seed-treated followed by drenching with different fungal isolates. Bootstrapped confidence intervals of the means. Dashed line represents the control treatment lower limit as visual reference. Means grouped by Scott-Knott test, after significant differences (p<0.001) in anova.

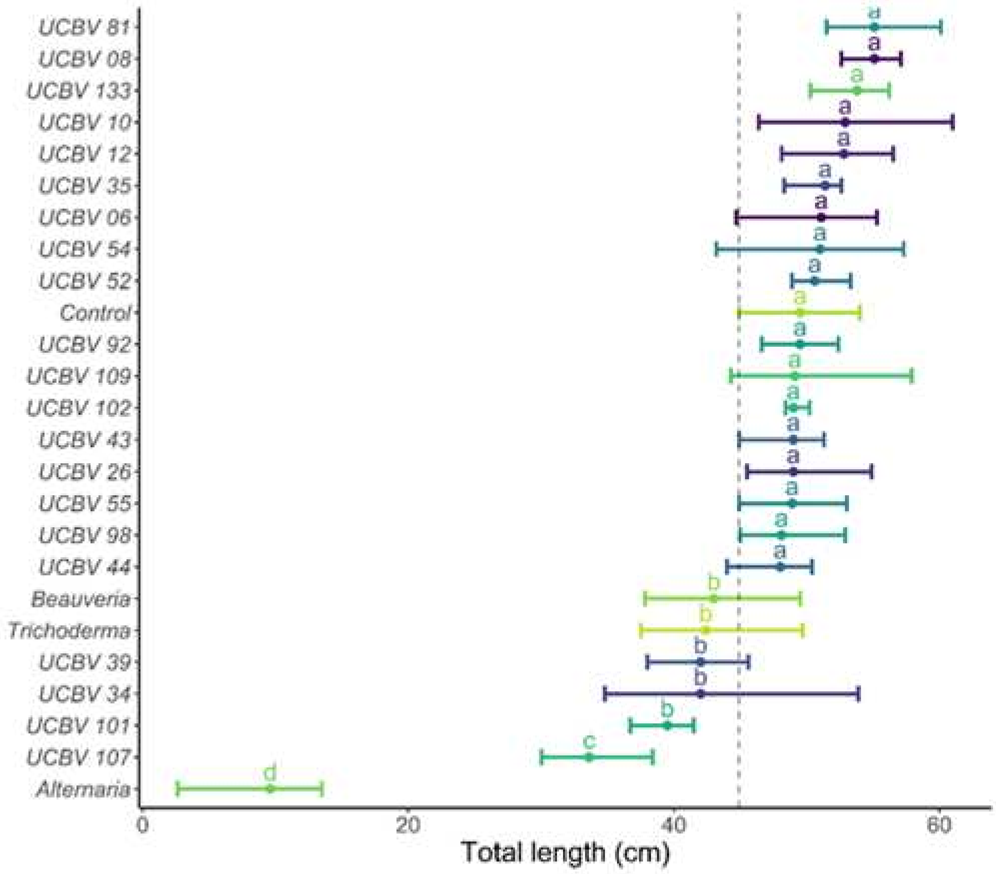


Figure 9: Total plant length (in cm) of tomato plants seed-treated followed by drenching with different fungal isolates. Bootstrapped confidence intervals of the means. Dashed line represents the control treatment lower limit as visual reference. Means grouped by Scott-Knott test, after significant differences (p<0.001) in anova.

The simple effect size of total mass increase in the five best treatments ranged from 20.9% with UCBV 133 to 33.16% with UCBV 35 (Figure 10). Nine *Clonostachys* treatments resulted in increased total plant length, with simple effects ranging from 6.8% to 11.5% for the five top treatments. No increases in dry mass or length happened with plants treated with *Beauveria bassiana* and *Trichoderma* sp.

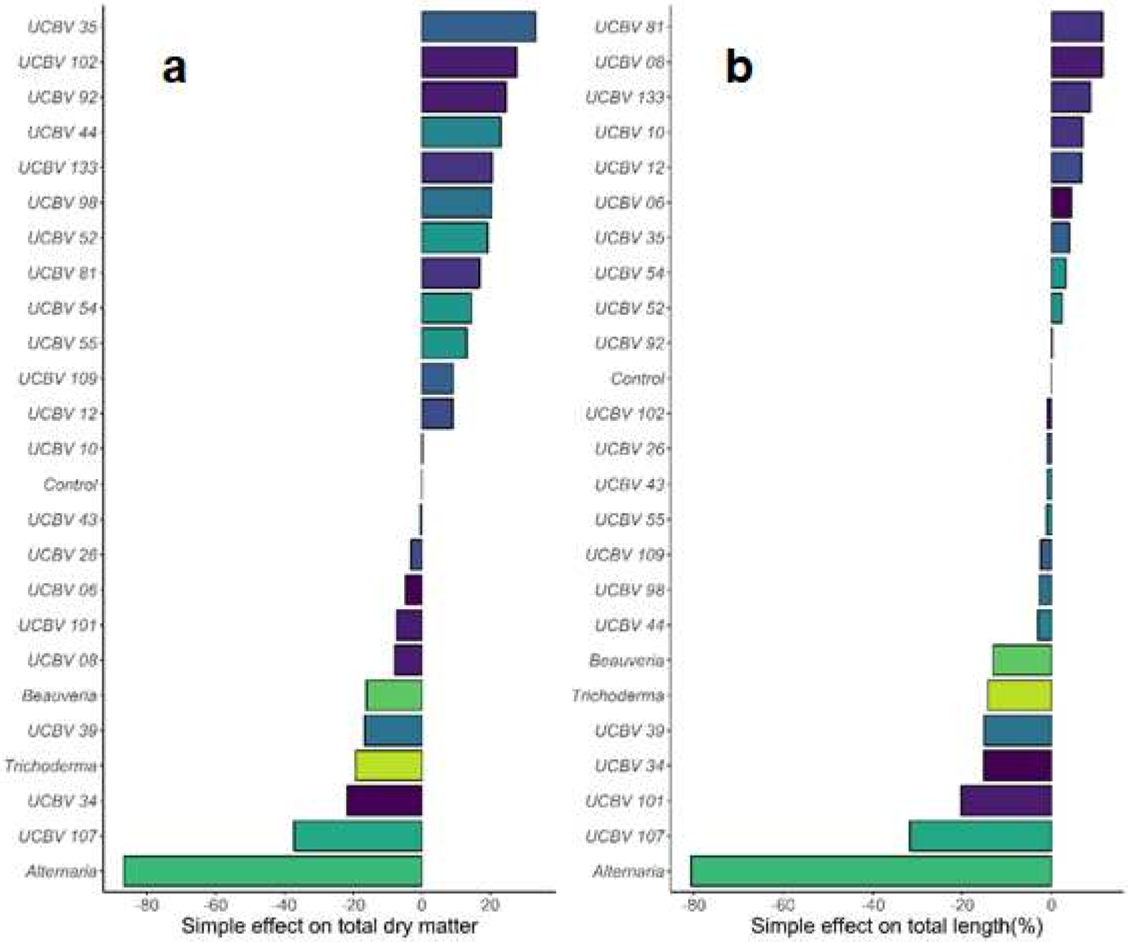


Figure 10: Simple effect size on total dry matter and length, relative to control treatment, of tomato plants seed-treated followed by drenching with different fungal isolates.

3.5 Discussion

The single-step seed microbiolization was not efficient in promoting measurable plant growth effect, since most *Clonostachys* treatments did not differ from the control in terms of accumulated dry matter or only showed slight increases for root and total lengths. The isolates established themselves on the seeds during the 18-hour imbibition, as shown by the complete recover of all isolates from microbiolized seeds after disinfection and plating on culture medium. However, the fungal biomass retained on seeds was probably insufficient for posterior establishment and rhizosphere colonization, since no satisfactory effects occurred during plant development.

Additionally, seedlings were not cultivated in axenic condition, but rather in a mixture of commercial substrate and soil. The naturally occurring microbiota also might have overtaken the competition against seed-surface conidia for rhizosphere space.

In contrast, seed microbiolization followed the soil drenching with 1 mL of saturated conidial suspension per seedling had strong positive effect on plant dry matter accumulation and on root length. Here, BCA's were more likely to dominate competition for the seedling young rhizosphere. The key seems to lie in the biodrenching, since a single application of conidial suspension of *C. rosea* (UCBV 133) seven days after sowing resulted in growth promotion in tomato (Macedo, 2011).

Taking these two experiments together, it can be inferred that a single step procedure with biodrenching the selected isolates would be enough for promoting plant growth for tomato seedlings or for other horticultural crops under intensive cultivation system, in which seedling production preceding transplantation is a common practice (Lorito et al., 2004; Shoresh; Harman; Mastouri, 2010; Tucci et al., 2011). For major crops using extensive cultivation system, seed coating with BCA's or other inoculants is strategic and it is expected that applied inocula will be able to survive and outcompete resident microbiota, successfully becoming established in rhizosphere (Chen et al., 2017).

A group of seven isolates from five species consistently and significantly outperformed the control treatment in terms of plant biomass, namely UCBV 35 (*C. byssicola*), UCBV 44 (*C.* aff. *compactiuscula*), UCBV 55 (*C.* aff. *setosa*), UCBV 81 (*C. rosea*), UCBV 92 (*C. rogersoniana*), UCBV 102 (*C. rogersoniana*) and UCBV 133 (*C. rosea*). The isolate UCBV 133 (=NCR61/F) has been investigated for the biocontrol of grey mold in different plant hosts (Cota et al., 2008; Morandi; Sutton; Maffia, 2000; Saraiva; M; Borges, 2014) and was included in this work as a reference BCA.

Preliminary data on the plant stimulant effects by UCBV 133 agree with our results, showing that it can induce biomass accumulation on ox-eye daisy, soybean and tomato (Macedo, 2011). Just as mycoparasitism is a common characteristic in the *Clonostachys* genus, with varying degrees among species and isolates (Moreira, 2012), so it seems to be the case for plant growth promotion ability.

Variation in BCA's traits of interest renders selection of isolates for agricultural use an obviously not straightforward task. Very distinct results were observed between some isolates within the same species, such as the positive effect on total dry matter for UCBV 98 and the complete opposite for UCBV 39, both *C. candelabrum.* Also, the chosen *Trichoderma* sp. isolate performed poorly, again revealing that even within the most studied growth-promoter fungal genus, refinement of isolates is mandatory. This process goes both ways: after choosing a set of candidates for plant growth promotion, screening of other hosts is a necessary refinement in search for a good generalist plant symbiont. It seems to be the case for *Clonostachys*, which has presented positive effects not only for economically important angiosperms, but also for the gymnosperm *Pinus radiata* (Moraga-Suazo; Sanfuentes, 2017).

On the other hand, one of the most consistent responses was the death of seedlings drenched with *Alternaria linariae* suspension. Only three out of sixteen total seedlings survived and, although the pathogen was not reisolated from dead tissues, subsequent deaths occurred along the days exclusively for this treatment. We can imply that the fungicide coating of the commercial seeds no longer had preventive action one week after sowing.

In conclusion, *Clonostachys* spp. can directly affect plants by improving their growth and not only act as antagonists of other fungi, indicating that growth promotion is a common feature in the genus. Apart from frequently studied *C. rosea*, several lesser-known species - or until now untested - remain to be explored for the same agriculturally beneficial features. Hence, subsequent steps will include testing plant grown promotion with other crops, associating this benefit with biocontrol of soilborne diseases.

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4. General conclusions

- *Cladosporium* spp. and *Clonostachys* species do not seem to act synergistically in terms of enhancing biocontrol over tomato early blight by their combined application.
- There is no evidence that the *Clonostachys* species exerts a curative effect on plants against *Alternaria linariae.*
- *Clonostachys chloroleuca* (UCBV 12) is able to reduce tomato early blight.
- *Clonostachys* species can provide benefits for plant growth and biomass accumulation.
- Delivering the plant growth-promoting fungi to tomato seedlings yields satisfactory results by biodrenching the substrate, but not by single-step seed soaking.
- *C. byssicola* isolate UCBV 35 yielded the highest plant biomass gain.
- Just as mycoparasitism, plant growth promotion seems to be a common feature in the *Clonostachys* genus.