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Bioactive secondary metabolites extracted from the plant growth promoting bacteria *Paraburkholderiatropica*

Donia S. Helal, Hussein El-khawas, Tarek R. Elsayed*

Department of Agricultural Microbiology, Faculty of Agriculture, Cairo University,12613 Giza, Egypt.

Abstract

Biological control agents and plant growth promoting rhizobacteria (PGPR) are widely applied as a substitute to chemical pesticides and fertilizers. In the current study, we characterized the plant growth promoting and biological control agent *Paraburkholderiatropica* bioactive secondary metabolites. Furthermore, we evaluated the *in vitro* plant growth promoting related functions and biological control activities against *Ralstonia solanacearum* and *Fusarium oxysporum*. Gas chromatography-mass spectrometry (GC-MS) analysis of the ethyl acetate extracts of bacterial culture supernatant proposed the potential production of Hexadecenoic acid, cyclohexanone, tetradecanol, and octadecane which are considered antimicrobial compounds with activity against plant pathogenic bacteria and fungi and have a positive outcome on plant growth of tomato seedlings. Root colonization ability was determined on tomato plant roots using viable bacteria counts of rifampicin resistant mutations generated for *Paraburkholderiatropica* isolate, while the colonization was confirmed and localized via scanning electron microscope (SEM). *Paraburkholderia tropica*isolate tested in this study and its extracted bioactive secondary metabolites can be used as an environmentally friendly substitution of chemical fertilizers and fungicides against fungal phytopathogens.

Keywords: Tomato, PGPR, *Paraburkholderiatropica*, Root colonization, GC-MS, SEM

1. Introduction

Plant Growth Promoting Rhizobacteria (PGPR) and biological control agents are widely applied as a substitute to chemical fertilizers to reduce the negative impact on the agricultural ecosystem [1] and to reduce environmental pollution. *Burkholderia* is considered among the most effective PGPR [2, 3] due to the wide ecological niches it can occupy such as the soil, plants, and animals. *Burkholderia* is the predominant bacteria in soil and is characterized by highly rhizocompetence in the rhizosphere [3, 4]. Consequently, *Paraburkholderia tropic*a (syn *Burkholderia tropica*)is a common rhizosphere colonizer of the most important crops, for instance, maize, rice, sugarcane, wheat, tomato, potato, and others [5, 6]. *P. tropica*is a free-living nitrogen fixing bacteriaisolated from the rhizosphere, rhizoplane, and regularly isolated from diverse surface sterilized plants [7, 8, 9]. Also, it is considered as phosphate solubilizing bacteria via organic acid production [10]. Furthermore, it can promote plant growth and antagonize plant pathogens via the production of a large variety of compounds with potent antifungal activity [11]. In addition, its ability to produce siderophore, indole-acetic acid (IAA), and several antimicrobial substances [12]. *P. tropica*can inhibit the growth of several fungal pathogens such as *Sclerotium rolfsii*, *Fusarium culmorum*, and *Fusarium oxysporum*. *P. tropica* has been known to form a biofilm on both biotic and abiotic surfaces [13]. Moreover, members of the genus *Burkholderia* are among the soil microbial community; however, they are highly affected by soil edaphic factors such as soilpH [14- 17].*Burkholderia* strains can also tolerate soil acidity, so they can persist in acidic soils. In this regard, the major aim of this study is to characterize the plant growth promoting and biological control agent

*Corresponding author e-mail: tarek.ragab@agr.cu.edu.eg

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Paraburkholderiatropica bioactive secondary metabolites and their *in vitro* plantgrowth promoting related functions and biological control activities against *R. solanacearum*and *F. oxysporum*.

2. Materials and methods:

The bacterial culture used in this study was *Paraburkholderiatropica* strain TRP-46 isolated from the rhizosphere of two months old grown tomato plants (*Solanum lycopersicum* cv. super strain-B) [Helal et al. under publication].

2.1. Confirmation of the plant growth promoting and biological control related functions for *Paraburkholderiatropica* isolate

2.1.1 Nitrogen fixation

The ability to fix atmospheric nitrogen was confirmed after several steps of subculturing on CCM semi-solid nitrogen free medium [18]. The CCM medium contained (g/L) Glucose 2.0; malic acid 2.0; mannitol 2.0; sucrose 1; K_2HPO_4 0.4; KH_2PO_4 0.6; Mg_2SO_4 0.2; NaCl 0.1; $MnSO_4$ 0.2; yeast extract 0.2; Biotin 0.5 mg; KOH 1.5; $CaCl_2$ 0.02; $FeCl_3$ 0.015; Na_2MoO_4 0.002; $CuSO_4$ 0.08 mg; $ZnSO_4$ 0.25; sodium lactate (50% v/v) 0.6 ml and agar 3 g. The pH of the medium was 7.0 (±0.2). The development of subsurface growth showed positive results.

2.1.2 Phosphate solubilization

The ability of bacteria to solubilize phosphate was tested usingNational Botanical Research Institute's Phosphate (NBRIP) agar medium [19] contains (g/L) dextrose 10; $Ca_3(PO_4)_2$ 5; $MgCl_2.6H_2O$ 5; $MgSO_4.7H_2O$ 0.25; KCl 0.2; $(NH_4)_2SO_4$ 0.1; and Agar 15. The pH of the medium was 7.0 (±0.2). The development of a clear zone around the bacterial growth after incubation yielded positive results.

2.1.3 Antibacterial activity

The antimicrobial activity was tested according to Xue et al.[20] against *Ralstonia solanacearum* as a representative for bacterial phytopathogens. Nutrient agar medium (NA) [21] was seeded, at 50°C before solidification, with 10% of 24 hr grown *R. solanacearum* culture then mixed and poured into Petri dishes. *R. solanacearum* seeded medium was spot inoculated with each bacterial isolate and incubated at 30°C for 24-48 hrs. Bacterial isolates surrounded by *R. solanacearum* free zones recorded a positive result.

2.1.4 Antifungal activity

The antifungal activity was tested against *Fusarium oxysporum* using a dual culture plate assay. Potato Dextrose Agar medium (PDA) was inoculated by a 6 mm mycelial agar disc of 7 days old fully grown *F. oxysporum* fungi at the center of the plate. A loopful of each of the bacterial isolates from an overnight culture was inoculated by streaking 3 cm away against the fungal mycelia disc. Plates were incubated for 5 days at 25°C and inhibited fungal growth was recorded as a positive result: either as a contact inhibition (C) or an inhibition zone (mm).

2.1.5 Antifungal activity of culture filtrate andethyl acetate extract

Paraburkholderia isolate was grown onto nutrient broth culture for 3 days at 30°C and centrifuged at 5000 rpm for 10 minutes, then the supernatant was sterilized by passing it through a millipore membrane filter (0.45 µm pore size). 250 ml from *Paraburkholderia*culture supernatant was mixed with the same volume of ethyl acetate. The mixture was shaken for one hour at room temperature, then the mixture was transferred to a separatory funnel and allowed to stand until the aqueous phasewas separated from the solvent phase. Ethyl acetate was then evaporated at room temperature and the remaining precipitate was dissolved on dimethylsulfoxide (DMSO) for antifungal activity measurement. Antifungal activity of ethyl acetate extract was detected qualitatively by agar well diffusion assay using 100 µl of the extract, while DMSO alone served as a negative control.

2.1.6 Protease activity

Protease activity was estimated as well, by streaking bacterial isolates onto nutrient agar medium supplemented with skim milk (10%), the formation of clear zones around the bacterial growth after incubation was considered as protease positive isolate.

2.2 Determination of the root colonization ability of *Paraburkholderiatropica*

Tomato seeds (*Lycopersicon esculentum*. cv. super strain-B) were inoculated by soaking for 15 min on *Paraburkholderiatropica* culture suspension and transferred to glass tubes filled with MS (Murashige and Skoog) medium [22] 16 hr light at 28°C. Tomato plants were collected 30 days after inoculation, where three plants were used to count the numbers of rifampicin resistant bacteria. CFU counts were enumerated by plating onto nutrient agar medium supplemented with rifampicin (50 µg/ml). Results were obtained after 48 hrs of incubation at 28°C and related to gram root fresh mass (rfm).

2.3 Scanning Electron Microscopy of tomato root samples inoculated with*Paraburkholderiatropica* isolate

One-centimeter root pieces of the inoculated plants with *Paraburkholderia tropica* were mounted on scanning electron microscopy studs. Furthermore,

samples were coated with a thin layer of gold by ion sputtering [23, 24]. Samples were then examined using a JEOLJSM-5200 scanning electron microscope (Tokyo, Japan).

2.4 Phylogeneticanalysisof*Paraburkholderiatropica* isolate

The evolutionary history of the *Paraburkholderiatropica* isolate was inferred using the Neighbor-Joining method. The phylogenetic tree involved bacterial nucleotide sequences of which 1 sequence of 16S rRNA gene amplified from bacterial isolates used in the current study, while 12 sequences representing the closest hits were obtained from the NCBI GenBank database. The tree was computed using the Maximum Composite Likelihood method, evolutionary analyses were conducted using MEGA version 5 software [25], and the phylogenetic tree architecture was confirmed via bootstrap analysis (1000 replicates) (Fig. 1).

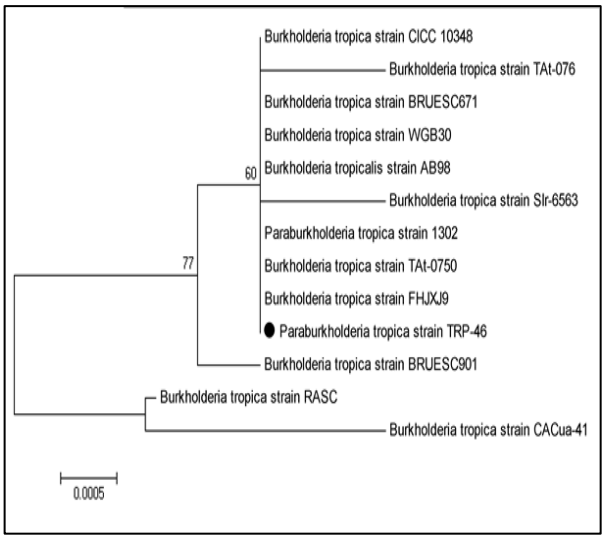


Fig. 1: A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences. Dark circles represent bacterial isolates used in this study; bootstrap values are indicated at each node.

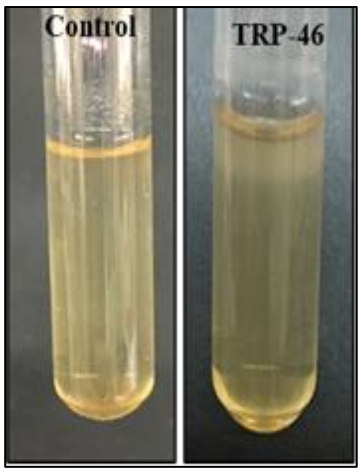
2.5 GC -MS analysis

The chemical composition of *Paraburkholderia tropica* culture supernatant ethyl acetate extract was performed using Trace GC Ultra-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was held at 50°C and then increased by 5°C /min to 180°C withhold 3 min then to 280°C by 10°C /min withhold 5min. The injector temperature was kept at 250°C. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 2 min and diluted samples of 1 µl were injected automatically using Autosampler AS3000 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–650 in full scan mode. The ion source and transfer line temperatures were set at 200 and 250°C respectively. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database.

3. Results and discussion

3.1. Nitrogen fixation

The ability of *Paraburkholderiatropica* TRP-46 to fix atmospheric nitrogen was confirmed after several steps of sub-culturing on a CCM semi-solid nitrogen-freemedium. *Paraburkholderiatropica*TRP-46 isolate was capable of developing subsurface growth and considered as a positive result (Fig. 2), this is in agreement with the previous study of Silva et al. [26] in which they reported that *Paraburkholderiatropica*is a nitrogen-fixing endophytic plant-associated bacterium regularly observed in sugarcane. Moreover, it has been observed that *P. tropica* is efficient in fixing nitrogen and can be detected in different spheres and crops, for instance, the rhizosphere, rhizoplane, stem, and internal tissues of corn, sugarcane, and corn plants [27, 28].



3.2. Phosphate Solubilization

The ability of bacteria to solubilize phosphate was determined using the NBRIP agar medium. *Paraburkholderia tropica* TRP-46 isolate developed a clear zone around the bacterial growth after incubation and showed positive results (Fig. 3). In light of this, numerous studies reported that*Paraburkholderia tropica* shows different *in vitro* abilities involved in plant growth promotion, for instance, phosphate

solubilization via organic acid production [27- 30]. Moreover, Kaur et al. [31] reported that *P. tropica* strain P-31 is a phosphate-solubilizing bacterial strain isolated from the rhizosphere of pomegranate (*Punica granatum*).

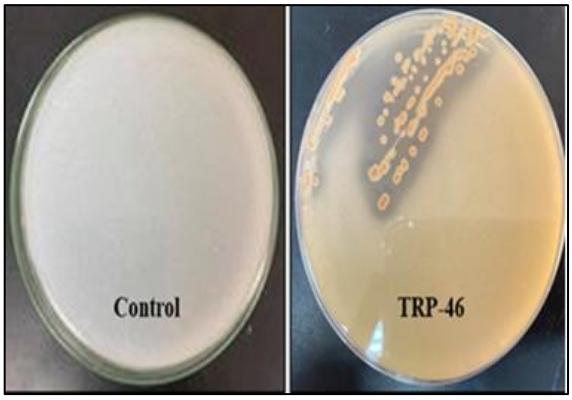


Fig. 3: Phosphate solubilizationactivity ofTRP-46

3.3. Antibacterial activity

The antimicrobial activity was tested against *Ralstonia solanacearum* as a representative for bacterial phytopathogens. *Paraburkholderiatropica* was not able to stop the growth of *R. solanacearum* and was recorded as a negative result. Moreover, no studies have been conducted to investigate this activity in *Paraburkholderia*sp..

3.4. Antifungal activity

The antifungal activity was tested against *Fusarium oxysporum* using a dual culture plate assay. Plates were incubated for 5 days at 25°C, and inhibited fungal growth yielded a positive result with 6 mm as inhibition zone. In this regard, *Burkholderia* species have been reported to form even antagonistic or mutualistic interactions with fungiand can produce a broad range of antifungal compounds that are able to suppress many phytopathogens such as *Rhizoctonia solani*, *Pythium* sp., and *Fusarium* sp. Therefore, it can improve plant vigor [32]. Moreover, several studies described the potentiality of *Burkholderiatorpica*as a biological control agent [27, 29, 33], and its ability to inhibit the growth of diverse phytopathogenic fungi, for instance, *Colletotrichumgloesporioides*,*Sclerotiumrolffsi*, *Fusariumculmorum*, and*Fusariumoxysporum* in maize plants and consequently limit the fungal attack [34]. Also, Barrera-Galicia et al. [35] reported that the *Burkholderia* strainshave the capacity to defeat the growth of *Fusarium* phytopathogens.

3.5.Antifungal activity of*Paraburkholderia tropica* ethyl acetate extract

Antifungal activity of ethyl acetate extract was detected qualitatively by agar well diffusion assay using 100 µl of the extract and it inhibited the fungal growth and was recorded as a positive result. The antagonistic performance of *Burkholderia* species is well known and is reliant on the production of a various range of antifungal compounds [32, 36]. Furthermore, the antagonistic role of *B. tropica* against phytopathogenic fungi can be via thesecondary metabolites and volatile compounds which appear to play an essential role in the inhibition of fungal growth, and therefore, they can protect plants against phytopathogenic fungi [34]. This is in agreement with the previous study of Elshafie and Camele [37] in which they reported that *Burkholderia* sp. reveals a significant source of antibiotics and bioactive secondary metabolites, therefore, they can be applied as biocontrol agents for phytopathogenic fungi.

3.6. Protease activity

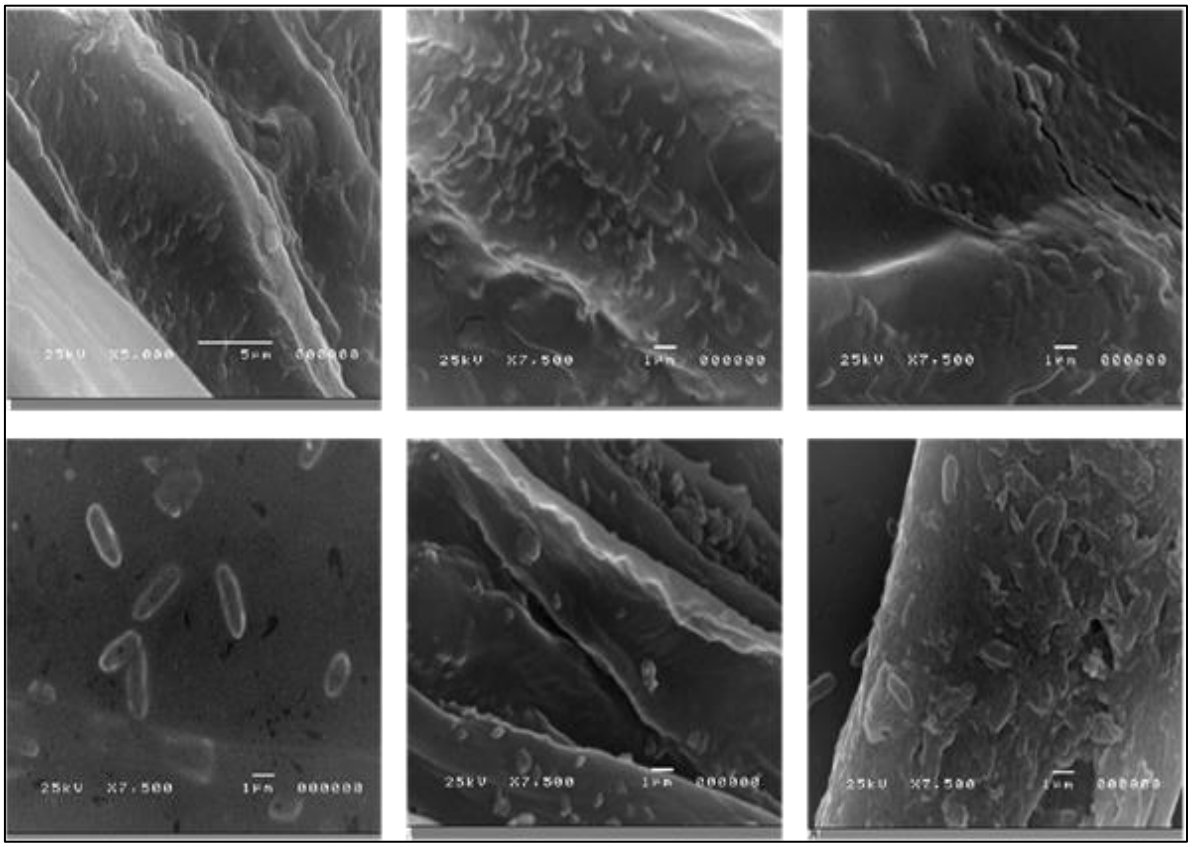
Protease activity was estimated by streaking bacterial isolates onto nutrient agar medium supplemented with skim milk (10%). *Burkholderia tropica* isolate formed clear zones and was considered protease positive.Several studies have described the potentiality of production of different fungal cell wall degrading enzymes such as chitinase, protease,and secondary metabolites which are considered common mechanisms that bacteria use to inhibit fungal pathogens [38- 40]. Additionally, protease enzymes play a significant role in degrading the fungal cell to limit the fungal growth [41].*Burkholderia* showed high capability to produce several extracellular hydrolytic enzymes such as chitinase, protease, cellulase, amylase, and glucanase [42- 44], which have important uses in both agricultural and pharmaceutical industries [37].

3.7. Scanning electron microscopy of tomato root samples for the observation of *Paraburkholderia tropica* TRP-46 colonization

One month after inoculation, bacterial cells were mostly observed on root surface. SEM further showed numerous bacterial cells on the root hairs. They were homogeneously distributed along the root (Fig. 4). This is in agreement with the previous study of Li et al. [45] where they reported that *B. multivorans* WS-FJ9 bacterium could colonize poplar rhizosphere, as well as the poplar root tissues and cells. Moreover, the colonization of *Paraburkholderia tropica* MTo-293 obtained by SEM of the roots in the

inoculated barley plants indicated the presence of a large number of bacterial cells on the root surfaces [13].

Fig. 4: Scanning electron and fluorescencemicroscopy images of primary roots colonized by *Paraburkholderia tropica* TRP-46, 30 days after inoculation



3.8. Evaluating the root colonization of *Paraburkholderia tropica* TRP-46 intomato rhizosphere

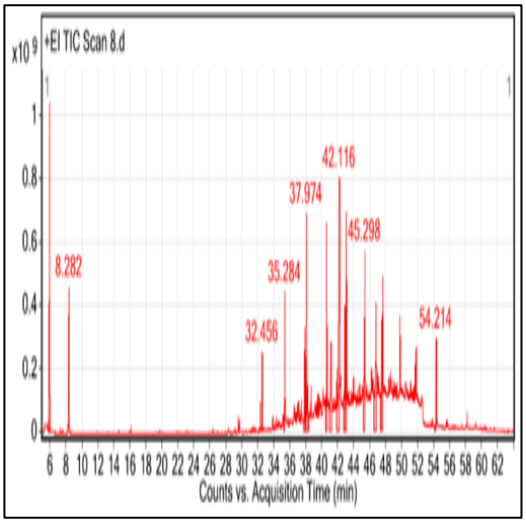
The root colonization potentiality of the plant growth promoting rhizobacteria on the tomato rhizosphere was determined utilizing CFU counts. The Rif^r CFU counts of PGPR one month after inoculation showed colonization densities of (Log_{10} CFU g^{-1} rfm = 4.8). This is in agreement with the previous study of [13] where they reported that the *Paraburkholderia tropica* MTo-293 grew as a biofilm on both abiotic and biotic surfaces and efficiently colonized barley roots and stems in plants grown in flasks in the presence of other microorganisms. *Paraburkholderia tropica* S39-2 possesses numerous plant growth promotion characteristics and can colonize and encourage the growth of oil palm and Jatropha [46]. It has been reported that *P. tropica* can colonize the surface and internal root tissues of wheat plants after seed inoculation under gnotobiotic growth conditions [28, 47]. Another point is that the ability to colonize roots is considered as the major reason that determines the inoculum effectiveness in crop production, also root colonization was associated with the ability to produce antibiotics which plays a significant role in the biological control of many phytopathogens [47, 48]. In the light of this, *Paraburkholderia tropica* canbe used as an efficient bioinoculant to reduce fertilizer use and improve crop production in agriculture.

3.9.Identification of potentially bioactive compounds of *Paraburkholderia tropica*TRP-46 by GC-MS

The chemical identification of the bioactive compounds present in the ethyl acetate extracts of TRP-46 was performed by GC-MS based on the retention time, peak areas, molecular weight, and molecular formula. The major compounds identified by GC-MS analysis in ethyl acetate extracts of TRP-46 were Hexadecenoic acid, 2.3-dihydroxypropyl ester which represent the highest percentage (14.65 %) is reported to express antifungal properties as a component found in *Jatropha curcas* leaf extracts [49, 50]. Also, Aliet al. [51] have reported that Hexadecenoic acid has antifungal, and antibacterial activity. Additionally, cyclohexanone is considered an antimicrobial

compound against plant pathogenic bacteria and fungi [52]; 1-Tetradecanol has a positive outcome on plant growth of tomato seedlings [53], and Octadecane has an antibacterial and antifungal activity [54]. Moreover, Butyl acetate, Hexadecane, 11-Decyldocosane, 2,2-Dideuterooctadecanal, 7-Methyl-Z-tetradecen-1-ol acetate, Tetraneurin A, 2,2,3,3,4,4-Hexadeutero Octadecanal,2-(4-Nitrobutyryl)cyclooctanone, 2-myristynoyl pantetheine, andBis(2-ethylhexyl)phthalate all were reported to have antimicrobial activity [55- 61] (Fig. 5and Table 1).

Fig. 5. GC-MS analysis of the ethyl acetate extract of TRP-46



4. Conclusion

This study showed that *Paraburkholderia tropica* isolates are very promising plant growth promoting inoculants due to their in vitro activities as well as their high root colonization abilities. The GC-MS analysis showed the potential presence of several bioactive compounds known for their antifungal activity in *Paraburkholderia tropica* culture supernatant, which could be used as a substitution of the currently used chemical fungicides

5. Conflicts of interest

"There are no conflicts to declare".

6. Acknowledgments

We thank Dr. Ghada Aboul Hassan for carefully reading the manuscript.

7. References

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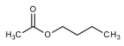
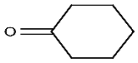
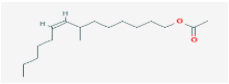
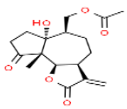
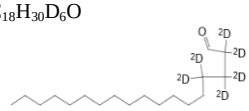
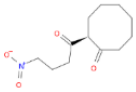
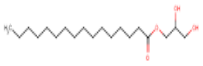
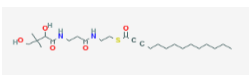
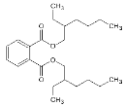
Table 1. GC GC-MS analysis of the ethyl acetate extracts of TRP-46

RT	Percentage area	Compound name	Molecular formula	Molecular weight g/mol	Proposed function
5.865	8.83	Butyl acetate	$C_6H_{12}O_2$ 	116.16	Antimicrobial activity (55)
8.282	4.26	Cyclohexanone	$C_6H_{10}O$ 	98.14	Antimicrobial activity against plant pathogenic bacteria and fungi (52)
32.235	0.78	1-Tetradecanol	$C_{14}H_{30}O$	214.39	Positive effect on plant growth of tomato seedlings (53)
32.456	1.94	Hexadecane	$C_{16}H_{34}$	226.41	Antimicrobial (56)
35.284	3.74	11-Decyldocosane	$C_{32}H_{66}$ 	450.9	Antagonistic activity (57)
37.78	3.21	2,2-Dideutero octadecanal	$C_{18}H_{34}D_2O$ 	268	Antimicrobial activity (58)
37.974	5.38	Octadecane	$C_{18}H_{38}$ 	254.5	Antibacterial and antifungal activity (54)
38.131	3.37	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$ 	268.4	------
38.314	0.6	Tetraneurin A	$C_{17}H_{22}O_6$ 	322.4	Antimicrobial activity (59)
40.527	7.01	2,2,3,3,4,4-Hexadeutero Octadecanal	$C_{18}H_{30}D_6O$ 	268	----------
41.075	6.4	2-(4-Nitrobutyryl) cyclooctanone	$C_{12}H_{19}NO_4$ 	241.28	--------
42.116	14.65	Hexadecenoic acid, 2.3-dihydroxypropyl ester	$C_{19}H_{38}O_4$ 	330.5	---------
46.691	8.79	2-myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$ 	484.7	---------
54.214	2.54	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$ 	390.6	Antimicrobial activity (60, 61)

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الملخص العربي

نواتج التمثيل الغذائي الثانوية النشطة حيويا المستخلصة من البكتيريا *Paraburkholderia tropica* المشجعة لنمو النباتات

دنيا سعد الدين هلال,حسين مصطفى الخواص, طارق سيد رجب

قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة القاهرة – الجيزة – مصر

ان البكتيريا المستخدمة في المكافحة الحيويةوالمشجعة لنمو النبات المصاحبة لمنطقة الريزوسفيرتستخدم علي نطاق واسع كبديل للمبيدات والاسمدة الكيميائية, ولذلك فان هذه الدراسة تستهدف توصيف ودراسة النواتج الثانوية النشطة بيولوجيا الخاصة بالبكتريا *Paraburkholderia tropica*المشجعة لنمو العديد من النباتاتوالتى لها دور في المكافحة الحيوية. بالاضافه الي انه تم تقييم الصفاتالمرتبطة بتشجيع نمو النباتاتوالمكافحة الحيويه ضد*Ralstoniasolanacearum* و*Fusarium oxysporum*. لقد اظهرت نتائج GC-MSلراشح البكتيريا المستخدمة في هذه الدراسة الإنتاج المحتمل حمض الهيكساديكانويك (حمض البالميتيك) والتيتراديكانول والأوكتادكان حيث ان هذه المركبات ذات نشاط مضاد للميكروباتونشاطضد البكتيريا والفطريات المسببة للأمراض النباتية بالاضافه الي قدرتها على تشجيع النمو النباتي لشتلات الطماطم.تم تقدير قدرة-TRP 46*Paraburkholderia tropica*علي استعمار جذور نبات الطماطم وذلك باستخدام طفرات مقاومة للريفامبيسين وتم تاكيدالنتائج من خلال استخدام الميكروسكوب الالكتروني الماسح. ومن خلال هذه النتائج فإن46-TRP*Paraburkholderiatropica*تعتبر بكتيريا واعدة لإستخدامها كلقاحات ميكروبية من اجل تشجيع نمو النبات وإستخدامها في المكافحة الحيوية.