SCREENING, OPTIMIZATION AND EVALUATION OF ACTINOBACTERIA FOR SIDEROPHORE PRODUCTION AND ITS EFFECT ON PLANT GROWTH.

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Abstract

Iron plays significant role in activation of enzymes, chlorophyll formation and carotenoid synthesis in plants. Red colour of chilli and Tomato is mainly due to carotenoid pigment. Direct. . Siderophore are low molecular weight compound which facilitate uptake of iron in plant. Siderophore are structurally and functionally diverse compounds The objective of this study was to screen, and optimize Actinobacterial isolates for siderophore production and evaluate growth promotion by potential SPB (Siderophore producing bacteria) in Chilli (*Capsicum annum* L.) and tomato (S*olanum lycopersicum*) using pot study. Total of 33 isolates were screened for siderophore production by the universal Chrom Azurol Sulphonate assay (CAS),. Quantitative analysis of siderophore was carried out using both CAS liquid assay as described by Payne, 1994 and iron perchloroate assay . Out of33 isolates 11 isolate showed the production of siderophore. Quantitative estimation of a siderophore by (CAS) shuttle assay revealed the yield of more than 70% siderophore unit. Out of 11 isolates six Actinobacterial strains produced more than 100 µg siderophore in iron per chlorate assay .To investigate the response of siderophore rich isolates on growth parameters. Pot study was carried out using isolates showing higher siderophore production quantitatively. Six isolates were used for pot study. Pot study revealed significant increase in overall growth parameters by isolate A2 and H2 compared to control. These isolates could be the promising isolates for preparation of Fe fertilizer.

Index terms-Siderophore, Chilli, Tomato, CAS assay, Fertilizer

I INTRODUCTION

Siderophores are recognized as lower molecular weight, ferric ion specific chelating agents produced by bacteria and fungi especially when grown in low stress of ferric ion. The major function of these compounds is to search off the available iron from environment and to make them available as minerals to microbial cells. Since so many years, research is going on in this field and several aerobic and anaerobic microorganisms found to be synthesizing number of siderophores. Due to aerobic atmosphere on earth, surface iron has been converted to oxyhydroxide polymers of very sparing solubility. At neutral pH, concentration of free ferric ion has been dictated by solubility product constant of Ferric hydroxide. Based on value of this constant, the maximum amount of uncomplexed ferric ion in solution at any biological pH is certainly not more than 10^{-18}M [1] Microorganisms consume vital iron under aerobic conditions for number of activities such as synthesis of ATP, formation of heme, reduction of ribonucieotide precursors of DNA, and others. Overall every cell requires at least one micromole iron for its optimum growth. With several environmental constrains and biological complex city, microorganisms receives that hydroxyl ion for ferric state of iron by producing some bio-molecules such as siderophore.

In recent time along with soil improvement, concept of phytoremediation also holds its potential *in situ* for the treatment of heavy metal contaminated soils. Presence of siderophore- producing bacteria (SPB) with plants are useful for metal removal from contaminated soils. Presence of metal resistant SPB assumes successful survival and linked growth of plants in heavily contaminated soils by alleviating the

metal toxicity and supply plants with nutrients, especially the iron.[2] Not only iron bacterial siderophores are capable to bind number of metals along with iron to enhance their bioavailability in the rhizosphere of plants. It was found that hydroxymate siderophores are mainly formed by ectorhizosphere and rhizoplane bacteria. The siderophores producing bacteria reduced the toxicity of metals and enhanced the phytoremediation. Siderophores treatment improved the growth of plants in the biological assay, growing on two different soils: one highly contaminated with heavy metals and the second strongly alkaline soil.

Iron is one of the significant micronutrient required for the activation of enzymes, oxidation reduction reactions and chlorophyll synthesis in plants. Chilli (*Capsicum annuum* L.) is an important vegetable crop cultivated in India. It is important ingredient of spices.Red colour of the chilli is mainly determined by carotenoid pigments Capsanthin and capsorubins. Iron has significant role in synthesizing this carotenoid pigment. Demand of chilli as natural coloring agent is increasing day by day. So the present study focuses on evaluation of actinobacterial siderophores as plant growth promoting agents. Byadgi chilies widely grown on Vertisols in northern Karnataka have exclusive property of low pungency and high colour value. These soils are slight to moderately calcareous and bioavailable iron is likely to be deficient in these soils. Lycopene is the chief constituent of carotenoid representing 98% of carotenoid imparting the characteristic red color to the fruit of Tomato crop. Keeping this view attempts were made in this investigation to determine the role of siderophore producing actinobacteria as Fe fertilizer for Chilli and Tomato (*Solanum lycopersicum*)

II MATERIALS AND METHODS

2.1 Production of siderophore from isolated actinomycetes.

Isolated actinomycetes were separately inoculated in liquid succinate medium and flasks were incubated at 30^{o}C for 3 to 5 days with shaking at 200 rpm. After incubation obtained supernatant was centrifuges at 10000 rpm for 20 minutes. Supernatant was then assayed for siderophore production by universal chemical assay (CAS).[3]

2.2 Qualitative analysis of siderophore: - universal chrome azurol sulphonate [CAS] assay for siderophore detection.

In a well prepared in CAS agar about 75 µl culture supernatant was added and allowed to incubate for 2-3 days at room temperature. After given time any change in color from blue to orange or yellow or purple indicated the presence of siderophore.

2.3 The Arnow test for catechole type siderophore detection [4]

In a one ml of supernatant about 1.1 ml of 0.5N HCL, 2.1ml nitrate molybdate reagent's , 3.1ml 3N NAOH was added. Formation of red color indicated positive test with absorption maxima at 510 nm.

2.4 The fecl3 test for hydroxamate type siderophore detection and classification[5]

1ml supernatant was mixed with 200 µl of 100mM $FeCl_3$ in 0.1M HCL. In results color form as orange or pink indicated the presence of hydroxamate type siderophore.

2.4 The Tetrazolium test for hydroxamate type siderophore detection:-[6]

A pinch of Tetrazolium salt was added with 1-2 drop of 2N NAOH and 0.1 ml test sample. The observed colour deep red indicate positive test.

2.5 Spectrophotometry analysis for carboxylates [7]

To 1 mL culture filtrate, 1 mL of 250 µM $CuSO_4$ and 2 mL of acetate buffer having pH 4 were added, observed for formation of blue colored copper complex

2.6 Quantitative estimation of siderophore using CAS shuttle assay described by Payne (1994)[8]

Culture was separately inoculated in succinate medium (SM) and then incubated on rotary shaker for 120 rpm at 28ºC. After incubation supernatant was collected. Supernatant (0.5ml) was then added with 0.5ml of CAS reagent and assayed at 630 nm against control uninoculated succinate medium as reference. % De-colourization was calculated by using following formula.

% Siderophore Units = [(Ar - As)/Ar] x 100.

Where, Ar - Absorbance of Reference, As - Absorbance of Sample

2.7 Quantitative estimation of siderophore using iron perchlorate assay [9]

This is a colorimetric assay which estimates hydroxamate type siderophore using deferoxymine/ferrichrome as standard.In a process 0.5 ml of culture supernatant was added with 2.5 ml of 5 mM $Fe(ClO_4)_3$ (iron perclorate) in 0.1 M $HCLO_4$ (perchloric acid) solution and allowed to incubate at room temperature for 5 minutes. Absorbance was measured at 480nm with un-inoculated medium as blank.

2.8 Factors affecting siderophore production.[3]

Influence of growth medium

Various media was used to study the siderophore secretion viz. Chemically Defined Low Iron Medium (CDLIM), Glycerol Asparagine Medium (GAM), Succinate Medium(SM), Casein Starch Medium (CSM). Siderophore production was checked after completion of 120 h of incubation at 28^0C on a rotary shaker at 220 rpm.

Influence of carbon source

The effect of carbon sources such as glucose was studied on siderophore production by *isolated strain **A2***. Carbon source was varied in the range of 0.05 – 0.4 M and its influence on siderophore production was studied.

Influence of nitrogen source

Effect of urea was studied at concentration varied in the range of 0.05 – 0.5 M. CDLIM was fortified.

Influence of phosphate concentration

In CDLIM, $K2HPO_4$ is the source of phosphate. Different concentrations of $K2HPO_4$ in the range of 0.005 – 0.05 M were used to study the effect of phosphate on siderophore production.

Influence of iron concentration

Iron content of CDLIM was varied by the addition of ($fecl_3$.) ferric chloride. Effect of iron on siderophore production was studied in the range of 0-60 µM concentration.

Influence of pH

The pH of CDLIM was adjusted in the range of 5-10 to check the influence of varying pH on siderophore production.

Influence of temperature

To check the influence of different incubation temperatures on siderophore production culture to be grown in CDLIM was incubated at different temperatures in the range of 20-44ºC for 120 h at 220 rpm

2.9 Siderophore detection using thin layer chromatography (TLC): Culture supernatant or concentrated samples of siderophore were spotted on 20 × 20 silica gel 60 F_{254} plates and spots were allowed to dry. The plates were then run in an n-butanol: acetic acid: dH2O (12:3:5) solvent system until the solvent front reaches the top of the plate. Plates were then dried and sprayed with 0.1 M FeCl3 in 0.1 N HCl. The formation of a wine-colored spot indicates a hydroxamate-type siderophore, while a dark gray spot indicates production of a catechol-type siderophore. [10]

2.10 Pot study

Sterilized seed [11] were immersed for 20 min in siderophore rich broth (90%) of six potential Actinobacteria producing higher percentage of siderophore (A2, A1,H2,H3,H4,H5) grown in Succinate Media for 72 hrs then seed were removed and allowed to dry. Vertisol calcareous soil sample brought from Dharwad region of Karnataka was sterilized and used for pot trial of chilli while slight sandy soil was used for pot trial of Tomato. Uninoculated seed were used as control. Water was added in equal quantity in the pot as per daily requirement and observed for shoot length, root length and with respect to control after 8 days till 28 days .The treatment was arranged in a randomized block design with three replicate for each treatment.

Anatomical Study of both plant was done with 10 day grown plant to observed for the development of the vascular bundles, and number and diameter of xylem vessels as the result of application of siderophore producing microorganism. Transverse cuts of the shoot samples were stained with solution (safranin -1%) and mounted on semi-permanent plates. Observed under binocular microscope.[12]

Micronutrient analysis using ICP mass spectrophotometry The plant were carefully dug out with the entire root system washed and divided in to root , shoot .The plant organs were oven dried at 60^0c for 48 hrs weighed and ground in to fine powder for the analysis of micronutrient by ICP mass spectrophotometry.

III RESULT AND DISCUSSION

3.1 Universal chemical assay (CAS)

As per CAS assay when 33 Actinobacterial islates screened on CAS agar plates formation of yellow to orange zone around the central inoculation was observed for 11 isolates indicated the presence of Siderophore

3.2 Arnow test

As per Arrow test after addition of the nitrate molybdate and sodium hydroxide in supernatant no particular color change was observed indicated negative test. This concludes absence of catachole type of siderophore

3.3 FeCl3 test

Presence of Hydroxymate type siderophore was confirmed after addition of the 100 mM $FeCl_3$ reagent in test supernatant with the formation of yellow colour in all sets.

3.4 Tetrazolium test

In a Tetrazolium test also positive reaction was apparent with deep red colour formed in a strong alkali conditions. This also proved hydroxyl type of Siderophore formation.

2.5 Spectrophotometry analysis for carboxylates

Blue colored copper complex was not formed indicating absence of carboxylates type of siderophores.

3.5 CAS shuttle assay (Payne, 1994)

In case of CAS shuttle assay also control remained blue and all experimental sets found to be yellow or pink indicated the positive test for siderphore. Quantitatively percent siderophore units were calculated by liquid Chrome Azurol Sulphonate assay (CAS assay) as described by Payne, 1994. Siderophore production by tested 11 isolate is found in the range of 67 % to 81% .Highest siderophore production of 81.22 is found in A2 Isolate.

3.6 Iron perchlorate assay

Siderophore production was found to be in the range of 62 µg\ml to 212 µg\ml .Highest concentration of siderophore recorded in isolate A2

3.7 Influence of various parameters on siderophore production.

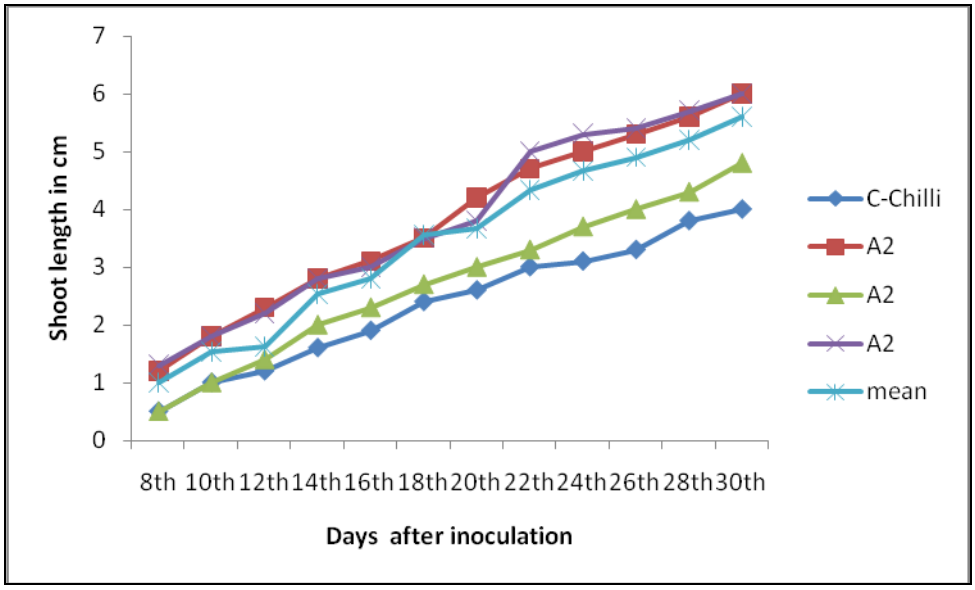
When an isolate A2 allowed to grow on four different media, maximum siderophore % was recorded with SM medium (81.22%) and least by the GAM media(72%) . With the change in glucose concentration (0.1M to 0.4M) no defined changes were recorded with marginally high siderohpore % was recorded at 0.2 M glucose concentration. Highest siderophore concentration found at 0.1M. With the variable concentration of phosphate (K_2PHO_4) Maximum siderophore % production was found at 0.2M. Iron treatment (10-40 µM) found to be inhibiting the siderophore production with the increase in the concentration. Maximum siderophore% was recorded in pH range from 7 ,8 and 9 .Then there is decrease in production of siderophore. Siderophore production in differential temperature at range by isolate A2 gets reduced as temperature increases from from 40^oC.

3.7 Thin layer chromatography

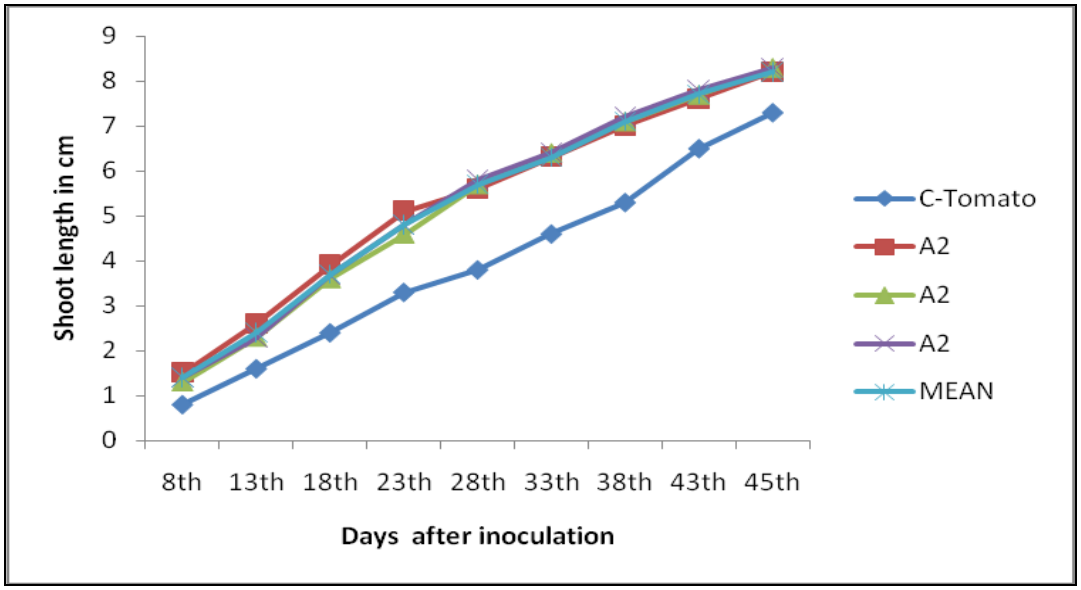
Development of wine red coloured spot with R_f value comparable with standard. This confirmed dihyroxymate type of siderophore

3.9 Pot study

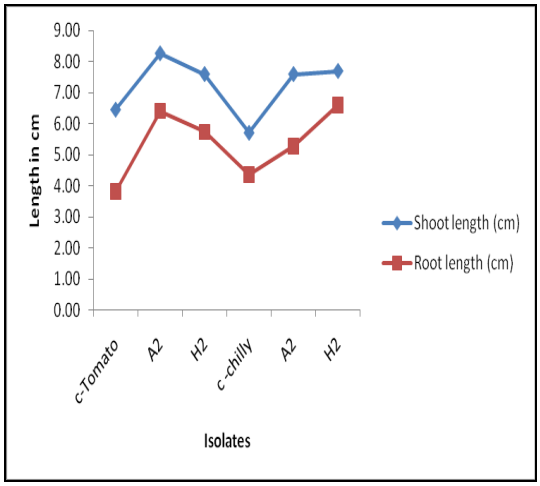
In one of the success story Maize seeds when bacterized with siderophore-producing pseudomonas identified as Fluorescent *Pseudomonas spp*. GRP3A, PR and *P. chloroaphis* ATCC 9446 in standard succinate and citrate media; they were found to be producing siderophore at 216.23µg/ml rate and later on found to be improving the maize germination percentage and overall plant growth [13] .This investigation has revealed production of 212 µg/ml by isolate A2 which has also improved growth parameters of chilli significantly .Total 6 treatments were set , out of which isolates A2, H2 and H5 isolates with siderophore rich broth significantly promoted growth of chili plant and isolate A2 and H2 for Tomato plant.Statistical analysis of the parameters such as root dry weight, shoot dry weight,root and shoot length by ANOVA revealed significance of treatment.

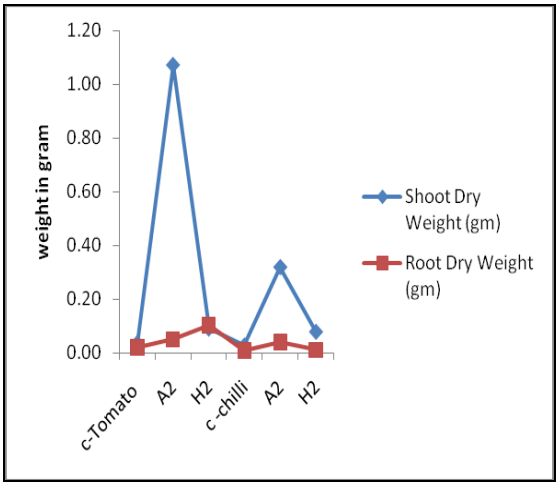


Graph -1Effect siderophore rich broth of A2 on Chilli plant shoot length, C-control

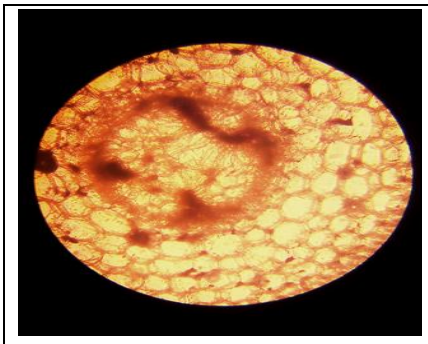


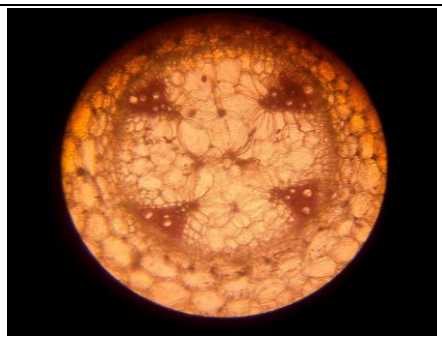
Graph-2 Effect of siderophore rich broth A2 on Tomato plant length ,C-Control





Graph 3and 4 Effect of siderophore rich broth A2 and H2 on shoot and root length, shoot and dry weight





T.S.of control shoot and treated tomato shoot Siderophore rich A2 broth (10 days old plant)

Treated tomato plant TS show increase xylem vessels size with compare control each vascular bundle has 4 xylem cells and 4 phloem cells and well developed cortex.T.S. Of stem of untreated tomato plant has not well developed vascular bundles on the same day. The results of anatomical study indicates the role of siderophore producing Actinobacteria on development of vascular bindles in plants. Nutrient analysis of A2 treated plants by ICP revealed increased uptake of iron as compared to control. This reflects the relevance of siderophore producing microorganisms in improvement of plant growth .So this investigation confirms the role of Siderophore producing Actinobacterial isolate A2 as Fe fertilizer.

Table 1: Result of Micronutrient analysis of plants by ICP mass Spectrophotometry

Parameter	Cu(ppm)	F (ppm)	Mn(ppm)	Zn(ppm)
Control chili	34	19000	284	30
Treated chili	50	30900	350	60
Control tomato	56	20600	350	64
Treated tomato	138	21100	362	66

IV SUMMARY

The inoculation of bacteria with siderophores on plant seeds caused a significant increase of shoot dry weight compared to untreated plants sustaining the proposed theory of the bacteria increasing absorption potential of the plant. Nutritional analysis of dry powder of plants confirmed the improved uptake of not only iron but Cu, Mn and Zinc by siderophore-treated plants. This denotes the application of SPB as Fe fertilizer and bioremediation of metal contaminated soils.

IV CONCLUSION

Siderophore produced by Actinobacteria may facilitate the solubility of nutrients such as Fe and other metal and utilization of microbial siderohpore by *Capsicum annum* L.plant and S*olanum lycopersicum* to improve the micronutrient availability.

V ACKNOWLEDGEMENT

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