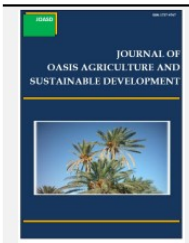

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Prospecting bacterial volatile organic compounds antifungal activities against postharvest diseases

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Abstract

Chemical pesticides have a plenty of negative impacts on human health and on the environment. Thus, modern agriculture cropping systems are moving towards more eco-friendly alternatives. This study aims to investigate the bioprotective effect of three volatile organic compounds (VOCs: N-Ethylaniline, 2-Heptanone and 3-Methylbutan-1-ol) produced by endophytic bacteria against 21 phytopathogenic fungal strains and their bioprotective effect on horticulture products i.e. tomato and lemon fruits and Potato tubers. The results showed that N-Ethylaniline and 3-methylbutan-1-ol had better antagonistic activity against the fungal strains by inhibiting the mycelia growth of the studied fungal strains at different concentrations. The N-Ethylaniline showed the lowest effective concentration (EC50) against *B. cinerea* strain S5 (0,258 mL/L headspace), *Fusarium solani* strain SB4.15.1 (0,496 mL/L headspace) and *Colletotrichum gloeosporioides* strain ManS3Fr02 (0,206 mL/L headspace). At EC50 this compound significantly reduced *B. cinerea* and *C. gloeosporioides* infections on tomato and lemon fruits, respectively. However, N-Ethylaniline didn't showed significant effect on *F. solani* infection on Potato tubers. This study showed the broad spectrum of in vitro antifungal activity of N-Ethylaniline and its effect to reduce postharvest infections of some fungal diseases suggesting its potential use as a biofumigant.

1. INTRODUCTION

In Tunisia, the agricultural sector is of great economic and socio-political importance because of its contribution to the achievement of the national objectives in terms of food security, income creation, employment, regional balance and management of natural resources. The main challenge facing the agricultural sector in Tunisia today is to improve product quality to better meet consumer expectations in terms of food safety (Chebbi et al. 2019).

The production and conservation of agricultural products are always confronted to numerous abiotic and biotic constraints. Bio-aggressors (fungi, bacteria, etc.) are one of the main causes of the deterioration of agricultural products during storage and transport (Benmeddour and Fenni, 2018). Several methods have been used to fight against these bio-aggressors with the predominance of the use of chemicals. However, the use of chemical pesticides has adverse effects on human health and on the environment (Coulibaly et al. 2021). For this reason the global trend is moving towards searching for more effective and environmentally friendly solutions such as biological methods based on the use of living organisms (parasites, fungi, bacteria, plant extracts, etc.) and their extracts to prevent or reduce the damage caused by diseases to an economically acceptable level (Dougoud et al. 2018).

Bacteria and their bioactive substances are among the most used living organisms in biological control (Ryan et al. 2008). They have several antimicrobial properties and they are used in crop protection in the field as well as in greenhouses and storage conditions (Arrarte et al. 2017). Among the bacterial substances with antifungal effects, volatile organic compounds (VOCs) showed a potential of application in agriculture sector. The VOCs have the ability to modulate the metabolome, and the proteome in both plants and microorganisms, making them excellent biostimulants and bioprotectants even

in open-field circumstances (Kanchiswamy et al. 2015; Chung et al. 2016). They have the ability to modulate plant physiological and hormonal pathways in order to boost biomass and yield output through increased root volume, leaf number, leaf size, and flower number, resulting in increased fruit and seed production (Sharifi and Ryu, 2018; Tyagi et al. 2018). Furthermore, they have antifungal, antibacterial, oomyceticidal, and nematicidal actions, as well as the ability to induce plant immunity (Bitas et al. 2013; Schalchli et al. 2016). In literature various bacterial VOCs with antifungal are cited. The 3-methyl-1-butanol, 2-methyl-1-butanol, Dimethyldisulfide, dimethyltrisulfide , chloroacetic acid, benzenamine, phenylethyl alcohol and N-Ethylaniline have antagonistic effect against fungi such as *Ceratocystis fimbriata*, *Botrytis cinerea*, *Colleotrichum gloeosporioides*, *Rhizoctonia solani* and *Aspergillus flavus* (Hernández-León et al. 2015; Rajaofera et al. 2019; Yang et al. 2019; Zhang et al. 2019; Chaouachi et al. 2021; Marzouk et al. 2021).

So, this work focuses on the study of the effect of bacterial VOCs i.e. N-Ethylaniline, 2-Heptanone and 3-Methylbutan -1-ol produced by endophytic bacteria (Chaouachi et al. 2021; Marzouk et al. 2021) on the *in vitro* growth of some phytopathogenic fungi, as well as their ability to protect tomato and lemon fruits and potato tubers against some fungal pathogens during storage.

2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Plant Material

Potato tubers (Spunta variety) and lemon (four-season lemon) and tomato fruits were purchased from the market.

2.1.2. Pure volatiles

The pure volatile compounds 3-Methylbutan-1-ol (Merck), 2-Heptanone (Sigma) and N-Ethylaniline (Sigma) were used in this study.

2.1.3. Fungal Strains

Twenty one fungal strains isolated from different plants and belonging to different fungal species

Table 1. Fungal strains collection used in this study

Fungal species	Fungal strain	Host plant	References
Alternaria sp.	KB2MR	Alfalfa	Ben Alaya *et al.* 2021
Alternaria sp.	SA1MST2.1	Alfalfa	Ben Alaya *et al.* 2021
Botrytis cinerea	S2	Tomato	Chaouachi *et al.* 2021
Botrytis cinerea	S5	Tomato	Chaouachi *et al.* 2021
Colletotrichum gloeosporioides	ManS3Fr02	Lemon	Aouani *et al.* 2022 (Unpublished data)
Colletotrichum gloeosporioides	ThS3Fr02	Lemon	Aouani et al. 2022 (Unpublished data)
Fusarium culmorum	FC2	Durum wheat	Kammoun *et al.* 2009
Fusarium oxysporum	DZ1.1MSR2.1.2	Alfalfa	Ben Alaya *et al.* 2021
Fusarium oxysporum	DZ1.1MSR2.2.1	Alfalfa	Ben Alaya *et al.* 2021
Fusarium solani	FS	-	Ayed *et al.* 2021
Fusarium solani	SB4.15.1	Potato	Khiari *et al.* 2022 (Unpublished data)
Rhizoctonia solani	RS1.2	Potato	Djébali *et al.* 2014
Rhizoctonia solani	RS5.2	Potato	Djébali *et al.* 2014
Rhizoctonia solani	KB2MSC2	Alfalfa	Ben Alaya *et al.* 2021
Rhizoctonia solani	SA1MST3.1	Alfalfa	Ben Alaya *et al.* 2021
Rhizoctonia solani	Collet1.1	Potato	Marzouk *et al.* 2022 (Unpublished
Rhizoctonia solani	Collet1.2	Potato	Marzouk *et al.* 2022 (Unpublished
Phoma medicagins	Pm8	Barrel Medic	Djébali 2013
Verticillium alfalfae	GF1.17	Potato	Khiari et al. 2022 (Unpublished data)
Verticillium alfalfae	GF1.18	Potato	Khiari *et al.* 2022 (Unpublished data)
Verticillium dahliae	je3.10	Potato	Khiari *et al.* 2022 (Unpublished data)

were used in this study (Table. 1).

2.2. Antifungal activity tests of pure volatiles

The antifungal activity of bacterial VOCs was assessed using the double Petri dish method (Chaouachi et al. 2021). A disc of 7 mm in diameter of a 7 days old fungal culture (in the dark at 25 °C) was transplanted onto PDA medium and confronted with a disc of whatman paper soaked with various VOCs volumes in order to have the following concentrations 0, 0.0625, 0.125, 0.3125, 0.625, 1.25, and 2.5 mL/L head space for N-Ethylaniline and 2-Heptanone and the concentrations of 0, 0.03125, 0.0625, 0.125, 0.3125, 0.625, and 1.25 mL/L head space for 3-Methylbutan-1-ol. Both Petri dishes were sealed face to face with cellophane paper to prevent the loss of the VOCs. In the control Petri dishes, the fungal cultures were placed in front of Whatman paper disc without VOCs. The diameter of the fungal colonies was measured at 7 and 15 days of confrontation. The Area under the growth progression curve (AUGPC) of the fungal colony was determined to appreciate the antifungal activity of the studied VOCs and then the lowest effective concentration that inhibit 50% of fungal growth (EC50) for each VOC was calculated.

2.3. *In vivo* Bioprotection test of pure volatiles

Tomato and lemon fruits as well as potato tubers used in this study were selected free of wounds and lesions. They were sterilized by soaking in 3 % bleach for 30 min for the potato tubers and 15 min for the lemon and the tomato fruit, and rinsed 5 times with sterile distilled water and dried on filter paper under a laminar flow hood. A hole of 7mm diameter was made at the level of the epidermis of the fruit or tuber in which a disc of fungal mycelium or a disc of PDA medium (control) was placed. The fruits or tubers were placed in plastic boxes on filter paper soaked with sterile distilled water to maintain sufficient humidity. The volumes of N-Ethylaniline corresponding to EC50 and to double EC50 (2 EC50) were poured onto filter paper discs in the plastic boxes. The plastic boxes were sealed with cellophane to prevent the release of VOCs and incubated at 25 ±2°C in the dark. The degree of fungal infection was estimated by measuring the diameter and depth of the rotting area due to fungal colonization of the tissues at 5 days for tomato, 8 days for lemon and 10 days for potato. A split-plot design was used in this test; each plastic box contains two treatments (non infected and infected fruit or tuber). The test was performed 3 times with two repetitions per treatment.

2.4. Statistical analysis

Linear mixed models were implemented for analyzes of variance using the lmerTest R packages. Multiple means comparisons were performed using Dunnett's or Tukey's at a value of $p \leq 0.05$. All statistical analyzes were performed using Rx64 4.0.2 software.
Hierarchical grouping of fungal strains based on their response to different volatile compounds was calculated based on Pearson correlations and complete linkage. Z-scores were calculated and used to generate the heatmap. The blue color indicates a low EC50 and the dark red color indicates a very high EC50. The dendrogram on the left shows fungal strains grouped into 10 different clusters according to the EC50 of each compound. The graph was obtained using the "heatmap" function of the "complex heatmap" package of the R software.

3. RESULTS

3.1.1. Effect of pure volatile compounds on the *in vitro* growth of fungal strains

The analysis of variance showed that the effect of pure volatile compounds on the mycelia growth of the 21 fungal strains depends on the fungal strain, the nature of VOCs and its concentration and their interactions. The N-Ethylaniline and 3-Methylbutan-1-ol showed a better antifungal activity in comparison to 2-Heptanone. Indeed, even at high concentrations (2.5 ml/L of head space), 2-Heptanone could not inhibit the growth of most fungal strains studied (Fig. 1). The analysis of the AUGPC at different concentrations (Fig. 2, 3 and 4) as well as the calculation of the EC50 showed a specificity of the compounds N-Ethylaniline and 3-Methylbutan-1-ol to the fungal strains. Hierarchical clustering analysis showed the existence of 10 groups of fungi based on the EC50 of each VOC (Fig. 1).

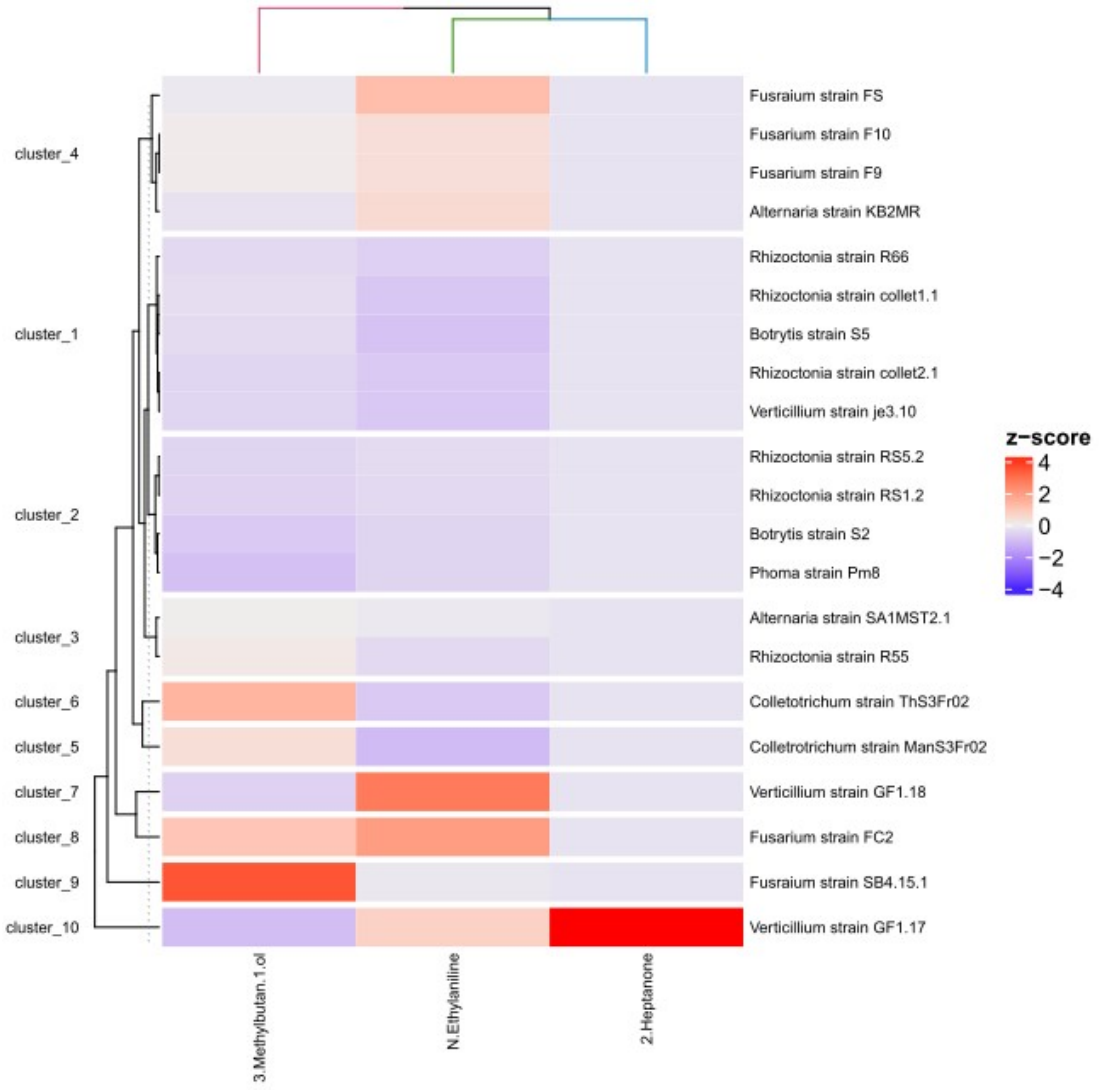

Fig. 1. Heatmap of the minimal effective concentration of VOCs that inhibit 50% of fungal growth (EC50) of phytopathogens.

The N-Ethylaniline has an a board spectrum antifungal activity inhiting the growth of all used fungal strains at different degrees (Fig. 2). It has the lowest EC50 for 10 fungal strains (cluster 1, 3, 5, 6 and 9) (Fig. 1) belonging in particular to the genera *Alternaria* (SA1MST2.1 with EC50 = 0.503 mL/L of head space), *Botrytis* (S5 with EC50 = 0.258 mL/L head space), *Colletotrichum* (ManS3Fr02 and ThS3Fr02 with EC50 equal to 0.206 and 0.294 ml/L head space respectively), *Fusarium* (SB4.15.1 with EC50 = 0.496 mL/L of head space), *Rhizoctonia* (collet1.1, collet1.2, KB2MSC2 and SA1MST3.1 with EC50 equal to 0.283, 0.294, 0.41 and 0.344 mL/L of head space air respectively) and Verticillium (je3.10 with EC50 = 0.288 mL/L of head space).

The 3-Methylbutan-1-ol inhibited the growth of all studied fungal strains at different extent (Fig. 2). It was able to inhibit 9 fungal strains (cluster 2, 4, 7, 8 and 10) (Fig. 1) at concentrations not exceeding 0.625 mL/L of head space. These fungal strains belong to the genera *Alternaria* (KB2MR with EC50 = 0.497 mL/L of head space), *Botrytis* (S2 with EC50 = 0.316 mL/L of head space), *Fusarium* (DZ1.1MSR2.1.2, DZ1.1MSR2.2.1 and FS with EC50 equal to 0.610, 0.610 and 0.545 mL /L of head space respectively), *Rhizoctonia* (RS1.2 and RS5.2 with EC50 equal to 0.393 and 0.402 mL/L of head space respectively), *Phoma* (Pm8 with 0.256 mL/L of head space) and *Verticillium* (GF1.17 and GF1.18 with EC50 equal to 0.239 and 0.378 mL/L of head space, respectively). The 3-Methylbutan-1-ol also inhibited the FC2 strain (cluster 9) (Fig. 1) of *Fusarium* with a lower EC50 (0.946 mL/L of head space) in comparison to N-Ethylaniline.

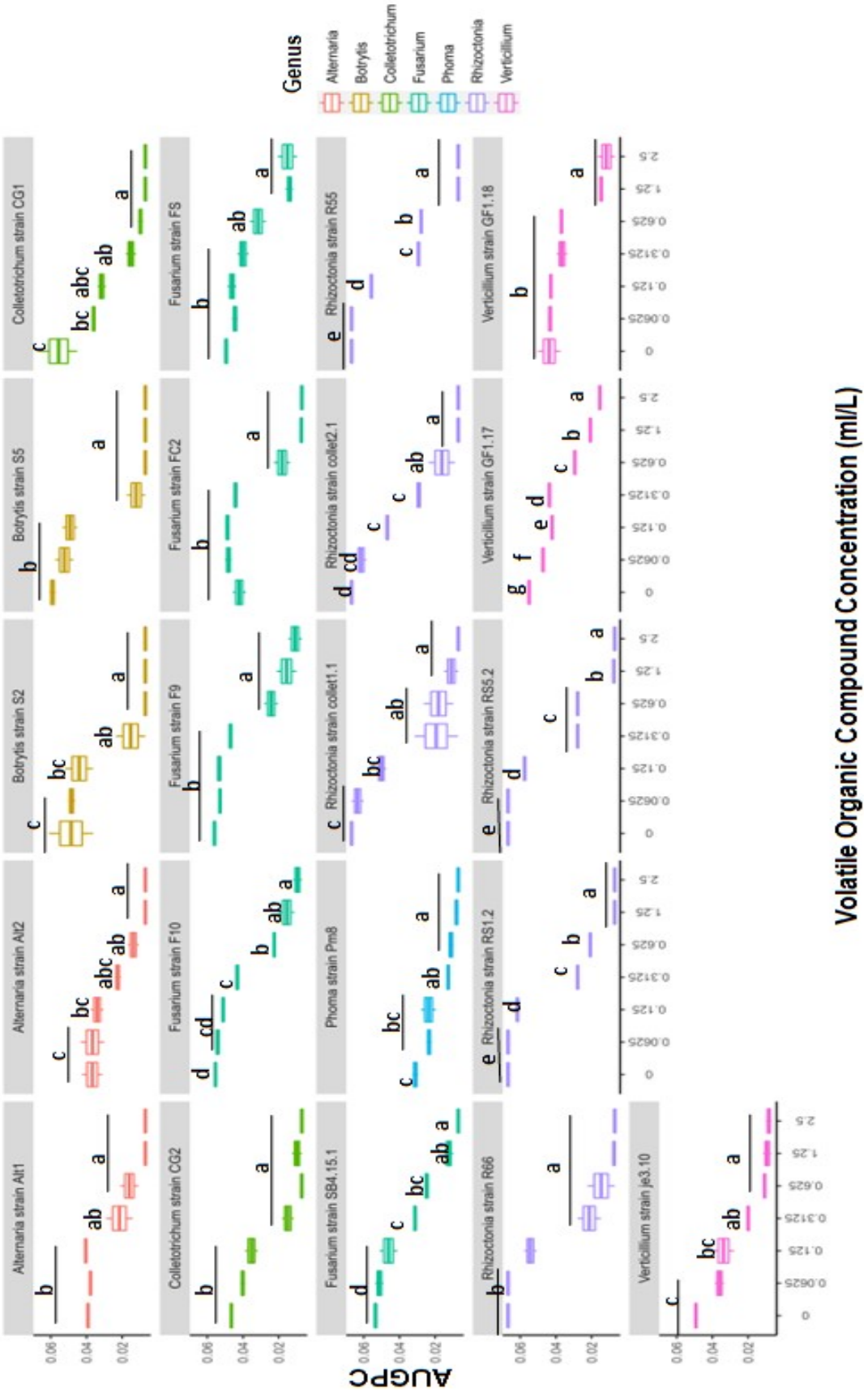

Fig. 2. *In vitro* antifungal activity of N-Ethylaniline against a collection of phytopathogenic fungal strains.

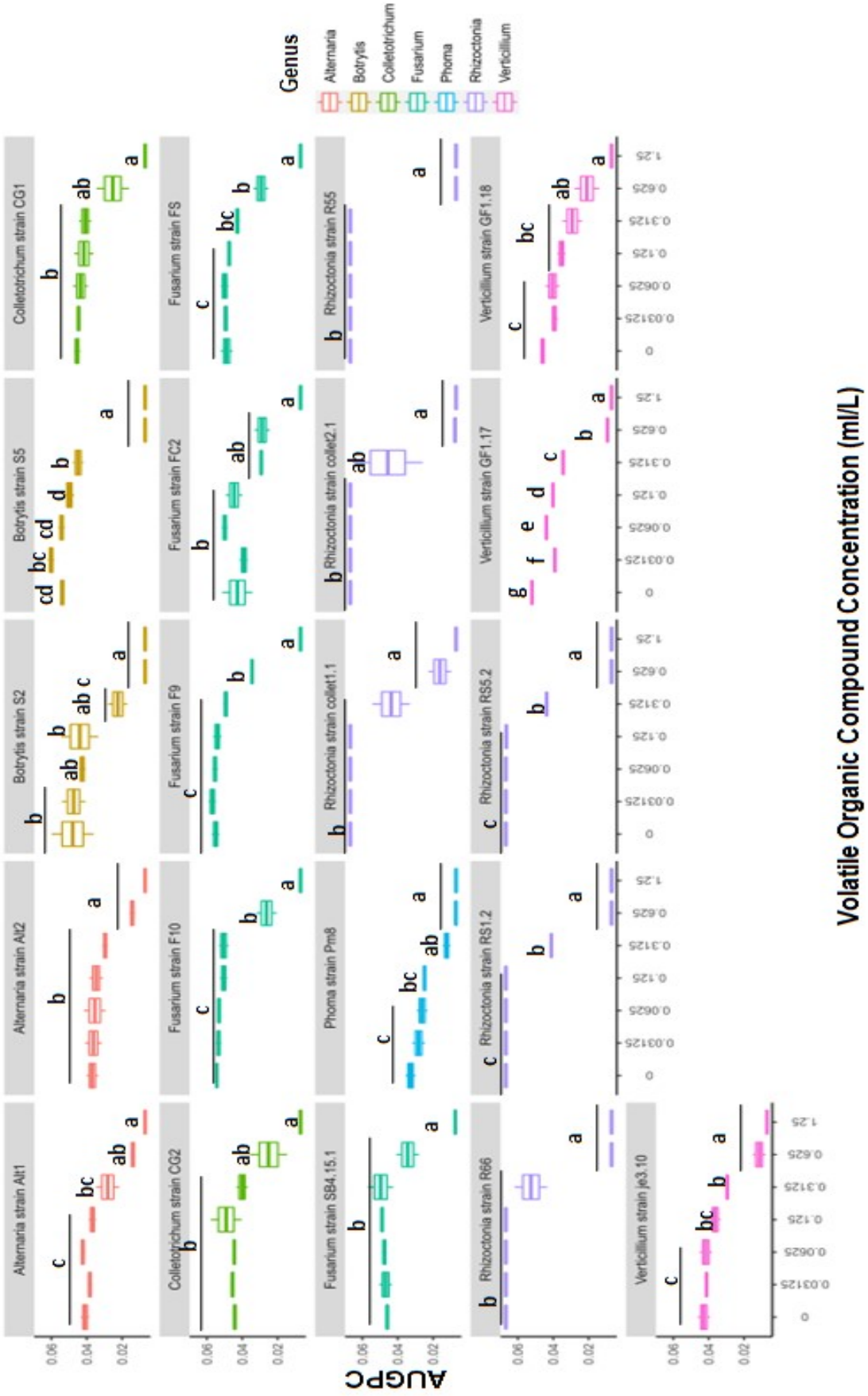

Fig. 3. *In vitro* antifungal activity of 3-Methylbutan-1-ol against a collection of phytopathogenic fungal strains.

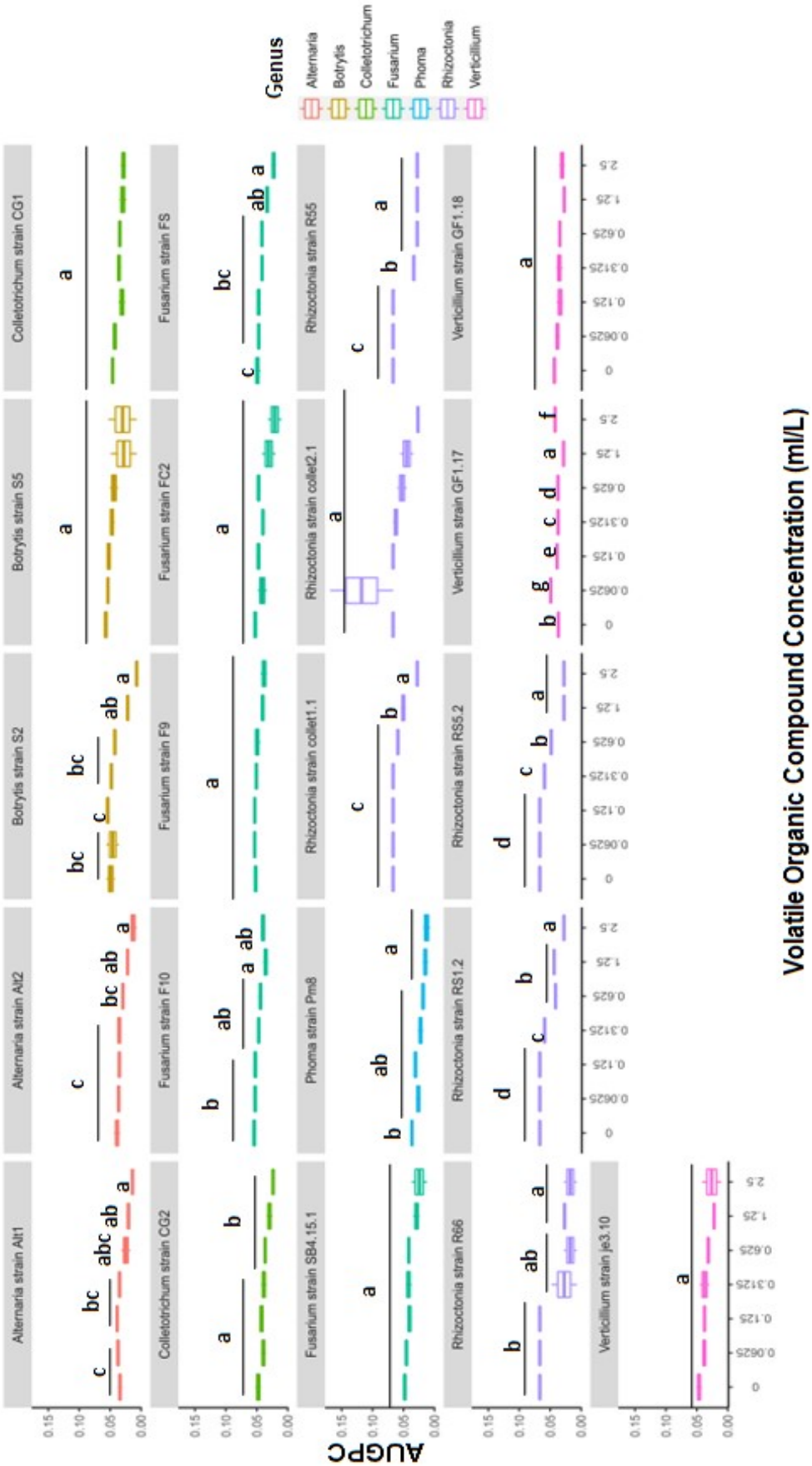

Fig. 4. *In vitro* antifungal activity of 2-Heptanone against a collection of phytopathogenic fungal strains.

3.1.2. Bioprotective effect of N-Ethylaniline against fungal diseases

3.1.2.1. Bioprotective effect of N-Ethylaniline on tomato fruit against *Botrytis cinerea*

The analysis of variance of the diameter and the depth of *B. cinerea* rot on tomato fruits in presence of N-Ethylaniline at 5 days after infection mainly depend on the concentration of this compound. The N-Ethylaniline highly decreased the rot diameter and depth of *B. cinerea* at EC50 and 2EC50 with no visible symptoms of phytotoxicity on tomato fruits were observed (Fig. 5 A, B and C).

3.1.2.2. Bioprotective effect of N-Ethylaniline on lemon fruit against *Colletotrichum gloeosporioides*

The analysis of the variance of the diameter and depth of *Colletotrichum gloeosporioides* rot on lemon fruits in the presence of N-Ethylaniline at 8 days after infection mainly depends on the concentration of this compound. The N-Ethylaniline highly ($P<0.001$) decreased significantly the rotting diameter and the depth of *Colletotrichum gloeosporioides* infcetion at EC50 and 2EC50with no visible symptoms of phytotoxicity on lemon fruits (Fig. 6 A, B and C).

3.1.2.3. Bioprotective effect of N-Ethylaniline on potato against *Fusarium solani*

Analysis of the variance of the diameter and the depth of rotting caused by *Fusarium solani* strain SB4.15.1 on potato tubers as well as the number of buds in the presence of N-Ethylaniline at 10 days post infection depends mainly on the concentration of this compound. The N-Ethylaniline did not significantly affecte the rot diameter of *Fusarium solani* at EC50 and 2 EC50,

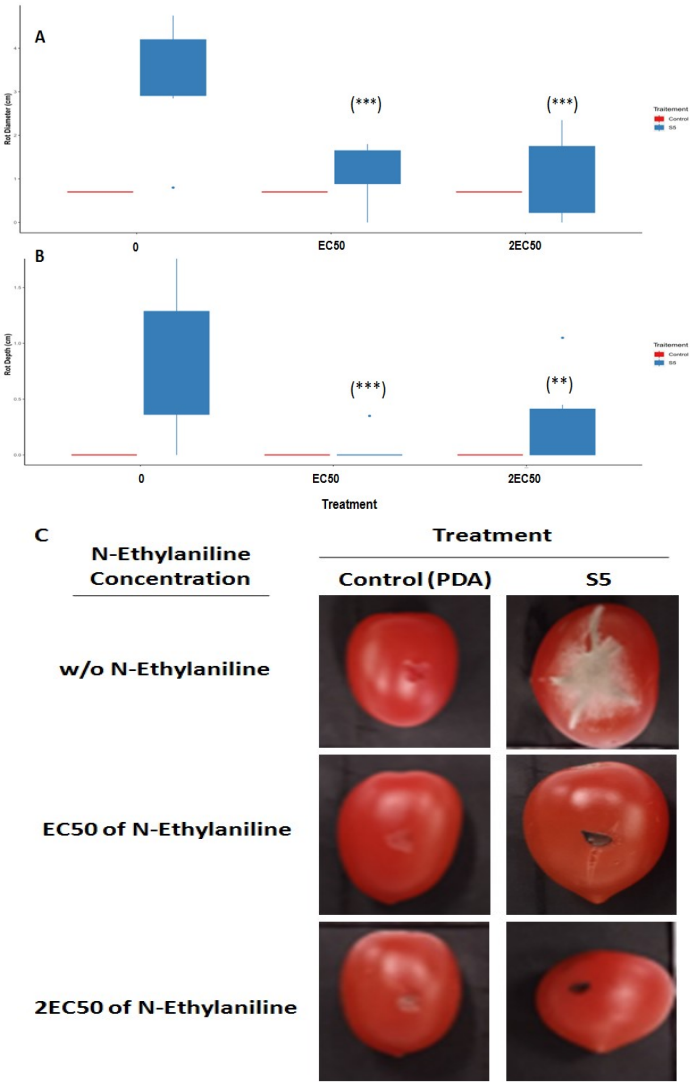

Fig. 5. Effect of N-Ethylaniline on *Botrytis cinerea* infection on tomato fruits. The boxplots represent the diameter (A) and the depth (B) of rot caused by S5 of *Botrytis cinerea* or control condition exposed or not to N-Ethylaniline. (C) Photos of tomato fruits at 5 days after infection with strains of *B. cinerea*

whereas it increased the depth of rot significantly at 2 EC50 (P=0.001) (Fig. 7 A and B). On the other hand, N-Ethylaniline decreased significantly (P<0.001) the number of buds at EC50 and 2EC50 (Fig. 7C).

4. DISCUSSION

According to the FAO reports, global agriculture systems are hampered by the degradation of natural resources and the loss of biodiversity in general and the spread of plant pests and diseases, some of which are becoming resistant to pesticides. Since the end of the last century, researchers have been moving towards the development of safer and more environment friendly control alternatives to fight against plant diseases in the field and after harvest. Promising results of biological control methods have emerged, especially after the successful use of different antagonistic biological control agents (BCAs) from the genera of *Pseudomonas* spp.,

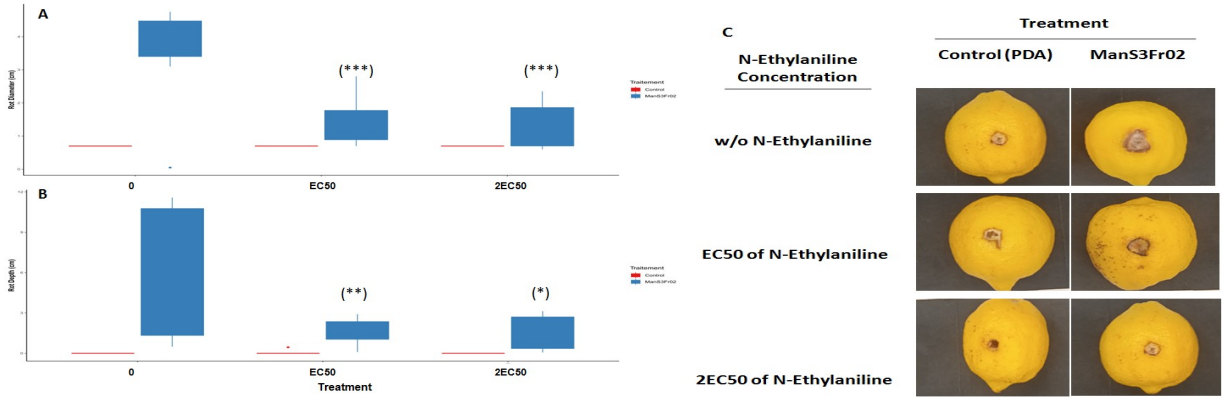

Fig. 6. Effect of N-Ethylaniline on *Colletotrichum gleosporioides* infection on lemon fruit. The boxplots represent the diameter (A) and the depth (B) of rot caused by strain ManS3Fr02 of *Colletotrichum sp.* or control condition exposed or not to N-Ethylaniline. (C) Pictures of lemon fruits at 8 days after infection with *Colletotrichum sp.* and exposed or not to N-Ethylaniline.

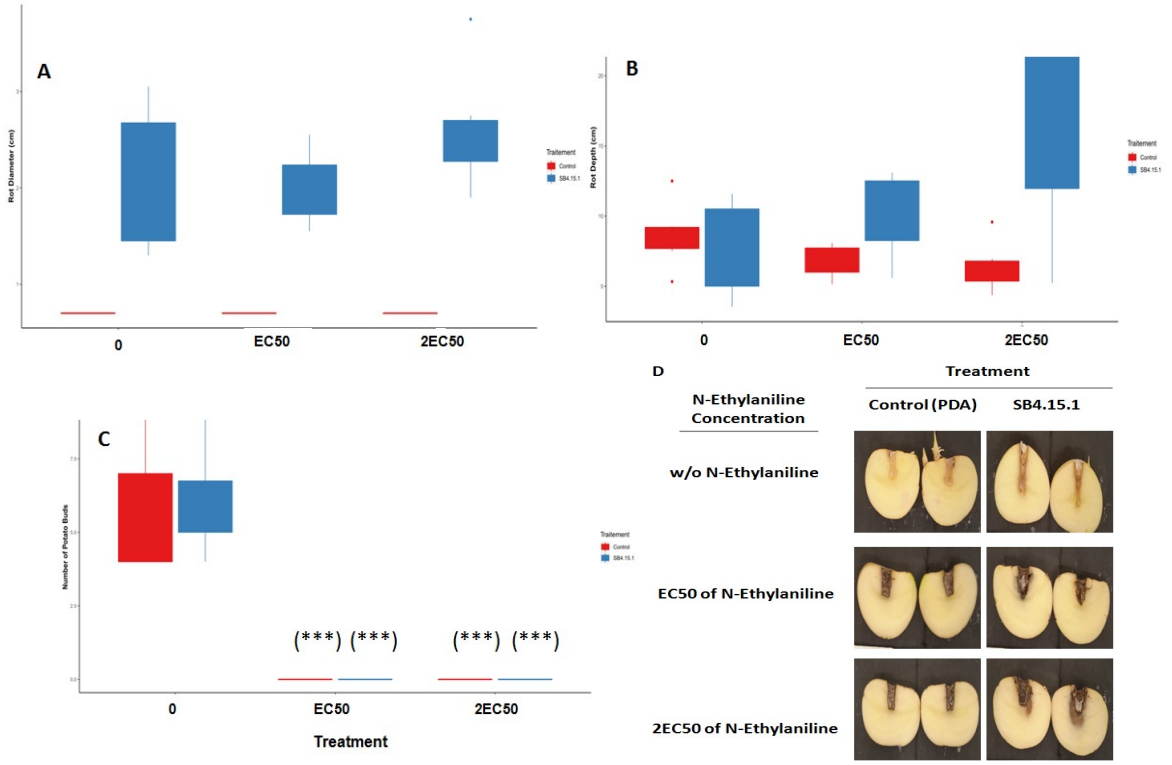

Fig. 7. Effect of N-Ethylaniline on *Fusarium solani* infection on potato tubers. Boxplots represent diameter (A) and depth (B) of rot caused by *Fusarium solani* strain SB4.15.1 or control condition exposed or not to N-Ethylaniline. (C) Effect of N-Ethylaniline on the number of buds that appeared after treatment at 10 days after infection on potato tubers exposed or not to N-Ethylaniline. (D) Pictures of potato tubers at 8 days after infection with *Colletotrichum sp.* and exposed or not to N-Ethylaniline.

Bacillus spp., *Burkholderia spp.* and *Trichoderma spp.* against disease-causing pathogens such as *B. cinerea*, *Colletotrichum* sp. , *Erwinia* spp., *Fusarium* spp., *R. solani*, *Phytophthora* spp., *Pythium* spp. (Compant et al. 2005; Ruiu, 2018). The use of bioactive substances of these biocontrol agents has also shown their effectiveness against plant pathogens such as lipopeptides and lytic enzymes (Ben Slimene et al. 2012; Slimene et al. 2015), as well as volatile organic compounds (Chaouachi et al. 2021; Marzouk et al. 2021). These latter cmpounds have shown an antifungal and bio-protective effect of agricultural products against various phytopathogens such as *B. cinerea* (Calvo et al. 2020), *Fusarium* spp. (Lee et al. 2017) and *R. solani* (Elkahoui et al. 2015). This work showed the ability of VOCs produced by endophytic bacterial strains i.e. N-Ethylaniline, 2-Heptanone and 3-Methylbutan-1-ol to inhibit the growth of several phytopathogenic *in vitro* and *in vivo* on horticulture products of economic interest in Tunisia such as tomato and lemon fruits and potato tubers. The results showed that N-Ethylaniline and 3-Methylbutan-1-ol were the most effective in reducing the *in vitro* growth of 21 fungal strains belonging to *Alternaria sp.* (KB2MR and SA1MST2.1), *B. cinerea* (S2 and S5), *C. gloeosporioides* (MANS3FR02 and THS3FR02), *F.culmorum* (FC2), *F. oxysporum* (DZ1.1MSR2.1.2 and DZ1.1MSR2.2.1), *F. solani* (FS and SB4.15.1) , *R. solani* (collet1.1., collet2.1, RS55, RS66, RS1.2 and RS5.2), *P. medicaginis* (Pm8), *V. alfalfae* (GF17.1 and GF18.1) and *V. dahliae* (je3.10). The Hierarchical grouping of fungal strains based on their response to different volatile compounds showed that these two compounds have specific antifungal strains profiles. In one hand, the 3-Methylbutan-1-ol was able to inhibit the *in vitro* growth of the strains belonging to the genera *Alternaria* (KB2MR), *Botrytis* (S2), *Fusarium* (DZ1.1MSR2.1.2, DZ1.1MSR2.2.1 and FS), *Rhizoctonia* (RS1.2 and RS5 .2), *Phoma* (Pm8) and *Verticillium* (GF1.17 and GF1.18) with an EC50 ranging from 0.239 to 0.610 ml/L of head space. This compound was reported as the most effective compound against *B. cinerea* (Calvo et al. 2020; Di Francesco et al. 2020) and *Penicillium chrysogenum* (Syrokou et al. 2022). In the other hand, the N-Ethylaniline showed the lowest EC50 against *Alternaria* (SA1MST2.1), Botrytis (S5), *Colletotrichum* (MANS3FR02 and THS3FR02), *Fusarium* (SB4.15.1), *Rhizoctonia* (collet1.1, collet1.2, KB2MSC2 and SA1MST3.1) and *Verticillium* (i3.10) ranging from 0.206 to 0.503 ml/L of head space. N-Ethylaniline was described as an antifungal substance for the first time in the study by Marzouk et al. (2021). Our results therefore prove that this compound has a broad spectrum antifungal activity. So based on their antifungal profiles against fungal strains, the combination of these two VOCs may have complementary activities against fungal pathogens.

The *in vivo* test was carried out using the N-Ethylaniline which reduced the infection of gray mould disease caused by *B. cinerea* and the anthracnose of citrus caused by *C. gloeosporioides* at EC50 of 0.258 and 0.206 mL/L of head space respectively without causing any phytotoxic effect on the quality of the fruit. To our knowledge, this is the first report of the in vivo effectiveness of N-Ethylaniline in reducing postharvest diseases of gray mould and anthracnose of tomato and lemon fruit, respectively. In other studies the effect of volatile compounds of *Streptomyces salmonis* PSRDC-09 reduced *C. gloeosporioides* growth and infection which was due to L-linalool production (Boukaew et al. 2021). Guevara-Avendaño et al. (2019) linked the antifungal activity of *Bacillus mycoides* against *C. gloeosporioides* to its production of VOCs such as acetones, pyrazines and sulfur compounds.

Tests on potato tubers showed that N-Ethylaniline increased the depth of *F. solani* rotting, whereas it reduced its in vitro growth. This is can be explained by the oxidation of potato tissues observed in presence of N-Ethylaniline which may probably enhanced the infection of the pathogen. Such tri-partite interaction between the VOCs-plant tissues-pathogen must be studied in deep to better characterize the effectiveness of the VOCs in postharvest disease management. Nevertheless, the fungal growth stimulatory effect of VOCs was previously reported by Briard et al. (2016) showing that the volatiles released by *P. aeruginosa* enhanced the growth of *Aspergillus fumigatus*. Finally, the N-Ethylaniline was able to inhibit bud formation on potato tubers, suggesting its potential use in reducing potato tuber germination for potato destinated for human consumption.

This study showed both the ability of the volatile compounds tested to inhibit the *in vitro* growth of a large number of phytopathogenic fungi infceting horticulture products and in particular the effectiveness of N-Ethylaniline to preserve the quality of tomato and lemon fruits and to reduce rotting symptoms of some postharvest

diseases suggesting its possible use as a biofungicide.

5. CONLUSION

The volatile organic compounds N-Ethylaniline and 3-Methylbutan-1-ol are reported here for the first time to have antifungal activities against various plant pathogens belonging to the genera *Alternaria*, *Botrytis, Colletotrichum*, *Fusarium*, *Rhizoctonia*, *Phoma* and *Verticillium*. The ability of N-Ethylaniline to inhibit infection due to *B. cinerea* on tomato and *C. gloeosporioides* on lemon was confirmed. So, this work provided evidence of the biocontrol capacity of VOCs and their potential use for the preservation of horticultural products in postharvest stage and in particular for N-Ethylaniline suggesting its possible use as a biofumigant.

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