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5. Study of Different Parameters of Plant Growth Induced by Plant Growth Promoting Fungi (PGPF) for Sustainable Agriculture

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5.1 Introduction:

Plant rhizosphere is a micro ecosystem at the vicinity of plant root system comprising of hot spot zone of microbial community (like fungi, bacteria, virus, insect, etc.) interacting with each other by several mechanisms influenced by root exudates. The fungal community which are non-pathogenic and promote growth of plants are called plant growth promoting fungi (PGPF), is a major focusing area for sustainable agriculture. It was experienced that non-judicious application of numerous chemical fertilizer and fungicides is liable to environmental pollution, deterioration of soil health and human health hazards in course of biological magnification due to non-degradable nature of synthetic fungicides. Presently, plant growth promoting fungi (PGPF) are getting much more attention for sustainable agriculture as it is supposed to economically viable due to its effectiveness, harmless to environment and perform

different mechanisms for promoting growth of plant such as the production of plant growth substances (e.g. Indole acetic acid, soluble phosphate, etc.), antagonistic activity against pathogen (through antibiosis, competition and mycoparasitism).

5.2 Seed Priming:

Seed priming is a pre-sowing treatment which leads to a physiological state that enables seed to germinate more efficiently. The majority of seed treatments are based on seed imbibition allowing the seeds to go through the first reversible stage of germination but do not allow radical protrusion through the seed coat. Seeds keeping their desiccation tolerance are then dehydrated and can be stored until final sowing.

During subsequent germination, primed seeds exhibit a faster and more synchronized germination and young seedlings are often more vigorous and resistant to abiotic stresses than seedlings obtained from unprimed seeds.

Priming often involves soaking seed in predetermined amounts of water or limitation of the imbibition time. The imbibition rate could be somehow controlled by osmotic agents such as PEG and referred as osmopriming.

Halopriming implies the use of specific salts while "hormopriming" relies on the use of plant growth regulators. Some physical treatments (UV, cold or heat) also provide germination improvement thus suggesting that priming effects are not necessarily related to seed imbibition.

A better understanding of the metabolic events taking place during the priming treatment and the subsequent germination should help to use this simple and cheap technology in a more efficient way.

5.2.1 Seed Priming and Calculation of Vigor Index (VI):

A. Requirements:

- Viable seed (100-200)
- Mercuric chloride (0.1%)
- Biotic agent/Abiotic agents [Conidial suspension (10^8 cfu ml^{-1}) as biotic agent]
- Petriplate
- Sterile distilled water
- Measuring scale

B. Methodology:

Plant seed was collected and then surface sterilized with 0.1% mercuric chloride for 2-3 min and then rinsed three times with distilled water.

Then 100-200 seeds were treated with 10ml of conidial suspension (10^8 cfu ml^{-1}) of biocontrol agent on petriplates. Five replicates were maintained for each treatment.

Treated seeds were kept at normal temperature for germination and growth observation. Seeds treated with sterile distilled water were served as untreated control. After 15 days, percent of germination, root length and shoot length were recorded and vigor index (**Table 5.1)** was calculated following the method of Baki and Anderson, 1973 (Abdul-Baki & Anderson, 1973). Vigor Index (VI) = Percent of Seed Germination x [Mean of Root Length + Mean of Shoot Length.

Table 5.1: Vigor Index after treatment with spore suspension of bio-control agent

Treatment/ Replica	% Seed Germination	Shoot length	Root length	Vigor Index Seed Germination (%) x [Mean of Root Length + Mean of Shoot Length]
	(10^8 cfu ml^{-1})	(10^8 cfu ml^{-1})	(10^8 cfu ml^{-1})	(10^8 cfu ml^{-1})
A	94	7.21	9.37	1558.52
B	93	5.98	9.39	1429.41
C	92	5.98	7.39	1230.04
D	93	5.37	6.39	1093.68

5.3 Antagonistic Activity:

Antagonists can be active through several mechanisms, such as mycoparasitism, antibiosis or other inhibitory substances, competition for nutrients or space or induced resistance.

Bio control agent (BCA) performs antagonistic activity for a specific pathogenic microorganism. Some biocontrol agents are *Trichoderma harzianum, Gliocladium virens, G. cathenulatum,* non-pathogenic *F. oxysporum, Coniothyrium minitans, Streptomyces griseoviridis* and *Bacillus subtilis.* A lot of research has also been done on *Pseudomonas* species which are good antagonists

In competition, BCA may grow faster and utilize food sources more efficiently at its niche than the soil borne pathogenic microorganism, thereby reduction of population of soil borne pathogenic microorganism. The most common reason for the death of many microorganisms growing in the vicinity of BCA is the starvation and scarcity of limiting nutrients source.

For antibiosis, antagonistic microorganisms may release some antimicrobial compound that slows down or completely inhibit the growth of pathogenic microorganisms. In *Trichoderm*a, small size diffusible compounds or antibiotics are produced that inhibit the growth of other microorganisms (Benitez et al. 2004).

In case of mycoparasitism, BCA may feed on pathogenic microbes directly. Mycoparasitism is one of the main mechanisms of *Trichoderma*. The process apparently include, chemotropic growth of *Trichoderma*, recognition of the host by the mycoparasites, secretion of extra cellular enzymes, penetrations of the hyphae and lysis of the host (Zeilinger et al. 1999).

Trichoderma recognizes signals from the host fungus, triggering coiling and host penetrations. Gajera et al. (2013) also reported in his study about the constitutive secretion of exochitinases by *Trichoderma* at low level which could degrade fungal cell-walls releasing oligomers played a central role in growth inhibition of pathogenic fungal strains.

5.3.1 Screening for Antagonistic Activity:

A. Requirements:

- Microbial agents (suspected as bio-control agent)
- Microbial pathogen (Bacteria/Fungus) causing disease
- Petriplate
- Potato Dextrose Media (PDA)
- Cork borer
- Measuring Scale
- Laminar Chamber
- BOD Incubator

B. Methodology:

Different microbial agents (bacteria or fungi) were used for screening of their antagonistic activity against other microbial pathogens by dual culture method as described by Skidmore and Dickinson (1976).

In dual culture method bio-control fungal disc (5mm) using sterile corn borer from 7 days old culture on PDA plate was taken and placed on PDA plate (petridish) in the periphery.

At the same time 5mm disc of pathogen was placed towards the opposite side on the same petri plate and incubated at 28 ± 2^0C for 7 days.

For each isolate three replicates were maintained. For the control, only pathogen was allowed to grow.

In antagonistic activity, percent inhibition of radial growth (PIRG) of pathogen against bio-control agent was studied after 3-10 days of incubation following the formula given by Skidmore and Dickinson 1976.

$$PIRG = \frac{R_1 - R_2}{R_1} X\ 100$$

Where PIRG stands for percentage of inhibition of radial growth of pathogen, R_1 means radial growth of pathogen without bio-control agent (Control), R_2 means radial growth of pathogen with bio-control agent as treatment (**Figure 5.1 and Table 5.2**).

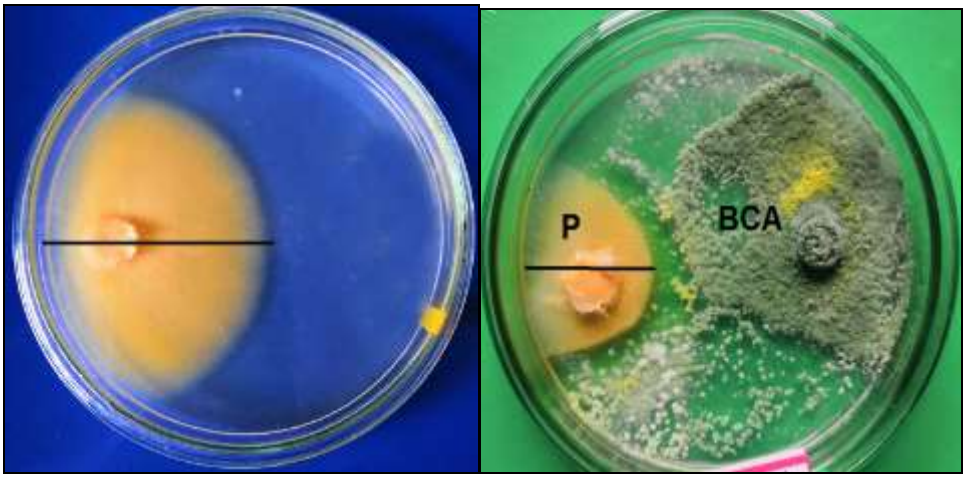

Figure 5.1: Dual culture method. P indicates pathogen; BCA indicate Bio-control agent. Black line indicate diameter of radial growth.

Table 5.2: Dual culture method for determination of Percent Inhibition of Radial Growth (PIRG) of a pathogen

Bio-control agents	Redial diameter (R_1) of pathogen without bio-control agent (mm)	Redial diameter (R_2) of *pathogen against* bio-control agent (mm)	PIRG = (R_1–R_2)/R_1x100
A	35	12	65.71
B	35	24	31.42
C	35	06	82.85
D	35	15	57.14
E	35	08	77.14

Where PIRG stands for percentage of inhibition of radial growth of pathogen, R_1 means radial growth of *pathogen* without bio-control agent (Control), R_2 means radial growth of pathogen with bio-control agent as treatment.

5.3.2 Antimicrobial Activity:

Plants are an extremely rich source of diverse organic compounds which are antifungal. In general, the interest in such antifungal compounds depends upon the concentration required for activity and the biological spectrum of activity. Some of the compounds are preformed and are located in external plant tissues, i.e., bark, peel, and cuticle; others are located throughout the plant, often in vacuoles; and still others are produced by plants in response to physiological stress or infection. In the latter group are the phytoalexins, low molecular weight organic compounds produced by plants in response to infection or stress and localized at the site of infection or stress. This group of compounds is usually lipophilic and includes compounds as diverse as simple phenols, flavonoids, isoflavonoids, coumarins, isocoumarins, sesquiterpenoids, polyenes, stilbenes, furanoterpenoids, and derivatives of these compounds.

Screening of antimicrobial activity by innovative Agar block method (Zerald *et al.*, 2020)

A. Requirements:

- Abiotic agents (. plant extract/ fungal metabolites/ chemical suspected as antimicrobial)
- Sterile agar solution (2%)
- Sterile petriplates
- Sterilized cork borer
- Measuring Scale
- BOD Incubator

B. Methodology:

Antimicrobial *in-vitro* screening of different abiotic agents was carried out following innovative agar block method by Zerald *et al.*, 2020. For agar block method, 10ml of 2% agar solution was prepared in 20ml culture tube, capped and then sterilized at 121°C with 15lb in^{-2} for 15 minutes. After sterilization, abiotic agent (i.e. plant extract/ fungal metabolites/ chemical) were added and poured on sterile petriplates aseptically, uniform layer was made and solidified. Then 5 mm diameter agar block containing abiotic agent was cut using sterilized corn borer. The agar blocks containing fungicides were placed in the periphery on a freshly prepared PDA plates. Fungus pathogen of 7 days old culture on PDA medium were cut as a fungal disc using sterilized corn borer and placed onto the centre of test plates. It was then incubated at 25°C for 10 days. Bioactive potentials of different abiotic agents were evaluated by measuring Percent of Inhibition of Radial Growth (PIRG) against the test fungal pathogen. In control, sterilized water was used in place of abiotic agent on agar block. PIRG was calculated using the following formula: $PIRG = (R_1–R_2)/R_1x100$. Where, R_1 = diameter of colony of test pathogen in the control; R_2 = diameter of colony of test pathogen (cm) on treated plates.

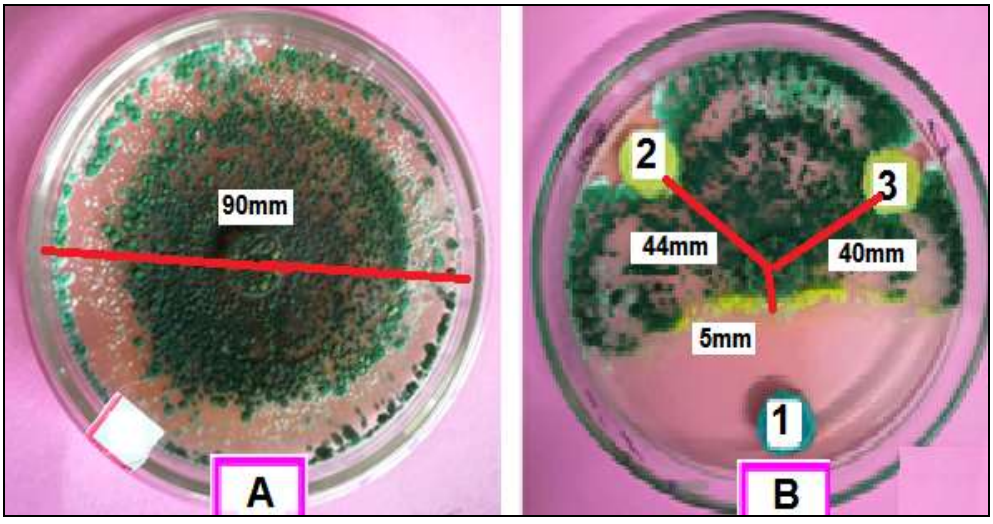

Figure 5.2: Innovative Agar block method for screening antimicrobial effect of abiotic agents (1, 2 and 3). A indicates radial growth of pathogen without treatment (Control). B indicates radial growth of pathogen with treatment of abiotic agents (1, 2 and 3).

Table 5.3: Agar block method for determination of percent of Inhibition of Radial Growth (PIRG) of test fungal pathogen against abiotic agents

Abiotic agents (plant extract/ fungal metabolites/ chemical)	Diameter of colony of test pathogen in the control plate R_1 (mm)	Diameter of colony of test pathogen on treated plates R_2 (mm)	Percent of Inhibition of Radial Growth [PIRG = $(R_1 – R_2)/R_1 x100$]
1	90	10	88.88
2	90	88	2.22
3	90	80	11.11

Where, R_1 = diameter of colony of test pathogen in the control; R_2 = diameter of colony of test pathogen (cm) on treated plates.

5.4 Biochemical Characterization:

5.4.1 Indole Acetic Acid (IAA) Production:

Indole-3-acetic acid (IAA) mediates an enormous range of development and growth responses including embryo symmetry establishment, initiation of cell division, promote vascular differentiation, root initiation and apical dominance.

PGPR all over the world have been reported to enhance the plant growth by the production of phytoharmones. *Azotobacter* and *Azospirillum* produced IAA ranged from 19.4-30.2µg/ml.

Pseudomonas fluorescens AK1 and *Pseudomonas aeruginosa* AK2 were tested for their ability to produce indole-3-acetic acid in the presence and absence of tryptophan and revealed that for both strains, indole production increased with increase in tryptophan concentration.

A. Requirements:

- Bio-control agent (7 days old)
- PDA broth
- Conical flask
- Sterile cork borer
- Test tube/Culture tube
- Tryptophan (500µg/ml) as the precursor of IAA
- Clinical Centrifuge for 10,000 rpm
- BOD Incubator
- Shaker for 120 rpm at 28 ± 2^0C
- Salkowski reagent (50ml, 35% of $HClO_4$, 1ml 0.5 m $FeCl_3$ solution)
- Orthophosphoric acid (10mM)

B. Methodology:

Indole acetic acid (IAA) production was detected following the method of Bric *et al*., 1991 (Bric, Bostock, & Silverstone, 1991; Shahab, Ahmed, & Khan, 2009). Bio-control agents were on grown PDA media and incubated at 37^0C for seven days. After seven days of incubation, fungal discs (5mm) using corn borer were transferred in conical flasks (250ml) containing PDA broth (100ml) amended with 500µg/ml tryptophan as the precursor of IAA and incubated in a shaker at 120 rpm at 28 ± 2^0C for 3-5 days. Control was set up without tryptophan. Fully grown cultures were centrifuged at 10,000 rpm for 10min. 1ml of the supernatant was mixed with 4ml of the Salkowski reagent (50ml, 35% of $HClO_4$, 1ml 0.5 m $FeCl_3$ solution). Further two drops of 10mM orthophosphoric acid was added to it and kept in dark for colour formation. Three replicates for each isolate were maintained. Appearance of pink color in test tubes indicates a positive result for IAA production. The amount of IAA production was expressed by + and – sign. The sign minus (–) indicates no IAA production; one plus (+) indicates faint pink colour and small amount of IAA production; double plus (++) indicates pink colour and medium amount of IAA production; triple plus (+++) indicates dark pink colour and high amount of IAA production.

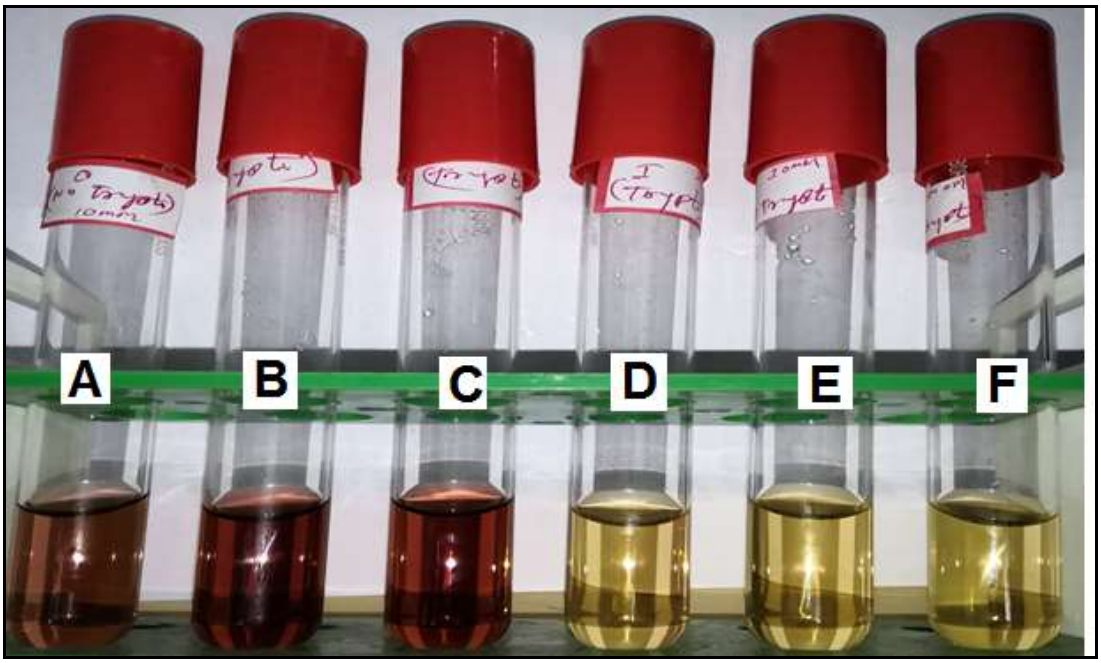

Figure 5.3: Indole Acetic Acid (IAA) production by bio-control agents (A-F).

Table 5.4: Amount Indole Acetic Acid (IAA) production by Bio-control agents

Bio-control agents	Amount of IAA production
A	++
B	+++
C	+++
D	-

Bio-control agents	Amount of IAA production
E	-
F	-

The sign minus (–) indicates no IAA production; one plus (+) indicates faint pink colour and small amount of IAA production; double plus (++) indicates pink colour and medium amount of IAA production; triple plus (+++) indicates dark pink colour and high amount of IAA production.

5.5 Solubilisation of Inorganic Phosphate:

Phosphorus is among the primary essential nutrient elements required by the plant in optimum amount for its proper growth and development. Although P content in an average soil is 0.05%, only 0.1% of the total P present is available to the plants because of its immobilization, chemical fixation and low solubility. Under such conditions, Phosphate solubilizing microorganisms (PSM) offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants through the secretion of various organic acids and enzymes. Further, most of the phosphate solubilising microorganisms were identified as *Trichoderma* sp., *Furarium* sp, *Bacillus* spp., *B. pantothenticus, B. megaterium, Flavobacterium* spp., *Klebsiella* spp., *Enterobacter alvei*, *Pseudomonas* spp., *Azotobacter* spp., *Rhizobium* spp. and *Azospirillum* spp.

A. Requirements:

- Culture of Bio-control agent (suspected for phosphate-solubilizing efficacy)
- Petriplates
- Sterile cork borer
- Pikovskaya's agar medium
- Tri-calcium phosphate/Rock phosphate
- BOD-Incubator
- Measuring Scale

B. Methodology:

For detection of phosphate-solubilizing efficacy, a loop full of actively growing culture of Bio-control agent were cut using sterile corn borer and placed on the centre of petriplates containing Pikovskaya's agar amended with tri-calcium phosphate and incubated at 28±2°C for 7 days (Pikovskaya, 1948).

Three replicates for each treatment were maintained. In control set without tri-calcium phosphate for each isolates were maintained. Incubated plates showing clear zone around the growth of bio-control agent was considered as positive. The performance of each fungus was marked by assigning them – and + sign (**Figure 5.4 & Table 5.5**).

The – indicates no P solubilisation; + small amount of P was dissolved; ++ medium amount of P was dissolved; +++ high amount of P was dissolved. Percent solubilisation efficiency and phosphate solubilisation index was calculated as:

Solubilization Index (SI) = (R+Z)/R; Solubilization Efficiency (SF) = (R+Z)/R x100; Where, SI = Solubilization Index, SE= Solubilization Efficiency, Z = Clear zone diameter (mm), R = Colony diameter (mm).

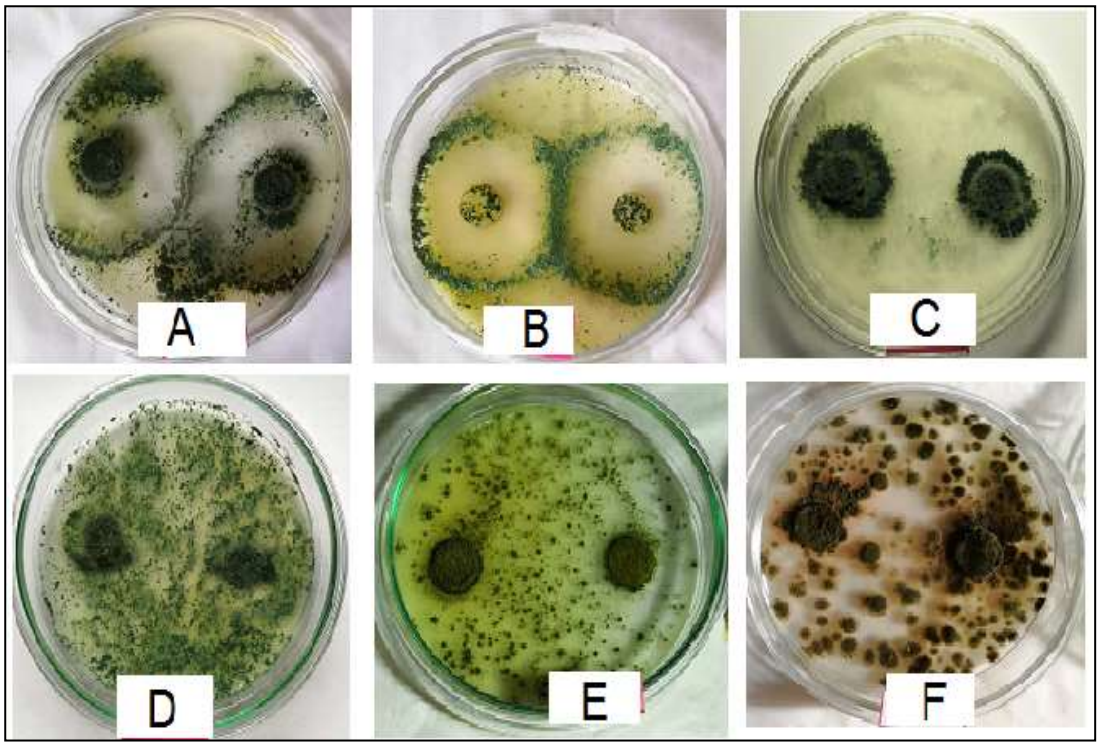

Figure 5.4: Phosphate Solubilization ability by different bio-control agents (A-F).

Table 5.5: Study of Phosphate Solubilization efficiency of different Bio-control agents on Pikovskaya's solid agar medium

Bio-control agents	Clear zone diameter (Z) mm	Colony diameter (R) mm	Solubilization Index	Solubilization Efficiency
A	25	55	1.454545455	145.4545455
B	32	58	1.551724138	155.1724138
C	5	28	1.178571429	117.8571429
D	0	24	1	100
E	0	24	1	100
F	0	28	1	100

Solubilization Index (SI) = (R+Z)/R; Solubilization Efficiency (SF) = (R+Z)/R x100

Where, SI = Solubilization Index; SE= Solubilization Efficiency; Z = Clear zone diameter (mm); R = Colony diameter (mm).

5.6 Hydrogen Cyanide (HCN) Production *in Vitro* Assay:

Cyanide forms a stable complex with the essential elements (Cu^{2+}, Fe^{2+}, and Mn^{2+}) for the protein function and therefore is considered as a toxic substance to most living organisms.

Hydrogen cyanide (HCN) is a volatile secondary metabolite that is synthesized by many bio-control agents and has a powerful effect on many pathogenic microorganisms.

HCN inhibits the electron transport and disrupts the energy supply to the cell, which leads to death of living pathogenic microorganisms.

Many bio-control agents (fungi and bacteria) have the ability to produce HCN including species of *Trichoderma* sp. *Bacillus* sp., *Pseudomonas* sp., and *Rhizobium* sp.

A. Requirements:

- Bio-control agents [Suspected for cyanide (HCN) production]
- Potato dextrose agar (PDA)
- Glycine (4.4 g/L)
- Petriplates
- Whatman number 1 filter paper
- Picric acid (5%
- 2% sodium carbonates (w/v)
- Parafilm paper
- BOD incubator

B. Methodology:

Bio-control agents were screened for hydrogen cyanide (HCN) production following the method of Lorck (1948) with some modification.

5mm disc of each isolate from seven days old culture were inoculated on petriplates containing sterilized potato dextrose agar (PDA) medium amended with glycine (4.4 g/L).

Controls were prepared using the similar setup but without glycine amended potato dextrose agar (PDA) medium.

Whatman number 1 filter paper previously soaked in a specific solution [0.5% picric acid and 2% sodium carbonate (w/v)] was properly adjusted underneath the petriplate's lid.

Plates were then sealed with parafilm paper tightly and incubated at 25°±2°C for 7 days.

After 7 days of incubation, the appearance of orange to red colour on whatman paper indicated the production of hydrogen cyanide.

Three replicates were prepared for each isolate. The amount of HCN production was expressed by + and – sign.

Thus sign minus (-) indicates no HCN production; one plus (+) indicates small amount of HCN production with light red colour; double plus (++) indicates moderate amount of HCN production with brown colour; triple plus (+++) indicates high amount of HCN production with red colour.

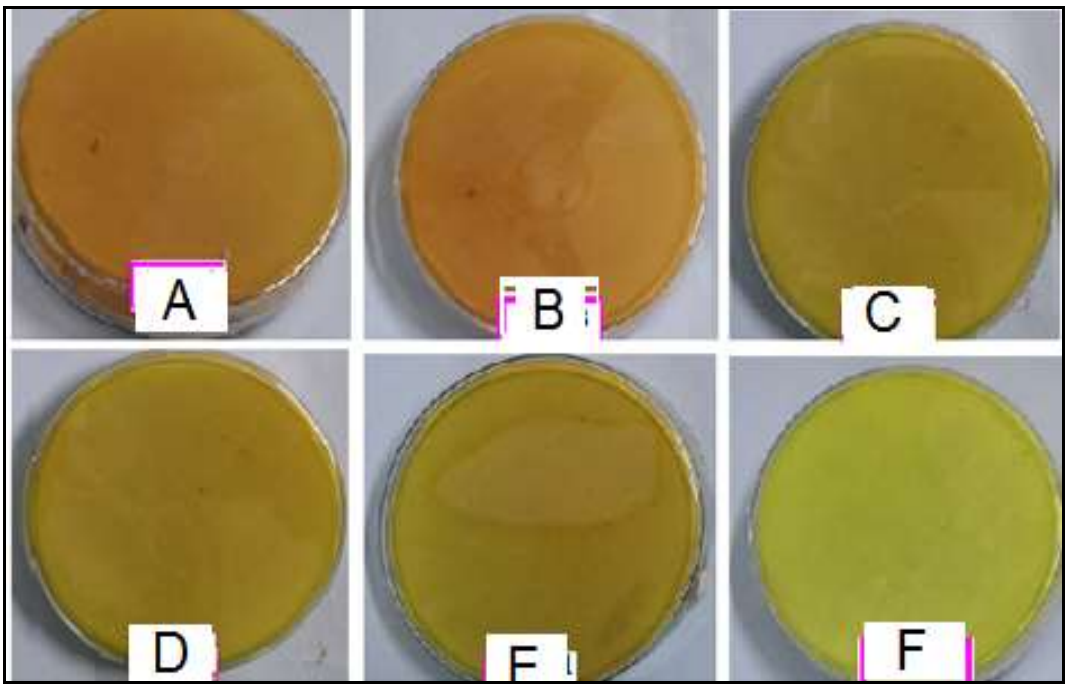

Figure 5.5: Hydrogen cyanide (HCN) producing by different Bio-control agents (A-F). No colour change indicates no HCN production; light red colour indicates small amount of HCN production; brown colour indicates moderate amount of HCN production; red colour indicates high amount of HCN production.

Table 5.6: Assay of hydrogen cyanide (HCN) production by different Bio-control agents

Bio-control Agents	Degree of HCN Production
A	+++
B	+++
C	++
D	+
E	+
F (Control)	-

The amount of HCN production was expressed by + and – sign. Thus sign minus (-) indicates no HCN production; one plus (+) indicates small amount of HCN production with light red colour; double plus (++) indicates moderate amount of HCN production with brown colour; triple plus (+++) indicates high amount of HCN production with red colour.

5.7 Siderophore Production:

Iron is perhaps, the most important micronutrient used by microorganisms (fungi and bacteria) and is essential for their metabolism, being required as a cofactor for a large number of enzymes and iron-containing proteins.

Siderophores are low molecular weight, iron chelating ligands synthesized by microorganisms that restrict the growth of pathogenic microorganisms by limiting the iron availability as it bind to the available form of iron (Fe2+) in the rhizosphere. Under iron limiting conditions, Plant Growth Promoting fungi (PGPF) and Plant Growth Promoting Rhizobacteria (PGPR) produce siderophores to competitively acquire ferric ions.

A. Methodology:

The siderophore production efficiency of isolates was studied following the method described by Schwyn and Neilands, 1987 using Chrome Azurol S (CAS) agar media.CAS agar plates were inoculated with 6mm fungal disc from actively growing region of seven days old bio-control fungal culture on PDA and incubated at 28 ± 2^0C for 7days. Isolates showing orange hollow zone following the incubation period were recognised as siderophore producing isolates.

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