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Formulation of Cost Effective and Locally Available Bio-Fertilizer for Increased Production and Faster Germination

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ABSTACT: Globally, the market for Bio-Fertilizers has been increasing owing to the environmental awareness, enhancing soil fertility and increasing demand for organic food. Bio fertilizers generate plants nutrients like nitrogen and phosphorous through their role in the soil or rhizosphere and make available to plants in a gradual manner. Recent progress in our understanding on PGPR diversity along with the mechanism of action should facilitate their application as reliable part in sustainable agricultural system. The progress to the data in using these PGPR in a variety of applications related to agriculture or gardening improvement along with the mechanism of action, carrier used, formulation of bio-fertilizer, pot trials and plant growth promoting traits are summarized and discussed in this paper.

Key words: -Bio-fertilizer, carrier, fertility, formulation, PGPR, plant growth promoting trait

I. INTRODUCTION: -

Agriculture contributes to a major share of national income and export earnings in many developing countries, while ensuring food security and employment. Sustainable agriculture is vitally important in today's world because it offers the potential to meet our future agricultural needs, something that conventional agriculture will not be able to do. Recently there has been a great interest in eco-friendly and sustainable agriculture. The rhizosphere is the volume of soil surrounding and under the influence of plant roots, and the rhizoplane is the plant root surfaces and strongly adhering soil particles (Kennedy, 2005). Often, studies of the microbial ecology of the rhizosphere also include the rhizoplane. The term rhizosphere will be used to refer to both zones. In the rhizosphere, very important and intensive interactions take place between the plant, soil, microorganisms and soil micro fauna.

The presence of rhizobacteria in the rhizosphere can have a neutral, detrimental or beneficial effect on plant growth. The presence of neutral rhizobacteria in the rhizosphere probably has no effect on plant growth. Deleterious rhizobacteria are presumed to adversely affect plant growth and development through the production of metabolites like phytotoxins or phytohormones but also through competition for nutrients or inhibition of the beneficial effects of mycorrhizae (Nehl et al., 1996; Sturz and Christie, 2003).

What are plant growth promoting rhizobacteria?

About 2 to 5% of rhizobacteria, when reintroduced by plant inoculation in a soil containing competitive microflora, exert a beneficial effect on plant growth and are termed plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). PGPR are free-living bacteria (Kloepper et al., 1989), and some of them invade the tissues of living plants and cause unapparent and asymptomatic infections (Sturz and Nowak, 2000). These rhizobacteria are referred to as endophytes, and in order to invade roots they must first be rhizosphere competent. It is important to note that the term edndo-rhizosphere, previously used in studies of the root zone microflora, is semantically incorrect and should not be used (Kloepper et al.,1992).

PGPR are known to improve plant growth in many ways when compared to synthetic fertilizers, insecticides and pesticides. They enhance crop growth and can help in sustainability of safe environment and crop productivity. The rhizospheric soil contains diverse types of PGPR communities, which exhibit beneficial effects on crop productivity. Several research investigations are conducted on the understanding of the diversity, dynamics and importance of soil PGPR communities and their beneficial and cooperative roles in agricultural productivity. Plant growth in agricultural soils is influenced by many abiotic and biotic factors. There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root

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activity made metabolism which is known as rhizosphere. The rhizosphere concept was first introduced by Hiltner to describe the narrow zone of soil surrounding the roots where microbe populations are stimulated by root activities. The recognition of plant growth-promoting rhizobacteria (PGPR), a group of beneficial plant bacteria, as potentially useful for stimulating plant growth and increasing crop yields has evolved over the past several years to where today researchers are able to repeatedly use them successfully in field experiments.

The concept of PGPR has now been confined to the bacterial strains that can fulfil at least two of the three criteria such as aggressive colonization, plant growth stimulation and biocontrol (Weller et al. 2002; Vessey 2003).

PGPR as Biocontrol Agents:

PGPR produce substances that also protect them against various diseases. PGPR may protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as by induction of host resistance. In recent years, the role of siderophore-producing PGPR in biocontrol of soil-borne plant pathogens has created great interest. Microbiologists have developed techniques for introduction of siderophore producing PGPR in soil system through seed, soil or root system. PGPR that indirectly enhance plant growth via suppression of phytopathogens do so by a variety of mechanisms. These include:

• The ability to produce siderophores (as discussed above) that Chelate iron, making it unavailable to pathogens

• The capacity to synthesize anti-fungal metabolites such as Antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens

• The ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce Systemic resistance.

Direct mechanisms:

Biological nitrogen fixation:

All organisms require nitrogen (N) to synthesize bio-molecules such as proteins and nucleic acids. Nitrogen is provided to agricultural lands by the application of urea and ammonium nitrate as chemical fertilizers. Microorganisms having the ability of biological nitrogen fixation (BNF) are responsible for the reduction of N_2 to ammonia (NH_3). Rhizobium is the best example of nitrogen fixer which fixes nitrogen in a sustainable manner. These microorganisms were traditionally considered to be responsible for legume infection process, though rhizobia can also behave as endophytes in nodules and frequent isolation of rhizobial strain from nodule often promotes growth of plant.

A number of bacterial species belonging to genera Azospirillum, Alcaligenes, Arthrobacter, Acinetobacter,Bacillus, Burkholderia, Enterobacter, Erwinia,Flavobacterium, Pseudomonas, Rhizobium and Serratia are associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth. The important role is played by plants in selecting and enriching the types of bacteria by the constituents of their root exudates. Thus, the bacterial community in the rhizosphere develops depending on the nature and concentrations of organic constituents of exudates, and the corresponding ability of the bacteria to utilize these as sources of energy.

Since associative interactions of plants and microorganisms must have come into existence as a result of co evolution, the use of latter group as bio inoculants must be pre-adapted, so that it fits into a long term sustainable agricultural system. PGPR are commonly used as inoculants for improving the growth and yield of agricultural crops and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. The use of bio-fertilizer and bioenhancer such as N_2 (nitrogen) fixing bacteria and beneficial micro-organism can reduce chemical fertilizer applications and consequently lower production cost. Utilization of PGPR in order to increase the productivity may be a viable alternative to organic fertilizers which also helps in reducing the pollution and preserving the environment in the spirit of an ecological agriculture.

Phosphorus solubilisation:

Phosphorus is an essential macronutrient for growth and development of plants involved in important metabolic pathways like photosynthesis, biological oxidation, nutrient uptake and cell division. Worldwide soils are supplemented with inorganic P as chemical fertilizers to support crop production but repeated use of fertilizers deteriorates soil quality. Therefore, the present scenario is shifting towards a more sustainable agriculture. Revealed that phosphorous is mainly absorbed by plants in two forms which are soluble forms: the monobasic (H_2PO_4) and the dibasic (HPO4). Though, a large amount of phosphorous is present in insoluble forms and is readily not accessible for plant growth. Reported that soluble components such as aluminium in acid soils (Ph <5) and calcium in alkaline soils (pH > 7) showed

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the high reactivity with phosphorous and that results in low level of P in soil. Organic (incorporated into biomass or soil organic matter) and inorganic compounds, primarily in the form of insoluble mineral complexes, are major sources of available P in the soil. Phosphate-solubilizing bacteria and fungi constitute approximately 1-50% and 0.1-0.5%, respectively, of the total population of cultivable microorganisms in the soil. Phosphate-solubilizing bacteria solubilize inorganic soil phosphates, such as $FePO_4$, $Ca_3(PO_4)_2$, and $AlPO_2$, through the production of siderophores, organic acids, and hydroxyl PGPB in agricultural soils. Endophytic bacteria possess the capacity to solubilize phosphates, and the endophytic bacteria from soybean may also participate in phosphate assimilation. Application of phosphate-solubilizing bacteria increases soil fertility due to their ability to convert insoluble P to soluble P by releasing organic acids, chelation and ion exchange. The positive effect of P solubilizers has been reported on food and fodder crops.

Potassium solubilisation:

Potassium (K) is the third vital nutrient required for plant growth and endophytic bacteria are able to solubilize insoluble form of potassium. Potassium solubilizing microorganisms present could provide an alternative technology to make potassium available for uptake by plants. A wide range of bacteria namely Pseudomonas, Burkholderia, Acidothiobacillusferrooxidans, Bacillus mucilaginosus, Bacillus edaphicus, B. CirculansandPaenibacillussp. Have been reported to release potassium in accessible form from potassium-bearing minerals in soils. These potassium solubilizing bacteria (KSB) were found to dissolve potassium, silicon and aluminium from insoluble K-bearing minerals such as micas, illite and orthoclase's, by excreting organic acids which either directly dissolved rock K or chelated silicon ions to bring K into the solution. Thus, application of K solubilizing bacteria as biofertilizer for agriculture improvement can reduce the use of agrochemicals and support eco-friendly crop production.

Siderophore production:

In plant growth promoting bacteria, iron in Fe^{3+} siderophore complex on bacterial membrane is reduced to Fe^{2+} which is further released into the cell from the siderophore via a gating mechanism. Binding of the siderophore to a metal increases the soluble metal concentration. On the alleviation of high level of heavy metal contamination bacterial siderophore's are released and plants assimilate iron from bacterial siderophores by means of different mechanisms, for instance, chelate and release of iron, the direct uptake of siderophore-Fe complexes, or by a ligand exchange reaction. Numerous studies of the plant growth promotion vis-a`-vis siderophore-mediated iron-uptake as a result of siderophore producing rhizobacterial inoculations.

Production of indolic compounds:

Microbial synthesis of the phytohormone auxin has been well-known for a long time. Reported that 80% of microbes isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites. Indole acetic acid (IAA) affects division of plant cells, extension, and differentiation; stimulates tuber and seed germination; increases the rate of root and xylem development; initiates lateral; controls processes of vegetative growth and adventitious root formation; pigment formation, biosynthesis of various metabolites, mediates responses to light, gravity and florescence; affects photosynthesis, and resistance to stressful conditions. IAA produced by plant growth promoting bacteria possibly; delay the above physiological processes of plants by changing the plant auxin pool. Currently, bacterial strains exhibiting ACC deaminase activity have been identified in a wide range of genera such as Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia, Serratia and Rhizobium etc. Such bacterial endophytes trap the ethylene precursor ACC and convert it into 2-oxobutanoate and ammonia, showed that some forms of stress are dismissed by enzyme ACC deaminase producers, such as effects of phytopathogenic microorganisms (viruses, bacteria, and fungi etc.), and heavy metals, radiation, wounding, insect predation, high salt concentration, draft, extremes of temperature, high light intensity, and flooding resistance to stress from polyaromatic hydrocarbons.

Indirect mechanisms:

Production of metabolites:

Microorganisms are used to control various diseases, which is known as biological and also an environment friendly Approach and these microbes are known as biocontrol agents. In biological controls chief activities employed by PGPR and competition for niche exclusion, nutrients, induced systemic resistance and antifungal metabolites. Reported that many rhizobacteria have been reported to produce

antifungal metabolites like pyrrolnitrin, phenazines, 2, 4-diacetylphloroglucinol, pyoluteorin, HCN, viscosinamide and tensin. Few species of bacteria produces and excrete hydrogen cyanide (HCN) which is a powerful inhibitor of cytochrome C oxidase and many other metalloenzymes. HCN is a metabolite and it has no role in primary metabolism. Proteobacteria have HCN synthase which is a membrane bound flavoenzyme that oxidizes glycine, producing HCN and CO_2.

Siderophore production:

Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition. Under iron-limiting conditions PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion. Siderophores (Greek: "iron carrier") are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses. Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe3+ complexes that can be taken up by active transport mechanisms. Many siderophores are non-ribosomal peptides, although several are biosynthesised independently. Siderophores are also important for some pathogenic bacteria for their acquisition of iron. Siderophores are amongst the strongest binders to Fe3+ known, with enterobactin being one of the strongest of these. Distribution of siderophore producing isolates according to amplified ribosomal DNA restriction analysis groups, reveals that most of the isolates belong to Gram- negative bacteria corresponding to the Pseudomonas and Enterobacter genera, and Bacillus genera are the Gram-positive bacteria found to produce siderophores.

PGPR in HCN production:

One group of microorganisms which acts as biocontrol agents of weeds include the Deleterious Rhizobacteria that can colonize plant root surfaces and able to suppress plant growth. Cyanide is a dreaded chemical produced by them as it has toxic properties. Although cyanide acts as a general metabolic inhibitor, it is synthesized, excreted and metabolized by hundreds of organisms, including bacteria, algae, fungi, plants, and insects, as a mean to avoid predation or competition. The host plants are generally not negatively affected by inoculation with cyanide-producing bacterial strains and host-specific rhizobacteria can act as biological weed-control agents. Bacterium produces hydrogen cyanide (HCN) and the seed bacterization with the isolate significantly increases the percent germination, rate of germination, plant biomass and nutrient uptake of wheat seedlings.

Resistance to drought and salinity stress:

Yield losses due to drought and salinity stress are increasing mainly due to climate change and intensive agriculture that leads to soil degradation. It has recently been discovered that some microbial inoculants known to have a positive effect on plant development can also help plants overcome or tolerate abiotic stress conditions, thereby reducing potential yield losses. Increases in soil salinity have become a serious problem for agriculture crops in the last years. Application of some bacteria, such as Rhizobium spp. And Azospirillum spp., increased plant tolerance to salinity conditions (Cordovilla et al. 1999; Hamaoui et al. 2001). Applications of a strain of A. Lipoferum to wheat plants reduced the negative effect of saline conditions (Bacilio et al. 2004). Drought stress limits growth and production of crop plants, particularly in arid and semiarid areas (Kramer and Boyer 1997). Greenhouse studies revealed that inoculation of maize seedlings with A. Brasilense resulted in the mitigation of many negative effects of drought stress (Casanovas et al. 2002). Among the specific effects were increased water content, reduction in the decrease of water potential, increased foliar area.

Resistance to abiotic stress:

PGPR's have been shown to alleviate a variety of abiotic stresses including drought, salinity, and temperature extremes (Nabati et al. 1994; Zhang and Ervin 2004; Mancuso et al. 2006; Khan et al. 2009; Craigie 2011). Our current understanding of how plants respond to environmental stresses has been informed by recent advances in genomics and transcriptomics. Response is mediated via an intricate network of signals that perceive the stress and set in motion molecular, biochemical, and physiological processes that may be unique to each stress (Hirayama and Shinozaki 2010; Krasensky and Jonak 2012; dos Reis et al. 2012).

The mode of action of PGPR`s in enhancing stress tolerance in plants is not well understood, but the presence of bioactive molecules in the extracts, such as betaines (Blunden et al. 1997) and cytokinin's (Zhang and Ervin 2004), may play a role. PGPR`s also increase the endogenous concentrations of stress-related molecules, such as cytokinin's, proline, antioxidants, and antioxidant enzymes in treated

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plants (Zhang and Ervin 2004; 2008; Zhang et al. 2010; Aziz et al. 2011; lolaluz et al. 2013; Fan et al. 2013).

Soil samples:

Rhizosphere soil samples (2 nos.) Were procured each from the following fields at different places.

1. Cotton field (Rairi village, district Buldhana, Maharashtra)
2. Soyabean field (Rairi village, district Buldhana, Maharashtra)
3. Sugarcane field (Rairi village, district Buldhana, Maharashtra)
4. Wheat field (Charholibudruk, district Pune, Maharashtra)are used for the isolation of PGPR's.

Materials

Media used for isolation of PGPR are Nutrient agar medium, LGI medium, Ashby's mannitol medium, King's B medium, Pikovskaya's medium

Gram characters:

Isolates obtained	Gram character	Motility	Shape
Isolate 1	Negative	Motile	Rods
Isolate 2	Negative	Motile	Rods
Isolate 3	Negative	Motile	Rods
Isolate 4	Positive	Motile	Rods
Isolate 5	Negative	Motile	Rods
Isolate 6	Negative	Motile	Rods
Isolate 7	Positive	Non-motile	Cocci
Isolate 8	Negative	Motile	Rods

IAA production-

IAA is the most common plant hormone of the auxin class and it regulates various aspects of plant growth and development.

Procedure-

The IAA production was estimated by growing the isolate in Nutrient broth (Himedia, India) containing 100µg/ml Tryptophan for 72 h at 30 °C and kept on shaking at 180 rev/min. Following incubation culture was centrifuged at 8,000 g and 1 ml of supernatant was mixed with 2 ml of Salkowsky s reagent and kept at room temperature for 20 min. Optical density was measured spectrophotometrically at 530 nm. The concentration of IAA in each sample was determined from the standard curve of IAA. Un-inoculated media was used as a control.

Colour change observed from colourless to light reddish or pink

(**Fig.** Colour change observed after addition of salkowasky's reagent. Control is water treated with salkowasky's reagent and isolates from 1 to 8 shown after treatment with reagent showing IAA production)

Siderophores are low molecular weight iron chelating compounds produced by micro organisms to combat low iron stress and the function of which is to solubilize and transport iron into microbial cells via cognate transport system.

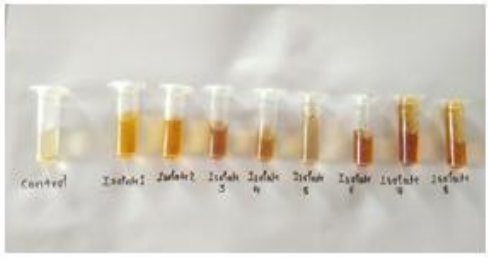
Procedure-

In $FeCl_3$ test, a red to brown colour is generated upon coordination of the metal ions with the siderophores.

Fe (yellow) + siderophore (aq.)

Fe- siderophore (aq.) Red complex

To 1 ml of supernatant, 5 ml of 2% ferric chloride solution was added. The formation of red or purple colour indicated the presence of siderophores. The ferric complex was easy to follow for its red compared to deferricompounds.



Nitrogen fixation-

Nitrogen (N) is the most vital nutrient for plant growth and productivity. Although, there is about 78% N2 in the atmosphere, which is unavailable to the growing plants.

Nitrogen fixing organisms are generally categorized as (a) symbiotic N2 fixing bacteria including members of the family rhizobiaceae which forms symbiosis with leguminous plants (e.g. rhizobia) (Ahemad and Khan, 2012d; Zahran, 2001) and non-leguminous trees (e.g. Frankia) and

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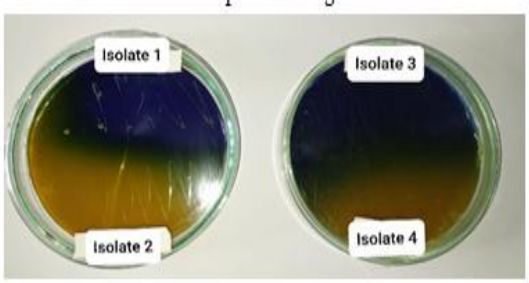
(b) non-symbiotic (free living, associative and endophytes) nitrogen fixing forms such as cyanobacteria (Anabaena, Nostoc), Azospirillum, Azotobacter, Gluconoacetobacterdiazotrophicus and Azocarus etc. (Bhattacharyya and Jha, 2012). However, non-symbiotic nitrogen fixing bacteria provide only a small amount of the fixed nitrogen that the bacterially-associated host plant requires (Glick, 2012). PGPR that fix N2 in non-leguminous plants are also called as diazotrophs capable of forming a nonobligate interaction with the host plants (Glick et al., 1999).

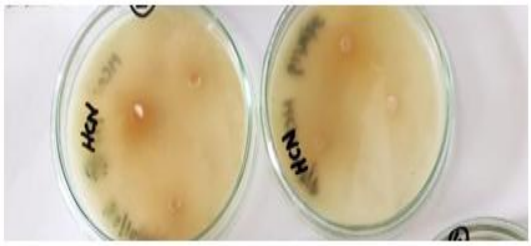
Procedure-

Nitrogen fixation ability was evaluating by growing on N- free LGI medium. Bacterial isolate was streaked on semi solid LGI medium and incubated at 28+_ °C for 7 days. Observation of bacterial growth on plate was observed as qualitative evidence of atmospheric nitrogen fixation. The colour change observed around the colonies from orange to blue can be observed which indicates nitrogen fixation.



HCN production-

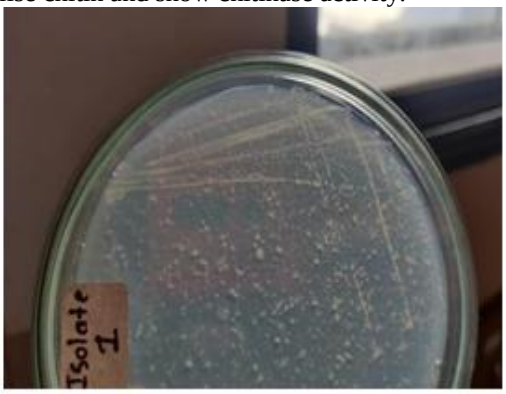
For qualitative estimation of HCN, isolate was streaked on nutrient agar plate supplemented with 4% glycine. A whatmann filter paper soaked in a solution of 2% Na2CO3 in 0.5% picric acid was placed between base and lid of petri plate and incubated at 28 ± 2 °C in inverted position for 96 h and observed for colour change from yellow to orange brown.



Chitinase activity-

Chitin is not readily water soluble, chitin is often chemically modified to form colloidal chitin, with a small particle size that is more readily manipulated to obtain homogenous distribution in agar media, compared to use of physically modified, finely ground chitin that can be difficult to obtain. 20g of chitin powder was measured and taken in a 1000ml beaker. It was then treated with 150ml of 12M HCl by the slow addition of HCl along the sides and was then stirred every 5min for 60min. The chitin – HCl mixture was filtered through eight layers of cheese cloth and the filtrate obtained was treated with 2 litres of ice-cold distilled water to allow precipitation of colloidal chitin. After overnight incubation, the filtrate was then passed through three layers of Whatmann filter paper kept in a Buchner funnel seated in a vacuum filtration flask. Around three litres of tap water were passed through the colloidal chitin using this assembly to increase the pH and this is continued till pH 7 is attained. The colloidal chitin obtained was autoclaved and was stored 4°C until further use. For the isolation of chitin utilizing bacteria, a solid agar medium of chitin was used which provided the bacteria with chitin as the primary carbon source. Since chitin is a complex polymer other carbon sources along with the chitin media was avoided so that the bacteria won't depend primarily on the other carbon source. Thus, a media was prepared supplemented with chitin. The media composed of moist Colloidal chitin, K_2HPO_4, KH_2PO_4, NaCl, Agar.

Further the media is prepared using half strength carbon source and chitin inoculated with each of the isolates separately. Media was kept in incubator for 3-4 days at 37± 2^0 C and further were streaked on modified chitin agar plates. The chitin agar plates were prepared without any carbon source but only chitin. The growth observed on plates could suggest that the isolates were able to utilise chitin and show chitinase activity.



Phosphate solubilizing Activity:

Phosphates, widely distributed in nature in both organic and inorganic forms, are not readily available to plants. Bacteria are widely distributed in the rhizosphere of tropical and subtropical

grasses. Many soil bacteria are reported to solubilize these insoluble phosphates through various processes. A few reports have also indicated the P-solubilizing activity of some nitrogen fixers. Many soil bacteria such as Pseudomonas, Rhizobium, Enterobactor, Bacillus etc possess the ability to solubilize insoluble inorganic phosphates and make them available to the plants.

These organisms are also known to produce amino acids, vitamins and growth promoting substances like Indole Acetic Acid (IAA) and Gibberellic Acid (GA), which results in better growth of plants. Addition of these phosphate solubilizing organisms saves almost fifty percent of phosphorus fertilizers applied to the fields.

Procedure-

All the suspected colonies were screened for phosphate solubilization on Picovskaya's medium. Isolates showing phosphate solubilizing ability were spot inoculated at the centre Picovskaya's plate and incubated at 37^0 C.Diameter of clearance zone was measured successively after 24 hours, up to 7 days.

Salt tolerance-

Soil salinity in arid regions is frequently an important limiting factor for cultivating agricultural crops. Although many technologies have been implicated in the improvement of salt tolerance, only PGPR-elicited plant tolerance against salt stress has been previously studied.

Procedure-

Isolate was screened for their ability to tolerate the abiotic stress particularly salt. A 20µl of overnight grown culture was inoculated into nutrient broth amended with 0.5% to 7 % salt. After 24 h, absorbance of the culture was determined at 600nm. Following the growth of the isolate for 24 h, the absorbance of the culture was taken at 600nm using uninoculated broth as a blank.

pH tolerance-

Soil microbial community structure is influenced by both biotic and abiotic factors prevailing in the soil milieu. The pH is one of the noticeable abiotic factors that affect soil microbial community. Different species prefer different range of pH for their optimal growth; however, they can tolerate a wide range around acidic, neutral or alkaline pH. Microbes with broad range of pH tolerance i.e. from acidic to alkaline soil have better survival rate or opportunity as compared to other microbes which have narrow range of pH tolerance.

Procedure-

The soil pH was measured according to the protocol of Jimenez et al. (2011) and pH tolerance was carried out at different pH ranges. Different ranges of pH (3 to 13) Burk's media were prepared by adjusting the media pH (NaOH or HCL); one set with pH 7.0 were maintained as control.

Isolate was screened for their ability to tolerate the abiotic stress particularly pH. A 20µl of overnight grown culture was inoculated into nutrient broth. After 24 h, absorbance of the culture was determined at 600nm. The ability of the isolate to sustain the pH was tested by growing in a varying degree of pH from pH 5.0 to pH 13.0 in the nutrient broth medium. Following the growth of the isolate for 24 h, the absorbance of the culture was taken at 600nm using uninoculated broth as a blank.

Pot trials:

PGPR`s are usually added to the soil (direct soil application), the seed (seed-applied inoculant), or the plant (e.g., foliar spray and root dipping) (Adholeya et al., 2005; Mahmood et al., 2016). Each inoculation method has advantages and disadvantages, depending on the number of inoculants, availability of equipment, type of seed (e.g., size, shape, and fragility), the presence of inhibiting compounds in the seed (e.g., fungicides, micronutrients, and PBM), and cost (Deaker et al., 2004; Bashan et al., 2014).

The most basic coating treatment is seed dressing, which refers to the application of finely milled solids dusted onto the surface of seeds in small amounts, and it is normally used for pesticide application (Scott, 1989).

Biofertilizers are supplied as carrier-based microbial inoculants which are added to the soil to enrich the soil fertility. The carrier is a medium that can carry the microorganisms in sufficient quantities and keep them viable under specified conditions, easy to supply to the farmers. The use of ideal carrier material is necessary in the production of good quality biofertilizer.

The formulation of microbial inoculants generally consists of three basic elements: the selected microorganism, a suitable carrier (that can be solid or liquid), and different additives. It is worth to note that factors such as incorrect inoculant formulation or limited shelf-life (i.e., inoculant viability on the seed surface) can hamper a wider use of seed coating (O'Callaghan, 2016).

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Formulation has a major impact on the microbial survival during the process of product elaboration, storage, and application, in its efficiency once applied on the target plant and in the economic feasibility of the application (John et al., 2011; Herrmann and Lesueur, 2013). Although the formulation of microbial inoculants is a critical issue, little research on this topic has been conducted (Parnell et al., 2016). Georgakopoulos et al. (2002) evaluated pre-selected bacterial and fungal antagonists responsible for biological control of damping-off in sugar beet and cucumber with the intention of developing potential commercial formulations based on a peat carrier material for seed coating.

Carrier material:

Various types of material are used as carrier for seed or soil inoculation. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10 -40 µm. According to the "Handbook for Rhizobia" (Somasegaran and Hoben, Springer, 1994), the properties of a good carrier material for seed inoculation are: (1) non-toxic to inoculant bacterial strain, (2) good moisture absorption capacity, (3) easy to process and free of lump-forming materials, (4) easy to sterilize by autoclaving or gamma-irradiation, (5) available in adequate amounts, (6) inexpensive, (7) good adhesion to seeds, and (8)good pH buffering capacity. Needless to say, (9) non-toxic to plant, is another important property.

Other essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered. (1) Survival of the inoculant bacteria on seed. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying condition until placed into soil. (2) Survival of the inoculant bacteria during the storage period. (3) Survival of the inoculant bacteria in soil. After being introduced into the soil, the inoculant bacteria have to compete with native soil microorganisms for the nutrient and habitable niche, and have to survive against grazing protozoa. Such carrier materials that offer the available nutrient and/or habitable micro-pore to the inoculant bacteria will be desirable. In this sense, materials with micro-porous structure, such as soil aggregate and charcoal, will be good carrier for soil inoculant.

Here, in this project we have used cow dung as a carrier.

Sterilization:

Sterilization of carrier material is essential to keep high number of inoculant bacteria on carrier for long storage period. The carrier i.e. cow dung as well as the soil used in pot trials is sterilized properly before pot trials. The way of carrier sterilization is autoclaving. Carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 ºC. Cow dung and soil is autoclaved and placed in incubator overnight to stimulate the growth of spores if present and autoclaved again to sterilize properly. This step was performed thrice and thus completely sterile soil and carrier was used for further use.

Formulation:

A range of commercial biofertilizer formulations are available and different strategies have been applied to ensure maximum viability of the microorganisms used in such formulations. These strategies comprise: (i) optimization of biofertilizer formulation, (ii) application of thermo-tolerant/ drought-tolerant/ genetically modified strains and, (iii) application of liquid biofertilizer. For convenience of application, a carrier material is used as a vehicle for the microorganisms to be used as biofertilizer. Moreover, such materials may have a role in maintaining the viability (shelf-life) of the microorganisms prior to its release into the field as well as they also provide a suitable micro environment for rapid growth of the organisms upon their release. A carrier could be a material, such as peat, vermiculite, lignite powder, clay, talc, rice bran, seed, rock phosphate pellet, charcoal, soil, paddy straw compost, wheat bran or a mixture of such materials. In common practice, for better shelf-life of biofertilizer formulation, a carrier or a mixture of such carrier materials are selected based on the viability of the microorganisms mixed with them. Similarly, pre-sterilization of the carrier material and its enrichment with nutrient is the other strategy to improve the shelf-life by allowing the microorganism to maintain/ grow in a non-competitive microenvironment. Sucrose, maltose, trehalose, molasses, glucose and glycerol are some supplementary nutrients and/cell protectants commonly used with a carrier material to ensure maximum cell viability and extended shelf-life.

Liquid biofertilizer formulation could be considered as one potential strategy for improving the shelf-life of biofertilizer. Unlike solid carrier based biofertilizers, liquid formulations allow the manufacturer to include sufficient amount of nutrients, cell protectant, and inducers responsible for cell/spore/cyst formation to ensure prolonged shelf-life.

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Preparation of microbial formulation –
Liquid formulation:

Liquid Microbial formulation is also very effective and helpful in managing the plant growth, health and productivity. In liquid microbial formulation various compounds such as glycerol, vermicompost wash, indole acetic acid, Malic acid or even formaldehyde have been used. Since the maintenance of quality and type of liquid formulations influence the shelf life of the formulations, the amendments are, however, required to manage the quality and storage process according to the inoculum's conditions.

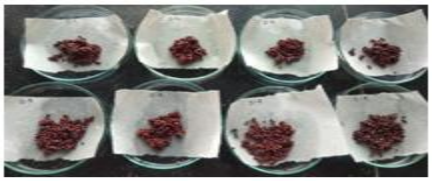
Types-

- Seed treatment
- Root dipping
- Soil application

Seed treatment –

we have used broth of different isolates that we have isolated, and further these broths are used to coat seeds. The wheat and mustard 0256seeds are dipped in the individual broth separately and the further they were allowed to dry. These seeds are used to sow in the soil for pot trails.

Broth of each isolate obtained and characterized and allowed to grow for 3- 4 days at 37℃ in shaker incubator at 130 rpm.



Wheat seeds (7-8 grams) soaked in broth of each isolate to get dried and coated with isolates

Mustard seeds (7-8 grams) soaked in broth of each isolate to get dried and coated with isolates

Solid formulation –

Solid microbial formulations are prepared by using solid inert carrier such as talc, bentonite, charcoal, chitin, chitosan, lignite, neem cake, vermicompost and/or alginate.

Carrier based formulations –

Various types of material are used as carrier for seed or soil inoculation. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10 -40 µm. According to the "Handbook for Rhizobia" (Somasegaran and Hoben, Springer, 1994), the properties of a good carrier material for seed inoculation are: (1) non-toxic to inoculant bacterial strain, (2) good moisture absorption capacity, (3) easy to process and free of lump-forming materials, (4) easy to sterilize by autoclaving or gamma-irradiation, (5) available in adequate amounts, (6) inexpensive, (7) good adhesion to seeds, and (8) good pH buffering capacity. Needless to say, (9) non-toxic to plant, is another important property.

Here, we have used cow dung farmyard manure as carrier. Cow dung sample used was sterilised completely packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 ºC. 150 ml of enriched broth was mixed with carrier i.e. cow dung sample. This carrier treated with broth was mixed (50 grams) with soil sample in each pot. This mixture was used for pot trial application.

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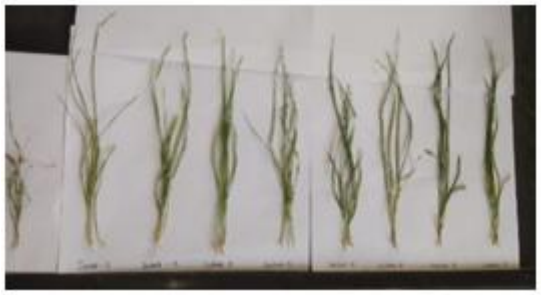
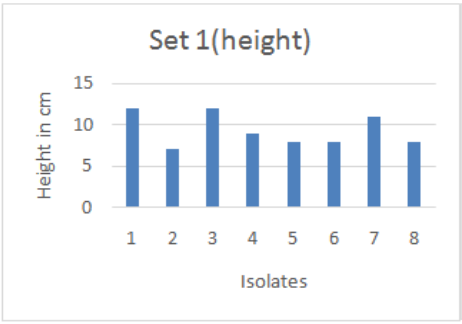
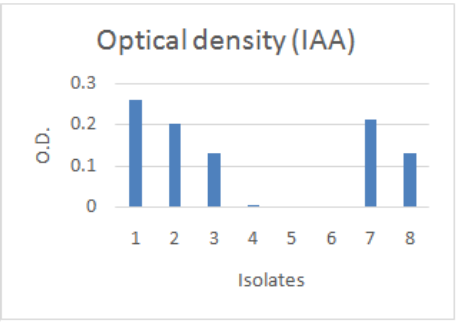
Seedlings of wheat after 9 days Seedlings of mustard (with carrier i.e. cow dung)

DISCUSSION-

Auxin has been shows to play a central role in many aspects of plant morphogenesis and response to environmental stimulations.







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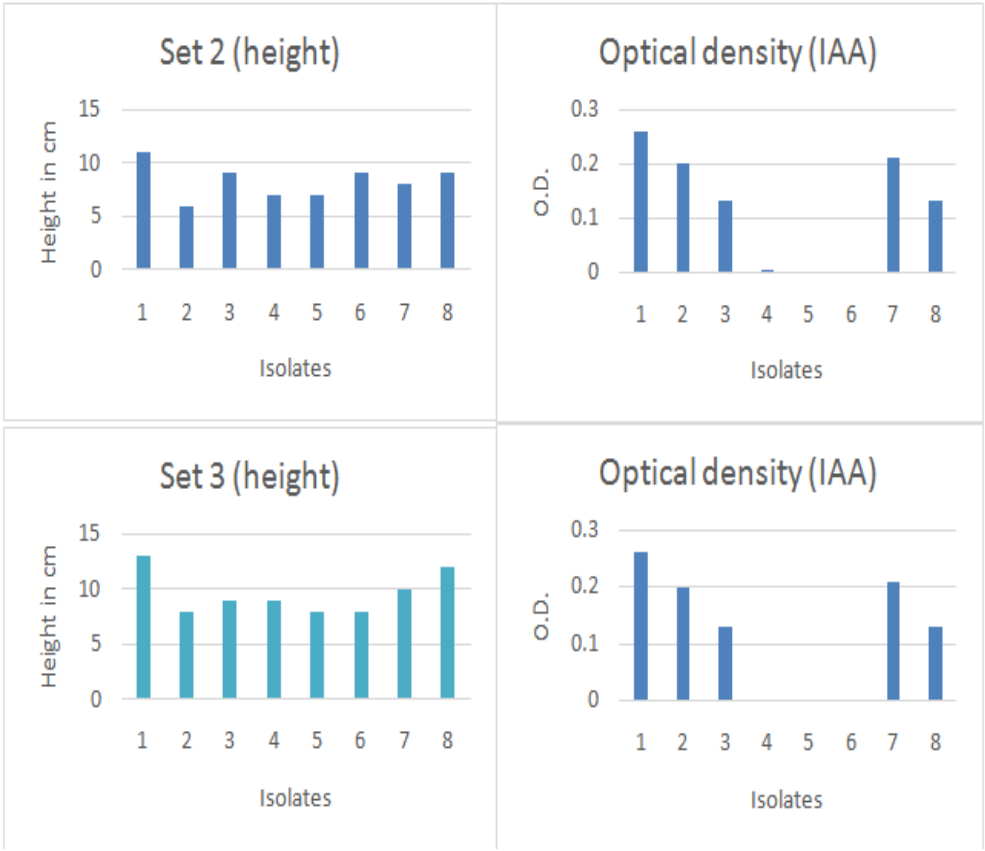

Comparing the graph, we can say that where the optical density was zero i.e. the isolates which were not able to produce IAA showed declined pattern in height measure. The isolates which were able to produce maximum IAA showed increased height pattern. Thus, IAA produced by the isolates is directly correlated with height and growth pattern of the plants.

Siderophores are metabolites produced by isolates when they are starved of iron. These metabolites have a wide application in almost all fields such as healthcare, agriculture and environment. Siderophore producing microorganisms can be isolated from various sources such as soil, air and water.

The effects of PGPR`s on the growth of plants are well known. However, little is known about the effects of these bacteria on the nodulation process and biological nitrogen fixation. Some studies indicated that certain PGPR`s can positively affect symbiotic nitrogen fixation by enhancing both root nodule number or mass and increasing the nitrogenase activity.

The inoculations by these isolates promoted various plant growth parameters like germination %, plant height and tiller number. Increasing the number of tillers is very important with respect to sugarcane yield. Significant changes in various plant growth parameters such as plant height, tiller number, dry matter yield and N uptake have been shown by the inoculation of various nitrogen fixing and plant growth promoting bacteria.

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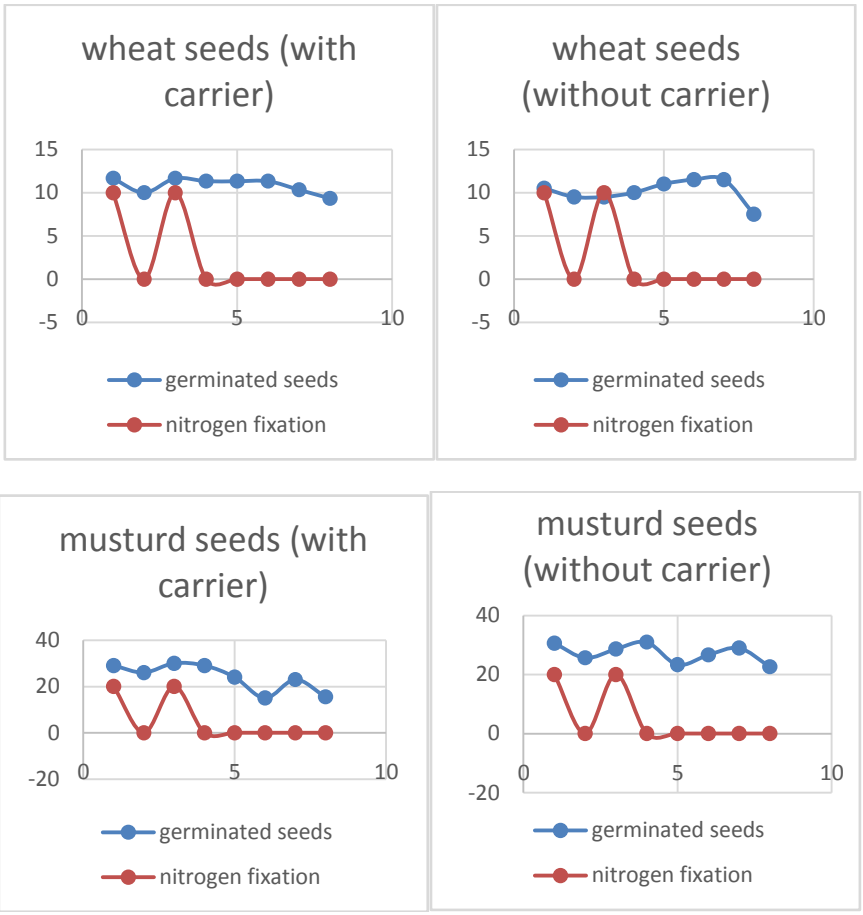

The scatter plot shows that isolates which were able to fix atmospheric nitrogen showed higher trend of gemination specifically in seed coating method and greater number of leaves after 9 days of growth.

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Biological control of plant pathogens and deleterious microbes can improve significantly plant health, as evidenced by increases in seedling emergence, vigor and yield (Antoun and Kloepper, 2001). Isolate 1 and isolate 5 was estimated as better producer of HCN and siderophores than other isolates and assumed to be better in promoting growth.

Chitinase can be used as a potential alternative to chemical fungicides. In biological control of fungal phytopathogens, application of agents containing various metabolites of microorganisms, including CHIs, appears to be the most efficient, since they show stronger fungicidal activity than purified chitinolytic enzymes. The use of agents constituting a consortium of chitinolytic microorganisms seems to bring better results in fighting fungal phytopathogens.

Large proportion of phosphorus in soil is insoluble and therefore unavailable to plants (Singh and Kapoor, 1994). Plant rhizospheric bacteria Bacillus and Pseudomonas are able to solubilize phosphates in vitro and most of them act as PGPR. Isolate 1, isolate 2 and isolate were able to solubilize inorganic phosphate and thus have potential to be used as PGPR's.

Obtained maximum isolates were not able to sustain extreme acidic pH but first 4 isolates were able to sustain extreme alkaline pH conditions. For stressed condition considering salt isolate 1 was able to sustain extreme salt conditions as well i.e. 7g/L. Other isolates were not able to sustain extreme salt conditions. These halotolerant and pH tolerant PGPR have potential to work as defensive agents of plants by enhancing growth, productivity, tolerance and defense system under saline and acidic or alkaline environments.

II. CONCLUSION-

With increasingconcern about the natural environment and the understanding about large scale chemical fertilizer usage should come to an end. PGPR are excellent model systems which can provide with bioactive compounds having diverse effect in agriculture and environmental sustainability. The productive efficiency of specific PGPR may be further enhanced with optimization and acclimatization according to soil conditions. Further research and understanding of mechanism of these PGPR and increasing the shelf life of these bio-fertilizer needs to be studied.

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