Chemical Identification of Secondary Metabolites from Rhizospheric Actinomycetes Using LC-MS Analysis: In Silico Antifungal Evaluation and Growth-Promoting Effects

Hazem S. Elshafie , Laura De Martino , Carmen Formisano , Lucia Caputo , Vincenzo De Feo , Ippolito Camele *

Posted Date: 7 April 2023

doi: 10.20944/preprints202304.0118.v1

Keywords: antimicrobial activity; natural products; microbial metabolites; plant diseases; biological control

Article

Chemical Identification of Secondary Metabolites from Rhizospheric Actinomycetes Using LC-MS Analysis: In Silico Antifungal Evaluation and Growth-Promoting Effects

Hazem S. Elshafie [1], Laura De Martino [2], Carmen Formisano [3], Lucia Caputo [2], Vincenzo De Feo [2] and Ippolito Camele [1,*]

[1] School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, 85100 Potenza, Italy; hazem.elshafie@unibas.it
[2] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Salerno, Italy; ldemartino@unisa.it (L.D.M.); lcaputo@unisa.it (L.C.); defeo@unisa.it (V.D.F.)
[3] Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, Via Montesano 49, 80131 Naples, Italy; carmen.formisano2@unina.it
* Correspondence: ippolito.camele@unibas.it; Tel.: +39-0971-205544; Fax: +39-0971-205503

Abstract: The rhizosphere is a rich source of actinomycetes which can produce several potential biologically-active secondary metabolites. The principal goals for this research are to extract, purify and characterize the bioactive secondary metabolites produced by three different strains of actinomycetes isolated from the rhizosphere of rosemary, black locust and olive. The plant growth-promoting effect (PGPE) of the studied strains of actinomycetes on *Ocimum basilicum* L. (basil) and disease-control effect on necrotic stem lesions of "black leg" caused by *Fusarium tabacinum* on basil, were *in silico* evaluated. The cell-free culture filtrates from the studied actinomycetes isolates were evaluated *in vitro* for their antimicrobial activity against some common phytopathogens. The secondary metabolites obtained from the cell-free culture filtrates have been chemically characterized using high-resolution electrospray ionization of liquid-chromatography/mass-spectrometric detection (ESI-(HR)Orbitrap-MS). Results of *in silico* trial showed that all studied isolates demonstrated PGPE on basil seedlings improving some *Eco*-physiological characteristics and reduced the disease incidence of *F. tabacinum*. The extracted metabolites from the studied actinomycetes demonstrated antimicrobial activity in Petri-plates assay. The chemical analysis revealed the presence of totally 20 different components. This research emphasizes how valuable the examined isolates are for producing bioactive compounds, indicating their putative antimicrobial activity and their potential employment as fungal-biocontrol agents. In particular, the obtained results revealed the possibility of green-synthesis of some important secondary metabolites such as N-Acetyl-L-histidinol, Rhizocticin A and Eponemycin from actinomycetes. The bioactive metabolites may be successively used to develop novel bio-formulations for both crop protection and/or PGPE.

Keywords: antimicrobial activity; natural products; microbial metabolites; plant diseases; biological control

1. Introduction

Bioactive substances are abundantly produced by soil microorganisms [1]. Due to bacteria's capacity to produce a variety of useful products, such as antibiotics, fungicides, herbicides, hydrolytic enzymes, antitumor, antivirals and immune-suppressants, there is increased interest in employing them for medical and agricultural applications [2–4]. Recently, the pathogen resistance demands to discover novel antimicrobial substances that are effective against serious phytopathogens; so, a high interest to screen new microbes from various environments, with antimicrobial activity, was arised to explore novel potential medications against infections that are resistant to drugs. Among these new isolates are actinomycetes, unicellular filamentous gram-positive bacteria, found throughout nature in a wide range of environments. Actinomycetes are prominent and significant producers of

numerous biological by-products such as antibiotics and plant growth promoting substances [5]. Actinomycetes are very similar to fungi due to their ability to form a mycelium (hyphae), but their hyphae are much smaller than fungal ones [5,6].

Actinobacteria is regarded as one of the major groups of actinomycetes, which includes the genus *Streptomyces* which produces several know antibiotics [4]. According to Berdy [7], the majority of the discovered bioactive substances are produced by genus *Streptomyces*. Girão et al. [8] reported the presence of several bioactive compounds, which account for around 45% of all known microbial bioactive metabolites, have been isolated from actinobacteria from terrestrial sources. Girão et al. [8] investigated the ability of several actinobacteria isolated from *Laminaria ochroleucahe* to control *Candida albicans* and *Staphylococcus aureus*. Therefore, new actinomycetes can be isolated for discovering novel bioactive compounds for agriculture or medicine purposes.

Ocimum basilicum L. (basil) is a culinary herb of *Lamiaceae* family. It is native of tropical zones of central Africa and southeast Asia and can be also found in Mediterranean region [9]. Basil can be infected by several phytopathogens such as *Fusarium* sp. (wilt disease), *Pythium* sp (damping off), *Botrytis* sp. (gray mold), *Colletotrichum* sp. (black-spot) and *Peronospora* sp. (downy mildew) [10]. In particular, *F. tabacinum* W. Gams (Beyma) causes the necrotic stem lesions "black leg" disease on basil [11].

The primary goals of the present research were: i) extraction and the purification of the extracts produced from three studied actinomycetes isolates; ii) chemical characterization of the metabolites of the three isolates using HR-LC-ESI-Orbitrap-MS; iii) *in vitro* evaluation of their antimicrobial activity against some phytopathogenic strains; iv) *in silico* evaluation of the promoting effect of the three actinomycetes strains on *O. basilicum* and controlling "black leg disease" on basil caused by *F. tabacinum*.

2. Results

2.1. Actinomycetes isolation and indentification

The morphological examination using the optical microscope of the three pure-cultures showed the typical like-mycelia structure of actinomycetes characterized by branching and filamentous growth. In particular, the colony of isolate Act1 produced well-developed vegetative radial-hyphae ranged between(1.5 – 2.0 µm Ø, consisting of long-semi-straight filaments bearing colonies arranged vertically in whorls with irregular shape (Figure 1A,B). The isolate Act2 showed a dense radial-hyphae ranged between 1.5 – 2.0 µm Ø and consists of long-semi-straight filaments bearing colonies arranged vertically in whorls with irregular shape (Figure 1C,D). The isolate Act3 showed a radial-hyphae consist of long straight filaments bearing colonies with regular coccoid-shape (Figure 1E,F).

The sequences of the amplified DNA have been carried out in BMR Genomics (Padova, Italy) as previously reported by Elshafie and Camele [12]. BLAST software has been used for comparing the obtained sequences with those available in GenBank. The analysis demonsrated strong similarities percentages with *Streptomyces* sp., *Streptomyces atratus* and *Arthrobacter humicola* present in GenBank. One sequence for each studied isolate was deposited in the NCBI GenBank with the following accession numbers ON241810, ON241816 and ON241806 for *Streptomyces* sp. (Act1), *Streptomyces atratus* (Act2) and *Arthrobacter humicola* (Act3), respectively.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 April 2023 doi:10.20944/preprints202304.0118.v1

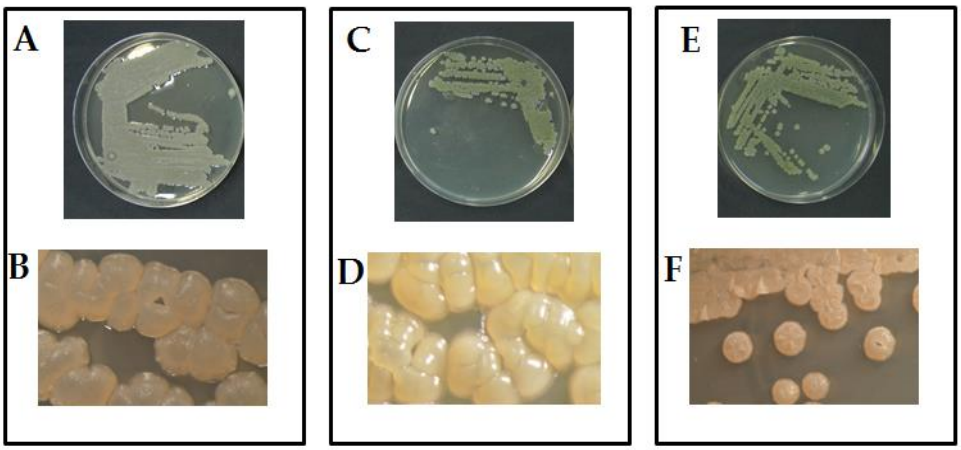

Figure 1. Microscopic-morphological features of the three studied actinomycetes isolates. A,C,E are cultures of *Streptomyces* sp. (Act1), *Streptomyces atratus* (Act2) and *Arthrobacter humicola* (Act3) grown in PY-CA media, respectively. B,D,F are the colonies of *Streptomyces* sp. (Act1), *Streptomyces atratus* (Act2) and *Arthrobacter humicola* (Act3), respectively examined under stereo-Microscope (1000x).

2.2. Growth-promoting and disease-control effects

2.2.1. Eco-physiological characteristics

All bacterized plants with the actinomycetes isolates resulted able to promote the growth of basil seedlings and showed higher values of *Eco*-physiological characteristics compared to the negative control (non-bacterized plants) (Table 1). In particular, seedlings inoculated with Act1 and Act3 showed the higher significant values of NL, SL and TDwS (Table 1). In addition, Act1 and Act2 showed the higher significant values regarding TFwS (Table 1).

On the other hand, the Table 2 reports the *Eco*-physiological characteristics of bacterized basil infected with *F. tabacinum.* In particular, seedlings inoculated with Act1 and Act3 demonstrated higher significant values of NT, SL and TFwS (Table 2). In addition, Act1 showed the higher significant values regarding TDwS (Table 1). Furthermore, Act2 *showed* a moderate PGPE on basil seedlings especially NT, SL and TDwS (Table 2).

Table 1. Eco-physiological characteristics of basil post-actinomycetes inoculation (healthy plant).

Actinomycetes isolates	***Eco*-Physiological Characteristics**				
	NL (n)	**NT (n)**	**SL (cm)**	**TFwS (g)**	**TDwS (g)**
Control	89±4b	8±2a	27±2b	85±12b	5±2c
Act1: *Streptomyces* sp.	113±12a	4±2b	39±4a	114±15a	23±2a
Act2: *A. humicola*	105±5a	7±1a	41±6a	99±9b	18±6a
Act3: *S. atratus*	85±7b	3±1b	27±2b	111±27a	14±2b

NL: number of leaves; NT: number of twigs; SL: shoot length; TFwS: total fresh weight of shoot; TDwS: total dry weight of shoot. Values followed by different letters in each vertical column are significantly different at $p < 0.05$ according to SPSS software. Data are expressed as mean of 3 replicates ± SDs.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 April 2023 doi:10.20944/preprints202304.0118.v1

Table 2. Eco-physiological characteristics of basil post-actinomycetes inoculation and (infected plants).

Actinomycetes isolates	*Eco*-Physiological Characteristics				
	NL (n)	NT (n)	SL (cm)	TFwS (g)	TDwS (g)
Cont -ve	85±9c	2±1ab	34±4b	75±4c	15±2b
Act1: *Streptomyces* sp.	146±7a	7±1a	45±5a	241±13a	40±4a
Act2: *A. humicola*	111±10b	5±1a	47±3a	246±7a	31±4ab
Act3: *S. atratus*	109±9b	3±1ab	34±4b	191±5b	20±3b

NL: number of leaves; NT: twigs count; SL: stem length; TFwS: total fresh weight of shoot; TDwS: total dry weight of shoot. Values followed by different letters in each column are significantly different at $p < 0.05$ according to SPSS software. Values are mean of 3 replicates ± SDs.

2.2.2. Disease-control effect

The bacterized-plants with *Streptomyces* sp. and *A.* humicola exhibited no foliar or radical symptoms due to *S. sclerotiorum* infection. In fact, the disease indexes of *Streptomyces* sp. and *A. humicola* were 0.3 and 0.2 %, respectively whereas the control effects were 99.2 and 99.5 %, respectively (Table 3). On the other hand, *S. atratus*-bacterized seedlings had a moderate disease index higher than 7 % and control effect higher than 79 % (Figures 2 and 3).

Results showed also that the C+ve (plants only inoculated with *F. tabacinum*) developed leaf chlorosis 20 DPI and later turns necrotic. In addition, a high percentage of leaf wilt and root necrosis were also observed 35 DPI. In particular, a significant high percentage of symptomatic leaves was observed in the case of seedlings infected with *F. tabacinum*, where the disease-index was higher than 36 % compared to C-ve (plants not inoculated with *F. tabacinum* or actinomycetes isolates) and plants bacterized with actinomycetes isolates (Figure 2, Table 3). *F. tabacinum* was always re-isolated from the infected-plants.

Table 3. Determination of disease-index and control-effect on basil after different applications.

Treatments	Disease-index	Control-effect
	DI %	CE %
Act1: *Streptomyces* sp.	2.3±0.8c	96.9±10.6a
Act2: *A. humicola*	0.7±0.1d	99.1±7.6a
Act3: *S. atratus*	8.3±0.9b	89.1±6.4ab
Cont. +ve (F)	76.4±9.5a	0.0±0.0c
Cont. -ve (H_2O)	0.0±0.0e	100.0±0.0a

Where: Cont. +ve (F): control positive (treated plants fungal pathogens); Cont. -ve (H_2O): control negative (healthy plants). DI% = disease-index; CE% = control-effect. Values followed by different letters in each column are significantly different at $p < 0.05$ according to SPSS software. Values are mean of 3 replicates ± SDs.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 April 2023 doi:10.20944/preprints202304.0118.v1

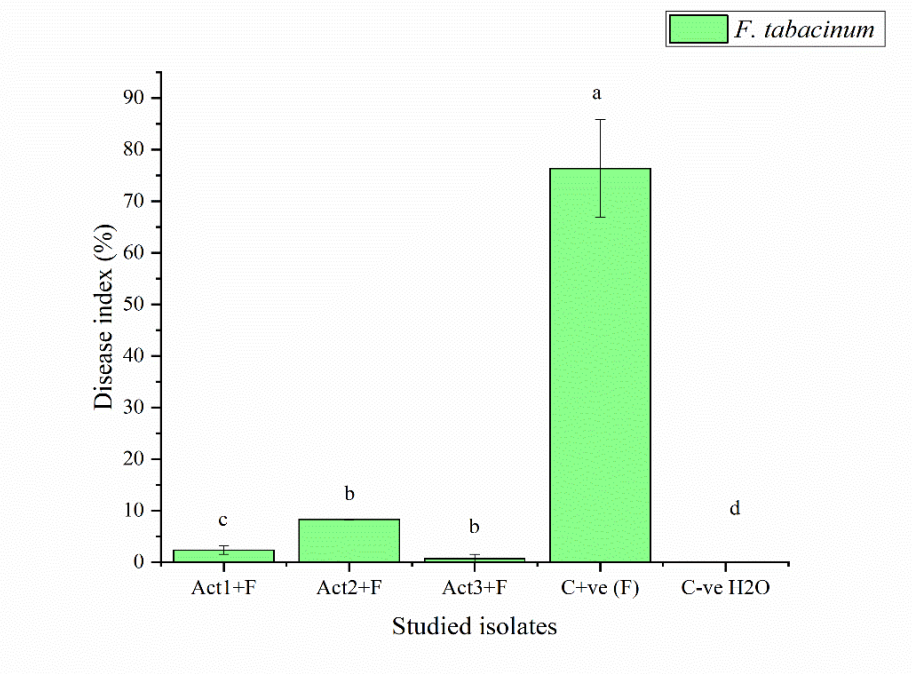

Figure 2. Disease-index of basil inoculated with *F. tabacinum.* Where: Act1+F; Act2+F; Act3+F are inoculated plants with *Streptomyces* sp., *A S. atratus* and *humicola,* respectively. Bars with different letters between different treatments are significantly different at $p < 0.05$ according to SPSS software. values are mean of 3 replicates ± SDs.

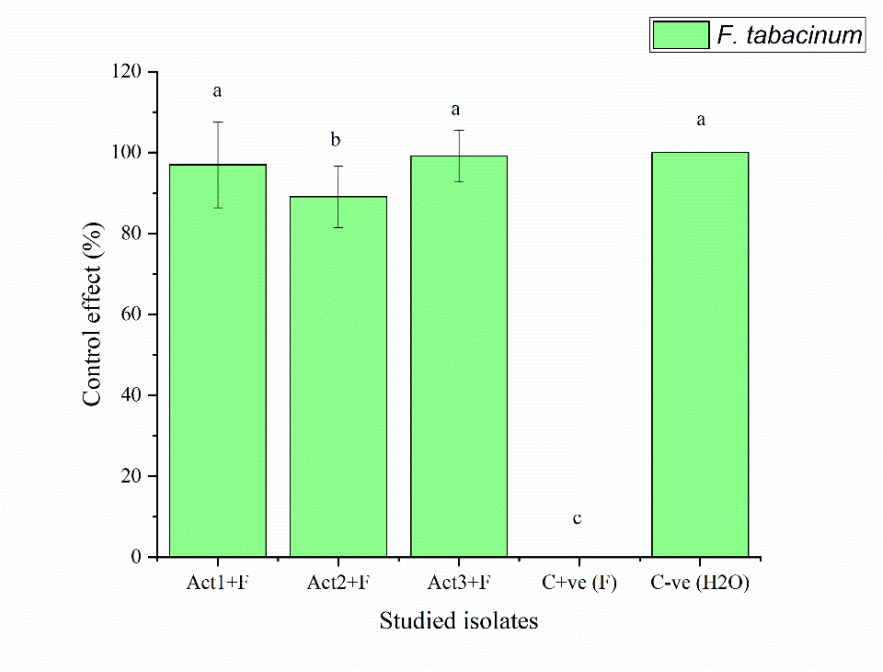

Figure 3. Control effect of basil after infection with pathogen. Bars with different letters between different treatments are significantly different at $p < 0.05$ according to SPSS software. Values are mean of 3 replicates ± SDs.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 April 2023 doi:10.20944/preprints202304.0118.v1

2.3. Antimicrobial assay of metabolites

2.3.1. Antifungal activity

The extracts have been evaluated for their antifungal activity against some food and phytopathogenic fungi following the incorporation method (Figure 4). *A. humicola* showed the highest antifungal effect against all tested fungi. *Streptomyces* sp. showed a moderate antifungal effect higher than *S. atratus* particularly against *M. fructigena*, *R. solani*, *F. oxysporum*, *P. expansum*, *P. digitatum*. On the other hand, *S. atratus* showed a promising effect against *S. sclerotiorum* and *P. commune*.

2.3.2. Antibacterial activity

Regarding the antibacterial activity, the extracts have been evaluated against some food and plant pathogenic bacteria following the disc diffusion method (Figure 5). The highest significant activity of *S. atratus* has been observed against *P. aeruginosa* and *X. campestris* showing BGI% at 88.9 and at 76.7%, respectively. *A. humicola* showed the highest activity against *P. fluorescens*, *X. campestris*, *P. syringae* and *P. aeruginosa* showing BGI% at 58.9, 57.8, 55.6 and 51.1%, respectively. *Streptomyces* sp. showed moderate activity against *P. aeruginosa*, *P. syringae* and *X. campestris* with BGI% at 36.7, 27.8 and 24. %, respectively. In addition, the lowest activity has been observed in case of *S. atratus* against *B. cereus* and *E. coli* with BGI% at 21.1 and 20.0%, respectively. On the other hand, *Streptomyces* sp. resulted not active against *E. coli*, *B. cereus* and *B. megaterium*. In case of *A. humicola* no activity was observed against *E. coli* and *B. megaterium*.

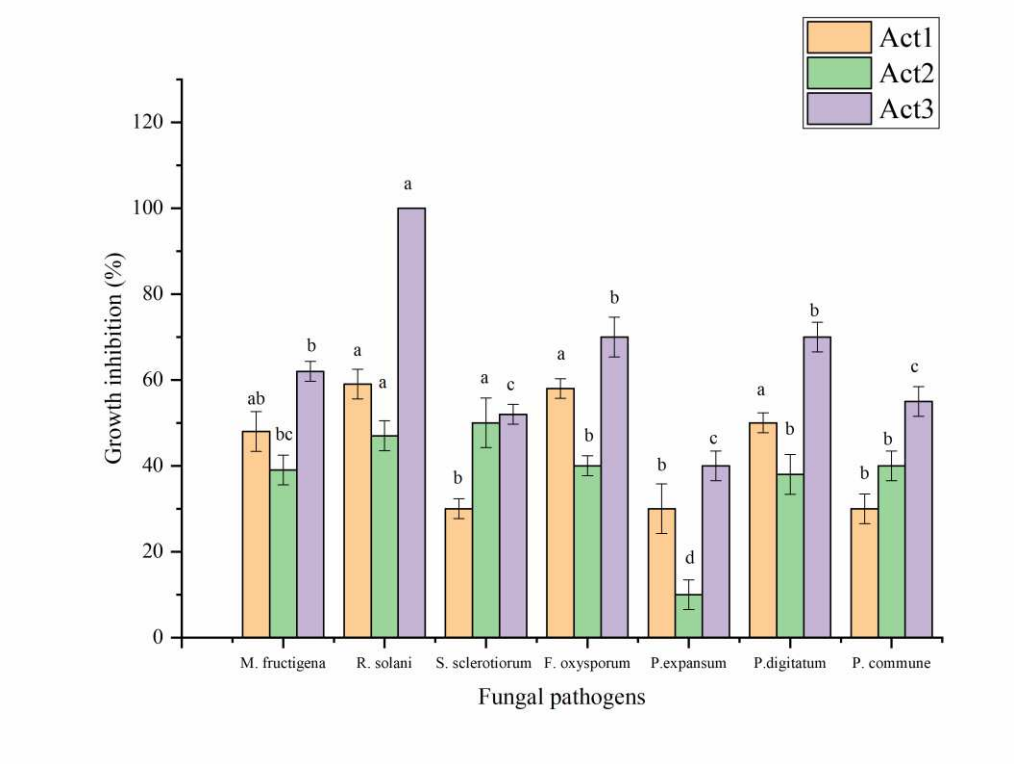

Figure 4. Antifungal activity of extracted metabolites. Bars with different letters between different phytopathogenic fungi for each treatment are significantly different at $p < 0.05$ according to SPSS software. Values are mean of 3 replicates ± SDs.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 April 2023 doi:10.20944/preprints202304.0118.v1

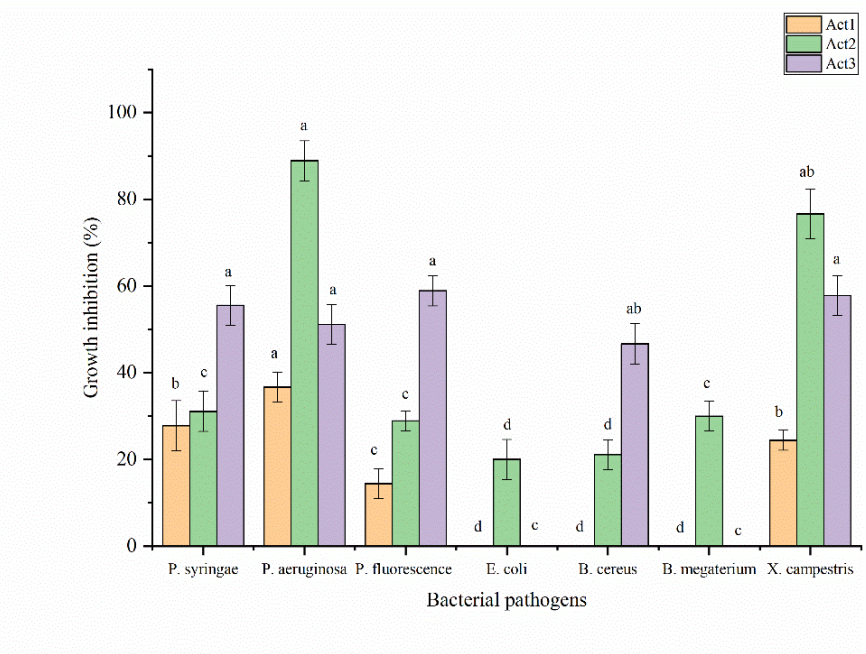

Figure 5. Antibacterial activity of extracted metabolites. Bars with different letters between different phytopathogenic bacteria for each treatment are significantly different at $p < 0.05$ according to SPSS software. Values are mean of 3 replicates ± SDs.

2.4. ESI/(HR)orbitrap/MS metabolic profiles

The chemical analysis of three studied actinomycetes isolates revealed the presence of 20 components, as illustrated in Table 4, (Figures S1–S3). Particularly, compound **1**: N-Acetyl-L-histidinol, identified both in *Streptomyces* sp. (Act1) and *S. atratus* (Act2) extracts, which eluted at a retention time of 0.87 min, showed a peak $[M+H]^+$ ion at m/z 184.1076, corresponding to a protonated molecular ion. From the spectrum, a produced ion at m/z 166 corresponding to a loss of oxydrile group, was also evident. We noted that the compound generating the protonated ion at 184.1076, is a prominent peak in all the extracts and this compound was previously isolated from *Streptomyces coelicolor* [13].

Compound **2:** N,N′-Diacetyl-2-deoxystreptamine, was identified only in *A. humicola* (Act3) extract, has molecular ion $[M+H]^+$ at m/z 247.1283. 2-Deoxystreptamine antibiotics constitute a large group of aminoglycoside antibiotics to which over 20 members have been assigned to date, including the paromomycins, the kanamycins, the gentamicins, the nebramycins, ribostamycin, the lividomycins, validomycin and ambutyrosin [14].

Compound **3**: Indolacatm V, displaying sodiated ion of 324.1659 which is consistent with Indolacatm V, only identified in *Streptomyces* sp. (Act1) extract, with retention time 1.18 min. This compound was previously isolated from *Streptomyces blastmyceticus* showing antagonistic activity toward protein kinase C [15].

Compound **4:** Hexahydro-2*H*-pyrido[1,2-*a*]pyrazin-3(4H)-one, plue protonated ion $[M+H]^+$ (m/z 155.117), detected only in *S. atratus* (Act2) extract. This compound was previously found in a *Streptomyces* extract, as reported by Nithya et al [16].

Compounds **5,6**: Compound **5** corresponds to valyldetoxinine with molecular ion $[M+H]^+$ at m/z 275.159, whereas **6** corresponds to paromomycin, with potassium molecular ion $[M+K]^+$ at m/z 654.265. These two compounds were found only in *A. humicola* (Act3) extract. Valyldetoxinine was a member of detoxin complex, a group of depsipeptide metabolites produced by *Streptomyces caesputosus* var. *detoxus*, present in the soil [17]. The detoxins exhibit antimicrobial activity against some microorganisms. Paromomycin is a naturally occurring aminoglycoside antibiotic, produced by *Streptomyces rimosus* sp. *paromomycinus*, that affects both prokaryotic and eukaryotic through the

chelation with A-site of ribosome [18,19]. Valyldetoxinine and paromomycin hadn't ever been previously reported in *A. humicola*.

Compound **7:** N-[3-[5-(2-methylpropyl)-3,6-dioxo-2-piperazinyl]propyl]-N′-nitro-,(2S-cis)-Guanidine, present in both extracts of *Streptomyces* sp. (Act1) and *S. atratus* (Act2), was eluted at 7.28 min, with a hydrate molecular ion $[M+H_2O]^+$ at m/z 332.1812 [20]. This compound was a cyclic dipeptide (CDP), widely biosynthesized during the cyclo-dipeptide synthsis cycle either within prokaryotic and/or eukaryotic cells [21]. This compound was also found in *Streptomyces* strains [22].

Compound **8**: *n*-Hexyl-β-D-glucoside, detected in *Streptomyces* sp. (Act1) and *S. atratus* (Act2) extracts, with a $[M+H]^+$ ion at m/z 265.1654; it is a substrate of β-glucosidases, hydrolytic enzymes, normally present in *Streptomyces* strains, that cleave β-glycosidic bonds of carbohydrates [23].

Compounds **9-11**: Nonactic acid **(9)** and homononactinic acid **(11)** identified only in *S. atratus* (R3) extract, with $[M+H]^+$ ion at m/z 203.1274 and m/z 217.1430 corresponding to protonated molecular ions, respectively. These two compounds have been isolated from *Streptomyces* and assayed against a panel of cancer cell lines as reported by Lu et al. [24].

Compound **10**: (11aS)-1,2,3,11a-Tetrahydro-8-hydroxy-7-methoxy-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one, also known as antibiotic DC81, present in Streptomyces sp. (Act1) and S. atratus (Act2), with m/z 247.1073, previously reported as antitumor and antibiotic compound originated from some actinomycetes [25].

Compounds **12-15**: these compounds were identified only in *Streptomyces* sp (Act1) extract. Compound **12** gave a $[M+H]^+$ ion at m/z 307.1646 attributed to phthoxazolins B, C or D. These compounds were isolated from the culture broth of *Streptomyces* sp., as reported by Shiomi et al. [26], but the limitation of LC-MS is that metabolites are structural isomers which cannot be distinguished. Compound **13** was identified as Chandrananimycin D, based on accurate mass at m/z 301.0811, attributing to a protonated molecular ion $[M+H]^+$: the phenoxazinone chandrananimycin D was firstly characterized and isolated from *Streptomyces griseus*; it also reported for its antiproliferative activity [27]. The compound **14** gave a $[M+H]^+$ ion at m/z 299.0659, attributed to a protonated molecular ion of carboxyexfoliazone. This compound was firstly report from a wild-type of *Streptomyces* strain, as reported by Abdelfattah et al. [28]. Compound **15** eluted at 10.52 min, had a molecular ion [M+H]+ at m/z 283.0705 corresponding to a protonated molecular ion of phencomycin and previously reported by Chatterjee et al. [29].

Compounds **16-18**: these compounds were detected only in *A. humicola* (Act3) extract, giving $[M+H]^+$ ion at m/z 288.2889 and $[M+H]^+$ ion at 346.2220, respectively. Particularly, compound **16** was attributed to a protonated molecular ion of 2-amino-3-hydroxyhexadecanoic acid, and has been already reported previously in *Arthrobacter* genus [30]. Compound **18** was attributed to a protonated molecular ion of Maoxianamides A or B. These two compounds were isolated from *Streptomyces maoxianensis*, as reported by Li et al., [31]. Compound **17**: 1,1-Dimethylethyl 2-[2-(ethoxycarbonyl)-1-cyclopenten-1-yl]diazenecarboxylate, found only in *S. atratus* (Act2) extract, gave a $[M+H]^+$ ion at m/z 269.14923.

Compound **19:** this compound was attributed to a protonated molecular ion Rhizocticin A, was identified both in *Streptomyces* sp. (Act1) and *S. atratus* (Act2) extracts, gave a $[M+H]^+$ ion at m/z 352.30490. This compound is a natural phosphonate antibiotic produced by the bacterial strain *Bacillus subtilis* [32] and it is very similar to plumbemycin, isolated from *Streptomyces plumbeus,* for amino acid (Z)-2-amino-5-phosphono-3-pentenoic acid, present in both antibiotics [33].

Compound **20**: this compound was attributed to a protonated molecular ion of Eponemycin, was identified in all three studied isolates, gave a $[M+H]^+$ ion at m/z 399.24997. This compound was reported to be produced by *Streptomyces hygroscopicus* and showed potent growth inhibition against various tumor cells [34]. Recently, Fitri et al. [35] reported that this compound is a potential candidate for a new antimalarial drug due to its efficacy against *Plasmodium berghei*.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 April 2023 doi:10.20944/preprints202304.0118.v1

Table 4. Chromatographic analysis of extracted secondary metabolites from studied actinomycetes isolates.

No.	Retention time (min)	Measured m/z	Molecular formula	Identification	Act1	Act2	Act3
1	0.87	184.1075	$C_8H_{13}N_3O_2$	N-Acetyl-L-histidinol	X	X	
2	1.17	247.12834	$C_{10}H_{18}N_2O_5$	N,N′-Diacetyl-2-deoxystreptamine			X
3	1.18	324.16592	$C_{17}H_{23}N_3O$	Indolactam V	X		
4	1.19	155.11748	$C_8H_{14}N_2O$	Hexahydro-2*H*-pyrido[1,2-*a*]pyrazin-3(4*H*)-one		X	
5	2.30	275.15955	$C_{12}H_{22}N_2O_5$	Valyldetoxinine			X
6	7.11	654.2653	$C_{23}H_{45}N_5O_{14}$	Paromomycin			X
7	7.28	332.18118	$C_{12}H_{22}N_6O_4$	Guanidine, N-[3-[5-(2-methylpropyl)-3,6-dioxo-2-piperazinyl]propyl]-N′-nitro-, (2S-cis)	X	X	
8	7.73	265.16544	$C_{12}H_{24}O_6$	*n*-Hexyl-β-D-glucoside	X	X	
9	7.88	203.12737	$C_{10}H_{18}O_4$	Nonactinic acid		X	
10	8.47	247.10730	$C_{13}H_{14}N_2O_3$	(11a*S*)-1,2,3,11a-Tetrahydro-8-hydroxy-7-methoxy-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one	X	X	
11	8.61	217.14296	$C_{11}H_{20}O_4$	Homononactinic acid		X	
12	8.81	307.16461	$C_{16}H_{22}N_2O_4$	phthoxazolins B, C and D	X		
13	9.43	301.08112	$C_{15}H_{12}N_2O_5$	Chandrananimycin D	X		
14	9.75	299.06592	$C_{15}H_{10}N_2O_5$	Carboxyexfoliazone	X		
15	10.52	283.07053	$C_{15}H_{10}O_4N_2$	Phencomycin	X		
16	11.32	288.2889	$C_{16}H_{33}NO_3$	2-Amino-3-hydroxyhexadecanoic acid			X
17	11.45	269.14923	$C_{13}H_{20}N_2O_4$	1,1-Dimethylethyl 2-[2-(ethoxycarbonyl)-1-cyclopenten-1-yl]diazenecarboxylate		X	
18	12.36	346.2220	$C_{17}H_{31}O_6N$	Maoxianamide A or B			X
19	12.20	352.30490	$C_{11}H_{22}N_5O_6P$	Rhizocticin A	X	X	
20	14.09	399.24997	$C_{20}H_{34}N_2O_6$	Eponemycin	X	X	X

 doi:10.20944/preprints202304.0118.v1

3. Discussion

The obtained results are promising for biocontrol of *F. tabacinum* where the treatments with actinomycetes isolates showed a high reduction of disease symptoms on *O. basilicum* seedlings, demonstrating the control effect exerted by *Streptomyces* sp. and *Arthrobacter humicola* against *F. tabacinum*. Furthermore, the treatment with the three studied isolates of actinomycetes may induce also the resistance effect against *F. tabacinum*. In addition, the obtained results of the current study are in agree with the results reported by Elshafie and Camele [12], where the same studied strains showed the capacity to stimulate the development of *S. lycopersicum* and reduced the disease incidence of *S. sclerotiorum*. The results agree with several research reporting that many actinomycetes from the soil can inhibit some harmful phytopathogenic fungi [36–38].

The PGPE of the studied isolates showed also significant influences on the *Eco*-physiological characteristics of basil plants which may be due to synthesis of phytohormones such as gibberellic acid, Indole 3-acetic acid and zeatine (Z) produced by symbiotic and saprophytic actinomycetes [39].

Chaudhary et al. [1] investigated the antagonistic behavior of some actinomycetes isolates obtained from various niche environments in India, and observed the bioactivity of some of studied isolates against *Bacillus cereus*, *Shigella dysenteriae* and Methicillin-resistant *Staphylococcus*. The same authors also noted that, none of the studied isolates were able to stop mycelium from growing inside cells, however were all able to stop the extracellular growth of filaments from the examined bacteria [1].

Our results are in agreement with Odumosu et al. [40], who reported that various *Streptomyces* species demonstrated efficient antibacterial activity against a variety of human and food-borne diseases including *Staphylococcus*, *Escherichia coli* and *Salmonella*. The same authors used GC-MS to examine the secondary metabolites generated by the species under study. The analysis which revealed the presence of some bioactive substances with antibiotic properties, therefore a potential source of novel antibiotics. On the other hand, the bioactive secondary metabolites produced by *Streptomyces* strains with antifungal activity, such as isoikarugamycin, produced by *Streptomyces zhaozhouensis* against *C. albicans*, may be in charge of the antibacterial activity of these microorganisms [41].

The chemical analysis of the bioactive extracts revealed the presence of several components, including phenoxazinones and detoxin complex, to which the bioactivities could have In particular, chandrananimycin D, was reported in literature [42] for its antimicrobial activity. Valyldetoxinine, a member of detoxin complex, was distinguished by its remarkable detoxifying effect against the antibiotic blasticidin S, both in animal and plant cells [43]. Paromomycin, an antibiotic depsipeptide, can be used as biocontrol agent to suppress the soilborne diseases and as plant protection agent, in particular against *Pectobacterium carotovorum*, the responsible for bacterial soft rot producing pectolytic enzymes that hydrolyze pectin between individual plant cells, and against *Phytophthora capsici*, the responsible for blight and fruit rot of peppers and other important commercial crops [44].

Jizba et al. [45] reported stimulatory effects of nonactic and homonanctinic acids on *Cucumis sativus* growth: the substances were also considered later as pesticidal metabolites by Jizba and Skibova [46]. Li et al. [31] reported a moderate antifungal activity of maoxianamide A and B against *S. sclerotiorum*, the fungal phytopathogen responsible for white mold, ubiquitous and highly destructive disease. Also, rhizocticin A was reported for its effect on inhibition of *Rhizoctonia solani*, the fungal pathogen causing brown patch [47].

4. Materials and Methods

4.1. Actinomycetes isolation and indentification

The three studied actinomycetes used in this study, have been isolated from the rhizosphere of black locust (*Robinia pseudoacacia* L.) (Act1), rosemary (*Rosmarinus officinalis* L.) (Act2) and olive (*Olea europaea* L.) (Act3) at Potenza city (Basilicata region, Sothern Italy), following the Membrane Filter Technique [48]. The morphological identification of the three actinomycetes isolates has been carried out previously based on their microscopic features using light microscope (Axioskop—ZEISS,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 April 2023 doi:10.20944/preprints202304.0118.v1

Oberkochen, Germany) and also by molecular methods-based PCR techniques as reported by Elshafie and Camele [12]. The isolates have been sub-cultured and conserved in peptone yeast calcium agar (PY-CA) nutrient media contains (g/L) peptone 5, yeast extract 3 and calcium chloride 0.7.

4.2. Plant growth-promoting & disease-control effects

A greenhouse-trial was undertaken out to evaluate the PGPE of the three isolates for basil as well as their disease-control effect (DC) against the necrotic stem lesions "black leg" caused by *F. tabacinum*. Basil seeds were surface sterilized with ethanol (70%), rinsed three times with sterile distilled water and then have been sowed in polystyrene seed-trays. The greenhouse's temperature and relative humidity were adjusted 24±2°C and 60-70 %, respectively, during the entire experiment.

Regarding actinomycetes treatment, a suspension 106 CFU.mL-1 of each isolate obtained from 5 days-fresh PY-Ca culture and inoculated into Minimal Mineral (MM) media [10.5 dipotassium phosphate, 4.5 potassium dihydrogen phosphate, 1.0 Ammonium sulfate, 0.5 trisodium Citrate Dihydrate, 0.2 Magnesium sulfate, 5.0 dextrose (g/L), pH 7] and left in Rotary-*Incubator* for 8 days at 180 rpm under constant temperature at 28°C. Broth culture (100 ml/pot) were poured into the basil-rhizosphere, 15 Days Post-Seed germination (DPSg).

For artificial fungal-infection, a conidial-suspension (10^8 spore/mL) of *F. tabacinum* was inoculated in potato dextrose broth (PDB) flask and incubated in agitation (180 rpm) for 7 days at 22 °C. Fifty mL broth was inoculated into the basil-rhizosphere 10 days after actinomycetes treatment. Twenty seedlings for each experiment i) untreated health; ii) treated only with actinomycetes; and iii) treated only with fungi, have been used.

At the end of the trial, plant growth was examined for the *Eco*-physiological characteristics 40 DPSg, following the method explained by Elshafie et al [49], going to measure stem length (SL), leaf number (NL), twigs number (NT), total shoot fresh-weight (TFwS) and total shoot dry-weight (TDwS). The disease incidence was monitored daily for 15 days post-infection (DPI), using the following scale: 0= less than 5 % symptomatic leaf; 1= 6 to 20 % of symptomatic leaf; 2= 21 to 50 % of symptomatic leaf; 3= 51 to 80 % of symptomatic leaf; 4 ≥ 80 % of symptomatic leaf [12]. Using Formula 1, the infection proportion (IP%) was calculated, whereas the Formula 2 and 3 were used for evaluating the disease-index (DI%) and control-effect (CE%), respectively [50].

$$\text{IP}\ (\%) = \frac{\text{NSL}}{\text{TLN}} \text{x}\ 100; \quad (1)$$

$$\text{DI}\ (\%) = \left[\Sigma \quad \frac{(\text{Scale value x N. S.L.})}{\text{Hi.S x TL}}\right] \text{x}\ 100; \quad (2)$$

$$\text{CE}\ (\%) = \left[\frac{\text{Di.P} - \text{Di.B}}{\text{Di.P}}\right] \text{x}\ 100; \quad (3)$$

Where: NSL= Number of Symptomatic Leaves; TLN= Total Leaf Number; Hi.S = Highest Scale; DI-P = Disease Index of Pathogen treatment; DI-B = Disease Index of actinomycetes-treated.

4.3. Extraction of metabolites

The secondary metabolites of the three studied strains were obtained following the method of Lavermicocca et al. [51] with little changes as follows: an Erlenmeyer-flask filled with 140 mL of MM broth and seeded with 2.0 mL of each actinomycete suspension at 10^7 CFU/mL and left in *Rotary-Incubator* for 8 days at 180 rpm under constant temperature at 28°C. The broth culture of each isolate was centrifuged at 20,000 *g*/10 min, the precipitate was discarded and the upper-phase was filtered using Millipore 0.22 µM. The purified filtrate was extracted in equal volume of suitable organic solvent (ethyl-acetate) and shacked for 5 min using a separator funnel. The combined organic fractions were concentrated by using an Eevaporator (Heidolf 2000, Germany) at 180 rpm/80°C for 20 min. The dried extracts were resuspended in 1 mL of sterile distilled water [52].

 doi:10.20944/preprints202304.0118.v1

4.4. Microbicidal test

4.4.1. Antifungal

The *in vitro* antifungal activity of the extracted metabolites from the three studied isolates was evaluated against the following phytopathogenic fungi, *Monilinia fructigena*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Penicillium expansum*, *P. digitatum* and *P. commune*, using the incorporation method [53,54]. Ten µL of each extract, at concentration 100 and 50 %, was deposited on a Potato Dextrose Agar (PDA) [55] pre-inoculated with each tested fungal disc. To assess the antifungal activity, the diameter of mycelium grew in Millimetre. The inhibition percentage of fungal growth (FGI %) was calculated following Formula (4) [56]. Cycloheximide was used as positive control (C+ve) at 100 µg/mL.

$$FGI\ (\%) = D.MGt / D.MGc \times 100 \quad (4)$$

where: FGI (%), mycelium inhibition percentage: D.MGt, mean diameter of fungal mycelium in treated Petri dish (mm); D.MGc, mean diameter of mycelium control Petri dish (mm).

4.4.2. Antibacterial assay

The *in vitro* antibacterial activity of the extracts was evaluated against the following pathogenic bacteria, *Pseudomonas syringae*, *P. fluorescence*, *P. aeruginosa*, *E. coli*, *Bacillus cereus*, *B. megaterium* and *Xanthomonas campestris*, using the disc diffusion method [57–59]. King'B (KB) nutrient media was used for reculturing the studied bacterial strains [60]. The bacterial suspensions were prepared in sterile distilled water (SDW) and adjusted at 10^6 CFU/mL. Four mL of each bacterial suspension diluted in soft agar (0.7 %) at 9:1 *v*/*v*, were poured into a KB Petri dish (90 mm). Fifteen µL of each extract, at concentration 100 and 50 %, was deposited on filter discs (6 mm-OXOID) previously placed on plates and left for 30 min under laminar flow. The eventually bactericidal effect was evaluated measuring the diameter of inhibition zone (D.Iz) in Millimetre compared to Tetracycline (1,600 µg/mL), used as C+ve. The bacterial growth inhibition percentage (BGI %) was calculated following Formula (5). All tested treatments were carried out in triplicates ± standard deviations (SDs).

$$BGI\ (\%) = D.Iz / D.Cc \times 100 \quad (5)$$

where: BGI (%) represents the bacterial inhibition percentage; D.Iz, mean diameter of inhibition zone in treated Petri dish (mm); D.Cc, mean diameter of bacterial grown in control Petri dish (mm).

4.5. ESI/(HR)orbitrap/MS metabolic profiles

The qualitative analysis of three studied actinomycetes extracts was performed by High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) using LTQ-XL Ion Trap mass spectrometer equipped with an Ultimate 3000 HPLC. Chromatographic separation was obtained using a Kinetex Polar C_{18} column (100 x 3.0 mm, 100Å, 2.6 µm). The injection volume was 0.5 mL/min and a mobile phase consisting of a combination of A (0.1% formic acid in water, *v*/*v*) and B (0.1% formic acid in acetonitrile MeCN); a linear gradient ranged between 5 to 60% B in 25 min, from 60 to 95 % B in 10 min, and held at 95 % B for 5 min was used. The mass spectrometer was set in positive ion mode. ESI source conditions were the following: capillary voltage −48 V; tube lens voltage −176.47; capillary temperature 280°C; sheath 15 and auxiliary gas flow (N_2) 5, sweep gas 0, spray voltage 5. MS spectra were obtained, at 30 000 resolutions, by full-range acquisition with scan range between 150− 1500 m/z.

5. Conclusions

This research revealed the biological activity of actinomycetes especially *Streptomyces* and *Arthrobacter*. This study highlighted also the value of the new studied strains in terms of their capacity to produce significant bioactive by-products that can be employed as biocontrol agents against several serious fungi, including *Fusarium* species. In conclusion, some metabolites, detected in the extracted of the studied isolates, were recognized as effective against phytopathogenic fungi and bacteria, which may represent the potential for their uses in future sustainable strategies to control

outbreaks in the vast range of crops. In fact, this research demonstrated the possibility of green-synthesis of some important secondary metabolites such as N-Acetyl-L-histidinol, rhizocticin A and Eponemycin from actinomycete. In particular, N-Acetyl-L-histidinol (detected in the current study from *Streptomyces* sp. and *S. atratus*), is considered an important derivative of the primary metabolite L-Histidinol, in agree with Ballio et al [13] who reported that N-acetyl-L-histidinol was produced in cultures of *Streptomyces coelicolor*. Regarding rhizocticin A (detected in the current study from *Streptomyces* sp. and *S. atratus*), is considered a natural phosphonate antibiotic and hydrophilic phosphono-oligopeptides extracted from different biocontrol agents such as *Bacillus subtilis* [47]. Eponemycin (detected in the current study from the three studied isolates) is considered an important antibiotic with specific *in vivo* antitumor effect against B16 melanoma [34]. Nevertheless, these potential bioactive compounds may be used to develop new commercial formulations either as plant-growth promoters or for crop protection.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, Hazem Elshafie and Ippolito Camele; Data curation, Vincenzo De Feo; Formal analysis, Carmen Formisano; Funding acquisition, Ippolito Camele; Investigation, Laura De Martino, Lucia Caputo and Ippolito Camele; Methodology, Hazem Elshafie, Laura De Martino, Carmen Formisano and Lucia Caputo; Supervision, Vincenzo De Feo and Ippolito Camele; Writing – original draft, Hazem Elshafie; Writing – review & editing, Hazem Elshafie, Laura De Martino, Vincenzo De Feo and Ippolito Camele.

Funding: This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chaudhary, H.S.; Yadav, J.; Shrivastava, A.R.; Singh, S.; Singh, A.K., Gopalan, N. Antibacterial activity of actinomycetes isolated from different soil samples of Sheopur (A city of central India). *J. Adv. Pharma. Technol. Res*. **2013**, *4*, 118-23.
2. Vikineswary, S.; Nadaraj, P.; Wong, W.H.; Balabaskaran, S.. Actinomycetes from a tropical mangrove ecosystem – antifungal activity of selected strains. *Asia Pac. J. Mol. Biol. Biotechnol*. **1997**, *5*, 81-86.
3. Jeffrey, L.S.H.; Norzaimawati, A.N.; Rosnah, H. Prescreening of bioactivities from actinomycetes isolated from forest peat soil in Sarawak. *J. Trp. Agric. Fd. Sci*. **2011,** *39*, 245-254.
4. Manivasagan, P.; Kang, K.-H.; Sivakumar, K.; Li-Chan, E.C.; Oh, H.M.; Kim, S.K. Marine actinobacteria: an important source of bioactive natural products. *Environ. Toxicol. Pharmacol*. **2014**, *38*, 172–188.
5. Pepper, I.L.; Gentry, T.J. Chapter 4 – "Earth Environments" in: *Environmental Microbiology (Third Edition)* – **2015** pp. 59-88.
6. Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T.; Williams, S.T. Bergey's manual of determinative bacteriology (9th ed). Williams and Wilkins, Baltimore. **1994**.
7. Berdy, J.. Bioactive microbial metabolites. J. **2005**Antibiotics 58, 1.
8. Girão, M.; Ribeiro, I.; Ribeiro, T.; Azevedo, I.C.; Pereira, F.; Urbatzka, R.; Leão, P.N.; Carvalho, M.F. Actinobacteria Isolated from *Laminaria ochroleuca*: A Source of New Bioactive Compounds. *Front. Microbiol*. **2019**.*10*, 683, 1-13
9. Simon, J.E. Basil. Center for New Crops & Plant Products, Department of Horticulture, Purdue University, West Lafayette, IN. **1998**.
10. Garibaldi, A.; Minuto, A.; Minuto, G.; Gullino, M.L. First Report of Downy Mildew on Basil (*Ocimum basilicum*) in *Italy. Plant Dis*. **2004**.*88*, 312.
11. Matta, A. *Fusarium tabacinum* (Beyma) W. Gams patogeno in natura su basilico e pomodoro. Rivista di Patologia Vegetale, S. **1978,** *IV*, 14, 119-125.

12. Elshafie, H.S.; Camele, I. Rhizospheric actinomycetes revealed antifungal and plant-growth-promoting activities under controlled environment. *Plants 11*, 1872. **2022.**
13. Ballio, A.; Russi S.; Vlasić, D. Isolation and identification of N-acetyl-L-histidinol in cultures of mutants of *Streptomyces coelicolor* requiring histidine. *Annali Dell'istituto Superiore di Sanita*. ***1966***,*2(4*), 523-530.
14. Seligman SJ. Frequency of resistance to kanamycin, tobramycin, netilmicin, and amikacin in gentamicin-resistant gram-negative bacteria. Antimicrob Agents Chemother. **1978,** Jan;13(1):70-3. doi: 10.1128/AAC.13.1.70. PMID: 626492; PMCID: PMC352186.
15. He, F.; Takahiro, M.; Iori, M.; Hitomi, N.; Miroslava, A.; Shotaro, H.; Takayoshi, A.; Ikuro. A. Molecular basis for the P450-catalyzed C-N bond formation in indolactam biosynthesis. *Nature Chem. Biol*. **2019**,*15*, 1206-1213.
16. Nithya, K.; Muthukumar, C.; Biswas, B.; Alharbi, N.S.; Kadaikunn, S.; Khaled, J.M.; Dhanasekaran, D. Desert actinobacteria as a source of bioactive compounds production with a special emphasis on Pyridine-2,5-diacetamide a new pyridine alkaloid produced by *Streptomyces* sp. DA3-7. Microbiol. Res**2018**, *207*, 116–133.
17. Li, W.R.; Han, S.Y.; Joullie, M.M. Total synthesis of (+)-valyldetoxinine and (-)-detoxin D1. *Tetrahedron* **1993**, *49*, 785-802.
18. El-Housseiny, G.S.; Ibrahim, A.A.; Yassien, M.A.; Aboshanab K.M. Production and statistical optimization Paromomycin by *Streptomyces rimosus*
NRRL 2455 in solid state fermentation. BMC Microbiol. **2021**, 21, 34.
19. Cho, R.M.; Kogan, H.V.; Elikan, A.B.; Snow, J.W. Paromomycin Reduces Vairimorpha (Nosema) ceranae Infection in Honey Bees but Perturbs Microbiome Levels and Midgut Cell Function. *Microorganisms* **2022**,*10*, 1107.
20. Furukawa, T.; Akutagawa, T.; Funatani, H.; Uchida, T.; Hotta, Y.; Niwa, M.; Takaya, Y. Cyclic dipeptides exhibit potency for scavenging radicals. **Bioorganic & Med. Chem. 2012**, *20*, 2002-2009.
21. Simon, G.; Berube, C.; Voyer, N.; Grenier, D. Anti-biofilm and anti-adherence properties of novel cyclic dipeptides against oral pathogens. *Bioorganic & Medicinal Chem.* **2019**, *27*, 2323–2331.
22. Tatsuta, K.; Tsuchiya, T.; Umezawa, S.; Naganawa, H.; Umezawa, H. Revised structure for arglecin. *J. Antibiotics* **1972**, *25*, 674-676.
23. Kumar, P.; Ryan, B.; Henehan, G.T.M. b-Glucosidase from Streptomyces griseus: Nanoparticle immobilization and application to alkyl glucoside synthesis. *Protein Exp. Purif.* **2017**, *132*, 164-170.
24. Lu, S.; Hu, J.; Xie, X.; Zhou, R.; Li, F.; Huang, R.; He, J. Secondary Metabolites with Cytotoxic Activities from *Streptomyces* sp. BM-8 Isolated from the Feces of Equusquagga. *Molecules* **2021**, *26*, 24, 7556.
25. Chen, C.Y.; Chen, Y.K.; Wang, J.J.; Hsu, C.C.; Tsai, F.Y.; Sung, P.J.; Lin, H.C.; Chang, L.S.; Hu, W.P. DC-81-enediyne induces apoptosis of human melanoma A375 cells: involvement of the ROS, p38 MAPK, and AP-1 signaling pathways. *Cell Biol. Toxicol.* **2013**, *29*, 85–99.
26. Shiomi, K.; Arai, N.; Shinose, M.; Takahashi, Y.; Yoshida, H.; Iwabuchi, J.; Tanaka, Y.; Omura, S. New antibiotics phthoxazolins B, C and D produced by Streptomyces sp. KO-7888. *J. Antibiot.* **1995**, *48*(7), 714-9.
27. Gomes, P.B.: Nett, M.; Dahse, H.M.; Hertweck, C.P. Structural merger of a phenoxazinone with an epoxyquinone antibiotic. *J. Nat. Prod.* **2010**, *73*, 1461-1464.
28. Abdelfattah, M.S. A new bioactive aminophenoxazinone alkaloid from a marine-derived actinomycete. *Nat. Prod. Res*. **2013**, 27, 2126–2131.
29. Chatterjee, S.; Vijayakumar, E.K.; Franco, C.M.; Maurya, R.; Blumbach, J.; Ganguli, B.N. Phencomycin, a new antibiotic from a Streptomyces species HIL Y-9031725. *J. Antibiot.* **1995**, *8*(11):1353-4.
30. Hidetsugu, N.; Hitoshi, E.; Koji, K.; Okumura, K. Biological method of producing serine and serinol derivatives. U.S. Patent No 3871958A. Washington, DC: U.S. Patent and Trademark Office. **1975**.
31. Li, J.M.; Yan, K.; Zhang, H.; Qi, H.; Zhang, J.; Xiang, W.S.; Wang, J.D.; Wang, X.J. New aliphatic acid amides from *Streptomyces maoxianensis* sp. nov. *J. Antibiotics* **2017**, *70*, 187-189.
32. Gahungu, M. ; Arguelles-Arias, A. ; Fickers, P. ; Zervosen, A. ; Joris, B. ; Damblon, C. ; Luxen, A. Synthesis and biological evaluation of potential threonine synthase inhibitors: Rhizocticin A and Plumbemycin A. *Bioorganic & Med. Chem.* **2013**, *21*, 4958-4967.
33. Fredenhagen, A.; Angst, C.; Peter, H.H. Digestion of rhizocticins to (Z)-L-2-amino-5-phosphono-3-pentenoic acid: revision of the absolute configuration of plumbemycins A and B. *J. Antibiotics* **1995**, *48*, 1043-1045.
34. Sugawara; K.; Hatori M.; Nishiyama, Y.; Tomita, K.; Kamei, H.; Konishi, M., Oki, T. Eponemycin, a new antibiotic active against B16 melanoma. I. Production, isolation, structure and biological activity. *J. Antibiot.* (Tokyo). **1990**, *43*(1):8-18.
35. Fitri, L.E.; Cahyono, A.W.; Nugraha, R.Y.B.; Alkarimah, A.; Ramadhani, N.N.; Laksmi, D.A.; Trianty, L.; Noviyanti, R. Analysis of eponemycin (α'β' epoxyketone) analog compound from Streptomyces hygroscopicus subsp Hygroscopicus extracts and its antiplasmodial activity during Plasmodium berghei infection. *Biomed. Res.* **2017**, *28*, 164-172.

36. Suwan, N.; Wassamon, B.; Sarunya, N. Antifungal activity of soil actinomycetes to control chilli anthracnose caused by *Colletotrichum gloeosporioides*. *J. Agric. Technol*. **2012**, *8*, 725-737.
37. Sacramento, D.R.; Coelho, R.R.R.; Wigg, M.D.; Wigg, M.D., Linhares, L.F.T.; Santos, M.G.M.; Semêdo, L.T.A.; Silva, A.J.R. Antimicrobial and antiviral activities of an actinomycete (*Streptomyces* sp.) isolated from a Brazilian tropical forest soil. *World J. Microbiol. Biotechnol.* **2004**, *20*, 225–229.
38. Prapangdee, B.; Kuekulvong, C.; Mongkolsuk, S. Antifungal Potential of Extracellular Metabolites Produced by *Streptomyces hygroscopicus* against Phytopathogenic Fungi. *Int. J. Biol. Sci.* **2008**, *4*, 330-337.
39. Solans, M.; Vobis, G.; Cassa´n, F.; Luna, V.; Wall, L.G. Production of phytohormones by root-associated saprophytic actinomycetes isolated from the actinorhizal plant *Ochetophila trinervis*. *World J. Microbiol. Biotechnol.* **2011**, *27*, 2195–2202.
40. Odumosu, B.T.; Buraimoh, O.M.; Okeke, C.J.; Ogah, J.O.; Michel, Jr. F.C.; Antimicrobial activities of the *Streptomyces ceolicolor* strain AOBKF977550 isolated from a tropical estuary. *J. Taibah Univer. Sci*. **2017**, *11*, 836–841.
41. Lacret, R., Oves-Costales, D.; Gómez, C.; Díaz, C.; de la Cruz, M.; Pérez-Victoria, I.; Vicente, F.; Genilloud, O.; Reyes, F. New ikarugamycin derivatives with antifungal and antibacterial properties from *Streptomyces zhaozhouensis*. *Mar. Drugs* **2014**, *13*, 128–140.
42. Zhang, X.M.; Liu, X.; Wang, Z.; Tian, Z.H.; Xie W.D. Viridobrunnines A B, antimicrobial phenoxazinone alkaloids from a soil associated *Streptomyces* sp. Heterocycles 2015, 91(9), 1809-1814.
43. Ogita, T.; Seto, H.; Ōtake, N.; Yonehara, H. The Structures of Minor Congeners of the Detoxin Complex. Agricul. Biol. Chem. **1981**, *45*, 2605-2611.
44. Park, J.W.; Lee, S.W., Balaraju, K.; Kim, C.J.; Park, K. Chili - Pepper Protection from Phytophthora capsici and *Pectobacterium carotovorum* SCC1 by Encapsulated Paromomycin Derived from Streptomyces sp. AMG-P1. *J. Pure & Appl. Microbiol.* **2012**, *6*, 1517-1522 .
45. Jizba, J.; Pfukrylova, V.; Ujhelyova, L.; Varkonda, S. Insecticidal properties of nonactic acid and homononactic acid, the precursors of macrotetrolide antibiotics. *Folia Microbiol*. **1992**, *37*, 299-303.
46. Jizba, J.; Skibova, I. Regulation of biosynthesis of pesticidal metabolic complexes in Streptomyces griseus. *Folia Microbiologica* **1994**, *39*, 119-28.
47. Kugler, M.; Loeffler, W.; Rapp, C.; Kern, A.; Jung, G. Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: biological properties. *Arch Microbiol.* **1990**, *153*(3), 276-81.
48. Njenga, W.P., Mwaura, F.B., Wagacha, J.M., Gathuru, E.M., 2017. Methods of Isolating Actinomycetes from the Soils of Menengai Crater in Kenya. Arch Clin Microbiol. 8, 3:45.
49. Elshafie, H.S.; Sakr, S.; Bufo, S.A.; Camele, I. An attempt of biocontrol the tomato-wilt disease caused by *Verticillium dahliae* using *Burkholderia gladioli* pv. *agaricicola* and its bioactive secondary metabolites. *Int. J. Plant Biol.* **2017**, *8*, 57-60.
50. Lee, K.J.; Kamala-Kannan, S.; Sub, H.S.; Seong, C.K.; Lee, G.W. *World J. Microbiol. Biotecnol*. **2008**, *24*, 1139-1145.
51. Lavermicocca, P.; Iacobellis, N.S.; Simmaco, M.; Graniti, A. Biological properties and spectrum of activity of *Pseudomonas syringae* pv. *syringae* toxins. *Physiol. Mol. Plant Pathol.* **1997**, *50*, 129-140.
52. Elshafie, H.S.; Viggiani, L.; Mostafa, M.S.; El-Hashash, M.A.; Bufo, S.A.; Camele, I. Biological activity and chemical identification of ornithine lipid produced by Burkholderia gladioli pv. agaricicola ICMP 11096 using LC-MS and NMR analyses. *J. Biol. Res*. **2017**, *90*, 96-103.
53. Elshafie, H.S.; Amato, M.; De Feo, V.; Camele, I. Chemical composition and antimicrobial activity of Chia (Salvia hispanica l.) Essential oil. *Europ. Food Res. Technol*. **2018**, *244*, 1675–1682.
54. Elshafie, H.S.; Gruľová, D.; Baranová, B.; Caputo, L.; De Martino, L.; Sedlák, V.; Camele, I.; De Feo, V. Antimicrobial activity and chemical composition of essential oil extracted from Solidago canadensis L. growing wild in Slovakia. *Molecules* **2019**, *24*, 1206.
55. Aryal, S. Potato Dextrose Agar (PDA)- Principle, Uses, Composition, Procedure and Colony Characteristics. Microbiol. Info. Com. 2018.
56. Zygadlo, J.A.; Guzman, C.A.; Grosso, N.R. Antifungal properties of the leaf oils of *Tagetes minuta* L. and *Tagetes filifolia* Lag. *J. Essent. Oil Res.* **1994**, *6*, 617–621.
57. Bhunia, A.; Johnson, M.C., Ray, B. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by Pediococcus acidilactici. *J. Appl. Bacteriol.* **1988**, *65*, 261–268.
58. Camele, I.; Elshafie, H.S.; Caputo, L.; Sakr, S.H.; De Feo V. *Bacillus mojavensis*: Biofilm formation and biochemical investigation of its bioactive metabolites. *J. Biol. Res.* **2019**, *92*, 39-45.
59. Gruľová, D.; Caputo, L.; Elshafie, H.S.; Baranová, B.; De Martino, L.; Sedlák, V.; Camele, I. De Feo, V. Thymol chemotype *Origanum vulgare* L. essential oil as a potential selective bio-based herbicide on monocot plant species. *Molecules* **2020**, 25(3), 595.
60. King, E.O.; Ward, M.K.; Raney, D.E. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **1954**, *44*, 301–307.