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Siderophore Producing *Aspergillus* spp as Bioinoculant for Enhanced Growth of Mung Bean

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Abstract Two fungal species *A. niger* and *A. parasiticus* isolated from agriculture field were used in the present study. These two fungal isolates were studied for the siderophore production on the modified CAS agar medium. On daily observation it was observed that the growth was increased along with the production indicating the change in colour of CAS dye from blue to purplish red by *A. niger* and Yellow by *A. parasiticus*. Quantitative determination of siderophore production was further studied using liquid medium. After 10 days of incubation *A. niger* and *A. parasiticus* produce 80% and 73% units of siderophore. Chemical determination showed that both the fungal isolates showed hydroxamate type of siderophore. Optimization of type of inoculum showed that block inoculum produced maximum siderophore compared to loop and monosporic inoculation in both the fungal isolates. Neutral pH 7.0 showed maximum siderophore production in both the isolates. Decrease in iron concentration increases the siderophore production by the fungal isolates. Maximum siderophore production 80% and 74% units was observed in *A. niger* and *A. parasiticus* respectively when there was no addition of iron in the medium. Pot studies were carried out to observe the effect of fungal inoculum on mungbean plant. Inoculation with fungal isolates showed increase in number of leaves, number of branches, number of lateral roots, plant height, fresh weight and dry weight of plant compared to control. Thus both the fungal isolates proved to be potential bioinoculant for improving the growth of mungbean plant.
Keywords *Fungi; Mungbean; Siderophore*

1. Introduction

Siderophores are low molecular weight (<10,000 Da), virtually ferric-specific ligands produced by microbes as scavenging agents in order to combat low iron stress. Main function of siderophore is to acquire ferric iron from insoluble hydroxides or iron bound to surfaces. They can also complex iron from other both soluble and insoluble iron compounds, such as ferric citrate, ferric phosphate, Fe-transferrin, ferritin (iron bound to sugars), plant flavone pigments, glycosides, and even artificial chelators, such as EDTA (Winkelmann, 2002). Schwyn and Neilands, (1987) developed a universal method to detect and determine siderophores using high affinity for iron (III). The ternary complex chrome azurol S/ iron (III)/ hexadecyltrimethylammonium bromide, with an extinction coefficient of

approximately 100,000 m^{-1} cm^{-1} at 630 nm, serves as an indicator. When a strong chelator removes the iron from the dye, its color turns from blue to orange. Milagres et al., (1999) developed a modified method of CAS agar plate assay which was made by incorporating the CAS blue dye in a medium with no contact with the micro-organisms tested. Half of each plate used in our experiments was filled with the most appropriate culture medium for each type of microorganism and the other half with CAS-blue agar.

Fungi have been shown to produce siderophores under aerobic conditions during iron limitation with the notable exception of certain Saccharomyces species. Most fungi secrete hydroxamate-type siderophores including fusarinines, coprogens, and ferrichromes. Many strains have been shown to simultaneously synthesize more than one type of hydroxamate siderophore. Although most fungi produce hydroxamate-type siderophores, zygomycetes have been shown to produce the carboxylate siderophore rhizoferrin (Thieken et al., 1992). A few fungi also secrete phenolate type compounds under iron limitation, but the relevance of these in iron uptake is uncertain (Fekete et al., 1989).

Plant growth mechanisms can be grouped as direct like fixation of atmospheric nitrogen, solubilization of minerals such as phosphates and production of plant growth regulators like auxins, gibberellins, cytokinin and ethylene. Indirect mechanisms include production of HCN, antibiotics, siderophores, synthesis of cell wall lysing enzymes and competitions with detrimental microorganisms for colonization on plant roots. Similar to PGPR (Plant growth promoting rhizobacteria), some rhizosphere fungi able to promote plant growth upon root colonization are functionally designated as 'Plant Growth Promoting Fungi' (PGPF). PGPF belongs to genera *Penicillium*, *Trichoderma*, *Fusarium*, *Aspergillus* and *Phoma* etc. PGPF which are non-pathogenic soil inhabiting saprophytes, have been reported to be beneficial to several crop plants not only by promoting their growth but also by protecting them from disease (Pandya and Saraf, 2010).

The use of siderophore producing fungi can help to improve iron deficiency, plant growth and the yield of economically important crops. Siderophore apart from their role in active transport of iron may act as growth antagonists by means of sequestering iron from the environment, restricting the growth of pathogens (Berg et al., 2002). Wolfgang et al., (2000) recorded that siderophore mixture from some fungi are an excellent sources for Fe nutrition of non-leguminous plants. They found that siderophore mixture from *P. chrysogenium* and *Rhizopus arrhizus* significantly improved the iron status of cucumber, maize and tomatoes plants as measured by chlorophyll concentration to the same degree as high as from Fe EDTA supply.

The main objective of the present work was to study the effect of these siderophore producing fungal isolates as biofertilizers for enhancement of Mung bean plants under greenhouse experiments.

2. Materials and Methods

2.1. Culture Collection and Maintenance

The fungal species used in the present study were *Aspergillus niger* PT1 and *A. parasiticus* PT6. These fungal cultures were isolated from agricultural fields during the diversity study. Stock cultures of fungal species were maintained on 2% malt extract agar (MEA) plates at 4^{o}C.

2.2. Siderophore Production using modified CAS agar medium

Modified CAS assay was used to test the potential of fungal species to produce siderophore in solid medium (Schwyn and Neilands, 1987). MEA medium was prepared. After solidifying, the medium was cut into halves, one of which was replaced by CAS-blue agar. The halves containing culture medium (MEA) were inoculated with fungal culture. The inoculum was placed as far as possible from the

borderline between the two media. The plates were incubated at 28^{o}C for 6 days. Fungal growth rates were monitored daily and expressed as the number of days required by the microorganism mycelia to cover the halves of Petri plates containing MEA medium. The CAS-agar colour changed from blue to orange or purple.

2.3. Quantitative estimation of siderophore

A 1.0 ml aliquot of supernatant of fungal liquid cultures was mixed with 1.0 ml of CAS assay solution prepared according to (Schwyn and Neilands, 1987). A reference was prepared with uninoculated broth and CAS solution. The sample (s) and reference (r) absorbance at 630 nm were measured after 1 h of incubation at room temperature. The percentage of iron-binding compounds of the siderophore type was calculated by subtracting the sample absorbance values from the reference. Amount of siderophore units was calculated as

% Siderophore Unit = (Ar-As)/Ar x 100

whereas, Ar = absorption of reference

As = absorption of sample

2.4. Detection of chemical nature of siderophore

Isolates that gave positive reaction in chrome azurol S (CAS) assay indicating the siderophore production were grown in 100 ml of 2% liquid malt extract in 250 ml Erlenmeyer flasks, which were inoculated with one block of agar mycelium (0-5 cm diameter) obtained from stock plates. The flasks were incubated at 28ºC for 6 days with constant shaking at 120rpm and the culture supernatants were examined for hydroxamates by $FeCl_3$ test (Neilands, 1981), catecholates by Arnow's test (Arnow, 1937) and carboxylates type of siderophore by the method of Shenker et al., (1992).

2.5. Effect of type of fungal inoculum

The effect of type of inoculum was carried out with three types of inoculation: monosporic, loop and block (0.5 cm diameter). Growth of species and CAS reaction were evaluated daily. The blocks were obtained from stock plates which contained MEA medium and fungal mycelium.

2.6. Effect of pH

The effect of pH between 5.0 to 10.0 on siderophore production was studied in 2% malt extract medium by adjusting the pH before inoculating the strain with 1N HCl or 1N NaOH. Following the inoculation and incubation at 28ºC for 6 days in CAS medium with constant shaking at 120rpm, the siderophore contents were estimated.

2.7. Effect of different iron concentrations

To determine the effect of iron concentration on siderophore production, 2% malt extract medium was supplemented with iron at concentration between 5 to 20 µM in different sets, for both the PT1 and PT6 separately. Following the inoculation and incubation at 28ºC for 6 days in CAS medium with constant shaking at 120rpm, the siderophore contents were estimated.

2.8. Growth promotion study of Mung beans

The pot study was carried out in triplicate on Mung beans. Seeds were surface sterilized by gently shaking with 70% ethanol (5 min) and 20% sodium hypochlorite solution followed by three rinses in sterile distilled water. Thereafter, seeds were soaked overnight in sterile distilled water and

germinated on sterile cotton-covered Petri dishes. After 3 days of germination, seeds were planted in the pots. All fungal isolates were grown on 2% Malt extract broth at 28°C for 7days, spore count was carried out and adjusted 10^8 spores/ml was used. Finally 10ml suspensions were mixed with the soil per pot. One control was kept that did not contain any fungal culture. After 30 days of plant growth, plants were carefully uprooted from soil. Plants were washed carefully under running tap water to remove adhering sand particles. Intact root system was carefully uprooted to prevent breakage. The following parameters were recorded: Length of root and shoot (cm), Number of lateral roots, Number of leaves, Fresh weight of plant (g), Dry weight of plant (g) (Pesqeira et al., 2006).

2.9. Estimation of iron from soil

Soil samples were taken immediately after 30 days and 60 days and transferred for storage in sealed plastic bags. Once in the laboratory, the soils were sieved (<2 mm), and stored at 4◦C for not more than one week before analyses. Soil iron was measured by Atomic Absorption Spectrophotometer (Lindsay and Norvell, 1978).

2.10. Estimation of iron in Mung bean plant

After 60 days of plantation, Mung bean plants were harvested. The roots were washed and fresh and dry weights of leaves and roots were recorded. Plant tissues were ground before chemical analysis. The analysis of iron content in the plant was carried out by digesting the samples in a di-acid digestion mixture (nitric acid (HNO3): perchloric acid (HClO4): 9:4). Iron content was measured by atomic absorption spectrophotometer (Bhargava and Raghupathi, 1993).

3. Results

3.1. Detection of siderophore production on the CAS-agar plate

Modified CAS-agar plate assay was used for the detection of siderophore by different fungal strains. After 15 days of incubation the results were qualitatively distinct in terms of color of CAS reaction and changes from blue to yellow and purplish red color. *Aspergillus niger* produced a color change from blue to purplish red in the CAS – agar medium. The distinct responses of color change CAS reaction (purple or orange) observed with the different microorganisms could be related to structural differences in the types of siderophores secreted. The siderophore production in solid medium by *A. niger* was 70mm followed by *A. parasiticus* (Figure 1).

3.2. Quantitative determination of siderophore production

In liquid medium, the siderophore production was evaluated by the universal CAS-liquid assay (Schwyn and Neilands 1987). In quantitative CAS assay, percent siderophore units were estimated as the production of CAS color shifted. *A. niger* and *A. parasiticus* produced 80% and 73% siderophore units after 15 days of incubation (Figure 1).

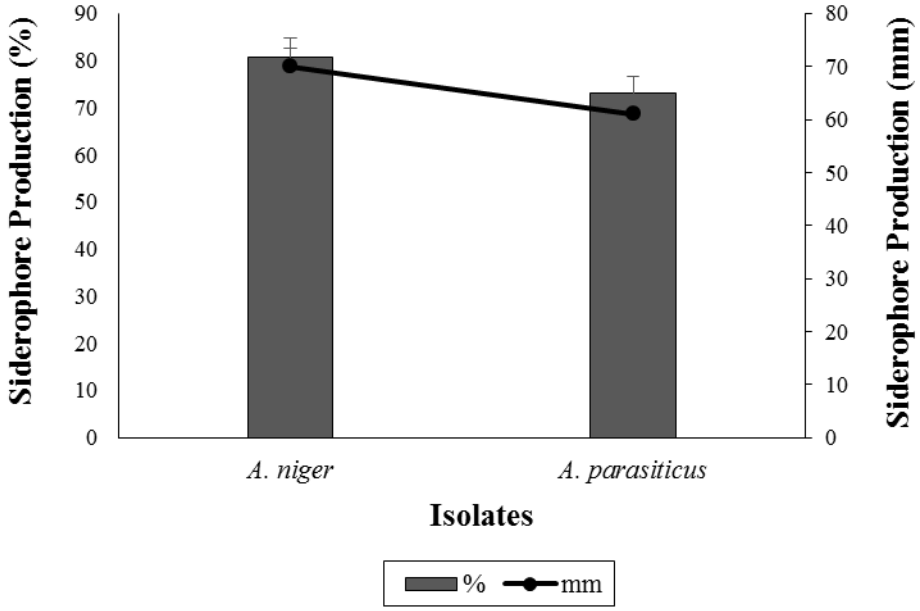

Figure 1: *Siderophore production on modified CAS agar medium*

3.3. Chemical characterization of siderophore

Fungal isolates showed presence of hydroxymate type of siderophores. None of them produced catecholate and carboxylate type of siderophores (Table 1). Also these two fungal isolates showed peak between 425 to 450 nm in spectrophotometric analysis which indicated their hydroxymate type of siderophore.

Table 1: *Chemical characterization of siderophore produced by fungal isolates*

	$FeCl_3$ test (Hydroxamate)	Arnow's test (Catecholate)	Shenker's test (Carboxylate)
Aspergillus niger	+	-	-
Aspergillus parasiticus	+	-	-

3.4. Optimization of siderophore production at different conditions

3.4.1. *Effect of type of fungal inoculum*

The type of inoculum effect the growth of the species and therefore production of secondary metabolites (such as siderophores). From various types of inoculums, the most significant influences were observed using blocks as inocula (Figure 2). The maximum CAS-reaction rate was obtained using block inoculum with *A. niger* (8 mm per day) compared to monosporic and loop inoculation. The CAS reactions produced by *A. parsiticus* (7.5 mm per day) were similar for all the types of inocula tested. However, the lowest reaction was obtained using loop inoculum. So it is clear that the block type of inoculums was optimum for highest CAS reaction for both species.

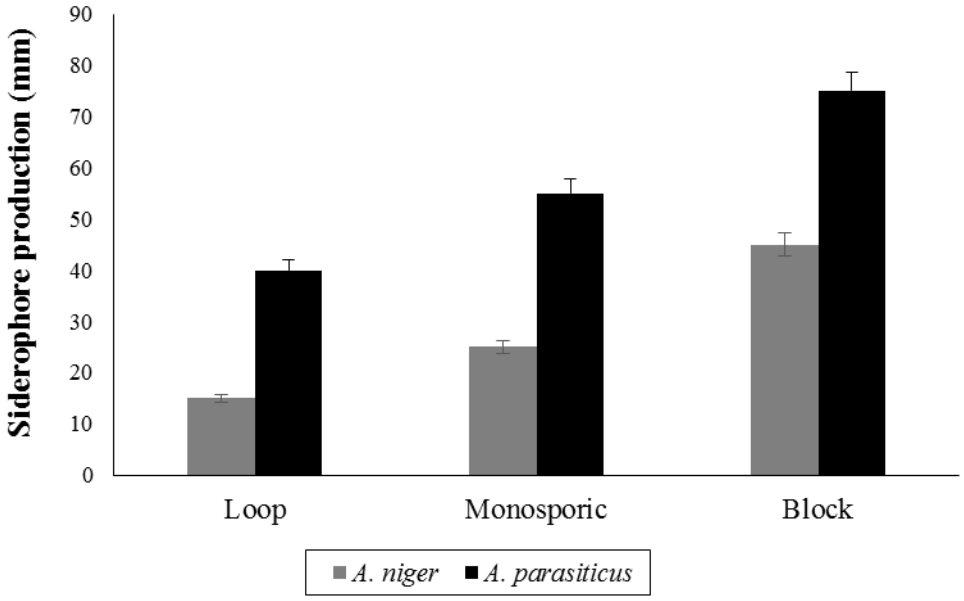

Figure 2: *Effect of type of inoculum on siderophore production*

3.5. Effect of pH

pH plays an important role in the solubility of iron and thereby its availability to the growing organism in the medium. Influence of pH on siderophore production is depicted in Figure 3, for both species, the optimum pH for siderophoregenesis was found to be 7.0. Isolate *A. niger* and *A. parasiticus* showed maximum siderophore production (80 and 75% units) at pH 7.This may be because of iron is present in insoluble form at neutral pH.

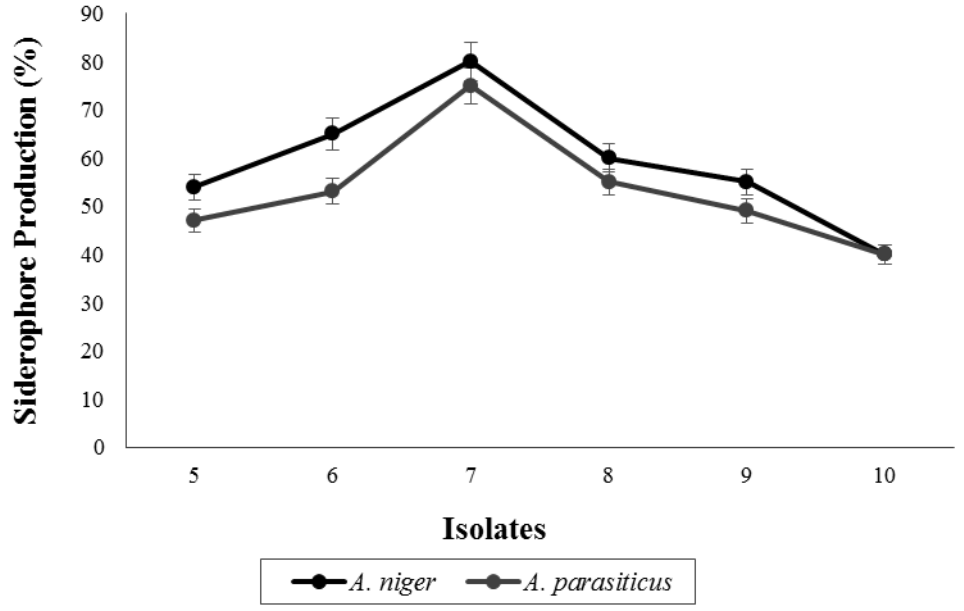

Figure 3: *Effect of pH on siderophore production*

3.6. Effect of Iron

As depicted in Figure 4, in both the cultures *A. niger* and *A. parasiticus*, the maximum siderophore production (80 and 74 % units) was observed in 2% Malt extract medium, without the addition of iron. The siderophore production was inversely proportional to the iron concentration. In 20 µM iron-supplemented cultures siderophores were detected only in *A. niger* extracts. In this condition, *A. parasiticus* did not produce siderophores in sufficient amounts to be detected by CAS liquid assay (responses<10%).

3.7. Influence of fungal treatments on growth and development of Mung bean plant under Greenhouse experiment condition

Study of Mung bean under the influence of two selected fungal isolates i.e. *A. niger* and *A. parasiticus*, showed increased growth of plants in terms of root length, shoot length, number of leaves and fresh weight as well as dry weight as compared to un-inoculated control. The effect of fungal inoculation resulted in more shoot length compared to un-inoculated control plants. Isolates *A. niger* and *A. parasiticus* increased 18.18% and 4.54% shoot length compared to control. The fungal isolates significantly increased the root length and Number of lateral roots of mung bean plants (Figure 7). The isolate *A. niger* produced the highest root length 66.66 % and isolate *A. parasiticus* 33.33% compared to control. The number of lateral roots was increased in both isolates compared to control. Isolate *A. niger* and *A. parasiticus* increased in number of roots 66.66% and 45.45% compared to control.

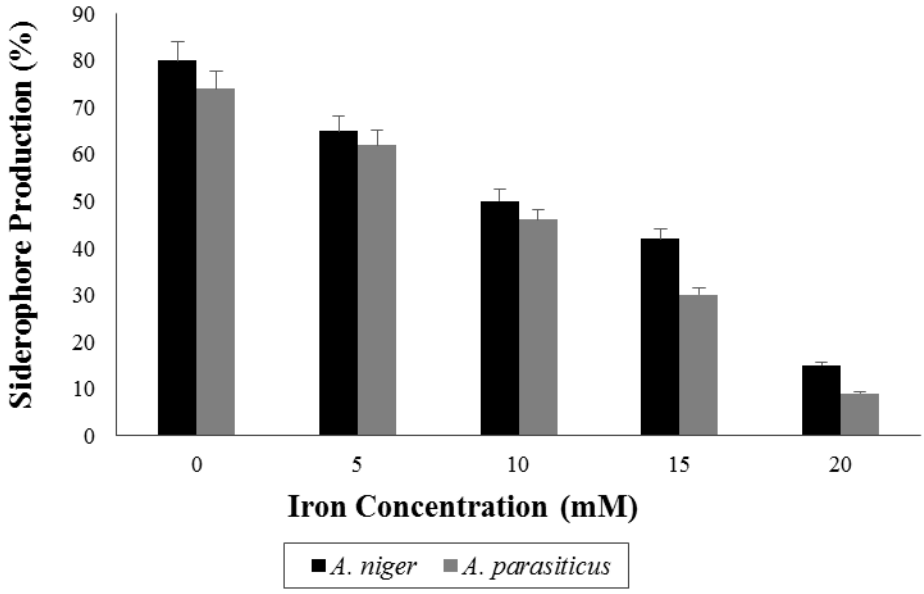

Figure 4: *Effect of iron concentration on siderophore production*

The fungal isolates significantly increased the number of leaves of mung bean plants. The isolate *A. niger* and *A. parasiticus* produced the highest number of leaves 86.20% and 37.93% compare to control. Results were obtained in the study of fresh weights that plants treated with fungal isolates increased in compared to control. *A. niger* isolate increased maximum fresh weight 81.69% compared to other isolate *A. parasiticus* 21.83%. Fungal isolates also increased in dry weight compared to control. Isolate *A. niger* and *A. parasiticus* increased in dry weight 42.5% and 25% compare to control (Table 2).

Table 2: *Biometric observation of mungbean plant inoculated with fungal isolates after 60 days of plantation*

Vegetative Parameters	Control	*A. niger*	*A. parasiticus*
No. of leaves	29 ± 1.21 ^{ns}	54.2 ± 1.37 ^{ns}	40 ± 1.32 *
No. of lateral roots	33.4 ± 1.19 ^{ns}	55 ± 1.42 ^{ns}	48 ± 1.33 ^{ns}
Root length (cm)	3 ± 0.17 ^{ns}	5 ± 0.35 *	4.5 ± 0.29 ^{ns}
Shoot length (cm)	22 ± 1.15 ^{ns}	26.5 ± 1.2 ^{ns}	23.3 ± 1.25 ^{ns}
Fresh weight (g)	1.4 ± 0.04 ^{ns}	2.58 ± 0.07 ^{ns}	1.73 ± 0.05 ^{ns}
Dry weight of (g)	0.8 ± 0.01 ^{ns}	1.4 ± 0.03 ^{ns}	1 ± 0.02 ^{ns}

3.8. Iron content in soil

Soil analysis of 0 day, 30 days and 60 days showed that Fe content decreased in soil inoculated with fungal isolates compared to control (Table 3).

3.9. Iron content in plant

Iron content increased in mung bean plants when grown in association with *A. niger* and *A. parasiticus* species. This increase was due to the production of siderophores by fungal strains. Maximum iron content in plant was observed by *A. niger* (6.65ppm) and *A. parasiticus* (6.25ppm). The control showed only (5ppm) of iron content in plants (Table 3).

Table 3: *Determination of iron from soil and mungbean plant after 60 days of plantation*

Treatment	Soil (ppm)			Mung bean Plant after 60 days (ppm)
	0 Day	30 Days	60 Days	
Control	6.94	6.92	6.84	5
A. niger	6.94	6.58	6.04	6.65
A. parasiticus	6.94	6.66	6.26	6.25

4. Discussion

In the present study modified CAS agar medium was used which increases the growth of fungal isolates by nullifying the toxic effect of detergent hexadecyltrimethylammonium bromide (HDTMA) used in the media preparation. Fungal isolates grow rapidly in the plate-half containing the medium, while it fails to grow in the plate-half containing CAS-blue agar medium. Siderophore produced by the *Aspergillus niger* and *A. parasiticus* diffused through the CAS-blue agar producing a colour change from blue to yellow, orange, purple or purplish red. *A. niger* showed colour change from blue to purplish red and *A. parasiticus* showed colour change to yellow. The siderophore production in solid medium expressed as CAS-reaction rate was correlated with siderophore production in liquid medium. Both the fungal isolates *A. niger* and *A. parasiticus* showed siderophore production 80% and 73% after 10 days of incubation. *Wolfiporia cocos* and *T. versicolor* were used as representatives of brown-and white-rot fungi, cultivated in malt extract liquid medium, under low iron concentration condition (approximately less than 15μM iron) for 30 days at 28ºC. Liquid cultures of *W. cocos* were positive in the CAS assay after 10 days of growth. The maximum siderophore production (68%) occurred after 25 days of cultivation, while *T. versicolor* produced a low CAS reaction and a peak was only observed on day 10 (Machuca et al., 2001).

The response of colour change on CAS agar medium by different microorganisms could be correlate to structural difference in the type of siderophore produced. Chemical characterization and colour change produced by the isolates *A. niger* and *A. parasiticus* showed hydroxamate type of siderophore. Vala et al., (2006) also reported marine *Aspergillus versicolor* was found to be the largest siderophore producer (182.5 microg/mL desferrioxamine mesylate equivalent).

Optimization study revealed that both the fungal isolates showed maximum siderophore production at block inoculation compared to loop and monosporic inoculation, at pH 7.0 and at low concentration of iron. Similar results also studied by Machuca et al., (2003). The siderophore production by *Aspergillus* fungi in liquid medium without iron supplementation showed highest percentages of siderophore unit. In 2mM/l iron-supplemented cultures, siderophore production decreased in *A. niger* extracts. In this condition, *A. tamarii* and *A. flavus* did not produce siderophores in sufficient amounts to be detected by CAS liquid assay (responses< 10%). The optimum pH for production of siderophores remained at neutral pH level though the range varied from pH 6.0–8.0. The optimum range of the concentration of Fe (III) required for siderophore production was recorded to be 1.5–21.0 µM.

Effect of fungal isolate *A. niger* and *A. parasiticus* in our study was then observed in pot trials using mungbean. Siderophore producing fungal isolates were inoculated as plant growth promoting fungi (PGPF) and compared to plants not treated with PGPF. After 60 days of study it was observed that both the fungi were able to promote the growth of the mungbean plant compared to untreated control. Plants inoculated with *A. niger* and *A. parasiticus* showed increase in seed germination, no. of leaves, no. of branches, no. of lateral roots, shoot length, fresh weight and dry weight of the plants compared to control. Similarly, inoculation with the fungal isolates resulted in increase in plant height, number of leaves, number of lateral roots, fresh weight, dry weight and total chlorophyll as compared to control. *Penicillium* sp. (S12B) and *Aspergillus* sp. (S11) showed increase in plant growth compared to other fungal isolates in 2% saline soils (Pandya and Saraf, 2010). Yadav et al., (2011) investigated plant growth-promoting activities of fungi and siderophores produced by *Aspergillus niger, Penicillium citrinum* and *Trichoderma harzianum* were found to increase the shoot and root lengths of chickpeas (*Cicer arietinum*).

The response of *A. niger* and *A. parasiticus* in terms of siderophore production and bioavailability of iron to plants was proved by determination of iron in soil and plant. Decrease in iron content from soil and increase of iron in plants revealed production of siderophore by fungal isolates and uptake of iron by mungbean plants compared to control.

5. Conclusion

Use of modified CAS agar medium the present study revealed the ability of *A. niger* and *A. parasiticus* for the production of hydroxamate type of siderophore. The significant feature of the study is the increase in vegetative growth parameters of mungbean plant upon inoculation with fungal isolates. Thus it can be concluded that *A. niger* and *A. parasiticus* can be a potential plant growth promoting fungi (PGPF) for sustainable agriculture.

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