Muscodor spp. controls tomato wilt disease by Ralstonia solanacearum and increases yield and total soluble solids content in tomatoes

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Short Report

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Abstract

Tomato is one of the most economically important crops in Brazil. However, several diseases limit tomato production, among which phytobacterial diseases are responsible for the greatest losses, such *Ralstonia solanacearum*, the pathogen most relevant to the crop. Endophytic fungi of the genus *Muscodor* are well known for the bioactivity of their volatile organic compounds (VOCs) against phytopathogens and also have the potential to promote plant growth, as in commum beans. Thus, in this study, we hypothesized that isolates of *Muscodor* spp. can be used for *Ralstonia solanacearum* control, and to increase tomato yield and total soluble solids content. To test our hypothesis, seven *Muscodor coffeanum* and two *Muscodor* sp. isolates associated with *Coffea arabica* were screened *in vitro* and *in vivo. In vitro*, using divided Petri dishes to evaluate the effect of VOCs, it was shown that the isolate of *M. coffeanum* CML 4009 promoted greater inhibitory effects on the growth of *R. solanacearum* race 3. *In vivo*, we inoculated seeds with *Muscodor* spp. isolates and later transplanted tomato seedlings into soil infested with *R. solanacearum*, and observed that the isolate CML 4009 reduced the severity of bacterial wilt. *Muscodor* isolates promoted an increase in fruit production and total soluble solids in tomatoes. Inoculation with *Muscodor* spp. is a plausible strategy for tomato seed treatment to control *R. solanacearum* diseases and increase tomato yield. The future of the *M. coffeanum* CML 4009 isolate as a biocontrol agent and plant growth promoter is promising.

Fulltext

The tomato (*Solanum lycopersicum*) is an economically important crop in Brazil. In 2019, the country ranked tenth among all countries in tomato production, with 3,917,967 metric tons (FAOSTAT, 2021). Goiás and São Paulo states are the largest producers, responsible for 28.9% and 25.6% of Brazilian production, respectively (IBGE, 2021). However, bacterial diseases can affect tomato productivity, cause significant losses. Different bacterial diseases have been described in tomato plants, such as, bacterial wilt, caused by *Ralstonia solanacearum*; bacterial speck, caused by *Pseudomonas syringae* pv. *tomato*; and bacterial black spot, caused by *X. vesicatoria*, *X. perforans* and *X. gardneri* (Jones et al., 2004).

Ralstonia solanacearum is one of the soil-borne pathogens most relevant to the tomato crop, occurring mainly in rainy summers and protected crops; and in the north and northeast regions, it is a problem throughout most of the year (Lopes, 2009; Lopes et al., 2015). *R. solanacearum* ranks second among the 10 bacterial plant pathogens causing the most significant economic losses worldwide (Mansfield et al., 2012). It induces rapid and fatal wilt, causing losses of up to 91% in tomato production (Yuliar et al., 2015). Control is difficult because it persists in the soil for a long period after its initial manifestation in the field (Lopes 2009). Bacterial diseases that affect aboveground plant parts lead to a loss of the photosynthetic surface and promote flower falling, and the affected fruits lose their quality and exhibit a reduced flavor (Preston, 2000; Quezado-Duval & Lopes, 2010; Kolomiets et al., 2017). Fungicides, such as copper, have been used to inhibit the growth of tomato bacterial agents (La Torreet al., 2018). However, agrochemicals are often costly and hazardous to both human and animal health and the environment (Koskey et al., 2021).

The control of plant diseases caused by bacteria is difficult because conventional control measurements do not present satisfactory efficiency and there is little availability of resistant cultivars. Therefore, the use of antagonistic microorganisms has been proposed (Marcuzzo et al., 2015; Kolomiets et al., 2017). Biological control agents are accepting solutions that have been gaining much interest among researchers and rural producers because of their diverse suppression mechanisms and ability to promote plant growth and provide increased agricultural productivity and environmental protection in addition to generating financial gains (Yuliar et al., 2015; Koskey et al., 2021).

Endophytic fungi of the genus *Muscodor* have been reported to be efficient biocontrol agents for the synthesis of volatile organic compounds (VOCs), and some species are commercially available as biological control agents (Strobel et al., 2001; Worapong et al., 2001; Mercier & Jimenez 2004, Mercier & Smilanick 2005; Grimme et al., 2007). In 2020 the species of *Muscodor* were transferred to *Induratia* genus and which was based on two fungal species with teleomorphs strikingly similar to *Induratia apiospora*, *M. thailandicus* (≡ *I. thailandica*) and *M. ziziphi* (≡ *I. ziziphi*) (Samarakoon et al., 2020). However, in this study, it did not include any sequences of *Induratia* owing to a lack of generic DNA data for comparison, thus, its phylogenetic affinities have remained unresolved. Recently, an ex-holotype strain of *Induratia apiospora* was found among the ATCC collection, enabling detailed morphological and molecular phylogenetic investigations. Thus, phylogenetic analyses of multigene sequence data revealed a close relationship of *Induratia apiospora* to the Barrmaeliaceae, whereas *Muscodo*r, which was described as *Induratia*, showed no relationship with *I. apiospora* (Cedeño-Sanchez et al., 2023). Therefore, the genus *Muscodor* was resurrected and belongs to the family Xylariaceae, and several *Induratia* species according Samarakoon et al. (2020) were formally transferred to *Muscodor*.

Several *Muscodor* species have been reported in Brazil, including *Muscodor coffeanum, Muscodor* sp. *Muscodor yucatanensis*, *Muscodor vitigenus* isolated from the stems and leaves of *Coffea arabica* (Hongsanan et al., 2015; Monteiro et al., 2017; Guimarães et al., 2021; Mota et al, 2021; Gomes et al., 2023), and *Muscodor brasiliensis*, isolated from the leaves of *Schinus terebinthifolius* (Pena et al., 2019). The profile of VOCS produced by *M. coffeanum* and *Muscodor* sp., was reported by Guimaraes et al. (2021) and Gomes et al. (2023), these species produced different VOCs as Pyrimidine 2-chloro-4-ethyl-6-methyl-, Cyclosativene, Tricyclo[4.3.1.1(3,8)] undecane- 3-methoxy-, 4-Amino-3,5-diethylpyridine, Imidazo [5,1-f][1,2,4] triazine-2,7-diamine, Dimethyl-[4-[2-(3-methylisoxazol-5-yl)vinyl] phenyl]amine, 4(1H)-Pyrimidinone, 2,3-dihydro-1-methyl- 6-(4-pyridinyl)-2-thioxo-, 2-Phenyl-6-chloro-benzofuran,1-Methyl-2-nitro-4-(1,2,2-trimethyl-cyclopentyl)-benzene.

Muscodor species from Brazil produce VOCs with antifungal activity against *Rhizoctonia solani*, *Fusarium oxysporum*, *Phoma*spp., *Fusarium solani*, *Fusarium verticillioides*, *Cercospora coffeicola*, *Pestalotia longisetula*, *Aspergillus ochraceus* (Monteiro et al., 2017), *Penicillium digitatum* (Pena et al., 2019), *Botrytis cinerea* (Guimarães et al., 2021), *Colletotrichum lindemuthianum*, *Sclerotinia sclerotiorum*, and *Pseudocercospora griseola* (Mota et al., 2021). Gomes et al. (2023) showed that *Muscodor coffeanum* isolates from coffee plants presented antimicrobial activity against *Aspergillus ochraceus*, *A. sclerotiorum, A. elegans, A. foetidus, A. flavus, A. tamari, A. tubingensis, A. sydowii, A. niger, A.*

caespitosus, A. versicolor, and *Penicillium expansum*, In addition, *M. coffeanum* showed nematicidal activity against *Meloidogyne incognita* and antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, and *E. faecium*, and even reduced preformed biofilms of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Guimarães et al., 2021). Moreover, *Muscodor* species are also able to produce non-volatile secondary metabolites, including extracellular amylase, cellulase, lipase, pectinase, phytase, protease, endo β-1,4-glucanase, as well as exo β-1,4-glucanase, and molecules that modulate enzymes that act in human hemostasis (Bastos et al., 2020; Monteiro et al., 2020; Cardoso et al., 2021). Hayashibara et al. (2022) showed that inoculation of common bean seeds with endophytic species of *Muscodor* (syn. *Induratia*) from *Coffea arabica* was effective in enhancing grain yield, and may be a promising management strategy to improve bean productivity and plant health. This was a pioneering study because it showed for the first time that it is possible to successfully introduce and establish *Muscodor* spp. on a plant that is different from the original host.

Given the importance of the *R. solanacearum*/tomato pathosystem and the potential for biocontrol of fungi of the genus *Muscodor*, we hypothesized that isolates of *Muscodor* spp. could be used to control bacterial wilt caused by *R. solanacearum* and to increase productivity and total soluble solids in tomatoes.

In this study, we evaluated seven *Muscodor coffeanum* (CML 4009, CML 4010, CML 4011, CML 4012, CML 4014, CML 4016, CML 4017) and two *Muscodor* sp. (CML 4013, CML 4015), deposited in the from the Coleção Micológica de Lavras, Laboratório de Sistemática e Ecologia de Fungos, Universidade Federal de Lavras, Brazil (http://www.dfp.ufla.br/cml). They wereisolated from fresh and healthy leaves and stems of coffee plants (*Coffea arabica*) growing spontaneously in a secondary forest in Mata do Paraíso, Zona da Mata region, Viçosa, Minas Gerais, Brazil (Guimarães et al., 2021). *Muscodor* isolates were grown on potato dextrose agar (PDA) at 25°C for 15 days. The *R. solanacearum* race 3 isolate was originally obtained from infected tomato plants and was provided by the Laboratory of Plant Bacteriology, Universidade Federal de Lavras, Brazil. The bacterium was activated on 523 solid medium (Kado & Heskett, 1970), at 28°C for 72 hours.

The production of volatile organic compounds (VOCs) by *Muscodor* isolates was evaluated using Petri dishes (9cm) with a divider. Each fungus was grown for 7 days in PDA medium at 25°C. On the other hand, 100 µL of a bacterial suspension ($OD_{600\ nm}$ = 0.1 ≈ 10^8 CFU mL^{-1}), growth on 523 medium broth (Kado & Heskett, 1970) for 24 h at (120 rpm at 28°C), was transferred to 523 solid medium (Kado & Heskett, 1970), followed by incubation for 72 hours. The experiment was carried out in a randomized design, with ten treatments in four replicates each. The control was a bacterial suspension in a 523 solid medium. To confirm the bactericidal effect, the fungus was removed, and only the pathogen was left for another 72 hours of incubation.

To assess the effect of seed treatment by *Muscodor* isolates on tomato plant growth and R. solanacearum control, ten superficially disinfested seeds (hybrid N-901) were arranged equidistantly from each other and 1 cm from the fungal mycelial disk in PDA medium (-1.2MPa). Seeds were placed slightly

after inoculation of the fungus into the culture medium, and both were incubated at 25°C for 10 days in the dark. Inoculated tomato seeds were sown in expanded polystyrene trays consisting of 5 × 10 cells with a cell size of 3.5 × 3.5 cm^2 filled with Tropstrato™, two seeds per cell, and grown in a greenhouse for 30 days. Then, the plants were transplanted to 4 L pots, and after 15 days (plants 45 days old), they were infected via the soil through root injury followed by irrigation with 30 mL of *R. solanacearum* bacterial suspension ($OD_{600\ nm}$ = 0.1 ≈ 10^8 UFC ml^{-1}). Noninoculated seeds were used as control. The plants were maintained in a greenhouse with controlled humidity (≈ 65%) and temperature (≈ 27°C). They were cultivated in a semi-hydroponic system and fertigated with a nutritive solution daily and trained on a single stem. Fertigation (100 mL per pot) was alternated with manual localized irrigation to pot capacity and electrical conductivity below 3 dS cm^{-1}. The experiment was carried out in a randomized design, with ten treatments in four replicates each. Disease severity was evaluated at 15 days after inoculation using the Nielsen and Haynes (1960) disease index scale, as follows: 1, healthy plant; 2, plant with 1/3 of leaves wilted; 3, plant with 2/3 of leaves wilted; 4, totally wilted plant; and 5, dead plant. The scores were converted to bacterial wilt index (BWI) values as proposed by Empig et al., (1962). BWI = (CxP)/Nx5, where C = assigned grade in each class of symptoms, P = number of plants in each symptom class and N is total number of plants and 5 is the maximum severity degree. This index was then used to verify whether the *Muscodor* isolates could reduce disease severity. The infected fresh fruit yield and total soluble solid content (°Brix) of the infected plants were also evaluated. The °Brix value was evaluated in naturally ripened fruits by collecting the pulp of each fruit produced, followed by digital refractometer readings.

For all assays, the experimental design was completely randomized; the data were subjected to ANOVA, and the means were compared with the Scott–Knott test at 5% probability using the statistical software SISVAR version 5.6. All experiments were performed twice to confirm the results.

Muscodor coffeanum (CML 4009, CML 4011, and CML 4014) and *Muscodor* sp. (CML4013) produced VOCs with antibacterial activities against *R. solanacearum* (data not shown). Highlight for the isolate CML 4009 produced VOCs with lethal activity against this pathogen (Fig. 1). In addition, plants inoculated with *M. coffeanum* (CML 4009) reduced *bacterial wilt disease* severity (Fig. 2A, B). *M. coffeanum* (CML 4009, CML 4012, and CML 4017) and *Muscodor* sp. (CML 4013 and CML 4015) increased the total soluble solids (°Brix) of the inoculated plants compared with those of the uninoculated control. Furthermore, the isolate *M. coffeanum* CML 4009 exhibited a 26.8% increase in fruit yield, *M. coffeanum* CML 4011 ranked second with an increase of 17.3% and the *M. coffeanum* isolates (CML 4012, CML 4014) and *Muscodor* sp. (CML 4015) ranked third with an increase of 17.1% (Fig. 2C).

This work reports the ability of VOCs from the *M. coffeanum* isolate (CML 4009) to efficiently inhibit the growth of *R. solanacearum* race 3 and its potential to protect tomato plants against this pathogen, besides of the ability of *Muscodor* spp. to promote the improvement of tomato productivity and total soluble solids content, an extremely relevant agronomic attribute related to the high economic return of tomatoes for processing. Thus, as observed for common bean plants (Hayashibara et al., 2022), M. *coffeanum* inoculated in tomato seeds also promoted better productivity and adaptation of the fungus to

species other than the host *C. arabica.* Previous work on the characterization of VOCs using SPME–GC-MS (Guimarães et al., 2021), showed that among the many compounds produced by *M. coffeanum* isolates, butanoic and propanoic acids, have already been reported to exhibit antifungal and antibacterial activities (Strobel et al., 2008). *M. coffeanum* (CML 4009) also produces various alcohols as 1-Propanol, 2-methyl-; 1-Butanol, 3-methyl-;1-Butanol, 2-methyl-; Phenylethyl Alcohol and Ethyl alcohol, and aldehydes such Hexanal. According to Mari et al. (2016), hexanal and many other alcohols also have antifungal and antibacterial activities. In addition to these compounds, isolate CML 4009 also produced terpenes (β-Phellandrene, α-Bergamotene, and Sesquiterpene) and esters (Methyl 2-methylpropanoate, Methyl 2-methylbutanoate, Butyric acid, thio-, S-methyl ester).

Here, we show that endophytic fungi can be used as biological control agents against phytobacteria. However, most studies on the biological control of plant disease-causing bacteria have been based on the use of bacteria as biocontrol agents. Indeed, Raza et al. (2016), studying the responses of the tomato wilt pathogen to VOCs produced by a biocontrol strain *Bacillus amyloliquefaciens*, observed significant inhibition of motility characteristics; antioxidant, enzyme and exopolysaccharide production; biofilm formation; and tomato root colonization. Kolomiets et al. (2017) also recommended biological preparations based on the bacteria *Bacillus subtilis* to restrict the development of bacterial black spots (*Xanthomonas vesicatoria)* and bacterial cancer (*Clavibacter michiganensis* subsp. *michiganensis*).

Furthermore, the antibiosis of antibacterial VOCs, *M. coffeanum* (CML4009) may have controlled bacterial wilt disease by *R. solanacearum*, since a reduction in disease severity was observed compared to that in the negative control. Another study by our group (Monteiro et al., 2020), showed that *M. coffeanum* isolates can produce the extracellular enzymes cellulase, pectinase, protease, phytase, lipase, amylase endo β-1,4-glucanase and exo β-1,4-glucanase. These enzymes can cause degradation of the host tissue, leading to the emission of danger signals called damage-associated molecular patterns (DAMPs), which include cell wall or extracellular protein fragments, peptides, nucleotides, and amino acids. Biological control agents may induce resistance or prime enhanced resistance against pathogen infections in plant tissues without directly interacting with the pathogen (Conrath et al., 2015). The specific molecular signatures of biological control agents, microbe-associated molecular patterns (MAMPs) and DAMPs, are recognized by the corresponding extracellular pattern-recognition receptors (PRRs), which relay these signals to multiple intracellular signalling modules (Köhl et al., 2019). Thus, MAMPs and DAMPs activate immune signalling pathways, such as pattern-triggered immunity (PTI), which plays an important role in reducing pathogen invasion and maintaining homeostasis of endophytic leaf microbiota of plants (Li et al., 2016; Köhl et al., 2019; Hou et al., 2019). Indeed, molecular patterns such as chitin and enzymes of endophytic fungi can act as elicitors, induce plant resistance and stimulate other endophytes (fungi and bacteria) to produce bioactive secondary metabolites with potential influences on host protection (Zabalgogeazcoa, 2008; Gao et al., 2010; Kusari et al., 2012).Our results showed that *M. coffeanum* isolates can protect tomato plants against *R. solanacearum*, and future studies will confirm the outcome of elicitation of PTI by *Muscodor* isolates, elucidating the potential interactions of these isolates with the plant and of the induced plant with the pathogen under standardized conditions to verify the specific signal transduction pathway that is promoted.

Notably *Muscodor* spp. improved tomato yield and total soluble solids content. Endophytic fungi can stimulate plant growth by increasing the availability of nutrients, the action of extracellular enzymes or soil pH regulation, and even by inducing the production of hormones in the host. They may also act directly by producing bioactive compounds (phytohormones), such as indole-3-acetic acid (AIA), gibberellins (GAs), and cytokinins (Shoresh et al., 2010; Kanchiswamy et al., 2015; Suwannarach et al., 2015). These compounds act as signals that control plant growth and development and modulate plant responses to environmental changes (Harllen & Bettiol 2009, Khan et al., 2015).

We proved the hypothesis that *M. coffeanum* (CML 4009) can be used *R. solanacearum* control strategy, as well as to promote the improvement of tomatoes yield and total soluble solids content.

Declarations

Authors' contributions Santos I.A.F.M, Cardoso P.G. and Resende L.V. designed the experiments; Santos I.A.F.M, Nunes P.S.O, Mengez G.A.L. and Monteiro M.C.P. performed the experiments; Guimarães S.S.C analysed the generated data and wrote the manuscript; Cardoso P.G. revised the manuscript.

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Conflicts of interest The authors declare that there are no conflicts of interest.

Research involving human participants and/or animals This study does not include experiments with either human participants or animals.

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Figures

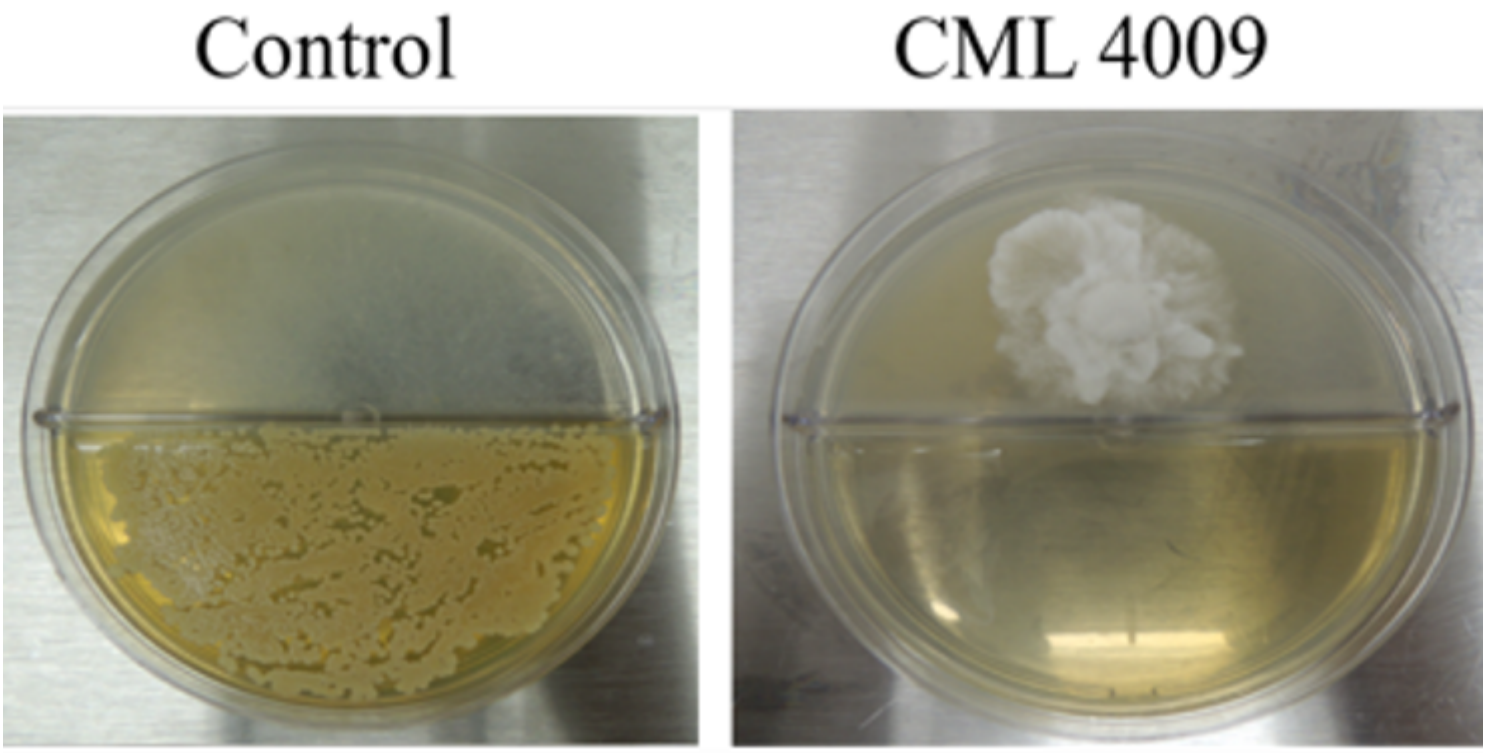

Figure 1

Effect of exposure to the VOCs of *Muscodor coffeanum* (CML 4009) on *R. solanacearum* growth.

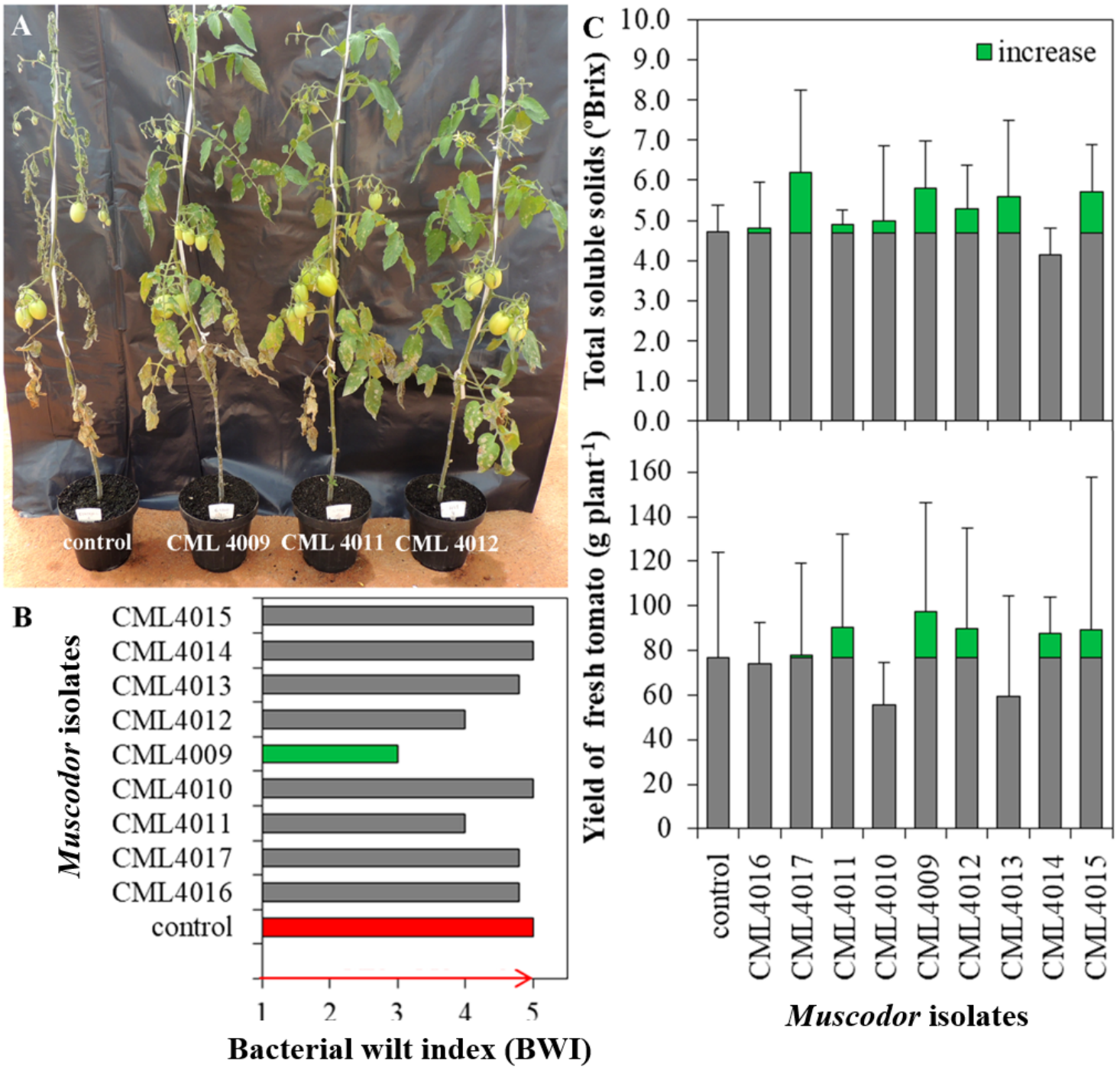

Figure 2

Protection of tomato plants (hybrid N-901) against *R. solanacearum* by *Muscodor* spp. and improvement of agronomic attributes. (A) Control (plants non inoculated with *Muscodor* spp.) and inoculated plants at 15 days. (B) Bacterial wilt index (BWI) of plants treated with the pathogen: 1, healthy plant; 2, plant with 1/3 of leaves wilted; 3, plant with 2/3 of leaves wilted; 4, totally wilted plant; and 5, dead plant. (C) Total soluble solids of fruits at the complete maturation stage and yield of fresh tomato plants under pathogen infection. Values indicate the means, and error bars indicate the standard deviation. The averages presented no significant difference (ANOVA, Scott–Knott test at 5%).