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Exploring siderophore and hydroxamate production by fungi in the gut of Thai earthworms and their agricultural applications

Kraisittipanit, R.[1], Charerntantanakul, W.[5], Aumtong, S.[4], Meechai, T.[7], Prathum, N.[1] and Tancho, A.[2,3,4,6*]

[1]Department of Molecular biology, Faculty of Dentistry, Bangkokthonburi University, Bangkok, 10170, Thailand; [2]Natural Agriculture Research and Development Center, Maejo University, Chiang Mai 50290, Thailand; [3]Fellow Member of the Academy of Science, the Royal Society of Thailand; [4]Faculty of Agricultural Production, Maejo University, Thailand; [5]Program of Biotechnology, Faculty of Science, Maejo University, Thailand; [6]Organic Fertilizer and Hydroponics Fund, Royol Project Foundation, mae Hia, Chiang Mai 50100, Thailand; [7]Department of Chemistry, Faculty of Dentistry, Bangkokthonburi University, Bangkok, 10170, Thailand.

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Abstract Sixty-nine fungal isolates were cultured on PDA and YMA media, with 42 testingpositive for siderophore production via the CAS blue agar assay. The highest producers—VL4, RC5, RY1, Mpe9, and RL3—exceeded 80% siderophore units (SU), with RC5 achieving 92.73 ± 0.56% SU. The ferric perchlorate test showed RL3 had the highest hydroxamate siderophore concentration at 1,060.92 ± 3.00 µg/ml. Siderophore production decreased when $FeSO_4$ was added to the culture medium, and varying media impacted the levels of siderophore and hydroxamate production. Samples from earthworm guts, vermicompost, and organic agricultural soil demonstrated low siderophore production (<60%) and hydroxamate concentrations (<10 µg/ml). Notably, siderophores significantly improved iron uptake in plants, evidenced by increased iron content and shoot height in rice. Morphological and ITS1 region analyses identified RL3 as *Aspergillus* sp. (closely related to *Aspergillus niger*) and RC5 as Mucor sp. (related to *Talaromyces angelicus*). The study concluded that fungi from economic and agricultural earthworm sources are found to be potent producers of siderophores and hydroxamate siderophores, offering potential applications for enhancing plant iron uptake and soil health.

Keywords: Siderophore, Hydroxamate, Earthworm gut, Fungi, Iron, Vermicompost

Introduction

The role of fungi in soil ecosystems is increasingly recognized, particularly their capacity to produce siderophores, which are high-affinity iron-chelating

* **Corresponding Author**: Tancho, A; **Email:** Arnat009@yahoo.com

compounds. Siderophores play a crucial role in the bioavailability of iron, an essential nutrient for plant growth and microbial activity. In the context of agricultural systems, where soil health is vital for sustainable crop production, understanding the interactions between soil microorganisms and plants is paramount (Schmidt *et al.,* 2016).

Earthworms are known as "ecosystem engineers" due to their profound influence on soil structure, nutrient cycling, and microbial communities (Lavelle *et al*., 1997). The intestines of earthworms create a unique microenvironment that supports a diverse microbial community, including fungi. Recent studies have highlighted the presence of various fungal species within the intestinal tract of earthworms, demonstrating their potential for producing beneficial compounds such as siderophores (Li *et al*., 2020).

Fungal siderophores are particularly interesting because they enhance the availability of iron in the soil, which is often limited due to its low solubility, especially in alkaline and neutral pH conditions (Baker *et al*., 2013). By facilitating iron uptake, these fungi can significantly influence plant health and growth, thus contributing to higher agricultural productivity. The isolation of siderophore-producing fungi from earthworm intestines not only broadens our understanding of soil microbiology but also offers potential applications in sustainable agriculture.

Recent research has focused on the identification and characterization of siderophore-producing fungi isolated from earthworms. For instance, studies have shown that these fungi can synthesize different types of siderophores, such as hydroxamate and catecholate types, each exhibiting varying affinities for iron (Meyer *et al*., 2018). Moreover, the interaction between earthworms and fungi may create a synergistic effect, enhancing nutrient availability and improving soil health.

This study aimed to investigate the diversity of siderophore-producing fungi isolated from the intestines of earthworms by analyzing their production capabilities and the types of siderophores synthesized for understanding their ecological roles and potential applications in promoting plant growth and soil fertility.

Materials and methods

Fungal isolation agar

For the isolation of fungal strains, two types of media were utilized. The first medium was Potato Dextrose Agar (PDA), which was prepared by dissolving 2.4 g of Potato Dextrose Broth (PDB) powder (Himedia) in 100 ml of sterile water. To suppress bacterial growth, 30 mg/L of Rose Bengal and 30 mg/L

of Chloramphenicol were added to the PDA solution. Rose Bengal acts as a selective agent by inhibiting excessive fungal colony growth, allowing for easier isolation of fungi, while Chloramphenicol is an antibiotic used to prevent bacterial contamination in the fungal cultures.

Siderophore test

Screening siderophore on CAS agar

The screening for siderophore production was conducted using Chrome Azurol S (CAS) agar, with Gauze No. 1 medium as the base. The medium was composed of glucose (20.0 g), KNO_3 (1.0 g), NaCl (0.5 g), K_2HPO_4 (0.5 g), $MgSO_4$ (0.5 g), $FeSO_4$ (0.01 g), and 1.5% agar in 1 liter of distilled water, with the pH adjusted to 7.2. This medium facilitates the detection of siderophore activity, where a color change around fungal colonies on the CAS agar indicates siderophore production. Siderophores chelate iron from the CAS dye, resulting in a visible color shift, which serves as a qualitative marker for siderophore-producing strains. This screening is essential for identifying fungi capable of producing siderophores, vital for iron acquisition in iron-limited environments.

CAS siderophore solution test

The Chrome Azurol Sulphonate (CAS) solution and CAS blue agar were prepared for siderophore screening, following the method adapted from Schwyn and Neilands (1987). The CAS indicator was prepared using three separate solutions: Solution 1, which consisted of 0.0729 g hexadecyltrimethylammonium bromide (HDTMA) dissolved in 50 ml of sterile water; Solution 2, containing 0.0605 g CAS dye dissolved in 50 ml of sterile water; and Solution 3, which included 83.3 µl of HCl, 0.027 mg of $FeCl_3$, and 50 ml of sterile water. These three solutions were mixed in a 40 ml: 50 ml: 10 ml ratio, respectively, resulting in a blue CAS indicator solution, which was subsequently sterilized by autoclaving. To prepare the CAS blue agar, the CAS indicator solution was mixed with Gauze No. 2 medium (containing 20.0 g glucose, 1.0 g KNO_3, 0.5 g NaCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, and distilled water to a total volume of 1.0 L, with the pH adjusted to 7.2) in a 1:1 ratio.

For the quantification of siderophores, the CAS-Shuttle assay (Fekete *et al*., 1989) was employed. In this assay, 150 µl of CAS solution was mixed with 150 µl of the culture supernatant (obtained from a 10-day incubation in Gauze No. 2 medium) in a 1:1 ratio. A positive result was indicated by a color shift from blue to another color, such as red, green, purple, or orange. The percentage of siderophore production (siderophore units: %SU) was calculated using the

equation provided by Tailor and Joshi (2012). All experiments were performed in triplicate, and the standard error of the mean was calculated for accuracy.

$$\text{Siderophore unit} = \left[\frac{Ar - As}{Ar}\right] \times 100 \qquad \text{(Equation 1)}$$

Where Ar as "Absorbance of reference" at 630 nm.
As as "Absorbance of sample" at 630 nm.

Hydroxamate siderophore test

0.5 mL of the supernatant is mixed with 0.5 mL of 0.1 M ferric perchlorate solution. The mixture is allowed to react for 5–10 minutes at room temperature. A reddish-brown color indicates the presence of hydroxamate siderophores, which can be quantified by measuring absorbance at 530 nm using a spectrophotometer. The intensity of the color is proportional to the siderophore concentration, and a standard curve is used for precise quantification. Controls using uninoculated media ensure the specificity of the reaction. This method provides both qualitative and quantitative data on siderophore production in fungal cultures.

Fungi identification

The molecular identification of fungi was performed by extracting genomic DNA from mycelia grown on PDA plates incubated at 30°C, which were harvested after 3 days. DNA extraction was carried out using the Plant DNeasy Minikit (QIAgen GmbH, Hilden, Germany) following the manufacturer's protocol. The extracted DNA was then subjected to electrophoresis on a 0.8% (w/v) agarose gel at 220 volts for 35 minutes. The concentration and purity of the DNA were measured using a spectrophotometer at an absorbance ratio of 260/280 nm. The DNA samples were subsequently diluted to a concentration of 5.0 ng, making them ready for downstream experiments. The internal transcribed spacer (ITS) region, specifically the ITS1 and ITS4 areas of the rRNA gene cluster, was amplified using PCR with ITS1 and ITS4 primers. The PCR protocol involved an initial 5 cycles of denaturation at 94°C for 5 minutes, annealing at 35°C for 1 minute, and extension at 72°C for 1 minute. This was followed by 35 cycles of denaturation at 94°C for 5 minutes, annealing at 52°C for 1 minute, and extension at 72°C for 10 minutes. After amplification, 5 µl of the PCR product was mixed with 1 µl of red-safe color detector (Ultrapower, Japan) and separated via electrophoresis on a 1.8% (w/v) agarose gel at 220 volts for 70 minutes. The PCR product bands were visualized using an LED light source and photographed with a digital camera. The PCR products were then sequenced, and the resulting sequences were submitted to the NCBI GenBank database for comparison with existing sequences using the BLASTn tool.

Siderophore bioassay and data analysis

Prior to testing, the soil used for the experiment was analyzed to assess its fertility by sending it to the Center for Research and Development of Natural Agriculture at Maejo University. The experiment aimed to evaluate the effect of a siderophore extract with a concentration of 80% SU on corn growth. The study employed a Completely Randomized Design (CRD) with 10 replications. The siderophore extract was applied as a foliar spray to the underside of the corn leaves at 1 month of age, repeated at 7-day intervals. The spraying continued for 3 months and was then discontinued. The control group received no siderophore treatment. After 4 months, growth parameters, including plant height and corn cob weight, were measured. The average values were calculated, and statistical analysis was performed using SPSS at a significance level of 0.01.

Results

CAS blue agar screening siderophore

The experiment assessed siderophore production by fungal isolates using the CAS agar assay, with color changes (yellow or pink) (shown in figure 1) and hydrolysis indices indicating siderophore activity. High siderophore production was seen in isolates such as RY2 (index 3.95), Mpe2 (3.84), MP1 (3.62), and Pe6 (3.46), typically correlated with pink coloration. Moderate activity was found in YC2 (2.25), RC5 (2.42), and RD4 (3.01), while lower production levels were observed in MG4 (1.07) and YL3 (1.23) (shown in table 1). Pink reactions generally indicated stronger siderophore activity, whereas yellow-producing isolates showed a range from moderate to low activity. These results suggest diverse siderophore production capabilities among the isolates, highlighting potential applications in enhancing plant growth and soil health through improved iron availability.

The quantification of siderophore and hydroxamate siderophore

A quantitative analysis of siderophore production by various fungal isolates sourced from earthworm guts, agricultural soil, and vermicompost is shown in Table 2. Among the isolates, RL3 exhibited the highest siderophore production, measuring 90.54 ± 0.41ab% SU, accompanied by a red-colored supernatant. Other notable producers, such as Mpe9 and RC5, also showed elevated siderophore activity, exceeding 80% SU. In contrast, isolates from vermicomposting systems and earthworm guts demonstrated moderate to lower siderophore production, with values ranging between 30.48% and 57.59%.

Table 1. 69 isolates positive siderophore producing fungi on CAS agar test

Samples	Isolates	CAS agar assay	Hydrolysis index	Samples	Isolates	CAS agar assay	Hydrolysis index
EW	YC2	yellow	2.25	EW	RC5	pink	2.42
EW	YC6	yellow	2.26	EW	RC8	yellow	1.15
EW	YC7	yellow	2.10	EW	RC9	yellow	1.65
EW	YC8	yellow	1.60	VCL	VL2	yellow	2.03
EW	YC9	yellow	1.52	VCL	VL4	yellow	2.54
EW	YC11	pink	2.42	EW	Pe1	yellow	2.79
EW	RY1	yellow	2.22	EW	Pe2	yellow	1.94
EW	RY2	pink	3.95	EW	Pe3	yellow	2.86
EW	RY3	yellow	2.32	EW	Pe5	pink	2.59
EW	RD4	pink	3.01	EW	Pe6	pink	3.46
EW	RD5	pink	3.30	EW	MG2	yellow	1.90
EW	YD10	yellow	1.84	EW	MG3	pink	2.57
EW	RK2	yellow	1.46	EW	MG4	pink	1.07
EW	RK4	pink	3.18	EW	MG5	pink	2.51
EW	RK7	yellow	1.47	EW	Mpe2	yellow	3.84
EW	YL2	yellow	1.81	EW	Mpe3	pink	3.31
EW	YL3	yellow	1.23	EW	Mpe5	pink	3.29
EW	RL1	pink	2.75	EW	Mpe8	pink	3.22
EW	RL3	yellow	2.65	EW	Mpe9	yellow	1.80
EW	RC1	yellow	2.51	EW	MP1	pink	3.62
EW	RC2	yellow	1.30	EW	MP6	pink	2.80

EW = earthworm gut, VCL = Vermicompost liquid

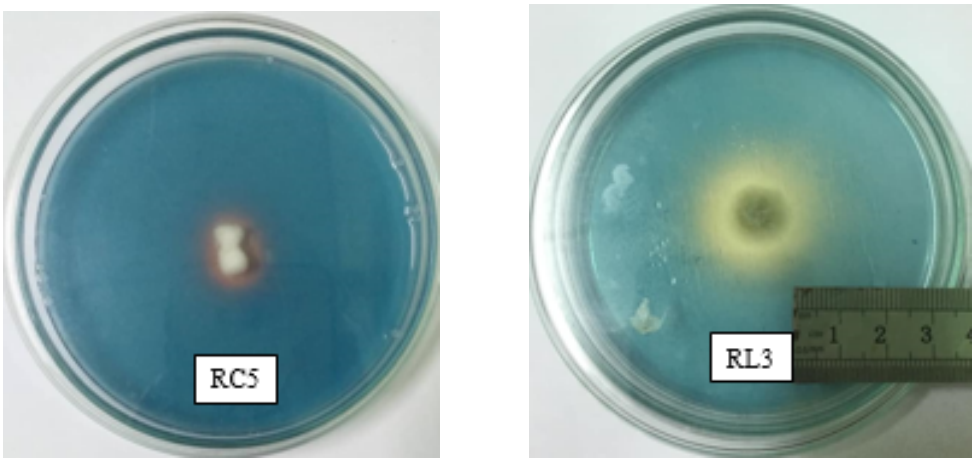

Figure 1. Two different highest siderophore test from CAS assay: the fungal isolate RC5 showed pink clear zone and isolate RL3 showed in yellow

Table 2. Quantitative production of siderophores and hydroxamate siderophore by fungi isolated from earthworm guts, agricultural soil and earthworm production

	codes/name	supernatant color	Siderophore unit (% SU) ± SD
Fungal isolates	VL4	red	82.05 ± 0.79a
	RC5	red	82.73 ± 0.56a
	RY1	yellow	80.29 ± 0.57b
	Mpe9	red	87.01 ± 0.78ab
	RL3	red	90.54 ± 0.41ab
Gut and others	vermicompost	dark-green	57.59 ± 0.30c
	vermicomposting liquid	dark-green	50.72 ± 0.17cd
	agricultural soil	green	57.60 ± 0.19c
	Metaphire peguana gut	green	30.48 ± 0.22d
	Metaphire posthruma gut	green	51.53 ± 0.64cd
	Perionyx sp. 1 gut	blue-green	26.97 ± 0.24d

$P < 0.01$

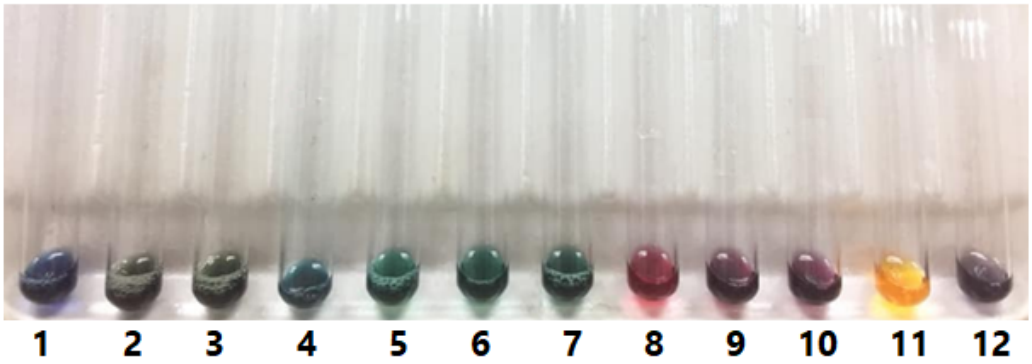

Figure 2. The qualitative test of siderophore: 1 = negative control, 2 = vermicompost liquid, 3 = vermicompost, 4 = Perionyx ap. 1 gut, 5 = Metaphire peguana gut, 6 = Metaphire posthruma gut, 7 = agricultural soil, 8 = RL3, 9 = RC5, 10 = Mpe9, 11 = RY1 and 12 = VL4

The quantification of hydroxamate siderophores produced by the five highest fungal isolates in two different media is illustrated in Table 3 and Figure 3). The isolates include VL4, RC5, RY1, Mpe9, and RL3, with RL3 showing the highest hydroxamate siderophore production at 1,060.92 ± 3.00 µg/ml with a red supernatant color. Other isolates, such as Mpe9 and RC5, also exhibited substantial siderophore activity with values of 145.49 ± 4.38 µg/ml and 89.56 ± 1.95 µg/ml, respectively. The ferric perchlorate test confirmed the presence of hydroxamate siderophores, as indicated by the varying colors in the test tubes. Notably, lower concentrations (<10 µg/ml) did not react with the ferric

perchlorate test, and some samples like vermicompost and earthworm gut isolates did not produce significant levels. It showed the distinct color changes corresponding to the siderophore concentrations in the different fungal isolates (Figure 2).

Table 3. The quantitation test of hydroxamate siderophore produced by the five highest fungal hydroxamate in two different media

	codes/name	supernatant color	Hydroxamate siderophore (µg/ml) ± SD
Fungal isolates	VL4	yellow	276.25 ± 2.89b
	RC5	yellow	89.56 ± 1.95d
	RY1	pink	43.96 ± 2.77e
	Mpe9	pink	145.49 ± 4.38c
	RL3	red	1,060.92 ± 3.00a
Gut and others	vermicompost	**	*
	vermicomposting liquid	**	*
	agricultural soil	**	*
	Metaphire peguana gut	**	*
	Metaphire posthruma gut	**	*
	Perionyx sp. 1 gut	**	*

$P < 0.01$, * less than 10 µg/ml is resulted non-reactive with ferric perchlorate test., ** clear color

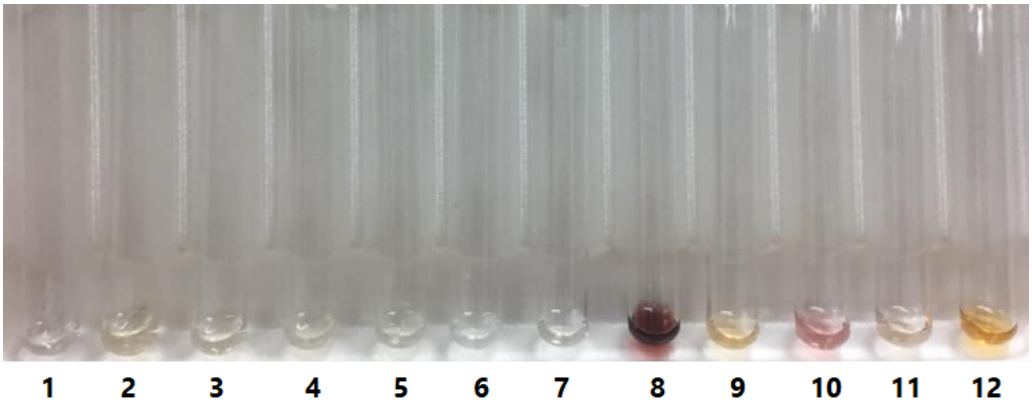

Figure 3. The Hydroxamate siderophore from ferric perchlorate ($FeCl_3$) test: 1 = negative control, 2 = vermicompost liquid, 3 = vermicompost, 4 = Perionyx ap. 1 gut, 5 = Metaphire peguana gut, 6 = Metaphire posthruma gut, 7 = agricultural soil, 8 = RL3, 9 = RC5, 10 = Mpe9, 11 = RY1 and 12 = VL4

The phylogenetic tree of the RL3 fungal isolate based on ITS1 sequencing, which revealed that RL3 is closely related to *Aspergillus niger* as shown in Figure 4. The phylogenetic tree compares RL3 with several other fungi, where RL3

exhibited strong siderophore production, indicated by the clear blue halo surrounding the fungal colony. This analysis is confirmed RL3's classification and its significant potential for siderophore production, making it an important candidate for further research on iron-chelation in fungi.

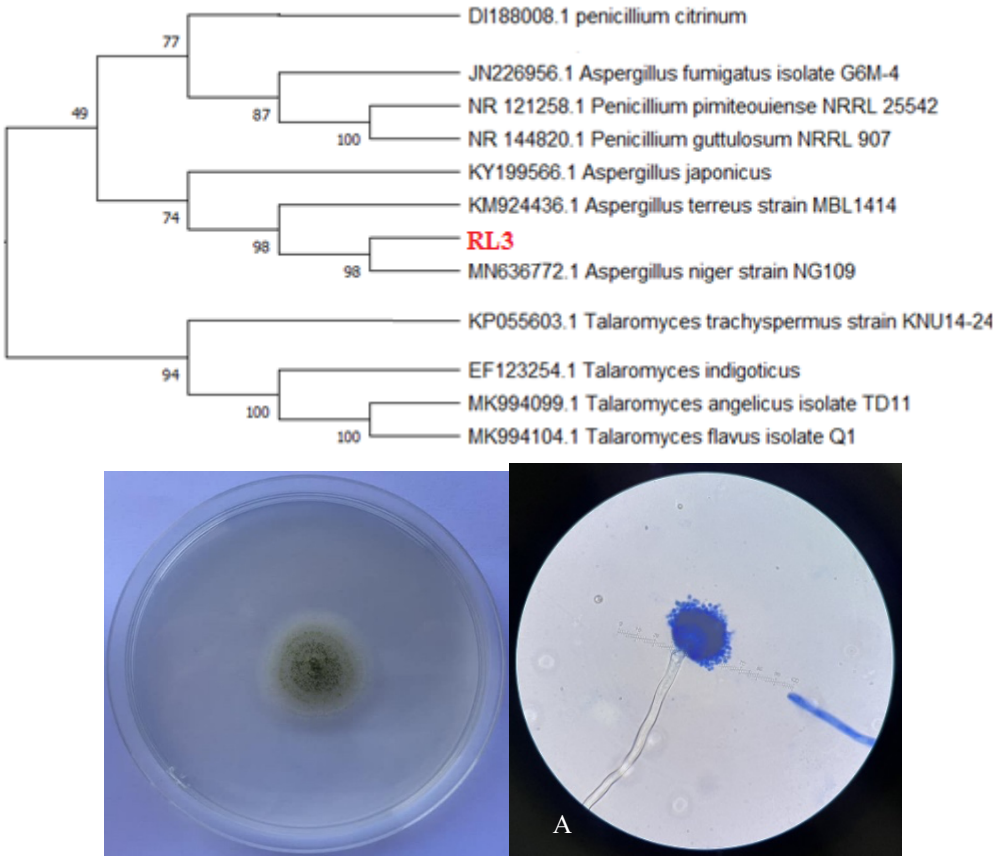

Figure 4. Phylogenetic tree of RL3 fungal isolate

The effect of Siderophore from RL3 crude extract to corn

Soil parametersincluding general characteristics, trace elements, minor elements, and heavy metals is shown in Table 4. EC (Electrical Conductivity) is 111 µS/cm, indicating low salinity suitable for rice cultivation, while the pH is slightly acidic at 6.3, ideal for rice growth. The organic matter content is 2%, reflecting moderate soil fertility. Nitrogen (N) is relatively low at 0.06 ppm, with Phosphorus (P) at 13 ppm and Potassium (K) at 153 ppm. Magnesium (Mg), Calcium (Ca), and Copper (Cu) levels are 350 ppm, 3,043 ppm, and 26 ppm, respectively, all essential for plant health. Iron (Fe) and Manganese (Mn) are 1.87

ppm and 234 ppm, while Zinc (Zn) is undetected. Notably, toxic metals like Arsenic (As), Mercury (Hg), Lead (Pb), and Cadmium (Cd) are absent, indicating safe soil conditions. Although the soil is suitable for rice cultivation, additional Nitrogen and Zinc may be required for optimal growth.

Table 4. Soil parameters before rice plantation

Parameter pf soil	Value
EC (µs/cm)	111
pH	6.3
%OM	2
N (ppm)	0.06
P (ppm)	13
K (ppm)	153
Mg (ppm)	350
Ca (ppm)	3,043
Cu (ppm)	26
Fe (ppm)	1.87
Zn (ppm)	ND
Mn (ppm)	234
As (ppm)	ND
Hg (ppm)	ND
Pb (ppm)	ND
Cd (ppm)	ND

ND = not detection for test

The results demonstrated a clear and significant positive effect of siderophore on corn growth. Corn plants treated with siderophore achieved an average shoot height of 1.54 ± 0.61 m, markedly higher than the 1.02 ± 0.72 m observed in untreated plants. Similarly, the corn weight in treated plants was 0.71 ± 0.82 kg, compared to 0.52 ± 0.99 kg in the control group (Shown in table 5), indicating improved biomass production. These findings are further substantiated by visual evidence, where siderophore-treated plants appeared taller, healthier, and produced fuller, more robust corn cobs (Shown in figure 5). The statistical significance of these differences, as confirmed by the F-test, underscores the efficacy of siderophore in enhancing corn growth and productivity.

Table 5. The effect of RL3 siderophore on iron concentration and early growth of corn over 4 months

Treatments	shoot height (without siderophore)	shoot height (added siderophore)
Height (m)	1.02 ± 0.72b	1.54 ± 0.61a
Corn weight (kg)	0.52 ± 0.98b	0.71 ± 0.82b
%CV	1.56	1.75
F-test	**	**

Figure 5. The effect of RL3 siderophore on early growth of corn over 4 months A = Corn without/with siderophore, B = Corn without siderophore, C = With siderophore

Discussion

This study highlighted the remarkable ability of *Aspergillus niger* (RL3) to promote iron uptake, and enhanced chlorophyll production, and improved corn growth through hydroxamate siderophore secretion. RL3 exhibited exceptional performance, producing 90.54 ± 0.41% siderophore units (SU) and 1,060.92 ± 3.00 µg/ml hydroxamate siderophores. Beyond supporting nutrient uptake, siderophores influenced plant hormone regulation, contributing to the observed increases in shoot height and biomass.

Siderophores facilitate iron uptake, which is essential for processes such as photosynthesis, respiration, and nitrogen fixation (Rout and Sahoo, 2015). Enhanced iron availability plays a key role in regulating plant hormones—particularly auxins, gibberellins, and cytokinins—thereby boosting shoot height and biomass. Auxins regulate cell elongation and root development. The enhanced iron uptake facilitated by RL3 promotes auxin biosynthesis, resulting in extended root systems and improved water and nutrient absorption (Chen *et*

al., 2010). This aligns with the robust root development observed in RL3-treated corn, leading to increased plant height and biomass.

Gibberellins (GAs) also drive shoot elongation and stimulate cell division. Iron ensures optimal GA biosynthesis, which promotes rapid stem elongation and cell expansion. The significant increase in shoot height from 1.02 ± 0.72 m to 1.54 ± 0.61 m in RL3-treated corn reflects this process (Bashir *et al*., 2017). Similarly, cytokinins, which delay leaf senescence and promote chlorophyll synthesis, play a key role in increasing photosynthetic activity. Enhanced cytokinin activity, driven by improved iron uptake, elevated chlorophyll-a and chlorophyll-b levels in treated plants (1.1401 ± 0.0125 µg/l and 0.9684 ± 0.0104 µg/l, respectively), contributing to prolonged photosynthesis and higher biomass production (Gupta *et al*., 2015).

RL3 produced significantly higher levels of hydroxamate siderophores (1,060.92 ± 3.00 µg/ml) compared to other isolates like Mpe9 (145.49 ± 4.38 µg/ml). Hydroxamate siderophores exhibit high affinity for iron, ensuring efficient iron uptake even in nutrient-deficient soils (Marschner, 2012). This finding aligns with Ahmed *et al*. (2020), who demonstrated that *A. niger* enhances maize growth by increasing iron availability in iron-deficient conditions.

Phylogenetic analysis confirmed that RL3 is closely related to *A. niger*, a species known for its high siderophore production and agricultural applications. *A. niger* regulates siderophore production through the SREA system, which modulates output based on environmental iron levels to ensure efficient resource allocation (Haas, 2014). This regulatory mechanism explains RL3's ability to maintain high siderophore production under iron-deficient conditions, as observed in this study.

The results demonstrated a significant improvement in corn growth due to RL3 treatment. The shoot height increased from 1.02 ± 0.72 m to 1.54 ± 0.61 m, and biomass rose from 0.52 ± 0.98 kg in untreated plants to 0.71 ± 0.82 kg in RL3-treated plants. These results aligned with Bashir *et al.* (2017), who reported enhanced crop yields from microbial inoculants by improving nutrient uptake. Although the increase in chlorophyll levels was not statistically significant ($P \leq 0.05$), the trend supports enhanced photosynthesis. Iron plays a crucial role in chlorophyll synthesis, further promoting plant growth and vitality (Sharma *et al*., 2013).

Environmental factors such as pH and soil composition also influenced RL3's performance. The soil pH of 6.3 provided favorable conditions for RL3 growth, consistent with findings by Tailor and Joshi (2012), who reported that *A. niger* thrives between pH 3.0 and 8.0. However, low nitrogen (0.06 ppm) and undetectable zinc levels highlight the need for additional fertilization. Ahmed *et*

al. (2020) similarly emphasized that microbial inoculants enhance the availability of essential micronutrients, further supporting crop performance.

This study confirms that *A. niger* (RL3) produces high levels of hydroxamate siderophores, facilitating iron uptake and regulating hormonal pathways that drive shoot elongation and biomass production. Phylogenetic analysis highlights RL3's evolutionary significance, positioning it as an ideal candidate for developing bioinoculants for sustainable agriculture. Future research should explore the potential synergy between *A. niger* and other beneficial microorganisms to optimize crop productivity and promote sustainable farming practices.

This study is confirmed that *Aspergillus niger* (RL3) possesses exceptional siderophore production capabilities, leading to enhanced iron uptake, increased growth in corn. The hydroxamate siderophores produced by RL3 not only facilitate iron transport but also influence plant hormonal pathways, including auxins and gibberellins, contributing to increased shoot height and biomass. The phylogenetic analysis is further established RL3's evolutionary significance, positioning it as a model microorganism for sustainable agriculture. Future research should explore the synergistic potential of combining *A. niger* with other beneficial microbes to develop microbial consortia that maximize crop productivity while promoting sustainable farming practices.

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Conflicts of interest

The authors declare no conflict of interest.

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