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PRODUCTION AND PLANT GROWTH EFFECT OF SIDEROPHORE PRODUCED BY *PSEUDOMONAS* RSML-24

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ABSTRACT:

Iron plays a vital functional role in overall metabolic reactions of plants required for production and utilization of energy. The lime-induced iron chlorosis is a characteristic problem associated with plants cultivated in the calcareous soils. The iron chlorosis is responsible for loss in vigor, stunned growth and thereby decreased crop yields. The provision of iron to the plants through various synthetic chemical chelators like foliar spray of EDTA-Fe and EDDHA-Fe complex is routinely used. Low absorption and toxicity to plants are the problems associated with these practices.

The present study is carried out to study fermentative production of siderophore, its purification, characterization and to test its plant growth promoting potential under iron stress conditions. The rhizospheric soil isolate *Pseudomonas* RSML-24 has produced the parrot green coloured pyoverdin; a conjugate of hydroxamate and catecholate type of siderophore, which is characterized by its strong Csaky test, weak Arnow test and absorption peak at 404 nm. The preliminary pot trials in alkaline calcareous soil exhibited a promising plant growth promoting potential of the siderophore in grape vines. The foliar application of siderophore-iron complex increased foliar iron content than EDTA-iron in grape vines. Based on the *in vivo* observations, an effective, eco-friendly and economically reasonable biological Fe chelator could be developed.

KEYWORDS: Production, Siderphore, *Pseudomonas,* Pyoverdin, Grape vine

INTRODUCTION:

Iron plays an important functional role in overall metabolic reactions of plants required for production and utilization of energy. The energy required for plant physiological function is obtained through photosynthesis and respiratory processes. Iron exists in aqueous solution in ferrous and ferric states; however, the ferric forms are not readily utilizable by plants and microbes because they frequently form insoluble oxides or hydroxides which limits their bio accessibility (Desai and Archana 2011; Zuo and Zhang 2011). In chlorotic plants, Fe concentrations can be higher than, equal to, or lower than those in normal plants. This disorder on calcareous soils is not always attributable to Fe deficiency. It may be a condition

known as lime-induced Fe chlorosis, which is a characteristic problem associated with cultivation in the calcareous soils, which comprise over one third of the world's land surface area and specifically the regions that receive less than 500 mm of annual rainfall (Vose, 1982 ; Gildersleeve and Ocampaugh, 1989).It is known that microbial siderophores make available plants with Fe nutrition to enhance their growth under iron deficient conditions (Crowley, 2006), by an incompletely understood mechanism. The two possible mechanisms are: (i) Microbial siderophores with high redox potential can be reduced to donate Fe (II) to the transport system of the plant. In this mechanism, it has been hypothesized that the microbial Fe (III) – siderophores are transported to the apoplast of the plant root where siderophore reduction may occur. Consequently, Fe (II) is trapped in the apoplast, which leads to high iron concentrations in the root (Mengel, 1995; Kosegarten *et al*., 1999). (ii) Microbial siderophores can scavenge iron forming a ligand which is exchanged with phytosiderophores (Masalha *et al*., 2000).

The provision of iron to the plants using various synthetic chemical compounds (e.g., DTPA, EDGA, EDTA, EDDHA, etc) as foliar spray or soil inoculation, have already been attempted but biodegradability of these chemicals is found to be considerably low as compared to the microbial siderophores (White, 2001). As a consequence, such chemical chelators can be toxic to the plants (Chen and Cutright, 2001). Iron is considerably less soluble than Zinc or Manganese in calcareous soils having alkaline pH values; thus, under such conditions the inorganic iron chelates contribute relatively little to the iron nutrition of plants. Bacterial siderophores, the organic iron chelates, may serve as a remedy to lime-induced chlorosis in plants grown in calcareous soils **(**Jurkevitch, *et al*., 1988)**.**

With only a few exceptions such as lactobacilli, all aerobic and facultative anaerobic microorganisms require iron for growth and proliferation (Wrigglesworth and Baum 1980; Pandey *et al*., 1994). In microbial world, the most prevalent and thriving mechanism of exploiting all available iron sources, independent of their nature, is the secretion and use of small-molecule compounds called siderophores. To acquire and transport iron under such conditions, the bacteria and other microorganisms frequently complex iron with organic chelates that combine with inorganic iron and significantly enhance its solubility (Emery, 1977). These chelates, which were designated earlier as siderochromes, sideramines, and sideromycins, are now conveniently termed as Siderophores (meaning in Greek: sideros = iron and phores = bearer) (Lankford, 1973). Siderophores are technically defined as the ferric iron specific, low molecular weight (< 1500) compounds, which solubilize and transport iron in to the cell (Neilands, 1981, Crowley *et al*. 1991). A large number of Siderophores produced by different microorganisms have been documented. Siderophore production is one of the mechanisms of plant growth promotion by plant growth promoting rhizobacteria (PGPR) either directly by provision of chelated iron to plants or indirectly by depriving the pathogen for iron availability (Duffy, 2001; Kloepper, 1996; Sindhu *et al*.1997; Chincholkar *et al*. 2000). The most common Siderophores can be classified as phenol-catecholates and hydroxamates, depending upon the chemical moieties that are involved in coordination of the ferric iron (Neilands, 1981), while

siderophores that contain neither of these ligand systems have classified as carboxylate siderophores. (Hofte, 1993; Winkelmann and Drechsel, 1997).

The present study deals with biosynthesis and characterization of siderophore from a rhizospheric bacterial strain Pseudomonas RSML24. The plant growth promoting potential of produced siderophore is also tested by pot trial method on grape vine (*Vitis vinifera*) planted in calcareous soil having alkaline pH.

MATERIALS AND METHODS:

The grape vine growing in the calcareous soil (pH.7.8) was uprooted. The roots were washed with sterile distilled water to remove loosely associated soil. A uniform suspension of the root-embraced soil was obtained by shaking the roots in sterile saline at 200 rpm (Steelmet, Pune) for 30 min. on a rotary shaker. The suspension was serially diluted. A 0.1 ml from each dilution was inoculated on nutrient agar and *Pseudomonas* isolation agar. The plates were incubated at 28 ± 2^{o} C for 48 hours. The isolates were maintained on nutrient agar slants, in the refrigerator for further studies.

Identification of bacterial genera was done following cultural, morphological and biochemical characterization according to Bergey's manual of systematic bacteriology.

The bacterial isolates were used for primary screening of their siderophore production ability. The isolates were grown in 100 ml of succinate medium consisting (g L^{-1}) of Succinate (4), (NH4) 2SO4 (1), MgSO4.7H2O (0.2), K_2HPO_4 (6), KH_2PO_4 (3), pH-7 (Meyer and Abdallah, 1978) in 250 ml Erlenmeyer flask. It was incubated at 28 ± 2^{o} C for 48 hours with constant shaking at 200 rpm on a rotary shaker.

The cultures were centrifuged at 10,956 g (10,000rpm) for 20 min. The supernatant was adjusted to pH 7 and was analyzed for appearance of siderophore by Universal Chemical assay i.e. Chrome Azurol Sulphonate (CAS) assay (Schwyn and Neilands, 1987). For this, 0.5ml culture supernatant and 0.5ml CAS reagent were mixed. It was observed for disappearance of blue color and read at 630 nm against respective sterile medium used for base line correction. A standard was prepared mixing sterile medium and CAS reagent. The siderophore units responsible for percent decolorization of blue colored CAS reagent for each isolate were determined using the following formula. (Payne, 1994)

$$\% \text{ Siderophore units} = \frac{Ar - AS}{Ar} \times 100$$

Where, Ar = OD of standard at 630 nm

As = OD of test at 630 nm

The culture supernatants were subjected for the detection of siderophore type following the Csaky test for hydroxamate type and Arnow's test for catecholate-phenolate type of siderophore (Payne, 1994). The culture supernatant was also scanned within the wavelength 200 nm to 500 nm, against un-inoculated

respective media using UV-Visible spectrophotometer (Shimadzu, 1601 Model, Japan), to determine the λ max.

The inoculum of bacterial isolate *Pseudomonas* RSML-24showing highest siderophore production is inoculated at 05% (v/v) for siderophore production in 03L of Succinate medium, (Barbhaiya and Rao 1985), consisting (g L^{-1}) of Succinate (4), (NH4) 2SO4 (1), MgSO4.7H2O (0.2), K_2HPO_4 (0.1), KH_2PO_4 (3), pH-7 in a 05L fermenter (Dyna biotech, Pune), at 28^{o}C and 150rpm for 36hrs.

The visible yellow green pigmentation and bright fluorescence developed in the culture medium indicated siderophore production, which was confirmed by the Universal Chemical Assay (Schwyn and Neilands, 1987). The ferment was then centrifuged at 10000 rpm for 20 min at 4 ^{o} C. The cell free supernatant thus obtained was subjected to extraction and purification of siderophore.

The extract obtained in solvent extraction method was subjected for determination of absorption maxima by scanning between the wavelengths 200 nm to 500 nm on UV-visible spectrophotometer.

The siderophore present in the cell free supernatant (1000 ml) was first complexed with iron by addition of 2M $FeCl_3$. The resultant chelate was extracted with 0.5 volume of a phenol-chloroform mixture (1:1 W/ V) as described earlier (Stinzi and Meyer, 1994). The aqueous fraction obtained was used for treatment of seed cuttings in pot trials on grape vine (*Vitis vinifera*) planted in calcareous soil having pH 7.8. The EDTA solution complexed with 2M $FeCl_3$ was also applied as a control.

RESULTS AND DISCUSSION:

Out of the 11 Gram negative isolates obtained on *Pseudomonas* isolation agar plates, 08 bacterial isolates were observed to produce water-soluble fluorescent green pigment. The Universal chemical assay (CAS test) was used for detection and quantification of percent units of siderophore produced (Schwyn and Neilands, 1987; Pyane, 1994). Decolorization of the blue colored CAS reagent and appearance of wine red color yielded positive test for all the tested eight bacterial isolates. The percent units of siderophore produced by all isolates also were calculated. The isolate RSML-24 showed maximum i.e. 79.20% siderophore units. (Table- 1)

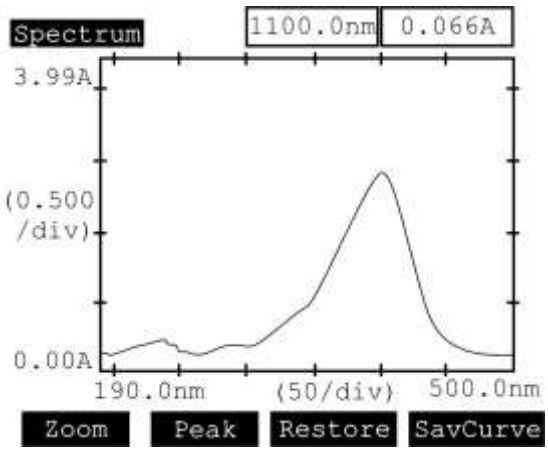
Table 1: Screening of bacterial isolates for siderophore production

Sr. No.	Isolate	% Siderophore units
1	RSML- 1	76.46
2	RSML-8	50.12
3	RSML- 7	56.30
4	RSML- 15	51.12

5	RSML- 22	62.12
6	RSML-37	60.02
7	RSML-24	79.20
8	RSML-35	75.51

Appearance of parrot green color in fermented broth (Fig.A-01) pointed toward siderophore synthesis, positive Csaky test and absorption maxima at 404 nm (fig-1) indicated production of hydroxamate type of siderophore by isolate RSML-24(Pyane, 1994; Budzikiewicz, 1993). Similar nature of UV absorption spectra of culture supernatant with peaks at about 400 nm was reported for pyoverdin type of siderophores produced by different strains of *Pseudomonas* (Bultreys *et al*, 2003).

Fig-1: Scanning of cell free extract on UV- Visible spectrophotometer.



The Arnow's test of cell-free supernatant was found weakly positive. The result also pointed towards appearance of siderophore that may be a conjugate of hydroxamate and catecholate such as pyoverdin of fluorescent *Pseudomonas* species. In pyoverdin, chelation of iron involves two hydroxamate functions derived from two hydroxyaminoacyl residues of the peptide chain and a catecholate group of the chromophore. (Budzikiewicz, 2001).

The preliminary pot trials exhibited the plant growth promoting effect of siderophore on germination and growth of plant when applied to the seed cuttings when compared with EDTA –Fe and Untreated (Fig.A-2).

The role of microbial siderophores in provision of iron to the plants has been extensively studied and documented (Crowley *et al*, 1987). The soil application of PGPR is known to increase the uptake of nutrients including iron in sugarcane (Umate, 2003). Naik, G. R. (2002) has reported improvement in height, number of tillers, stem girth and leaf area in Fe-efficient variants of sugarcane grown in typical calcareous soil. Utilization of microbial siderophores for iron acquisition by oat; a monocot graminaceous

plant has been reported (Crowley *et al*, 1988). Iron uptake by oat is reported to be more efficient from naturally occurring chelates like ferrichrome than from synthetic chelates like EDDHA. (Reid and Crowley, 1984). Considerable increase in percent germination, shoot, root length and dry weight of maize seeds upon bacterization with siderophore producing strains of *Pseudomonas* spp. under iron limiting conditions in calcareous soil system is reported (Sharma and Johri, 2003). Radzki, *et, al* (2013) have reported the efficiency of bacterial siderophores in provision of iron to iron-starved tomato plants in hydroponics culture.

It is concluded that the siderophore pyoverdin produced by rhizospheric *Pseudomonas* RSML-24 when chelated with iron has potential to improve growth of grape vine growing in calcareous soil.

Fig.A-1 Cell free centrifugate- pyoverdin

A B

Fig.A-2: Pot trials, Treatment with A) Siderophore B) EDTA-Fe

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