ISOLATION AND *IN VITRO* SCREENING OF SILICATE SOLUBILIZING BACTERIA FROM PADDY RHIZOSPHERE

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by

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2020

DECLARATION

I, hereby declare that this thesis entitled **"ISOLATION AND *IN VITRO* SCREENING OF SILICATE SOLUBILIZING BACTERIA FROM PADDY RHIZOSPHERE"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"ISOLATION AND *IN VITRO* SCREENING OF SILICATE SOLUBILIZING BACTERIA FROM PADDY RHIZOSPHERE"** is a record of research work done independently by **Ms. Akhila P. Subhash (2018-11-093)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.	And other co-workers
cm	Centimeter
cfu	Colony forming unit
CRD	Completely randomized design
CD	Critical difference
cv.	Cultivar
°C	Degree celsius
Fig.	Figure
g	Gram
ha	Hectare
hrs	Hours
kg	Kilogram
L	Litre
µg	Microgram
µL	Microliter
µm	Micrometer
mg	Milligram
mL	Milliliter
mm	Millimetre
min	Minutes
M	Molar
viz.,	Namely
nm	Nanometre
pH	Negative logarithm of hydrogen ions
No.	Number
OD	Optical density
ppm	Parts per million

pv.	Pathovar
%	Per cent
rpm	Rotations per minute
Sl.	Serial
SSB	Silicate Solubilizing Bacteria
sp. or spp.	Species (singular and plural)
SE (m)	Standard error (Mean)
subsp.	Subspecies
i.e.	That is
t	Tonnes
var.	Variety

INTRODUCTION

1. INTRODUCTION

Silicon (Si) is the second most abundant element on earth's crust and more than 90 per cent of earth's crust is composed of silicate minerals. It is a major component of sand, silt and clay minerals. Due to its abundance and non-essential nature, typically Si has not been considered as a limiting factor in soil fertility. Integrated management of six macronutrients *viz.*, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) as well as the seven micronutrients iron (Fe), manganese (Mn), boron (B), copper (Cu), zinc (Zn), chlorine (Cl) and molybdenum (Mo) are considered as essential for sustainable crop yields. However, under certain special crop or soil conditions there are some beneficial elements, like Si that will enhance crop yield by promoting several desirable plant physiological processes.

Numerous field studies have shown that supplying crops with adequate plant-available Si can suppress plant diseases, reduce insect attack, improve environmental stress tolerance and increase crop productivity. Silicon benefits the plants in several other ways by accelerating growth, conferring rigidity to leaves thus maximizing leaf surface area for photosynthesis and mitigating the effects of abiotic stresses like drought, salt and metal toxicity in several plants including rice, wheat, sugarcane, cucumber, citrus, tomato and barley (Adatia and Besford, 1986; Ma and Yamaji, 2006). Furthermore, Si appears to interact with defense related signaling pathways and Si status seems to regulate a range of physiological activities (van Bockhaven *et al.,* 2013; Mao *et al.,* 2013).

Among the plants, silica concentration is found to be higher in monocots than in dicots and its level increased in an ascending order in legumes, fruit crops, vegetables, grasses and grain crops (Thiagalingam *et al.*, 1977). Grasses can accumulate 2 to 20 per cent foliar dry weight as hydrated polymer or silica gel.

Rice is the most important cereal crop in the world and is the staple food for almost half of the world's population including India. But there are various constraints in rice production like incidence of pests and diseases, low soil fertility and fertilizer use efficiency, water management and poor agronomic practices which drastically reduce its

yield. Indiscriminate use of fertilizers and pesticides to improve the yield are also harming the natural ecosystem. However, Si helps in alleviating all these stresses without having any negative impact on the environment.

Desilication, which is a leaching process, leads to continuous loss of Si from soil. As desilication process is high in tropical and subtropical soils, they are generally low in plant-available Si and would benefit from Si fertilization. So, the need for proper Si management to increase yield and sustain crop productivity is necessary in temperate and in tropical countries. Silicon diminution in the soil can also occur in intensive cultivation practices and continuous monoculture of high-yielding cultivars. As a result, these soils are generally low in plant-available Si (Juo and Sanchez, 1986; Foy, 1992).

Despite its abundance on earth's crust, Si is mostly present in insoluble forms that cannot be readily absorbed by plant roots (Rodrigues and Datnoff, 2005; Vasanthi *et al.,* 2012). The purpose of application of Si source is to provide soluble Si to plants. Therefore, a good source must have much of the Si in its readily soluble form in the soil solution. This characteristic is the most important and most difficult aspect to fulfil. Because Si is always combined with other elements like aluminium silicate or potassium silicate and most natural sources being insoluble, finding a soluble source that has good characteristics is not easy.

Bacteria are found plentiful in soil but only few of them have the capacity to solubilize silicate minerals, releasing silica. Studies of mineral dissolution with cultures of bacteria and fungi have shown dramatic increase in the dissolution rates of minerals (Rogers and Bennett, 2004). Silicate solubilizing bacteria (SSB) play an efficient role in solubilizing insoluble forms of silicates thereby increasing soil fertility and enhancing plant defense mechanisms (Vasanthi *et al.,* 2012).

Efficient silicate solubilizing bacteria can help in the release of other essential nutrients in soil. Dissolution of silicate will help in rendering phosphorus available for plant absorption as Si competes with phosphorus fixation sites; silica acts like a substitute for phosphorus in plants (Janardhan, 2014). The potassium solubilization potential of

silicate solubilizing bacterial isolates has also been reported (Naureen *et al*., 2015). Thus, SSB enhances silicate, phosphorus and potassium content in soil, plant as well as grain yield and it finds wide application in rice ecosystem.

In Kerala, continuous monocropping with high Si accumulator species such as rice results in the removal of plant-available Si which is superior than the supply via natural practices releasing it into the soil unless fertilized with Si (Rao and Yadav, 2018). Silicon is not much a mobile nutrient in soil. Therefore, a continued supply of Si would be required predominantly for a healthy and productive development of plants during all growth stages (Savant *et al.,* 1997; Epstein, 2001). Recent studies suggest that if adequate silica is not available, the yield from rice may be seriously affected (Savant *et al.*, 1997; Esser, 2002).

Although, silicate solubilizing bacteria are involved in the dissolution of naturally available insoluble silicate minerals in agricultural soils, studies on these bacteria are limited. Hence, isolation, screening and characterization of silicate solubilizing bacteria especially from paddy rhizosphere need to be studied in detail for the maximization of growth and yield in rice.

With this background the present investigation was undertaken with the following objectives

i. To isolate silicate solubilizing bacteria from paddy rhizosphere.
ii. To assess their silicate, phosphorus and potassium solubilization potential under *in vitro* conditions.
iii. To assess the antagonistic efficiency of superior SSB isolates under *in vitro* conditions.
iv. To characterize the superior SSB isolates based on morphological and biochemical characters.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Plants absorb various mineral elements as nutrients for their ideal growth. Among them, nitrogen (N), phosphorus (P) and potassium (K) are considered as the major elements that are required for plant survival. Similarly, the beneficial effect of silicon (Si) for healthy crop production has been reported (Farooq and Dietz, 2015). Silicon which is considered as a wonder element, has proved its beneficial role in quality production especially in the case of hyper-Si accumulator plants such as rice.

Considering the present-day climate change scenario, crops are more frequently exposed to a number of biotic and abiotic stresses. Silicon benefits the plants in many ways by mitigating these stresses. But it is mostly present in the unavailable form in the soil that cannot be readily absorbed by the plants. Microorganisms play a major role in making this Si available to the plants.

2.1. SILICON IN SOIL

Silicon, a beneficial element is present on the earth's crust in abundance after oxygen (Ehrlich, 1981). It is considered as the eighth most abundant element in universe. Silicon dioxide (SiO_2 or silica) comprises about 50-70 per cent of the soil mass. Its content in soils vary greatly and ranges from <1 to 45 per cent by dry weight (Sommer *et al.*, 2006).

In nature, Si generally occurs in the form of silicates, including ferromagnesian silicates (e.g. olivine, pyroxenes, and amphiboles), aluminosilicates (e.g. feldspar, mica, and clays), and silicon dioxide (e.g. amorphous silica, quartz). In general, silicate minerals consist of Si in a tetrahedral fashion where Si is surrounded by four oxygen atoms (Gauger *et al.*, 2016).

The solid phase, the liquid phase and the adsorbed phase are the three different fractions of Si in soil (Matichencov and Bocharnikova, 2001; Sauer *et al.*, 2006). In the

solid phase Si forms are divided into three primary groups: the amorphous forms, the poorly crystalline and microcrystalline forms and the crystalline forms. Previously, the crystalline form consisted only of the primary and the secondary crystalline silicates, which are abundant in mineral soils that developed from rocks and sediments. The silica materials consist primarily of quartz and disordered silica. The amorphous and poorly crystalline and microcrystalline forms are also components of the Si fractions in the solid phase. The components of Si in the liquid and the adsorbed phases are similar, with exception that those in liquid phase are dissolved in the soil solution, while those in the adsorbed phase are held onto soil particles as the iron and aluminium oxides or hydroxides.

2.2. SILICON CYCLE IN SOIL

The solid, liquid, and adsorbed phases of Si are the key components of the Si cycle in soil. The silicic acid *i.e.* H_4SiO_4 and the polymerized and complexed silicic acid in soil solution are the major components of the liquid phase. The only form of Si that is absorbed by plants and microorganisms is the uncharged form of H_4SiO_4. Later, within the plant tissues or the cell structure of the microorganisms these absorbed Si gets deposited in the form of polymerized silica. These polymerized silica bodies return to the topsoil in the litter fall and the remains of microorganisms and eventually enter the highly soluble biogenic silica pool that contributes to the Si in the soil solution (Farmer *et al*., 2005; Saccone *et al*., 2007; Fraysse *et al*., 2010). Along with the application of manures and compost, Si is also added to soils and the decomposition of Si rich manure can increase the available soil Si level (Song *et al*., 2013).

The chemistry of Si in the liquid phase is regulated by a number of processes: (a) the dissolution of Si that contains primary and secondary minerals, (b) the absorption of H_4SiO_4 in the soil solution by the vegetation and microorganisms, (c) the Si adsorption on and the desorption from various solid phases, (d) the preservation of the stable Si in the

soil profile (silica polymorphs), I leaching and (f) addition (*i.e.,* fertilization, irrigation, atmospheric, plant litter, animal manure, and remains of microorganisms).

The different forms of Si including ionic, molecular and aggregate Si may be present in the natural irrigation water. Silicon is also added to the soil as atmospheric deposition via wind-blown dust and phytolith particles from savanna fires (Kurtz *et al*., 1987; Street-Perrott and Barker, 2008; Opfergelt *et al*., 2010). However, the atmospheric contribution of Si to the soil solution is very low when compared with the other Si inputs to the soil-plant system (Street-Perrott and Barker, 2008). Bio cycling of silica in the soil also occurs through microbial activities that involve bacteria, fungi, and actinomycetes. Thus, plants and microbes, through their interplay with soil minerals, contribute appreciably to the global Si cycle.

2.3. SILICON DEFICIENCY

The development of strong leaves, stem and roots may be affected due to Si deficiency. Also, the formation of a thick silicate epidermal cell layer is affected due to its deficiency and makes the rice plants susceptible to bacterial and fungal diseases and insect and mite pests. Severe Si deficiency reduces the number of panicles and the number of filled spikelets per panicle. Silicon deficient plants are also particularly susceptible to lodging (Dobermann and Fairhurst, 2000). The symptoms noticed include:

- Soft and droopy leaves and culms, thus increasing mutual shading.
- Increased lodging.
- Lower or reduced grain yields.
- Reduced photosynthetic activity.
- Increased occurrence of diseases such as blast and brown spot.

2.4. SILICATE SOLUBILIZING MICROORGANISMS (SSB)

Silicate solubilizing microorganisms (SSB) play an efficient role in solubilizing the insoluble form of silicates thus increasing soil fertility and enhancing plant defense mechanisms (Vasanthi *et al*., 2012).

Silicate solubilizing bacteria are distributed in soil, water, aquatic sediments and in silicate minerals, but their population is comparatively low than the total bacteria indicating their uniqueness. A higher SSB population was observed in pond sediments with 40×10^4 cfu g^{-1} followed by sugarcane field soil which recorded 31×10^4 cfu g^{-1} dry weight. In different silicate minerals, phyto-sil showed the highest SSB population followed by muscovite. Silicate solubilizing bacterial population was very low in quartz and illite. Magnesium trisilicate was more easily solubilised than, quartz, or muscovite (Vasanthi *et al.,* 2016).

Saxena (1989) has proposed a procedure for the estimation of available silica and Anthoniraj (1999) reported that SSB release silica (SiO_2) into solution in the form of soluble silicates and this principle is employed in isolating SSB from soil and other materials. Isolation of SSB from soil and water in Bunt and Rovira medium containing 0.25 per cent magnesium trisilicate was carried out earlier by Muralikannan and Anthoniraj (1998).

Vasanthi *et al*. (2013) determined the silicate solubilization potential of bacteria by evaluating the different types of media containing an insoluble silicate mineral. However, no specific medium was recommended either for isolation or for enumeration and screening. This study reported that for enumeration, soil extract agar medium containing 0.25 per cent magnesium trisilicate is more ideal while the plain glucose medium with 0.25 per cent magnesium trisilicate is ideal for screening the isolates as there is rapid solubilization and larger clearing zone.

Microorganisms like *Bacillus caldolytyicus*, *Bacillus mucilaginosus* var. *siliceous*, *Proteus mirabilis*, *Pseudomonas* and *Penicillium* were found to release silica from natural silicates (Lauwers *et al*., 1974; Avakyan *et al*., 1986). Several microbes like *Burkholderia*, *Collimonas*, *Dyella*, *Janthinobacterium*, *Aminobacter*, and *Frateuria* have been reported to solubilize the biotite, which contains considerable amounts of silicate minerals (Uroz *et al.*, 2009).

Muralikannan (1996) and Muralikannan and Anthoniraj (1998) suggested that soil contains a variety of microorganisms but a few are capable of solubilizing silicate. A virulent silicon solubilizing bacterium (Si SOL B) was isolated and tested on a variety of crops in different soils. The release of soluble silicates from silicate minerals and soil was observed with the inoculation of SSB. This bacterium was found to enhance the growth, suppression of pests and diseases thus increasing the yield and was used as biofertilizer.

In rice, the field trials conducted with the inoculation of SSB showed that this bacterium enhanced the growth, yield, chlorophyll content, 1000 grain weight, number of filled grains and biomass (Avakyan *et al.*, 1986). Soil incubation studies revealed that inoculation of SSB to sterile and unsterile soil solubilized silica in water and enhanced the available silica in soils (Muralikannan, 1996).

Gopal *et al.* (2005) isolated silicate solubilizers from the rhizosphere of wilt diseased (caused by Phytoplasma) and wilt tolerant palms and recorded significantly higher population (6×10^2 cfu g^{-1} of dry soil) in field tolerant coconut palms than in wilt diseased palms (1×10^2 cfu g^{-1} of dry soil). They reported *Bacillus* spp., *Pseudomonas* spp., *Aspergillus* spp. And *Pencillium* spp. Solubilizing silicates.

In rice ecosystem, the positive role of *Bacillus*, as silicate solubilizing bacterium has been reported by many workers (Norkina and Pumpyanskaya, 1956; Duff and Webley, 1959; Webly *et al.*, 1960). *Bacillus mucilaginosus*, as a member of *Bacillus* play a significant role in enhancing the Si nutrition, plant growth promotion and biocontrol of

Pyricularia oryzae, the causal organism of blast disease in rice crop (Datnoff *et al*., 1991).

Bacillus mucilaginosus, a common soil bacterium and also a silicate solubilizing and a model microorganism in research on silicate mineral weathering has been reported (Malinovskaya *et al*., 1990; Basak and Biswas, 2009). Extensive studies on this bacterium were mainly focused on releasing Si from the soil minerals and the application of the same as plant growth promoting rhizo bacteria (PGPR) seems to be a promising approach in increasing the rice productivity growing under lowland condition.

Shu *et al.* (2008) studied the effect of *Bacillus mucilaginous* on weathering of phosphorite and reported that analysis of different proteins was of significance in exploring the molecular mechanisms in bacterial process.

Xia *et al.* (2008) isolated a silicate mineral solubilizing bacterial strain Q12 and identified it as *Bacillus globisporus* Q12 based on the 16S rDNA gene sequence analysis. The strain showed better growth on silicate mineral, Biotite than on Feldspar and Muscovite. Solubilization of Si and K from silicate minerals by the strain resulted mostly from the action of organic acids. Gluconic and acetic acid were likely involved in the solubilization of Si and K in Feldspar.

Vijayapriya and Muthukkaruppan (2010) isolated ten bacterial isolates from soil, identified and characterized as *Bacillus mucilaginosus*. Among the 10 isolates tested, four isolates viz., SSB 3, SSB 5, SSS 8 and SSB 9 were found to be very efficient in silicate solubilization and recorded 11.0 mm, 11.5 mm, 15.4 mm and 15.0 mm zone of solubilization.

Vijayapriya *et al.* (2019) isolated, purified and studied the Si solubilization of 20 silicate solubilizing organisms. Among the isolates, SSB 17 was found to be a superior isolate and recorded much significant values in the Si solubilization compared with other

isolates. The best isolate showed much significant values in the form of clearing zone in the degradation of organic siliceous materials.

Sulizah *et al.* (2018) isolated five bacterial isolates OS4, OS5, OS7, OS12 and OS13 from paddy rhizosphere soil that can solubilize silicate in Bunt and Rovira agar and broth. The highest silica solubilization capacity in Bunt and Rovira Agar was obtained by solubilizing index of OS7 by 1.10, while the highest silica solubilizing activity in Bunt and Rovira broth were found in OS12 with silica concentrate 1.053 ppm.

Lee *et al.* (2019) isolated a high Si and P solubilizing bacterial strain *Enterobacter ludwigii* GAK2 through 16S rRNA gene sequence analysis. This strain produced organic acids such as citric acid, acetic acid, and lactic acid as well as indole-3-acetic acid (IAA), and gibberellic acid (GA1, GA3) in Luria-Bertani media. In addition, GAK2 inoculation promoted seed germination in a gibberellin deficient rice mutant *Waito-C* and rice cultivar *'Hwayoungbyeo'*. Overall, the isolate GAK2 increased root length, shoot length, fresh biomass, and chlorophyll content of rice plants which revealed that *E. ludwigii* GAK2 is a potential Si and P bio-fertilizer.

Silicate solubilizing organisms promote the growth and yield in rice by secreting certain plant growth promoting substances like IAA and GA in the rhizosphere region. Hence, the development of this organism, as agricultural bio-inoculant needs to be exploited in detail for the maximization of growth and yield in rice (Bin *et al*., 2002).

2.5. MECHANISM OF ACTION OF SSB

Bacteria help in the dissolution of silicates by producing excess proton, hydroxyl anion, organic ligands, extra cellular polysaccharides and enzymes (Berthelin and Belgy, 1979; Barker *et al*., 1998). The production of organic acids such as 2 keto-gluconic acid, alkalies and polysaccharides by bacterial isolates can solubilize insoluble minerals such as silicates, phosphates and potash into soluble form (Joseph *et al*., 2015). They supply H^+ ion to the medium and promote hydrolysis. Natural acids like oxalic acid, citric acid,

keto acids and hydroxy carbolic acids which form complexes with cations, promote their elimination and retention within the medium in a dissolved state.

The most accepted mechanism of weathering silicate minerals is by acidolysis (Xia *et al.,* 2008). The solubilization of SiO_2 originated from quartz have been reported by certain bacterial isolates such as *Burkholderia cenocepacia* KTG, *Aeromonas unctate* RJM 3020, and *Burkholderia vietnamiensis* ZEO3. These isolates were capable of solubilizing silicates and produced acid as detected by yellow halo formation on solid Luria Bertani media containing 0.2 per cent (v/v) bromophenol blue (Santi and Didiek, 2017).

Many bacteria in soil are able to solubilize unavailable form of silicate mineral such as quartz by excreting organic acid, which either directly dissolves rock K or chelate Si ions to bring the K and Si into solution. It was postulated that the reaction responsible for bacteria promoted K and Si solubilization may involve a combination of proton attack and complexation reaction by organic acid (Xia *et al*., 2008). The analysis of organic acids produced in the medium containing feldspar and quartz by *Bacillus flexus* and *B. mucilaginosus* showed variation with the minerals. The release of silica in solution serve as a nutrient for life forms (Vasanthi *et al.,* 2016).

2.6. SILICON ABSORPTION BY PLANTS

The solubilization of silica by bacteria is considered as a source of supply for crops. On an average, plants absorb from 50 to 200 kg of Si ha^{-1}. The solubilized Si is absorbed in the form of orthosilicic acid (H_4SiO_4) along with water. Sugarcane (300–700 kg of Si ha^{-1}) is the largest absorber of Si followed by rice (150–300 kg of Si ha^{-1}) and wheat (50–150 kg of Si ha^{-1}) (Bazilevich, 1993). Such values of absorbed Si cannot be fully explained by passive absorption (such as diffusion or mass flow) because the upper 20 cm soil layer contains only an average of 0.1–1.6 kg Si ha^{-1} as monosilicic acid (Matichenkov *et al.,* 1997; Matichenkov *et al.,* 2000).

The three classes of Si absorbers include (a) Si accumulator crops such as rice, wheat, millet, sugarcane as they require large quantity of Si (b) Si non-accumulator crops – Snapdragon (*Antirrhinum majus*) and (c) Si excluder – soybean (van der Vorm, 1980; Marschner, 1995).

Generally, Si absorption in graminaceous plants is much higher than its absorption in other plant species. For example, rice is a common Si-collector that absorbs Si in active progression (Jian *et al.,* 2006), as other graminaceous plants do including barley (*Hordeum vulgare* L.) (Barber and Shone, 1966), ryegrass (*Lolium perenne*) (Jarvis,1987), wheat (*Triticum* spp.) (Casey *et al.,* 2004), maize (*Zea mays* subsp. *Mays*) (Bakhat *et al.,* 2009) and some cyperaceous plants.

Majority of dicotyledon plants, such as cucumbers (*Cucumis sativus*), melons, strawberries, and soybeans (*Glycine max* L. Merr) absorb Si inertly (Mitani and Ma, 2005). Nonetheless, some plants especially dicotyledon, such as tomatoes (*Solanum lycopersicum*), beans and other plants, are not able to absorb Si from soil (Mitani and Ma, 2005; Yongchao *et al.*, 2005; Yongchao *et al.,* 2006; Miroslav *et al.,* 2007).

2.7. SILICON UPTAKE MECHANISMS IN PLANTS

Takahasi *et al*. (1990) categorized plant species that rely primarily on active or passive mechanisms as high, intermediate or low accumulators, respectively based on the mechanisms of Si uptake.

The plants in the high-accumulator category have a Si content in the shoot that ranges from 1.0 per cent to 10 per cent dry weight and are primarily monocotyledons such as rice, wheat, sorghum, barley, bamboo, and sugarcane (Ma *et al*., 2001; Yongchao *et al*., 2007). The dryland Gramineae with shoot Si contents that range between 0.5 per cent and 1.5 per cent dry weight mostly form the intermediate-accumulator plants. The dicots, which accumulate <0.2 per cent shoot dry weight Si, form the low-accumulator group.

In the plant, Si is transported from the root to shoot by the transportation stream in the xylem (Ma, 2009). The different mechanisms by which Si is taken up by plants includes active and passive mode of transportation (Qiong *et al*., 2015). The amount of Si uptake by the active mechanism is typically larger than that predicted based on the mass flow and is attributed to the density of Si transporters in the roots and shoots that facilitate the absorption process across the membranes of root cells.

Rice depends on the availability of silicic acid on all growth stages as well as for protection from abiotic stresses such as drought, salinity, and metal toxicity and also biotic stresses such as rice yellow stem borer, *Scirpophaga incertulas* (Walker), and blast (*Pyricularia grisea*) (Kim *et al.,* 2002; Rodrigues *et al.,* 2003; Ranganathan *et al.,* 2006). Thus, the evolution of the rice plant has ensured mechanisms for uptake of silicic acid. Also, mechanisms for controlled transport of silicic acid exist (Mengel *et al.,* 2006) and its deposition occurs in all parts of plant (Yoshida *et al.,* 1962).

In rice, both the radial transport and the xylem loading of Si are mediated by transporters (Mitani and Ma, 2005). Moreover, these transporters were recently identified and were coded by low-Si genes such as the *Lsi1* and *Lsi2* in roots and the *Lsi6* in shoots (Mitani and Ma, 2005; Ma *et al*., 2006; Yamaji *et al*., 2008). The *Lsi1* may encode a membrane protein similar to the water channel proteins, also known as aquaporins (Ma *et al*., 2006). The amount of Si uptake by the plant via the passive mechanism is likely to be entirely driven by mass flow.

2.8. SILICON DEPOSITION IN PLANTS

Silicon is deposited in the epidermal tissue as a fine layer of Si–cellulose membrane and is associated with pectin and calcium ions. By this, the double-cuticular layer can protect and mechanically strengthen plant structures. With increasing Si concentration in the plant sap, monosilicic acid gets polymerized. Increased mechanical strength of the culm helps reduce crop lodging (Savant *et al.*, 1997a).

Silicon is accumulated in the form of silica gel and is deposited in epidermal cells, sclerenchyma, vascular bundles and in inflorescence brackets in cereals (Lanning *et al*., 1958).

Rice accumulates about 4 to 20 per cent Si in straw and almost every part of it contains this element which is not at all added exogenously as fertilizer as done with nitrogen, phosphorus and potassium. In rice leaves, Si is deposited in "dumbbell" type structures that increase in size as the plant leaf matures, preceded by lignification (Guoliang *et al.,* 2013). Silicon in rice is preferentially deposited in epidermal cell walls, where it polymerizes to form Si-cuticle double layers (Natsumi *et al.,* 2015).

Increased Si nutritional content of leaves induced lignin production, oxidative cross-linking in cell walls and phytoalexin production (Wanchun *et al.,* 2010). Silicon deposits in the lumen of the cells through needle-like silica structures, molding the inner cell walls (Guoliang *et al.,* 2013).

Fleck *et al.* (2011) studied the role of Si in root formation and function and found that Si nutrition increased suberization of the exodermis and lignification of the sclerenchyma, as well as reduced the zone of radial oxygen loss by roots. This study also showed that genes involved in suberin and lignin formation were differentially regulated by Si nutrition.

2.9. ROLE OF SILICATE SOLUBILIZING BACTERIA

Efficient SSB can help in the release of several nutrients in soil. This can be directly due to solubilization of other minerals by SSB or indirectly due to solubilized Si. It has been previously reported that the solubilized Si improves availability of P to plants by competing with P fixation sites in soil (Muralikannan and Anthoniraj, 1998). Thus, Si acts as a substitute for P in plant system.

Naureen *et al*. (2015) reported the K solubilization of silicate and phosphate solubilizing bacterial isolates. The deficiency of K in soil can be successfully managed by the application of biofertilizers based on bacterial isolates that can solubilize K in soil.

2.9.1. EFFECT ON ABIOTIC STRESSES

Silicon nutrition alleviates many abiotic stresses including physical stress like lodging, drought, high temperature, UV and chemical stress like salt, metal toxicity, nutrient imbalance and many others (Epstein, 1999).

2.9.1.1. EFFECT ON DROUGHT

Drought is one of the most limiting environmental stresses for crop production (Kramer and Boyer, 1995). The growth and development of plants experiencing occasionally periods of drought depend on the ability of stomata to control water loss. Drought stress can damage plant cell membranes and cell wall architecture, as well as inhibit photosynthesis and cell division (Hsiao, 1973; Taiz and Zeiger, 2006).

Plants respond to drought by closing their stomata, which reduces leaf transpiration and prevents excessive water loss in their tissues. The control of leaf stomata closure is a crucial mechanism for plants since it is essential for both CO_2 acquisition and desiccation prevention (Dodd, 2003).

Leaf water potential and water content decrease substantially when plants are exposed to drought (Farooq *et al.,* 2009). Application of Si can significantly improve water status in non-irrigated crops. Based on research, the application of silicate solubilizing bacteria (SSB-bio-silica) on oil palm seedling had a significant effect on stomatal opening in the period after drought stress treatment (Santi *et al,* 2018).

Silicon deposits 2.5 μm thick between the cuticle (generally 0.1 μm thick in rice) and endodermal cells have been found in rice (Ma and Takahashi, 2002). This action occurs owing to a reduction in the diameter of stomatal pores and consequently, a

reduction in leaf transpiration. Silicon can reduce the transpiration rate by 30 per cent in rice, which has a thin cuticle (Ma, 2004).

A well-thickened layer of silica gel associated with the cellulose in the epidermal cell walls, which reduces water loss, while an epidermal cell wall with less silica gel will allow water to escape at an accelerated rate (Wong *et al.,* 1972).

Silicification of trichomes has been observed in plants (Sangster *et al*., 1983; Hodson *et al*., 1985). It is possible that Si-fortified trichomes act as antennae that absorb shortwave radiation and emit long-wave radiation to aid in the cooling of leaves. However, it is also possible that Si-fortified trichomes increase the leaf-atmosphere boundary layer, thus creating a larger energy transfer gradient.

2.9.1.2. EFFECT ON HEAVY METAL TOLERANCE

Among the abiotic factors affecting plants, heavy metal stresses have received increasing attention over the last several decades. The term heavy metal refers to any metallic element with relatively high density that is toxic even at low concentration. In general, heavy metals relate to a group of metals and metalloids with greater than 4 g cm^{-3} atomic density (Hawkes, 1997).

The heavy metals include cadmium (Cd), nickel (Ni), lead (Pb), iron (Fe), zinc (Zn), cobalt (Co), arsenic (As), chromium (Cr), silver (Ag) and platinum (Pt) and the majority of them do not play an essential role in plants. Although naturally present in the soil, concentration of these heavy metals increases as a result of geologic and anthropogenic activities causing a harmful effect on both plants and animals (Chibuike and Obiora, 2014). Heavy metals retard plant growth by marginalizing the cellular functions of proteins, lipids, and elemental components of thylakoid membranes (Kim *et al.*, 2014).

Silicon derived enhancement in plant tolerance to heavy metal toxicity is well documented, and the beneficial role of Si in detoxification can be ascribed to both

external (growth media) and internal plant mechanisms (Cocker *et al*., 1988; Sahebi *et al*., 2015).

The external mechanism of elevating heavy metal tolerance is mainly due to the increased pH by silicate application resulting in metal silicate precipitates that decrease the metal phyto-availability, regulation of activities of metal transporters and co-deposition along with metals on growth media whereas internal mechanisms include stimulation of enzymatic and non-enzymatic antioxidants, co-precipitation of heavy metals in plants, metal ions chelation, compartmentalization of excess heavy metals into the vacuoles, inhibition of heavy metal transports from root to shoot (Bhat *et al.*, 2019).

Hammond *et al. (*1995) reported the exclusion of aluminium (Al) from the subtending tissue as a result of Si deposition at the epidermis, restricting total overall Al uptake into the barley roots. Formation of hydroxyaluminosilicates in the apoplast of the corn root apex helps in reducing the mobility of apoplastic Al (Wang *et al.*, 2004).

Silicon was also effective in alleviating Fe toxicity in rice (Okuda and Takahashi, 1962). Silicon enhanced the oxidative power of rice roots, resulting in enhanced oxidation of Fe from ferrous iron to insoluble ferric iron. Therefore, excess Fe uptake was indirectly prevented by Si application. For upland plants, excess Fe stress is not a problem.

Iwasaki *et al*. (2002) reported that the interaction of Si with phenolic substances maintains the apoplast in reduced state preventing the oxidation of manganese in cowpea. Enhanced production of enzymatic and non-enzymatic antioxidants resulted in reduced membrane lipid peroxidation in cucumber (Shi *et al*., 2005).

Cell wall bound Si inhibit apoplastic Cd uptake in rice by covalently bonding with Cd and trapping Cd as it diffuses through the cell wall and intracellular spaces (Nwugol and Huerta, 2008). In rice Si competes with arsenate ions for root entry points (Seyfferth and Fendorf, 2012).

2.9.1.3. EFFECT ON SALINITY

Salinity stress, a major yield restraining factor in dry and semidry areas, can be repressed by increasing Si (Tahir *et al*., 2006). Silicon application to the plants under salt stress limits the transpiration ratio and increases root activities. As consequence of root activities, plants can increase the nutrients uptake and decrease salt toxicity. Silicon absorption by plants leads to increased Ppase and ATPase activities in vacuoles, which reduces Na^+ uptake and enhances K^+ uptake by the cell membrane. Separation of salt ions into the vacuoles and increasing the K^+/Na^+ ratio in the cells of the roots and leaves decrease Na^+ toxicity.

Silicon can increase the antioxidant enzyme activity of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in plants under salt-stress (Yongchao *et al.*, 2003; Zhujun *et al.*, 2004). The induced oxidative damage by salt can be decreased through decreasing in level of lipid peroxidation, and H_2O_2 content (Zhujun *et al.*, 2004). This enzymatic protection mechanism helps plants to overcome salinity stress damage.

Oxidative damage in tomato leaves decreases with increasing Si (Richmond and Sussman, 2003), resulting in increased activity of catalase and superoxide dismutase enzymes, increased protein content in the tomato leaves, decreased ascorbate peroxidase enzyme, decreased malondialdehyde concentration and decreased H_2O_2 levels (Khalid *et al.*, 2005).

The roots and shoots of Si-treated rice plants under salinity stress notably improved when compared to control plants (Matoh *et al.*, 1986). Silicon indirectly reduces the oxidative damage of cucumber tissues under salt stress through the activities of guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase, dehydroascorbate reductase, and glutathione reductase (Zhujun *et al.*, 2004).

2.9.1.4. EFFECT ON RADIATION

Radiation injures plants. Silicon seems to protect plants from radiation injury. When rice seedlings (30 days old) were irradiated with different doses of gamma-rays, the decrease in the dry weight was less appreciable in the Si-supplied plants than in the Si plants that had not been treated with Si, suggesting that Si increases the resistance of rice to radiation stress (Takahashi, 1966). Furthermore, when the plant was supplied with Si after radiation treatment, the growth recovery was faster compared to that of the plants without Si supply.

2.9.2. EFFECT ON BIOTIC STRESSES

The accumulated Si not only improves growth and yield of plants but also involved in induction of systemic resistance against pest and diseases (Vijayapriya and Muthukkaruppan, 2010). The deposition of silica on epidermal layers offers a physical barrier to insects and pests (Sahebi *et al*., 2015).

2.9.2.1. EFFECT ON PESTS

Silicon deposition patterns within plant tissues act as a physical barrier to insect feeding, as silica makes plant tissues difficult for insects to efficiently chew, penetrate and digest. Sucking pests and leaf eating caterpillars have a low preference for the silicified plant tissues. As Si is involved in toughening plant tissues, it indirectly helps in delaying insect penetration of host tissues and thus increasing the duration of insect exposure to natural enemies, adverse environmental conditions and chemical controls.

In addition to this, Si can also reduce pest damage by enhancing the induced chemical defences of plants following insect attack. Silicon acts as an elicitor of systemic stress signals, mediated by phytohormone pathways, leading to the efficient synthesis of defensive compounds (Fauteux *et al*., 2005). Not only shoots but also roots can defend against insect attacks.

High root Si concentrations can effectively reduce the feeding and relative growth rate performance of the sugarcane root-feeding insect, the greyback cane grub (*Dermolepida albohirtum*) (Frew *et al*., 2016).

Silicon suppresses insect pests such as stem borer, brown planthopper, rice green leafhopper, and white backed planthopper, and non-insect pests such as leaf spider and mites (Savant *et al.*, 1997b).

Stems attacked by the rice stem borer were found to contain a lower amount of Si (Sasamoto, 1961). In a field study, a positive relationship between the Si content of rice and resistance to the brown planthopper has been observed (Sujatha *et al.,* 1987).

Enhanced leaf Si also provides resistance to plant hoppers (Ma and Takahashi, 2002). Yoshihara *et al.* (1979) found that elevated silicic acid in the phloem inhibited brown plant hopper (*Nilaparvata lugens*) feeding. Another study, that of Wenqiang *et al.* (2015), found that increased plant silicic acid content caused a decrease in plant hopper residence time on the plant, a decrease in fertility and a decrease in honeydew production.

It appears that Si defense against insects is threefold: (a) enhanced physical protection of the leaf for attack/colonization, (b) the leaf being a poorer quality substrate as Si makes it less digestible and results in lower macro-nutrient content and (c) the phenology of the insect's life-cycle is slowed down, also making it more prone to predation. Silicon content regulation of biochemical pathways may also play a role (van Bockhaven *et al.,* 2013).

2.9.2.2 EFFECT ON DISEASES

The mechanism for Si-induced resistance to diseases is due to (i) Si acting as a physical barrier and (ii) soluble Si acting as a modulator of host resistance to pathogen. Si is deposited beneath the cuticle to form a cuticle-Si double layer which mechanically impede penetration of fungi and thus disrupt the infection process. The soluble Si can produce phenolics and phytoalexins in response to infection by pathogen.

Dual culture antagonistic assays of SSB revealed the potential antagonistic activity of bacterial isolates against four plant pathogenic fungi – *Magnaporthae grisae*, *Rhizoctonia solani*, *Alternaria alternata* and *Macrophomina phaseolina*. Mean zone of inhibition of these bacterial isolates against the four pathogenic fungi ranged between 4 mm to 39 mm (Naureen *et al.*, 2015).

Silicon stimulates chitinase activity and rapid activation of peroxidases and polyphenol oxidases after fungal infection (Cherf *et al.*, 1994). Glycosidically bound phenolics extracted from Si amended plants when subjected to acid or ß-glucosidase hydrolysis displayed strong fungistatic activity.

Datnoff *et al.* (1997) reported that relatively large amounts of plant available Si appear to be very important for both robust growth and fungal disease resistance to rice. Rodrigues *et al.* (2001) studied effect of Si and host resistance on sheath blight (*Rhizoctonia solani* Kuhn) development in rice and reported significant reduction in the severity of sheath blight by fertilization with Si along with reduction in infected tillers.

Datnoff *et al.* (2005) reported that Si can reduce several important diseases of rice, including blast, brown spot, sheath blight, leaf scald and grain discolouration. Levels of control are equal to that achieved by fungicides used for diseases such as blast and brown spot. Hence, number of fungicide applications and rates can be reduced significantly. Residual activity of Si was effective for disease control in second year crop and was comparable to first year Si application or a full rate of a fungicide. Silicon enhanced performance of partially resistant cultivars for both blasts and sheath blight. These findings suggest that Si could be employed in integrated disease management systems for reducing fungicide use and enhancing host plant resistance for the control of important rice diseases worldwide.

Ranganathan *et al.* (2006) reported that application of metasilicate at 100 and 150 mg kg^{-1} to the rice crop at fortnightly interval throughout the growth period resulted in

significant accumulation in leaf bundle sheath cells imparting resistance against blast disease.

2.9.3. EFFECT ON CROP YIELD

Meharg *et al.* (2015) revealed that biotic as well as abiotic stress mitigation, waste valorization and grain nutritional qualities which are mainly achieved through secondary metabolites and signalling molecules, enhanced structural rigidity, altered cell wall chemistry, competition for uptake and internal sequestration of toxic ion due to Si nutrition has led to an increase in the crop yield.

Silicon was found to activate certain enzymatic reactions resulting in greater sucrose production in sugarcane and reduction in phosphatase and provide greater supply of essential high energy precursors needed for cane growth (Tisdale *et al.,* 1993). The application of Si mobilizing bacteria and calcium silicate significantly produced higher cane yields in fresh plantings and also in ratoon crops (Brindavathy *et al*., 2012).

Better filling of grains by the application of silica was reported by Vijayakumar (1977) who found an increase in thousand-grains weight by the application of silica. Application of silica to rice was found to increase the grain yield under both upland and waterlogged conditions (Datta and Shinde, 1985). Silicon supply increased the photo assimilation of carbon and also promoted the assimilated carbon to the panicle in rice (Lin *et al.,* 2002). Silicon plays an important role in regulating grain nutrition in rice by assimilation of the hazardous toxins such as arsenic, antimony and cadmium (Li *et al.,* 2009).

Snyder *et al.* (1986) showed that application of calcium silicate increased rice yields. Shashidhar *et al.* (2008) reported that application of calcium silicate at 2 t ha^{-1} was found to be effective in increasing plant height, number of tillers per hill and panicle length over the control, and resulted in 25 -30 per cent higher grain yield.

Ghanbari *et al.* (2011) reported that Si application increased the number of filled spikelets and decreased blank spikelets. Wattanapayapkul *et al.* (2011) also reported increase in grain yield of paddy due to Si application.

Aarekar (2013) found that the plant height, total number of productive tillers per pot and dry matter per hill of paddy were found to increase growth and yield due to Si application through different sources (rice husk ash, bagasse ash, fly ash and calcium silicate) in a pot culture experiment.

Wader (2013) found that the application of Si through calcium silicate to upland paddy at 240 kg ha^{-1} was found to be optimum for increasing the growth and yield attributing characters of paddy. Patil *et al.* (2017) reported significant increase in height of plant, number of tillers, number of panicles per hill 1000 grains weight and yield of low land paddy with application of calcium silicate.

From this, the role of SSB in solubilizing the insoluble silicate sources and thereby mitigating many biotic and abiotic stresses like drought, salinity, heavy metal toxicity, incidence of pests and diseases are well understood. By mitigating these stresses, they also indirectly help in enhancing plant growth and yield potential. Hence, the isolation and *in vitro* screening of SSB especially from paddy rhizosphere need to be studied furthermore.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiment on "Isolation and *in vitro* screening of silicate solubilizing bacteria from paddy rhizosphere" was carried out in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during 2018-2020. The details of the materials used and methods followed in the study are described below.

3.1. ISOLATION OF SILICATE SOLUBILIZING BACTERIA

3.1.1. Soil Sampling

Soil samples were collected from different upland and lowland paddy fields of Kerala. Healthy plants were identified at different sites in the paddy field, and they were gently uprooted and 50 g of soil samples were collected from the rhizosphere region. These soil samples were pooled to get a representative sample. The samples were brought into the laboratory in sterile polythene bags for further studies.

3.1.2. Isolation of Silicate Solubilizing Bacteria (SSB)

Bacteria capable of solubilizing the insoluble silicate compound *i.e.* magnesium trisilicate were isolated from rhizosphere soils collected from different paddy fields by serial dilution and plating technique using Bunt and Rovira medium (Bunt and Rovira, 1955) supplemented with 0.25 per cent insoluble magnesium trisilicate. The medium was sterilized by autoclaving at 121°C for 15 min and cooled to 45°C before pouring into sterile Petri plates.

The soil samples collected were air dried, ground and sieved through 0.5 mm sieve so as to obtain fine sized particles. Ten gram of this sample was weighed and transferred to 250 mL conical flask containing 90 mL sterile distilled water and vortexed to mix the contents well and get 10^{-1} dilution. One mL of this dilution was transferred to 9 mL sterile distilled water followed by vortexing to get 10^{-2} dilution. Similarly, dilutions

were made upto 10^{-9}. Hundred µL of the serially diluted soil samples were spread uniformly using a sterilized glass spreader on Petri plates containing Bunt and Rovira agar supplemented with 0.25 per cent magnesium trisilicate as a sole source of silicate for selective screening of the silicate solubilizing bacteria. The plates were incubated at room temperature for 3 days. Based on the clear halo zone formed around the bacterial colonies, they were identified as Silicate Solubilizing Bacteria (SSB).

3.1.3. Purification of Silicate Solubilizing Bacteria (SSB)

SSB obtained by the serial dilution technique were further purified by the streak plate method on Bunt and Rovira agar medium supplemented with 0.25 per cent magnesium trisilicate and the well isolated colonies on the plates were preserved on Nutrient agar slants at 4°C in a refrigerator for further use.

3.2. ASSESSING THE SOLUBILIZATION POTENTIAL OF BACTERIAL ISOLATES UNDER *IN VITRO* CONDITIONS

The silicate solubilization potential of all the bacterial isolates obtained were assessed by Plate assay and Broth assay.

3.2.1. Plate assay

All the isolates were checked for silicate solubilization on Bunt and Rovira agar medium supplemented with 0.25 per cent magnesium trisilicate as per the method described by Vasanthi *et al*. (2013).

The sterilized Bunt and Rovira medium supplemented with 0.25 per cent insoluble silicate compound *i.e.* magnesium trisilicate was poured into the sterilized Petri plates to a thickness of 3 mm. After solidification of the media, spot inoculation of all the SSB isolates was done in the center of the agar plates with the help of a flame sterilized loop under aseptic conditions. The plates were incubated at room temperature for 7 days. The

clearance zone formed around the bacterial colonies were observed and measured in mm. Two replications were maintained for each treatment.

3.2.2. Broth assay

All the bacterial isolates obtained were tested for magnesium trisilicate solubilization quantitatively in sterilized Bunt and Rovira broth supplemented with 0.25 per cent insoluble magnesium trisilicate. Each conical flask containing 100 mL of sterile broth was inoculated with a loopful of pure SSB bacterial culture and incubated at room temperature for 7 days. Uninoculated control was also maintained. Two replications were maintained for each treatment. The silicate solubilization in the broth was assessed quantitatively by the method described by Santi and Didiek (2017).

The culture broth was filtered through Whatman filter paper No. 1. Two mL of above filtered sample was taken in 50 mL test tubes and 1 mL dilute HCl (1:1) and 2 mL of ammonium molybdate reagent was added and allowed to stand for 5 minutes. Then 2 mL of oxalic acid solution and 2 mL of reducing agent was added (prepared by dissolving 500 mg 1 amino 2-napthol 4-sulphonic acid and 1 g sodium sulfite in 50 mL distilled water and this was added to a solution of 30 g sodium bisulfite in 150 mL distilled water) and volume made up to 50 mL with distilled water. The solution was allowed to stand for 5 min. The absorbance was measured in Spectronic-20 Spectrophotometer at 650 nm against reagent blank. From the optimum absorbance of sample and from slope of standard graph, the available silica content in the broth was calculated in mg L^{-1}.

Stock solution was prepared by dissolving 4.34 g magnesium trisilicate in distilled water and volume made up to 1000 mL. Standard concentrations (40, 80, 120, 200, 240 and 300 mg L^{-1}) was prepared using stock solution (2, 4, 6, 10, 12 and 15 mL) by following the same procedure described above. From the optimum absorbance of sample and from slope of standard graph, the available silica content in the broth was calculated in mg L^{-1}.

3.3. ASSESSING THE PHOSPHATE AND POTASH SOLUBILIZATION POTENTIAL OF BACTERIAL ISOLATES UNDER *IN VITRO* CONDITIONS

All the SSB isolates obtained were assessed qualitatively and quantitatively for phosphorus and potassium solubilization in Pikovskaya's medium and in Aleksandrov medium respectively.

3.3.1. Plate assay for Phosphate Solubilization by SSB Isolates

The ability of the SSB isolates to release phosphorus was assessed by the method described by Yasmin *et al*. (2004).

The isolates were tested for their ability to solubilize the insoluble tricalcium phosphate present in the Pikovskaya's agar medium (Pikovskaya, 1948; Gupta *et al*., 1994). A loopful of pure culture was placed in the center of the agar plates of 3 mm thickness and incubated at 28°C for 5 days. The clearance zone formed around the bacterial colonies were observed and measured in mm. Two replications were maintained for each treatment.

3.3.2. Broth assay for Phosphate Solubilization by SSB Isolates

Phosphorus solubilization by SSB isolates was assessed quantitatively by the method described by Clescerie *et al*. (1998).

Cultures were inoculated into 50 mL of sterile Pikovskaya's broth and incubated at 28°C for 10 days. The broth was centrifuged and 5 mL of the supernatant was collected in a screw capped vial. Five ml of Vanadomolybdate solution was added to the supernatant. The volume was made up to 25 mL and incubated overnight for the development of yellow colour. The absorbance was measured by using spectrophotometer at 430 nm wavelength. Uninoculated control was also maintained. Two replications were maintained for each treatment. Using the standard curve for phosphorus, the available phosphorus was calculated in mg L^{-1}.

3.3.3. Plate assay for Potassium Solubilization by SSB Isolates

The ability of bacterial cultures to release potassium into the Aleksandrov medium were assessed as per the procedure described by Lu and Huang (2010).

Sterilized Aleksandrov medium containing potassium aluminosilicate as the sole source of potassium was poured into sterilized Petri plates to a thickness of 3 mm. After solidification of the media, the plates were spot inoculated in the center with each bacterial culture and incubated at 28°C and was assayed visually up to 7 days. The clearance zone formed around the bacterial colonies were observed and measured in mm. Two replications were maintained for each treatment.

3.3.4. Broth assay for Potassium Solubilization by SSB Isolates

The solubilization of potassium by SSB isolates were assessed by using flame photometry described by Sugumaran and Janarthanam (2007).

One mL of overnight culture of each isolate was inoculated into 50 mL of sterile Aleksandrov broth and then incubated for 12 days at 28°C. After incubation, the broth cultures were centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the cells and insoluble potassium. One mL of the culture supernatant was taken in a 25 mL volumetric flask and the volume was made to 25 mL with distilled water and mixed thoroughly. After that the solution was fed to flame photometer and potassium content was determined. Two replications were maintained for each treatment.

3.4. QUALITATIVE ASSESSMENT OF ACID PRODUCTION BY SILICATE SOLUBILIZING BACTERIAL ISOLATES

Acid production by the five superior SSB bacterial isolates selected based on the plate and broth assay of silicate in Bunt and Rovira agar medium, were tested qualitatively as per the procedure described by Naureen *et al.* (2015).

The sterilized Bunt and Rovira medium amended with 0.2 per cent bromophenol blue was poured into the sterilized Petri plates. After solidification of the media, spot inoculation of all the SSB isolates was done in the center of the agar plates with the help of a flame sterilized loop under aseptic conditions. The plates were incubated at room temperature for 24 to 72 hrs. The colour change in the media was observed. Two replications were maintained for each isolate.

3.5. EVALUATION OF ANTAGONISTIC EFFICIENCY OF THE ISOLATES AGAINST PHYTOPATHOGENS UNDER *IN VITRO* CONDITIONS

Antagonistic activity of the five superior bacterial isolates were checked against major pathogens of paddy such as *Rhizoctonia solani*, *Magnaporthe grisea*, *Helminthosporium oryzae and Xanthomonas oryzae* pv. *oryzae* using dual culture technique as described by Naureen *et al.* (2009) and Hassan *et al*. (2010). The pathogens *viz.*, *Rhizoctonia solani*, *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae* were obtained from the culture collection of Department of Agricultural Microbiology, College of Agriculture, Vellayani and *Helminthosporium oryzae* was obtained from the culture collection of Department of Plant Pathology, College of Agriculture, Vellayani. Mixed media of Potato Dextrose Agar and Bunt and Rovira medium in the proportion of 1:1 was used for testing the antagonistic activity of SSB isolates against fungal pathogens. For testing the antagonistic activity of SSB isolates against bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*, mixed medium of 1:1 proportion of Potato Sucrose Peptone Agar medium and Bunt and Rovira agar medium was used in the investigation.

3.5.1. Antagonistic Activity of SSB Isolates Against *Rhizoctonia solani, Magnaporthe grisea* and *Helminthosporium oryzae*

Dual culture technique for fungal pathogens *viz.*, *Rhizoctonia solani*, *Magnaporthe grisea* and *Helminthosporium oryzae* was done on mixed medium (1:1 proportion of Potato Dextrose Agar and Bunt and Rovira agar medium). Mycelial discs (4

mm) of fungal pathogens were placed at centre of Petri plates. Two streaks using fresh suspension of bacterial isolates were made equidistantly (3 cm apart from the center) on both sides of the mycelial disc in each plate. Plates were incubated at 30^0 C for 7 days. The size of inhibition zone was measured in mm. Four replications were maintained for each isolate.

3.5.2 Antagonistic Activity of SSB Isolates Against Bacterial Pathogen *Xanthomonas oryzae* pv. *oryzae*

Antagonistic activity against bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* was done on mixed medium (1:1 proportion of Potato Sucrose Peptone Agar medium and Bunt and Rovira agar medium). Bacterial pathogen was spread on the plate using spread plate method. Sterile disc was dipped in 7 days old liquid culture (10^7 cfu mL^{-1}) of SSB and placed at the center of the plates aseptically. Plates were incubated at 30^0 C for 7 days. After incubation, size of inhibition zone was measured in mm.

3.6. CHARACTERIZATION OF THE SSB ISOLATES

The five superior SSB isolates were characterized based on morphological and biochemical characters.

3.6.1 Morphological Characterization of SSB Isolates

The colony morphology including colony colour, shape, texture, margin, elevation and transparency on agar plates were studied. A loopful of pure culture of each bacterial isolate were streaked on Nutrient Agar medium and incubated at room temperature for 24 to 36 hrs and the single colonies obtained were analyzed.

The other morphological characters including cell shape, motility, Gram staining and endospore staining were also studied.

3.6.1.1 Cell Shape

The purified SSB cultures at log phase were observed microscopically for the cell morphological characters.

3.6.1.2 Cell Motility

The 72 h old cells were observed microscopically on cavity slide for their motility using hanging drop technique.

3.6.1.3 Gram Reaction

Gram staining was carried out as per modified Hucker's method (Rangaswami and Bagyaraj, 1993) and observed under the microscope.

3.6.1.4 Endospore Staining

The endospore staining was carried out as per the method described by Collins *et al.* (1995) and observed under the microscope.

3.6.2 Biochemical Characterization of SSB Isolates

Biochemical characterization of five superior SSB isolates was done by performing various biochemical tests by using readymade Himedia© kits KB013 (HiBacillus™ Identification kit).

The kit was opened aseptically and the sealing foil was peeled off. Each well was inoculated with 50 µL of culture suspensions of the five superior SSB isolates by surface inoculation method. It was then incubated at room temperature for 24 to 48 hrs. The results were interpreted as per the standards given in the Results Interpretation Chart of the Manufacturer.

Various biochemical tests performed were Voges Proskauer's, Citrate utilization, ONPG, Nitrate reduction, Catalase, utilization of Malonate, Arginine, Sucrose, Mannitol, Glucose, Arabinose and Trehalose.

3.6.2.1 Growth of Superior SSB Isolates in Different pH

The effect of pH on the growth of five superior silicate solubilizing bacterial isolates were tested in Luria Bertani broth. The pH of the broth was adjusted to three different levels *i.e.*, acidic (4.0), neutral (7.0) and alkaline (9.0) values using 0.1 N HCl or NaOH. Each conical flask containing 100 mL of sterile broth was inoculated with a loopful of pure SSB bacterial culture and incubated at room temperature for 48 hrs and the growth was observed visually. Two replications were maintained for each isolate.

3.6.2.2 Growth of Superior SSB Isolates at Different Temperature

The effect of temperature on the growth of five superior silicate solubilizing bacterial isolates were tested in Luria Bertani broth. Each conical flask containing 100 mL of the sterile broth was inoculated with a loopful of pure SSB bacterial culture and incubated at three different temperature *i.e.*, at low temperature (4°C), at room temperature (28°C) and at high temperature (45°C) for 48 hrs and the growth was observed visually. Two replications were maintained for each isolate.

3.6.2.3 Growth of Superior SSB Isolates in Different NaCl concentrations

The ability of five superior silicate solubilizing bacterial isolates to tolerate three different NaCl concentrations were assessed in Luria Bertani broth. The NaCl concentration of the broth was adjusted to 0.034 *M* (2 g L^{-1}), 0.17 *M* (10 g L^{-1}) and 0.85 *M* (50 g L^{-1}). Each conical flask containing 100 mL of this sterile broth was inoculated with a loopful of pure SSB bacterial culture and incubated at room temperature for 48 hrs and the growth was observed visually. Two replications were maintained for each isolate.

3.7. STATISTICAL ANALYSIS

The data obtained from the studies conducted under laboratory were subjected to analysis of variance (ANOVA) after appropriate transformations wherever needed. In the case where the effects were found to be significant, critical difference values were calculated at 5 per cent level of significance. Then the significance of treatments was compared with the critical difference values. All the data were analysed in ‘OPSTAT’ developed by CCS Haryana Agricultural University.

RESULTS

4. RESULTS

The present study on "Isolation and *in vitro* screening of silicate solubilizing bacteria from paddy rhizosphere" was conducted during 2018-2020 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. Studies were carried out to isolate and screen bacteria which are capable of solubilizing insoluble form of silicate. The results based on statistically analyzed data pertaining to the experiments conducted during the course of investigation are presented below.

4.1. ISOLATION OF SILICATE SOLUBILIZING BACTERIA

4.1.1. Isolation of Silicate Solubilizing Bacteria (SSB) Associated with Paddy

Bacteria capable of solubilizing silicates were isolated from the rhizosphere soils collected from different upland and low land paddy fields by serial dilution and plate count method using Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate (Plate1 and 2).

Twenty seven isolates of bacteria capable of solubilizing insoluble form of silicate (magnesium trisilicate) were obtained from different locations. These isolates were allotted code numbers from SSB 1 to SSB 27 as shown in Table 1.

4.2. ASSESSING THE SOLUBILIZATION POTENTIAL OF THE BACTERIAL ISOLATES UNDER *IN VITRO* CONDITIONS

All the twenty seven isolates obtained from different locations were subjected to both plate assay and broth assay in Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate for assessing the silicate solubilization potential.

Table 1. Different locations of soil sample collection

Sl. No	Isolate code No.	Place
1	SSB 1	Karamana, Thiruvananthapuram.
2	SSB 2	Karamana, Thiruvananthapuram.
3	SSB 3	Karamana, Thiruvananthapuram.
4	SSB 4	Karamana, Thiruvananthapuram.
5	SSB 5	Karamana, Thiruvananthapuram.
6	SSB 6	Karamana, Thiruvananthapuram.
7	SSB 7	Karamana, Thiruvananthapuram.
8	SSB 8	Vilakkudy, Kollam.
9	SSB 9	Vilakkudy, Kollam.
10	SSB 10	Vilakkudy, Kollam.
11	SSB 11	Vilakkudy, Kollam.
12	SSB 12	Vilakkudy, Kollam.
13	SSB 13	College of Agriculture, Vellayani
14	SSB 14	College of Agriculture, Vellayani.
15	SSB 15	College of Agriculture, Vellayani
16	SSB 16	College of Agriculture, Vellayani
17	SSB 17	College of Agriculture, Vellayani.
18	SSB 18	College of Agriculture, Vellayani
19	SSB 19	College of Agriculture, Vellayani.
20	SSB 20	College of Agriculture, Vellayani.
21	SSB 21	College of Agriculture, Vellayani.
22	SSB 22	College of Agriculture, Vellayani.
23	SSB 23	College of Agriculture, Vellayani.
24	SSB 24	Onattukara, Kayamkulam
25	SSB 25	Onattukara, Kayamkulam.
26	SSB 26	Onattukara, Kayamkulam.
27	SSB 27	Onattukara, Kayamkulam.

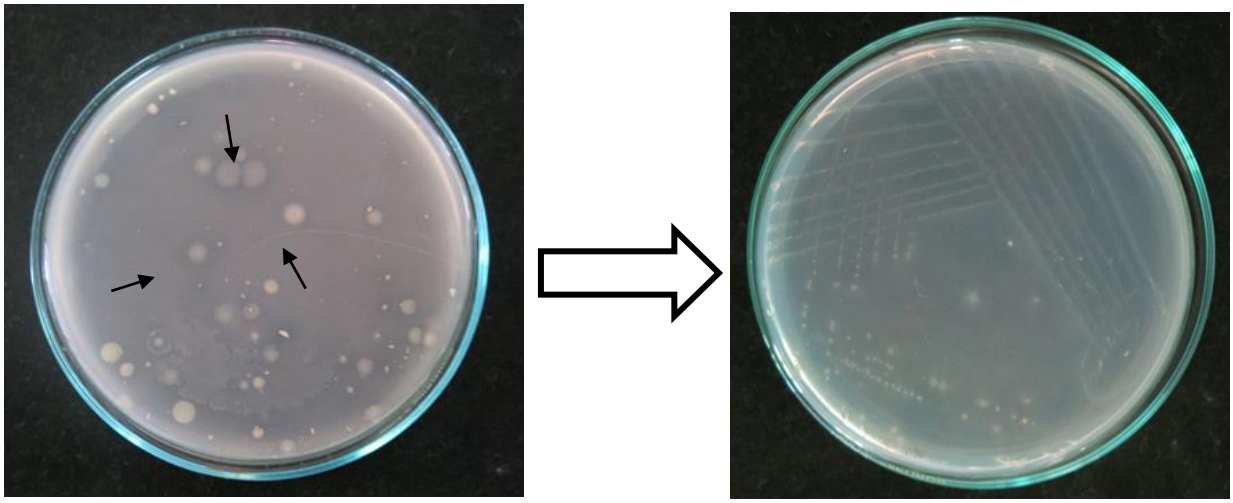


Plate 1. Silicate Solubilizing Bacterial isolates (SSB) on Bunt and Rovira medium supplemented with 0.25% magnesium trisilicate

(A) (B)

Plate 2. (A) Liquid cultures of SSB isolates (B) Maintenance of SSB isolates in Nutrient Agar slants

All the SSB isolates selected could effectively solubilize the insoluble silicate compound, magnesium trisilicate, under the assay conditions. The silicate solubilization in broth as well as clearance zone in plate was found to be high in majority of the isolates (Table 2).

4.2.1. Plate assay

The ability of bacteria to solubilize magnesium trisilicate varied with each isolate. The size of clearance zone ranged from 3 mm to 13 mm in Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate.

After spot inoculation on Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate, the maximum clearance zone of 13 mm was recorded with the isolate SSB 14 which was significantly superior to all other isolates. This was followed by SSB 18 which produced a clearance zone of 9 mm and was found significantly superior to all other isolates except SSB 14 which recorded the highest. The lowest mean value was observed with the isolate SSB 12 with a clearance zone of 3 mm which was on par with SSB 4 (3.12 mm), SSB 19 (3.12 mm), SSB 5 (3.75 mm), SSB 24 (3.75 mm), SSB 10 (4 mm), SSB 26 (4 mm) and SSB 27 (4 mm). The data are presented in Table 2.

Based on plate assay, SSB 14 (13 mm), SSB 18 (9 mm), SSB 20 (6.75), SSB 9 (6.25), SSB 3 (6 mm), SSB 11 (6 mm), SSB 13 (6 mm) and SSB 22 (6 mm) were selected as the superior SSB isolates.

4.2.2. Broth assay

The SSB isolates showed wide variation for magnesium trisilicate solubilization in the Bunt and Rovira broth supplemented with 0.25 per cent magnesium trisilicate and it ranged from 23.08 mg L^{-1} to 94.65 mg L^{-1}. The results are presented in Table 2.

Table 2. Silicate solubilization potential of SSB isolates

Sl. No.	Isolate code No.	Zone of clearance (mm)*	Solubilization (mg L^{-1}) *
1	SSB 1	5.25	41.46
2	SSB 2	5.50	41.31
3	SSB 3	6.00	73.25
4	SSB 4	3.12	56.07
5	SSB 5	3.75	55.63
6	SSB 6	4.50	37.09
7	SSB 7	5.75	33.12
8	SSB 8	5.25	36.32
9	SSB 9	6.25	66.40
10	SSB 10	4.00	43.31
11	SSB 11	6.00	39.27
12	SSB 12	3.00	55.40
13	SSB 13	6.00	39.18
14	SSB 14	13.00	84.28
15	SSB 15	5.00	41.68
16	SSB 16	5.00	24.99
17	SSB 17	4.50	23.08
18	SSB 18	9.00	77.39
19	SSB 19	3.12	59.77
20	SSB 20	6.75	94.65
21	SSB 21	5.00	40.93
22	SSB 22	6.00	70.39
23	SSB 23	5.25	30.54
24	SSB 24	3.75	58.22
25	SSB 25	4.50	57.42
26	SSB 26	4.00	25.05
27	SSB 27	4.00	47.05
	CD (0.05)	1.067	1.667
	Sem (±)	0.38	0.57

*Mean of 2 replications

Among the isolates, SSB 20 showed the highest silicate solubilization of 94.65 mg L^{-1} which was significantly superior to all other isolates. This was followed by SSB 14, SSB 18, SSB 3 and SSB 22 with a solubilization of 84.28 mg L^{-1}, 77.39 mg L^{-1}, 73.25 mg L^{-1} and 70.39 mg L^{-1} respectively. The lowest solubilization in the broth was observed in case of SSB 17 which was 23.08 mg L^{-1}. Based on broth assay, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 were selected as the superior isolates.

Based on plate as well as broth assays of all the twenty seven bacterial isolates obtained from the paddy rhizosphere of different locations, five isolates, *viz.*, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 which showed the maximum clearance zone in plate and silicate solubilization in the broth were selected as the superior SSB isolates (Plate 3).

4.3. ASSESSING THE PHOSPHATE AND POTASH SOLUBILIZATION POTENTIAL OF THE BACTERIAL ISOLATES UNDER *IN VITRO* CONDITIONS

All the isolates obtained were subjected to plate assay and broth assay for phosphate solubilization in Pikovskaya's medium and potassium solubilization in Aleksandrov medium.

The ability to solubilize the insoluble phosphate compound, tricalcium phosphate and insoluble potassium compound, potassium aluminosilicate varied with each bacterial isolate. Among all the isolates tested, some could effectively solubilize these insoluble compounds under the assay conditions and data are given in Table 3 and 4.

4.3.1. Plate assay for Phosphate Solubilization by SSB Isolates

Among the twenty seven SSB isolates obtained, fourteen isolates showed phosphate solubilization. The clearance zone in plates ranged from 0.87 mm to 5.5 mm in Pikovskaya's medium. The results are presented in Table 3.

After spot inoculation for phosphate solubilization in Pikovskaya's medium, maximum clearance zone of 5.5 mm was recorded with the isolate SSB 22 which was on par with SSB 23 with 5 mm clearance zone (Plate 4). This was followed by SSB 18 with a clearance zone of 4.25 mm which was significantly superior to all other isolates except SSB 22 and SSB 23. The lowest clearance zone was observed with the isolate SSB 20 (0.87 mm) which was on par with SSB 4 (1.25 mm) and SSB 10 (1.25 mm).

4.3.2. Broth assay for Phosphate Solubilization by SSB Isolates

All the isolates showed phosphate solubilization in Pikovskaya's broth and it ranged from 0.68 mg L^{-1} to 41.92 mg L^{-1}. Among them, SSB 22 showed the highest solubilization of 41.92 mg L^{-1} which was significantly superior to all other isolates. This was followed by SSB 23 with 34.10 mg L^{-1} solubilization. The lowest solubilization in the broth was observed with the isolate SSB 5 (0.68 mg L^{-1}) which was on par with SSB 25 (0.75 mg L^{-1}). The results are presented in Table 3.

4.3.3. Plate assay for Potassium Solubilization by SSB Isolates

Among the twenty seven SSB isolates obtained, twelve isolates showed potassium solubilization. The clearance zone in plates ranged from 2.25 mm to 5.5 mm in Aleksandrov medium. The data are shown in Table 4.

On spot inoculation of all the isolates for potassium solubilization on Aleksandrov medium, maximum clearance zone of 5.5 mm was recorded with the isolate SSB 8 which was significantly superior to all other isolates (Plate 5). This was followed by SSB 16 (4.25 mm) and SSB 21 (4.25 mm) which was found to be on par with SSB 18 (4 mm), SSB 12 (3.5 mm) and SSB 13 (3.5 mm). The lowest clearance zone was recorded with the isolate SSB 22 with a clearance zone of 2.25 mm which was on par with SSB 7 (2.75 mm), SSB 1 (2.87 mm), SSB 6 (2.87 mm) and SSB 2 (3 mm).

Table 3. Phosphate solubilization potential of SSB isolates

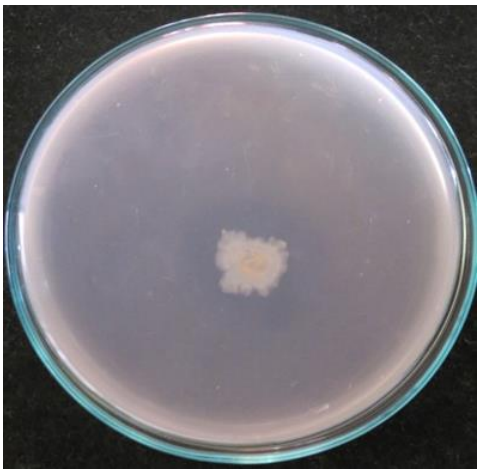
Sl. No.	Isolate code No.	Zone of clearance (mm)*	Solubilization (mg L^{-1}) *
1	SSB 1	0.00	3.93
2	SSB 2	1.62	12.92
3	SSB 3	2.00	32.04
4	SSB 4	1.25	14.37
5	SSB 5	0.00	0.68
6	SSB 6	3.37	14.76
7	SSB 7	1.50	14.14
8	SSB 8	1.62	13.50
9	SSB 9	0.00	5.88
10	SSB 10	1.25	13.01
11	SSB 11	0.00	11.53
12	SSB 12	0.00	10.97
13	SSB 13	2.50	23.33
14	SSB 14	0.00	13.55
15	SSB 15	0.00	2.57
16	SSB 16	2.37	16.94
17	SSB 17	0.00	9.73
18	SSB 18	4.25	14.26
19	SSB 19	0.00	2.60
20	SSB 20	0.87	14.71
21	SSB 21	1.75	19.00
22	SSB 22	5.50	41.92
23	SSB 23	5.00	34.10
24	SSB 24	0.00	7.50
25	SSB 25	0.00	0.75
26	SSB 26	0.00	7.54
27	SSB 27	0.00	2.91
	CD (0.05)	0.623	1.475
	Sem (±)	0.21	0.50

*Mean of 2 replications

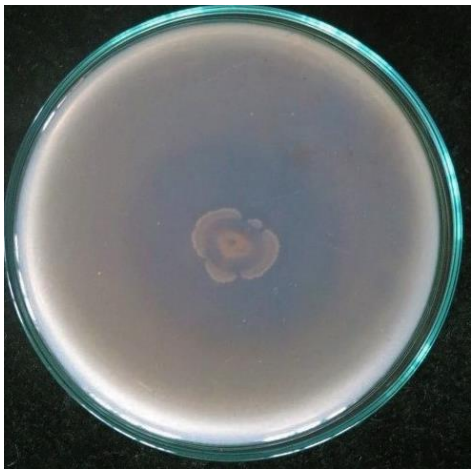
Table 4. Potassium solubilization potential of SSB isolates

Sl. No.	Isolate code No.	Zone of clearance (mm)*	Solubilization (mg L^{-1}) *
1	SSB 1	2.87	37.50
2	SSB 2	3.00	37.50
3	SSB 6	2.87	12.50
4	SSB 7	2.75	37.50
5	SSB 8	5.50	37.50
6	SSB 12	3.50	12.50
7	SSB 13	3.50	37.50
8	SSB 16	4.25	12.50
9	SSB 18	4.00	37.50
10	SSB 20	3.50	12.50
11	SSB 21	4.25	37.50
12	SSB 22	2.25	37.50
	CD (0.05)	0.861	23.275
	Sem (±)	0.29	7.98

*Mean of 2 replications



SSB 3



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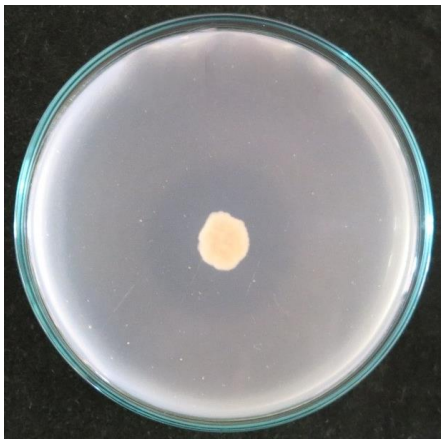
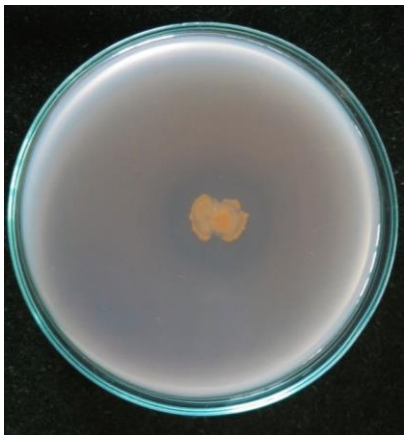


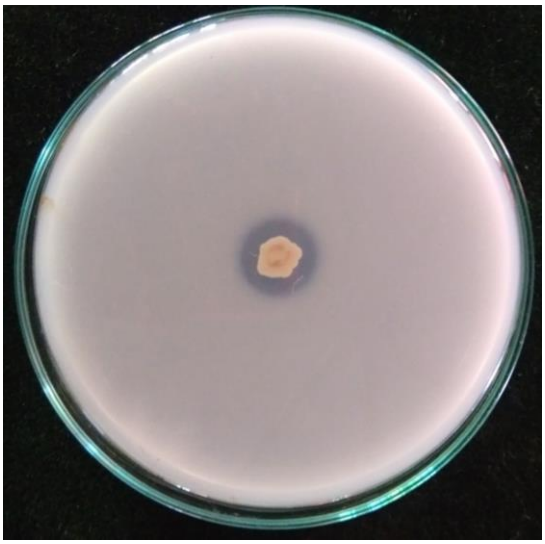
SSB 18

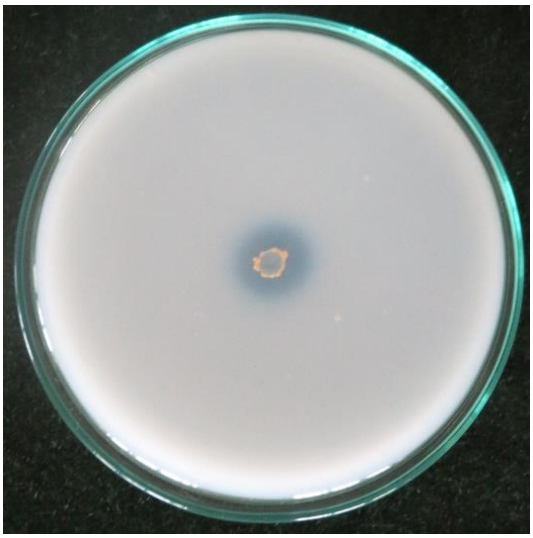


SSB 20

SSB 22

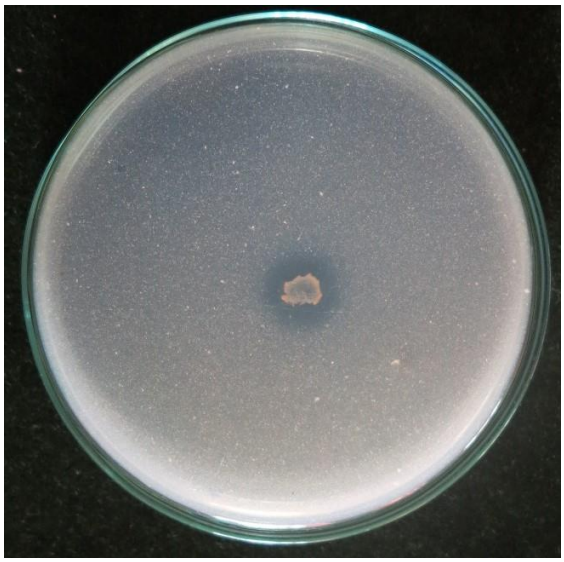
Plate 3. Superior SSB isolates on Bunt and Rovira medium supplemented with 0.25% magnesium trisilicate





SSB 22 SSB 23

Plate 4. Phosphate solubilization of SSB on Pikovskaya's agar medium



SSB 8

Plate 5. Potassium solubilization of SSB on Aleksandrov agar medium

4.3.4. Broth assay for Potassium Solubilization by SSB Isolates

Among the twenty seven isolates, twelve isolates showed potassium solubilization in Aleksandrov broth. The highest potassium solubilization was shown by SSB 1, SSB 2, SSB 7, SSB 8, SSB 13, SSB 18, SSB 21 and SSB 22 with a solubilization of 37.5 mg L^{-1} which were found to be statistically on par. This was followed by SSB 6, SSB12, SSB 16 and SSB 20 with a solubilization of 12.5 mg L^{-1} which was also found to be statistically on par. The isolates SSB 3, SSB 4, SSB 5, SSB 9, SSB 10, SSB 11, SSB 14, SSB 15, SSB 17, SSB 19, SSB 23, SSB 24, SSB 25, SSB 26 and SSB 27 did not show any potassium solubilization in the medium.

4.4. QUALITATIVE ASSESSMENT OF ACID PRODUCTION BY SILICATE SOLUBILIZING BACTERIAL ISOLATES.

Acid production by the five superior silicate solubilizing bacterial isolates SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 was detected as blue media turned yellow around the bacterial colonies. All the five isolates tested showed positive results for the acid production (Plate 6). The yellow zone indicated the ability of these isolates to produce organic acids in the Bunt and Rovira medium amended with 0.2 per cent bromophenol blue. The results are presented in Table 5.

4.5. EVALUATION OF ANTAGONISTIC EFFICIENCY OF THE ISOLATES AGAINST PHYTOPATHOGENS UNDER *IN VITRO* CONDITIONS.

The antagonistic activity of the five superior silicate solubilizing bacterial isolates SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 were assessed against the major fungal pathogens of paddy *viz., Rhizoctonia solani*, *Magnaporthe grisea*, *Helminthosporium oryzae* and one bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* following dual culture method and the results are furnished below.

4.5.1. Antagonistic Activity of SSB Isolates Against *Rhizoctonia solani*

Three out of five isolates tested, inhibited *Rhizoctonia solani*. SSB 18 exhibited the maximum zone of inhibition (ZOI) of 9.65 mm which was significantly superior to all other isolates. It was followed by the isolates SSB 22 and SSB 20 with a zone of inhibition of 5.27 mm and 3.65 mm respectively. The other isolates SSB 3 and SSB 14 did not show any inhibition against *Rhizoctonia solani* (Table 6) (Plate 7).

4.5.2. Antagonistic Activity of SSB Isolates Against *Magnaporthe grisea*

Out of five isolates tested, three isolates showed antagonistic activity against the rice blast disease causing pathogen *Magnaporthe grisea* (Table 7) (Plate 8). Among them, SSB 18 showed maximum ZOI of 14.45 mm which was significantly superior to all other isolates. It was followed by the isolate SSB 22 and SSB 3 with a zone of inhibition of 10.65 mm and 3.45 mm respectively. All other isolates, SSB 14 and SSB 20 did not show any inhibition to *Magnaporthe grisea.*

4.5.3. Antagonistic Activity of SSB Isolates Against *Helminthosporium oryzae*

Four out of the five isolates tested showed inhibition against *Helminthosporium oryzae,* the fungal pathogen causing brown spot disease in rice. SSB 18 showed maximum antagonistic activity with the maximum ZOI of 10.80 mm which was significantly superior to all other isolates. It was followed by the isolate SSB 22 with a ZOI of 8.47 mm which was found to be on par with SSB 20 (7.37 mm). SSB 14 did not show any inhibition against *Helminthosporium oryzae* (Table 8) (Plate 9).

4.5.4. Antagonistic Activity of SSB Isolates Against *Xanthomonas oryzae* pv. *oryzae*

Among the five isolates tested, three isolates showed inhibition to the bacterial pathogen of rice *Xanthomonas oryzae* pv. *oryzae.* SSB 18 exhibited maximum antagonistic activity with a clearance zone of 11.50 mm which was significantly superior to all other isolates (Table 9) (Plate 10). It was followed by the isolates SSB 20 with a

Table 5. Qualitative assessment of acid production by SSB isolates

Sl. No.	Isolate code No.	Acid production
1	SSB 3	+
2	SSB 14	+
3	SSB 18	+
4	SSB 20	+
5	SSB 22	+

Table 6. Antagonistic activity of SSB isolates against *Rhizoctonia solani*

Sl. No.	Isolate code No.	ZOI (mm)*
1	SSB 3	0.00[d]
2	SSB 14	0.00[d]
3	SSB 18	9.65[a]
4	SSB 20	3.65[c]
5	SSB 22	5.27[b]
	CD (0.05)	0.683
	Sem (±)	0.22

*Mean of 4 replications

Table 7. Antagonistic activity of SSB isolates against *Magnaporthe grisea*

Sl. No.	Isolate code No.	ZOI (mm)*
1	SSB 3	3.45[c]
2	SSB 14	0.00[d]
3	SSB 18	14.45[a]
4	SSB 20	0.00[d]
5	SSB 22	10.65[b]
	CD (0.05)	0.732
	Sem (±)	0.24

*Mean of 4 replications

Table 8. Antagonistic activity of SSB isolates against *Helminthosporium oryzae*

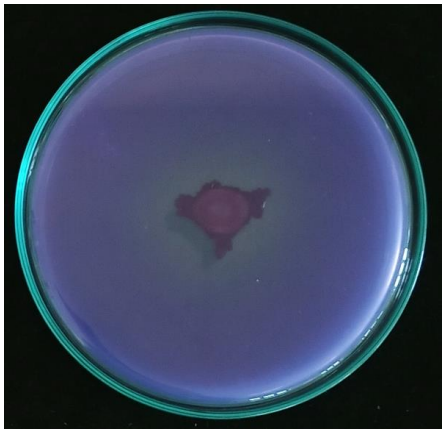
Sl. No.	Isolate code No.	ZOI (mm)*
1	SSB 3	5.00[c]
2	SSB 14	0.00[d]
3	SSB 18	10.80[a]
4	SSB 20	7.37[b]
5	SSB 22	8.47[b]
	CD (0.05)	1.337
	SEm (±)	0.44

*Mean of 4 replications

Table 9. Antagonistic activity of SSB isolates against *Xanthomonas oryzae* pv. *oryzae*

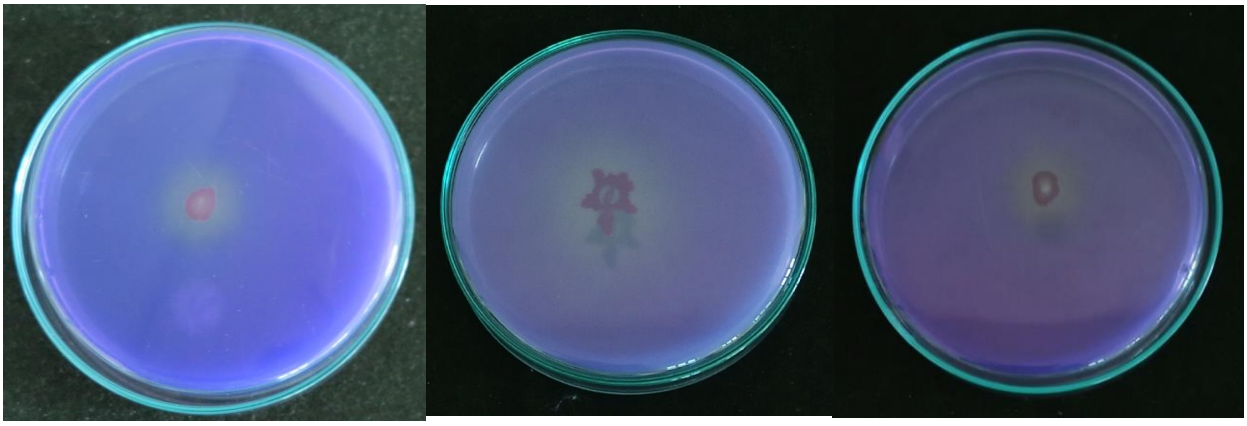
Sl. No.	Isolate code No.	ZOI (mm)*
1	SSB 3	0.00[c]
2	SSB 14	0.00[c]
3	SSB 18	11.50[a]
4	SSB 20	10.50[b]
5	SSB 22	10.00[b]
	CD (0.05)	0.555
	SEm (±)	0.18

*Mean of 4 replications



SSB 3

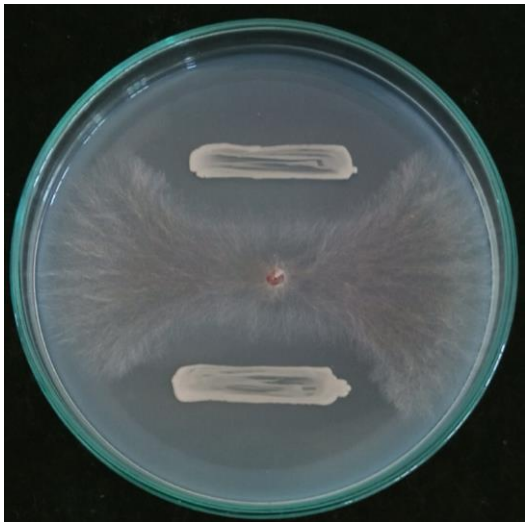
SSB 14



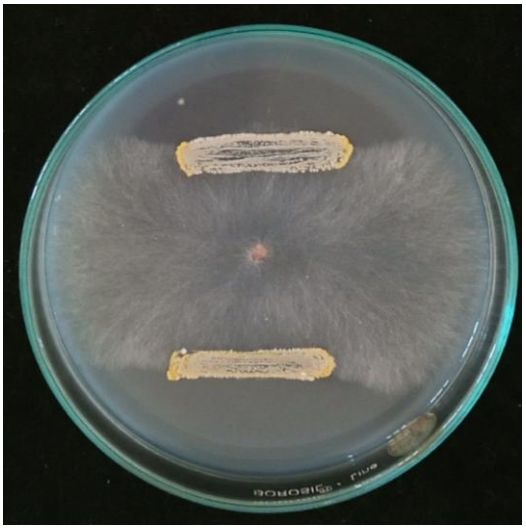
SSB 18 SSB 20 SSB 22

Plate 6. Acid production by superior SSB isolates on Bunt and Rovira medium supplemented with 0.2 % bromophenol blue

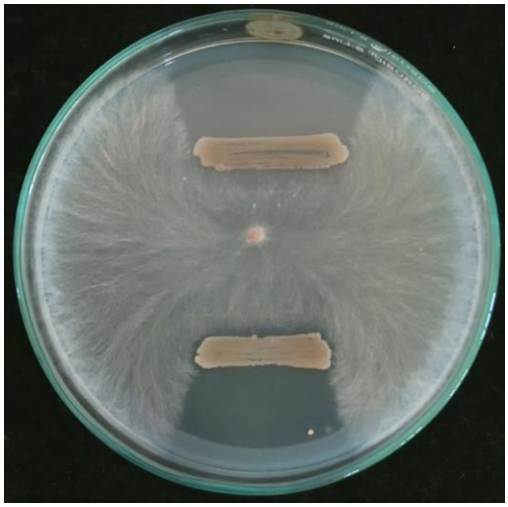
Control



SSB 18

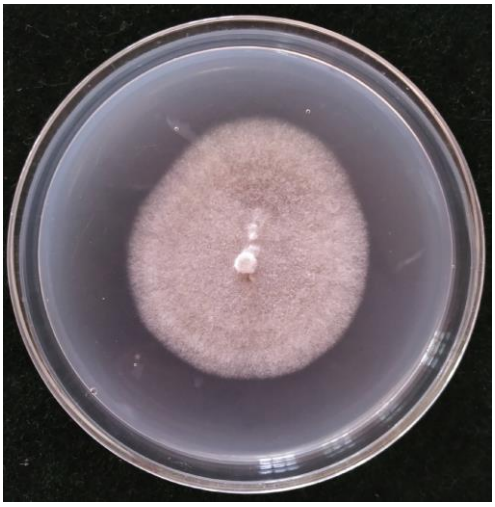


SSB 20



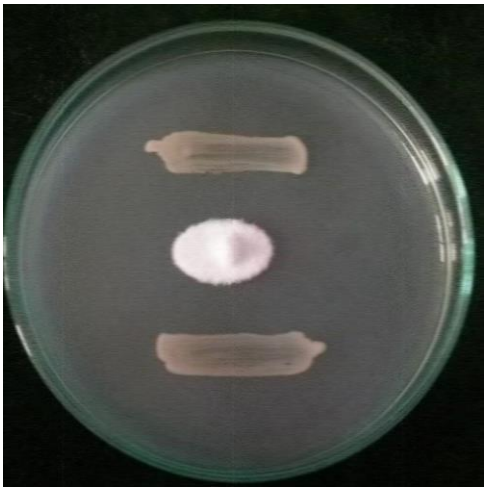
SSB 22

Plate 7. Antagonistic activity of SSB isolates against *Rhizoctonia solani*

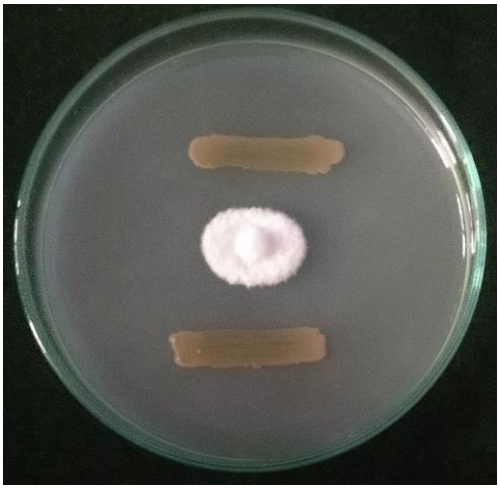


Control

SSB 3

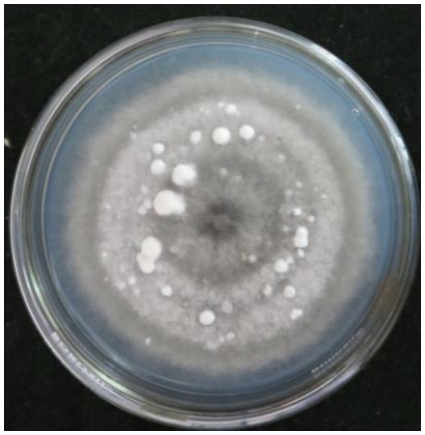


SSB 18



SSB 22

Plate 8. Antagonistic activity of SSB isolates against *Magnaporthe grisea*



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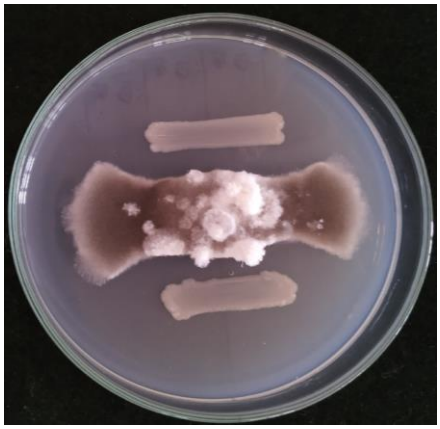

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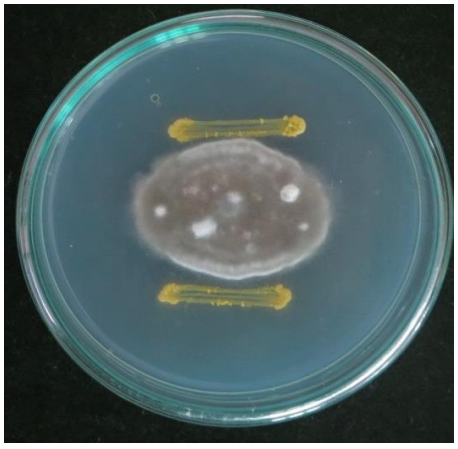
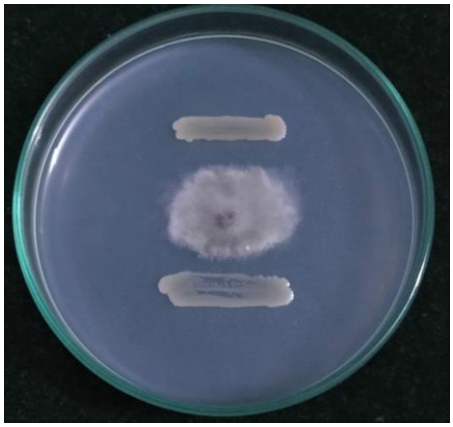
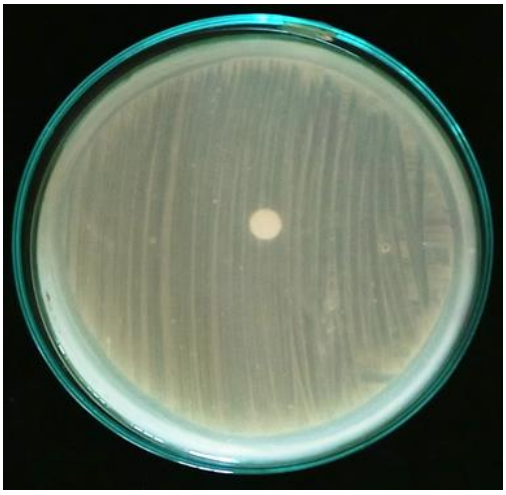


SSB 20



SSB 22

Plate 9. Antagonistic activity of SSB isolates against *Helminthosporium oryzae*



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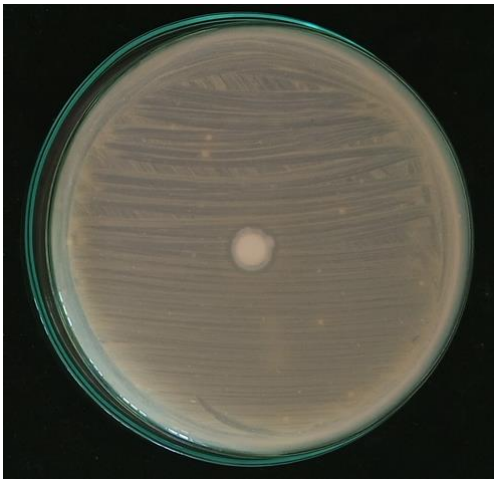


SSB 18

SSB 20

SSB 22

Plate 10. Antagonistic activity of SSB isolates against *Xanthomonas oryzae* pv. *oryzae*

clearance zone of 10.50 mm which was found to be on par with SSB 22 (10 mm). All other isolates SSB 3 and SSB 14 did not show any inhibition against *Xanthomonas oryzae* pv. *oryzae.*

4.6. CHARACTERIZATION OF THE ISOLATES.

The five superior isolates SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 were characterized based on morphological and biochemical characters.

4.6.1. Morphological Characterization of Superior SSB Isolates

The five superior isolates were subjected to morphological characterization comprising colony colour, shape, texture, margin, elevation and transparency.

Out of the five superior isolates, SSB 20 and SSB 22 exhibited light yellow and yellowish green coloured opaque colonies, SSB 14 exhibited transparent off-white colonies and SSB 3 and SSB 18 showed creamy mucoid colonies on Nutrient Agar medium (Plate 11). Details of the colony morphology of these isolates are presented in Table 10.

The results of cell characteristics revealed that all the five superior isolates were rod shaped and stained Gram positive. Out of the five superior isolates tested, three were found motile, while the other two non-motile and all of them were endospore formers. The results are presented in Table 11.

4.6.2. Biochemical Characterization of Superior SSB Isolates

For further characterization, the five superior isolates were subjected to a series of biochemical tests. All the isolates were found to be negative for Voges-Proskauer's test and positive for citrate, ONPG, nitrate reduction and glucose. The data are shown in Table 12 (Plate 12).

4.6.2.1 Growth of Superior SSB Isolates in Different pH

The effect of pH on the growth of five superior silicate solubilizing bacterial isolates were tested and the results are presented in Table 13 (Plate 13). The results revealed that they are unable to grow in low pH value (4.0), while they showed vigorous growth in neutral pH (7.0). SSB 3 and SSB 18 showed moderate growth, while SSB 14 and SSB 20 showed vigorous growth and SSB 22 did not show any growth in high pH value (9.0).

4.6.2.2 Growth of Superior SSB Isolates at Different Temperature

The effect of temperature on the growth of five superior bacterial isolates were tested, and the results are presented in Table 14 (Plate 14). The results have shown that none of the isolates were able to grow at low temperature (4°C), while they exhibited vigorous growth at room temperature (28°C). SSB 3 and SSB 22 showed moderate growth at high temperature (45°C), while SSB 14, SSB 18 and SSB 20 exhibited vigorous growth.

4.6.2.3 Growth of Superior SSB Isolates in Different NaCl concentrations

The ability of bacterial isolates to tolerate different NaCl concentrations were assessed and the results are shown in Table 15 (Plate 15). The results revealed that three out of the five superior isolates tested, SSB 3, SSB 14 and SSB 18 showed vigorous growth in 0.034 *M* (2 g L^{-1}) NaCl concentration, while SSB 20 and SSB 22 showed moderate growth. One out of five isolates tested, SSB 3 exhibited vigorous growth in 0.17 *M* (10 g L^{-1}) NaCl concentration, while SSB 14, SSB 18 and SSB 20 showed moderate growth and SSB 22 did not show any growth. SSB 3 and SSB 20 showed moderate growth in 0.85 *M* (50 g L^{-1}) NaCl concentration, while SSB 14, SSB 18 and SSB 22 did not show any growth.

Table 10. Colony characteristics of superior SSB isolates

Sl. No.	Isolate code No.	Colour	Shape	Margin	Elevation	Texture	Optic
1	SSB 3	Creamy	Irregular and spreading	Irregular	Flat	Smooth ang glossy	Opaque
2	SSB 14	Off-white	Irregular and spreading	Irregular	Flat	Smooth ang glossy	Transparent
3	SSB 18	White Gummy	Round	Entire	Raised	Smooth	Opaque
4	SSB 20	Light yellow	Round	Entire	Raised	Smooth	Opaque
5	SSB 22	Yellowish green	Round	Entire	Flat	Smooth ang glossy	Opaque

Table 11. Morphological characterization of superior SSB isolates

Sl. No.	Isolate code No.	Cell shape	Motility	Gram reaction	Endospore formation
1	SSB 3	Rod	Motile	Positive	Endospore formers
2	SSB 14	Rod	Motile	Positive	Endospore formers
3	SSB 18	Rod	Non motile	Positive	Endospore formers
4	SSB 20	Rod	Non motile	Positive	Endospore formers
5	SSB 22	Rod	Motile	Positive	Endospore formers

Table 12. Biochemical characterization of superior SSB isolates

Sl. No	Biochemical Tests	SSB 3	SSB 14	SSB 18	SSB 20	SSB 22
1	Malonate utilization	+	+	+	-	+
2	Voges Proskauer's	-	-	-	-	-
3	Citrate utilization	+	+	+	+	+
4	ONPG	+	+	+	+	+
5	Nitrate reduction	+	+	+	+	+
6	Catalase	+	+	-	+	+
7	Arginine	-	+	+	-	+
8	Sucrose	-	-	+	-	+
9	Mannitol	-	+	+	+	+
10	Glucose	+	+	+	+	+
11	Arabinose	-	-	-	+	+
12	Trehalose	-	-	-	+	+

Table 13. Growth of superior SSB isolates in different pH

Sl. No.	Isolate code No.	4.0	7.0	9.0
1	SSB 3	-	++	+
2	SSB 14	-	++	++
3	SSB 18	-	++	+
4	SSB 20	-	++	++
5	SSB 22	-	++	-

(-) No growth, (+) Moderate growth, (++) Vigorous growth

Table 14. Growth of superior SSB isolates in different temperature

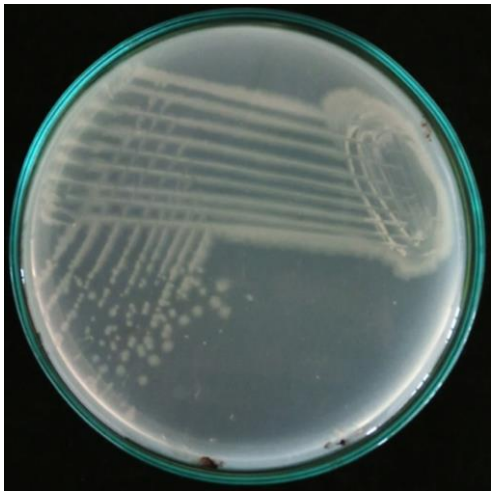
Sl. No.	Isolate code No.	4°C	28°C	45°C
1	SSB 3	-	++	+
2	SSB 14	-	++	++
3	SSB 18	-	++	++
4	SSB 20	-	++	++
5	SSB 22	-	++	+

(-) No growth, (+) Moderate growth, (++) Vigorous growth

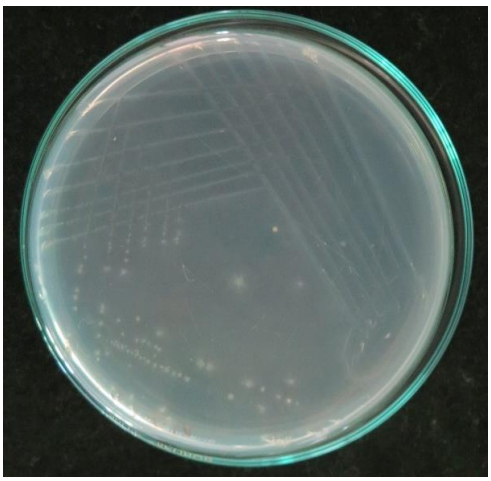
Table 15. Growth of superior SSB isolates in different concentrations of NaCl

Sl. No.	Isolate code No.	0.034 *M* (2 g L^{-1})	0.17 *M* (10 g L^{-1})	0.85 *M* (50 g L^{-1})
1	SSB 3	++	++	+
2	SSB 14	++	+	-
3	SSB 18	++	+	-
4	SSB 20	+	+	+
5	SSB 22	+	-	-

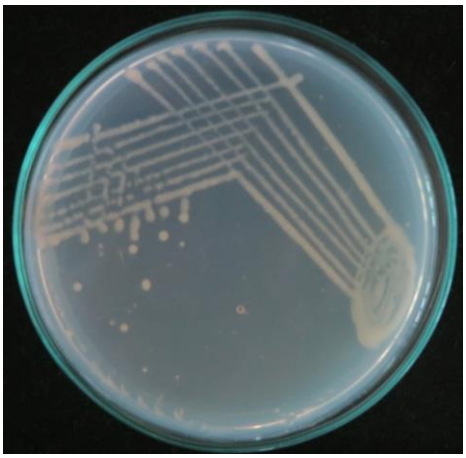
(-) No growth, (+) Moderate growth, (++) Vigorous growth



SSB 3

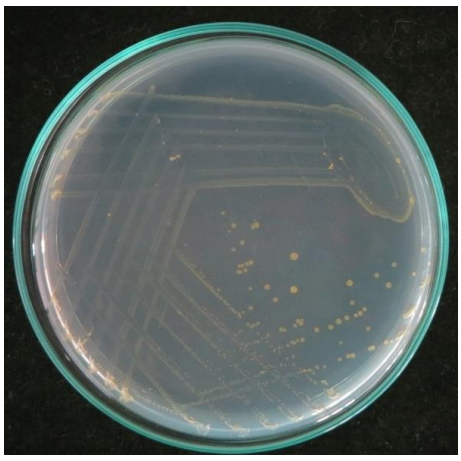


SSB 14



SSB 18

SSB 20



SSB 22

Plate 11. Colony characteristics of superior SSB isolates on Nutrient Agar medium

Plate 12. Biochemical characterization of superior SSB isolates

pH 4.0

pH 7.0

pH 9.0

Plate 13. Growth of superior SSB isolates in different pH

Temperature - 4°C

Temperature - 28°C

Temperature - 45°C

Plate 14. Growth of superior SSB isolates in different temperature

NaCl concentration - 0.034 *M*

NaCl concentration - 0.17 *M*

NaCl concentration - 0.85 *M*

Plate 15. Growth of superior SSB isolates in different concentrations of NaCl

DISCUSSION

5. DISCUSSION

Intensive crop cultivation practices have led to the depletion of plant available silicon in the soil which has become one of the possible limiting factors that contribute to yield decline in several crops. Even though silicon is present abundantly on the earth's crust, it is unavailable to plants. So, their increasing demands for plants are met by external fertilizers application. Inorganic materials such as quartz, clay, mica and feldspars are rich in silicon but acts as poor silicon fertilizer because of the low solubility of the silicon. However, the indiscriminate use of chemical fertilizers has several negative effects like environmental pollution, destruction of soil microorganisms, soil nutrient balance and often leads to poor soil quality. A suitable biological substitute that helps to solubilize insoluble silicate and thereby enhance plant growth without affecting the environment has become essential. The importance of silicate solubilizing bacteria in solubilizing insoluble silicate minerals and thereby promoting plant growth are gaining attention nowadays.

Silicon benefits many plants, especially rice which is the major food grain and staple food for majority of the world's population, in several ways by accelerating the growth, mitigating abiotic stresses like drought, salinity, heavy metal toxicity and biotic stresses like incidence of pests and diseases. Hence the present investigation envisaged to isolate and screen the best silicate solubilizing bacteria from paddy rhizosphere.

In the present study, twenty seven bacterial isolates capable of solubilizing insoluble form of silicate were obtained during the initial isolation process from the rhizosphere soils of different upland and lowland paddy fields. Many reports have shown the association of silicate solubilizing bacteria with various crops, *viz.*, rice, wheat and sugarcane, as these crops are high silicate accumulators (Muralikannan and Anthoniraj, 1998; Brindavathy *et al.*, 2012; Naureen *et al.*, 2015; Kang *et al.*, 2017).

In rice ecosystem, the positive role of *Bacillus* as silicate solubilizing bacterium has been reported by many workers (Norkina and Pumpyanskaya, 1956; Duff and Webley, 1959; Webly *et al.,* 1960). Kang *et al.* (2017) isolated and characterized a novel silicate solubilizing bacterial strain *Burkholderia eburnea* CS4-2 that promotes growth of japonica rice. Lee *et al.* (2019) also isolated and characterized a high silicate and phosphate solubilizing novel strain *Enterobacter ludwigii* GAK2 that promoted growth of rice plants.

Bunt and Rovira medium was used to isolate silicate solubilizing bacteria as it enabled detection of solubilization of the insoluble silicate compound, magnesium trisilicate, through the formation of a clearance zone around the bacterial colonies on agar plates. Sulizah *et al.* (2018) obtained five bacterial isolates from paddy rhizosphere soil which could solubilize silicate in Bunt and Rovira agar and broth.

All the twenty seven isolates obtained in the isolation process were subjected to plate assay and broth assay for magnesium trisilicate solubilization in Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate under *in vitro* conditions. After spot inoculation in the center of agar plates, clearance zone was noticed which ranged from 3 mm to 13 mm. Earlier studies conducted by Naureen *et al.* (2015) with silicate bacterial strains isolated from different locations also showed similar results with solubilization zone up to 54 mm in plate assay. Related results were also retrieved in studies conducted by Vijayapriya *et al.* (2019) with *Bacillus mucilaginosus* strains. They obtained twenty silicate solubilizing bacterial isolates from lowland paddy rhizosphere soils, of which three isolates namely SSB 8, SSB 11 and SSB 17 were more efficient in silicate solubilization and recorded clearance zone of about 15 mm and thirteen SSB isolates showed medium silicate solubilizing efficiency of 10 to 14.99 mm clearance zone and remaining four isolates recorded below 10 mm clearance zones. Similar results were also obtained by Vasanthi *et al.* (2016) who isolated silicate solubilizing bacteria from paddy soils, sugarcane fields, pond sediments and sea water which showed good growth

and solubilization zone in magnesium trisilicate and the zone of clearance ranged from 5 mm to 15 mm. Among them *Bacillus flexus*, *Bacillus megaterium* and *Pseudomonas fluorescens* showed larger clearing zone than others.

However, in the present study the isolates showed wide variations for magnesium trisilicate solubilization in culture broth ranging from 23.08 mg L^{-1} to 94.65 mg L^{-1}. The results are also in accordance with the findings of Janardhan (2014) with a solubilization that ranged from 14.39 mg L^{-1} to 76.31 mg L^{-1} in silicate broth.

The superior isolates which showed maximum clearance zone on Bunt and Rovira agar plates produced maximum silicate solubilization in broth when compared to others. This result indicated that there is a correlation between silicate solubilizing activity in broth and solubilization zone on Bunt and Rovira agar medium as observed by Vasanthi *et al.* (2013).

In the present investigation, five superior SSB isolates, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 were selected based on plate and broth assay which showed maximum clearance zone in agar plates and silicate solubilization in broth (Fig. 1 and Fig. 2).

The silicate minerals dissolution mechanism by microbes was studied by Hiebert and Bennett (1992) which showed that surface adhering bacteria created a reaction zone in their immediate vicinity in which organic acids, produced within the cell and released out extracellularly were concentrated. This resulted in a chemical potential gradient between cell surface and the surrounding bulk fluid. High concentrations of these complex organic acids chelated SiO_2 at the mineral surface and dissolved the mineral even though the bulk pore water was supersaturated with respect to the dissolving mineral. Silica, thus chelated and in solution, is available for transport away from the dissolution site along the ground water flow path. Their examination of mineral surfaces from the microcosms also showed the evidence of biological colonization and chemical

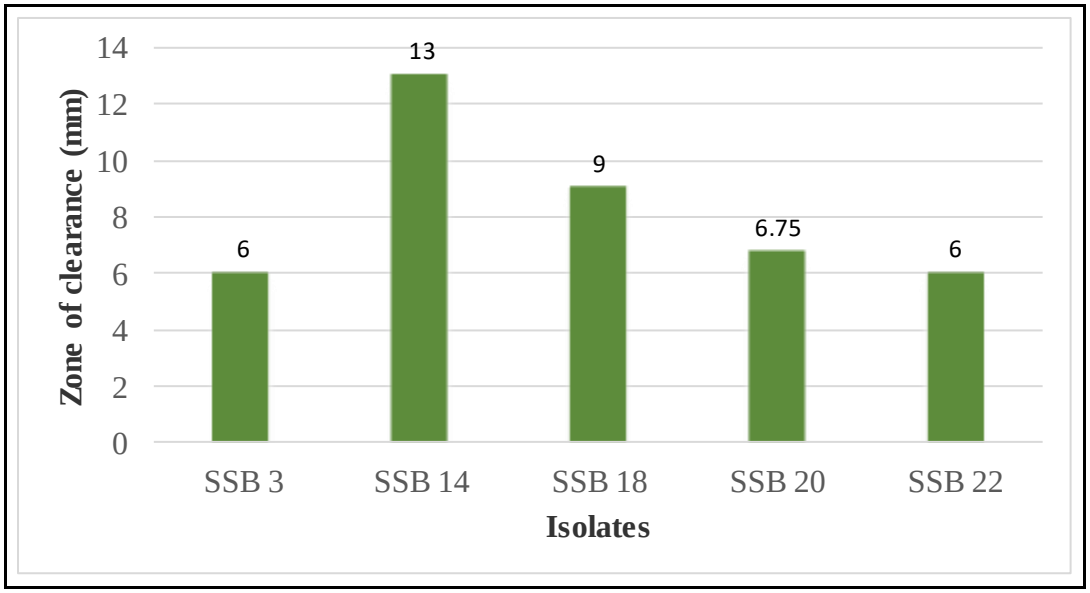


Fig. 1. Silicate solubilization potential of five superior SSB isolates on Bunt and Rovira agar plate assay

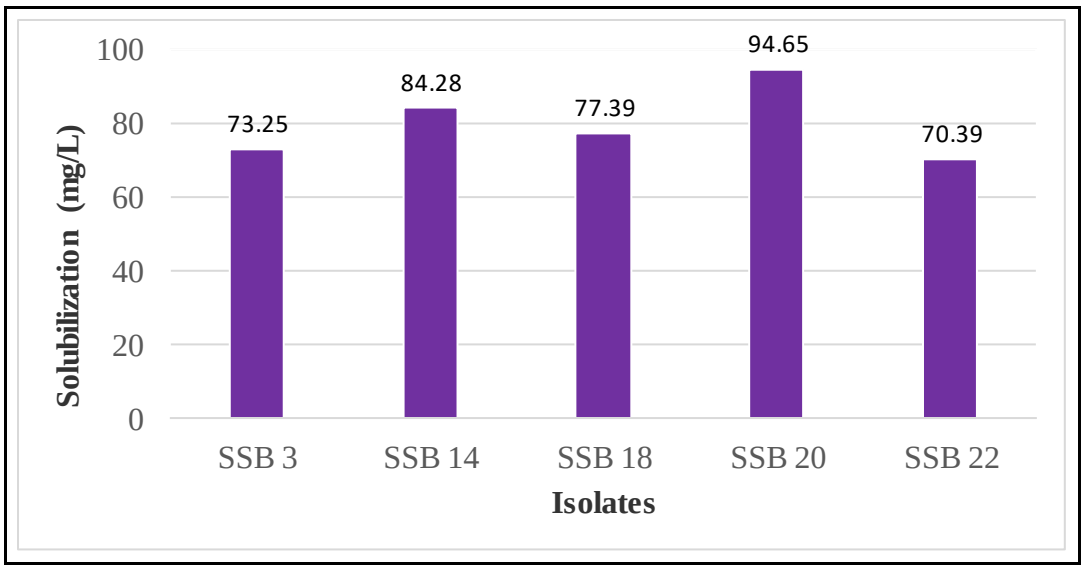


Fig. 2. Silicate solubilization potential of five superior SSB isolates in Bunt and Rovira broth assay

alteration. They observed the bacterial cells in a variety of morphologies which colonized the mineral surfaces, primarily as individual cells and small patches.

The results of Malinovskaya *et al.* (1990) showed that mixtures of microbial extracellular polymers and low molecular weight ligands had a synergistic effect on silicate mineral weathering. Experimental weathering studies conducted by Welch and van devivere, (1994) detected that microbial extracellular polymers can react chemically with mineral surfaces and mineral ions, increasing dissolution rate up to several orders of magnitude.

Barker *et al.* (1998) also reported that silicate dissolution was due to the production of excess proton, organic ligands, hydroxyl anion, extra cellular polysaccharides (EPS) and enzymes. Their dissolution experiments demonstrated the microbial colonization of surfaces, production of organic and inorganic acids and extracellular polymers which greatly accelerates mineral weathering reactions and releases up to two orders of magnitude more material into solution than abiotic controls. But, the magnitude of these enhancement effects in natural environments and the mechanisms involved are still poorly understood. It is difficult to extrapolate *in vitro* results to natural conditions where nutrient concentrations, rates of microbial metabolism, microbial community structure, and mineral weathering rates vary widely.

In addition to magnesium trisilicate solubilization, several studies also proved the phosphorus and potassium solubilization potential of the SSB isolates. This can be directly due to solubilization of other minerals by silicate solubilizing bacteria or indirectly due to solubilized silicon. It has been previously reported that the solubilized Si improves availability of phosphorus to plants by competing with phosphorus fixation sites in soil and silicon acts as a substitute for phosphorus in plant system (Muralikannan and Anthoniraj, 1998). Sahebi *et al.* (2015) also reported that silicon increases the availability of phosphorus indirectly by decreasing the availability of iron and manganese in plants.

The experiment conducted by Grudev (1987) indicated that potassium can be released from silicate minerals and he proposed that the formation of mucilaginous capsules consisting of exopolysaccharides by the bacteria enhanced mineral dissolution. Naureen *et al.* (2015) proposed that potassium is mostly present in soil in the form of silicates hence silicate solubilizing bacteria can play an efficient role here by liberating soluble potassium in to soil. Hence, the present investigation also monitored the qualitative and quantitative estimation of phosphorus and potassium by the SSB isolates under *in vitro* conditions.

All the isolates obtained were subjected to plate assay and broth assay for phosphorous and potassium solubilization. Fourteen SSB isolates were found to solubilize the insoluble phosphorus compound, tricalcium phosphate and the clearance zone ranged from 0.87 mm to 5.5 mm in Pikovskaya's agar plates and solubilization in Pikovskaya's broth ranged from 0.68 mg L^{-1} to 41.92 mg L^{-1} (Fig. 3 and Fig. 4). Twelve isolates could solubilize the insoluble potassium compound, potassium aluminosilicate which produced clearance zone ranging from 2.25 mm to 5.5 mm in Aleksandrov agar plates and solubilization in Aleksandrov broth which ranged from 12.5 mg L^{-1} to 37.5 mg L^{-1} (Fig. 5 and Fig. 6). Similar results were reported with silicate solubilizing bacterial isolates by Naureen *et al.* (2015) with clearance zone that ranged from 5 mm to 55 mm for phosphorus solubilization in Pikovskaya's agar plates and the clearance zone for potassium solubilization ranged from 2 mm to 11 mm in Aleksandrov agar plates.

Wuxing *et al.* (2006) reported that leaching of SiO_2 and potassium from silicate minerals by *Bacillus mucilaginosus* occurred as a result of the combination of both exopolysaccharides and organic acids. *B. mucilaginosus* produces organic acids such as oxalate and citrate that can form bidentate complexes with metal ions which tend to be more effective in enhancing dissolution than monodentate ligands, such as those formed by acetate or propionate. At the same time the bacterium produces polysaccharides which can strongly adsorb the organic acids and these can combine with the minerals and form

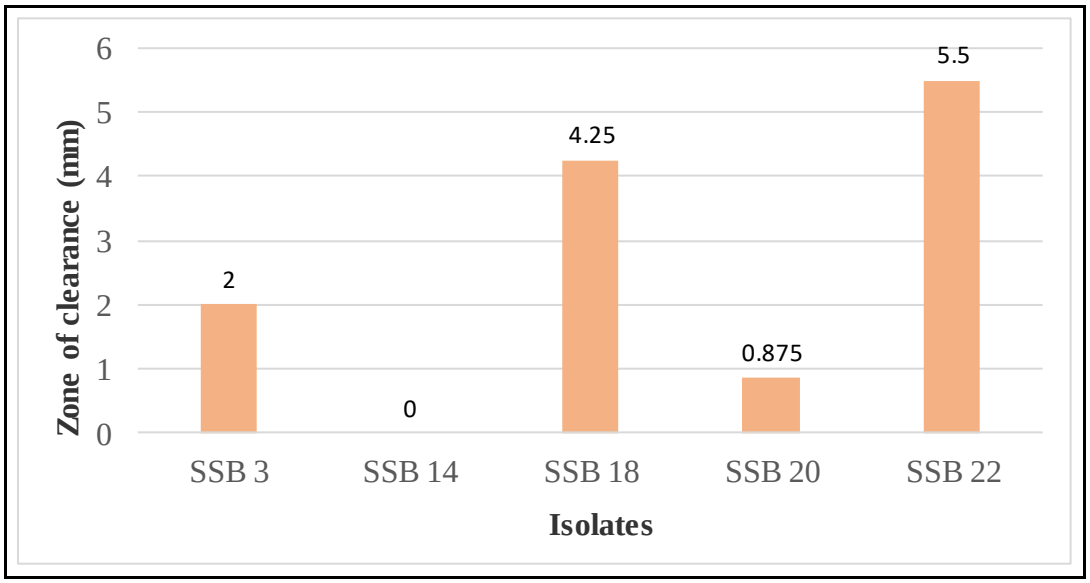


Fig. 3. Phosphate solubilization potential of five superior SSB isolates on Pikovskaya's agar plate assay

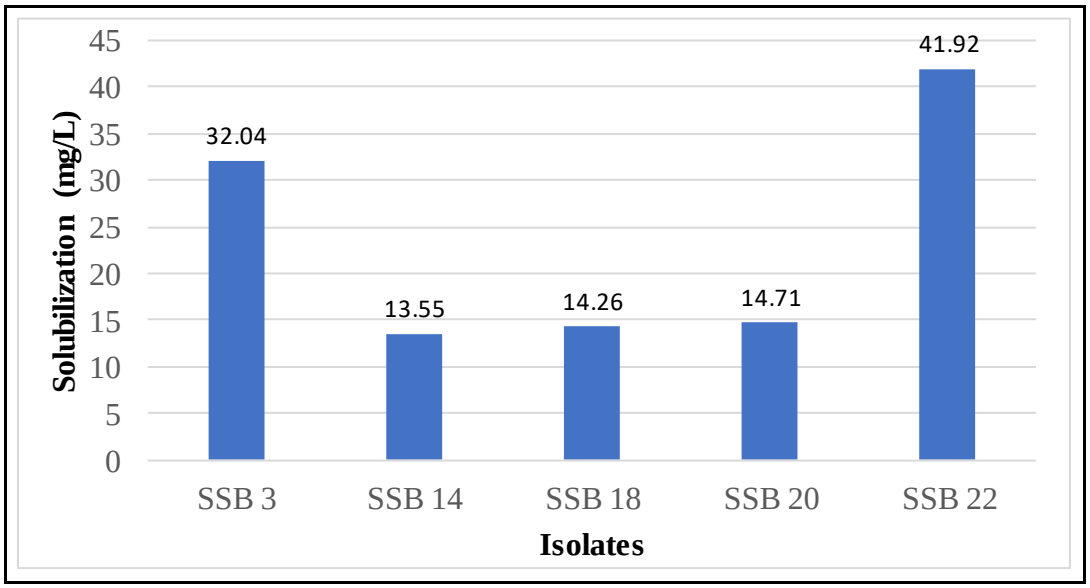


Fig. 4. Phosphate solubilization potential of five superior SSB isolates in Pikovskaya's broth assay

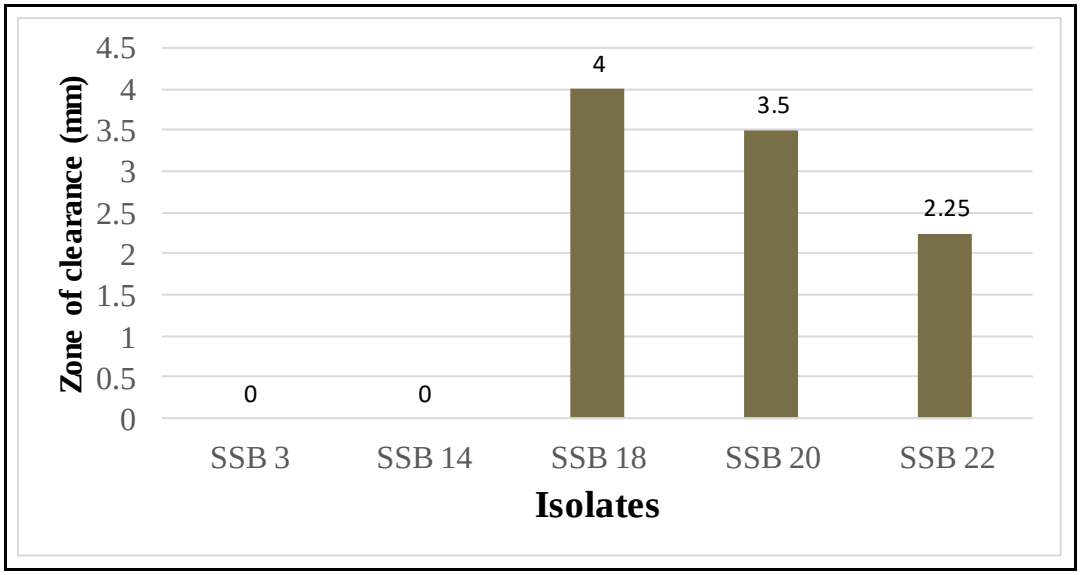


Fig. 5. Potassium solubilization potential of five superior SSB isolates on Aleksandrov agar plate assay

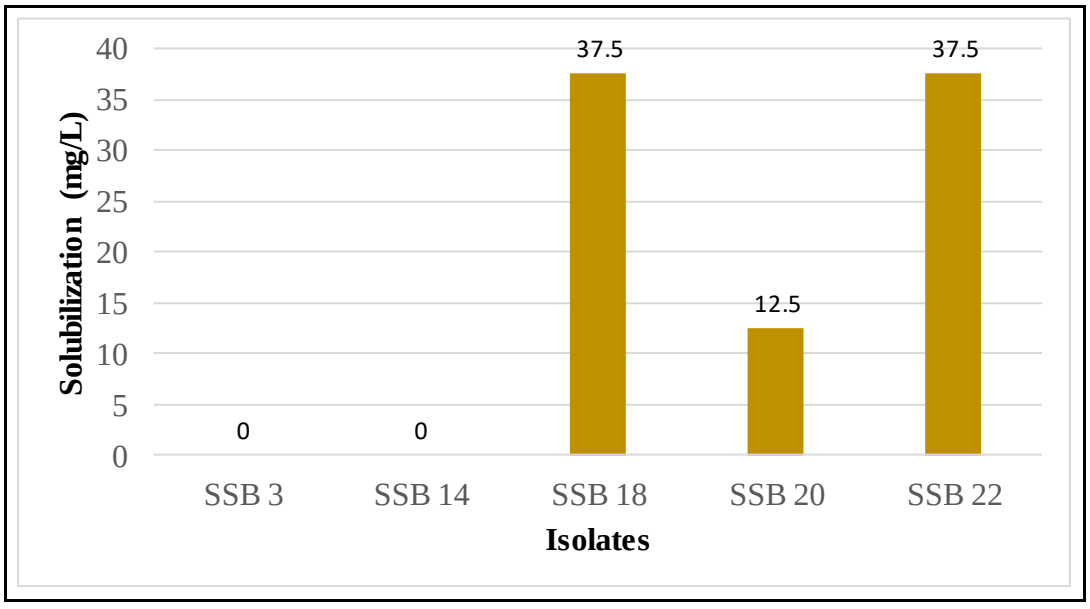


Fig. 6. Potassium solubilization potential of five superior SSB isolates in Aleksandrov broth as

bacterial–mineral complexes and as a result minerals are partially degraded. On the other hand, the polysaccharides also absorb SiO_2. The resulting alteration of the concentration of SiO_2 will affect the equilibrium between the mineral and fluid phases, leading to a reaction toward SiO_2 and potassium solubilization, which finally leads to further degradation of the minerals.

Vainberg *et al.* (1980) proposed that dissolution of minerals was caused by the formation of organic acids in the culture media. Hence, the present study also included the qualitative assessment of acid production. Five superior SSB isolates were tested qualitatively for their ability to produce organic acids in Bunt and Rovira medium amended with 0.2 per cent bromophenol blue. All the five isolates tested showed positive results for acid production. The yellow zone formed around the colonies indicated their ability to produce organic acids. Related findings were reported by Naureen *et al.* (2015) who observed that the bacterial isolates capable of solubilizing phosphates and silicates produced acid as detected by yellow halo formation on media containing bromophenol blue. The results obtained are also in accordance with the findings of Santi and Didiek (2017) who stated that certain bacterial isolates such as *Burkholderia cenocepacia* KTG, *Aeromonas punctata* RJM 3020, and *Burkholderia vietnamiensis* ZEO3 were capable of solubilizing silicates and produced acid as detected by yellow halo formation on solid Luria Bertani media containing 0.2 per cent (v/v) bromophenol blue. They also reported that colour change on the medium amended with 0.2 per cent bromophenol blue from blue into yellow or greenish-yellow was due to pH drop through the release of organic acid into medium by SSB inoculants.

The study conducted by Xia *et al.* (2008) using silicate mineral solubilizing bacterial strain *Bacillus globisporus* Q12 revealed gluconic acid as the most active agent for solubilization of three silicate minerals, *viz.*, muscovite, biotite and feldspar whereas gluconic and acetic acids were responsible for solubilization of silicon and potassium from feldspar.

Several ways have been reported by Naureen *et al.* (2009) and Hassan *et al.* (2010) by which silicate solubilizing bacteria can antagonise fungal pathogens which includes production of hydrolytic enzymes, siderophores, hydrogen cyanide and antibiotics. Vijayapriya and Muthukkaruppan (2010) reported the efficiency of the SSB on plant growth stimulation and on the augmentation of induced systemic resistance against *Pyricularia oryzae* that yields added advantage in the biofertilizer application of the same to harness maximum plant growth and biocontrol benefits in rice. Out of the ten SSB isolates obtained by them, four isolates, *viz.,* SSB 3, SSB 5, SSB 8 and SSB 9 were found to be efficient in silicate solubilization and antagonistic activity against *Pyricularia oryzae*.

The present study also evaluated the antagonistic efficiency of five superior SSB isolates and the results revealed their ability to inhibit the mycelial growth of major fungal pathogens of paddy, *viz., Rhizoctonia solani, Magnaporthe grisea, Helminthosporium oryzae,* causing sheath blight, blast and brown spot diseases respectively and bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* causing bacterial blight of paddy.

Out of the five isolates tested, three isolates inhibited rice sheath blight disease causing pathogen, *Rhizoctonia solani*. SSB 18 exhibited the maximum zone of inhibition (ZOI) of 9.65 mm which was significantly superior to all other isolates (Fig. 7). Three out of five isolates tested showed antagonism against the rice blast pathogen, *Magnaporthe grisea*. Among them, SSB 18 was found superior with the maximum ZOI of 14.45 mm (Fig. 8). These results are in agreement with the studies of Naureen *et al.* (2015) who reported the inhibition of phytopathogenic fungi by silicate solubilizing bacterial isolates with a ZOI that ranged from 5 mm to 33 mm and from 4 mm to 39 mm for *Rhizoctonia solani* and *Magnaporthe grisea* respectively. Four isolates inhibited *Helminthosporium oryzae,* the brown spot pathogen of rice. SSB 18 showed maximum antagonistic activity with the ZOI of 10.80 mm which was significantly superior to all other isolates (Fig. 9).

The bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* was inhibited by three isolates and SSB 18 was found superior with a clearance zone of 11.50 mm (Fig. 10).

Morphological studies of the superior SSB isolates revealed that they show variation in colony characters *i.e.* creamy, off-white, white gummy, light yellow or yellowish green colour with irregular and spreading or round shape, irregular or entire margin, flat or raised elevation, smooth or smooth and glossy texture, opaque or transparent colonies. Earlier studies on morphological characters conducted by Osman (2009) reported same results. Similar results were also observed on the colony characteristic studies conducted by Naureen *et al.* (2015).

The results of cell characteristics showed that all the superior SSB isolates are rod shaped, Gram positive endospore formers. Three out of five superior isolates exhibited motility. All the five superior isolates were further subjected to a series of biochemical tests. All the isolates were found to be negative for Voges-Proskauer's test and positive for citrate, ONPG, nitrate reduction and glucose. These results are in accordance with the findings of Osman (2009) who studied morphological and biochemical characteristics of silicate bacterial isolates *viz.*, *Bacillus circulans* (transparent isolate) and *Bacillus mucilaginosus* (milky white isolate) which are rod shaped, Gram positive endospore formers.

Based on morphological and biochemical characterization of five superior SSB isolates, the tentative taxonomic position of these isolates were identified as *Bacillus* spp.

The present study also evaluated the ability of the five superior SSB isolates to grow in different pH, temperature and salinity levels. The results showed that they are unable to grow in low pH value (4.0), while they showed vigorous growth in moderate pH value (7.0) and their growth exhibited variation in high pH value (9.0). The effect of temperature on their growth revealed that they were unable to grow at low temperature (4°C), while they exhibited vigorous growth at room temperature (28°C) and their growth

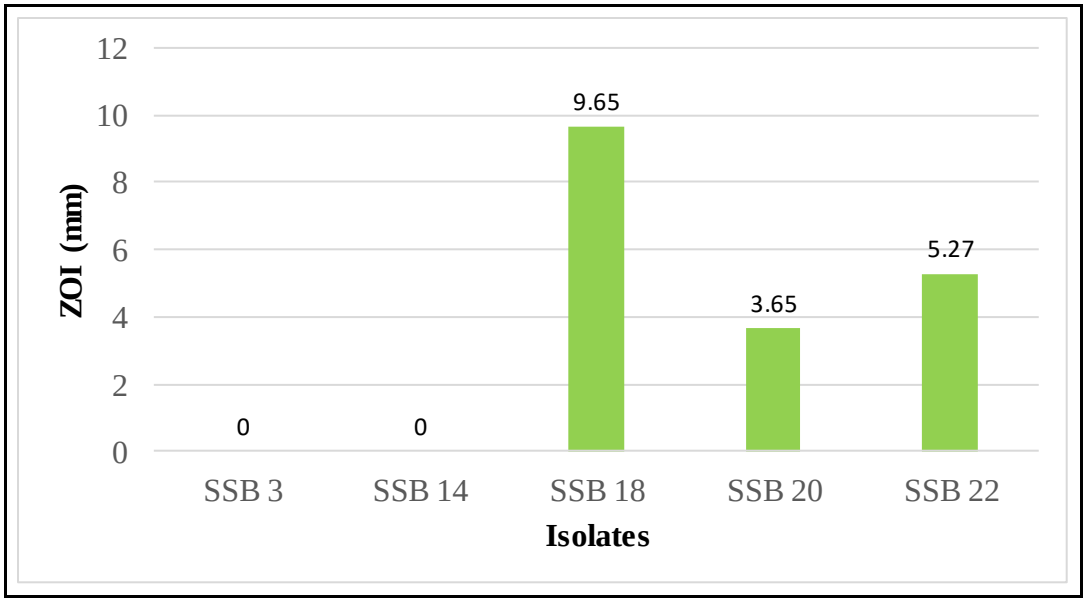


Fig. 7. Antagonistic activity of five superior SSB isolates against *Rhizoctonia solani*

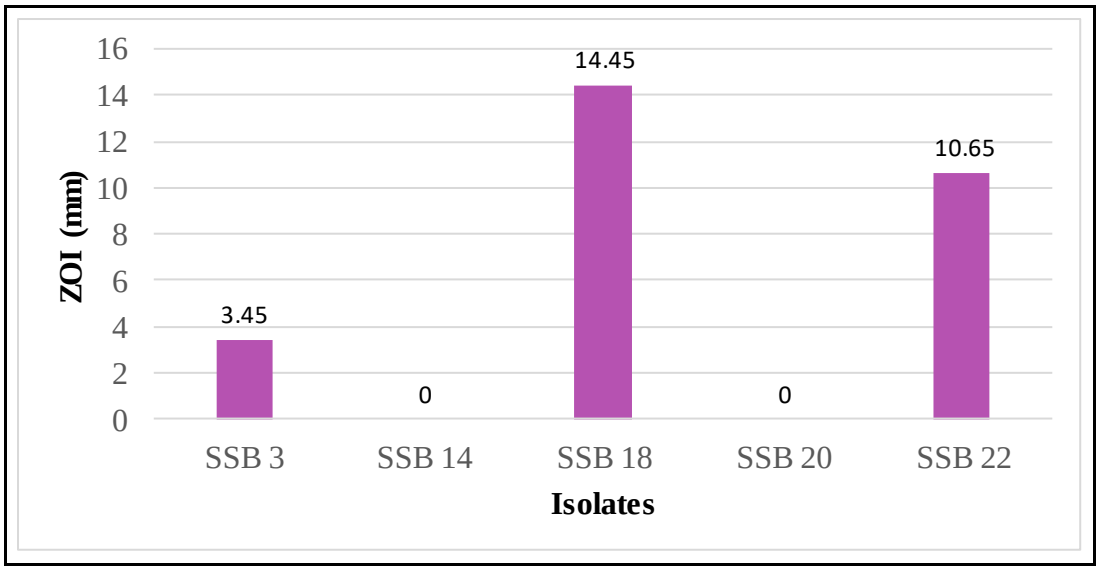


Fig. 8. Antagonistic activity of five superior SSB isolates against *Magnaporthe grisea*

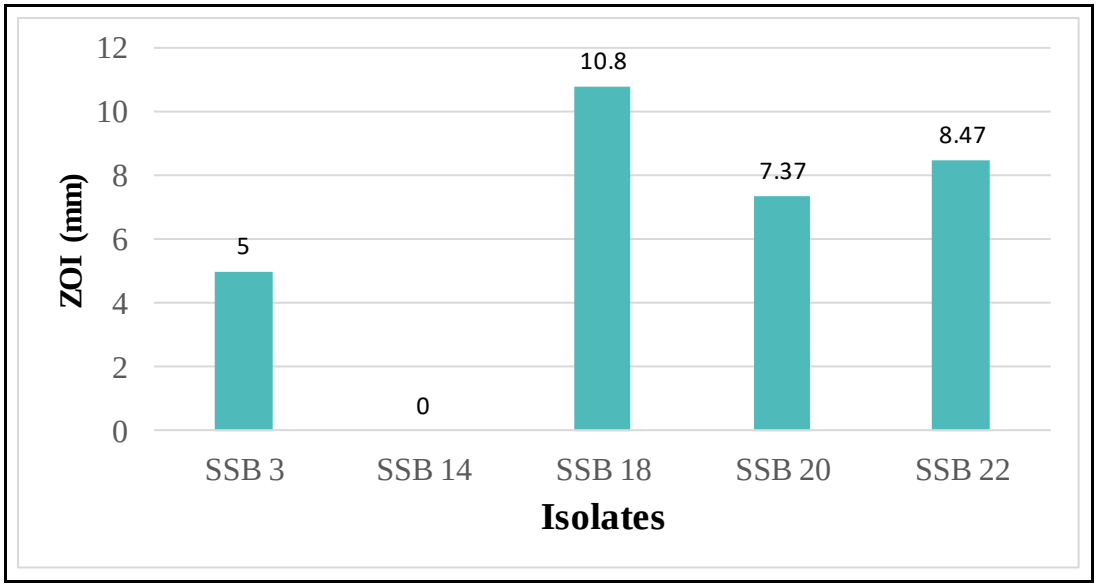


Fig. 9. Antagonistic activity of five superior SSB isolates against *Helminthosporium oryzae*

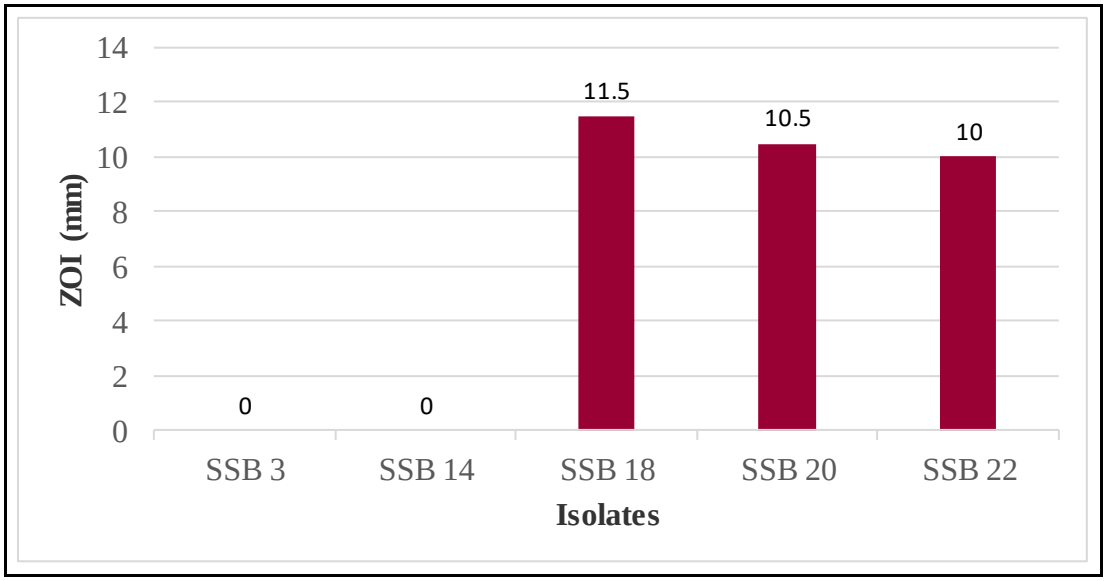


Fig. 10. Antagonistic activity of five superior SSB isolates against *Xanthomonas oryzae* pv. *oryzae*

showed variation at high temperature (45°C). The ability of superior SSB isolates to grow in different NaCl concentrations varied according to the isolates in 0.034 *M* (2 g L^{-1}), 0.17 *M* (10 g L^{-1}) and 0.85 *M* (50 g L^{-1}) NaCl concentrations. These results are in agreement with the study conducted by Osman (2009) with the exception that they are unable to grow at low temperature (4°C).

The present investigation could identify five superior bacterial isolates, *viz.*, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 capable of solubilizing magnesium trisilicate in plate and broth assay. Dual culture assay of these SSB isolates against major pathogens of paddy revealed that maximum antagonistic activity was exhibited by isolate SSB 18.

SUMMARY

6. SUMMARY

Silicon is one of the most important beneficial element that has a positive influence on plant growth. Unfortunately, the silica that occurs in soil is in an unavailable polymerized form and for its absorption by plants it has to be depolymerized and rendered soluble by means of biological or chemical process. However, the extensive use of chemical fertilizers has several harmful effects on environment like pollution, eutrophication, destruction of soil fertility and nutrient availability etc. Therefore, following of biological ways like the use of microorganisms that are capable of solubilizing silicate and making them available to the plants are gaining importance nowadays. In this context the programme entitled "Isolation and *in vitro* screening of silicate solubilizing bacteria from paddy rhizosphere", was conducted during 2018-2020, in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram.

The main objective of the present study was to isolate and screen efficient bacteria *in vitro* that are capable of solubilizing the insoluble form of silicate. The salient findings of the present study are summarized below.

Twenty seven isolates of bacteria capable of solubilizing insoluble form of silicate (magnesium trisilicate) was isolated from the rhizosphere soils collected from different upland and lowland paddy fields of Kerala by serial dilution and plate count method using Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate. These isolates were allotted code numbers from SSB 1 to SSB 27. All the twenty seven isolates when subjected to plate and broth assay effectively solubilized the insoluble silicate compound used, magnesium trisilicate. In plate assay, the size of the solubilization zone ranged from 3 mm to 13 mm and solubilization in broth ranged from 23.08 mg L^{-1} to 94.65 mg L^{-1}. The maximum clearance zone of 13 mm was recorded with the isolate SSB 14 which was significantly superior to all other isolates. In broth assay, SSB 20 showed the highest silicate solubilization of 94.65 mg L^{-1}.

Based on plate as well as broth assay of all the twenty seven isolates obtained, five isolates *viz.*, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 which showed the maximum clearance zone in plate and silicate solubilization in broth were selected as superior isolates.

All the isolates obtained were subjected to plate and broth assay for phosphate and potassium solubilization in Pikovskaya's medium and Aleksandrov medium respectively. Among them, fourteen isolates showed phosphate solubilization and twelve isolates showed potassium solubilization in plates. For phosphate solubilization, the clearance zone in plates ranged from 0.87 mm to 5.5 mm and solubilization in broth ranged from 0.68 mg L^{-1} to 41.92 mg L^{-1} in Pikovskaya's medium. The maximum clearance zone of 5.5 mm was recorded with the isolate SSB 22 which was on par with SSB 23 with 5 mm clearance zone in plate and highest solubilization of 41.92 mg L^{-1} in broth was shown by SSB 22 which was significantly superior to all other isolates. For potassium solubilization, the clearance zone in plates ranged from 2.25 mm to 5.5 mm and in broth it ranged from 12.5 mg L^{-1} to 37.5 mg L^{-1}. Maximum clearance zone of 5.5 mm was recorded with the isolate SSB 8 which was significantly superior to all other isolates. The highest potassium solubilization of 37.5 mg L^{-1} in broth was observed with isolates SSB 1, SSB 2, SSB 7, SSB 8, SSB 13, SSB 18, SSB 21 and SSB 22 which were found to be statistically on par.

Acid production by the five superior SSB isolates, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 was detected as a yellow halo around the bacterial colonies in bromophenol blue amended Bunt and Rovira medium. All the five superior SSB isolates tested showed positive results for acid production. The yellow zone indicates the ability of these isolates to produce organic acids in the Bunt and Rovira medium amended with 0.2 per cent bromophenol blue.

The antagonistic activity of the five superior SSB isolates were assessed against major fungal pathogens of paddy *viz., Rhizoctonia solani*, *Magnaporthe grisea*, *Helminthosporium oryzae* and one bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae*

following dual culture method. Out of the five isolates tested, three isolates (SSB 18, SSB 20 and SSB 22) inhibited *Rhizoctonia solani*. Among them, SSB 18 was found superior with the maximum zone of inhibition (ZOI) of 9.65 mm. Three isolates (SSB 3, SSB 18 and SSB 22) showed antagonism against *Magnaporthe grisea* and SSB 18 exhibited maximum ZOI of 14.45 mm. Four isolates (SSB 3, SSB 18, SSB 20 and SSB 22) inhibited *Helminthosporium oryzae* and the maximum ZOI of 10.8 mm was shown by SSB 18. The bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* was inhibited by three isolates (SSB 18, SSB 20 and SSB 22). Among them, SSB 18 was found superior with the maximum ZOI of 11.50 mm.

The five superior SSB isolates were characterized based on morphological and biochemical characters. All the isolates were rod shaped, Gram positive endospore formers.

In the present investigation, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 are selected as the superior silicate solubilizing bacterial isolates. Further studies on these efficient SSB isolates on soil as well as plants are required before developing commercial formulations. Hence, the further studies may be focused on the following.

1. Soil incubation studies on the ability of the superior isolates to release silicon from soil.
2. Studies on the effect of superior SSB isolates on growth and yield of paddy under pot culture and field condition.
3. Evaluation of antagonistic efficiency of the superior isolates under pot culture condition and field evaluation.
4. Development of commercial formulations of the superior SSB isolates.

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APPENDICES

APPENDIX - I

COMPOSITION OF MEDIA USED

1. Bunt and Rovira Media

Glucose	-	20.0 g
Peptone	-	1.0 g
Yeast extract	-	1.0 g
$(NH_4)_2SO_4$	-	0.5 g
K_2HPO_4	-	0.4 g
$MgCl_2$	-	0.1 g
$FeCl_3$	-	0.01 g
Mg trisilicate	-	2.5 g
Agar-agar	-	20.0 g
Distilled water	-	1000 mL
pH	-	6.6 – 7.0

Glucose, Peptone, Yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, $MgCl_2$ and $FeCl_3$ were dissolved in 500 mL distilled water and volume made up to 1000 mL. It was then distributed into 100 mL each in 250 mL flasks and 0.25 g Mg trisilicate was added into each flask and pH was adjusted. Two gram agar-agar was added into each flask and autoclaved at 15 lbs pressure and 121°C for 15 minutes.

2. Nutrient Agar

Peptone	-	5.0 g
Beef extract	-	3.0 g
NaCl	-	5.0 g
Agar-agar	-	20.0 g
Distilled water	-	1000 mL
pH	-	7.0

Peptone, Beef extract and NaCl were dissolved in 500 mL distilled water and volume made up to 1000 mL. Twenty gram agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 ºC for 15 min.

3. Pikovskaya's Media

Glucose	-	10.0 g
$Ca_3(PO_4)_2$	-	5.0 g
$(NH_4)_2SO_4$	-	0.5 g
Yeast extract	-	0.5 g
KCl	-	0.2 g
$MgSO_4$	-	trace
$FeSO_4$	-	trace
Agar-agar	-	20.0 g
Distilled water	-	1000 mL

Glucose, $Ca_3(PO_4)_2$, $(NH_4)_2SO_4$, Yeast extract, KCl, $MgSO_4$, and $FeSO_4$ were dissolved in 500 mL distilled water and volume made up to 1000 mL. Twenty gram agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 ºC for 15 min.

4. Aleksandrov Media

Aleksandrov agar	-	29.6 g
Distilled water	-	1000 mL
Agar-agar	-	5.0 g
pH	-	7.2 ± 0.2

Ready-made (Hi-media) Aleksandrov agar was dissolved in 500 mL distilled water and volume made up to 1000 mL and pH was adjusted. Five gram agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 ºC for 15 min.

5. Potato Dextrose Agar

Peeled and sliced potatoes	-	200.0 g
Dextrose	-	20.0 g
Agar-agar	-	20.0 g
Distilled water	-	1000 mL

Potatoes were boiled in 500 mL of distilled water and the extracts was collected by filtering through a muslin cloth. Agar-agar was dissolved separately in 500 mL of distilled water. The potato extract was mixed in the molten agar and 20 g of dextrose was dissolved in the mixture. The volume was made up to 1000 mL with distilled water and medium was sterilized at 15 lbs pressure and 121˚C for 15 min.

6. Potato Sucrose Peptone Agar

Peeled and sliced potatoes	-	300.0 g
Peptone	-	5.0 g
Na_2HPO_4	-	2.0 g
$Ca(N0_3)_2$	-	0.5 g
Sucrose	-	20.0 g
Agar-agar	-	20.0 g
Distilled water	-	1000 mL

Potatoes were boiled in 500 mL of distilled water and the extracts was collected by filtering through a muslin cloth. Agar-agar was dissolved separately in 500 mL of distilled water. The potato extract was mixed in the molten agar and Na_2HPO_4, $Ca(N0_3)_2$ and sucrose were dissolved in to the mixture. The volume was made up to 1000 mL with distilled water and medium was sterilized at 15 lbs pressure and 121°C for 15 min.

APPENDIX - II

COMPOSITION OF STAIN USED

1. Crystal violet

One volume saturated alcohol solution of crystal violet in four volumes of one per cent aqueous ammonium oxalate.

2. Gram's iodine

Iodine crystals	-	1.0 g
Potassium iodide	-	2.0 g
Distilled water	-	300 mL

3. Safranin

Ten ml saturated solution of safranin in 100 ml distilled water.

ISOLATION AND *IN VITRO* SCREENING OF SILICATE SOLUBILIZING BACTERIA FROM PADDY RHIZOSPHERE

by

AKHILA P. SUBHASH

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Abstract of the thesis

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ABSTRACT

The study entitled “Isolation and *in vitro* screening of silicate solubilizing bacteria from paddy rhizosphere”, was conducted during 2018-2020, in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, with the objective of isolation and *in vitro* screening of bacteria which are capable of solubilizing insoluble form of silicate.

Bacteria capable of solubilizing silicates were isolated from the rhizosphere soils collected from different upland and low land paddy fields by serial dilution and plate count method using Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate. Based on the clear halo zone formed around the bacterial colonies on solid media, they were identified as Silicate Solubilizing Bacteria (SSB).

Twenty seven isolates of bacteria capable of solubilizing insoluble form of silicate (magnesium trisilicate) were obtained from different locations and were allotted code numbers from SSB 1 to SSB 27. These isolates were subjected to plate and broth assay in Bunt and Rovira medium supplemented with 0.25 per cent magnesium trisilicate. After three days of incubation of test plates at room temperature, all the twenty seven isolates solubilized magnesium trisilicate and produced clearing zone around the bacterial colonies on solid media. The size of clearance zone ranged from 3 mm to 13 mm in plates. The maximum clearance zone of 13 mm was recorded with the isolate SSB 14 which was significantly superior to all other isolates. In broth culture, SSB 20 showed the highest silicate solubilization of 94.65 mg L^{-1}. Based on plate as well as broth assay of all the twenty seven isolates obtained, five isolates *viz.*, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 which showed the maximum clearance zone in plate and silicate solubilization in broth were selected as superior isolates.

All the isolates obtained were subjected to plate and broth assay for phosphate solubilization in Pikovskaya’s medium and potassium solubilization in Aleksandrov medium. Among them, fourteen isolates showed phosphate solubilization in plates and the clearance zone ranged from 0.87 mm to 5.50 mm. The maximum clearance zone of

5.50 mm was recorded with the isolate SSB 22 which was on par with SSB 23 with 5 mm clearance zone in plate and highest solubilization of 41.92 mg L^{-1} in broth was shown by SSB 22 which was significantly superior to all other isolates. Twelve isolates showed potassium solubilization in plates and the clearance zone ranged from 2.25 mm to 5.50 mm. Maximum clearance zone of 5.50 mm was recorded with the isolate SSB 8 which was significantly superior to all other isolates. The highest potassium solubilization of 37.50 mg L^{-1} in broth was observed with isolates SSB 1, SSB 2, SSB 7, SSB 8, SSB 13, SSB 18, SSB 21 and SSB 22 which were found to be statistically on par.

Acid production by the five superior SSB isolates, SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 was detected as a yellow halo around the bacterial colonies in bromophenol blue amended Bunt and Rovira medium. All the five superior isolates tested showed positive results for acid production.

The antagonistic activity of the five superior SSB isolates were assessed against major pathogens of paddy *viz., Rhizoctonia solani*, *Magnaporthe grisea*, *Helminthosporium oryzae* and *Xanthomonas oryzae* pv. *oryzae* following dual culture method. Out of the five isolates tested, three isolates (SSB 18, SSB 20 and SSB 22) inhibited *Rhizoctonia solani*. Three isolates (SSB 3, SSB 18 and SSB 22) showed antagonism against *Magnaporthe grisea* and four isolates (SSB 3, SSB 18, SSB 20 and SSB 22) inhibited *Helminthosporium oryzae.* The bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* was inhibited by three isolates (SSB 18, SSB 20 and SSB 22). Among all the five isolates tested against different phytopathogens, SSB 18 was found superior with the maximum zone of inhibition of 9.65 mm, 14.45 mm, 10.80 mm and 11.50 mm against *Rhizoctonia solani, Magnaporthe grisea, Helminthosporium oryzae* and *Xanthomonas oryzae* pv. *oryzae* respectively.

The five superior isolates were characterized based on morphological and biochemical characters. The results revealed that all the isolates were rod shaped, Gram positive endospore formers.

Based on the results of present study, it can be concluded that SSB 3, SSB 14, SSB 18, SSB 20 and SSB 22 are the superior silicate solubilizing bacterial isolates. Among them, SSB 18 showed the highest antagonistic activity against major pathogens of paddy.