

# Effective Biofertilizer *Trichoderma* Spp. Isolates with Enzymatic Activity and Metabolites Enhancing Plant Growth

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## Research Article

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# Abstract

Trichoderma species have been widely recognized as biofertilizer fungi for their ability to produce phytohormones and enhancing plant growth. In our current study, fifteen strains of *Trichoderma* spp (T1-T15) were screened for their capacity to produce phytohormones and metabolites eliciting plant growth. The strains were previously isolated from olive rhizosphere soil in northern Algeria. Plant growth promoting (PGP) potential of *Trichoderma* spp. was evaluated *in-vitro* through the production of phosphatases, iron chelators (siderophores), cyanhydric acid (HCN) and ammonia (NH<sub>3</sub>). Besides, Plant growth phytohormones such as gibberellic acid and indole-3-acetic acid (IAA) were assessed quantitatively by a colorimetric assay. Results showed an effective potential of *Trichoderma* isolates in PGP biomolecules production. Importantly, qualitative estimation of phosphate solubilization indicates that T10 gave the highest P-solubilization on medium Pikovskaya's with a solubilization index (SI) of 3, whereas, the high capacity nitrogen-fixing was related to T8. In other hand, quantitative analysis of IAA and gibberellic acid revealed a production varying between (1.30 µg mL<sup>-1</sup> – 21.15 µg mL<sup>-1</sup>) and (0.53µg/ml – 7.87µg/ml), respectively, the highest amount of both phytohormones was obtained by T11 isolate. Indeed, analysis of ethyl acetate extracts of T11 isolate by high-performance liquid chromatography (HPLC) revealed a high amount (71.19 mgL<sup>-1</sup>) of IAA. Overall, Results showed clearly that isolate T11 has promising plant growth promoting properties. Hence, this native Trichoderma isolate (T11) identified as *Trichoderma harzianum* strain (OL587563) could be used later as biofertilizer for sustainable olive crop agriculture.

## Introduction

Chemical fertilizers are not recommended in agriculture, considering the huge damage they cause to environment and human health. Therefore, a sustainable agriculture requires the utilization of biofertilizers (Ismail et al. 2016). Microorganisms associated to the plant roots rhizosphere are often beneficial to plants; they provide nutrients, protection against biotic and abiotic stresses and stimulate plant growth (Lynch et al. 2001; Filiz et al. 2021). Additionally, microorganisms with plant growth activity have many advantages as they are environmentally friendly, enhancing crop productivity and economically valuable (Renuka et al. 2018).

Searching for microorganisms with important growth promoting activity as well as antagonistic impact became imperative. Various fungi are known to produce numerous bioactive substances. Trichoderma species are free-living and/or entophytic fungi that grow vigorously in soil and plant root ecosystems; they have received much interest as economic and safe biocontrol agents for different pathogens and enhancers of plant defense mechanisms. They are widely applied in stimulation of plant growth because of their capacity to produce plant growth promoters (Saber et al. 2009; Hassan et al. 2013; Vinale et al. 2014; Ezzat et al. 2015). Furthermore, Trichoderma species are able to promote plant growth through various mechanisms such as solubilizing insoluble phosphate and production of plant hormone such as IAA and siderophore (Napitupulu et al. 2019)

Phytohormones play an important role in agriculture (Jolanta et al. 2019), they are synthesized by many soil and rhizosphere microorganisms, they have various roles such as modification of the physiological functions of plants to accelerate their growth by intensive cell division in callus tissue, promotion of phloem development, enhance lateral root development, plant growth stimulation, prevention of leaf aging by slowing down the breakdown of chlorophyll pigments in plants and improving metabolism even at low concentrations (Karadeniz et al. 2006; Emilie and Jean-Benoit 2016; Vokhid et al. 2020).

Gibberellins are diterpenoid plant hormones (MacMillan 2002; Hiroshi 2006; Hamayun et al. 2010). GAs exhibit biological activity and are the most widespread in nature. Also, participate in every aspect of plant growth and development including seed germination, stem extension, flowering aging and stimulate the formation of hydrolytic enzymes in germinating cereal grain (Tsavkelova et al. 2006; Pallardy 2008; Hamayun et al. 2010; Jaroszek et al. 2014). On the other hand, IAA is responsible for the division, extension, and differentiation of plant cells and tissues. Phytohormones stimulate seed and tuber germination; increase the rate of xylem and root formation; control processes of vegetative growth, tropism, fluorescence, and fructification of plants, affecting photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stress factors (Pallardy 2008; Tsavkelova et al. 2006). In addition, Production of antifungal substances by *Trichoderma* spp, such as iron chelators (siderophore) and hydrogen cyanide may also occur promotion of plant growth (Anke et al. 1991; Samuels et al. 2002; Dutta et al. 2006; Ushamailini et al. 2008); these metabolites can protect plants against phytopathogens (Benitez et al. 2000; Whipps 2001).

The aim of this study was to evaluate *in-vitro* the plant growth promoting (PGP) properties of fifteen indigenous strains of *Trichoderma* spp. isolated from olive rhizosphere soils in northern Algeria. Moreover, investigate their enzymatic activity in order to select the promising *Trichoderma* species. These species could be as a potential biofertilizer for olive crop.

## **Materials And Methods**

### **Fungal isolation and identification**

#### **Isolation of *Trichoderma* species**

*Trichoderma* spp. used in this study were evaluated previously for their biocontrol potential against the olive wilt pathogen; *Verticillium dahlia* (Reghmit et al. 2021). *Trichoderma* spp. were isolated on Potato Dextrose Agar (PDA) medium using the soil dilution plate technique as described by Johnson and Curl (1972),

#### **Phenotypic characterization of *Trichoderma* isolates**

After seven days of incubation, fungal colonies cultured on PDA medium were examined macroscopically and microscopically (Rifai 1969). Macroscopic and microscopic characterization of all isolates tested were described and illustrated in our previous study (Reghmit et al. 2021).

# Molecular characterization of *Trichoderma* isolate T11

*Trichoderma* isolates were cultured on PDA plates for 5 days at 25°C. Identification of *Trichoderma* isolates to the species level were confirmed using sequences of the internal transcribed spacer region of the ribosomal RNA gene (ITS rDNA). ITS was amplified using primer pairs ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3'), ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns 1993). DNA was extracted using DNA extraction commercial kit (NucleoSpin Plant II, Macherey-Nagel Germany). The polymerase chain reaction (PCR) was performed in a volume of 10µL. The PCR conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C and 52°C for 30 seconds respectively for ITS gene. Primer extension was done at 72°C for 45 seconds followed by final extension at 72°C for 7 min. PCR products were observed on 1,5% agarose gels stained with ethidium bromide under ultra violet light using the Gel doc biorad system (USA). PCR products were purified using a NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel, Düren, Germany). The purified PCR products were sequenced. Sanger sequencing was carried out using the Applied Biosystems Big Dye v3.1 kit and the PCR primers mentioned above. The sequenced data were compared against the Gene Bank database using the BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi> Blast) available at NCBI home page: <http://www.ncbi.nlm.nih.gov>). ITS sequence of isolate T11 was analyzed using MEGA11 software. Finally, the cross ponding species for each isolate was identified.

## Qualitative screening of enzymes

Enzymatic activity was evaluated by measuring the halo of hydrolysis zone around the disc inoculated as follows: - (no change): isolates showing no enzyme activity, + (halo zone less than 10 mm): isolates showing very low enzyme activity, ++ (halo zone of 10–30 mm): isolates showing low enzyme activity, +++ (halo zone of 40–60 mm): isolates showing high enzyme activity, ++++ (halo zone of 70 mm and above): isolates showing very high enzyme activity.

## Phosphate solubilization

**Phosphate solubilization was screened qualitatively according to the method described by Gupta et al. (1994) on the solid medium Pikovskaya agar containing tricalcium phosphate (TCA) as insoluble inorganic phosphorus source. Assay was based on inoculated 6mm agar disc cut from 5 day-old fungal culture of each strain into medium and incubated at 26 ± 2°C. Clear zone around fungal colony indicated positive solubilization of mineral phosphate (Noori and Saud 2012). The solubilization index (IS) was**

**calculated by the ratio of the total diameter (halo + colony) and the colony diameter (Edi-Premono et al.1996).**

## **Pectinase production:**

Pectinase screening was carried out on solid medium according to the method described by Hankin and Anagnostakis (1975). Pectinase Agar Medium containing: (Citrus Pectin 10g,  $(\text{NH}_4)_2\text{HPO}_4$  3g,  $\text{MgSO}_4$  0,1g, Agar 20g, Distilled water 1000 ml), pH was adjusted to 5.5. After three days of incubation, the plates were flooded with iodine potassium –iodine solution (Iodine 1g, Potassium iodine 5g in 330 ml distilled water). A clear zone around colonies indicates positive pectinase activity (Gueye et al. 2018).

## **Amylase production**

Amylase production was determined by using amylase detection medium (Abe et al. 2015) (Yeast extract 3 g, Peptone 5 g, Amidon soluble 2 g, Agar 20 g, Distilled water 1000 ml). After incubation at 28 °C for 72 hours, the plates were flooded with 5 ml of iodine solution (1% KI; 0.5% I<sub>2</sub> (v/v)) for 15 minutes. Amylase production was assessed by the formation of a light yellow halo around colonies.

## **Lipase production**

Qualitative lipase production was evaluated on culture medium containing Tween 20 (polyoxyethylene-sorbitan-monolaurate) as a lipid substrate (Abe et al. 2015). Medium used containing (Tween20 10 mL, Peptone 10 g, NaCl 5 g,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0,1g, Agar 20 g, Distilled water 1000 ml). Tween 20 was autoclaved separately and added to the sterile medium. Discs of 6 mm of *Trichoderma* cut from 5 day-old fungal culture of each isolate were inoculated in the center of the medium and incubated at  $26 \pm 2^\circ\text{C}$  for 5 days. The presence of opaque precipitate or an opaque white halo around the colonies refers to the production of lipases.

## **Screening for qualitative PGPF activity of Trichoderma species**

### **Hydrogen cyanide (HCN) production**

HCN production was screened qualitatively according to the method of Bakker and Schipper (1987). *Trichoderma* isolates were inoculated on solid medium containing Tryptic Soya Agar (TSA) supplemented with 4.4 g/L of glycine. A whatman filter paper impregnated by alkaline picric acid solution (2.5 g of picric acid; 12.5 g of  $\text{Na}_2\text{CO}_3$ ; 1000 ml of distilled water) was placed under the top cover of each plate. The plates were incubated at  $26 \pm 2^\circ\text{C}$  for 7 days. A change in the filter paper color from yellow to light brown, brown or reddish brown implies the production of HCN (Meera and Balabaskar 2012).

### **Nitrogen fixation activity**

In order to evaluate nitrogen-fixing ability, medium lacking nitrogen ( $\text{KH}_2\text{PO}_4$  0.2 g,  $\text{CaCO}_3$  5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g,  $\text{NaCl}$  0.3 g, Mannitol 10 g,  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  0.1 g, agar 20 g and 1000 ml of distilled water (pH 7.2) were used. Discs of 6 mm from pure culture of *Trichoderma* were inoculated in the center of a Petri dish containing nitrogen-fixing medium. After 3 days of inoculation, test of nitrogen-fixing ability was considered positive if the colony can grow normally on the selective medium (Zhang et al. 2017).

## **NH<sub>3</sub> production**

**Production of ammonia was tested in peptone water according the method of Bakker and Schipper (1987). Broth culture containing 10 ml peptone water was inoculated and incubated at 28°C for 72 h. Later; 1 ml of Nessler's reagent was added to each tube. Development of color from yellow to brownish orange was recorded as a positive test for ammonia production.**

## **Siderophore production**

**Siderophores production was evaluated according to the method of Hoyos-Carvajal et al. (2009) on malt extract agar medium containing 8-hydroxyquinoline ( $50 \text{ mg L}^{-1}$ ) as chelator. Growth of strains tested on this medium after 5 days of incubation at  $26 \pm 2^\circ\text{C}$  was recorded as a positive result for siderophores production.**

## **Quantitative estimation of gibberellins and indole-3-acetic acid (IAA) by the colorimetric assay**

### **Dosage of gibberellins**

Fungal isolates were screened for gibberellic acid production on medium containing (Yeast extract 5g, Sucrose 30g, Sodium nitrate 30g, Magnesium sulphate 0.5g, Potassium chloride 0.5g, Ferrous sulphate 0.01g, Dipotassium hydrogen phosphate 1g). pH was adjusted to 6.4 (Uthandi et al. 2010). Each isolate was grown on this broth medium for 5 days at 28 °C. After incubation period, the fermented media were centrifuged and the supernatant was used. The gibberellins were estimated calorimetrically by standard method (Holbrook et al. 1961; Sharma et al. 2018). 2 ml of zinc acetate reagent (21.9g zinc acetate + 1 ml of glacial acetic acid and volume was made upto 100 ml with distilled water) was added to 15 ml of

supernatant. After 2 minutes, 2 ml of potassium ferrocyanide (10.6% in distilled water) were added and centrifuged at low speed (2000 rpm) for 15 minutes. To 5 ml of supernatant 5 ml of 30 per cent HCl was added and mixture was incubated at 20°C for 75 min. For blank 5 ml of 5 per cent HCL was used. Absorbance was read at 254 nm concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA3) as standard.

## **Dosage of Indole acetic acid (IAA)**

Isolates of *Trichoderma* were screened for their efficient ability to produce Indole Acetic Acid (IAA) using L-tryptophan as precursor according to the method of Bric et al. (1991). The essays were done in potato dextrose broth medium (PDB), which was supplemented with 1 g/l of L-tryptophan. The culture was incubated in a shaker with 150 rpm at 28°C. After 3 days of incubation, production was qualitatively estimated by colorimetric assay. The filtrates were centrifuged and then 2 ml of each supernatant was added to 2 ml of Salkowski reagent (1ml of ferric chloride (0.5M) was added to 50ml of perchloric acid (35%)). The development of a pink or red color revealed the IAA production; the absorbance was measured at 530 nm using a spectrophotometer (ZUZI spectrophotometer model 4201/50).

## **Extraction of IAA**

Since isolate T11 and T12 showed high IAA production, they were subject to liquid-liquid extraction of IAA. Fermentation was performed in PDL medium supplemented with 1 g/l L-tryptophan. Extraction was carried out according to the method of Mohite (2013) with few modifications. After three days of fermentation, the fermentation broth was centrifuged at 9000×g for 15 min at 4°C. The supernatant obtained was acidified and adjusted to pH 2.5. The supernatant was collected and mixed with ethyl acetate (1:2). The funnel containing ethyl acetate and supernatant was vigorously shaken several times. Then, organic phase was separated from aqueous phase. The supernatant was extracted three times with ethyl acetate. The organic layers were combined and evaporated by a Rotavapeur at 50°C with a slight rotation. Extracts containing IAA, was filtered through a 0.22 µm Millipore membrane and stored at - 20°C for high-performance liquid chromatography (HPLC) analysis.

## **Thin layer chromatography (TLC) of IAA**

TLC slide was prepared with silica gel G and calcium carbonate with a solvent system containing Propanol: Water (8:2). The extracted sample and standard IAA (10mg/100ml) were spotted on TLC plate. Chromatogram was developed with the Salkowski's reagent (Kuang-Ren et al. 2003). Rf value calculated as the ratio between the distance run by the compound and distance run by the solvent front.

## **Quantification of IAA by high-performance liquid chromatography**

The production of IAA was analyzed by HPLC (Agilent 1200), with a C18 column (4.6×150 mm). Solvent A (methanol) and solvent B (water with 1% (v/v) acetic acid) (A/B = 60:40) at a flow rate of 1 ml min<sup>-1</sup>. IAA was separated at 254nm-280nm by UV-VIS detector and a column temperature of 30°C. A volume of 20

$\mu\text{L}$  was injected for each analysis. IAA levels were quantified based on a standard curve first established in the same conditions.

## Results

### Phenotypic and molecular identification of the promising strain T11

Macroscopic and microscopic examination of isolate T11 revealed that this isolate could be identified as *T. harzianum* strain, T11 showed dark green producing tufts, no concentric rings were observed. Conidial productions were restricted to the center of the colonies, diffused, and appeared with green color. Conidiophores character frequent branching and verticillate. Phialides were ampulliform and convergent. Conidia subglobose to obovoid shape. Formation of chlamydospore was in terminally and intercalary position (Fig. 2).

The obtained partial gene sequence of the strain T11 was 560 nucleotides in length (Genebank, OL587563). The comparison of the obtained 5.8S rRNA gene partial sequence of the strain T11 against the 18S ribosomal RNA sequences of ITS region available on database was performed by BLASTN. The closed sequences were imported into the MEGAX software and aligned. The phylogenetic analysis indicated that the strain T11 belong to the genus *Trichoderma*. A significant similarity for possible species relatedness (85%) was found with the validly described species *Trichoderma harzianum* isolate ARCF 497 (MZ477246.1). These obtained results strongly suggested that the strain T11 was identified as *Trichoderma harzianum* T11 strain.

The evolutionary history was inferred Neighbour-Joining method showing the phylogenetic position of *Trichoderma harzianum* strain T11 and representatives of certain other related taxa based on 5.8S rRNA sequences. Access numbers in the Genbank databases are given after the name of each strain.

### Results of qualitative screening of enzyme production

Results of qualitative screening showed that the majority of tested isolates were able to produce amylases, pectinases, lipases and phosphatases at different degrees. The enzymatic activities of different isolates were estimated according to the diameter of the clearing zone discolored around colonies or/and color intensity. Among the 15 tested isolates, three isolates namely, T6, T7 and T15 showed high lipolytic activity with the formation of opaque precipitate with a diameter of 50mm, 50mm and 40 mm, respectively, around the colonies after only 4 days of incubation (Table 1, Fig. 4).while, pectinase production was positive for all isolates tested with varying diameters of the hydrolysis zones. Importantly, isolate T5 showed very high pectinase activity with a hydrolysis diameter of 70 mm, besides, the four isolates T2, T10, T13 and T14 revealed a clear yellowish hydrolysis zone of 65 mm on the solid medium. On the contrary, other isolates have less degradation capacity; they showed hydrolysis diameters varying between 35mm and 62mm. Moreover, the production of amylases was revealed by the presence of a light yellow halo around the inoculated discs in the center after 72h of incubation at 28°C,



four tested isolates showed amylolytic activity. Results showed that isolate T10 has a good ability to degrade the starch present in the medium by developing a hydrolysis zone of 55mm, the starch hydrolysis zones shown by isolates T6, T12, and T15 were average, they are estimated at 40mm, 29mm and 35mm respectively. On the other hand, the smallest diameter of hydrolysis is recorded by isolate T1 (13mm).

Results of the qualitative estimation of phosphate solubilization indicate that the most strains of *Trichoderma* were able to solubilize insoluble inorganic phosphates. Two isolates of *Trichoderma* namely, T13 and T5 gave the highest P-solubilization on medium Pikovskaya's achieved a visible halo-zone on all mediums, followed by T12 which exhibited high P-solubilization activity showing a visible halo of 65mm (Fig. 4). In addition, 3 isolates T4, T6, and T10 showed higher solubilization phosphate with a solubilization index (SI) of 1.85, 1.85, and 3 respectively, followed by isolates T7, T12, and T13 with an SI ranged between 1.55 and 1.77. However, the other isolates showed a lower solubilization index ( $IS \leq 1.41$ ).

The results of quantitative screening of HCN production revealed that isolates of *Trichoderma* were able to produce HCN. The filter paper color varied from yellow to reddish brown, this observation indicated that the amount of the produced HCN was differed from less to important amount. Based on color variation, we noticed that T12 produces considerable amount of HCN comparing to T2 and T13 that showed a less important production of HCN. Additionally, isolates of *Trichoderma* grow on medium lacking nitrogen and high capacity nitrogen-fixing was related to T8 which showed important radial and aerial mycelial growth compared to other isolates, suggesting that these isolates had the ability of nitrogen-fixing. Furthermore, several isolates tested were able to produce ammonia, isolate T2, T4, T6, T11, T12 had been noted with the highest amount of  $NH_3$  production, while the others isolates produced less important amount. Further, among the 15 tested isolates for siderophores production on malt extract agar medium containing 8-hydroxyquinoline, only 5 isolates T5, T7, T8, T12 and T14 showed positive results. However, isolate T7, T8 and T12 showed important mycelial density compared to isolates T5 and T14.

Table 1  
Qualitative screening of *Trichoderma* spp. for active biomolecules.

<i>Trichoderma</i> isolates	Phosphatase Production	Lipase Production	HCN Production	NH <sub>3</sub> production	Siderophores production	Azote fixation
T1	++++	+++	+	+	-	++
T2	+++	-	+	++++	-	++
T3	++++	-	-	++	-	++
T4	+++	-	+	++++	-	++
T5	++++	-	-	++	++++	++
T6	+++	+++	-	++++	-	++
T7	+++	+++	+	+	++++	++
T8	++	-	+	+	+++	+++
T9	++++	-	-	++	-	++
T10	++++	-	-	++	-	++
T11	++++	-	+	++++	-	++
T12	+++	-	++++	++++	+++	++
T13	++++	-	++	+	-	+
T14	++	-	-	+	++	+
T15	++++	+++	-	+	-	-

- Isolates showing no enzyme activity, + Isolates showing very low enzyme activity, ++ Isolates showing low enzyme activity, +++ Isolates showing high enzyme activity, +++++ isolate showing very high enzyme activity

## Dosage of indole acetic acid (IAA)

The results of IAA production by the different isolates of *Trichoderma* are presented in Table 1. In our present investigation, all *Trichoderma* spp isolates were able to produce IAA. Although, their amount produced varied considerably, the highest IAA production was observed by T11 with 21.15  $\mu\text{g mL}^{-1}$  of IAA followed by T8 and T2 with 7.87 and 7.71  $\mu\text{g mL}^{-1}$  of IAA production respectively. The other isolates revealed less production and the amount of IAA produced by them ranged from 1.30  $\mu\text{g mL}^{-1}$  to 7.16  $\mu\text{g mL}^{-1}$  (Fig. 5). Similarly, all isolates of *Trichoderma* spp. produced gibberellic acid, the highest amount of gibberellic acid was produced by T11 (7.87  $\mu\text{g/ml}$ ) followed by T3 (4.64  $\mu\text{g/ml}$ ). Results of this studied indicated that *Trichoderma* spp. tested were a promising candidates for the production of various phytohormones such as IAA and GA (Table 2).

Table 2  
Production of IAA and GA by various isolates  
of *Trichoderma* species

Isolates	IAA ( $\mu\text{g mL}^{-1}$ )	GA ( $\mu\text{g mL}^{-1}$ )
T1	5,13	3,98
T2	7,71	3,93
T3	2,44	4,64
T4	6,85	0,67
T5	1,30	3,91
T6	3,06	1,12
T7	6,03	0,67
T8	7,87	3,97
T9	2,59	1,08
T10	6,77	3,11
T11	21,15	7,87
T12	7,16	1,92
T13	4,66	4,51
T14	5,48	0,53
T15	2,91	4,2

## Detection of IAA by thin layer chromatography

IAA extracted from culture filtrates was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed a pink color spot at the  $R_f$  corresponding to the authentic IAA (0.84) (Fig. 6). This result confirmed the ability of both isolates T11 and T12 to produce IAA.

## Quantification of IAA in fermentation solution by HPLC

Both extracts of isolates T11 and T12 were analyzed by HPLC. IAA extracted from the culture of T11 and T12 isolates showed a similar peak to that of the IAA standard. According to the chromatogram of IAA standard, the retention time of IAA was 28.6 min (Fig. 7), The regression equations of IAA standard curve and its correlation coefficients ( $r^2$ ) ( $y = 0.488x - 0.072$   $R^2 = 0.986$ ) was generated by concentration of IAA against the peak areas, Results show that IAA was detected in chromatograms of T11 and T12 extracts, revealing an amount of  $71.19 \text{ mgL}^{-1}$  and  $17.71 \text{ mgL}^{-1}$  respectively. (Fig. 8, 9). Representative HPLC

chromatograms of IAA (Tr at 28.6) obtained from standard and extracts of both isolates T11 and T12 were shown in (Fig. 8, 9).

## Discussion

*Trichoderma* spp. has been widely used in agriculture as plant growth promoting fungi and as biopesticide to control soil-borne diseases. In this study, plant growth promoting properties (PGP) of *Trichoderma* isolates have been demonstrated, *Trichoderma* tested were found efficient for the production of enzymes and phytohormones that induce plant growth, while the production of biomolecules was variable for each of the *Trichoderma* isolates used. *Trichoderma harzianum* strain (OL587563) showed the best performance regarding phytohormones' production. This result suggests that this strain could play a key role as biofertilizer candidate improving the plant growth. Furthermore, *Trichoderma* isolates tested were able to produce extracellular enzymes such as pectinase; this enzyme is involved in the decomposition of organic matter and can extract nutrients from the plant tissues, in addition it helps in the entry of fungal hyphae. The secretion of pectinase by many species of *Trichoderma* was also described by Cherkupally et al. (2017) and Nazia et al., (2003).

Besides, Phosphate solubilizing microorganisms have attracted the attention of agronomists; these microorganisms were used as soil inoculum to improve plant growth (Fasim et al. 2002). In same line, it has been shown that *Trichoderma* species have the ability to solubilize insoluble phosphate into soluble phosphate (Akintokun et al. 2007; Saravanakumar et al. 2013); these findings were similar to our results. Furthermore, López et al. (2019) reported that *Trichoderma atroviride* LBM 112 and *T. stilbohypoxylis* LBM 120 showed positive results for phosphate solubilization with formation of halo-zone on the solid medium containing insoluble inorganic phosphorus source. In previous studie, Li et al. (2018) reported that *Trichoderma asperellum* strain CHF 78 has several plant growth-promoting traits, such as the phosphate- solubilizing ability and the production of siderophores. Moreover, five *Trichoderma* isolates tested in this study were capable to produce siderophores, amount of siderophore produced by strain of *Trichoderma* spp varies considerably depending on the strain (Anke et al. 1991). Our result is in agreement with Ghosh et al. (2017) and Vinale et al. (2013) who revealed that antagonistic spp. of *Trichoderma* namely *T. viride*, *T. harzianum*, *T. longibrachiatum* and *T. asperellum* produced considerable amounts of siderophore. Whereas, López et al. (2019) reported that *Trichoderma atroviride* LBM 112 showed negative result for siderophores production, while *T. stilbohypoxylis* LBM 120 showed positive results on malt extract agar medium containing 8-hydroxyquinoline. Additionally, our results showed that *Trichoderma* isolates were able to produce HCN; which support the findings of previous studies that reported a positive production of HCN by *Trichoderma* spp. (Aarti and Meenu 2015; Ng et al. 2015; Mohiddin et al. 2017). Importantly, production of ammonia and nitrogen-fixing ability by *Trichoderma* isolates were demonstrated. Bach et al. (2016) revealed that nitrogen-fixing by microorganisms play a key role on growth-promoting. It has been suggested that the promotion effect on plant growth might be mediated by providing nitrogen trough Biological Nitrogen Fixation and hormones (Hemerly, 2016). Our result is supported also by, Ahemad and Kibret (2014) who reported that ammonia is useful for plant as directly or indirectly. Ammonia production by the *Trichoderma* isolates may influence plant growth

indirectly; ACC synthesized in plant tissues by ACC synthase is released from plant roots and taken up by neighboring micro-organisms. Then, *Trichoderma* may hydrolyze ACC (1-aminocyclopropane-1-carboxylic acid) to ammonia. Besides, the result of the production of ammonia is sustained with results obtained by Mohiddin et al. (2017) who reported that among 20 *Trichoderma* spp. isolated from chilli rhizosphere, 13 isolates were able to produce ammonia.

In addition, all *Trichoderma* isolates tested produced IAA at different levels. Our results revealed that some values of IAA obtained were in the same range compared to study of Lalngaihawmi and Bhattacharyya (2019) who reported that the amount of IAA produced by *Trichoderma* spp were ranging from  $6.32 \mu\text{g mL}^{-1}$  to  $13.38 \mu\text{g mL}^{-1}$ . Furthermore, four *Trichoderma* isolates tested in our study gave a high amount of IAA compared to the *Trichoderma* spp species tested by Mohiddin et al. (2017), which revealed that the amount of IAA produced by *Trichoderma* spp. ranged from  $1.538 \mu\text{g mL}^{-1}$  to  $6.605 \mu\text{g mL}^{-1}$ . So, the isolate T11 produced a high amount of IAA ( $21.15 \mu\text{g mL}^{-1}$ ) compared with previous studies. The amounts of IAA were analyzed by HPLC and the results showed variable amount of IAA produced by both isolates T11 and T12. From chromatograms of HPLC, the level of IAA in liquid extracts T11 was nearly five orders of magnitude higher than those observed in T12 extract. This result support the result found in the dosage of IAA where the amount of IAA produced in the supernatant of T11 was more important compared to the supernatant of T12.

## Conclusion

In conclusion, the present study clearly demonstrates that *Trichoderma* isolates obtained in ecosystem niche of olive showed high potential on PGP biomolecules production. *In-vitro* assays revealed that *Trichoderma* isolates examined in this work were able to produce biomolecules such as phytohormones, hydrolytic enzymes, siderophores, N<sub>2</sub> fixation, ammonia, and phosphatases. *Trichoderma harzianum* T11 (OL587563) were the most promising strain as alternative for application on olive crop. This strain could be a sustainable solution to improve olive crop growth. The results obtained *in-vitro* so far are very promising, nevertheless it is important to investigate the efficiency of the strain in field. Moreover, it is needed to evaluate *Trichoderma harzianum* T11 (OL587563) on soil–plant system and to elucidate their efficacy as effective plant growth-promoting fungi.

## Declarations

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### Competing Interests

**Conflict of interest** The authors declare that there is no conflict of interest.

The authors have no relevant financial or non-financial interests to disclose

## Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Reghmit Abdenaceur, Benzina-tihar Farida, Djeziri Mourad, Hadjouti Rima, Oukali Zahia and Sahir-Halouane Fatma. The first draft of the manuscript was written by Reghmit Abdenaceur and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University of M'hamed Bougara, Department of biology, Boumerdes 35000, Algeria (Date 10/02/2021/No number is not available).

## Consent to participate

Informed consent was obtained from all individual participants included in the study.

## Consent to publish

The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1, 2, 3, 4, 5, 6, 7, 8 and 9.

## Consent for publication

My manuscript doesn't contain any individual person's data in any form (including individual details, images or videos),

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## Availability of data and material

Please contact author for data requests

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## Figures



**Figure 1**

The different regions prospected in northern Algeria for the *Trichoderma* isolates collection. Illustration source: [www.google.com/earth](http://www.google.com/earth)



## Figure 2

Macroscopic and microscopic appearance of *Trichoderma* isolate T11 after 7 days of incubation

## Figure 3

The evolutionary relationships of *Trichoderma harzianum* strain (OL587563)

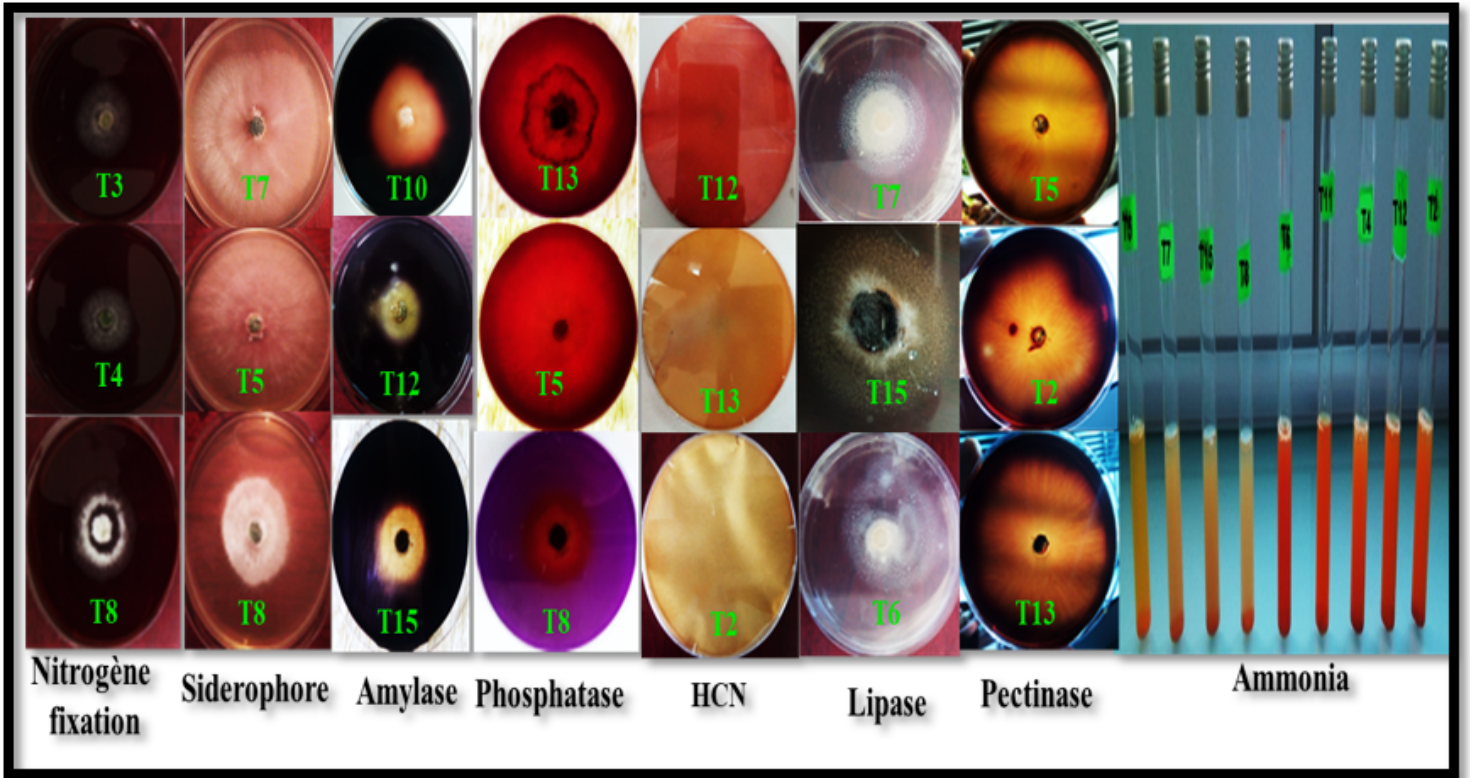


Figure 4

Qualitative screening of various isolates of *Trichoderma* for bioactive molecules

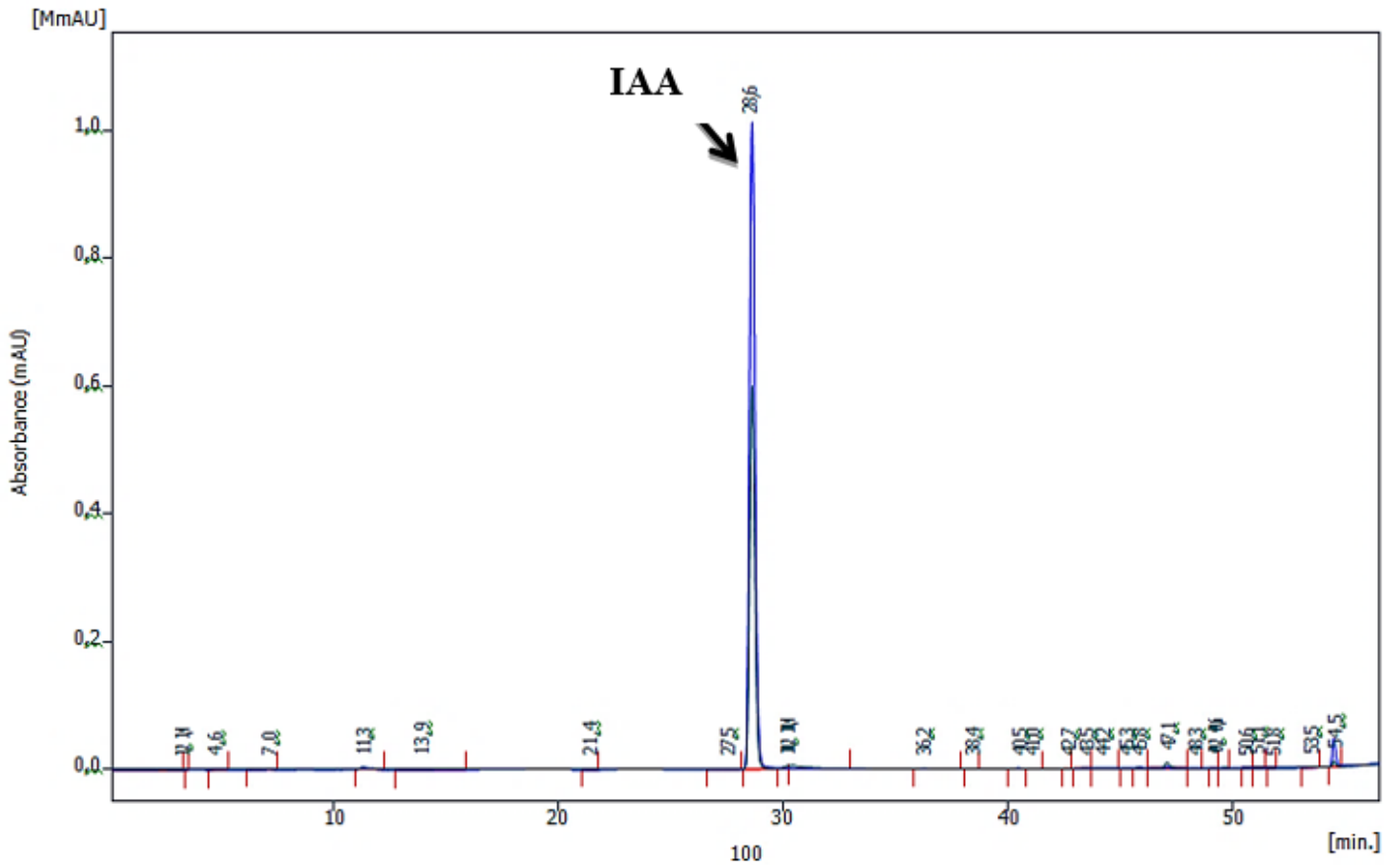


**Figure 5**

IAA production by *Trichoderma* isolates on liquid medium supplemented with tryptophan.

**Figure 6**

Thin layer chromatogram of IAA produced by T11 and T12 detected by Salkowski's reagent and compared with standard



**Figure 7**

HPLC chromatogram of IAA standard.

**Figure 8**

HPLC chromatogram of T11 ethyl acetate extract

**Figure 9**

HPLC chromatogram of T12 ethyl acetate extract