Harzianic acid: a novel siderophore from *Trichoderma harzianum*

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Abstract

Agriculture-relevant microorganisms are considered to produce secondary metabolites during processes of competition with other micro- and macro-organisms, symbiosis, parasitism or pathogenesis. Many different strains of the genus *Trichoderma*, in addition to a direct activity against phytopathogens, are well-known producers of secondary metabolites and compounds that substantially affect the metabolism of the host plant. Harzianic acid is a *Trichoderma* secondary metabolite, showing antifungal and plant growth promotion activities. This report demonstrates the ability of this tetramic acid to bind with a good affinity essential metals such as Fe\(^{3+}\), which may represent a mechanism of iron solubilisation that significantly alters nutrient availability in the soil environment for other microorganisms and the host plant.

Introduction

*Trichoderma* spp. are well-studied filamentous fungi commonly found in the soil community that are widely marketed as biopesticides, biofertilisers and soil amendments, due to their ability to protect plants, enhance vegetative growth and contain pathogen populations (Vinale et al., 2008a). To date, hundreds of different *Trichoderma*-based preparations are commercially used to protect and/or increase the productivity of various crops (Lorito et al., 2010).

One factor that contributes to the beneficial biological activities exerted by *Trichoderma* strains is the wide variety of secondary metabolites that they are able to produce (Sivasithamparam & Ghisalberti, 1998; Reino et al., 2008).

Fungal secondary metabolites may be considered a large and heterogeneous group of small molecules not directly essential for growth, but having an important role in signalling, development and interaction with other organisms (Song et al., 2006; Mukherjee et al., 2012).

*Trichoderma* metabolites may act by directly inhibiting the growth of pathogens, or by indirectly triggering the defence system in the host plant, thus increasing disease resistance, and by promoting plant growth (Vinale et al., 2012). The accumulation of *Trichoderma* metabolites varies according to the species or the strain used and is related to the biosynthesis and biotransformation rates (Vinale et al., 2009a).

Siderophores are low-molecular-weight metabolites produced for scavenging iron from the environment and have an high affinity for iron(III) (Hider & Kong, 2010). Fe\(^{3+}\)-chelating molecules can be beneficial to plants because they can solubilise the iron which is otherwise unavailable and can suppress the growth of pathogenic microorganisms by depriving the pathogens of this necessary micronutrient (Leong, 1986).

We have recently purified from the culture filtrate of a strain of *Trichoderma harzianum* a nitrogen heterocyclic compound named harzianic acid (HA) and demonstrated its growth promotion effect (Vinale et al., 2009b). Growth of different fungal pathogens and canola seedlings was shown to be affected by treatments with HA in a concentration-dependent manner, suggesting a role of this
metabolite in plant growth regulation, as well as in the antagonism against phytopathogenic agents.

In this work, we demonstrate that HA has a Fe³⁺-chelating activity. This secondary metabolite strongly binds essential metals such as iron, thus representing a previously unrecognised siderophore.

**Materials and methods**

**Microbial strain and culture conditions**

*Trichoderma harzianum* strain M10 was maintained on potato dextrose agar (PDA; SIGMA, St Louis, MO) slants at room temperature and subcultured bimonthly. Genomic DNA was isolated to analyse ribosomal DNA. Using PCR approach were amplified fragments containing the internal transcribed spacer 1 (ITS1-partial sequence), the internal transcribed spacer 2 (ITS2-healthy sequence), using primers SR6R (5′-AAGTAGAAGTCGAACAAGG-3′) and LR1 (5′-GGTGTTTCTTTCTCCT-3′).

The PCR products were gel electrophoresed for quantification and assessment of PCR specificity and finally sequenced. Sequence analysis of the internal transcribed spacers (ITS) of the rDNA indicated 99% similarity with GenBank sequences of *T. harzianum* confirming the identity of this species.

Ten 7-mm diameter plugs of M10 strain obtained from actively growing margins of PDA cultures were inoculated to 5-L conical flasks containing 1 L of sterile potato dextrose broth (PDB; SIGMA). The stationary cultures were incubated for 21 days at 25 °C. The cultures were filtered under vacuum through filter paper (Whatman No. 4, Brentford, UK).

**Extraction and isolation of HA**

The filtered culture broth (2 L) was acidified to pH 4 with 5 M HCl and extracted exhaustively with ethyl acetate (EtOAc). The combined organic fraction was dried with 5 M HCl and extracted exhaustively with ethyl acetate.

The red residue (EtOAc) and evaporated in vacuo at 35 °C. The combined organic fraction was dried with 5 M HCl and extracted exhaustively with ethyl acetate. The filtered culture broth (2 L) was acidified to pH 4 (EtOAc). The combined organic fraction was dried with 5 M HCl and extracted exhaustively with ethyl acetate.

**CAS agar plate assays**

The method to detect siderophore production was previously described by Schwyn & Neilands (1987). Orange halos around the colonies, growth on chrome azurol S (CAS) plates, are indicative of siderophore activity. CAS solution was also used for detection of siderophore production in culture filtrate (50 µL of culture was added to 950 µL of CAS solution, and after reaching equilibrium, the absorbance was measured at 650 nm). The CAS assay was also used to test the chelating properties of a 10⁻³ M HA solution in methanol.

The CAS assay was modified according to Milagres et al. (1999) to test the ability of strain M10 to produce iron-binding compounds eventually avoiding the growth inhibition caused by the toxicity of the CAS blue agar medium. Petri dishes (10 cm in diameter) were prepared with the malt extract agar (MEA) medium. After solidification, the medium was cut into halves, one of which was replaced by CAS blue agar. The halves containing culture medium (MEA) were inoculated with M10 plugs. The plates were incubated at 25 °C for 6 days. *Pseudomonas fluorescens* strain CHA0 (bacterial strain collection of prof A. Zoina – University of Naples) was used as a positive control (Maurhofer et al., 1994; Youard et al., 2007).

**Iron-binding affinity of HA**

To measure the iron-binding affinity of HA, the method of Kaufmann et al. (2005) was used with some modifications. Stock solutions of ferric chloride (10 mM) and HA (10 mM) were prepared with 4:1 MeOH/0.1 M NaOAc buffer solution (pH 7.4). Aliquots of both stock solutions were diluted, and the absorbances of the formed complexes were measured at 290 nm in triplicate in the presence and absence of EDTA (10 mM and saturated solution).

**LC/MS of HA–Fe(III) complex**

The Fe(III)-binding properties of the HA were investigated by adding 100 µL of a Fe(III) chloride solution
(10 mM) to 100 µL of 10 mM HA in MeOH. The solution turned red and was directly infused into the LC/MS system at 5 µL min⁻¹ using a syringe pump. Full scans in the range m/z 100–1200 were performed on a Bruker 6340 ion trap mass spectrometer equipped with an electrospray ionisation source (ESI) and operating in the positive ion mode. High-resolution spectra were recorded using a Waters Alliance 2695 HPLC connected to a Waters LCT Premier XE mass spectrometer with an ESI. Samples were injected using the onboard injector in 10 µL injection volumes and eluted with 20% acetonitrile/water at a flow rate of 0.3 mL min⁻¹ to the time-of-flight mass spectrometer. For the HA–Fe(III) complex, positive ESI-HRMS found m/z 491.0574 ([C₁₉H₂₇NO₆-FeCl₂]+ requires 491.0565).

Plant growth promotion assay

Tomato (Solanum lycopersicum cv. Roma) seeds were surface sterilised using 70% EtOH for 2 min, followed by 2% NaClO for 2 min, thoroughly washed with sterile distilled water, then placed on magenta box containing half-strength Murashige and Skoog salt (MS) medium (ICN Biomedicals, Irvine, CA) containing 1% agar and 1.5% sucrose, adjusted to pH 5.7 and vernalised for 2 days at 4 °C in the dark. Media with iron concentrations different from the standard 50 µM were prepared according to Murashige & Skoog (1962), except that defined amounts of Fe(III) were added (diluted 1/10 and 1/100 corresponding to 5 and 0.5 µM, respectively). Sterile solutions of HA were added to the substrate before the agar solidification to have final concentrations of 100, 10 and 1 µM. Untreated substrates were used as controls. Seedlings were grown in a growth chamber (16-h photoperiod); the temperature was maintained at 25 ± 1 °C with a relative humidity of 65–75%. Growth of stem height was measured daily. Each treatment consisted of 20 replicates, and the experiment was repeated four times. At the end of each experiment, the whole plants were dried and weighed. Data from the experiments were combined as statistical analysis determined homogeneity of variance (P ≤ 0.05).

Plant tissues (50–100 mg) were ground with liquid N₂, mineralised according to Beinert (1978) and the iron contents were determined using atomic absorption analysis.

Results

Purification and identification of HA

Trichoderma harzianum M10 was grown in PDB, and the culture filtrate was extracted with ethyl acetate, from which HA was purified as described above. The molecular mass of this compound, as determined by electrospray ionisation–mass spectrometry, was 365 Da, and its pattern corresponded to that of 2-hydroxy-2-[4-(1-hydroxy-octa-2,4-dienylidene)-1-methyl-3,5-dioxo-pyrrolidin-2-ylmethyl]-3-methyl-butyric acid (Fig. 1) described by Sawa et al. (1994) and Vinale et al. (2009b). The HA structure was confirmed by nuclear magnetic resonance.

Iron(III)-binding activity of M10 and HA

The assay described by Schwyn & Neilands (1987) and Milagres et al. (1999) was used for the detection of siderophores released in the substrate by M10 strain. The fungus grew on CAS blue agar and the iron(III)-chelating compounds, excreted by the microorganism and diffused through the medium producing a colour change from blue to orange. Purified HA decolorises CAS blue agar, indicating that it could form a complex with Fe(III) (Fig. 2). While the free compound in aqueous solution is pale orange, addition of Fe(III) results in the appearance of a red colour, indicating that an iron complex is formed.

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**Fig. 1.** Characterization of HA–Fe(III) complex by LC/MS. (a) HA and its mass spectrum; (b) mass spectrum of HA–Fe(III).
Characterisation of Fe(III)–HA complex

To investigate the formed Fe(III)–HA complexes, experiments were carried out adding FeCl₃ to a solution of HA. The uncomplexed HA molecule is detected as [M + H]⁺ (m/z 366.2), [M + Na]⁺ (m/z 388.2), [M + K]⁺ (m/z 404.2) and [M₂ + Na]⁺ (m/z 753.3; Fig. 1a). After adding Fe(III), the spectrum showed additional signals at m/z 455.1 and m/z 491.1 corresponding to two 1 : 1 Fe(III)-containing complexes, which were assigned the following signals based on the chloride isotope pattern, [M–H + Fe(III) + Cl₂ + H]⁺ (m/z 491.1) or [M–2H + Fe(III) + Cl + H]⁺ (m/z 455.1), as determined by isotopic distribution. The resulting mass spectrum is depicted in Fig. 1b. High-resolution spectrum of the Fe(III)–HA complex showed signals at m/z 491.0574 ([C₁₉H₂₇NO₆FeCl₂]⁺ requires 491.0565), confirming the 1 : 1 HA–Fe complex.

The apparent binding constant \(K_{\text{app}}\) was determined to measure the affinity of HA for iron (III). These experiments were performed using a previously described protocol based on the competition between HA and EDTA for iron and the detection of the complexes by measuring the characteristic absorption (Wang et al., 2002). The loss of Fe(III)–HA absorbance at 340 nm upon addition of EDTA was used to calculate the equilibrium constant, presuming the formation of a 1 : 1 Fe–HA complex, according to the following equation:

\[
\text{EDTA} - \text{Fe} + \text{HA} = \text{EDTA} + \text{Fe} - \text{HA}
\]

\[
K_{\text{eq}} = \frac{[\text{EDTA}][\text{Fe} - \text{HA}]}{[\text{HA}][\text{EDTA} - \text{Fe}]} = \frac{K_{\delta, \text{EDTA}}}{K_{\delta, \text{HA}}}
\]

Using the known affinity of EDTA for Fe(III) \((5.00 \times 10^{-25} \text{ M})\), we were able to determine the relative affinity \(K_{\delta, \text{app}}\) of HA for Fe³⁺ to be \(1.79 \times 10^{-25} \text{ M}\).

Plant growth promotion

The activity of HA as plant growth promoter was evaluated by analysing the effect on seed germination and measuring lengths, fresh or dry weights of tomato seedlings treated with metabolite solutions as compared to controls.

Seed germination was significantly affected by HA applications. In particular, when 100 or 10 μM HA solutions were used, the percentages of germinated seeds were 4–5 times higher than in control (Table 1). Shoot and root growth of tomato seedlings was enhanced by treatments with HA used at different concentrations. The highest increase of shoot and root length (+76% and +66%, respectively) was registered using 10 μM of HA compared with control (Fig. 3). A promotion effect on both fresh and dry plant weights was observed using HA at 10 and 1 μM. Conversely, a higher concentration of HA (100 μM) did not significantly affect plant weights or seedling lengths compared with the untreated control (Table 1, Fig. 3).

To further characterise the role of HA in iron availability, plants were supplemented with low concentrations of iron (5 and 0.5 μM). Root length of tomato seedlings grown in media containing the lowest iron concentration was improved by treatments with both 10 and 1 μM HA; conversely, no significant effect was detected in shoot growth (Fig. 4a) or in plant weight in comparison with control (data not shown). However, in

Table 1. Growth promotion of tomato seedlings treated with HA at different concentrations fresh and dry weights 12 days after treatment; germination 2 days after treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh weight (mg)</th>
<th>SD</th>
<th>Dry weight (mg)</th>
<th>SD</th>
<th>% of Germination SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46.9a</td>
<td>8</td>
<td>5.4a</td>
<td>0.4</td>
<td>16.5a</td>
</tr>
<tr>
<td>HA 100 μM</td>
<td>59.8a</td>
<td>7</td>
<td>6.3a,b</td>
<td>0.6</td>
<td>88.0b</td>
</tr>
<tr>
<td>HA 10 μM</td>
<td>73.7b</td>
<td>6</td>
<td>6.6b</td>
<td>0.5</td>
<td>71.5b</td>
</tr>
<tr>
<td>HA 1 μM</td>
<td>72.5b</td>
<td>8</td>
<td>6.5b</td>
<td>0.7</td>
<td>27.5c</td>
</tr>
</tbody>
</table>

Values are means of 10 replicates. SD: ±standard deviation. Values with the same letter do not differ significantly \((P < 0.05)\).
these growing conditions, iron levels increased in plants treated with HA, particularly when 1 μM HA was used (Fig. 4b).

**Discussion**

The interaction of *Trichoderma* spp. with plants confers several benefits for the associated host that include (1) the suppression of phytopathogens using direct antagonistic mechanisms (i.e. antibiosis, mycoparasitism, competition for nutrient and space); (2) plant growth promotion; (3) enhanced nutrient availability and uptake and (4) induction of plant host resistance (Harman *et al.*, 2004).

In addition, some *Trichoderma* strains produce compounds that can cause substantial changes in the metabolism of the host plant. The involvement of secondary metabolites in the ability of *Trichoderma* spp. to activate plant defence mechanisms and regulate plant growth has been investigated (Vinale *et al.*, 2008b). HA is one of these natural products that shows antifungal and plant growth promoting activities (Vinale *et al.*, 2009b). In the present study, we investigated the Fe³⁺-chelating properties of HA.

HA, isolated by RP-18 vacuum chromatography from a 2 M NaOH 2 M extract, showed the same ¹H and ¹³C parameters as those previously reported for 2-hydroxy-2-[(4-(1-hydroxy-octa-2,4-dienylidene)-1-methyl-3,5-dioxo-pyrrolidin-2-ylmethyl]-3-methyl-butyric acid, a compound belonging to the chemical class of tetramic acids.

The naturally occurring tetramic acid derivatives have attracted significant attention because these metabolites have a wide distribution and have been found to display a remarkable diversity of biological activities playing a significant role in ecological interactions. These activities may be enhanced significantly through the chelation of the tetramic acid nucleus with metal ions (important for transport across membranes in biological tissues). It was found that in some cases the metal complexes obtained revealed higher biological activity than their ligands (Ghisalberti, 2003; Schobert & Schlenk, 2008; Athanasellis *et al.*, 2010).

Both the living microorganism and the isolated metabolite HA when tested in the CAS blue agar plates caused a colour change of the substrate from blue to orange indicating that the iron(III)-binding properties of the fungus is related also to the production of the tetramic acid.
acid derivative. Moreover, the LC–MS analysis of a HA–Fe$^{3+}$ solution showed additional signals at 455.1 and 491.1 m/z corresponding to a 1 : 1 chloride-containing complex, $\left[ M-H+Fe(III)+Cl_{2}+H\right]^{+}$ (m/z 491.1) or $\left[ M-2H+Fe(III)+Cl+H\right]^{+}$ (m/z 455.1). As chloride is a coordinating ligand for iron, it is possible that the chloride anion is directly bound to the metal (Caudle et al., 1994).

The value of $K_D$ of Fe–HA complex ($1.79 \times 10^{-25}$ M) may be directly compared with that of other chelators showing a 1 : 1 Fe : ligand stoichiometry, such as desferrioxamine, EDTA, 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione (HPD), pyoverdin and pyochelin (Kaufmann et al., 2005). As shown in Table 2, HA has lower affinity to Fe(III) than desferrioxamine, pyoverdin and HPD, while it has a stronger affinity for iron (III) than EDTA and pyochelin. This data suggest that HA could compete for available iron in solution and provide a method for iron solubilisation.

Microbial siderophores are used as iron-chelating agents that can regulate the availability of iron in the plant rhizosphere. It has been assumed that competition for iron in the rhizosphere is controlled by the affinity of the siderophore for iron. The important factors, which participate, are concentration of various types of siderophores, kinetics of exchange and availability of Fe complexes to microorganisms as well as plants. Siderophores produced by beneficial agents may have important effects on both microbial and plant nutrition. Fe$^{3+}$-siderophore complexes can be recognised and taken up by several plant species, and this process is considered crucial for plant iron uptake, particularly in calcarceous soils (Sharma et al., 2003).

*Trichoderma* metabolites may help the plant to withstand pathogens by both promoting the growth and development of root and shoot systems, and stimulating the defence mechanisms (Vinale et al., 2008b). In the present work, we confirm a possible involvement of HA in plant growth regulation probably because of its Fe(III)-binding activity. In fact, HA affected the germination of tomato seeds and improved the growth of the seedlings even in iron-deficient conditions (increment of iron concentration into the plants was also registered). This finding supports the hypothesis that HA actively influences the growth of *Trichoderma*-colonised plants.

The isolation and application of bioactive compounds produced by beneficial microorganisms responsible for the desired positive effects on plants may be a promising alternative to the use of living antagonists.

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### References


