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Research Article

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A Novel Formulation of *Piriformospora indica, Trichoderma harzianum* and *Trichoderma longibrachiatum* and Microtiter Plate Method to Determine Siderophore Production

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Abstract

The paper focuses on novel synergistic interactive study of *Piriformospora indica*, *Trichoderma harzanium*, and *Trichoderma longibrachiatum* in petri dish with the microtiter plate method under salinity and drought conditions for siderophore production*in vitro.* Isolates of *Piriformospora indica, Trichoderma harzianum, and Trichoderma longibrachiatum* showed a positive result for siderophore production on CAS agar plate under varied range of sodium chloride (NaCl) and polyethylene glycol (PEG). The isolates were further selected for qualitative estimation of siderophore where it was estimated that maximum production of siderophore was observed by *Trichoderma harzianum* followed by *Trichoderma longibrachiatum* and least production was seen in *Piriformospora indica* which was confirmed by measuring the zone diameter and color intensity on microtiter plate. Besides, all strains were placed together on plates to see synergistic reflection among them under varying NaCl and PEG conditions. Mapping these isolates under varying saline and dehydrating conditions may be considered a worthwhile approach in agriculture sustenance under iron-deficient soil.

Keywords: Synergistic, agriculture, siderophore, microtiter, drought condition.

I. Introduction

Siderophore is derived from Greek words which mean iron carriers. Iron being one of the amplest elements present in the earth's crust but still, unobtainable by microorganisms due to its tendency to form insoluble complexes of $Fe(OH)_3$at the soil pH which exists on the earth's surface. To combat this issue microorganisms have developed sophisticated techniques to chelate the insoluble iron into a soluble form by secreting certain organic compounds known as siderophores. Siderophores are considered to be of low molecular mass which helps microorganisms in low iron conditions by solubilization of iron and carrying it into

microbial cells through a transport system (Chowdappa et al. 2020). While iron is one of the essential micronutrients for growth but excessive iron results in toxicity hence the process of evolution has created a specific method for specific chelation and regulated transport of iron with help of siderophores. (Loper & Buyer 1991).

Siderophores usually are of less than 1000 Daltons and are extracellular in nature binding to (Fe^{+3}) To battle plant disease microbes producing siderophore acts as biocontrol agents. Siderophore plays a crucial role in soil mineral weathering where minerals and nutrients are directly chelated from the soil among the neighboring microorganisms. Siderophore has also aligned special attention since its one of the essential micronutrients for aquatic life, also the concentration presence of the iron is low hence siderophore produced by certain planktons plays a crucial role in the biogeochemical cycling of Fe in oceans too(Ahmed & Holmstrom, 2014)(Ghosh et al. 2017).

Microbial siderophores are also being seen as one of bioremediation remedy for accidental hydrocarbon spillage conditions in marine environments. Diverse detection techniques are employed in the detection of siderophore however the most universal technique for detection remains the chrome azurol S assay through observing the color transition around the microbial colony from blue to orange. (Srivastava et al. 2018). The bases of CAS assay technique stand on the competition for iron between the ferric complex & the indicator dye.(Neilands & Schwyn, 1987)

II. Materials and Methods

For the present invention we cultured three species of fungus available in departmental culture collection namely, *Trichoderma harzanium, Trichoderma longibrachiatum* as well as *Piriformospora indica* which were grown on PDA (Potato Dextrose Agar) at 28^{o}C for 7 days in bio-oxygen demand (BOD) incubator.

2.1 Detection of siderophore *In vitro*:

Chrome Azurol S (CAS) agar method is generally used to detect the mobilization of iron. The respective strains of fungus were being cultured on potato dextrose agar (PDA) plates after their growth,5mm of fungal mats of each isolate were scooped out from PDA plate and was placed on CAS agar plate following the protocol of Schwyn and Neiland (1987). The glass wares were prewashed with 6N HCL to remove any iron contamination and were autoclaved before using CAS indicator gave the medium a characteristic blue color as shown in Fig.1

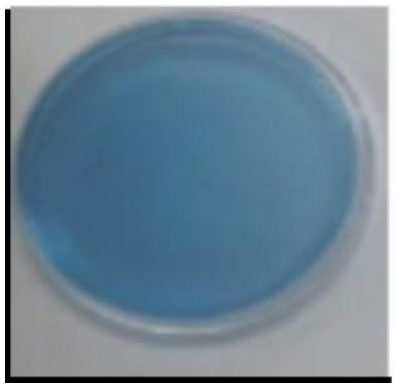


Fig.1: Blue PDA agar media before inoculation.

2.2 Detection of Siderophore with the help of Chrome Azurol Sulphonate (CAS) assay:

To make CAS Dye:

- Solution of 0.06g of Chrome Azurol Sulphonate dye (Himedia chemicals) was mixed with 50 ml of distilled water (Solution 1)
- Salt of 0.00027g $FeCl_3.6H_2O$ was mixed in 10 ml of 10 mM HCL (Solution 2)
- 0.073 gram of Hexadecyltrimethylammonium bromide is mixed with 40 ml of distilled water (Solution 3).

To make the resultant final solution: Solution 1 of CAS (Red Color) taken in 9ml is mixed with Solution 2. The final Solution is then mixed with Solution 3 by constantly stirring with finally blue color resultant solution. The blue dye was then autoclaved and stored in black plastic bottle away from direct light. This is to be noted that changing the pH as well as temperature of dye would result in blue color changing to pink color as shown in Fig.2

Fig.2: Change in Dye Color due to change in pH of the dye solution

2.3 To prepare CAS agar:

Glassware's were washed with 6N HCl, after drying20 grams of PDA was mixed in 500ml distilled water. The resultant solution was mixed on hotplate and pH was adjusted to 7 using 1N HCl and 1N NaOH. The resultant solution was then autoclaved after sealing the flask with cotton for 20 minutes at 121°C for 15psi. The solution is then allowed to cool at room temperature after which 50 ml of sterile chrome azurol sulphonate dye solution was mixed with 450 ml of potato dextrose agar. The final blue PDA agar media was then poured in glass petri plates under laminar air flow near the burner to avoid contamination.

The agar was then left to solidify, after which 5 mm fungal mats of each isolate were scooped out from PDA plate and was placed on CAS agar plate, total 3 fungus strains were placed on the petri plates. Plates were then closed and sealed using paraffin strips under aseptic environment. The petri plates were incubated at 28°C for 96hours in Incubator.

The Iron CAS complex present in agar shows the agar with a rich blue color whereas the siderophore production by the microorganism in iron starving condition gave orange color in plate. The orange halo around the fungus indicated the positive test for siderophore as shown in fig 3,4,5. The halo surrounding the fungus colony was then measured to determine the amount of siderophore production. (Arora & Verma, 2017)

Fig.3: Siderophore halo formation produced by *T. longibrachiatum*

Fig.4: Siderophore halo formation produced by *P. indica*

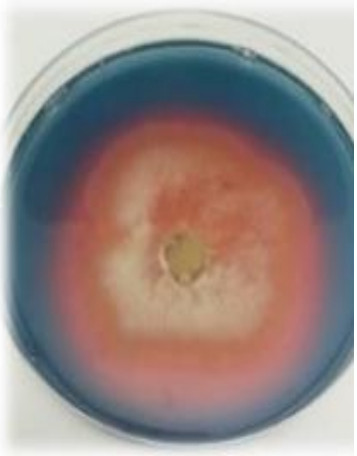


Fig.5: Siderophore halo formation produced by *T. harzanium*

2.4 Measurement of Solubilization Index:

All the isolates were screened under *in vitro* condition for their siderophore production activity on CAS agar medium. Three spot inoculation of fungal isolates was made onto the plates in triplicate under aseptic condition and incubated at 25–28°C for 96 hours. Uninoculated CAS agar plate served as control. Comparative solubilization index measurement was carried out from day 1 to day 4 of incubation by measuring clear zone and colony diameters in millimeters. Solubilization index was determined by using the following formula: ratio of the total diameter (colony + halo zone) divided by the colony diameter.

2.5 Petri dish method to determine the effect of NaCl & PEG condition on siderophore production.

For petri dish study the CAS agar plates were prepared according to the protocol devised by (Neilands & Schwyn, 1987). The potato dextrose agar was mixed with varying concentration of PEG and NaCl and was poured in petri plates in presence of CAS dye. All the three fungal isolates were being placed on the CAS media and the promotion or inhibitory action of all the three fungal isolates were being recorded as observed in fig 6.

2.6 Microtiter plate method to determine the effect of NaCl & PEG condition on siderophore production.

For microtiter plate study 12 gram of potato dextrose broth was added in 500 ml of water followed by autoclaving and preparing of 1%w/v, 2%w/v as well as 3%w/v concentration of solution with both NaCl as well as PEG(Polyethylene Glycol),Fungal mycelium of each strain were placed in their respective wells after which the potato dextrose broth was added in each well as a growth media simultaneously adding Chrome azurol s dye in each well whereas controls were placed without mycelium to check for any contamination which would hamper the continuity of the study. The whole microtiter plate was placed in shaker incubator at 95 RPM at 28 °C for 4 days. The result was observed and inference for the same was carried out (fig 8 & 9).

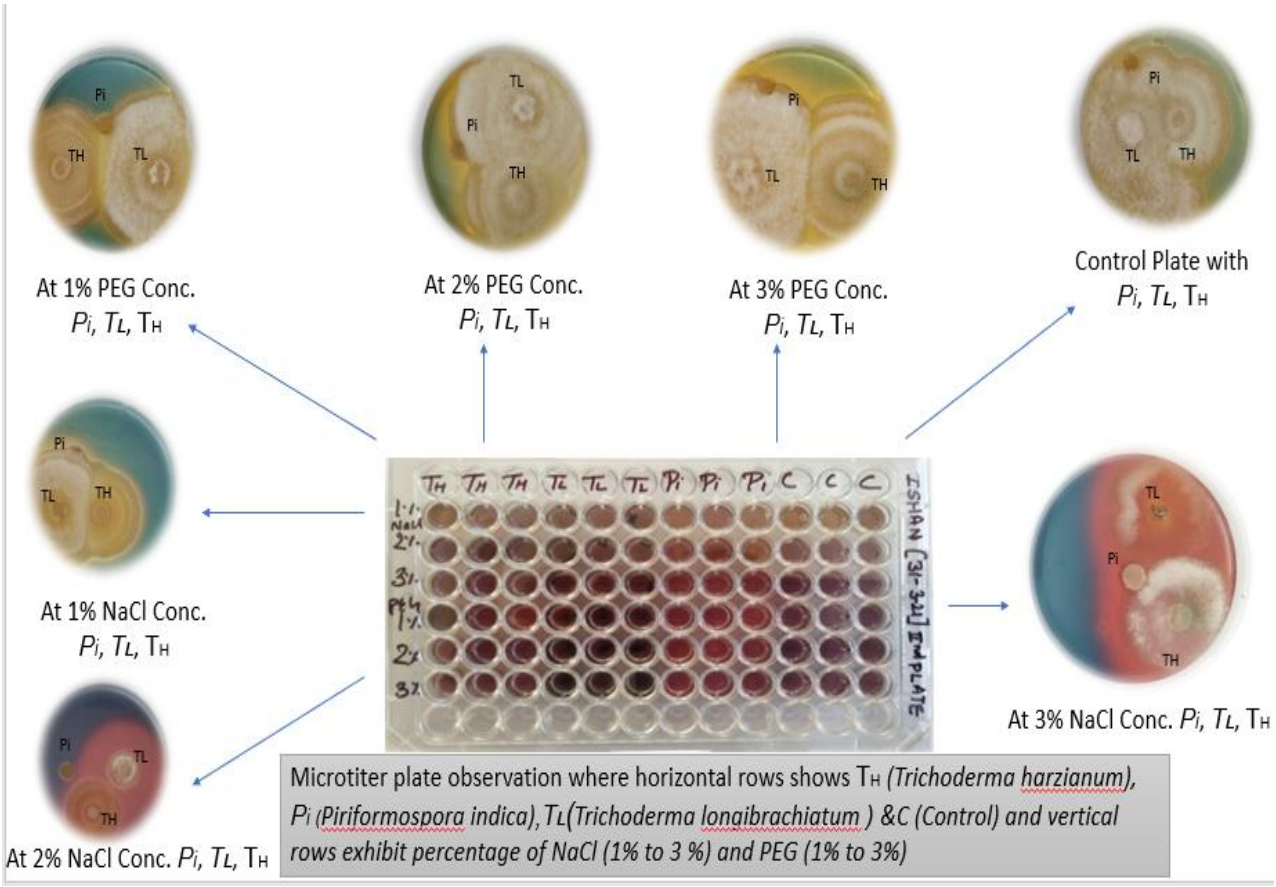


Fig.6: Demonstrating the effect of varying PEG & NaCl Conc. (1%, 2% & 3%) on the three fungal isolates on CAS Solid Media

III. Results and Discussion

3.1 Siderophore Detection:

It was observed that orange halo formation around the fungus colonies due to iron ligand complexformation which was anindication of siderophore production, among the strains kept under study *Trichoderma harzianum* showed maximum siderophore production within minimum time frame whereas moderate siderophore production was observed in *Trichoderma longibrachiatum* and followed by *Piriformospora indica* as seen in Fig 7.

The amount of siderophore production in *T. harzianum* strains shown in fig. 5, whereas Fig. 4 shows the amount of siderophore production in *P. indica* and figure 3 reflects siderophore production in *T. longibrachiatum*.

As per the results obtained, we can conclude that *T. harzianum is* a good and rapid producer of siderophore as compared to other fungal strains (fig.7), wherein the siderophore pattern observed as: *Trichoderma harzianum* > *Trichoderma longibrachiatum*> *Piriformospora indica*.

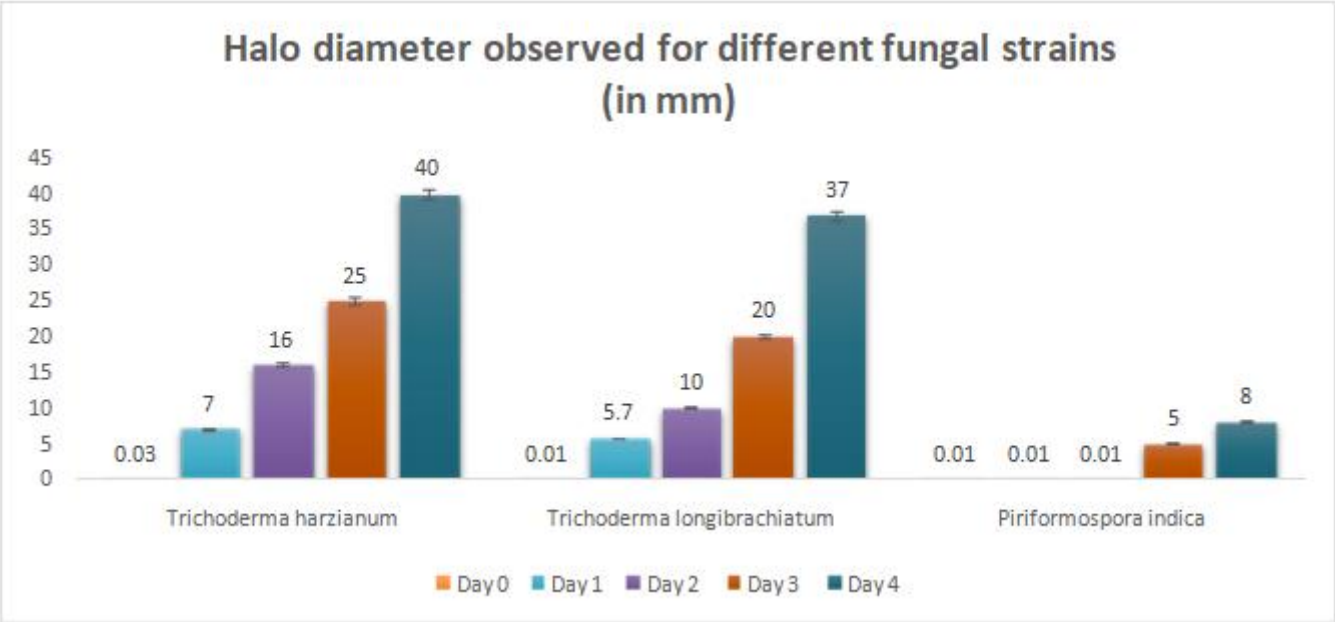
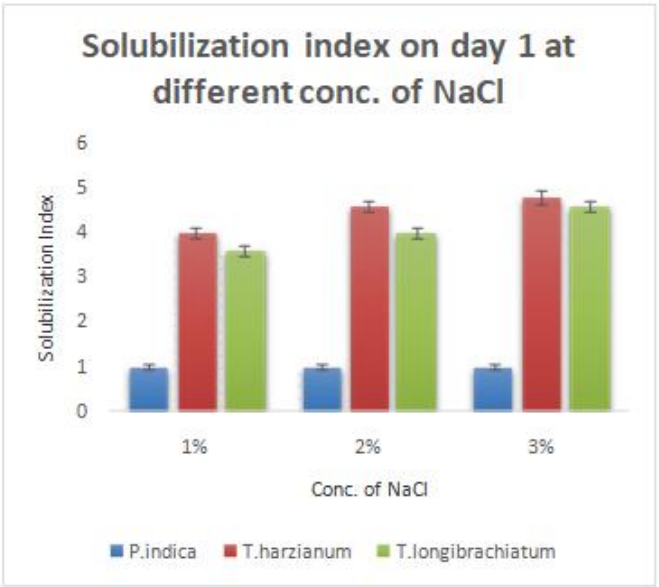
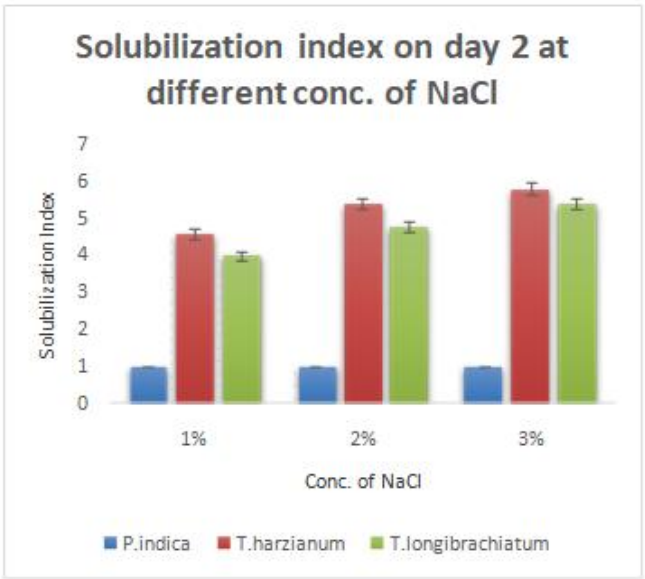


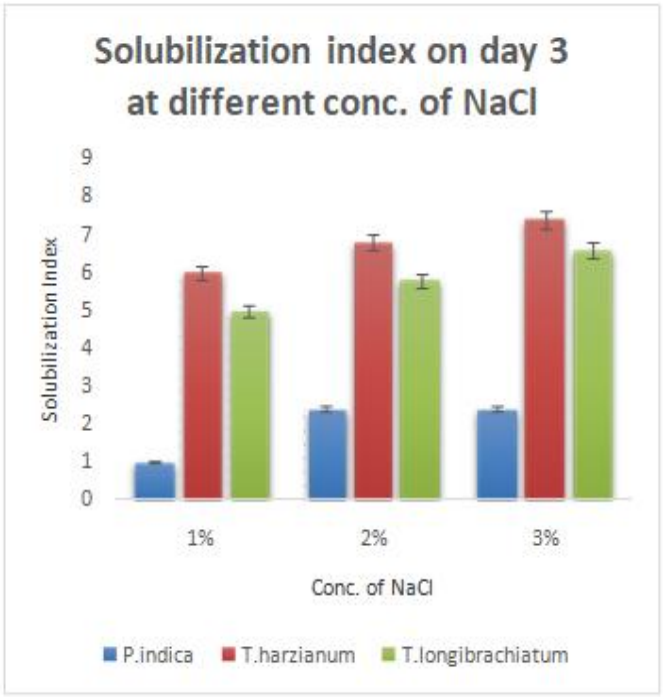
Fig.7: Demonstrating the effect of varying PEG & NaCl Conc. (1%,2% & 3%) on the three fungal isolates on CAS Solid Media.

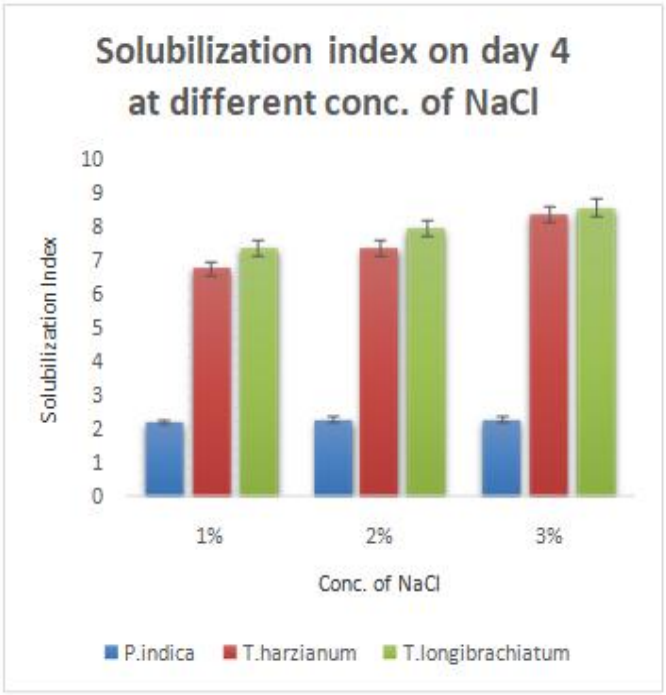
Based on solubilization index calculation it can be easily inferred that the positive production of siderophore was being observed under varying NaCl as well as PEG concentrations by all the three isolates and by comparing the various isolates, it is concluded that the *T. harzianum*has the strongest ability to produce siderophore as compared to *T. longibrachiatum*and *P. indica*. The solubilization index was found to be greatest in case of *T. harzianum*than *T. longibrachiatum*and *P. indica*varying stress conditions.

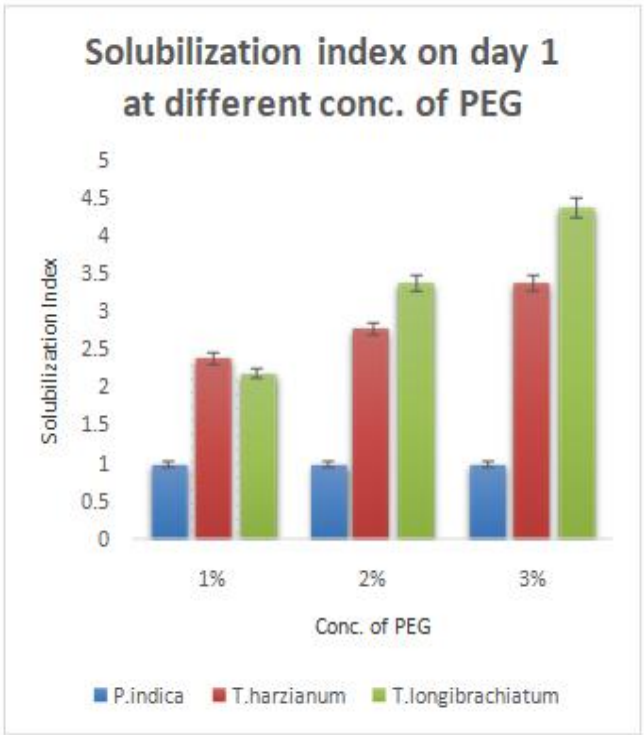
3.2 Graphs displaying Solubilization Index (SI) of fungal isolates on CAS solid Media at varying NaCl & PEG concentration:

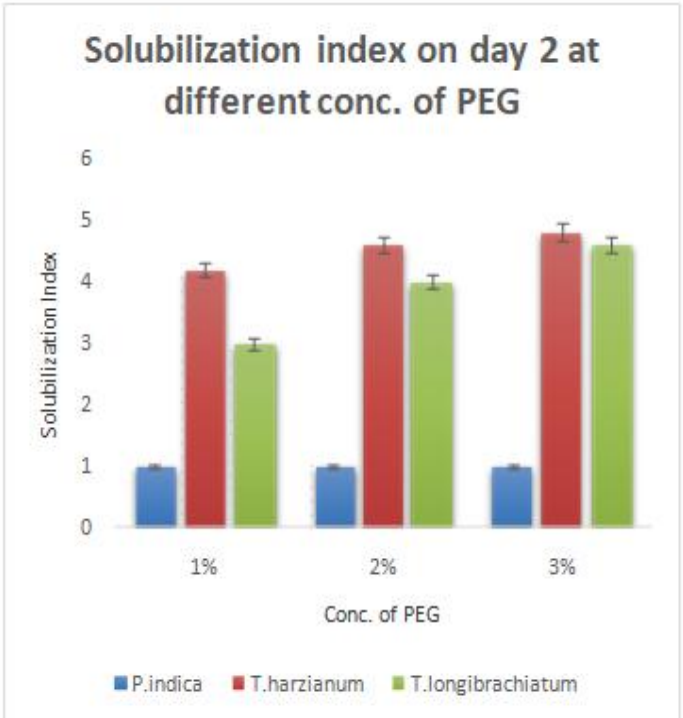


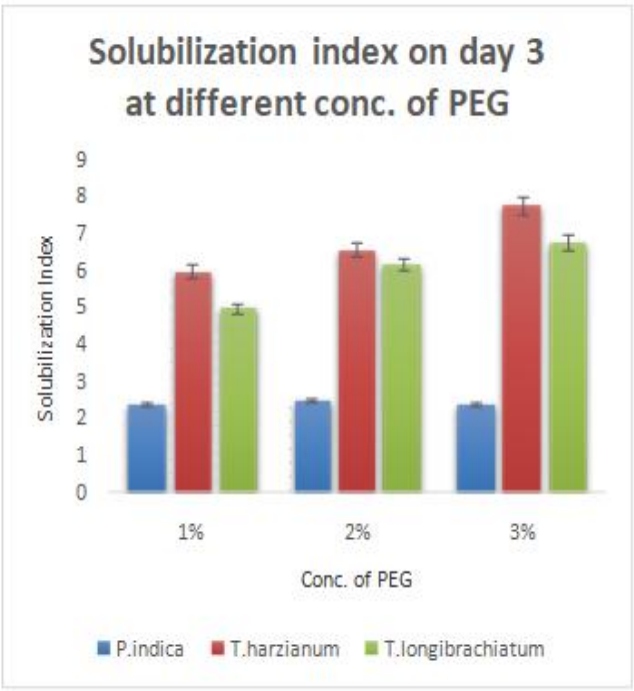


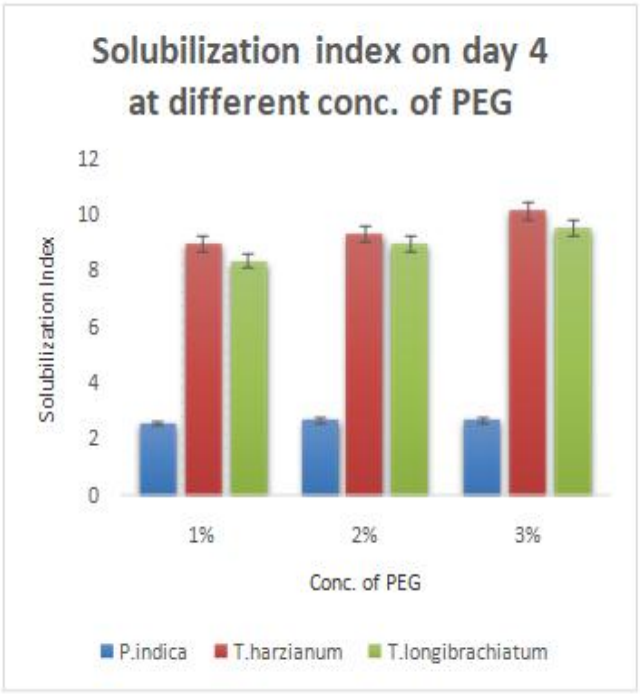

Solubilization index on day 3 at different conc. of NaCl
Solubilization Index
0
1
2
3
4
5
6
7
8
9
1%
2%
3%
Conc. of NaCl
P.indica
T.harzianum
T.longibrachiatum


Solubilization index on day 4 at different conc. of NaCl
Solubilization Index
0
1
2
3
4
5
6
7
8
9
10
1%
2%
3%
Conc. of NaCl
P.indica
T.harzianum
T.longibrachiatum


Solubilization index on day 1 at different conc. of PEG
Solubilization Index
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
1%
2%
3%
Conc. of PEG
P.indica
T.harzianum
T.longibrachiatum


Solubilization index on day 2 at different conc. of PEG
Solubilization Index
0
1
2
3
4
5
6
1%
2%
3%
Conc. of PEG
P.indica
T.harzianum
T.longibrachiatum





3.2 Microtiter Plate Experiment:

The positive result of the siderophore production has led to further verify the siderophore production in case of salinity and drought conditions, for the same 96 well microtiter plate was inoculated with the CAS dye and respective fungus mycelium were placed. The whole setup is shown in figure 8 as on first day.

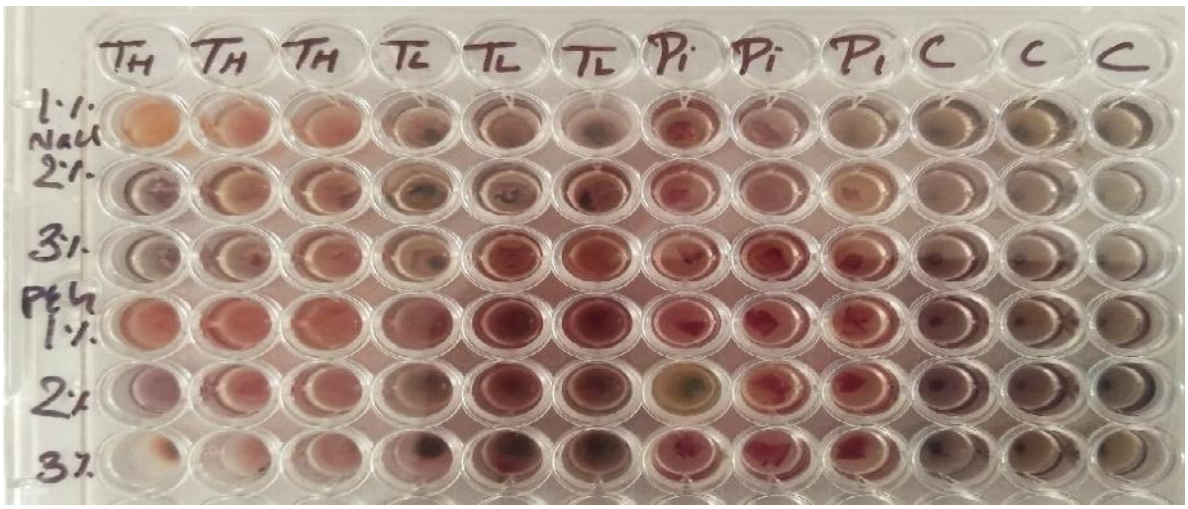


Fig.8: Initial Setup of microtiter plate assay to represent the siderophore production where horizontal rows shows : T_H *(Trichoderma harzianum),* T_L *(Trichoderma longibrachiatum),* P_i*(Piriformospora indica)*& C (Control) and vertical rows exhibits percentage of NaCl (1% to 3%) and PEG (1% to 3%)

The choice of microtiter plate setup was done keeping in mind that the experiment design has potential to save time producing rapid results and also is an easy way to detect siderophore production when compared to classical or traditional method.

The microtiter plate setup design reduced the chemicals utilization up to 70 percent costing less and saving manpower. Based on daily observation up to day 4 results concluded with the microtiter plate study for siderophore detection in presence of different concentrations of NaCl as well as PEG were surprising since the siderophore detection was observed in all the wells of the microtiter plate comparatively more or less at different concentrations as shown in figure 9.

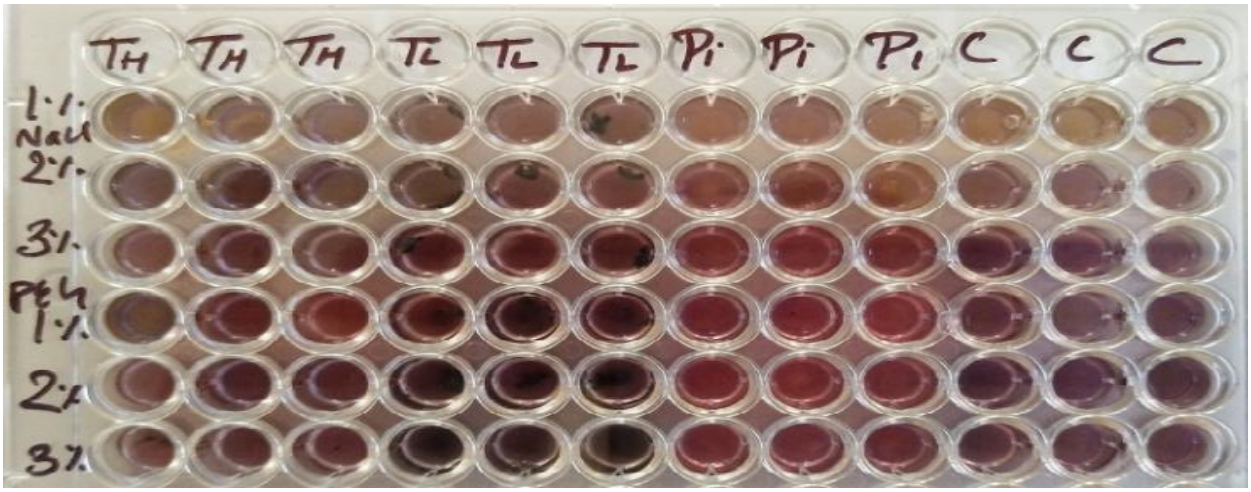


Fig.9: On Day 4 microtiter plate observation, where horizontal rows shows :T_H*(Trichoderma harzianum),* T_L *(Trichoderma longibrachiatum),* P_i*(Piriformospora indica)*& C (Control) and vertical rows exhibits percentage of NaCl (1% to 3%) and PEG (1% to 3%)

The lower concentrations of NaCl which is 1% w/v & 2%w/v NaCl showed poor siderophore production for all the three fungus (*T. harzianum, T. longibrachiatum, P.indica)*. The maximum amount of siderophore production was being observed by *T. harzianum* in concentrations with 2%w/v and 3%w/v PEG. Least siderophore production was observed in *P.indica* at 1% w/v and 2%w/v NaCl salt concentrations. *T. longibrachiatum* displayed moderate siderophore production in presence of different concentrations of salts such as NaCl and PEG, which can also be verified through the data presented in graphical format.

By comparing the overall efficacy of siderophore production qualitatively based on microtiter plate it can be concluded that *T. harzianum* can be considered as a good choice to be used in agricultural fields.

IV. Conclusion

The study conducted has novel approach by deploying synergistic impact of*T. harzianum, T. longibrachiatum, P. indica* to solve farmer issues with iron deficient soil in conjunction with agricultural productivity under varied range of saline and dehydrated conditions.

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