Characterization and Fungal Inhibition Activity of Siderophore from Wheat Rhizosphere Associated *Acinetobacter calcoaceticus* Strain HIRFA32

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Abstract *Acinetobacter calcoaceticus* HIRFA32 from wheat rhizosphere produced catecholate type of siderophore with optimum siderophore (ca. 92 % siderophore units) in succinic acid medium without FeSO₄ at 28 °C and 24 h of incubation. HPLC purified siderophore appeared as pale yellow crystals with molecular weight [M⁺]¹ *m/z* 347.18 estimated by LCMS. The structure elucidated by ¹H NMR, ¹³C NMR, HMQC, HMBC, NOESY and decoupling studies, revealed that siderophore composed of 2,3-dihydroxybenzoic acid with hydroxyhistamine and threonine as amino acid subunits. In vitro study demonstrated siderophore mediated mycelium growth inhibition (ca. 46.87 ± 0.5 %) of *Fusarium oxysporum*. This study accounts to first report on biosynthesis of acinetobactin-like siderophore by the rhizospheric strain of *A. calcoaceticus* and its significance in inhibition of *F. oxysporum*.

Keywords *Acinetobacter calcoaceticus* · Wheat rhizosphere · NMR · Acinetobactin-like siderophore · *Fusarium oxysporum*

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Introduction

Rhizosphere is the most effective niche for diverse bacteria with plant growth promoting (PGP) traits [1–3]. The rhizosphere and its inhabiting microorganisms fulfil important ecological functions and are responsible for plant growth and health [4]. These bacteria have several mechanisms for plant growth promotion and biocontrol of phytopathogens [5–7]. Siderophore production is considered as one of the indirect mechanisms for promotion of plant growth. Siderophores are low-molecular-weight molecules that are secreted by microorganisms to take up iron from the environment [8]. Rhizobacteria produce different types of siderophores and these siderophore/s help in better survival of the bacteria in the rhizosphere. The siderophores produced by rhizosphere bacteria can supply iron to plants under iron limiting conditions [9] many strains of rhizobacteria, which are effective in biocontrol, produce siderophore. Siderophore also can act as one of the factors for inducing systemic resistance of plants and preventing growth of certain pathogenic bacteria and fungi in iron deficient conditions [10–13]. Their modes of action in suppression of disease may be based on competition for iron with the pathogen [9]. Thus, siderophore is one of the important PGP traits found in competent rhizobacteria.

Members of genus *Acinetobacter* are ubiquitous in nature [2, 3, 14, 15]. They are commonly found in rhizosphere of wheat [1, 3, 16, 17]. These rhizospheric *Acinetobacter* strains exhibit PGP traits such as production of indole acetic acid [1], mineral solubilization [3], nitrogen fixation [18], antimicrobial activity [19] and synthesis of siderophores [2, 3, 18]. However, there is paucity of information on structure and role of siderophore from *Acinetobacter* found to be associated with rhizosphere of crop plant such as wheat. In case of genus *Acinetobacter*, siderophore

synthesis is intensely studied till date only in clinical strains of *A. baumannii* and *A. haemolyticus*. These clinical strains of *Acinetobacter* are known to produce two siderophores; namely acinetobactin and acinetoferrin, which have been characterized from *A. baumannii* and *A. haemolyticus*, respectively [20–22]. Thus, the objective of this investigation was to purify and perform structural characterization of siderophore from a strain of *A. calcoaceticus* previously isolated from rhizosphere of wheat. Further to investigate the role of this siderophore in suppression of *Fusarium oxysporum*, a fungal phytopathogen.

Materials and Methods

Characterization of *Acinetobacter* sp. HIRFA32

Isolate HIRFA32 previously isolated from the rhizosphere of wheat variety HI1535 during flowering stage [3] was identified by polyphasic approach, which included chromosomal transformation assay (CTA) [23], 16S rRNA gene sequencing, biochemical identification by API GN32 and BIOLOGTM system, physiological characterization, FAME analysis, determination of GC content and DNA–DNA hybridization with the closest affiliation. The 16S rRNA gene sequencing using universal primers for identification of the *Acinetobacter* isolate up to species level was performed by the method described previously [24]. Biochemical characterization of the *Acinetobacter* isolate was carried out using BIOLOGTM identification system (BIOLOGTM, USA) which was as follows: A pure culture of a bacterium was grown on a BIOLOGTM Universal Growth w/5 % sheep blood agar plate (BIOLOGTM, USA) for 18 h. The bacteria were swabbed from the surface of the agar plate, and suspended to a specified density in GN Inoculating Fluid (BIOLOGTM, USA). An aliquot (150 µl) of bacterial suspension was pipette into well of the GN2 MicroPlate (BIOLOGTM, USA). The MicroPlate was incubated at 30 °C for 24 h. The MicroPlates were read visually and compared to the GN Database (BIOLOGTM, USA), and a result was determined. Physiological characterization of the *Acinetobacter* isolate included scoring of growth at pH range (2–12), different temperature (10, 20, 28, 37, 42, 44 and 55 °C) and NaCl concentration (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 % w/v). FAME analysis of the *Acinetobacter* isolate was done by the Sherlock System using RTSBA6 method as described earlier [2]. The GC content of the *Acinetobacter* strain was determined by HPLC method (at DSMZ, Germany) as per the methodology described earlier [25], which included HPLC system (Shimadzu Corp., Japan) equipped with analytical column VYDAC201SP54, C18, 5 µm (250 × 4.6 mm) and a SPD-

6A UV spectrophotometric detector. The reference DNA used for this study was from *Bacillus subtilis* DSM 402, *Xanthomonas campestris* pv. *campestris* DSM 3586T and *Streptomyces violaceoruber* DSM 40783. The GC content was calculated according to the method of Mesbah et al. [26]. The DNA–DNA hybridization was performed (at DSMZ, Germany), which included disruption of cell by French pressure cell (Thermo Spectronic), purification of DNA by chromatography on hydroxyapatite as described by Cashion et al. [27] and further DNA–DNA hybridization was carried out as per the method described by De Lay et al. [28] under the modification described previously by Huss et al. [29] using a model Cary 100 Bio UV–Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian). The isolate was maintained on LB medium throughout the study.

Optimization of Siderophore Production

Siderophore production by *Acinetobacter* sp. HIRFA32 was determined by CAS blue plate method [30] and the siderophore units (SU) was calculated by the formulae described earlier [31, 32]. The type of siderophore produced by *Acinetobacter* strain was determined by employing methods such as Csaky, Arnow's and Hathway's method [32, 33]. Optimization studies for siderophore production by *Acinetobacter* sp. HIRFA32 included effect of type of iron restricted media (succinic acid medium, SAM [34]; glucose minimal medium, GMM [35]; mannitol minimal medium, MMM [35]), temperature (20, 28, 37 and 42 °C) and Fe³⁺ concentration (0.0, 1.0, 20.0 and 200 µM) on siderophore production. The optimum medium was supplemented with FeSO₄ in order to obtain the different concentrations of iron. For the optimization studies, the media were inoculated with the 100 µl of overnight grown cultures (ca. 10⁷ cell/ml) and kept at 30 °C, 200 rpm for 48 h. Growth was measured for each culture at OD 530 nm. The cell free filtrate was mixed in equal proportion with CAS reagent and allowed to stand at room temperature for 30 min. The absorbance was measured at 630 nm. The siderophore unit (% SU) was calculated [31, 32]. The siderophore production was also motioned at various time intervals up to 120 h at optimum conditions.

Partial Purification by XAD-2 Column Chromatography

Cell free supernatant was harvested and processed for purification of siderophore as follows: One percent inoculum (ca. 10⁷ cells/ml) was inoculated into a 500 ml flask

containing 200 ml of SAM. The cultures were grown for 24 h by incubating at 28 °C and 200 rpm. After incubation, supernatant was obtained by centrifuging the cultures at 10,000 rpm for 20 min. The supernatant was then acidified to pH 2 with conc. HCl. Ethyl acetate extraction (2 vol) of the acidified supernatant was done. The aqueous layer thus recovered was subjected to XAD-2 column. The column was prepared as follows: XAD-2 (80 gm) was activated by allowing it to stand in methanol for 15 min. Methanol was carefully decanted and the resin was left in distilled water for 10 min. The activated resin was then transferred to a 30 × 5 cm glass column containing distilled water and allowed to pack overnight. The bed volume so obtained was 130 ml. The loading and the elution rates were determined by the bed volume and the manufacturer's instructions for XAD-2. The column was packed (approximately 14 cm) with the prepared XAD-2 and equilibrated with two bed volumes of ddH₂O. Partial purification proceeded as follows: The acidified supernatant was passed through the column and the flow-through was collected. Once all supernatant had been run, the column was washed with two bed volumes of ddH₂O. This wash was also collected in a separate beaker. The column was then eluted with approximately 250 ml methanol 50 % (v/v) and approximately twelve fractions (20 ml) were collected. Fractions were collected until no colour was present in the flow-through. The column was then washed with four bed volumes of methanol, followed by four bed volumes of ddH₂O to re-equilibrate the column. All the fractions obtained were tested by CAS reagent. The fractions, which appeared yellow to brown in color, showed presence of siderophore and were pooled together and were concentrated in rotary evaporator (BUCHI Labortechnik AG CH-9230, Flawil, Switzerland) set at 50 °C [13]. The dried sample was re-dissolved in 2 ml ddH₂O and stored at 4 °C till further use.

HPLC purification

The partially purified siderophore was scanned on UV–Vis spectrophotometer (UV-1601, Shimadzu Labsolutions, Japan) in the range of 190–700 nm as per the method described previously [36]. Siderophore from HIRFA32 was further purified on preparative HPLC (Shimadzu) equipped with C-18 reverse phase column (particle size 5 µm, 250 × 4.6 mm) and UV detector. Methanol was used for elution. Gradient used was as follows: 10 % (v/v) methanol for 5 min and then from 10 to 50 % within 30 min, thereafter held for 15 min. The flow rate was 46.5 ml/min and detection was at 310 nm. The CAS positive fraction was dried by evaporation at 40 °C and the purified siderophore was used for further studies.

Structure Determination of the Siderophore

The pure siderophore was analyzed by FT-IR spectrophotometry (FT-IR-8400, Shimadzu). The powder of the pure siderophore was dissolved in D₂O and analysed by NMR (Varian Mercury 300 MHz). The chemical shifts were given in δ values (ppm) relative to HOD, and the coupling constants were expressed as *J* values in Hz. Standard programs were used for ¹H–¹H chemical shift correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC). Me decoupling at centre C-5' studies were also performed to elucidate the structure of the siderophore. Specific optical rotation was recorded using polarimeter (JASCO) and the molecular weight of the pure compound was determined by LCMS.

Determination of Siderophore Mediated In Vitro Inhibition of *F. oxysporum*

Fusarium oxysporum (MTCC 284) was a plant pathogen used in this part of study and antagonism was screened on King's B medium. An agar plug (1 × 1 cm) taken from an actively growing *F. oxysporum* culture and was placed on the centre of the medium. Simultaneously, HIRFA32 was streaked in the form of 'V' and 3 cm away from the agar plug at sides towards the edge of Petri plates. Plate inoculated with fungal agar plugs alone was used as control. The plates were incubated at 30 °C until fungal mycelia completely covered the agar surface in control plate [2, 37]. The percent mycelium inhibition was calculated by the formulae:

$$\text{Percent mycelia growth inhibition (\%)} = (dc - dt/dc) \times 100,$$

where dc is the average diameter of fungal colony in control and dt is the average diameter of fungal colony in treatment group.

Deposition of Strain HIRFA32

The culture was deposited to German Collection of Microorganisms and Cell Cultures, Germany and Bioresource Collection and Research Centre, Taiwan with accession number DSM 22318 and BCRC 80107, respectively.

Results and Discussion

Siderophores are low molecular weight (350–1500 Da) organic molecules, which can compete for ferric iron in ferric hydroxide complexes [38]. Microbial siderophores are important to plant pathology as determinants of

biocontrol activity and/or ecological factors influencing the iron nutrition of plants. As already mentioned, siderophores are important in induced systemic resistance. Several reports state the structure of siderophores produced by PGP rhizobacteria such as schizokinen produced by *Rhizobium leguminosarum* IARI 917 [13]; pyoverdine by *Pseudomonas fluorescens* [39]; protochelin by *Azotobacter vinelandii* [11]; Rhizobactin by *Rhizobium meliloti* [30] and many more. However, there is no report of structural characterization of siderophore from PGP strain of *Acinetobacter*. HIRFA32 a strain of *Acinetobacter* sp. possess other PGP traits along with siderophore production and has also exhibited plant growth promotion of wheat seedlings in pot experiments [3]. Thus, HIRFA32 was selected in the present study and polyphasic identification of this strain was performed followed by the structure elucidation of the siderophore produced by this strain.

Isolate HIRFA32 belonged to genus *Acinetobacter* which was confirmed by CTA and 16S rRNA gene sequencing. It was revealed that strain HIRFA32 had *Acinetobacter calcoaceticus* as its closest relative (96.93 % similarity). The partial 16S rRNA gene nucleotide sequence determined in this study has been deposited in GenBank database with the accession number EU921465. Physiological analysis showed that HIRFA32 can grow optimally at 28 °C, pH 6 and in the presence of 0.5 % (w/v) NaCl. The strain was able to grow in the temperature range of 10–37 °C and pH 6–10. It could grow at all the tested concentrations of NaCl. Biochemical characterization of HIRFA32 by done API identification system identified HIRFA32 as *A. baumannii* with % d as 96.0 (Data not shown). On the BIOLOG™ identification, it was found that the HIRFA32 was closest to *A. calcoaceticus* with SIM value of 0.387. Fatty acid composition of HIRFA32 was C_{21:0}, C_{12:0} 2OH, C_{12:0} 3OH, C_{14:0}, C_{15:0}, C_{16:1} ω7c alcohol, C_{16:0} N alcohol, C_{16:0}, C_{17:1} ω8c, C_{17:0} 10-methyl, C_{18:3} ω6c (6, 9, 12), C_{18:1} ω9c, C_{18:0}. The strain HIRFA32 was identified as *A. calcoaceticus* with a SI value of 0.282 by FAME analysis. The DNA GC content of HIRFA32 was 38.7 mol %. DNA–DNA hybridization studies were performed with the closest match; *A. calcoaceticus* (DSM30006^T) and the results showed HIRFA32 had 77.4 % DNA–DNA similarity with *A. calcoaceticus* (DSM30006^T).

Strain HIRFA32 previously isolated from rhizosphere of wheat was identified as *Acinetobacter* sp. based on 16S rRNA gene sequencing as it is known that comparative analysis of comprehensive databases of bacterial 16S rRNA sequences with appropriate software allows rapid identification of bacteria based on their rRNA sequence data and 16S rRNA gene sequencing is considered as a powerful tool for species level identification of bacteria [40–42]. However, it is considered that a 16S rRNA gene

sequence similarity of 97 % should become the boundary for delineation of prokaryotic species [43] and more recently, a more relaxed cut-off value of 98.7–99 % has been proposed after inspection of a large amount of recently published data [44, 45]. Since, HIRFA32 showed less than 97 % similarity with type strains of *Acinetobacter*; further characterized by a polyphasic approach which included physiological and biochemical characterization, chemotaxonomic characterization (FAME analysis), determination of base composition and DNA–DNA hybridization with the closest match was performed. Biochemical characterization of HIRFA32 was also done using API identification system (Biomereux Ltd, France) [46] which, is mainly meant for identification of clinical strains; as well as by BIOLOG system. However, the SIM value obtained for this strain in the BIOLOG system was very low suggesting the need for other techniques for identification of this strain. FAME analysis was used as a tool of chemotaxonomic identification which identified HIRFA32 as *A. calcoaceticus* but again with a very low SI index [47]. Further, genetic tools such as base composition and DNA–DNA hybridization were also employed. The GC content of *Acinetobacter* sp. HIRFA32 was found to be within the range reported for the genus *Acinetobacter* [15, 48]. Taking into consideration 70 % DNA–DNA similarity as the threshold value for the definition of new bacterial species [49], it was concluded that HIRFA32 is a strain of *A. calcoaceticus*. Thus, polyphasic approach could be used as a promising tools in absolute identification of members of *Acinetobacter* mainly those which are isolated from environmental source.

There are over 500 described siderophores that are classified based on their chelating group specific for ferric iron [50]. There are two main siderophore classes, the catechol-type and the hydroxamate-type. HIRFA32 in the present study was able to produce catechol-type siderophores based on a test selective for detection of catecholate siderophore [51]. Catechol-type siderophores bind ferric iron with adjacent hydroxyls of catechol rings, and are almost always derived from 2,3-dihydroxybenzoic acid (DHBA) [52]. The optimization of siderophore production by HIRFA32 was performed by using three different iron restricted media such as SAM, GMM and MMM in absence of iron, with iron present at low (0.1 μM FeSO₄), medium (20 μM FeSO₄) and high concentrations (200 μM FeSO₄) and at different temperatures; 20, 28, 37 and 42 °C. Previous reports on siderophore production suggest that these parameters affect the siderophore production [34, 35]. Optimization studies revealed that HIRFA32 produced highest siderophore (ca. 92 % siderophore units) in SAM without FeSO₄ at 28 °C (Table S1). The optimization studies revealed that the iron availability probably regulates the synthesis of siderophore in HIRFA32 as optimum

Fig. 1 Representative ^1H NMR spectrum of siderophore from *A. calcoaceticus* HIRFA32 ^1H NMR spectrum was recorded on D_2O (300 MHz). Scale represents δ values

Table 1 NMR spectral data for siderophore from *A. calcoaceticus* HIRFA32

Position	δH , mult, intrgt, (J in Hz) ^a	$\delta\text{C}(\text{mult})^b$	^1H – ^1H COSY	HMBC ^c
1		–		
2		129.88		C6
3	7.2, s, 1H	116.65	H5	C2
4		–		
5	8.45, s, 1H	133.56	H3	C3
6	2.95, t, 2H	22.55	H7	C7
7	3.65–3.85, m, 2H	43.77	H6	C6
8		–		
9		167.39		
1'		–		
2'		170.25		
3'				
4'	4.43, d, 1H	58.26	H5'	C5', C5'–CH ₃
5'	4.43, dq, 1H	78.70	H4', H5'–CH ₃	C4', C5'–CH ₃
5'–CH ₃	1.25, d, 1H	16.1	H5'	C5', C4'
1''		115.6		
2''		147.32		
3''		144.58		
4''	6.83, dd, 1H	(7.8, 1.1) 119.819	H5'', H6''	C5'', C6''
5''	6.62, t, 1H (8)	118.994	H4'', H6''	C4'', C6''
6''	7.02, dd, 1H (8.1, 1.1)	119.613	H4'', H5''	C4'', C5''

^a Spectra were recorded in D_2O at room temperature. Chemical shift values are in ppm relative to HOD

^b ^{13}C NMR taken as decoupled spectrum

^c Carbons correlated to carbon resonances in ^{13}C column

production of siderophore was obtained in media without Fe^{3+} (Table S1). Presence of iron in the media increased the growth of the strain HIRFA32 however; there was decrease in siderophore production as indicated by the percent siderophore units. It was observed that the carbon source in the growth medium also affected the siderophore production by strain HIRFA32. The media components affect the siderophore production by rhizobacteria as

reported earlier [53]. In the present study, the siderophore was produced optimally at 28 °C, which was the optimum temperature for the growth of the strain HIRFA32. In the growth and siderophore production studies (Fig. S1), it was also observed that siderophore production increased at 24 h of incubation and the amount of siderophore was sustained even after the death phase of the culture was reached suggesting that the produced siderophore was not being

Fig. 2 **a** Representative image depicting siderophore mediated inhibition of *F. oxysporum* by *A. calcoaceticus* HIRFA32 under iron limiting conditions. **A** Restricted growth of *F. oxysporum*; **B** Growth of *F. oxysporum* in absence of *A. calcoaceticus* HIRFA32 (Control), **b** Growth inhibition of *Fusarium oxysporum* by partially purified siderophore from *A. calcoaceticus* HIRFA32. **A** 20 µl of partially purified siderophore from *A. calcoaceticus* HIRFA32; **B** 10 fold diluted partially purified siderophore; **C** 20 µl of supernatant of 96 h grown *A. calcoaceticus* HIRFA32 in iron restricted media; **D** Solvent blank

degraded by the HIRFA32. Previous reports on time course of siderophore production by PGP *Pseudomonas* species showed similar results of increased siderophore production at 24–30 h however there was decline in amount of siderophore thereafter [53].

Several methods are reported for purification of siderophore. These methods mainly depend on the type of siderophore being produced. The extracellular siderophore produced by HIRFA32 was purified from the cell free supernatant. In the initial step of purification the cell free supernatant was subjected to solvent extraction at low pH with double volume of ethyl acetate which is reported to remove DHBA [32, 54]. The aqueous phase by then subjected to column chromatography which employed XAD resin which selectively binds to catechol siderophore. The partially column purified fraction which appeared yellow to brown in colour and was CAS positive was subjected to HPLC with C_{18} column for further purification. The conditions were optimized using analytical HPLC followed by

which preparative HPLC was performed. The pure siderophore showed retention time at 10.18 min at the HPLC profile (Data not shown). Similar methods were used previously to purify catechol type of siderophore from *P. fluorescens* [39] and strains of *Bacillus* spp. [55] followed by NMR studies for determination of siderophore structure.

The pure compound showed positive reaction with CAS reagent and was found to be UV active with λ_{max} at 310 nm recorded in MeOH. The IR spectrum (neat) indicated the presence of phenolic-OH ($3,140\text{ cm}^{-1}$), amide ($1,674\text{ cm}^{-1}$), aromatic double bonds ($1,587, 1,553\text{ cm}^{-1}$) and a vinyl alkyl ether group ($1,201, 1,062\text{ cm}^{-1}$). Molecular weight was determined by LCMS [M^{+1}] m/z 347.18, calculated for $C_{16}H_{18}N_4O_5$. Pure siderophore from HIRFA32 was obtained as pale yellow crystals (hygroscopic) with melting point as 187–188 °C. To elucidate the structural skeleton of the siderophore, analysis by 1H NMR (Fig. 1) and ^{13}C NMR techniques was performed and structure obtained by supported by HMQC, HMBC, NOESY and decoupling studies (Table 1). NMR studies confirmed presence of DHBA and revealed that two amino acids namely; hydroxyhistamine and threonine were present as the subunits. This study marks that siderophore produced by rhizospheric strain of *A. calcoaceticus* HIRFA32 was like that of acinetobactin which has been previously characterized from clinical strains of *A. baumannii* and *A. haemolyticus* [22, 56].

The bacteria in the rhizosphere produce iron chelators which have more affinity for ferric iron as compared to that produced by the fungi. Hence, siderophore production by rhizobacteria can sequester iron in the rhizosphere making it unavailable for pathogenic fungi and thus inhibiting their growth [2, 9]. This serves in biocontrol of soil borne pathogenic fungi and therefore the phytopathogenic fungal inhibition activity of siderophore produced by HIRFA32 was investigated in the present study. A phytopathogen *F. oxysporum* which causes wilting of wheat plant was used in this study. Strain HIRFA32 exhibited $46.87 \pm 0.5\%$ mycelium growth inhibition of a *F. oxysporum* under iron limited conditions (Fig. 2a) and similar inhibitory activity was also observed for the partially and completely purified siderophore (Fig. 2b), indicating the probable role of siderophore produced by HIRFA32 in suppressing the growth of phytopathogens by scavenging the trace iron from the growth medium. Similar fungal inhibition activity is reported for siderophore produced by strains of *Pseudomonas* and other PGP rhizobacteria [2, 8, 9]. This activity of acinetobactin-like siderophore produced by *A. calcoaceticus* HIRFA32 could be making the strain rhizosphere competent and its presence in rhizosphere of wheat could be serving as a front line defense for roots from the attack of pathogenic *F. oxysporum*.

Conclusions

The present investigation by the polyphasic approach of identification revealed that strain HIRFA32 from rhizosphere of wheat is a member of *Acinetobacter calcoaceticus*. Further investigation demonstrated that the strain HIRFA32 produces acinetobactin-like siderophore and also restricted growth of *F. oxysporum*. This is the first report on structural characterization of siderophore from a rhizospheric strain of *A. calcoaceticus* with the ability to inhibit growth of *F. oxysporum* indicating potential of HIRFA32 to support indirect plant growth promotion of the crop plant such as wheat.

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