SCREENING OF ACID-TOLERANT CONSORTIA OF *AZOSPIRILLUM* AND PHOSPHATE SOLUBILIZING BACTERIA FROM LATERITIC SOILS

By

ATHULYA, M. M.

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THESIS

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Faculty of Agriculture
Kerala Agricultural University, Thrissur

Department of Agricultural Microbiology

COLLEGE OF HORTICULTURE

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DECLARATION

I hereby declare that this thesis entitled **"Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara **Athulya, M.M.**

11/4/2014 2011-11-141

CERTIFICATE

Certified that this thesis entitled **"Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils"** is a record of research work done independently by **Athulya, M.M. (2011-11-141)** 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.

Vellanikkara
11/4/2014

Dr. K. Surendra Gopal
Chairman
Associate Professor (Microbiology)
Dept. of Agrl. Microbiology
College of Horticulture
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Athulya, M.M. (2011-11-141)** a candidate for the degree of **Master of Science in Agriculture** with major field in **Agricultural Microbiology,** agree that this thesis entitled **"Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils"** may be submitted by **Ms. Athulya, M.M.,** in partial fulfilment of the requirement for the degree.

Dr. K. Surendra Gopal
Associate Professor (Microbiology)
Dept. of Agrl. Microbiology
College of Horticulture
Vellanikkara, Thrissur
(Chairman)

Dr. D. Girija
Professor & Head
Dept. of Agrl. Microbiology
College of Horticulture
Vellanikkara, Thrissur
(Member)

Dr. P. Sureshkumar
Professor & Head
RTL, Vellanikkara
(Member)

Dr. S. Nimala Devi
Professor (Horticulture), AICVIP
Dept. of Olericulture
College of Horticulture
Vellanikkara, Thrissur
(Member)

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INTRODUCTION

1. INTRODUCTION

The existence of soil microbes capable of soil phosphorus transformation and nitrogen fixation from the atmosphere to available forms has been well established. Biofertilizers are agricultural inputs, which make use of microorganisms to fertile the soil. Their constituent micro-organisms biologically interact with the soil, root and seed of plants, promoting the growth of micro-flora that enhances soil fertility. As chemical fertilizers cause a deterioration of the soil vitality over time, biofertilizers are attractive alternatives, benefiting harvests and soil alike.

Biological Nitrogen Fixation (BNF) offers the most promising supplement to chemical nitrogenous fertilizers through associative, free living and symbiotic nitrogen fixation by rhizosphere microflora. These microbes are commonly found in association with the roots of diverse plants (Brown, 1975). In the living system, plants gain benefit when the bacteria die and release nitrogen to the surrounding environment, or when the bacteria are loosely associated with roots of plants. Bacteria of the genus *Azospirillum* are nitrogen fixers that live in close association with plants in the rhizosphere. The *Azospirillum*-plant association leads to the enhanced growth and yield of different host plants under appropriate growth conditions due to nitrogen fixation (Purushothaman *et al.*, 1980).

Phosphorus is another essential macronutrients, which is needed for the higher yield of crops. Most agricultural soil contains a large portion of soluble inorganic phosphate, which is rapidly immobilized as iron and aluminium phosphates in acidic soils and becomes unavailable to plants. Thus, farmers have to apply several fold excess phosphorus fertilizer inorder to overcome this problem. Therefore, the release of insoluble and fixed forms of phosphorus is important in increasing soil phosphorus availability. Phosphate solubilizing bacteria (PSB) have been considered as one of the possible alternatives for improving P use efficiency to get higher plant growth and yield. Seed or soil inoculation with PSB is found to increase crop yield by improving the

solubilization of fixed soil phosphorus and applied phosphates (Reena *et al.*, 2013).

In recent years, it is reported that the consortia of microbes in the rhizosphere act synergistically by stimulating each other through physical and/or biochemical process, provide essential nutrients to plants, and protect them from pathogens. Studies have shown that the beneficial effects of *Azospirillum* on plants can be enhanced by co-inoculation with other beneficial microorganisms. The consortia of *Azospirillum* and PSB will provide both nitrogen and phosphorus to plants simultaneously.

One of the major factors affecting the field performance of biofertilizers in Kerala is the soil pH. Kerala soils are acidic with low activity clays, gravelly with low water holding capacity and high phosphorus fixing capacity. The soil pH ranges from 4.5-6.2. Among the acidic soils, majority of the soils are lateritic in nature with poor availability of nitrogen and phosphorus and such soils needs to be enriched with nitrogen and phosphorus to get optimum plant growth. The Kerala has already declared its organic farming policy and the entire state will turn into "organic" in another ten years. Hence, there will be lots of demand for biofertilizers which are suitable for acidic soils of Kerala. Moreover, the consortia of *Azospirillum* and PSB will help to provide nitrogen and phosphorus simultaneously to crops. *Azospirillum* and phosphate solubilizing microorganisms (PSM) are very popular biofertilizers in Kerala.

There are no studies conducted in Kerala on the efficiency of native acid-tolerant consortia of *Azospirillum* and PSB for lateritic soils. The development of native acid-tolerant consortia of *Azospirillum* and PSB from lateritic soils will help to provide nitrogen and phosphorus simultaneously to the plants. Hence, the present study on "Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils" was undertaken. The main objectives of the present study were as follows:

- To isolate and characterize *Azospirillum* and PSB from lateritic soils
- To develop an efficient and native acid-tolerant consortium of *Azospirillum* and PSB for growth enhancement of okra.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Biofertilizer is one of the best modern tools for agriculture. Biofertilizer contains microorganisms, which promote the adequate supply of nutrients to the host plants and ensure their proper development of growth and regulation in their physiology. Living microorganisms are used in the preparation of biofertilizers. Role of biofertilizers on the crop yield has documented by Rana *et al.* (2013). Combined inoculation of *Azospirillum* and phosphate solubilizing bacteria on food crops gave significant increase in dry matter and yield over single inoculation (Alagawadi and Gaur, 1992).

2.1 *AZOSPIRILLUM* AS NITROGEN FIXER

Azospirillum is not the only the microorganism capable of colonizing vegetables and induces beneficial effects on them, but it congregates several characteristics present in different microorganisms, which makes it a valuable PGPR. Higher growth and yield observed in *Azospirillum*-inoculated subtropical grasses (*Zea mays*, *Oryza sativa*, *Saccharum officinarum*, *Sorghum bicolor* and forages such as *Digitaria* spp.) were primarily attributed to the biological N_2 fixation (BNF) exerted by the bacteria (Dobereiner *et al.,* 1976). Inoculation of *Azospirillum* in wheat and maize has indicated that 5-10% and up to 18% (Rennie, 1980) of the plant nitrogen was derived from N_2 fixation. In addition, inoculated plants grew normal with only a partial amount of the nitrogen fertilizer usually required for such growth (Kapulnik *et al.*, 1981). Furthermore, of the entire nitrogen fixed by the bacteria, less than 5% was incorporated into the host plants (Eskew *et al.*, 1981).

There was an increase in the total nitrogen content of shoots and grains of inoculated plants (Baldani *et al.*, 1983). Studies have also shown low or even negligible nitrogenase activity in plants positively responding to inoculation (Venkateswarlu and Rao, 1983). Evidence that N_2 fixation contributes to the nitrogen balance of plants is based on the common observation of an increase in

the nitrogenase activity within inoculated roots (Okon *et al*., 1983). The amounts of fixed nitrogen are insufficient to explain total increases in nitrogen content of inoculated plants. High nitrogen fertilization levels, which inhibit N_2 fixation, did not eliminate the plant response to inoculation of *Azospirillum* (Mertens and Hess, 1984).

Well-documented enzymatic activities are of sufficient magnitude to account for the increase in total nitrogen yield of inoculated plants if the entire fixed nitrogen is incorporated into the plants (Sarig *et al*., 1984). *Azospirillum* fixes atmospheric nitrogen under microaerophilic and nitrogen limiting conditions (Gallori and Bazzicalupo, 1985). N_2 fixation was naturally the first major mechanism of action suggested for the enhancement of plant growth by *Azospirillum*. Okon and Kapulnik (1986) reported the positive bacterial effects on morphological and physiological changes in the inoculated roots, which enhanced water and mineral uptake. All wild-type *Azospirillum* strains fix atmospheric nitrogen efficiently either as free-living bacteria or in association with plants and participate in several transformations in the nitrogen cycle (Heulin *et al*., 1989).

Nitrogen fixation possibility contributes to the plant with small amounts of nitrogen, which may be important in critical stages of plant development, such as the reproductive and the tillering stages (Bashan and Levanony, 1990). *Azospirillum* can convert atmospheric nitrogen into ammonium under microaerophilic conditions at low nitrogen levels, through the action of the nitrogenase complex. Nevertheless, to date no unique mechanism had been established to explain the growth promotion capability of *Azospirillum*. Instead, the most accepted hypothesis postulates that a sum of events accounts for the general plant growth promotion effect by *Azospirillum* sp. (Bashan and Holguin, 1997).

Eventhough, this characteristic could be extremely valuable in agriculture, subsequent field studies including those in which isotopic dilution techniques were used, failed to demonstrate a significant BNF in *Azospirillum*-inoculated

crops (Van de Broek *et al.*, 2000). Nitrogen fixation is performed by a nitrogenase complex, and occurs when the availability of nitrogen compounds and oxygen tension are low (Steenhoudt and Vanderleyden, 2000). Phytohormone production and nitrogen fixation are recognized as the processes involved in plant growth promotion by *Azospirillum* (Steenhoudt and Vanderleyden, 2000).

2.2 CHARACTERISTICS OF *AZOSPIRILLUM*

Tarrand *et al.* (1979) reported that *Azospirillum* are nitrogen fixers, exhibiting N_2 dependent growth under microaerobic conditions. Colonies on potato agar are typically light or dark pink, often wrinkled and non-slimy. In semi-solid nitrogen free malate (Nfb) medium, *Azospirillum lipoferum* develops predominantly into pleomorphic cells within 48 h in contrast to *A. brasilense,* which retains mainly vibroid form. On BMS agar media, after 1-2 weeks of incubation at 33-35^{0}C, colonies of *Azospirillum* are pink, opaque, irregular or round, often wrinkled and have umbonate elevation. Pigmentation is best on BMS agar medium incubated under the light.

Azospirillum sp. are gram negative curved rods of variable sizes which exhibit spirillar movement and polymorphism. The cells contain poly-β-hydroxy butyrate (PHB) granules and fat droplets. These are associative microaerophilic diazotrophs isolated from the roots and above ground parts of a variety of crop plants like forage grasses, cereals, legumes, millets and soils (Bashan *et al.*, 2004). *Azospirillum* species belong to facultative endophytic diazotrophic group which colonize the surface and interior of roots (Tejera *et al.*, 2005).

2.3 ACID-TOLERANT *AZOSPIRILLUM*

Rai (1991) have isolated five *Azospirillum brasilense* strains from the roots of finger millet plants grown in acid soils of Chhota Nagpur of Bihar. In another study, *Azospirillum* sp. were isolated from approximately 40% of soil samples from soil pH between 5.0 and 6.6 in wheat rhizosphere of Eastern Australia.

However, isolates of *Azospirillum* were rare in soil between pH 4.5 and 5.0 and absent below pH 4.5. When a medium buffered with 0.05 M malate and 0.05 M phosphate, it was found that all *Azospirillum* isolates had a lower minimum pH for growth when supplied with fixed nitrogen than when grown under nitrogen fixing conditions (New and Kennedy, 1989).

2.4 EFFICIENCY OF *AZOSPIRILLUM*

The production of plant growth promoting substances by *Azospirillum* has often been proposed as one of the key factors responsible for the plant growth promotion, as plant growth substances could be detected in the supernatant of *Azospirillum* cultures.

A. brasilense produced gibberellins and cytokinin like substances with tryptophan in a culture. The plant growth substances produced by *A. brasilense* induced proliferation of lateral roots and root hairs and thus increased greater rates of nutrient absorption, which in turn increased plant growth (Tien *et al.*, 1979).

It has been suggested that *Azospirillum* inoculation may promote availability of ions in the soil by helping the plant scavenge limiting nutrients (Lin *et al.*, 1983) which may explain accumulation of nitrogen compounds in the plant without any apparent N_2 fixation.

Combinations of different amounts of indole acetic acid, gibberellin and kinetin (0.001 to 0.05 µg ml^{-1}) produced changes in root morphology of pearl millet (*Pennisetum americanum* L.), similar to those produced by inoculation with *A. brasilense* (Jain and Patriquin, 1984). An alternative to nitrogen fixation for nitrogen accumulation due to *Azospirillum* inoculation in wheat plants is the bacterial nitrate reductase (NR) theory. Nitrate reductase activity of wheat leaves was decreased by inoculation with some *Azospirillum* strains. Inoculation of plants with NR^- mutants resulted in minimal plant response concomitant with an increase in leaf NR, compared with inoculation with the parental NR^+ strain. The parental strain aided nitrate reduction in the roots and thus decreased nitrate

translocation to the leaves, while inoculation with the NR^- mutant caused direct translocation and reduction of nitrate in the plant foliage (Ferreira *et al.*, 1987).

Varieties of auxins like indole-3-acetic acid (IAA), indole-3-pyruvic acid, indole-3-butyric acid and indole lactic acid, cytokinins (Cacciari *et al.*, 1989) and gibberellins (Bottini *et al.*, 1989) were detected, with auxin production being quantitatively most important. Studies on IAA production showed that it depends on the type of culture media and availability of tryptophan as a precursor. The principal mechanism by which *Azospirillum* enhances plant growth is undetermined. Nitrogen fixation, hormonal effects, general improvement in the growth of the entire root system and bacterial nitrate reductase activity in roots are the possible modes of action (Bashan and Levanony, 1990).

Among the strains tested, *A. brasilense* Cd produced the highest level of IAA (approx. 380 $\mu mol.l^{-1}$) (El-Khawas and Adachi, 1999). Morphological changes on the plant root due to *Azospirillum* inoculation could be mimicked by applying a combination of plant growth substances. Several investigations conducted with mutant strains altered IAA production to indicate the involvement of bacterial IAA in the promotion of root development.

A. brasilense SpM 7918, a very low-IAA producer, showed a reduced ability to promote root system development when compared to the wild type strain Sp6. Another mutant of *A. brasilense* with low production of phytohormones but high nitrogenase activity did not enhance root growth over uninoculated controls. In short, several evidences support the involvement of IAA produced by *Azospirillum* in the promotion of plant growth. However, there are no reports showing to what extent IAA produced in the rhizosphere originates from *Azospirillum* (Barassi *et al.*, 2007).

2.5 EFFECT OF *AZOSPIRILLUM* ON PLANT GROWTH

Azospirillum occurs as free living in the soil or associated with the roots of cereal crops, grasses and tuber plants (Tarrand *et al.*, 1979). The effect of

Azospirillum inoculation on the total yield increase in field-grown plants generally ranged from 10 to 30% (Watanabe and Lin, 1984).

Yield increases due to inoculation of *Azospirillum* were reported in 75% of all experiments using summer cereals and only in 50% of the experiments using spring wheat (Smith *et al.*, 1984; Schank and Smith, 1984). Okon (1985) evaluated the worldwide success of *Azospirillum* inoculation and concluded that positive effects on yield were obtained in approximately 65% of all field experiments.

Azospirillum can colonize roots externally and internally. In external colonization, the bacteria form mainly small aggregates, although many single cells may also be scattered on the root surface. These externally colonizing bacteria are embedded in the mucigel layer of the root surface (Bashan *et al.*, 1986). Both live and dead roots can be colonized (Bashan *et al.*, 1986; Bashan and Levanony, 1988). Plant inoculation with *Azospirillum* affected many foliage parameters directly due to positive bacterial effects on mineral uptake by the plant. Enhancement in uptake of NO_3^-, NH_4^+, PO_4^{2-} K^+, Rb^+, and Fe^{2+} by *Azospirillum* was proposed to cause an increase in foliar dry matter and accumulation of minerals in stems and leaves. During the plant reproductive period, these minerals could have been transferred to the panicles and spikes and finally resulted in a higher yield. Increased mineral uptake by plants has been suggested to be due to a general increase in the volume of the root system and not to any specific enhancement of the normal ion uptake mechanism (Murty and Ladha, 1988).

In internal colonization, *Azospirillum* cells can colonize roots by penetrating into the root intercellular spaces (Levanony *et al.*, 1989). However, the incidence of positive results may not be frequent enough to enable commercialization of the bacterial preparation, as negative or no-effect results of inoculation were rarely reported (Harris *et al.*, 1989). Visible enhancement in the growth of several vegetable plants have also been reported (Bashan *et al.*, 1989).

The plant may take up nitrogen more efficiently from the limited supply in the soil, resulting in a lower requirement of nitrogen fertilization to attain a certain yield (Bashan, 1990).

In addition to improved mineral uptake, *Azospirillum* inoculation improved water status in stressed sorghum plants. Inoculated plants were less water stressed, having more water in their foliage, higher leaf water potential and lower canopy temperature than uninoculated plants. It is likely that improved mineral and water uptake played a vital role in *Azospirillum*-plant association. There is evidence that some *A. brasilense* strains failed to improve uptake of several ions but, improved plant growth (Bashan *et al.*, 1990).

The above-ground plant responses to *Azospirillum* inoculation in cereals and non-cereal species were often reported with respect to increased total plant dry weight, amount of nitrogen in shoots and grains, total number of tillers, fertile tillers and ears, early heading and flowering time, increased number of spikes and grains per spike, increased grain weight, greater plant height and leaf size and higher germination rates (Bashan and Levanony, 1990). In addition, significant effect on development of the root system has frequently been observed.

The most marked effects of *Azospirillum* inoculation on plants are the various morphological changes in the root system. These changes are directly related to inoculum concentrations, which is higher than optimal levels had inhibitory effects, while low bacterial doses had no effect. The optimal inoculum level for seeds or seedlings of many cereals, vegetables, and other crop plants was 10^5-10^6 cfu ml^{-1}, for corn 10^7 cfu ml^{-1} and for tomato 10^8cfu ml^{-1}. However, an inoculums concentration of 10^8-10^{10} cfu ml^{-1} usually inhibited root development (Bashan and Levanony, 1990).

Positive effects of inoculation have been demonstrated on various root parameters, including increase in root length, number and length of lateral roots, root dry weight, the number, density, and early appearance of root hairs, root surface area, enhanced cell division in the root meristem, changes in cell

arrangements in the cortex and stimulation of root exudation (Bashan and Levanony, 1990).

Inoculation of plants with *A. brasilense* and *A. lipoferum* are usually done either by seed inoculation or by application of the bacteria directly to the soil as near as possible to the germinating seedlings. Although not a typical case for *Azospirillum*, the inoculation site is likely to determine its ultimate fate regarding root colonization and survival or death of cells (Bashan, 1999).

Apart from being a general plant colonizer (Bashan *et al.*, 2004), *Azospirillum* is remarkably versatile which can help plants minimize the negative effects of abiotic stresses. *Azospirillum* strains have no preferences for crop plants or weeds, or for annual or perennial plants, and can be successfully applied to plants that have no previous history of *Azospirillum* in their roots. It appears that *Azospirillum* is a general root colonizer and not a plant specific bacterium (Barassi *et al.*, 2007).

2.5.1. Azospirillum and Okra

Amrithalingam and Balakrishnan (1988) noticed increased plant height in bhendi cultivar K-1, when inoculated with *Azospirillum* + 75% recommended nitrogen. Similar results were noticed in Pusa Sawani cultivar of bhendi (Parvatham and Vijayan, 1989; Balasubramani, 1998).

Inoculation of *Azospirillum* to seed, soil and seedling increased the number of fruits per plant, fresh and dry weights of pod per plant and ascorbic acid content of bhendi (Balasubramani, 1998).

2.6 BACTERIA AS PHOSPHATE SOLUBILIZER

Higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non-rhizosphere soil (Katznelson *et al.*, 1962; Raghu and Mac Rae, 1966). Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds,

such as tri-calcium phosphate, di-calcium phosphate, hydroxy apatite and rock phosphate (Goldstein, 1986). *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important phosphate solubilizing bacteria (Subbarao, 1988). A large portion of soluble inorganic phosphate applied to soil is rapidly immobilized and becomes unavailable to plants (Dey, 1988).

Soil contains phosphate solubilizing bacteria which are capable of solubilizing insoluble phosphates through production of organic acids and chelating oxo acids from sugars (Halvorson *et al.*, 1990).

Most agricultural soils contain large reserves of phosphorus, a considerable part of which has accumulated because of regular applications of phosphatic fertilizers (Richardson, 1994). Concentration of bioavailable phosphorus in soil is very low (1.0 mg kg^{-1}soil) (Goldstein, 1994).

The phenomena of fixation and precipitation of phosphorus in soil is generally dependent on pH and soil type. Thus, in acid soils, phosphorus is fixed by free oxides and hydroxides of aluminium and iron, while in alkaline soils, it is fixed by calcium, causing a low efficiency of soluble phosphatic fertilizers (Goldstein, 1994). Efficiency of phosphatic fertilizer throughout the world is around 10 - 25% (Isherword, 1998). Population of PSB depends on different soil properties (physical and chemical properties, organic matter, and phosphorus content) and cultural characters (Kim *et al.*, 1998). Larger populations of PSB are found in agricultural soils (Yahya and Azawi, 1998).

High proportion of PSM is concentrated in the rhizosphere, and they are metabolically more active than from non-rhizosphere soils (Vazquez *et al.*, 2000). Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most efficient P solubilizers (Whitelaw, 2000).

Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa *et al.*, 2002). Inorganic forms of phosphorus are solubilized by a group of heterotrophic microorganisms excreting organic acids that dissolve phosphatic minerals and/or chelate cationic partners of the phosphorus ions i.e. PO_4^{3-} directly, releasing phosphorus into solution (He *et al.*, 2002). These bacteria in the presence of labile carbon serve as a sink for phosphorus by rapidly immobilizing it even in low phosphorus soils (Bünemann *et al.*, 2004).

Microbial community influences soil fertility through soil processes viz. decomposition, mineralization, and storage/release of nutrients. Microorganisms enhance the availability of phosphorus to plants by mineralizing organic phosphorus in soil and by solubilizing precipitated phosphates (Chen *et al.*, 2006). Crops can absorb phosphorus in the form of soluble orthophosphate ion. The solubility of phosphate is inhibited by the presence of iron and aluminium in acidic soils and calcium in neutral and alkaline soils. This leads to fixation of phosphorus, making it unavailable to crop plants. The phosphate solubilizing bacteria (phosphobacteria) secretes organic acids which act on insoluble phosphates and convert the same into soluble form (Ponmurugan and Gopi, 2006). Microbial biomass assimilates soluble phosphorus, and prevents it from adsorption or fixation (Khan and Joergesen, 2009).

2.7 ACID-TOLERANCE OF PSB

Pal (1999) reported an isolate (PAS 2) of pasture and waste land of pH 4.8 had highest phosphate solubilizing capacity (63%) which tolerated a wide range of soil acidity (pH 4.5-6.1). He also observed that strains of PSB isolated from soils of lower pH possessed greater acid tolerance than those isolated from higher pH ranges. Islam *et al.* (2007) have identified *Acinetobacter* sp. as an efficient P-solubilizer. They grew rapidly in the liquid medium at pH 5 and 7 but almost no growth occurred at pH 3.

2.8 EFFICIENCY OF PSB

Pseudomonas striata and *Bacillus polymyxa* solubilized 156 and 116 mg l^{-1} respectively (Rodríguez and Fraga, 1999).

Direct application of rock phosphate is often ineffective with in short period for most annual crops (Goenadi *et al*., 2000). Acid producing microorganisms are able to enhance the solubilization of rock phosphate (Gyaneshwar *et al*., 2002). The PSB in conjunction with single super phosphate and rock phosphate reduce the phosphorus dose by 25 and 50 %, respectively (Sundara *et al*., 2002).

The PSB solubilized the fixed soil phosphorus and applied phosphates resulting in higher crop yields (Gull *et al*., 2004). The PSB strains exhibited inorganic P-solubilization abilities ranging between 25-42 µg ml^{-1} and organic P mineralization abilities between 8-18 µg ml^{-1} (Tao *et al*., 2008).

The *Pseudomonas putida*, *P. fluorescens* Chao and *P. fluorescens* released 51, 29 and 62% phosphorus respectively with highest value of 0.74 mg/50 ml from Fe_2O_3 (Ghaderi *et al*., 2008). *P. fluorescens* solubilized 100 mg l^{-1} containing $Ca_3(PO_4)_2$ or 92 and 51 mg l^{-1} containing $AlPO_4$ and $FePO_4$ respectively (Henri *et al*., 2008).

2.9 EFFECT OF PSB ON PLANT GROWTH

Considerable evidence supports the specific role of phosphate solubilizing bacteria in the enhancement of plant growth. *Bacillus megaterium* var. *phosphaticum* was applied successfully in the former Soviet Union and India, but it did not show the same efficiency in soils in the United States. Simultaneous increases in phosphorus uptake and crop yields have been observed after inoculation with *Bacillus polymyxa* (Gaur and Ostwal, 1972), *Bacillus firmus* (Datta, 1982), and *Bacillus cereus* and others. Undoubtedly, the efficiency of the inoculation varies with the soil type, specific cultivar and other parameters. The P content of the soil is probably one of the crucial factors in determining the

effectiveness of the product. The production of chelating substances by microorganisms as well as the production of inorganic acids, such as sulphidric nitric and carbonic acid has been considered as the mechanisms involved in phosphate solubilization. The plant growth promotion by PSB is considered to be related to their ability to synthesize plant growth regulating substances (Sattar and Gaur, 1987). A strain of *Pseudomonas putida* stimulated the growth of roots and shoots and increased 32P-labeled phosphate uptake in canola.

Inoculation of rice seeds with *Azospirillum lipoferum* strain 34H increased the phosphate ion content and resulted in significant improvement of root length and fresh and dry shoot weights (Murty and Ladha, 1988).

Alternative possibilities other than organic acids for mineral phosphate solubilization have been proposed based on the lack of a linear correlation between pH and the amount of solubilized phosphorus (Asea *et al.,* 1988).

Complexing of cations is an important mechanism in P solubilization, if the organic acid structure favors complexation (Fox *et al.*, 1990). Rock phosphates are often too insoluble to provide sufficient phosphorus for crop uptake. Use of PSMs can increase crop yields up to 70 per cent (Verma, 1993). Chabot *et al.* (1993) demonstrated growth stimulation of maize and lettuce by several microorganisms capable of mineral phosphate solubilization.

Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, Pi may be released from a mineral phosphate by proton substitution for Ca^{2+} (Goldstein, 1994). The production of organic acids by phosphate solubilizing bacteria has been well documented. Type and position of the ligand in addition to acid strength determine its effectiveness in the solubilization process (Kpomblekou and Tabatabai, 1994).

Phosphate solubilization takes place through various microbial processes or mechanisms including organic acid production and proton extrusion (Surange *et al.,* 1995; Dutton and Evans, 1996; Nahas, 1996). Organic acids produced by PSB solubilized insoluble phosphates by lowering the pH, chelation of cations and

competing with phosphate for adsorption sites in the soil (Nahas, 1996). Carboxylic anions produced by PSB, have high affinity to calcium, solubilized more phosphorus than acidification alone (Staunton and Leprince, 1996). A strain of *Burkholderia cepacia* showed no indole acetic acid production, but displayed significant mineral phosphate solubilization and moderate phosphatase activity which improved the yield of tomato, onion, potato, banana and coffee.

Inorganic acids such as hydrochloric acid can also solubilize phosphate but they are less effective compared to organic acids at the same pH (Kim *et al.*, 1997). Phosphorus solubilizing activity is determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe *et al.*, 1998). Organic anions and associated protons are effective in solubilizing precipitated forms of soil phosphorus (like Fe and Al phosphates in acid soils and Ca phosphates in alkaline soils) (Jones, 1998).

Carboxylic acids mainly solubilize aluminium phosphate and iron phosphate (Henri *et al.*, 2008; Khan *et al.*, 2007) through direct dissolution of mineral phosphate as a result of anion exchange of PO_4^{3-} by acid anion or by chelation of both Fe and Al ions associated with phosphate (Omar, 1998). In certain cases, phosphate solubilization is induced by phosphate starvation (Gyaneshwar *et al.*, 1999). Higher crop yields resulted from solubilization of fixed soil phosphorus and applied phosphates by PSB (Zaidi, 1999).

Calcium phosphate (Ca-P) release results from the combined effects of pH decrease and carboxylic acids synthesis, but proton release cannot be the single mechanism (Deubel *et al.*, 2000). Moreover, carboxylic anions replace phosphate from sorption complexes by ligand exchange (Whitelaw, 2000) and chelate both Fe and Al ions associated with phosphate, releasing phosphate available for plant uptake after transformation.The PSB dissolve the soil P through production of low molecular weight organic acids mainly gluconic and keto-gluconic acids (Deubel *et al.*, 2000), in addition to lowering the pH of rhizosphere. The pH of rhizosphere

is lowered through production of proton or bicarbonate release (anion or cation balance) and gaseous (O_2/CO_2) exchanges. Phosphorus solubilization ability of PSB has direct correlation with pH of the medium.

Release of root exudates such as organic ligands can also alter the concentration of P in the soil solution (Hinsinger, 2001). Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and decrease the pH in basic soils (Kpomblekou and Tabatabai, 1994). Phosphate solubilizing bacteria application promoted P-uptake as well as the yields in several crops (Khalid *et al.*, 2004).

Phosphate solubilizing microbes can transform the insoluble phosphorus to soluble forms HPO_4^{2-} and $H_2PO_4^-$ by acidification, chelation, exchange reactions and polymeric substances formation (Delvasto *et al.*, 2006; Chang and Yang, 2009).

P-solubilizing activity of the PSB isolates was associated with the release of organic acids like citric acid, gluconic acid, lactic acid, succinic acid, propionic acid and three unknown organic acids. Their solubility increases with a decrease of soil pH. Phosphate solubilization is the result of combined effect of pH decrease and organic acids production (Fankem *et al.*, 2006). An inverse relationship between pH and P solubilized was also observed (Chen *et al.*, 2006).

The ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. The use of phosphate solubilizing bacteria as inoculants increased the P uptake by plants (Chen *et al.*, 2006). Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation (Ponmurugan and Gopi, 2006). *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soyabean crop (Son *et al.*, 2006).

Phosphate solubilizing bacteria (PSB) isolated from the crops grown in vertisols were tested for the production of Plant Growth Promoting Substances (PGPS) such as Indole Acetic Acid (IAA), Gibberellic Acid (GA) and organic acids and PSB were able to produce IAA and GA. The organic acids produced were gluconic acid, tartaric acid, citric acid, maleic acid, succinic acid, glyoxalic acid and a few unidentified acids (Vikram *et al.*, 2007).

Phosphate solubilizing ability is controlled by nutritional, physiological and growth conditions of the microbial culture (Reyes *et al.*, 2007), but it is mostly due to the lowering of pH alone by organic acids (Moghimi and Tate, 1978) or production of microbial metabolites (Abd-Alla, 1994).

Single and dual inoculation along with P fertilizer was 30-40% better than P fertilizer alone for improving grain yield of wheat, and dual inoculation without P fertilizer improved grain yield up to 20% against sole P fertilization (Afzal and Bano, 2008). Buffering capacity of the medium reduce the effectiveness of PSB in releasing P from tri-calcium phosphates (Stephen and Jisha, 2009). Greater efficiency of P solubilizing bacteria has been shown through co-inoculation with other beneficial bacteria and mycorrhiza (Khan *et al.*, 2009). Co-inoculation of PSM and PGPR reduced P application by 50% without affecting corn yield (Yazdani *et al.*, 2009).

2.10 EFFECT OF *AZOSPIRILLUM* AND PSB CONSORTIA ON PLANT GROWTH

The beneficial influence of phosphate solubilizing bacteria on survival of *Azotobacter* in the rhizosphere has been observed (Kundu and Gaur, 1980). Several studies demonstrated the beneficial influence of combined inoculation of phosphate-solubilizing bacteria and *Azotobacter* on yield, as well as on nitrogen and phosphorus accumulation in different crops. The effect of combined inoculation of rice with *Azotobacter* and phosphate solubilizing bacteria on yield,

nitrogen and phosphorus accumulation in plants were more significant than the effect of separate treatments (Kundu and Gaur, 1984).

Co-inoculation of sorghum with *Azospirillum* and *Glomus* significantly increased P, N, Zn, Cu and Fe content. Thus, co-inoculation may substitute partially as P and N fertilizer (Veeraswamy *et al.*, 1992). Co-inoculation of *Pseudomonas striata* and *Bacillus polymyxa* strains showing phosphate-solubilizing ability, with a strain of *Azospirillum brasilense*, resulted in a significant improvement of grain and dry matter yields, with an increase in N and P uptake, compared with separate inoculations with each strain (Alagawadi and Gaur, 1992).

Belimov *et al.* (1995) found that the inoculation of barley with bacterial mixtures (*A. lipoferum* 137 + *Agrobacterium radiobacter* 10 + *Arthrobacter mysorens* 7) provided a more balanced nutrition for the plants and the improvement in the root uptake of nitrogen and phosphorus was the major mechanism of interaction between plants and bacteria. Synergistic interactions on plant growth have been observed by co-inoculation of PSB with N_2 fixers such as *Azospirillum* (Belimov *et al.*, 1995) and *Azotobacter* or with vesicular arbuscular mycorrhizae (Kim *et al.*, 1998).

Phosphate-solubilizing *Agrobacterium radiobacter* combined with nitrogen fixer *Azospirillum lipoferum* produced improved grain yield of barley compared with single inoculations in pot and field experiments (Belimov *et al.*, 1995). Single and double inoculation with *Azotobacter*, *Azospirillum* and *Streptomyces* increased P, Mg and N content in wheat grains (Elshanshoury, 1995).

The applied mixed microbial consortium including AM fungi and rhizobacteria such as *Pseudomonas* sp., *Azospirillum* sp., *Pantoea* spp. increased growth and nutrient uptake of maize, wheat and legumes (Boddey and Germida, 1995). Two biofertilizer agents *Azospirillum amazonense* A10 and *Bacillus megaterium* P7, alone and in combination increased the grain yield of rice in autoclaved soil by 103-256% over control (Khan *et al.*, 2003).

El-komy (2005) studied phosphorus mobilization in wheat (*Triticum aestivum*) inoculated with *B. megaterium* or *A. lipoferum* 137 as single or mixed inocula in presence of Ca_3PO_4. They observed that wheat inoculated with mixed inocula exhibited high shoot dry weight, total nitrogen yield and the shoot phosphorus content increased by 37 and 53% compared to the plants inoculated with *A. lipoferum* and uninoculated ones, used as control, respectively. Maximum nitrogenase activity was observed in mixed inoculum treatment and was increased by 500 and 32% compared to uninoculated and *A. lipoferum* inoculated plants. Results demonstrated the beneficial influence of co-inoculation of *A. lipoferum* and *B.megaterium* for providing balanced N and P nutrition of wheat plants.

Investigations made by Raja *et al.* (2006) on the effect of individual and microbial consortium viz. *A. lipoferum*-Az 204, *Bacillus megaterium* var. *phosphaticum* and *Pseudomonas fluorescens* pf-1 on rice root exudates and plant growth under hydroponic culture conditions revealed that the bioinoculants consortium improved the colonization potential, sustainability within the inoculants and enhanced crop growth and confirmed the beneficial effects of microbial consortium over conventional single inoculants application method.

Raja *et al.* (2006) reported that the application of consortium of inoculants has brought more increase in plant growth promoting substances than any of the individual inoculants, but the increase in IAA content over individual treatments was minimal. Symbiotic relationship between PSB and plants is synergistic in nature as bacteria provide soluble phosphate and plants supply root borne carbon compounds (mainly sugars), that can be metabolized for bacterial growth (Perez *et al.*, 2007).

Askary *et al.* (2008) reported that, co-inoculation of wheat seeds with *A. brasilense* and *Rhizobium meliloti* had positive and significant effects on the grain yield and N, P, K content were compared to either single inoculation or control plants. Co-inoculation with symbiotic microorganisms to create a successful

system of biological nitrogen fixation in a non-leguminous crop can lead to many profits for plant.

Trimurtulu *et al.* (2011) observed the mixed microbial consortium of *Azospirillum* and *Azotobacter*, phosphate solubilizing bacteria, plant growth promoting rhizobacteria and AM fungi as the balanced combination of different microorganisms for achieving maximum output in the cultivation of chilli in vertisols of India.

Rajasekar and Elango (2011) tested the effect of microbial consortium of plant growth promoting rhizobacteria (PGPR) like *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus* separately and in combination on the growth of *Withania somnifera*. The combination of microbial consortium strains has a great potential for use as biofertilizer.

Rafi *et al.* (2012) reported that the panicle and seed weight of foxtail millet (*Setaria italica*) increased significantly due to inoculation with *Azospirillum* and PSB in combination compared to individual inoculum.

Mixed inoculants provided more balanced nutrition for the plants, and the improvement in N and P uptake was the major mechanism involved. This evidence points to the advantages of the mixed inoculations of PGPR strains comprising *Azospirillum* and phosphate-solubilizing bacteria.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

A study on "Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils" was carried out in Department of Agricultural Microbiology, College of Horticulture, Vellanikkara during the year 2011-13. Details of materials used and the methods followed are presented below.

3.1 COLLECTION OF SOIL SAMPLES FROM LATERITIC SOILS OF THRISSUR DISTRICT, KERALA

The rhizosphere and non-rhizosphere soil samples from lateritic soils were collected from ten different locations of Thissur district. The locations were selected at random based on the acidic nature of soil using agro-ecological map. Soil samples were collected at a depth of 5-10 cm using standard protocols. A 'V' shaped cut was made up to the plough layer and a thin slice of soil was taken from the cut ends to get soil from top to bottom of the pit. From each location, five random samples were collected and pooled together to get a representative soil sample by quartering technique. About 100 g of soil from each location was properly tagged, sealed and stored in refrigerator for further studies.

3.2 NUTRIENT STATUS OF SOIL COLLECTED FROM DIFFERENT LOCATIONS OF THRISSUR DISTRICT

The soil samples were analyzed for initial pH, organic carbon (%), available phosphorus and available potassium.

3.2.1. Soil reaction (pH)

The pH of the soil was determined in 1:2.5 soil-water suspensions. Ten gram of air-dried and 2 mm sieved soil was taken in a 50 ml beaker. Twenty five milliliter of distilled water was added, stirred well for about 5 min and kept for

half an hour and stirred well again and took the reading using the pH meter (Elico L1 120).

3.2.2. Organic carbon

The soil organic carbon was determined by using Walkley-Black wet digestion method (Walkley and Black, 1934). The soil was grinded to pass though a 0.5 mm sieve avoiding iron or steel mortars. Transferred 1.0 g of soil into a 500 ml wide mouth conical flask. 10 ml of 1N $K_2Cr_2O_7$ was added and swirled the flask gently to disperse the soil in the solution. Then 20 ml of concentrated H_2SO_4 was added rapidly. Immediately the flask was swirled gently until the soil and the reagents were mixed, vigorously for a total of one minute. The flask was allowed to stand on an asbestos sheet for about 30 min. Then 200 ml of water was added to the flask along with 3-4 drops of ferroin indicator and titrated the solution with 0.5 N ferrous ammonium sulphate.

As the end point approached, the solution attained a greenish cast and then changed to a dark green colour. At this point, the ferrous ammonium sulphate was added drop by drop until the colour changed sharply from blue to red. A blank determination was also made in the same manner, but without soil, to standardize the $Cr_2O_7^{2-}$.

$$\mathrm{OC}\ (\%) = \frac{\left(\mathrm{meq}\ K_2Cr_2O_7 - \mathrm{meq}\ Fe(NH_4)_2SO_4\right) \times 0.003 \times 100 \times 1.3}{\mathrm{weight\ of\ soil\ (g)}}$$

3.2.3. Available phosphorus

Available 'P' was extracted using Bray No. 1 (Bray and Kurtz, 1945), which consisted of 0.03 N NH_4F and 0.025 N HCl. Add five grams of soil to a 250 ml conical flask with 50 ml of Bray No.1 reagent and shake for five minutes. Filtering was done though Whatman No. 42 filter paper and to avoid interference of fluoride, 7.5 ml of 0.8 M (10 ml, 4%) boric acid (50 g H_3BO_3 per litre) was

added to 5 ml of the extract. Estimation was done by reduced molybdate blue colour method (Olsen *et al.,* 1954).

Five milliliter of the extract was pipetted out into a 25 ml volumetric flask and diluted to approximately 20 ml. Four milliliter of reagent B (Appendix Ii) was added and the volume was made up with distilled water and mixed the contents well. After 10 min, the intensity of colour was read at 660 nm. The colour was stable for 24 h and the maximum intensity developed within 10 min. The concentration of P in the sample was computed using standard curve.

For the preparation of standard curve, different concentrations of P at 1, 2, 3, 4, 5 and 10 ml of 2 μg ml^{-1} P solution was prepared in 25 ml volumetric flasks. Five milliliter of the extracting reagent (Bray No.1) was added and colour developed as described above by adding reagent B. The concentration vs. absorbance curve was plotted on a graph paper.

$$\text{Available P (mg/kg soil)} = \frac{\text{Absorbance for sample}}{\text{Slope of standard curve}} \times \frac{50}{5} \times \frac{25}{5}$$

3.2.4. Available potassium

Estimation was done by flame photometric method. Five gram of soil was mixed with 25 ml of neutral normal potassium acetate for five minutes and filtered immediately through a Whatman No. 42 filter paper. First few ml of the filtrate was discarded. Potassium concentration in the extract was determined using flame photometer after necessary settings and calibration of the instrument.

Standard curve for potassium was prepared by using standard solution of ammonium acetate. Measured aliquots were diluted from the standard solution using ammonium acetate solution to give concentrations of 5 to 20 μg ml^{-1} of K. After attaching the appropriate filter and adjusting the gas and air pressure, the reading was set in the flame photometer as zero for the blank (ammonium acetate)

and at 100 for 20 µg ml^{-1} of K. The curve was obtained by plotting the readings against the different concentrations (5, 10, 15 and 20 µg ml^{-1}) of K.

$$\text{Available K (mg kg}^{-1}\text{ soil)} = \mu\text{g K per ml of aliquot} \times \frac{25}{5}$$

3.3 ISOLATION AND ENUMERATION OF *AZOSPIRILLUM* ISOLATES

Test tubes containing 5.0 ml Nfb (Nitrogen free bromothymol blue) semi-solid medium (Appendix Ia) (Okon *et al.*, 1977) was inoculated with 100 µl of appropriate dilutions (10^{-3}, 10^{-4}, 10^{-5}) of soil suspension and enumeration was performed using most probable number (MPN) method (Appendix IV). White pellicle formation and blue colour development in the media were taken as positive for *Azospirillum*. After 5-7 days of incubation at 30±2^{0}C, 10 µl of pellicle forming culture was spread on Nfb-solid medium supplemented with ammonium chloride as nitrogen source. Morphologically divergent colonies were picked from the plates and transferred to fresh Nfb semi-solid medium. Colonies that showed white undulating pellicle formation with blue colour development were further purified and preserved.

3.4 CHARACTERIZATION OF *AZOSPIRILLUM* ISOLATES

Isolates obtained on Nfb semi-solid medium were identified through morphological characterization and biochemical tests.

3.4.1. Morphological characterization

The colonies of *Azospirillum* were streaked on BMS agar medium (Appendix-Ib) for pink colour development. The microscopic observations were made for gram reaction and motility. The isolates were inoculated on Nfb semi-solid medium for overnight growth and checked for the pellicle formation.

3.4.2. Biochemical tests

The tests for *Azospirillum*, such as phosphatase, oxidase, catalase, urease and starch hydrolysis were carried out (Tarrand *et al.*, 1979).

3.4.2.1. Phosphatase activity

The phosphatase activity was estimated from the amount of *p*-nitro phenol released, which was quantified based on the colour intensity at 430 nm in a spectrophotometer (Tabatabai and Bremner, 1969).

To 1.0 ml of the sample taken in a 50 ml conical flask, 1.0 ml of 1.0 per cent *p*-nitro phenol phosphate, 4.0 ml of distilled water and a few drops of toluene were added. The mixture was incubated for 24 h and one conical flask was maintained as control. After incubation, 5.0 ml of 0.1 N NaOH was added to the flask. The contents were mixed for a few seconds by shaking and microbial suspension was filtered through Whatman No. 1 filter paper. The development of yellow colour was taken as positive for phosphatase production.

3.4.2.2. Oxidase test

Oxidase test was carried out by spreading a well-isolated colony on oxidase disc (Hi-media). The reaction was observed within 5-10 seconds at 25-30^{0}C. Appearance of violet colour indicated positive reaction. A colour change later than 60 seconds or no change at all was considered as negative reaction.

3.4.2.3. Urease test

The urea broth (Appendix-Id) medium was inoculated with bacterial culture. The culture was incubated at 30±2^{0}C for 48 h. The phenol red indicator turned to pink due to alkaline nature of the medium because of ammonia production (Stuart *et al.*, 1945). Otherwise, indicator remained yellow at acidic range of pH which indicated no urease production.

3.4.2.4. Catalase test

Growth from an overnight culture was smeared on a glass slide. A drop of 3.0% hydrogen peroxide was added on it. Cultures showing immediate effervescence were treated as positive for catalase activity (Taylor and Achanzar, 1972).

3.4.2.5. Starch hydrolysis

Starch agar (Appendix Ie) plates were prepared and streaked with *Azospirillum* isolates. The isolates were allowed to grow at 32^0C for 48 h. Iodine solution was poured on to the plate. The blue-black colour appeared due to formation of starch-iodine complex (Priest, 1977). The clear zone around the colony indicated the degradation of starch, which occurred due to production of amylase.

3.5 ISOLATION AND ENUMERATION OF PHOSPHATE SOLUBILIZING BACTERIA (PSB)

Ten grams of soil sample was suspended in 90 ml of sterile water and serial dilutions of the suspension were made in sterile water blanks. One ml of 10^{-3}, 10^{-4} and 10^{-5} dilutions were plated on Pikovskaya's Agar medium (Appendix If) for obtaining microorganisms capable of dissolving insoluble phosphates. The plates were incubated for 4-5 days at 28 ± 2^0C. Transparent and clear zone around microbial colonies indicated the extent of phosphate solubilization (Sharma *et al.*, 2011). Such bacterial cultures were purified and maintained as PSB on Pikovskaya's agar slants for further studies. Number of colonies on the respective dilution was calculated and expressed as colony forming units per gram of soil (cfu g^{-1}).

3.6 SCREENING OF *Azospirillum* sp. AND PSB FOR ACID-TOLERANCE

3.6.1. *Azospirillum* isolates

3.6.1.1. Acid-tolerance of Azospirillum in liquid medium

Assessment of acid-tolerance of various *Azospirillum* strains in liquid medium were done in nutrient broth adjusted to pH 7.0, 6.5, 5.5, 4.5 and 3.5 (Pal, 1998). pH was adjusted before and after sterilization using either filter sterilized 0.1N NaOH or HCl. The 48 h old culture was inoculated @ 0.1 ml into nutrient broth with pH values 7.0, 6.5, 5.5, 4.5 and 3.5 respectively. O.D. values at 560 nm were taken after 72 h incubation. Growth in pH 6.5, 5.5, 4.5 and 3.5 were compared with pH 7.0 and percent change in acid-tolerance was calculated using the following formula.

$$\text{Per cent change in acid-tolerance over control} = \left[\frac{(\text{O.D. at pH 7.0 - O.D. at pH x})}{\text{O.D. at pH 7.0}}\right] \times 100$$

where, pH x = pH 3.5, 4.5, 5.5 or 6.5

The cultures having less per cent change in acid-tolerance were rated as the most acid-tolerant strains.

3.6.1.2. Acid-tolerance of Azospirillum on solid medium

Acid-tolerance test was carried out on solid medium. For this, pH of the nutrient agar medium was adjusted to 7.0, 6.5, 5.5 and 4.5 (Pal, 1998). pH of the media was adjusted before and after sterilization using either filter sterilized 1N NaOH or 1N HCl. The 48 h old culture was inoculated @ 0.1 ml on the nutrient agar media adjusted to pH 7.0, 6.5, 5.5 and 4.5 using spread plate method. Number of colonies at pH 6.5, 5.5 and 4.5 were compared with that of pH 7.0 and per cent relative population was calculated using the following formula.

$$\text{Percent relative population} = \left[\frac{\text{population at pH x}}{\text{population at pH 7.0}}\right] \times 100$$

Where, pH x = pH 4.5, 5.5 or 6.5. Values nearer to hundred were ranked as most acid-tolerant strains.

3.6.2. PSB isolates

3.6.2.1. Acid-tolerance of PSB in liquid medium

Assessment of acid-tolerance of various PSB strains in liquid medium were done in nutrient broth adjusted to pH 7.0, 6.5, 5.5, 4.5 and 3.5. The pH was adjusted before and after sterilization using either filter sterilized 0.1N NaOH or HCl. The 48 h old cultures were inoculated at 0.1 ml into nutrient broth having pH 7.0, 6.5, 5.5, 4.5 and 3.5. Optical density values at 560 nm were taken after 72 h incubation. Growth at pH 6.5, 5.5, 4.5 and 3.5 were compared with pH 7.0 and per cent change in acid-tolerance was calculated using the following formula.

$$\text{Per cent change in acid-tolerance over control} = \left[\frac{(\text{O.D. at pH 7.0 - O.D. at pH x})}{\text{O.D. at pH 7.0}}\right] \times 100$$

where, pH x = pH 3.5, 4.5, 5.5 or 6.5

The cultures having less per cent change in acid-tolerance were rated as the most acid-tolerant strains.

3.6.2.2 . Acid-tolerance of PSB on solid medium

Acid-tolerance test for PSB on solid medium was also carried out. For this, pH of the Pikovskaya's agar medium was adjusted to 7.0, 6.5, 5.5 and 4.5 respectively. The pH was adjusted before and after sterilization using either filter sterilized 1N NaOH or 1N HCl. 0.1 ml of 48 h old culture was inoculated on the Pikovskaya's agar medium having pH 7.0, 6.5, 5.5, 4.5 respectively using spread plate method. Number of colonies at pH 6.5, 5.5 and 4.5 were compared with that of pH 7.0 after 72 h incubation and per cent relative population was calculated using the following formula.

$$\text{Per cent relative population} = \left[\frac{\text{population at pH x}}{\text{population at pH 7.0}} \right] \times 100$$

Where, pH x = pH 4.5, 5.5 or 6.5. Isolates which showed more than fifty per cent relative population were ranked as most acid-tolerant strains.

3.7 SCREENING OF ACID-TOLERANT *Azospirillum* ISOLATES FOR ITS EFFICIENCY UNDER *IN VITRO*

3.7.1. Efficiency for N_2-fixation

Nitrogen fixation by each *Azospirillum* isolate was studied (Humphries, 1956). To a 250 ml conical flask, 100 ml of the nitrogen free semi-solid malate medium was dispensed and autoclaved at 15 lbs pressure for 15 min. The *Azospirillum* isolates were grown separately for 24 h in Nfb semi-solid medium and inoculated @ 2 ml/100 ml of the medium. Triplicate samples were kept for each isolate. The flasks were incubated at 32^0C for 14 days.

After 14 days of incubation, the culture was homogenized. Five mililitre of the homogenized culture was drawn and digested with 5 ml concentrated H_2SO_4 and 200 mg catalytic mixture (K_2SO_4:$CuSO_4$:Selenium in the ratio 100:10:1) until the contents become clear. After cooling, the volume was made upto 25 ml with distilled water. Then, 5 ml of aliquot was transferred to microkjeldhal distillation unit. An aliquot of 10 ml of 40% NaOH was added and steam distilled. Ammonia evolved was collected over 2% boric acid (20 ml) containing 2 drops of mixed indicator (83.3 mg bromocresol green+16.6 mg methyl red indicator dissolved in 10 ml of 95% ethanol) and back titrated against 0.05 N H_2SO_4. Total nitrogen content of the culture was determined and the results were expressed as mg of N fixed per gram of malate.

$$\text{Per cent nitrogen} = \frac{(TV \times N \times 0.014)}{W} \times \frac{25}{5} \times 100$$

Where,

TV	=	Titre volume
N	=	Normality of H_2SO_4
W	=	Weight or volume of sample used

3.7.2. Efficiency for indole acetic acid (IAA) production

In vitro auxin production by the selected isolates of *Azospirillum* was determined as indole acetic acid (IAA) equivalent in the presence of L-tryptophan (Khalid *et al.,* 2004). For this purpose, 10 ml nutrient broth were taken in 100 ml Erlenmeyer flasks, autoclaved and cooled. L-tryptophan was filter sterilized by passing though 0.2 µm membrane filters and added at the rate of 1.0 mg/ml to the liquid medium. The contents in the flask were inoculated with 1.0 ml of 3 days old bacterial broth adjusted to a population of 10^7-10^8 cfu/ml. The flasks were plugged tightly and incubated at 28 ± 2^0C for 10 days with non-inoculated media as control. After incubation, the contents were filtered though Whatman No. 2 filter paper. Auxin compounds (IAA equivalent) were determined by spectrophotometer. While measuring IAA equivalents, 2.0 ml filtrate was mixed with 2 drops of *o*-phosphoric acid and 4.0 ml of Salkowski reagent (2.0 ml of 0.5 M $FeCl_3$ + 98.0 ml of 35 % $HClO_4$). The contents in the test tubes were allowed to stand for half an hour for colour development. Similarly, colour was also developed in standard solutions of IAA. The intensity of colour was measured at 530 nm by using spectrophotometer. Standard curve was used for comparison to calculate auxin production by *Azospirillum* isolates.

3.8 SCREENING OF ACID-TOLERANT PSB ISOLATES FOR ITS EFFICIENCY UNDER *IN VITRO*

3.8.1. Quantitative estimation of phosphate solubilization

The bacterial isolates positive for P-solubilization on Pikovskaya's agar medium were subjected to quantification of inorganic phosphorus. The available P content of the supernatant was estimated by using phospho-molybdic blue colour method (Olsen *et al.*, 1954). The flasks containing 50 ml Pikovskaya's broth were inoculated with 500µl of overnight grown culture of each isolate and incubated for 10 days at 28±2^{0}C. The amount of Pi released in broth was estimated at 14 days of incubation along with the uninoculated control. The reduction in pH of the broth from the initial pH of 7.0 was also recorded after 14 days of incubation so as to determine the amount of acidity produced and correlate with the Pi released.

The Pikovskaya's broth cultures were centrifuged at 10,000 rpm for 10 min to separate the cells and insoluble phosphate. A known amount of supernatant was taken in test tube and volume of 8.6 ml made with distilled water. One milliliter of ammonium molybdate reagent was added followed by 0.4 ml of ANSA reagent. The contents were mixed for 10 min for colour development. Intensity of blue colour was read in a spectrophotometer at 660 nm. The amount of available 'P' present in the broth was calculated using standard graph of different known concentrations of P using KH_2PO_4.

3.8.2. Qualitative estimation of phosphate solubilization

Twenty microlitres of 24 h old PSB cultures were spotted on Pikovskaya's agar plate and incubated for seven days at 28±2^{0}C. The halo-zone and colony diameter were measured at 2, 5 and 7 days after incubation. The results were expressed as per cent solubilization efficiency (SE) (Nguyen *et al.*, 1992).

$$\text{Solubilization Efficiency (\%)} = \frac{SD}{CD} \times 100$$

Where, SD - Solubilization diameter

CD - Colony diameter

3.8.3. Efficiency for IAA production

Efficiency of phosphate solubilizing bacteria for IAA production was done as described in 3.7.2.

3.9 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF PSB ISOLATES

Most acid-tolerant and efficient six PSB isolates were subjected to its morphological and biochemical characterization such as gram reaction, cell shape, motility, oxidase, catalase, indole, methyl red, Voges-Proskauer, citrate utilization and urease tests.

3.10 COMPATIBILITY STUDIES BETWEEN *AZOSPIRILLUM* AND PSB ISOLATES

The cultures of *Azospirillum* and PSB were tested for their compatibility with each other by cross-streak assay method (Raja *et al.*, 2006) on nutrient agar medium. *Azospirillum* isolate was streaked at one end as a single streak and the PSB was streaked vertical to the test organism and plates were incubated at 32^0C for one week. Inhibition of the colonies of each isolate indicated non-compatibility between the isolates.

3.11 SELECTION OF *AZOSPIRILLUM* AND PSB ISOLATES FOR POT CULTURE STUDIES BASED ON ACID-TOLERANCE, EFFICIENCY AND COMPATIBILITY

Three most compatible, efficient and acid-tolerant consortia were selected for pot culture experiment. The combinations were selected based on compatibility and overall ranking for acid-tolerance and efficiency.

3.12 EVALUATION OF ACID-TOLERANT AND EFFICIENT CONSORTIA (*AZOSPIRILLUM* AND PSB) FOR GROWTH ENHANCEMENT OF OKRA

The most promising acid-tolerant, efficient and compatible consortia of *Azospirillum* and PSB were evaluated for its efficiency in enhancing nitrogen and phosphorus uptake in okra under pot culture studies. The experiment was conducted during February-April, 2013 at College of Horticulture, Vellanikkara. The treatment details of experiment were as follows:

Design : CRD

Test crop : Okra

Variety : Arka Anamika

Treatments : 7

Replication : 3

Number of plants per treatment: 15

T_1 : FYM 25 t/ha + N:P:K @ 110:35:70 kg ha^{-1} (POP recommendation)

T_2 : AND-4 + PMD-7 (*Azospirillum* + *Acinetobacter* sp.) + FYM 25 t/ha + 70 kg ha^{-1} K

T_3 : AMU-2 + PMD-7 (*Azospirillum* + *Acinetobacter* sp.) + FYM 25 t/ha + 70 kg ha^{-1} K

T_4 : AND-4 + POL-1 (*Azospirillum* + *Bacillus megaterium*) + FYM 25 t/ha+ 70 kg ha^{-1} K

T_5 : T_2 + T_3 + T_4 + FYM 25 t/ha + 70 kg ha^{-1} K

T_6 : Consortium - TNAU (Azophos) + FYM 25 t/ha+ 70 kg ha^{-1} K

T_7 : Absolute control

The consortia were applied (10^8 cfu ml^{-1} of each isolate) at the time of sowing as seed treatment based on KAU POP recommendation.

3.12.1. Weather data during crop growth

The weekly weather data for the period from February, 2013 to April, 2013 was provided by the Department of Agrl. Meteorology, College of Horticulture, Thissur.

3.12.2. Preparation of potting mixture and sowing

The potting mixture was prepared with sand:soil:cowdung in the ratio of 1:1:1. After seed treatment, seeds were directly sown in the pots.

3.12.3. Seed treatment

For treating 50-100 g seeds, 5 ml of culture was used. Seeds were moistened by sprinkling rice gruel water and 5 ml culture was taken in a petri-dish to which moistened seeds were added. They were mixed well, dried under shade for 30 min. and sown immediately.

3.12.4. Observations

Observations on germination percentage, plant height, number of leaves, days to flowering, number of fruits per plant, yield per plant, fresh and dry weight of shoot, root and plant and pest and disease incidence were recorded.

3.12.4.1. Germination percentage

Germination percentage was calculated using the following formula:

$$\text{Germination percentage} = \frac{\text{No. of plants germinated}}{\text{Total no. of seeds sown}} \times 100$$

3.12.4.2. Plant height

The distance from the base of the plant to the tip was taken as plant height at 30 days interval and expressed in centi meters.

3.12.4.3. Number of leaves

Total number of leaves per plant was counted from each plant and mean number of leaves were obtained.

3.12.4.4. Days to flowering

Number of days taken from sowing to opening of first flower was recorded and mean was found out.

3.12.4.5. Number of fruits per plant

Total numbers of fruits were counted from each plant and mean was obtained.

3.12.4.6. Fruit yield

Fruit yield was recorded at 60 das and at harvest (90 das). Per cent increase in fruit yield in different treatments over control was also calculated.

3.12.4.7. Pest and disease incidence

Pest and diseases on plants were recorded throughout the period of study.

3.12.4.8. Total dry matter production

Plant samples for the estimation of dry matter were collected at the time of harvest (90 das). Plants from each treatment were partitioned into shoot and root. These samples were oven dried at 65^0C for 72 h till a constant weight was

achieved. The dry weight of different plants was recorded and the total dry matter production was obtained in grams per plant.

3.12.5. Chemical analysis of plant

3.12.5.1. Collection and preparation of plant samples

Mature leaves were collected from the plant for nutrient analysis at the time of harvest. Samples from individual treatment were dried in an oven at 60^0C till constant weight was observed and further ground to fine powder using pestle and mortar. The powdered samples were used for nutrient analysis.

3.12.5.2. Nitrogen content in plant

Nitrogen content of plant was estimated by modified microkjeldhal method (Jackson, 1973) at harvest. Dried leaf samples (0.5 g) were digested with 5 ml of concentrated H_2SO_4 and 200 mg digestion catalyst (K_2SO_4:$CuSO_4$:Selenium in the ratio of 100:10:1) until the contents became clear. After cooling, the volume was made up to 25 ml with distilled water. Then, 5 ml of aliquot was transferred to microkjeldhal distillation unit. An aliquot of 10 ml of 40 per cent sodium hydroxide was added and steam distilled. Ammonia evolved was trapped in 2 per cent boric acid (20 ml) containing 2 drops of mixed indicator (83.3 mg bromocresol green and 16.6 mg methyl red indicator dissolved in 10 ml of 95 % ethanol) and back titrated against 0.05 N H_2SO_4. Nitrogen uptake was expressed as percentage.

$$\text{Nitrogen (\%)} = \frac{(TV \times N \times 0.014)}{W} \times \frac{25}{5} \times 100$$

Where,

TV	-	Titre Volume
N	-	Normality of H_2SO_4
W	-	Weight of sample used

3.12.5.3. Phosphorus content in plant

Phosphorus content in the plant sample was estimated by using vanado-molybdate reagent (Sarruge and Haag, 1974). Five milliliter of digested sample was transferred to 25 ml volumetric flask and added 5 ml of vanado-molybdate reagent. Volume was made up with distilled water and mixed the contents thoroughly. Absorbance of the solution was read after 30 min at 420 nm using spectrophotometer. The concentration (ppm) of P was found out using standard curve.

The standard curve was prepared by transferring 0, 1, 2, 3, 4 and 5 ml of standard P solution to 25 ml volumetric flask to get 0, 2, 4, 6, 8 and 10 ppm of P respectively. Five milliliter of vanado-molybdate reagent was added to each flask. Volume was made up and mixed it thoroughly. Absorbance was read at 420 nm after 30 min using spectrophotometer. Absorbance was plotted against concentration on a standard graph.

Per cent P in the sample

$$= \frac{\text{conc. from the graph (ppm)} \times \text{vol. of digested sample} \times \text{vol. made}}{\text{wt. of sample} \times \text{aliquot taken} \times 10^6} \times 100$$

3.12.5.4. Chemical analysis of soil sample

Soil samples were analysed at the time of harvest. Total nitrogen per cent and available phosphorus was analysed as desribed in 3.2.2 and 3.2.3.

3.13 16S rDNA SEQUENCE ANALYSIS OF EFFICIENT *AZOSPIRILLUM* AND PSB ISOLATES

After the evaluation of three most acid-tolerant, efficient and compatible consortia of *Azospirillum* and PSB under pot culture experiment, 16S rDNA

sequence analysis of the consortial isolates were carried out to identify the isolates.

3.13.1. Amplification of 16S rDNA gene

A colony was taken by micropipette tip, mixed with 10 µl sterile water. 2µl of the culture suspension was used as template for amplification of 16S rDNA gene. The details of primer used are given below.

Primer details	**Sequence 5'- 3'**	**Base pair**
8 F	AGAGTTTGATCCTGGCTCAG	20
1522 R	AAG GAG GTG ATC CAG CCG CA	20

Polymerase chain reaction was carried out in Eppendorf Master Cycler (Gradient) using PCR master mix 'Emerald Amp GT PCR'. The composition of the reaction mixture for PCR is as follows:

Component	**Per reaction volume required**
Master Mix	12.5 µl
Template	2.0 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
dH_2O	9.5 µl
Total	25.0 µl

The reaction was set in 200 µl microfuge tube chilled over ice flakes. A momentary spin was given to mix completely all reagents and set in master cycler for amplification. The details of master cycler programme are as follows:

No.	**Step**	**Temperature (^{0}C)**	**Time (min)**
1	Initial denaturation	95	3.00
2	Denaturation	94	1.30
3	Annealing	55	0.40
4	Primer extension	72	01.30
5	Steps 2 – 4	34 cycles	-
6	Final extension	72	20.00
7	Final hold	4	10.00

3.13.2. Agarose gel electrophoresis

The quality of isolated DNA was evaluated though agarose gel electrophoresis (Sambrook *et al.*, 1989). 1X TAE buffer was prepared from the 50X TAE (pH 8.0) stock solutions. Agarose (Genei, Low EEO) (1%) was weighed and dissolved in TAE buffer by boiling. Ethidium bromide prepared from a stock of 10 mg ml^{-1} was added to it at a concentration of 0.5 µg ml^{-1} and mixed well. The comb was placed properly and dissolved agarose was poured into the tray. The gel was placed in the electrophoresis unit after 30 min with well side directed towards the cathode. 1X TAE buffer was added to the buffer tank (Genei, Bangalore) so as to cover the well with a few millimeter of buffer. 2 µl of the PCR product was carefully loaded into the wells using a micro pipette. The Gene Ruler 1 kb DNA ladder was used as the molecular weight marker. The cathode and anode of the electrophoresis unit were connected to the power pack and the

gel was run at constant voltage of 100 V. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

3.13.3. Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using gel documentation imaging system.

3.13.4. Purification and sequencing of PCR product

The PCR product was purified and sequenced at Scigenom Pvt. Ltd. Cochin, using the primers 8F and 1522r.

3.13.5. Nucleotide sequence analysis

The blastn programme (http://blast.ncbi.nlm.nih.gov/Blast.) was used to find out the homology of the nucleotide sequences.

3.14 STATISTICAL ANALYSIS

Analysis of variance was done on the data collected using the statistical package MSTAT (Freed, 1986). Multiple comparisons among the treatment means were done using DMRT.

RESULTS

4. RESULTS

The experimental results obtained from the studies on “Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils” are presented below.

4.1 COLLECTION OF SOIL SAMPLES FROM LATERITIC SOILS OF THRISSUR DISTRICT, KERALA

Lateritic soil samples were collected from ten identified locations of Thrissur district (Table 1, Plate 1). Out of ten samples collected, five samples were from rhizosphere regions (banana, amorphophallus, coconut, cocoa, napier grass) and five samples were collected from non-rhizosphere regions of homesteads.

4.2 NUTRIENT STATUS OF SOIL COLLECTED FROM DIFFERENT LOCATIONS OF THRISSUR DISTRICT

4.2.1. Soil reaction (pH)

The pH of the soil samples were found to be acidic which ranged from 5.22 to 6.51 (Table 2, Fig. 2). Soil from Vellanikkara showed highest acidic pH (5.22) and Wadakkancherry (6.49) and Madakkathara (6.51) soils showed near neutral pH.

4.2.2. Organic carbon

Organic carbon content was found to be high in soil samples collected from Ollur (OL), Vellanikkara (VL), Elanad (EL), Chelakkara (CH) and Perumpilavu (PV) (2.26, 2.14, 2.0, 1.85 and 1.66 % respectively). It was medium in soil samples collected from Nadavarambu (ND), Koratty (KR), Madakkathara (MD),

Table 1. Details of soil samples collected from lateritic soils of Thrissur district in Kerala

Sl. No.	Location (Code)	Panchayat	Geographical Position	Rhizosphere/ Non-rhizosphere	Host
1	Chelakkara (CH)	Chelakkara	N 10.69494^0 E 076.34150^0	Non-Rhizosphere	-
2	Koratty (KR)	Koratty	N 10.26587^0 E 076.35012^0	Rhizosphere	*Musa* spp. (Banana)
3	Elanad (EL)	Pazhayannoor	N 10.61812^0 E 076.39014^0	Non-Rhizosphere	-
4	Madakkathara (MD)	Madakkathara	N 10.56236^0 E 076.26032^0	Rhizosphere	*Amorphophallus paeoniifolius* (Elephant foot yam)
5	Mulayam (MU)	Ollukkara	N 10.53029^0 E 076.28281^0	Rhizosphere	*Cocos nucifera* (Coconut)
6	Nadavarambu (ND)	Velukkara	N 10.32122^0 E 076.21890^0	Non-Rhizosphere	-
7	Ollur (OL)	Avinissery	N 10.48139^0 E 076.19782^0	Rhizosphere	*Theobroma cacao*(Cocoa)
8	Perumpilavu (PV)	Kadavalloor	N 10.69707^0 E 076.09410^0	Non-Rhizosphere	-
9	Vellanikkara (VL)	Madakkathara	N 10.54779^0 E 076.28381^0	Rhizosphere	*Pennisetum purpureum* (Napier grass)
10	Wadakkanchery (WD)	Wadakkanchery	N 10.70649^0 E 076.26331^0	Non-Rhizosphere	-

(- No host)

Table 2. Nutrient status of soil samples collected from different locations of Thrissur district

<table>
<tr><th rowspan="2">Location</th><th rowspan="2">pH</th><th colspan="3">Organic carbon (%)</th><th colspan="3">Available P (kg ha^{-1})</th><th colspan="3">Available K (kg ha^{-1})</th></tr>
<tr></tr>
<tr><td>Chelakkara (CH)</td><td>6.21</td><td colspan="3">1.85</td><td colspan="3">107.48</td><td colspan="3">310.24</td></tr>
<tr><td>Koratty (KR)</td><td>6.23</td><td colspan="3">1.32</td><td colspan="3">70.65</td><td colspan="3">464.80</td></tr>
<tr><td>Elanad (EL)</td><td>6.50</td><td colspan="3">2.0</td><td colspan="3">96.05</td><td colspan="3">331.52</td></tr>
<tr><td>Madakkathara (MD)</td><td>6.51</td><td colspan="3">1.28</td><td colspan="3">141.72</td><td colspan="3">918.40</td></tr>
<tr><td>Mulayam (MU)</td><td>6.29</td><td colspan="3">1.17</td><td colspan="3">62.68</td><td colspan="3">249.76</td></tr>
<tr><td>Nadavarambu (ND)</td><td>6.39</td><td colspan="3">1.45</td><td colspan="3">105.10</td><td colspan="3">534.24</td></tr>
<tr><td>Ollur (OL)</td><td>5.93</td><td colspan="3">2.26</td><td colspan="3">324.37</td><td colspan="3">321.44</td></tr>
<tr><td>Perumpilavu (PV)</td><td>5.48</td><td colspan="3">1.66</td><td colspan="3">75.60</td><td colspan="3">390.88</td></tr>
<tr><td>Vellanikkara (VL)</td><td>5.22</td><td colspan="3">2.14</td><td colspan="3">33.82</td><td colspan="3">420.0</td></tr>
<tr><td>Wadakkancherry (WD)</td><td>6.49</td><td colspan="3">1.02</td><td colspan="3">62.50</td><td colspan="3">752.64</td></tr>
<tr><td colspan="2" rowspan="2">Nutrient range</td><td><0.7</td><td>>0.7 to 1.5<</td><td>>1.5</td><td><10</td><td>>10 to 24<</td><td>>24</td><td><115</td><td>>115 to 275<</td><td>>275</td></tr>
<tr><td>L</td><td>M</td><td>H</td><td>L</td><td>M</td><td>H</td><td>L</td><td>M</td><td>H</td></tr>
</table>

L : Low

M : Medium

H : High

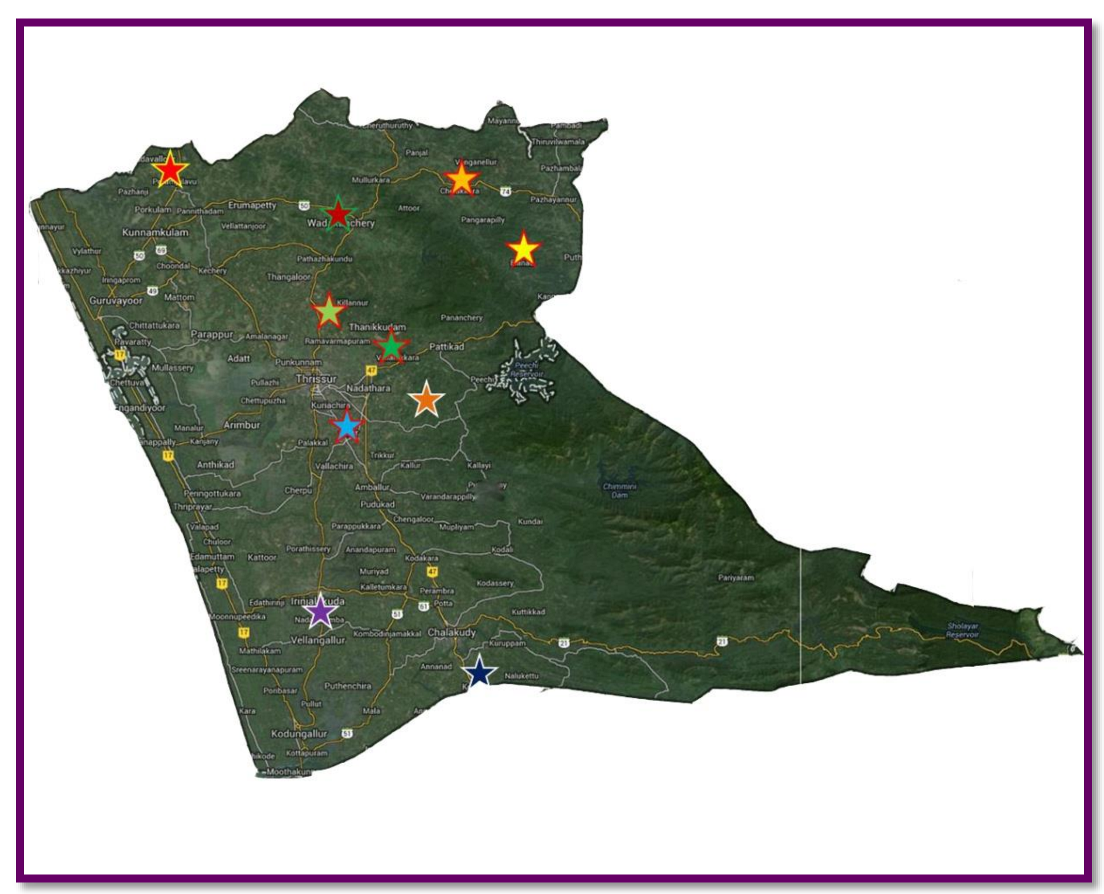


Plate1. Location map of soil samples collected from Thrissur district

Mulayam (MU) and Wadakkancherry (WD) (1.45, 1.32, 1.28, 1.17 and 1.02 per cent respectively) (Table 2).

4.2.3. Available phosphorus

Available phosphorus was found to be high in soils collected from all the ten locations, which ranged from 33.82 kg ha^{-1} to 324.37 kg ha^{-1} (Table 2). Highest available P was recorded in Ollur (OL) (324.37 kg ha^{-1}) and lowest was in Vellanikkara (VL) (33.82 kg ha^{-1}).

4.2.4. Available potassium

The available K in all the soil samples collected ranged from 249.76 kg ha^{-1} to 918.40 kg ha^{-1}. Available K was found to be high in all the locations except from Mulayam (MU) (249.76 kg ha^{-1}) which was in medium range (Table 2). Highest available K was recorded in Madakkathara (MD) (918.4 kg ha^{-1}).

4.3 ISOLATION AND ENUMERATION OF *AZOSPIRILLUM* ISOLATES

Of the ten locations, Madakkathara (MD) and Mulayam (MU) recorded highest population ($1.1x10^5$ MPN g^{-1}) whereas, Chelakkara (CH), Elanad (EL) and Perumpilavu (PV) showed lowest population ($0.9x10^4$ MPN g^{-1}) (Table 3, Plate 2).

A total of 32 isolates with subsurface white pellicle and blue colour development were further confirmed on BMS agar medium (Appendix 1b). Only six isolates (AMU-2, ACH-1, AND-4, AEL-3, AOL-4, AWD-1) were found to be positive for *Azospirillum* (Table 3). These six isolates were maintained on nutrient agar slants and used for further studies.

Table 3. Enumeration and confirmation of *Azospirillum* isolates from different lateritic soils

Sl. No.	Location	Total population of *Azospirillum* in each location (MPN g^{-1})	No. of isolates obtained from each location	Morpho types	Confirmation of *Azopsirillum* on BMS agar
1	Chelakkara	0.9×10^4	2	ACH – 1	+
2	Chelakkara			ACH – 2	-
3	Koratty	0.1×10^5	1	AKR – 1	-
4	Elanad	0.9×10^4	3	AEL – 1	-
5	Elanad			AEL – 2	-
6	Elanad			AEL – 3	+
7	Madakkathara	1.1×10^5	1	AMD – 1	-
8	Mulayam	1.1×10^5	2	AMU – 1	-
9	Mulayam			AMU – 2	+
10	Nadavarambu	0.1×10^5	9	AND – 1	-
11	Nadavarambu			AND – 2	-
12	Nadavarambu			AND – 3	-
13	Nadavarambu			AND – 4	+
14	Nadavarambu			AND – 5	-
15	Nadavarambu			AND – 6	-
16	Nadavarambu			AND – 7	-
17	Nadavarambu			AND – 8	-
18	Nadavarambu			AND – 9	-
19	Ollur	0.4×10^5	4	AOL – 1	-
20	Ollur			AOL – 2	-
21	Ollur			AOL – 3	-
22	Ollur			AOL – 4	+
23	Perumpilavu	0.9×10^4	4	APV – 1	-
24	Perumpilavu			APV – 2	-
25	Perumpilavu			APV – 3	-
26	Perumpilavu			APV – 4	-
27	Vellanikkara	0.1×10^5	2	AVL – 1	-
28	Vellanikkara			AVL – 2	-
29	Wadakkanchery	1.3×10^4	4	AWD – 1	+
30	Wadakkanchery			AWD – 2	-
31	Wadakkanchery			AWD – 3	-
32	Wadakkanchery			AWD – 4	-

'+' : Isolates which developed pink colour on BMS agar medium

'-' : No pink colour development on BMS agar medium

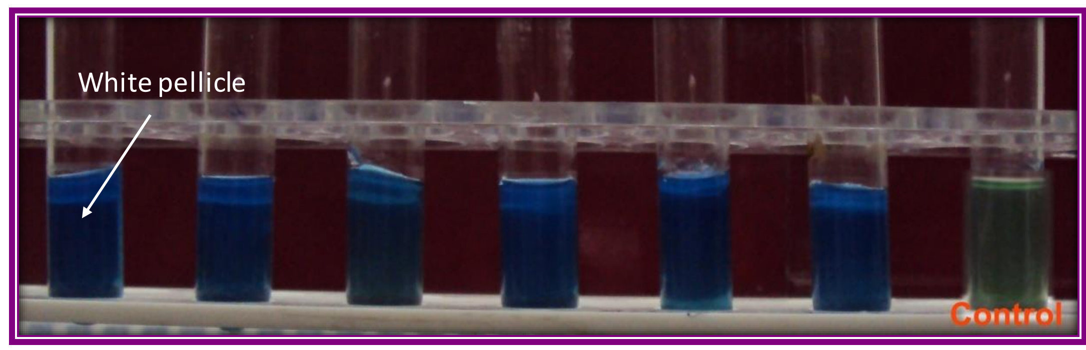


Plate 2. *Azospirillum* isolates in Nfb semi-solid media

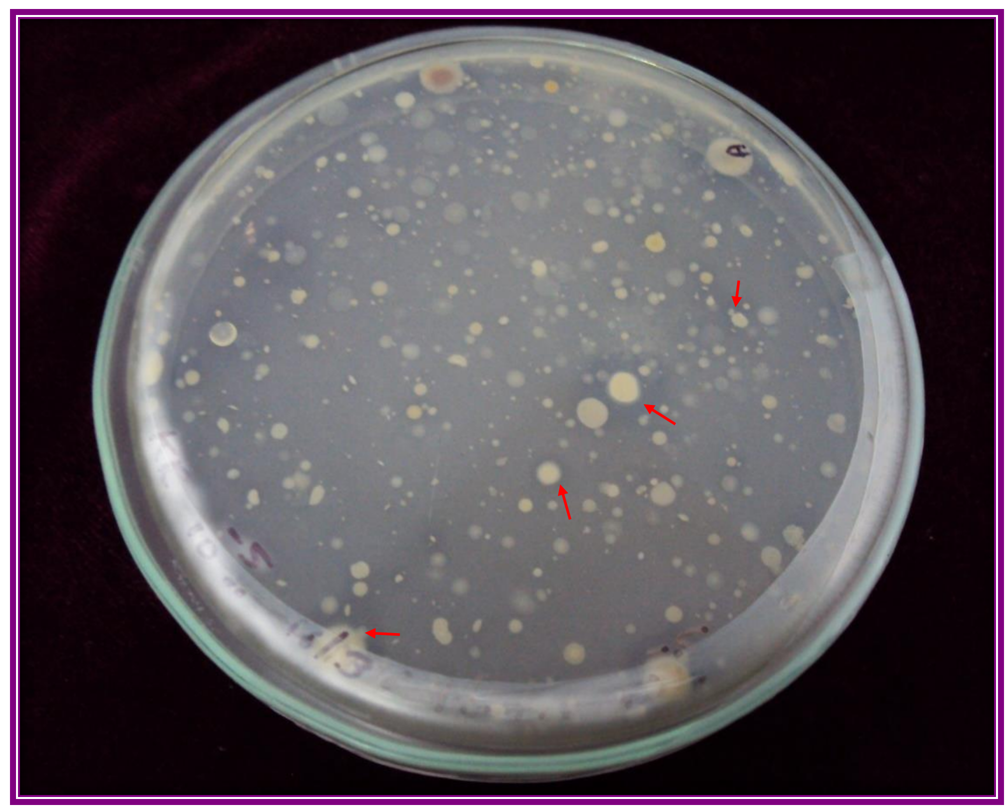


Plate 3. Colonies of phosphate solubilizing bacteria (PSB) on Pikovskaya's agar media

4.4 CHARACTERIZATION OF *AZOSPIRILLUM* ISOLATES

4.4.1. Morphological characterization

All the six isolates were found to be gram negative and motile. All the isolates formed sub-surface white pellicle with blue colour development on Nfb semi-solid media and developed pink colour on BMS agar medium with typical umbonate elevation (Table 4, Plate 4).

4.4.2. Biochemical tests

All isolates showed positive reaction for phosphatase, oxidase, urease, catalase and negative for starch hydrolysis (Table 4, Plate 5).

4.5 ISOLATION AND ENUMERATION OF PHOSPHATE SOLUBILIZING BACTERIA (PSB)

A total of 35 PSB strains were obtained from ten lateritic soil samples of Thrissur district (Table 5). Those isolate which exhibited clear zone around the colony on Pikovskaya's agar medium were purified and maintained as PSB on Pikovskaya's agar slants (Plate 3). Highest morpho-types of PSB isolates were obtained from Koratty (KR) (8 morpho-types) and lowest (one each) from Chelakkara (CH), Nadavarambu (ND) and Perumpilavu (PV) and Vellanikkara (VL). The population of PSB ranged from $100 x 10^5$ cfu g^{-1} to $0.1 x 10^5$ cfu g^{-1}. The PMU-2 isolate from Mulayam (MU) recorded highest population (10^7 cfu g^{-1}) and lowest in Wadakkanchery (10^4 cfu g^{-1}).

4.6 SCREENING OF *Azospirillum* sp. AND PSB FOR ACID-TOLERANCE

4.6.1. *Azospirillum* isolates

4.6.1.1. Acid-tolerance of Azospirillum in liquid medium (Nutrient Broth)

Table 4. Characteristics of *Azospirillum* isolates

Characteristics	**Isolates**					
	AMU-2	**ACH-1**	**AND-4**	**AEL-3**	**AOL-4**	**AWD-1**
Gram reaction	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Sub surface pellicle with blue colour development on semi-solid Nfb medium	+	+	+	+	+	+
Pink colour development on BMS agar	+	+	+	+	+	+
Urease activity	+	+	+	+	+	+
Phosphatase activity	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-
Oxidase activity	+	+	+	+	+	+
Catalase	+	+	+	+	+	+

'+' : Positive reaction

'-' : Negative reaction

AMU-2: *Azospirillum* from Mulayam

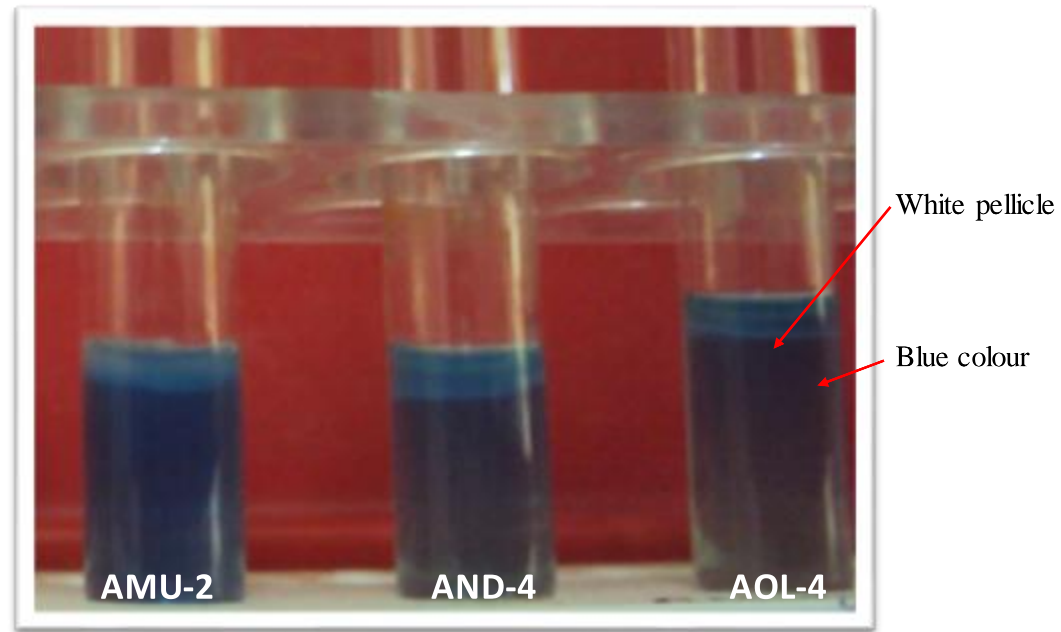
ACH-1 : *Azospirillum* from Chelakkara

AND-4 : *Azospirillum* from Nadavarambu

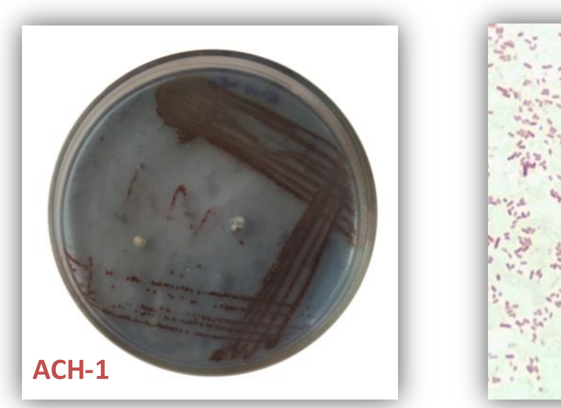
AEL-3 : *Azospirillum* from Elanad

AOL-4 : *Azospirillum* from Ollur

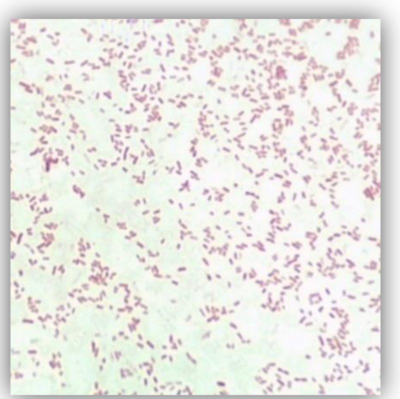
AWD-1: *Azospirillum* from Wadakkancherry



a. Subsurface white pellicle with blue colour development on Nfb semi-solid medium

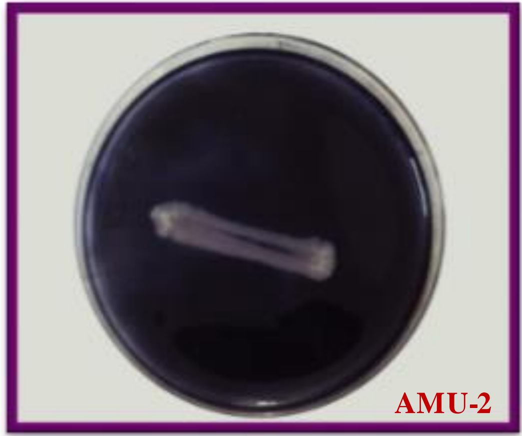


b. Pink colour colony on BMS agar medium

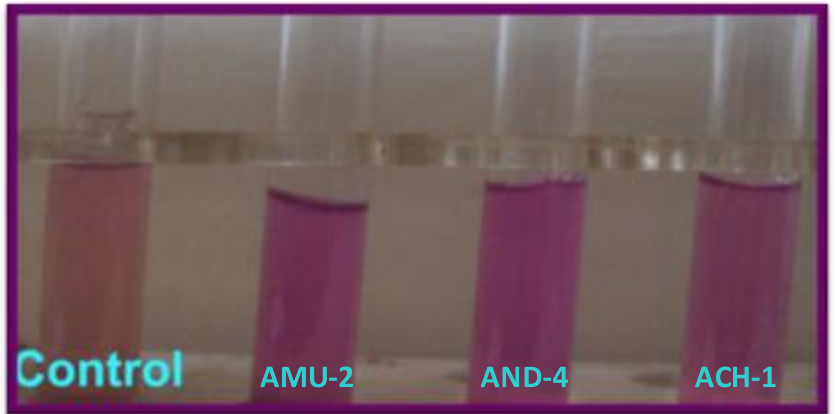


c. Gram reaction (Gram -ve)

Plate 4. Cultural characteristics of *Azospirillum* isolates



a. Starch hydrolysis



b. Urease test

c. Oxidase test

Plate 5. Biochemical characteristics of *Azospirillum* isolates

Table 5. Enumeration and morpho-types of phosphate solubilizing bacteria (PSB) from lateritic soils

Sl.No.	Location	Morpho-types	No. of isolates obtained from each location	x 10^5 cfu g^{-1} of soil
1	Chelakkara	PCH – 2	1	10.0
2	Koratty	PKR – 2	8	1.0
3	Koratty	PKR – 3		4.0
4	Koratty	PKR – 4		1.0
5	Koratty	PKR – 1		2.0
6	Koratty	PKR – 5		1.0
7	Koratty	PKR – 6		3.0
8	Koratty	PKR – 7		2.0
9	Koratty	PKR – 8		5.0
10	Elanad	PEL – 6	3	4.0
11	Elanad	PEL – 4		2.0
12	Elanad	PEL – 3		6.0
13	Madakkathara	PMD – 6	7	1.0
14	Madakkathara	PMD – 5		3.0
15	Madakkathara	PMD – 4		15.0
16	Madakkathara	PMD – 3		6.0
17	Madakkathara	PMD – 1		1.0
18	Madakkathara	PMD – 2		9.0
19	Madakkathara	PMD – 7		1.0
20	Mulayam	PMU – 1	4	10.0
21	Mulayam	PMU – 2		100.0
22	Mulayam	PMU – 3		7.0
23	Mulayam	PMU – 4		10.0
24	Nadavarambu	PND – 6	1	10.0
25	Ollur	POL – 1	4	10.0
26	Ollur	POL – 2		3.0
27	Ollur	POL – 3		4.0
28	Ollur	POL – 4		2.0
29	Perumpilavu	PPV-1	1	1.0
30	Vellanikkara	PVL – 3	1	14.0
31	Wadakkanchery	PWD – 1	5	1.0
32	Wadakkanchery	PWD – 2		1.0
33	Wadakkanchery	PWD – 3		7.0
34	Wadakkanchery	PWD – 4		0.10
35	Wadakkanchery	PWD – 9		4.0

All the six isolates selected for the studies were found to grow at pH ranging from 4.5 to 7.0 but, none were tolerant at pH 3.5 (Table 6). Only three isolates (AND-4, AMU-2, ACH-1) showed less than forty per cent change in population over neutral pH. Among the three isolates, AND-4 (Nadavarambu) was found to be more tolerant to acidic pH upto 4.5 followed by AMU-2 (Mulayam) (Plate 6a). AWD-1 (Wadakkanchery) was found to be least tolerant to acidic pH.

4.6.1.2. Acid-tolerance of Azospirillum on solid medium (Nutrient Agar)

Ranking of acid-tolerance was determined statistically based on per cent relative population. All the six isolates selected for studies were found to grow at pH ranging from 4.5 to 7.0 (Table 7). Only four isolates were (AND-4, AMU-2, ACH-1, AEL-3) able to show more than fifty per cent relative population at acidic pH to neutral pH. More per cent relative population was shown by AND-4 (82.87) followed by AMU-2 (74.32). Least per cent relative population at acidic pH when compared with neutral pH was shown by AWD-1 (35.42).

4.6.2. PSB isolates

4.6.2.1. Acid-tolerance of PSB in liquid medium (Nutrient Broth)

All the 35 isolates were found to grow at pH ranging from 4.5 to 7.0 (Table 8). But, none of the isolates were tolerant at pH below 4.5 except POL-3 (Ollur) which was tolerant upto pH 3.5 (Plate 6b). Among 35 isolates, only six isolates namely POL-3, POL-1, PMD-7, PKR-3, PKR-8 and PMD-2 were able to show less than forty per cent change in population at acidic pH over neutral pH.

4.6.2.2. Acid-tolerance of PSB on solid medium (Pikovskaya's Agar)

Ranking of acid-tolerance was done statistically based on the average of percent relative population at pH 5.5 and pH 4.5 to neutral pH. Among 35 PSB isolates, only six isolates were able to show more than fifty per cent relative

Table 6. Screening of *Azospirillum* sp. for acid-tolerance in nutrient broth

Isolates	**Per cent change in population over neutral pH**			**Ranking of acid-tolerant *Azospirillum***
	At pH 5.5	**At pH 4.5**	**Sum**	
AMU-2	7.69	12.82	20.51	2
ACH-1	2.34	20.47	22.81	3
AND-4	2.53	9.49	12.03	1
AEL-3	9.21	51.32	60.53	4
AOL-4	7.69	87.91	95.6	5
AWD-1	25.56	80	105.56	6

(Ranking was done statistically based on summation of per cent change in population at pH 5.5 and pH 4.5 over pH 7.0.)

Table 7. Screening of *Azospirillum* sp. for acid-tolerance on nutrient agar media

Isolates	**Per cent relative population**			**Ranking of acid-tolerant *Azospirillum***
	At pH 5.5	**At pH 4.5**	**Mean**	
AMU-2	88.52	60.11	74.32	2
ACH-1	92	30.29	61.14	3
AND-4	91.57	74.16	82.87	1
AEL-3	83.64	19.09	51.36	4
AOL-4	75	21.43	48.21	5
AWD-1	59.38	11.46	35.42	6

(Ranking was done statistically based on the average of per cent relative population at pH 5.5 and at pH 4.5 over pH 7.0)

Table 8. Screening of phosphate solubilizing bacteria isolates for acid-tolerance in nutrient broth

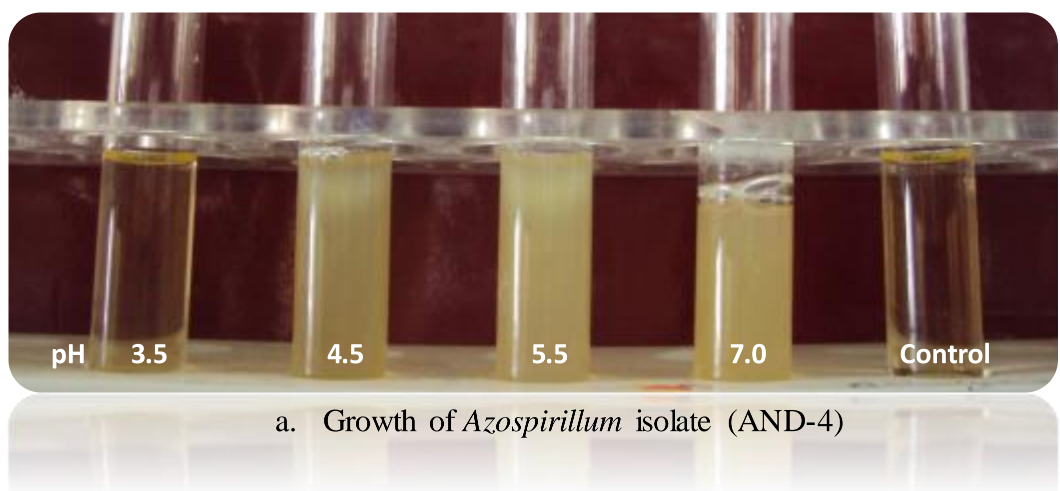
Isolates	Per cent change in population over neutral pH			Ranking of acid-tolerant PSB
	At pH 5.5	At pH 4.5	Sum	
PCH-2	63.08	81.54	144.62	32
PEL-3	19.35	35.48	54.84	12
PEL-4	30.95	36.90	67.86	17
PEL-6	25.93	65.43	91.36	23
PKR-1	41.77	86.08	127.85	31
PKR-2	17.65	23.53	41.18	8
PKR-3	10.27	21.23	31.51	4
PKR-4	42.86	62.50	105.36	25
PKR-5	13.70	75.34	89.04	22
PKR-6	74.68	74.68	149.37	33
PKR-7	3.30	52.75	56.04	14
PKR-8	9.83	24.28	34.10	5
PMD-1	27.00	52.00	79.00	21
PMD-2	13.54	25.00	38.54	6
PMD-3	59.02	91.80	150.82	34
PMD-4	12.73	85.45	98.18	24
PMD-5	25.42	88.14	113.56	28
PMD-6	6.12	44.90	51.02	11
PMD-7	8.87	21.77	30.65	3
PMU-1	21.05	26.32	47.37	10
PMU-2	12.24	28.57	40.82	7
PMU-3	8.93	60.71	69.64	19
PMU-4	26.60	81.91	108.51	26
PND-6	72.46	89.86	162.32	35
POL-1	8.09	19.65	27.75	2
POL-2	1.59	63.49	65.08	16
POL-3	5.74	21.31	27.05	1
POL-4	5.95	61.90	67.86	18
PPV-1	18.95	41.05	60.00	15
PVL-3	36.62	83.10	119.72	29
PWD-1	58.44	62.34	120.78	30
PWD-2	1.43	54.29	55.71	13
PWD-3	21.21	55.56	76.77	20
PWD-4	22.22	87.78	110.00	27
PWD-9	14.29	30.00	44.29	9

(Ranking was done statistically based on summation of per cent change in population at pH 5.5 and at pH 4.5 over pH 7.0.)

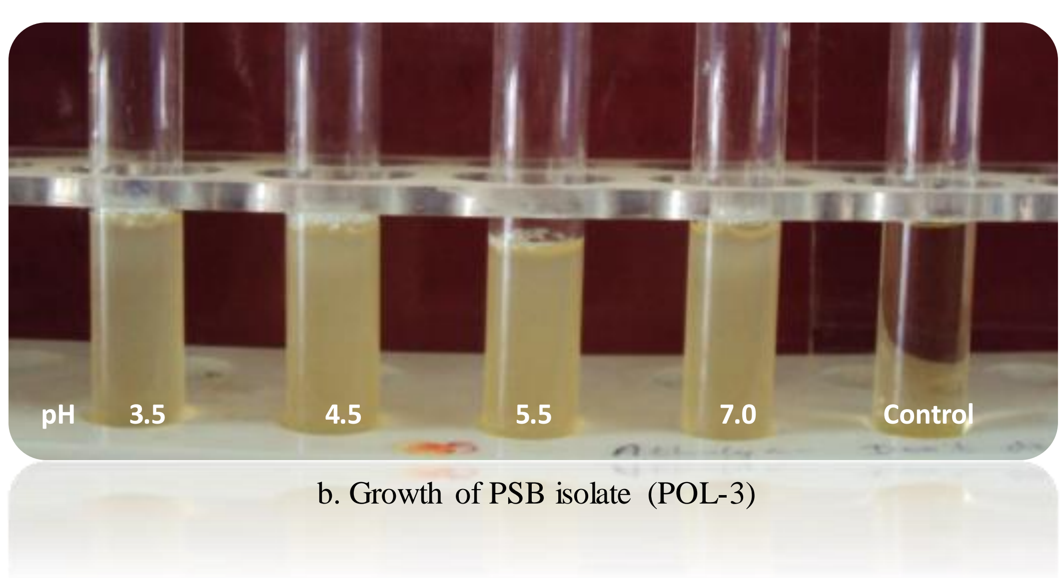
Table 9. Screening of different isolates of phosphate solubilizing bacteria for acid-tolerance on Pikovskaya's agar media

Isolates	Per cent relative population			Ranking of acid-tolerant PSB
	At pH 5.5	At pH 4.5	Mean	
PCH-2	13.04	4.35	8.70	31.0
PEL-3	10.26	2.56	6.41	33.0
PEL-4	16.28	4.65	10.47	27.0
PEL-6	25.00	0.00	12.50	22.0
PKR-1	15.63	3.13	9.38	30.0
PKR-2	3.64	0.00	1.82	35.0
PKR-3	62.77	54.15	58.46	4.0
PKR-4	18.42	5.26	11.84	24.0
PKR-5	25.00	5.00	15.00	19.0
PKR-6	14.29	0.00	7.15	32.0
PKR-7	17.65	5.88	11.77	25.0
PKR-8	86.00	22.00	54.00	5.0
PMD-1	28.57	0.00	14.29	20.0
PMD-2	57.14	17.86	37.50	8.0
PMD-3	11.11	0.00	5.56	34.0
PMD-4	15.52	6.90	11.21	26.0
PMD-5	21.05	10.53	15.79	18.0
PMD-6	60.47	13.95	37.21	9.0
PMD-7	78.43	72.55	75.49	2.0
PMU-1	41.38	13.79	27.59	14.0
PMU-2	80.00	20.00	50.00	6.0
PMU-3	28.57	4.76	16.67	17.0
PMU-4	21.74	4.35	13.05	21.0
PND-6	35.29	8.82	22.06	16.0
POL-1	70.27	56.76	63.52	3.0
POL-2	43.48	17.39	30.44	13.0
POL-3	86.67	76.67	81.67	1.0
POL-4	35.14	13.51	24.33	15.0
PPV-1	54.55	9.09	31.82	11.0
PVL-3	17.24	3.45	10.35	28.0
PWD-1	20.00	0.00	10.00	29.0
PWD-2	65.71	25.71	45.71	7.0
PWD-3	25.00	0.00	12.50	23.0
PWD-4	50.00	12.50	31.25	12.0
PWD-9	56.25	12.50	34.38	10.0

(Ranking was done statistically based on the average of per cent relative population at pH 5.5 and at pH 4.5 over pH 7.0)

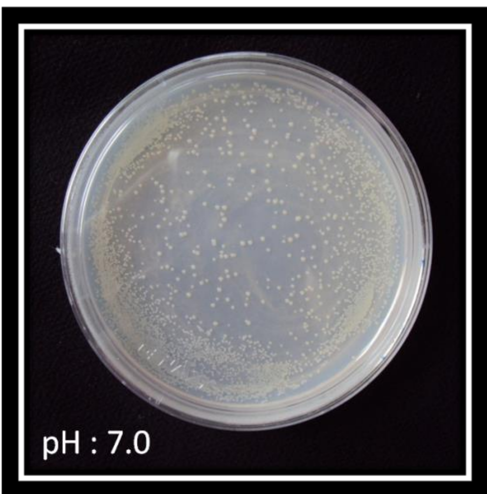


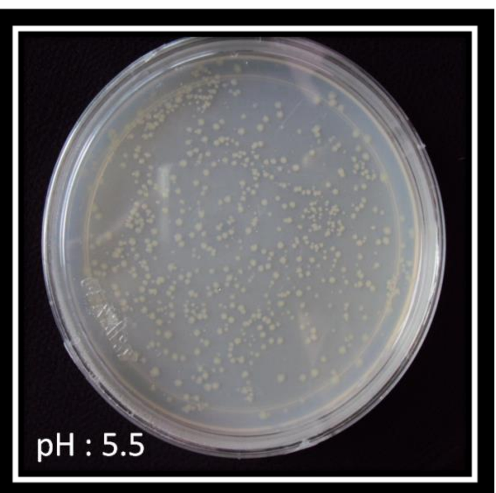
a. Growth of *Azospirillum* isolate (AND-4)

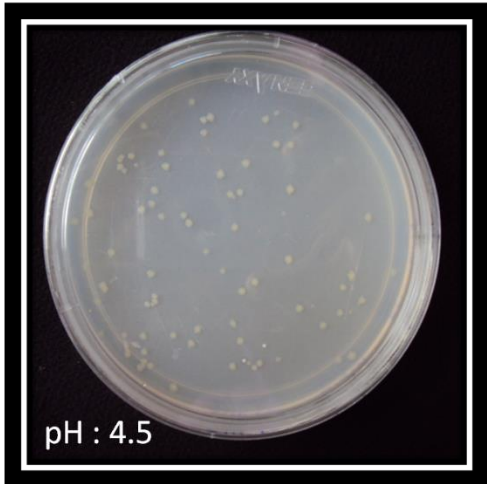


b. Growth of PSB isolate (POL-3)

Plate 6. Acid-tolerance of *Azospirillum* and PSB isolate in nutrient broth







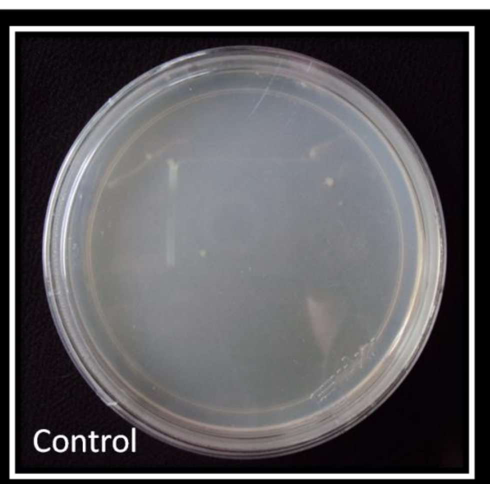


Plate 7. Acid-tolerance of PSB isolate (PMD-7) on Pikovskaya's agar

population at acidic pH to neutral pH (Table 9, Plate 7). POL-3 (Ollur) was found to be most acid-tolerant PSB strain, which showed maximum percent relative population of 81.67 at acidic pH to neutral pH followed, by PMD-7 (75.49) and POL-1 (63.52).

4.7 SCREENING OF ACID-TOLERANT *AZOSPIRILLUM* ISOLATES FOR ITS EFFICIENCY UNDER *IN VITRO*

4.7.1. Efficiency for N_2-fixation

The total nitrogen fixed by different isolates of *Azospirillum* was measured by Microkjeldhal method. Two isolates AND-4 (Nadavarambu) and AMU-2 (Mulayam) was found to fix more than 10 mg of N per g of malate utilized (Table 10). The isolate AND-4 showed highest nitrogen fixation (17.94 mg of N g^{-1} of malate) followed by AMU-2 (14.58 mg of N g^{-1} of malate). These two isolates also showed tolerance to acidic pH 4.5 in liquid and solid medium.

4.7.2. Efficiency for indole acetic acid (IAA) production

All the six isolates were tested for IAA production. All the isolates were found to produce IAA (Table 10, Plate 10a). Among the six isolates, AMU-2 and AND-4 were found to be the highest IAA producers. The isolate AMU-2 showed highest IAA production (51.95 µg ml^{-1}) followed by AND-4 (30.53 µg ml^{-1}).

4.8 SCREENING OF ACID-TOLERANT PSB ISOLATES FOR ITS EFFICIENCY UNDER *IN VITRO*

4.8.1. Quantitative estimation of phosphate solubilization

All the six acid-tolerant isolates were found to solubilize phosphorus in a range of 87 µg ml^{-1} to 207 µg ml^{-1} (Table 11, Plate 8). The isolates PMD-7 and POL-1 showed highest P-solubilization (207.22 µg ml^{-1} and 187.78 µg ml^{-1}

Table 10. *In vitro* screening of acid-tolerant *Azospirillum* isolates for efficiency in nitrogen fixation and IAA production

Isolates	**Amount of nitrogen fixed (mg of N g^{-1} of malate utilized)**	**Concentration of IAA (µg ml^{-1})**
AMU-2	14.58^{b}	51.95^{a}
ACH-1	3.26^{d}	21.13^{c}
AND-4	17.94^{a}	30.53^{b}
AEL-3	4.96^{cd}	12.16^{d}
AOL-4	5.25^{c}	8.16^{e}
AWD-1	3.68^{cd}	12.55^{d}

Means followed by the same letter do not differ at $p<0.05$

(Each value represents mean of three replications)

respectively). A reduction in pH of the broth was also observed. But, it was not proportionate to the amount of P solubilized.

4.8.2. Qualitative estimation of phosphate solubilization

Among the six acid-tolerant isolates, POL-1 was found to have highest solubilization efficiency (450 %) (Plate 9) compared to all other isolates followed by PKR-8 and PMD-7 (262.5 and 247.62 %) respectively after seven days of incubation (Table 11)

4.8.3. Efficiency for IAA production

All the six acid-tolerant PSB isolates were tested for its efficiency in IAA production (Table 11, Plate 10b). The isolates PMD-7 (33.07 μg ml^{-1}) and PKR-3 (32.43 μg ml^{-1}) showed highest IAA production.

4.9 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF PSB ISOLATES

Only one isolate was gram positive rod (POL-1), while others were gram negative rods. All the isolates were motile except PKR-3. All the six isolates were positive for catalase and citrate utilization and negative for indole and methyl red tests. Oxidase was positive only for PMU-2 while urease was positive only for POL-1 isolate. The isolates PKR-3, PKR-8 and PMU-2 were positive for Voges-Proskauer test. The PSB isolates PMD-7, PMU-2, POL-1 and POL-3 were identified as *Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp. and *Acinetobacter* sp. respectively. Two isolates (PKR-3 and PKR-8) could not be identified due to their unusual characteristics (Table 12).

Table 11. *In vitro* screening of different acid-tolerant PSB isolates for P-solubilization, solubilization efficiency and IAA production

Isolates	Quantity of P-solubilized (µg ml^{-1})	Reduction in pH*	Solubilization efficiency (%)	IAA production (µg ml^{-1})
PKR-3	97.44 (9.89[e])**	5.68	183.33 (13.55[c])**	32.43[a]
PKR-8	86.78 (9.34[f])	5.74	262.50 (16.21[b])	19.57[c]
PMD-7	207.22 (14.41[a])	4.35	247.62 (15.75[b])	33.07[a]
PMU-2	145.67 (12.08[c])	4.43	158.33 (12.60[d])	20.09[c]
POL-1	187.78 (13.72[b])	3.41	450.0 (21.21[a])	24.75[b]
POL-3	100.67 (10.05[d])	5.31	185.18 (13.60[c])	12.92[d]

Means followed by the same letter do not differ at $p<0.05$

(Each value represents the average of three replications)

* Initial pH was adjusted to 7.0

** Square root transformed values are given in bracket

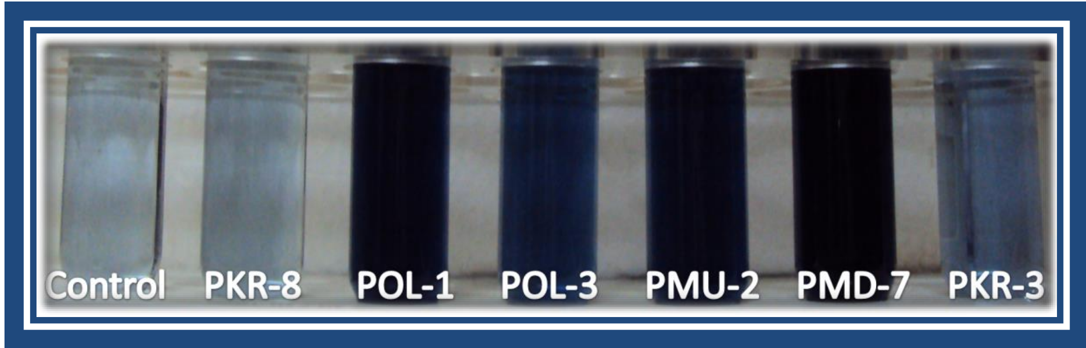
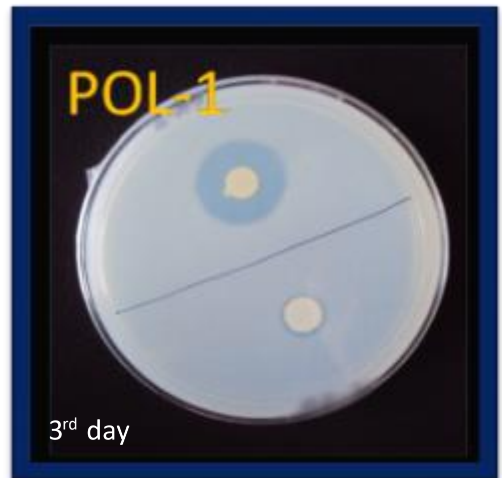


Plate 8. Quantitative estimation of phosphate solubilization



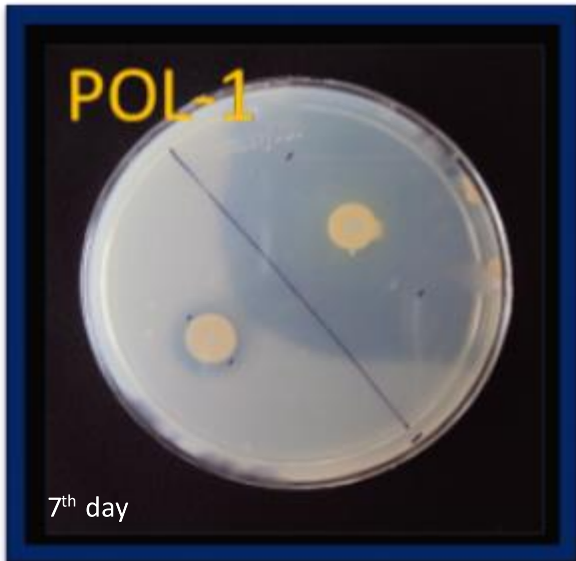
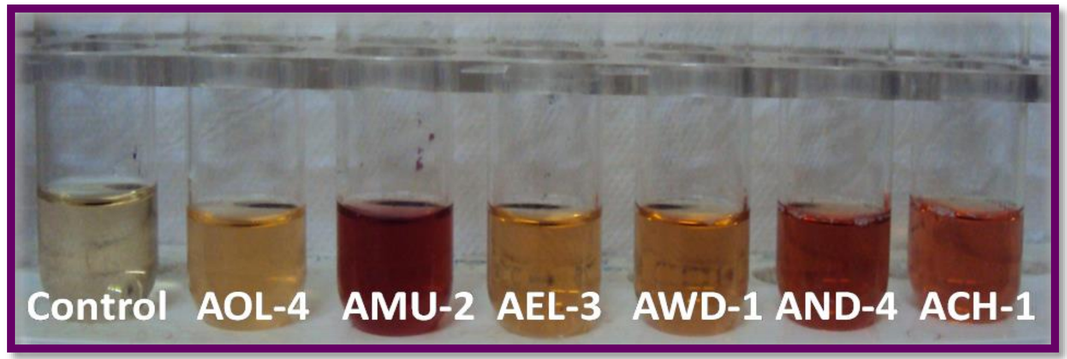
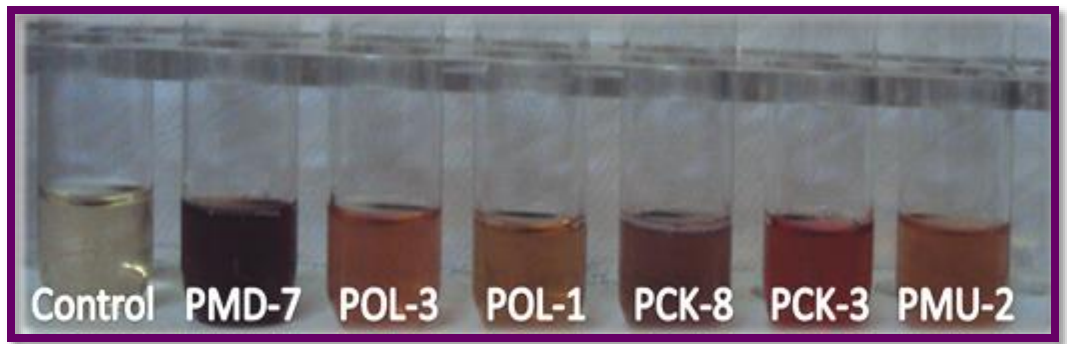


Plate 9. Solubilization efficiency of PSB isolate (POL-1)



a. *Azospirillum* isolates



b. PSB isolates

Plate 10. Indole acetic acid (IAA) production by *Azospirillum* and PSB isoaltes

Table 12. Characterization of phosphate solubilizing bacteria

Characters	**Isolates**					
	PKR-3	**PKR-8**	**PMD-7**	**PMU-2**	**POL-1**	**POL-3**
Gram reaction	-	-	-	-	+	-
Morphology	Rods	Rods	Rods	Rods	Rods	Rods
Motility	-	+	+	+	+	+
Oxidase	-	-	-	+	-	-
Catalase	+	+	+	+	+	+
Indole	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-
Voges-Proskauer	+	+	-	+	-	-
Citrate utilization	+	+	+	+	+	+
Urease	-	-	-	-	+	-
Possible organism	UI	UI	*Acineto-bacter* sp.	*Pseudo-monas* sp.	*Bacillus* sp.	*Acineto-bacter* sp.

UI : Unidentified

'+' : Positive for the test

'-' : Negative for the test

M

4.10 COMPATIBILITY STUDIES BETWEEN *AZOSPIRILLU* AND PSB ISOLATES

Based on the acid-tolerance and efficiency, six most acid-tolerant and efficient isolates each from *Azospirillum* and PSB were evaluated for compatibility. A total of 36 combinations were tested for its compatibility. Only sixteen combinations of *Azospirillum* and PSB were found to be compatible and twenty were incompatible (Table 13, Plate 11).

4.11 SELECTION OF *AZOSPIRILLUM* AND PSB ISOLATES FOR POT CULTURE STUDIES BASED ON ACID-TOLERANCE, EFFICIENCY AND COMPATIBILITY

Finally, after *in vitro* studies for acid-tolerance, efficiency and compatibility, three consortia were selected for pot culture experiment (Each consortium is a combination of compatible isolate of one *Azospirillum* and one PSB).

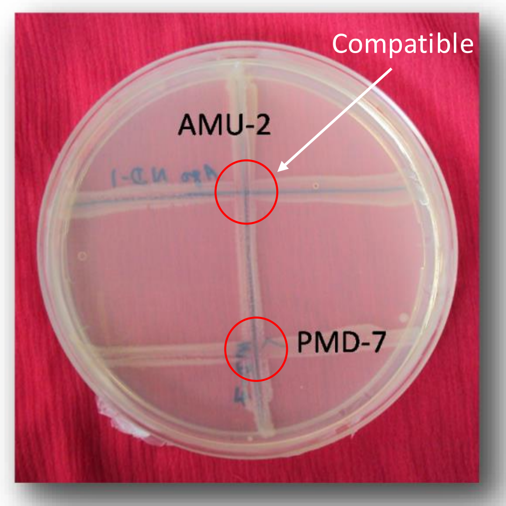
Based on the acid-tolerance, efficiency and compatibility, the combinations selected for the pot culture experiment were AND-4 + PMD-7 (consortium-1), AMU-2 + PMD-7 (consortium-2) and AND-4 + POL-1 (consortium-3). AMU-2 and AND-4 were *Azospirillum* isolates and PMD-7 and POL-1 were PSB isolates (Table 14) (Fig. 9).

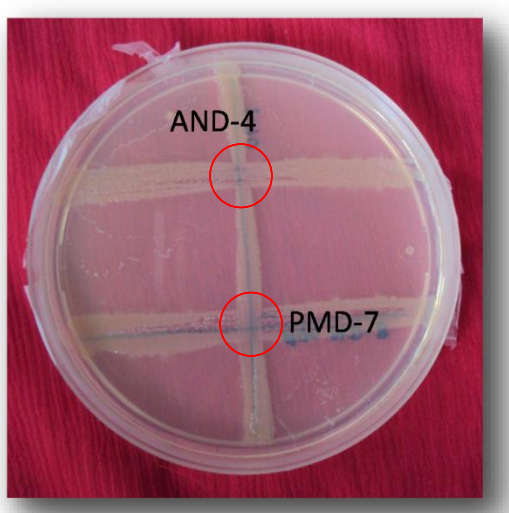
4.12 EVALUATION OF ACID-TOLERANT AND EFFICIENT CONSORTIA OF *AZOSPIRILLUM* AND PSB FOR GROWTH ENHANCEMENT OF OKRA

A pot culture experiment was conducted to evaluate the acid-tolerant consortia of *Azospirillum* and PSB on growth enhancement of okra (Plate 12). The pH of the potting mixture was 5.45. The total nitrogen per cent in the potting mixture was 0.093% and the available phosphorus was 10 mg kg^{-1}, which is

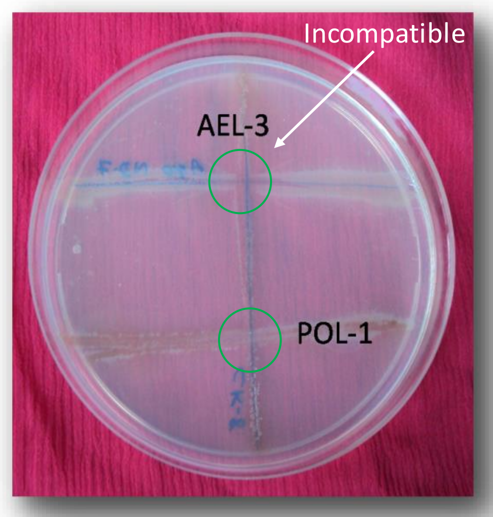
Table 13. Compatibility between the most acid-tolerant and efficient isolates of *Azospirillum* and phosphate solubilizing bacteria

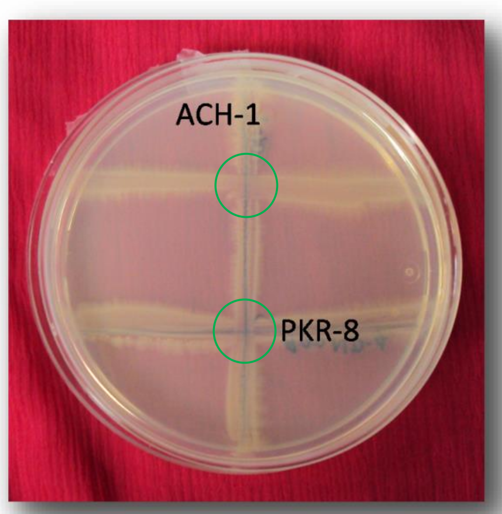
Sl. No.	*Azospirillum* + PSB combinations	Compatible/Incompatible
1	AMU-2 + PKR-3	Compatible
2	AMU-2 + PKR-8	Compatible
3	AMU-2 + PMD-7	Compatible
4	AMU-2 + PMU-2	Compatible
5	AMU-2 + POL-1	Compatible
6	AMU-2 + POL-3	Compatible
7	ACH-1 + PKR-3	Compatible
8	ACH-1 + PKR-8	Incompatible
9	ACH-1 + PMD-7	Incompatible
10	ACH-1 + PMU-2	Incompatible
11	ACH-1 + POL-1	Incompatible
12	ACH-1 + POL-3	Incompatible
13	AND-4 + PKR-3	Compatible
14	AND-4 + PKR-8	Compatible
15	AND-4 + PMD-7	Compatible
16	AND-4 + PMU-2	Compatible
17	AND-4 + POL-1	Compatible
18	AND-4 + POL-3	Incompatible
19	AEL-3 + PKR-3	Incompatible
20	AEL-3 + PKR-8	Incompatible
21	AEL-3 +PMD-7	Incompatible
22	AEL-3 + PMU-2	Incompatible
23	AEL-3 + POL-1	Incompatible
24	AEL-3 + POL-3	Incompatible
25	AOL-4 + PKR-3	Incompatible
26	AOL-4 + PKR-8	Compatible
27	AOL-4 + PMD-7	Incompatible
28	AOL-4 + PMU-2	Compatible
29	AOL-4 + POL-1	Compatible
30	AOL-4 + POL-3	Incompatible
31	AWD-1 + PKR-3	Compatible
32	AWD-1 + PKR-8	Incompatible
33	AWD-1 + PMD-7	Incompatible
34	AWD-1 + PMU-2	Incompatible
35	AWD-1 + POL-1	Incompatible
36	AWD-1 + POL-3	Incompatible





a. Compatible isolates





b. Incompatible isoaltes

Plate 11. Compatibility between *Azospirillum* and PSB isolates

Azospirillum / PSB			Ranking					
			1	2	3	4	5	6
			PMD-7	POL-1	POL-3	PKR-3	PKR-8	PMU-2
Ranking	1	AND-4	+(2)	+(3)	-	+(5)	+(6)	+(7)
	2	AMU-2	+(3)	+(4)	+(5)	+(6)	+(7)	+(8)
	3	ACH-1	-	-	-	+(7)	-	-
	4	AEL-3	-	-	-	-	-	-
	5	AOL-4	-	+(7)	-	-	+(10)	+(11)
	5	AWD-1	-	-	-	+(9)	-	-

Fig.9. Selection of three most efficient consortia based on overall ranking for acid-tolerance, efficiency and compatibility

‘+’ : Compatible

‘-’ : Incompatible

( ) : Sum of corresponding rank of *Azospirillum* and PSB

Table 14. Combinations of *Azospirillum* and PSB selected for consortium

Consortia	Isolates selected for consortium	
	Azospirillum	PSB
Consortium-1	AND-4	PMD-7
Consortium-2	AMU-2	PMD-7
Consortium-3	AND-4	POL-1

AMU-2 : *Azospirillum* from Mulayam

AND-4 : *Azospirillum* from Nadavarambu

PMD-7 : PSB from Madakkathara

POL-1 : PSB from Ollur

Plate 12. Overview of pot culture experiment

considered to be moderate. The initial population of *Azospirillum* and PSB isolates was 10^8 cfu ml^{-1} in the culture media before inoculation to the plants.

4.12.1. Germination percentage

There was significant difference between treatments. Highest germination percentage was recorded in T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) and T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (88.89%) which was on par with T_2 and T_5. Lowest germination percentage was recorded in T_7 (Absolute control) (71.11%) after five days of sowing (Table 15).

4.12.2. Plant height

Observations on plant height were recorded at 30 das, 60 das and 90 das (at harvest) (Table 16). Maximum plant height at all the three intervals were recorded in T_1 (POP recommendation) which was considered as positive control. The treatment T_1 was on par with T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) (13.71 cm) at 30 das. At 60 das, there were no significant differences among treatments except for treatment T_1 (POP recommendation). At 90 das, T_4 (*Azospirillum* sp. + *Bacillus megaterium*) recorded maximum plant height (54.77 cm) among the consortia. All other treatments were on par with control except T_1.

4.12.3. Number of leaves

Number of leaves per plant was recorded at 30 das, 60 das and 90 das (Table 17). At 30 das, maximum number of leaves was recorded on T_4 (*Azospirillum* + *Bacillus megaterium*) (7.90) and minimum number of leaves were recorded on T_6 (Azophos-TNAU consortium) (3.43). At 60 das, T_1 (POP recommendation) (20.46) had maximum number of leaves followed by T_4 (*Azospirillum* + *Bacillus megaterium*) (18.73) which were found to be on par and minimum was observed on T_6 (Azophos-TNAU consortium) (12.86). At 90 das, maximum number of leaves was recorded on T_2 (*Azospirillum*+*Acinetobacter* sp.)

Table 15. Effect of different treatments on germination percentage

Treatments	Germination percentage (%)
T_1 (POP recommendation)	80.00^{bc}
T_2 [*Azospirillum* (AND-4) + *Acinetobacter* sp. (PMD-7)]	84.44^{ab}
T_3 [*Azospirillum* (AMU-2) + *Acinetobacter* sp. (PMD-7)]	88.89^{a}
T_4 [*Azospirillum* (AND-4) + *Bacillus megaterium* (POL-1)]	88.89^{a}
T_5 (T_2+T_3+T_4)	82.22^{abc}
T_6 (Azophos-TNAU)	75.56^{cd}
T_7 (Absolute control)	71.11^{d}

Means followed by the same letter do not differ at $p<0.05$

Table 16. Effect of different treatments on plant height

Treatments	Plant height (cm)		
	30 das	60 das	90 das
T_1 (POP recommendation)	17.45[a]	43.89[a]	67.17[a]
T_2 (AND-4 + PMD-7)	10.75[b]	27.11[b]	47.90[bc]
T_3 (AMU-2 + PMD-7)	13.71[ab]	25.48[b]	50.03[bc]
T_4 (AND-4 + POL-1)	11.26[b]	26.53[b]	54.77[b]
T_5 (T_2+T_3+T_4)	9.81[b]	26.65[b]	46.46[bc]
T_6 (Azophos-TNAU)	7.72[b]	20.47[b]	44.7[bc]
T_7 (Absolute control)	9.50[b]	20.81[b]	39.83[c]

Means followed by the same letter do not differ at $p<0.05$

(das : days after sowing)

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

Table 17. Effect of different treatments on number of leaves

Treatments	Number of leaves/plant		
	30 das	60 das	90 das
T_1 (POP recommendation)	6.90^{a}	20.46^{a}	26.33
T_2 (AND-4 + PMD-7)	7.28^{a}	18.58^{ab}	28.52
T_3 (AMU-2 + PMD-7)	5.50^{ab}	16.06^{ab}	27.53
T_4 (AND-4 + POL-1)	7.90^{a}	18.73^{ab}	28.00
T_5 (T_2+T_3+T_4)	7.63^{a}	18.13^{ab}	28.00
T_6 (Azophos-TNAU)	3.43^{b}	12.86^{b}	25.53
T_7 (Absolute control)	5.70^{ab}	14.93^{ab}	24.60
			NS

Means followed by the same letter do not differ at $p<0.05$

(das : days after sowing)

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

(28.52) and minimum on T_7 (Absolute control) (24.60). But, statistically, there were no significant differences between treatments.

4.12.4. Number of days taken for first flowering

Early flowering was observed in T_1 (POP recommendation) (43.47 days) followed by T_4 (*Azospirillum* + *Bacillus megaterium*) (44.87 days). The treatment T_6 (Azophos-TNAU consortium) took maximum days for first flowering (53.07 days) (Table 18).

4.12.5. Number of fruits per plant

Number of fruits per plant was recorded on 60 das and 90 das (Table 19). Maximum number of fruits was recorded in T_1 (POP recommendation) at both 60 and 90 das and was found to be statistically superior to all other treatments at 60 das. Among the consortia, T_4 (*Azospirillum* + *Bacillus megaterium*) recorded highest number of fruits per plant (41 fruits/plant). However, total number of fruits was highest in T_1 (POP recommendation) (51.0/plant).

4.12.6. Fruit yield

Fruit yield was recorded at 60 das and 90 das. Total fruit yield was also calculated (Table 20).

At 60 das, maximum fruit yield was recorded in T_1 (POP recommendation) (49.30 g $plant^{-1}$) which was on par with T_4 (*Azospirillum* + *Bacillus megaterium*) (27.62 g $plant^{-1}$), T_5 (combination of three consortium) (23.45 g $plant^{-1}$) and T_2 (*Azospirillum* + *Acinetobacter* sp.) (22.22 g $plant^{-1}$). At 90 das, maximum fruit yield was recorded in T_1 (113.15 g $plant^{-1}$) which was followed by T_4 (*Azospirillum* + *Bacillus megaterium*). Total fruit yield was maximum in T_1

Table 18. Effect of different treatments on number of days taken for first flowering

Treatments	Number of days taken for first flowering
T_1 (POP recommendation)	43.47^{a}
T_2 [*Azospirillum* (AND-4) + *Acinetobacter* sp. (PMD-7)]	46.93^{ab}
T_3 [*Azospirillum* (AMU-2) + *Acinetobacter* sp. (PMD-7)]	48.73^{ab}
T_4 [*Azospirillum* (AND-4) + *Bacillus megaterium* (POL-1)]	44.87^{a}
T_5 (T_2+T_3+T_4)	46.87^{ab}
T_6 (Azophos-TNAU)	53.07^{b}
T_7 (Absolute control)	49.07^{ab}

Means followed by the same letter do not differ at $p<0.05$

Table 19. Effect of different treatments on number of fruits

Treatment	Number of fruits/plant		
	60 das	**90 das**	**Total**
T_1 (POP recommendation)	20.33[a]	30.67[a]	51.00[a]
T_2 (AND-4 + PMD-7)	11.00[bc]	20.67[ab]	31.67[abc]
T_3 (AMU-2 + PMD-7)	7.67[bc]	25.33[ab]	33.00[abc]
T_4 (AND-4 + POL-1)	13.33[b]	27.67[a]	41.00[ab]
T_5 (T_2+T_3+T_4)	9.33[bc]	21.00[ab]	30.33[abc]
T_6 (Azophos-TNAU)	4.67[c]	20.00[ab]	24.67[bc]
T_7 (Absolute control)	7.67[bc]	13.00[b]	20.67[c]

Means followed by the same letter do not differ at $p<0.05$

(das : days after sowing)

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

Table 20. Effect of different treatments on fruit yield

Treatments	**Fruit yield (g plant^{-1})**			**Per cent increase in yield over control**
	60 das	**90 das**	**Total**	
T_1 (POP recommendation)	49.30^{a}	113.15^{a}	162.45^{a}	181.21
T_2 (AND-4 + PMD-7)	22.22^{ab}	99.25^{a}	121.47^{ab}	110.26
T_3 (AMU-2 + PMD-7)	14.52^{b}	108.09^{a}	122.61^{ab}	112.25
T_4 (AND-4 + POL-1)	27.62^{ab}	112.71^{a}	140.33^{ab}	142.91
T_5 (T_2+T_3+T_4)	23.45^{ab}	92.66^{ab}	116.11^{abc}	100.99
T_6 (Azophos-TNAU)	14.44^{b}	72.31^{ab}	86.74^{bc}	50.16
T_7 (Absolute control)	13.53^{b}	44.24^{b}	57.77^{c}	0.00

Means followed by the same letter do not differ at $p<0.05$

(das : days after sowing)

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

(162.45 g plant$^{-1)}$ followed by T_4 (g plant^{-1}). Per cent increase in yield over control for T_1 was 181.21%. T_4 had a per cent increase in yield of 142.91%.

4.12.7. Pest and disease incidence

The plants were infected with viral disease during the crop period (Plate 13). The maximum yellow vein mosaic disease incidence (46%) was recorded in T_7 (Absolute control) at the time of harvest and it was lowest in the case of T_1 (POP recommendation) (13%). Among the consortia, the yellow vein mosaic disease ranged from 26 to 40% at the time of harvest. The pest such as mite and petiole maggots also affected the plants.

4.12.8. Fresh weight of shoot, root and plant

Fresh weight of shoot, root and plant was taken at the time of harvest (Table 21). Maximum fresh weight (g plant^{-1}) for shoot, root, and plant was recorded in T_1 (127.53, 24.61, 152.14 respectively). Lowest fresh weight for shoot, root and plant was recorded in T_7 (Absolute control) (81.48, 12.56, 94.04 respectively). There were no significant difference among the treatments.

4.12.9. Dry weight of shoot, root and plant

Maximum dry weight for shoot, root and plant was recorded in T_1 (POP recommendation) (31.24, 10.34, 41.58 g/plant respectively). Lowest dry weight was recorded in treatment T_7 (Absolute control) (18.60, 4.17, 22.78 for shoot, root and plant respectively). All the treatments were found to be on par (Table 22).

4.12.10. Population of *Azospirillum* and PSB in soil at harvest

Initial population of *Azospirillum* and PSB were found to be non significant among all the treatments (Table 23). At harvest stage, population of *Azospirillum* and PSB had increased in all the treatments except in T_7 (Absolute control). At

a. Yellow Vein Mosaic

b. Petiole Maggot

c. Red spider mite

Plate 13. Pest and disease incidence of okra

Table 21. Effect of different treatments on fresh weight of shoot, root and plant

Treatments	Fresh wt. (g plant^{-1})		
	Shoot	Root	Plant
T_1 (POP recommendation)	127.53^{a}	24.61^{a}	152.14^{a}
T_2 (AND-4 + PMD-7)	100.20^{ab}	14.43^{b}	114.64^{ab}
T_3 (AMU-2 + PMD-7)	96.44^{ab}	15.97^{ab}	112.42^{ab}
T_4 (AND-4 + POL-1)	114.23^{ab}	18.39^{ab}	132.62^{ab}
T_5 (T_2+T_3+T_4)	95.66^{ab}	16.90^{ab}	112.56^{ab}
T_6 (Azophos-TNAU)	100.30^{ab}	16.09^{ab}	116.40^{ab}
T_7 (Absolute control)	81.48^{b}	12.56^{b}	94.04^{b}

Means followed by the same letter do not differ at $p<0.05$

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

Table 22. Effect of different treatments on dry weight of shoot, root and plant

Treatment	Dry wt. (g $plant^{-1}$)		
	Shoot	Root	Plant
T_1 (POP recommendation)	31.24^{a}	10.34^{a}	41.58^{a}
T_2 (AND-4 + PMD-7)	22.73^{ab}	5.43^{b}	28.17^{b}
T_3 (AMU-2 + PMD-7)	23.83^{ab}	7.42^{ab}	31.25^{ab}
T_4 (AND-4 + POL-1)	23.70^{ab}	6.10^{b}	29.80^{ab}
T_5 (T_2+T_3+T_4)	21.25^{b}	5.83^{b}	27.08^{b}
T_6 (Azophos-TNAU)	19.44^{b}	5.42^{b}	24.87^{b}
T_7 (Absolute control)	18.60^{b}	4.17^{b}	22.78^{b}

Means followed by the same letter do not differ at $p<0.05$

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

Table 23. Effect of different treatments on the population of *Azospirillum* and PSB in soil at the time of harvest

Treatment	**Population of *Azospirillum* and PSB**			
	Azospirillum **($x10^3$ MPN g^{-1})**		**PSB ($x10^5$ cfu g^{-1})**	
	Initial status	**Final status**	**Initial status**	**Final status**
T_1 (POP recommendation)	7.36	11.40^{b}	3.03	1.02^{e}
T_2 (AND-4 + PMD-7)	9.21	17.15^{ab}	3.27	3.53^{c}
T_3 (AMU-2 + PMD-7)	8.10	23.38^{a}	3.27	6.43^{a}
T_4 (AND-4 + POL-1)	8.90	23.04^{a}	3.00	5.70^{ab}
T_5 (T_2+T_3+T_4)	7.14	13.76^{ab}	3.67	5.32^{b}
T_6 (Azophos-TNAU)	9.21	9.28^{b}	2.83	3.37^{c}
T_7 (Absolute control)	9.03	7.73^{b}	3.47	2.08^{d}
	NS		NS	

Means followed by the same letter do not differ at $p<0.05$

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

harvest, population of *Azospirillum* was found to be maximum in T_3 (*Azospirillum* + *Acinetobacter* sp.) (23.38x10^3 MPN g^{-1}) which was on par with T_4 (*Azospirillum* + *Bacillus megaterium*) (23.04x10^3 MPN g^{-1}) and minimum in T_7 (Absolute control) (7.73x10^3 MPN g^{-1}).

At harvest, population of PSB was found to be highest in T_3 (*Azospirillum* + *Acinetobacter* sp.) (6.43x10^5 cfu g^{-1}) which was on par with T_4 (*Azospirillum* + *Bacillus megaterium*) (5.70x10^5 cfu g^{-1}). Population of PSB was least in T_7 (Absolute control) (2.08x10^5 cfu g^{-1}).

4.12.11. Nutrient uptake by okra

Maximum N_2 uptake was recorded in T_1 (POP recommendation) (0.99 g $plant^{-1}$) followed by T_4 (*Azospirillum* + *Bacillus megaterium*) (0.81 g/plant) and T_3 (*Azospirillum* + *Acinetobacter* sp.) (0.80 g/plant) which were on par with T_1 (Table 24). Maximum uptake of phosphorus was recorded in T_1 (POP recommendation) (0.41 g $plant^{-1}$) which was on par with all consortia applied treatments. Minimum uptake of phosphorus was noticed in T_7 (Absolute control) (0.23 g $plant^{-1}$).

4.12.12. Nutrient status in soil at harvest

Per cent total nitrogen in soil was found to be non-significant among different treatments. Highest available phosphorus was recrded in T_1 (POP recommendation) (26.34 mg kg^{-1}) and minimum in T_6 (Azophos-TNAU) (15.63 mg kg^{-1}).

4.13 16S rDNA SEQUENCE ANALYSIS OF EFFICIENT *AZOSPIRILLUM* AND PSB ISOLATES

The most acid-tolerant and efficient consortial isolates of PSB under pot culture experiment were identified by 16S rDNA sequence analysis using polymerase chain reaction (PCR).

Table 24. Effect of different treatments on uptake of nitrogen and phosphorus

Treatment	Nutrient uptake ($g\ plant^{-1}$)	
	N_2	P_2O_5
T_1 (POP recommendation)	0.99^{a}	0.41^{a}
T_2 [*Azospirillum* (AND-4) + *Acinetobacter* sp. (PMD-7)]	0.66^{bc}	0.29^{ab}
T_3 [*Azospirillum* (AMU-2) + *Acinetobacter* sp. (PMD-7)]	0.80^{ab}	0.34^{ab}
T_4 [*Azospirillum* (AND-4) + *Bacillus megaterium* (POL-1)]	0.81^{ab}	0.36^{ab}
T_5 (T_2+T_3+T_4)	0.63^{bc}	0.35^{ab}
T_6 (Azophos-TNAU)	0.60^{bc}	0.29^{ab}
T_7 (Absolute control)	0.57^{c}	0.23^{b}

Means followed by the same letter do not differ at $p<0.05$

Table 25. Effect of different treatments on the soil total nitrogen per cent and phosphorus at the time of harvest

Treatments	Total N_2 (%)	P_2O_5 (mg kg^{-1})
T_1 (POP recommendation)	0.073	26.34^{a}
T_2 (AND-4 + PMD-7)	0.060	19.49^{ab}
T_3 (AMU-2 + PMD-7)	0.080	19.19^{ab}
T_4 (AND-4 + POL-1)	0.086	20.53^{ab}
T_5 (T_2+T_3+T_4)	0.106	18.45^{ab}
T_6 (Azophos-TNAU)	0.093	17.41^{ab}
T_7 (Absolute control)	0.093	15.63^{b}
	NS	
Initial content in potting mixture	0.093	16.60

Means followed by the same letter do not differ at $p<0.05$

AMU-2: *Azospirillum* sp.; AND-4 : *Azospirillum* sp.

PMD-7 : *Acinetobacter* sp.; POL-1 : *Bacillus megaterium*

4.13.1. Amplification of 16S rDNA gene

Amplification of 16S rDNA gene was carried out by colony PCR. The PCR product was checked on 1.0% (w/v) agarose gel and documented. Only one amplicon of about 1500 bp was obtained (Plate 14).

4.13.2. Purification and sequencing of PCR product

PCR products of the PSB isolates PMD-7 and POL-1 were purified and sequenced. The nucleotide sequences of the isolates are given in Appendix-II.

4.13.3. Nucleotide sequence analysis

Homology search of nucleotide sequences obtained from the isolates PMD-7 and POL-1 with other reported 16S rDNA gene sequences was carried out. PMD-7 showed homology with *Acinetobacter* sp. and POL-1 showed homology with *Bacillus megaterium*. The sequence analyses are given in plate 15, 16.

Azospirillum isolates AND-4 and AMU-2 were identified based on morphological and cultural characters as described by Tarrand *et al.* (1979).

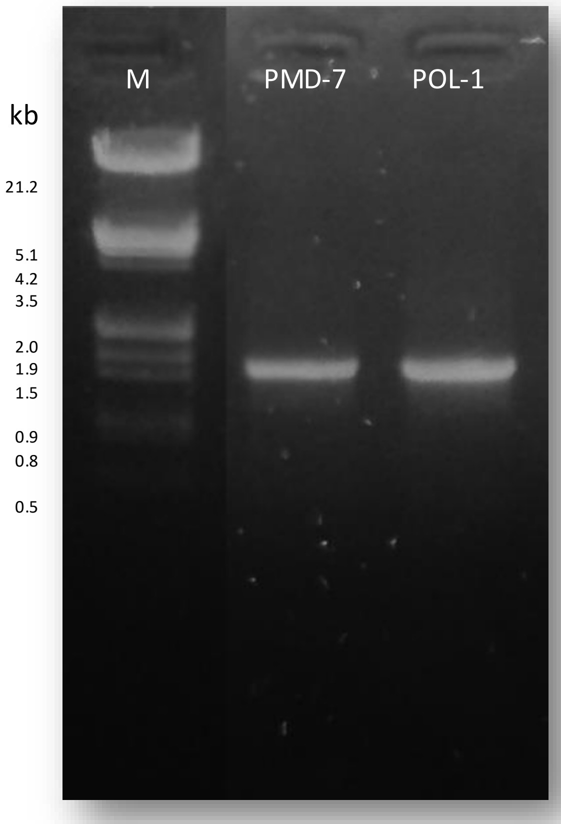
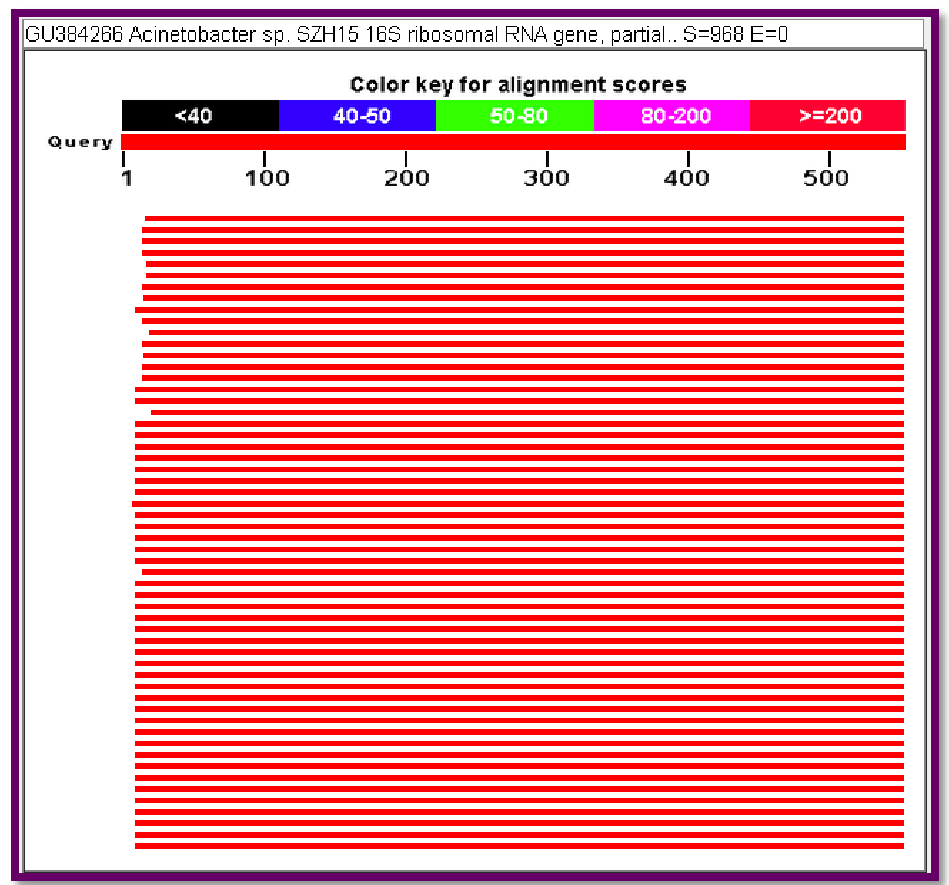
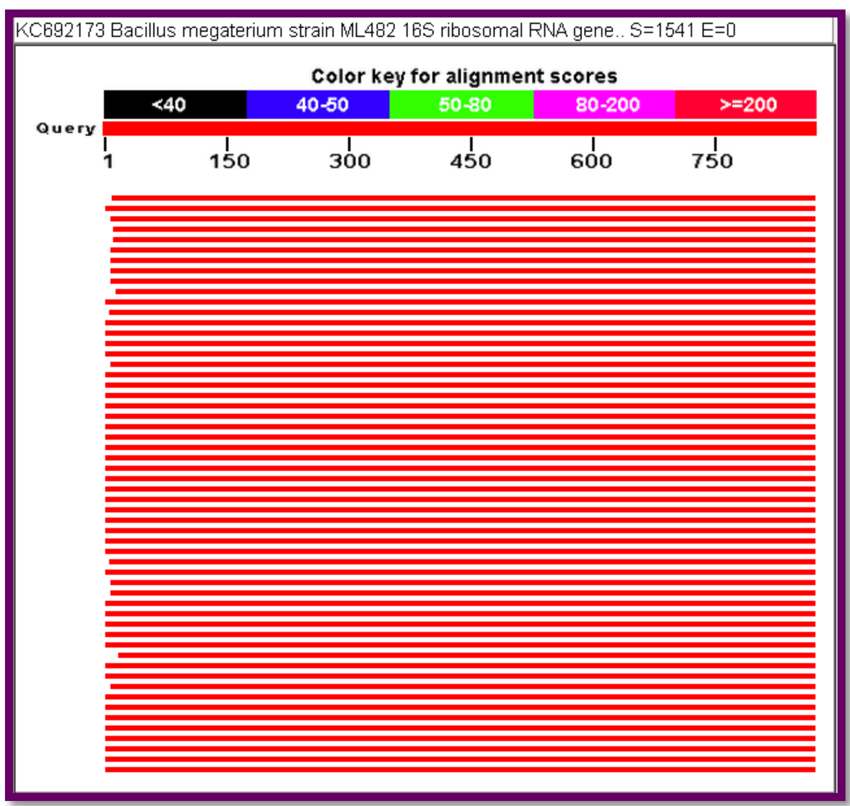


Plate 14. Amplification of 16S rDNA gene of PSB isolates



	Description	Max score	Total score	Query cover	E value	Ident	Accession
	Acinetobacter sp. TPS11 16S ribosomal RNA gene, partial sequence	970	970	97%	0.0	99%	FJ821604.1
	Acinetobacter baumannii strain OVC6 16S ribosomal RNA gene, partial sequence	968	968	96%	0.0	99%	JQ660722.1
	Acinetobacter sp. SZH15 16S ribosomal RNA gene, partial sequence	968	968	98%	0.0	99%	GU384266.1
	Acinetobacter baumannii strain NBRAJG89 16S ribosomal RNA gene, partial sequence	968	968	97%	0.0	99%	EU661706.1
	Acinetobacter sp. CHNSH-239 16S ribosomal RNA gene, partial sequence	966	966	96%	0.0	99%	JX965399.1
	Acinetobacter baumannii strain 6.1 16S ribosomal RNA gene, partial sequence	966	966	97%	0.0	99%	JX286667.1
	Acinetobacter baumannii strain OVC5 16S ribosomal RNA gene, partial sequence	966	966	96%	0.0	99%	JQ660721.1
	Acinetobacter baumannii strain LCR69 16S ribosomal RNA gene, partial sequence	966	966	97%	0.0	99%	FJ976578.1
	Acinetobacter baumannii strain ELA-28o 16S ribosomal RNA gene, partial sequence	966	966	97%	0.0	99%	FJ195018.1
	Uncultured Acinetobacter sp. clone X59 16S ribosomal RNA gene, partial sequence	965	965	98%	0.0	99%	KF003205.1
	Acinetobacter baumannii NCGM 237 DNA, complete genome	965	5768	98%	0.0	99%	AP013357.1
	Acinetobacter sp. BG-9-R4 16S ribosomal RNA gene, partial sequence	965	965	96%	0.0	99%	KF560364.1
	Acinetobacter baumannii strain 137A (BC30) 16S ribosomal RNA gene, partial sequence	965	965	98%	0.0	99%	KF254610.1
	Acinetobacter baumannii strain CCGGD201101 16S ribosomal RNA gene, partial sequence >dbj|AB859680.1| Acinetobacter bai	965	965	98%	0.0	99%	KF430814.1
	Acinetobacter baumannii strain AHBR4 16S ribosomal RNA gene, partial sequence	965	965	98%	0.0	99%	KF241517.1
	Acinetobacter baumannii BJAB0715, complete genome	965	5790	98%	0.0	99%	CP003847.1
	Uncultured bacterium clone A0 16S ribosomal RNA gene, partial sequence	965	965	98%	0.0	99%	JQ731890.1
	Uncultured bacterium clone M58 16S ribosomal RNA gene, partial sequence	965	965	98%	0.0	99%	KC894569.1
	Acinetobacter baumannii D1279779, complete genome	965	5779	98%	0.0	99%	CP003967.1

Plate 15. Sequence analysis of PMD-7 isolate



Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus megaterium strain ML482 16S ribosomal RNA gene, partial sequence	1541	1541	98%	0.0	99%	KC692173.1
Bacillus sp. 3571BBRRJ 16S ribosomal RNA gene, partial sequence	1535	1535	99%	0.0	98%	JF309241.1
Bacillus sp. S859 16S ribosomal RNA gene, partial sequence	1533	1533	98%	0.0	98%	KC523393.1
Bacillus megaterium strain QAP19 16S ribosomal RNA gene, partial sequence	1531	1531	98%	0.0	98%	KF419129.1
Bacillus megaterium strain GC61 16S ribosomal RNA gene, partial sequence	1531	1531	98%	0.0	98%	KF158230.1
Bacillus megaterium strain ML479 16S ribosomal RNA gene, partial sequence	1531	1531	98%	0.0	98%	KC692206.1
Bacillus megaterium strain ML257 16S ribosomal RNA gene, partial sequence	1531	1531	98%	0.0	98%	KC692200.1
Bacillaceae bacterium soil 01 16S ribosomal RNA gene, partial sequence	1531	1531	98%	0.0	98%	GU070741.1
Bacillus megaterium strain ML235 16S ribosomal RNA gene, partial sequence	1530	1530	98%	0.0	98%	KC692187.1
Bacillus subtilis strain tomatorhizoBAC1/2011 16S ribosomal RNA gene, partial sequence	1530	1530	97%	0.0	99%	JF304809.1
Bacillus aryabhattai strain GJM820 16S ribosomal RNA gene, partial sequence	1530	1530	99%	0.0	98%	HM209759.1
Bacterium enrichment culture clone 03 16S ribosomal RNA gene, partial sequence	1530	1530	98%	0.0	98%	GU294272.1
Bacillus sp. W2.09-240 16S ribosomal RNA (16S rRNA) gene, complete sequence	1528	1528	99%	0.0	98%	JX458450.1
Bacillus megaterium strain B12 16S ribosomal RNA gene, partial sequence	1528	1528	99%	0.0	98%	KF010350.1
Bacillus sp. IARI-HHS2-45 16S ribosomal RNA gene, partial sequence	1528	1528	99%	0.0	98%	KF054758.1
Bacillus aryabhattai strain JN162 16S ribosomal RNA gene, partial sequence	1528	1528	99%	0.0	98%	KF150411.1
Bacillus megaterium strain ML258 16S ribosomal RNA gene, partial sequence	1528	1528	98%	0.0	98%	KC692166.1
Bacillus aryabhattai strain M2 16S ribosomal RNA gene, partial sequence	1528	1528	99%	0.0	98%	KC934850.1
Bacillus megaterium strain VB21 16S ribosomal RNA gene, partial sequence >gb|KF054750.1| Bacillus megaterium strain IARI-	1528	1528	99%	0.0	98%	KC609020.1
Bacillus sp. R-42278 partial 16S rRNA gene, strain R-42278	1528	1528	99%	0.0	98%	HE603521.1
Bacillus aryabhattai partial 16S rRNA gene, strain AntCr18	1528	1528	99%	0.0	98%	HF570067.1
Bacillus aryabhattai partial 16S rRNA gene, strain AntCr1	1528	1528	99%	0.0	98%	HF570057.1
Bacillus sp. 6090 16S ribosomal RNA gene, partial sequence >gb|KF150401.1| Bacillus aryabhattai strain JN129 16S ribosoma	1528	1528	99%	0.0	98%	JX566650.1
Bacillus sp. MBEE60 gene for 16S rRNA, partial sequence	1528	1528	99%	0.0	98%	AB733549.1
Bacillus sp. IARI-J-17 16S ribosomal RNA gene, partial sequence	1528	1528	99%	0.0	98%	JX441880.1

Plate 16. Sequence analysis of POL-1 isolate

DISCUSSION

5. DISCUSSION

Nitrogen and phosphorus are the two major nutrients required by the plants. Insufficiency of these elements leads to stunted growth and yield loss. The application of chemical fertilizers to supply nitrogen and phosphorus is not feasible due to its high cost. The application of chemical fertilizers especially that of nitrogen and phosphorus source can increase the acidity of already acidic soils. Further, under tropical climate with high rainfall, the use efficiency of these fertilizers is very low due to leaching losses of nitrogen fertilizers and fixation of phosphorus. In this context, there is a need to find alternative sources of nutrients. Biofertilizers can be used as an alternative to chemical fertilizers because they are cheap, eco-friendly and plant growth hormone producers. In recent years, the uses of biofertilizers are gaining importance due to increasing popularity of organic farming and harmful effects of agrochemicals. Kerala state has announced an organic policy in 2010, in which stress has been given on organic farming, and it is declared that the entire state will be converted into organic state in another ten years. The Kasaragod district of Kerala has already been declared as organic district. The biofertilizers are the integral component of organic farming. As the state turns to organic farming, there will be high demand for biofertilizers and organic manures. The consortium of biofertilizers production as well as its use has not become popular due to the non-availability of native consortia of biofertilizers suitable for acidic soils of Kerala. Eventhough, the biofertilizers have been well accepted as a source of nutrients, its efficiency depends on the soil edaphic factors and the environment. Therefore, there is a need to develop a consortium of native biofertilizer to improve its effectiveness.

Kerala soils are acidic in nature and the efficiency of biofertilizers are greatly affected by the acidic pH of soil. Therefore, there is a need to develop native biofertilizers suitable for the acidic soils. Among the biofertilizers, *Azospirillum* and phosphate solubilizing bacteria are popular in Kerala. In recent years, it has also been reported that consortia of biofertilizers perform better than

individual isolates (Rafi *et al.*, 2012). At present, there is no availability of a consortium of biofertilizer in Kerala, which can supply nitrogen and phosphorus simultaneously to the plants under acidic soil pH. Hence, a study was undertaken to screen an acid-tolerant and efficient consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils of Thrissur district in Kerala. The objectives of the study were to screen the isolates of *Azospirillum* and PSB for acid-tolerance, efficiency and compatibility, and also to develop an efficient, acid-tolerant consortium of *Azospirillum* and PSB suitable for acidic soils.

The lateritic soils were collected from ten different locations of Thrissur district which showed pH in the range of 5.22 (Vellanikkara) to 6.51 (Madakkathara) (Fig. 1). The enumeration of *Azospirillum* sp. and PSB was done from each location to assess the pH preference of the isolates. Maximum population of *Azospirillum* ($1.1x10^5$ $MPNg^{-1}$) was recorded in Mulayam (pH 6.3) and Madakkathara (pH 6.5) (Fig. 2) which shows that *Azospirillum* sp. prefers near neutral or neutral pH. In a similar studies on *Azospirillum* associated with wheat, in Eastern Australia, *Azospirillum* was isolated from 40% of the samples with pH between 5.0 and 6.6 (New and Kennedy, 1989) which indicates that the *Azospirillum* sp. can be found in the acidic soils. In the present studies, *Azospirillum* sp. was found between pH 5.2 to 6.5.

All the isolates, which were having the characters of *Azospirillum* sp., were characterized further to confirm the identity of *Azospirillum* sp. using standard protocols (Krieg and Dobereiner, 1984). A total of 32 isolates of *Azospiriilum* were obtained from various locations of lateritic soils (Table 3). The colonies which showed pink colour on BMS agar medium indicated that the isolates were *Azospirillum*. In the present studies, only six isolates namely AMU-2, ACH-1, AND-4, AEL-3, AOL-4 and AWD-1 were confirmed as *Azospirillum*, which were in agreement with the characters described earlier by Tarrand *et al.* (1979). These confirmed isolates were found to be gram negative, motile with subsurface pellicle on Nfb semi-solid media, pink coloured colonies on BMS agar media, positive urease activity, phosphatase activity, oxidase activity, catalase activity

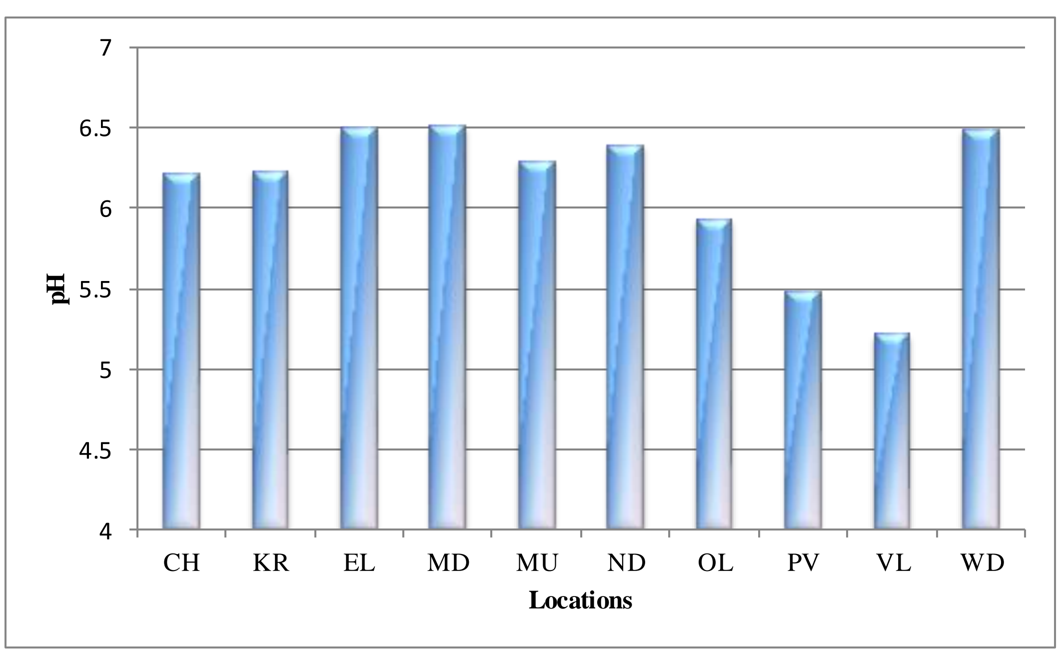


Fig. 1. pH of the soil samples collected from different locations of Thrissur district

CH : Chelakkara

KR : Koratty

EL : Elanad

MD : Madakkathara

MU : Mulayam

ND : Nadavarambu

OL : Ollur

PV : Perumpilavu

VL : Vellanikkara

WD : Wadakkancherry

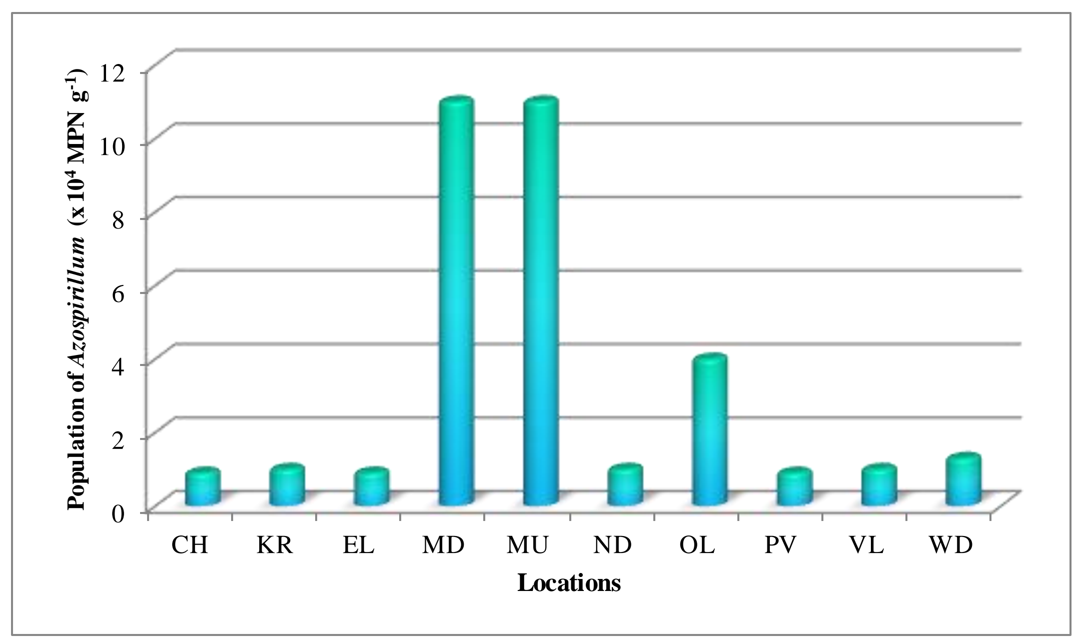


Fig. 2. Population of *Azospirillum* isolates from different lateritic soils

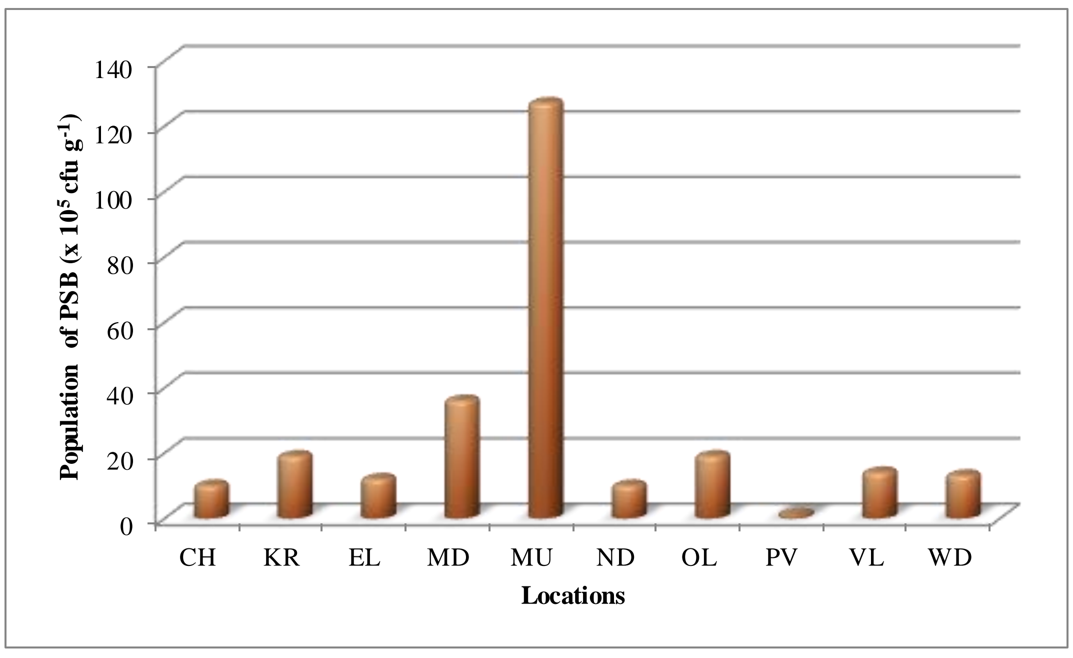


Fig. 3. Population of PSB isolates from different lateritic soils

and negative results for starch hydrolysis (Table 4). These results are in agreementwith the keys described for the identification of *Azospirillum* sp. (Tarrand *et al.*, 1979).

The six confirmed isolates of *Azospirillum* were screened for acid-tolerance between pH 3.5 and 7.0 in both solid and liquid media in order to assess their level of acid-tolerance. The isolate AND-4 (Nadavarambu) (soil pH 6.39) was found to be the most acid-tolerant upto pH 4.5 followed by AMU-2 (Mulayam) (soil pH 6.29) and ACH-1 (Chelakkara) (soil pH 6.21) (Table 6&7). In a similar studies, New and Kennedy (1989) reported that *Azospirillum* were absent in soil with pH below 4.5. However, those isolates could tolerate more acidic condition when grown in the presence of fixed nitrogen than in the absence. In the present study, all the six isolates were found to be more tolerant to pH 4.5 in the presence of a nitrogen source and hence the present results are in agreement with earlier studies.

Simultaneously, along with the isolation of *Azospirillum* sp., phosphate solubilizing bacteria were also isolated from ten different locations on Pikovskaya's agar media. A total of 35 isolates of PSB were obtained (Table 5). The maximum population (100x10^5 cfu g^{-1}) was recorded from Mulayam followed by Madakkathara (15x10^5 cfu g^{-1}) (Fig. 3). Pal (1999) reported that one of the PSB isolates PAS-2 from pasture and waste land (pH 4.8) had highest P-solubilizing capacity which tolerated a wide range of soil acidity ranging from pH 4.5 to 6.1. However, in the present studies, higher population of PSB was observed in soil samples with a pH ranging from 6.3 to 6.5, indicating that PSB prefers near neutral pH. Population of the PSB was found to be less in soils below pH 6.0. The higher population of PSB at Mulayam (soil pH 6.29) and Madakkathara (Soil pH 6.51) might be due to the rhizosphere effect of host plants. It has been well established that the rhizosphere microflora is greatly influenced by the host plant. Foster (1988) reported that bacterial populations residing in the rhizosphere are several orders of magnitude larger than those residing in bulk

soils. However, the present studies also indicated that *Azospirillum* sp. and PSB isolates can also be found in the soils with pH ranging from 5.22 to 6.0.

All the 35 isolates of PSB were screened for acid-tolerance in both solid and liquid media. All the isolates were able to tolerate acidic pH in the range of 4.5 to 6.5 (Table 8&9). However, only one isolate (POL-3) was able to tolerate pH upto 3.5. Pal (1999) reported that only 9 out of 23 bacterial isolates tolerated acidic pH under *in vitro*. He also reported that the strain PAS-2 isolated from pasture and wasteland of pH 4.8 had highest acid-tolerance. The present studies indicated that the PSB isolates obtained from acidic soils of pH 5.93 to 6.51 had high acid-tolerance.

The acid-tolerant isolates of *Azospirillum* and PSB were screened for their efficiency in nitrogen fixation and P-solubilization respectively under *in vitro* conditions. The six most acid-tolerant strains of *Azospirillum* namely (AMU-2, ACH-1, AND-4, AEL-3, AOL-4 and AWD-1) were screened for nitrogen fixation and indole acetic acid production under *in vitro* conditions.

The two isolates (AND-4 and AMU-2) were found to fix more than 10 mg of N g^{-1} of malate utilized (Fig. 4). However, AND-4 recorded highest nitrogen fixing ability (17.94 mg of N g^{-1} of malate). The nitrogen fixing ability of different isolates might be associated with soil type, environmental factors, nitrogen status of soil, crop and its varieties (Lakshmikumari *et al.*, 1976, Rai, 1991). In the present studies, AND-4 isolate of *Azospirillum* showed highest nitrogen fixation indicating that the acid-tolerant *Azospirillum* can fix very high amount of nitrogen which is important in the improvement of the plant growth.

In the present studies, AMU-2 recorded 51.95 μg ml^{-1} IAA which is very high with the acid-tolerant isolate. The IAA production of the native *Azospirillum* sp. isolates ranged from 8.16 μg ml^{-1} to 51.95 μg ml^{-1} (Fig. 5) which is more than the normal quantity of IAA production by *Azospirillum* sp. In a similar studies, Umali-Garcia *et al.* (1980) reported that IAA and other hormones produced by *Azospirillum brasilense* stimulated the growth of lateral roots and root hairs in

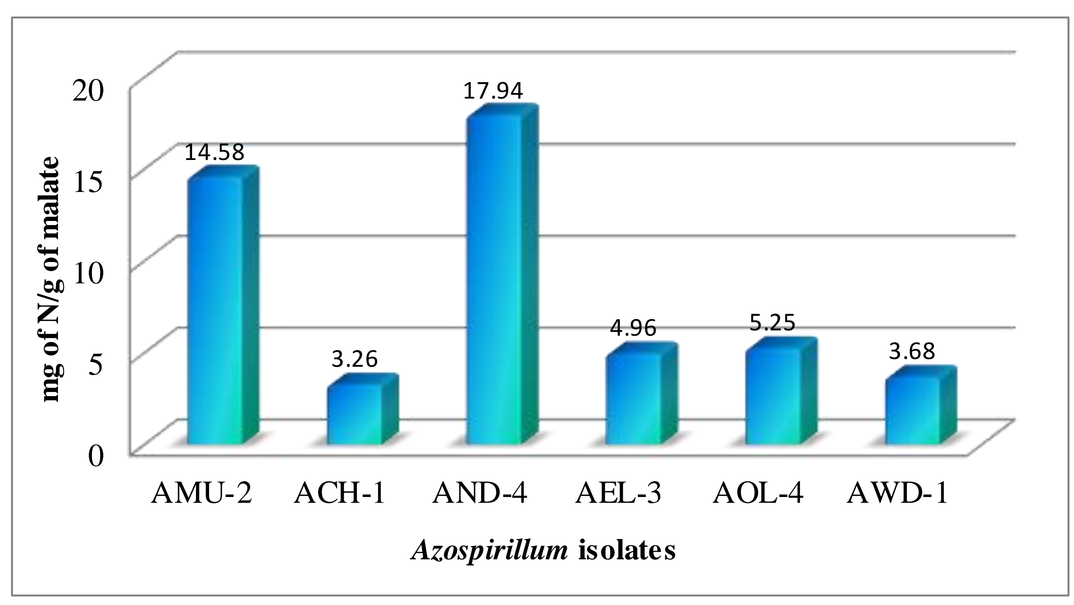


Fig.4. Nitrogen fixed by *Azospirillum* isolates

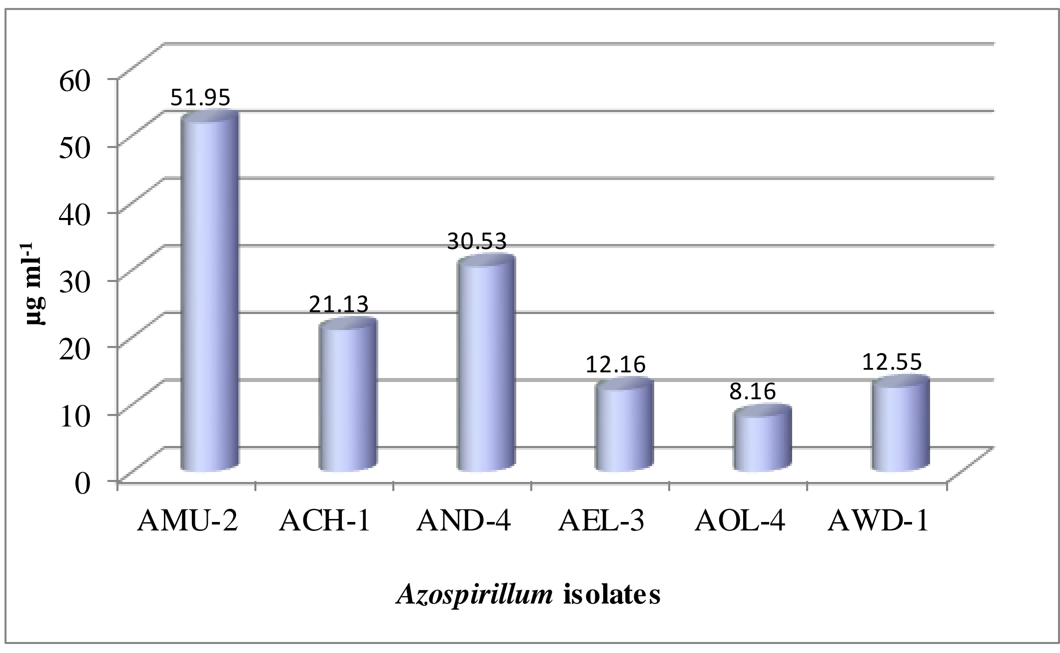


Fig.5. Indole acetic acid production by *Azospirillum* isolates

pearl millet. However, in the present studies, there was variation in the IAA production by different isolates of *Azospirillum* sp. which might be due to thedifferences in the tryptophan and IAA metabolic pathways. Hartmann *et al.* (1983) reported that the differences in the IAA production are due to differences in the tryptophan and IAA biosynthesis.

Similarly, the six most acid-tolerant phosphate-solubilizing bacteria (PKR-3, PKR-8, PMD-7, PMU-2, POL-1 and POL-3) were screened for efficiency in P-solubilization and indole acetic acid production. The amount of P-solubilized by the isolates ranged from 97 $\mu g\ ml^{-1}$ to 207 $\mu g\ ml^{-1}$ (Fig. 6). The highest P-solubilized was recorded in the case of PMD-7 (207 $\mu g\ ml^{-1}$) followed by POL-1 (187.78 $\mu g\ ml^{-1}$) isolates. Pal (1998) reported that the P-solubilizing capacity of PSB isolates isolated from Garhwal, Himalaya region ranged between 11.4 and 45 $\mu g\ ml^{-1} day^{-1}$, which were capable of growing in low pH (5.4-5.6) but, showed variation in their growth rate. Jena and Rath (2013) reported that the production of acidity in the medium is directly correlated to the reduction in pH of the medium due to the production of variety of organic acids. In the present study, PMD-7 isolate recorded highest P-solubilization indicating its potential as an efficient P-solubilizer there was a reduction in pH due to P-solubilization. However, it was not proportionate to the amount of P-solubilized. These results are in agreement with the studies conducted by Asea *et al.* (1988) who reported a lack of linear correlation between pH and the amount of P-solubilized in liquid media.

In the case of solubilization efficiency, which was determined based on the clear and halo zone of the Pikovskaya's agar media, the isolate POL-1 recorded maximum efficiency (450%) in the present studies (Fig. 7). However, there was no correlation between P-solubilization on the solid media and P-solubilization efficiency. In a similar study, Ostwal and Bhide (1972) reported contradictory results between solubilization efficiency and P-solubilization in liquid cultures. However, POL-1 was found to be the most acid-tolerant isolate and an efficient P-solubilizer in the present studies.

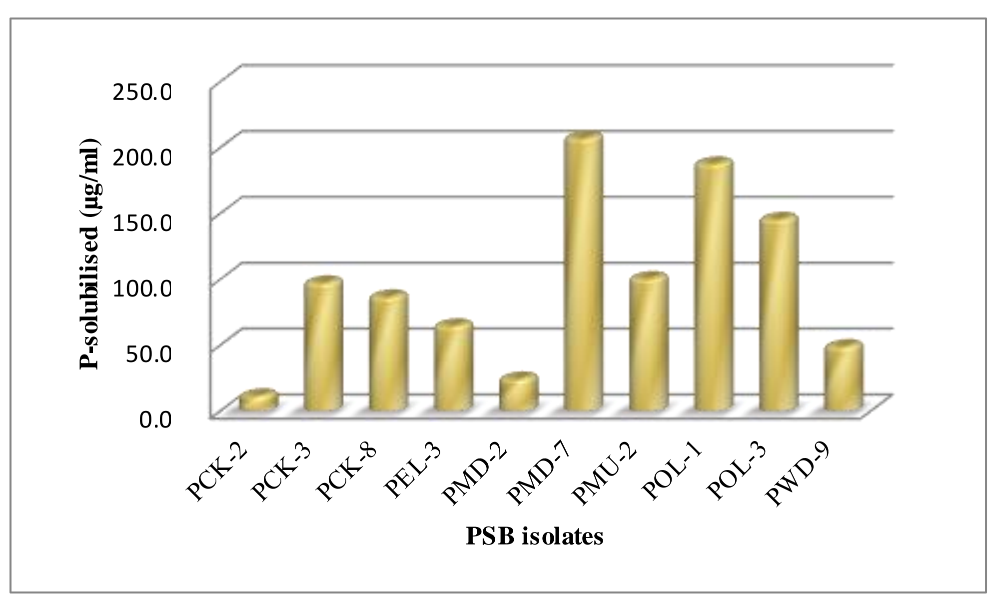


Fig.6. Phosphorus solubilization by PSB isolates

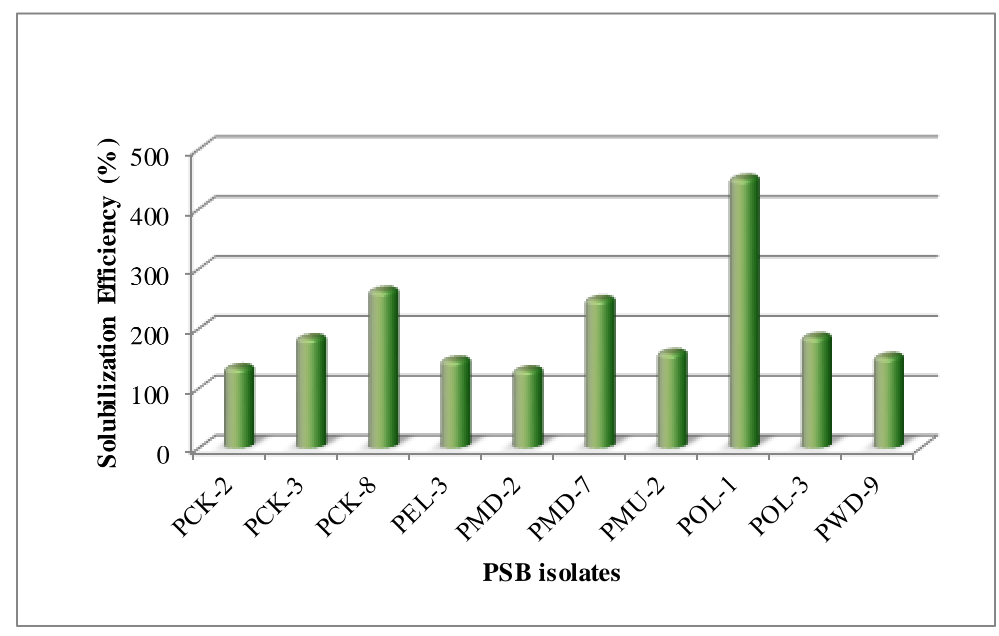


Fig.7. Solubilization efficiency of PSB isolates

The most acid-tolerant PSB isolates were also screened for IAA production. The PMD-7 isolate recorded highest IAA production (33.07 µg ml^{-1}) followed byPKR-3 (32.43 µg ml^{-1}) (Fig. 8). In a similar studies, Tien *et al.* (1979) reported that *Azospirillum* and PSB isolated from rhizosphere soil of pearl millet produced indole acetic acid and gibberellins and cytokinin like substances which enhanced the plant metabolism. However, production of IAA varied greatly among different species of bacteria and mainly influenced by culture conditions, growth stage and availability of substrate (Vijila, 2000) which is in agreement with the results of the present studies.

As the present studies involved the consortia of acid-tolerant *Azospirillum* sp. and PSB, the compatibility studies between the six most acid-tolerant *Azospirillum* sp. and PSB isolates were carried out. Out of the 36 combinations of *Azospirillum* sp. and PSB, sixteen isolates were found to be compatible and twenty were incompatible (Table 13). Raja *et al.* (2006) reported that the *Azospirillum* sp., *B. megaterium* and *Pseudomonas fluorescens* were compatible under *in vitro* conditions, which were in agreement with the results of present studies. However, some of the isolates were found to be incompatible, which might be due to the capacity of one isolate to multiply rapidly which will compete for space and nutrients.

Based on the acid-tolerance, nitrogen fixation, IAA production, P-solubilization, P-solubilization efficieny and compatibility between the isolates, the three most acid-tolerant, efficient and compatible isolates selected for the pot culture experiment were AND-4 (*Azospirillum* sp.)+PMD-7 (*Acinetobacter* sp.), AMU-2 (*Azospirillum* sp.)+PMD-7 (*Acinetobacter* sp.) and AND-4 (*Azospirillum* sp.)+POL-1 (*Bacillus megaterium*) (Table 14).

The results of the pot culture experiment indicated positive response in plant biometric parameters with consortia applied treatments. The maximum germination percentage was recorded in T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) and T_4 (*Azospirillum* sp. + *Bacillus megaterium*) with 88.89% which was higher than T_1 (KAU POP recommendation) (Fig. 10). The increase in germination

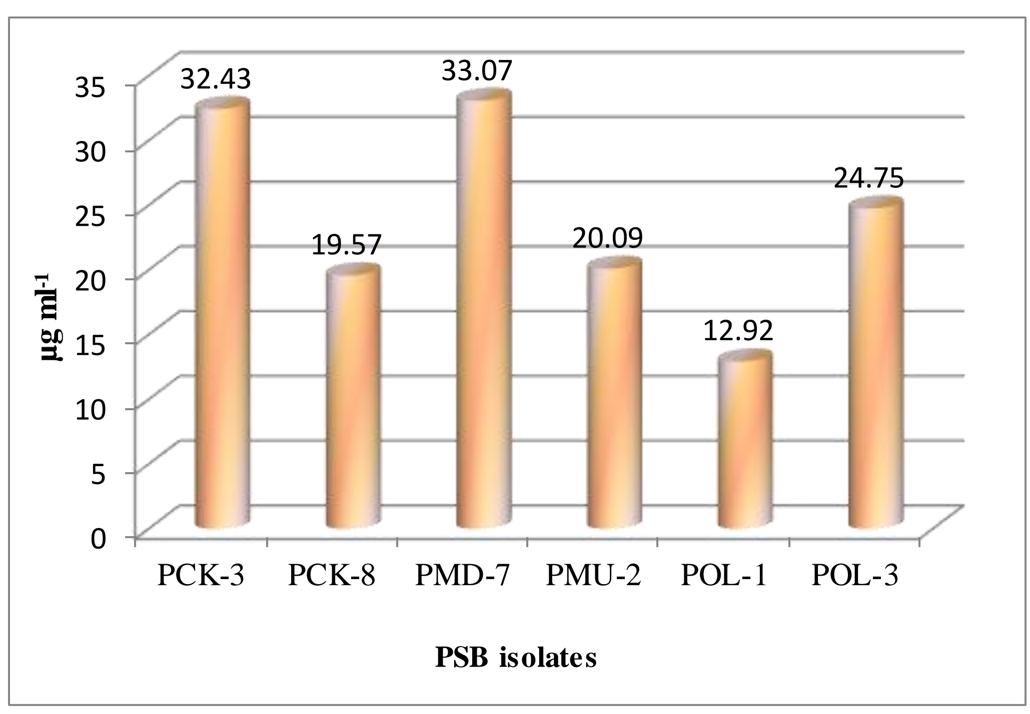


Fig.8. Indole acetic acid production by PSB isolates

percentage after five days of consortial inoculation might be due to the productionof indole acetic acid (Fallik *et al.*, 1989). Likewise, PSB also produced auxins, gibberellins and cytokinins which might have improved the plant growth and stimulated the microbial development (Sattar and Gaur, 1987).

Among the consortia treatments, the plant height was highest (54.77 cm) in the case of T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (Fig. 11). The maximum number of leaves were recorded in the treatment T_2 (*Azospirillum* sp. + *Acinetobacter* sp.) (28.52) followed by T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (28.0) (Fig. 12). The minimum days taken for flowering was recorded in T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (44.87) (Fig. 13). The maximum number of fruits per plant (41.0 fruits plant^{-1}) and fruit yield (140.33 g plant^{-1}) were recorded in the case of T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (Fig. 14&15). Based on the overall biometric and yield parameters among the consortia treatments, the most promising consortia was T_4 (*Azospirillum* sp. + *Bacillus megaterium*). However, among all the treatments, T_1 (KAU POP recommendation) performed better than the consortia treatments.

In a similar studies, Alagawadi and Gaur (1992) reported that combined inoculation of *Azospirillum brasilense* and *Pseudomonas striata* or *Bacillus polymyxa* showed significant increase in grain yield, N_2 and phosphorus uptake in sorghum. El-Komy (2005) reported the beneficial influence of co-inoculation of *Azospirillum lipoferum* and *Bacillus megaterium* for providing balanced nitrogen and phosphorus nutrition in wheat plants. Trimurtulu (2011) also reported that mixed microbial consortia of *Azospirillum, Azotobacter*, PSB, PGPR and AMF increased not only soil microbial population but also yield of chilli. Rafi *et al.* (2012) also reported that *Azospirillum lipoferum* and PSB inoculation in foxtail millet significantly increased plant height, shoot and root weight over control plants.

In the present studies, the consortium of *Azospirillum* sp. + *Bacillus megaterium* was found to be the most efficient in enhancing the growth of okra under acidic soils indicating that this consortium is suitable for acidic soils of

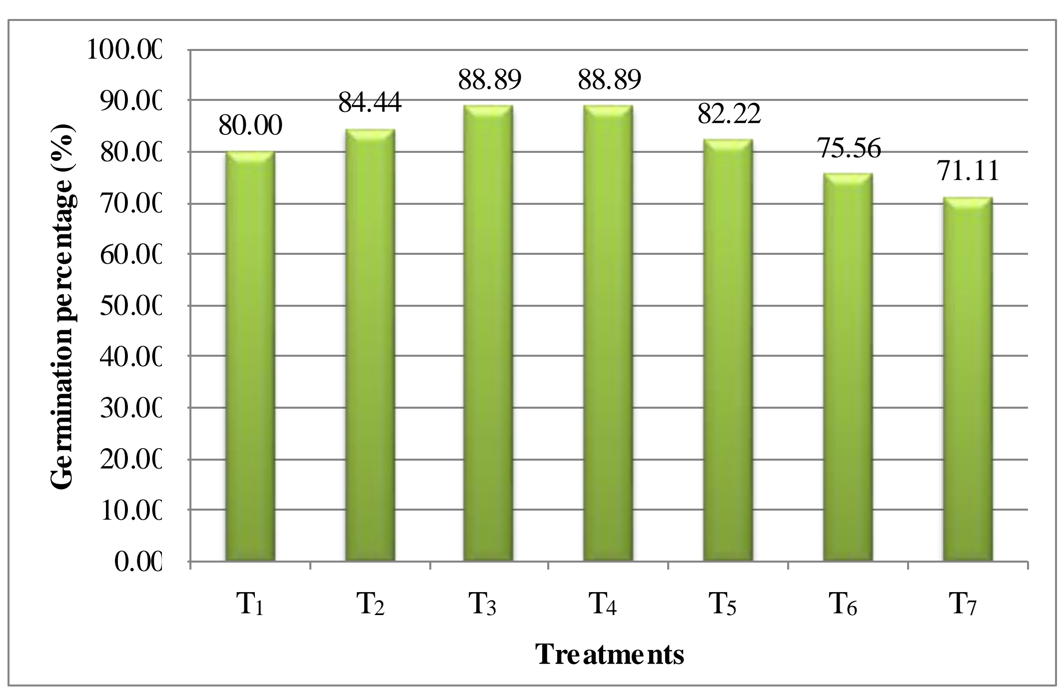


Fig.10. Effect of different treatments on germination percentage

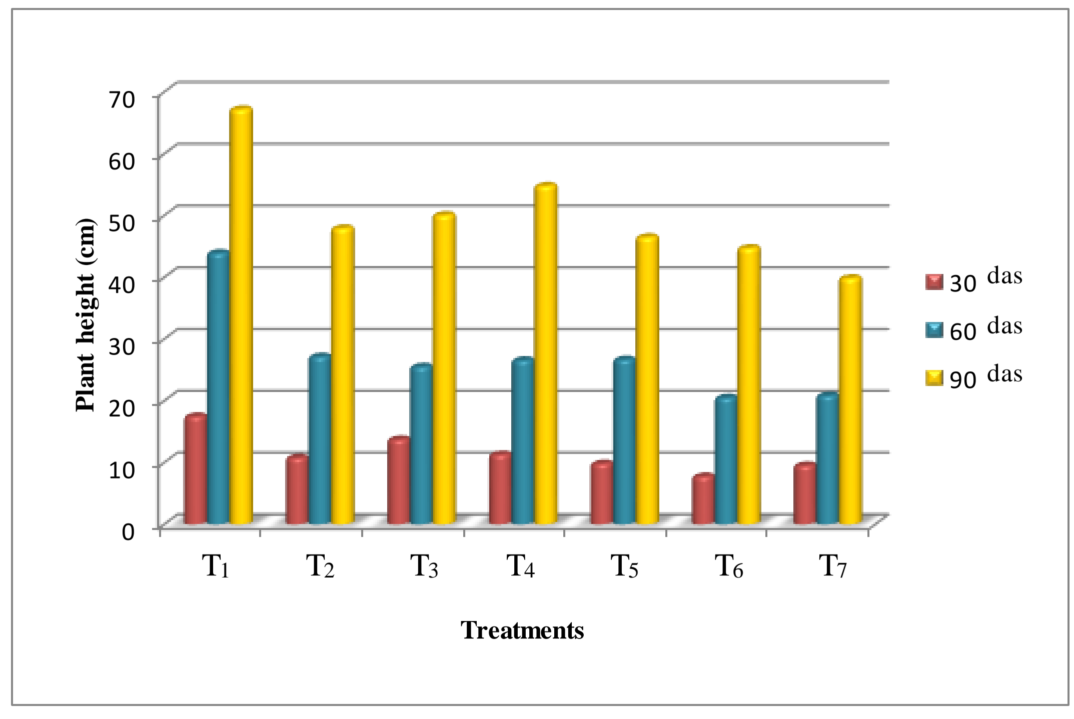


Fig.11. Effect of different treatments on plant height

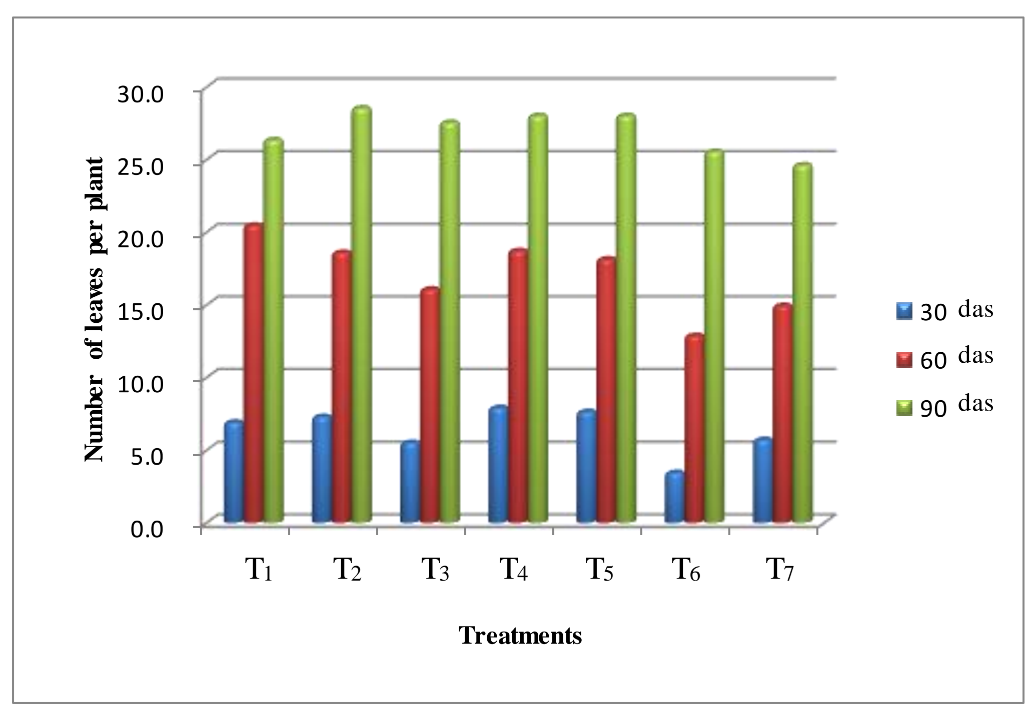


Fig.12. Effect of different treatments on number of leaves

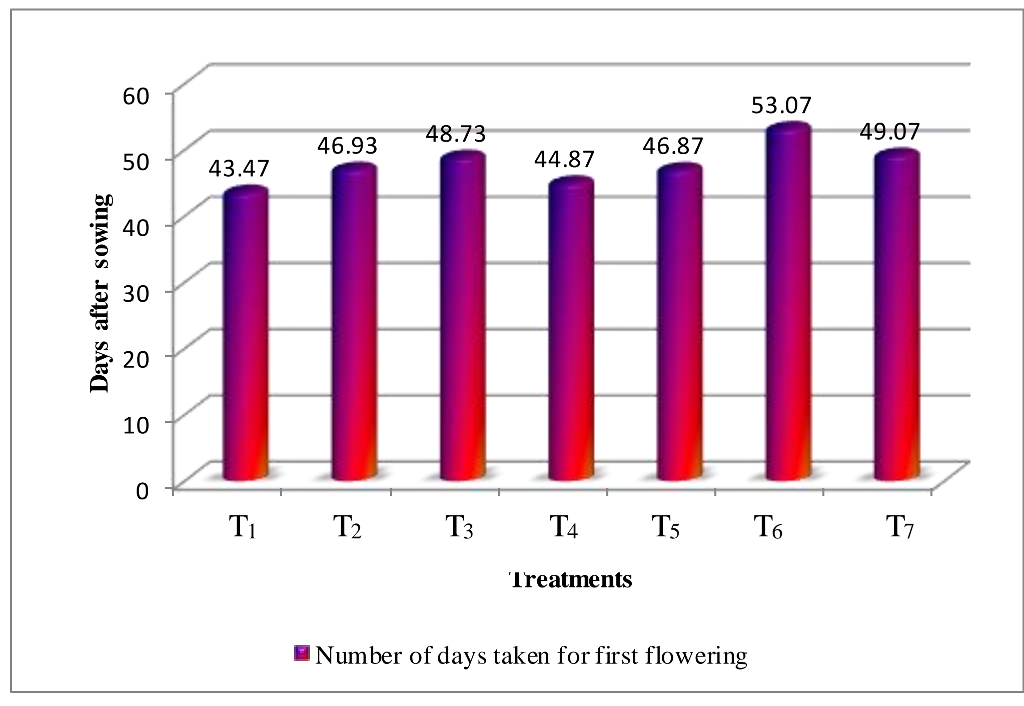


Fig. 13. Effect of different treatments on number of days taken for flowering

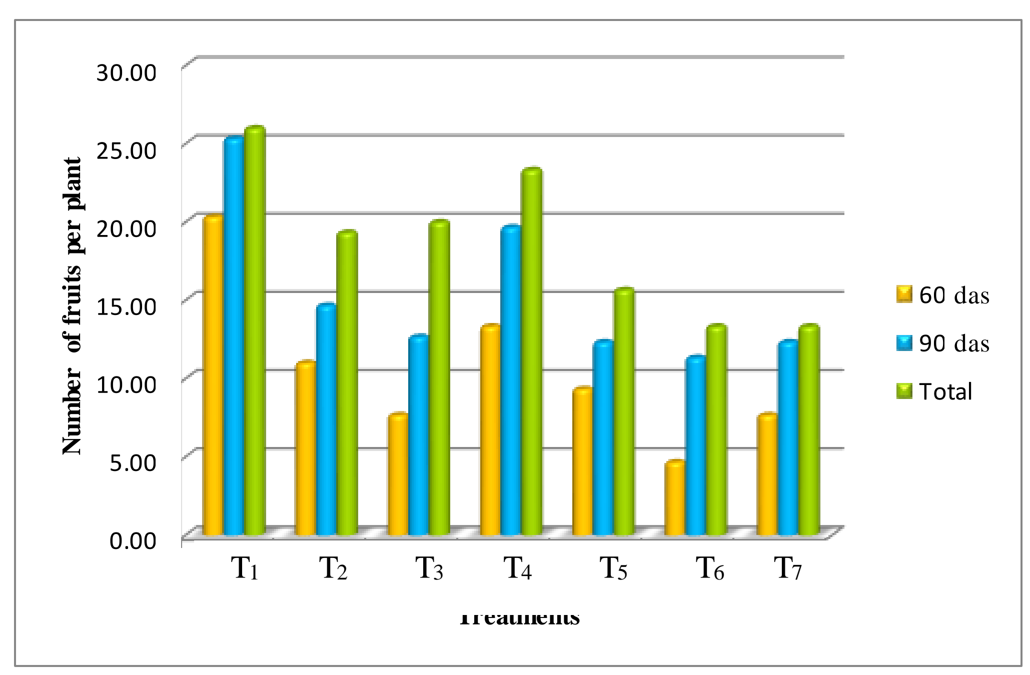


Fig.14. Effect of different treatments on number of fruits

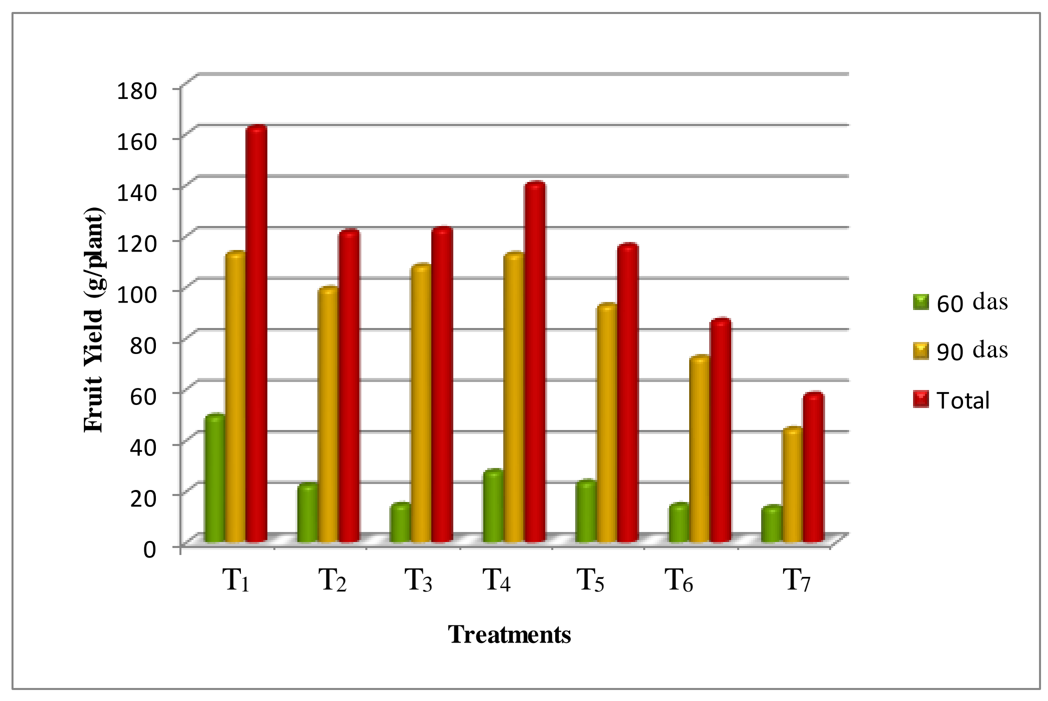


Fig.15. Effect of different treatments on fruit yield

Kerala. Moreover, the mixed microbial consortia are considered to be a balanced combination of different microorganisms for achieving maximum output in the cultivation of chilli. At the time of harvest, the population of *Azospirillum* and PSB increased in all the treatments when compared with control (Table 23). However, the treatments with chemical fertilizers (T_1) affected the growth of PSB. Alagawadi and Gaur (1992) reported that the population of *Azospirillum* and PSB in the rhizosphere of sorghum was higher than the uninoculated treatment which is in agreement with the present studies.

The okra plants were infected with pest and diseases during the crop period. The yellow vein mosaic disease was recorded two months after sowing. The highest disease incidence was recorded in T_7 (Absolute control) (46%). Among the consortia, the disease incidence varied between 26-40%, which might be due to high ambient temperature (33.9 to 36.3^{0}C) and low rainfall. Singh (1990) reported that dry, hot weather with little or no rainfall was conducive for disease development of okra yellow vein mosaic virus. In the present studies, the temperature ranged from 33 to 36^{0}C and very little rainfall was recorded. The results of the present studies are in agreement with earlier reports.

In addition to the diseases in the okra plants, there was mite and petiole maggot attack towards the end of the crop growth. The pest and disease incidence in the okra plants might be due to the climatic conditions, which caused the infections. Rachana *et al.* (2009) reported that the incidence of mite in okra had significant positive correlation with temperature, a significant negative correlation with relative humidity and rainfall. However, in the present studies, the pest and disease was higher in the control plants when compared with the other treatments which might be due to high temperature and scanty rainfall recorded in present studies (Appendix II).

The most efficient consortium of *Azospirillum* sp. + PSB (T_4 treatment) was identified using standard protocols. The PSB isolate of the consortium was identified by 16S rDNA sequencing. Based on the homology, the POL-1 (PSB

isolate) was identified as *Bacillus megaterium*. *Bacillus megaterium* is a widely used P-solubilizer which has been already commercialized (El-Komy, 2005).

The nitrogen and phosphorus uptake by okra was determined at the time of harvest in order to find the amount of nitrogen fixed by *Azospirillum* and solubilization of phosphorus by PSB isolates. The maximum nitrogen uptake was recorded in T_1 (POP recommendation) (0.99 g N_2 plant^{-1}) followed by T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (0.81 g N_2 plant^{-1}) which was on par with the plants treated with chemical fertilizers. In the case of phosphorus, the uptake was maximum in T_1 (POP recommendation) (0.41 g P plant^{-1}). In a similar study, *Azospirillum* treated tea plants showed increased total nitrogen and nitrogenase activity. Where as, PSB treated plants increased available phosphorus and phosphatase activity. The dual inoculation showed increased plant growth hormones in soil and population build up of respective organism (Fernataz *et al.*, 2007). Rafi *et al.* (2012) also reported that dual inoculation of *Azospirillum lipoferum* and phosphate solubilizers increased the uptake of nitrogen, phosphorus, zinc, copper and iron in the shoots of foxtail millet under field condition. These results are in agreement with the present studies.

The present studies clearly indicated that a consortium of *Azospirillum* and PSB increased the growth and yield of okra. The consortium of *Azospirillum* + *Bacillus megaterium* was found to be the most promising consortia for the acidic soils. Moreover, the consortial isolates were found to be tolerant upto pH 4.5 which means that these isolates can be a potential biofertilizer for the acidic soils. Since, majority of the Kerala soils are acidic in nature, the major constraint in the biofertilizer application is the acidic nature of the soil. In the present studies, a consortia consisting of nitrogen and phosphatic biofertilizers were identified which is not only acid-tolerant but also efficient under acidic pH. However, the consortium has to be evaluated under field conditions so as to commercialize this biofertilizer consortium for the benefit of farmers in Kerala.

SUMMARY

6. SUMMARY

The present study on "Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils" were carried out in the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara during 2011-2013. The major objectives were to develop acid-tolerant and efficient consortia of *Azospirillum* and phosphate solubilizing bacteria suitable for acidic soils of Kerala and to evaluate the consortia under pot culture experiment using okra as test crop. The important findings of the study are summarized below:

- The soil samples were collected from lateritic soils of Thrissur district. The pH ranged from 5.22 to 6.51 with maximum in the case of Madakkathara and minimum in Vellanikkara.
- A total of 32 isolates, which appeared to be similar to the characters of *Azospirillum* sp. on Nfb media and 35 PSB isolates were obtained from ten locations of Thrissur district.
- Highest population of *Azospirillum* was obtained from Madakkathara (MD) and Mulayam (MU) (1.1×10^5 MPN g^{-1}). Whereas, Chelakkara (CH), Elanad (EL) and Perumpilavu (PV) showed least population (0.9×10^4 MPN g^{-1}). Highest population of phosphate solubilizing bacteria was obtained from Mulayam (12.7×10^6 cfu g^{-1}).
- Six isolates out of 32 were confirmed as *Azospirillum* sp. based on morphological and biochemical characterization. These isolates were designated as AMU-2, ACH-1, AND-4, AEL-3, AOL-4 and AWD-1.
- Screening of *Azospirillum* sp. and PSB isolates for acid-tolerance were carried out in solid as well as in liquid media. The *Azospirillum* sp. AND-4 and AMU-2 were found to be the most acid-tolerant isolates upto pH 4.5. Among the PSB isolates, POL-3, POL-1 and PMD-7 were the most acid-tolerant isolates upto pH 4.5.

- *Azospirillum* isolate AND-4 fixed maximum amount of nitrogen (17.94 mg of Ng^{-1} of malate) and AMU-2 recorded highest IAA production (51.95 μgml^{-1}).
- PSB isolates were screened for its efficiency in P-solubilization both qualitatively and quantitatively. Quantitative estimation of P- solubilized by PSB isolates revealed PMD-7 as the most efficient P-solubilizer (207.22 $\mu g\ ml^{-1}$). However, POL-1 recorded maximum solubilization efficiency (450%). The PMD-7 isolate produced maximum IAA (33.07 $\mu g\ ml^{-1}$).
- The six most acid-tolerant and efficient isolates each from *Azospirillum* and PSB were subjected to compatibility test. Only sixteen combinations of *Azospirillum* and PSB were found to be compatible and twenty were incompatible.
- The selected combinations of consortium for pot culture experiment were AND-4 (*Azospirillum* sp.) + PMD-7 (*Acinetobacter* sp.), AMU-2 (*Azospirillum* sp.) + PMD-7 (*Acinetobacter* sp.) and AND-4 (*Azospirillum* sp.) + POL-1 (*Bacillus megaterium*).
- Maximum germination percentage was recorded in T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) and T_4 (*Azospirillum* sp.+ *Bacillus megaterium*) (88.89%).
- Among the consortia treatments, the plant height was highest (54.77 cm) in the case of T_4 (*Azospirillum* sp. + *Bacillus megaterium*). The maximum number of leaves were recorded in the treatment T_2 (*Azospirillum* sp. + *Acinetobacter* sp.) (28.52 leaves per plant) followed by T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (28.0 leaves per plant). The minimum days taken for flowering was recorded in T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (44.87 days). The maximum number of fruits per plant (41.0 fruits $plant^{-1}$) and fruit yield (140.33 g $plant^{-1}$) were recorded in the case of T_4 (*Azospirillum* sp. + *Bacillus megaterium*). Based on the overall biometric and yield parameters among the consortia treatments, the most promising consortia was T_4 (*Azospirillum* sp. + *Bacillus megaterium*). However, among

all the treatments, T_1 (KAU POP recommendation) performed better than the consortia treatments.

- At the time of harvest, the population of *Azospirillum* and PSB increased in all the treatments when compared with control. However, the treatments with chemical fertilizers (T_1) affected the growth of PSB.
- Maximum N_2 uptake was noticed in T_1(POP recommendation)(0.99 g plant^{-1}) followed by T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (0.81 g plant^{-1}) and T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) (0.80 g plant^{-1}). Maximum uptake of phosphorus was noticed in T_1 (POP recommendation) (0.41 g plant^{-1}) followed by T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (0.36 g plant^{-1}).
- The AND-4 and AMU-2 were identified as *Azospirillum* isolates based on keys described in Bergey's Manual of Systematic Bacteriology.
- 16S rDNA sequence analysis was carried out for the two most efficient PSB isolates (PMD-7 and POL-1). The PMD-7 showed homology with *Acinetobacter* sp. and POL-1 showed homology with *Bacillus megaterium*.
- The present study clearly showed that the acid-tolerant and efficient consortium can perform on par with chemical fertilizers. Among the three consortia tested in the study, T_4 (*Azospirillum* sp. + *Bacillus megaterium*) performed better with respect to biometric and yield parameters under pot culture experiment.

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APPENDICES

APPENDIX - I

(a) Nfb semi-solid medium

Malic acid	– 5g
K_2HPO_4	– 0.5g
KOH	– 4g
Mg $SO_4.7H_2O$	– 0.1g
NaCl	– 0.02g
$CaCl_2$	– 0.01g
$FeSO_4.7H_2O$	– 0.05g
Na_2MoO_4	– 0.002g
$MnSO_4$	– 0.01g
Bromothymol blue (BTB)	– 2 ml (0.5% alchohol solution)
Agar	– 2g
Distilled water	– 1000 ml
pH	– 6.8-7.2

(b) BMS agar

Washed, peeled potato	– 200g
L-Malic acid	– 2.5g
KOH	– 2g
Raw cane sugar	– 2.5g
Vitamin solution	– 1ml (from 0.01g biotin+0.02g pyridoxin prepared in 1000 ml)
Distilled water	– 1000 ml
Bromothymol blue (BTB)	– 2 ml (0.5% alchohol solution)
Agar	– 20g

(c) Rojo-Congo red medium

K_2HPO_4	– 0.5g
$MgSO_4 . 7H_2O$	– 0.2g
NaCl	– 0.1g
Yeast extract	– 0.5g
$FeCl_3 . 6H_2O$	– 0.015g
Malic acid	– 5g
KOH	– 4.8g
Agar	– 20g
Congo red	– 1g
Distilled water	– 1000 ml
pH	– 7.0

(d) Urea broth

Urea	– 20g
Yeast extract	– 0.1g
KH_2PO_4	– 9.0g
K_2HPO_4	– 9.5g
Phenol red	– 0.01g
Distilled water	– 1000 ml
pH	– 6.8

(e) Starch agar

Starch	– 20g
Beef extract	– 3g
Peptone	– 3g
Agar	– 20g
Distilled water	– 1000 ml

(f) Pikovskaya's agar

Glucose	– 10g
$Ca_3(PO_4)_2$	– 5g
$(NH_4)_2SO_4$	– 0.5g
NaCl	– 0.2g
$MgSO_4.7H_2O$	– 0.1g
KCl	– 0.2g
Yeast extract	– 0.5g
$MnSO_4.H_2O$	– 0.002g
$FeSO_4.7H_2O$	– 0.002g
Distilled water	– 1000 ml
pH	– 7.0

(g) Nutrient agar

Beef extract	– 3g
Peptone	– 5g
NaCl	– 5g
Agar	– 20g
Distilled water	– 1000 ml

(h) Nutrient broth

Beef extract	– 3g
Peptone	– 5g
NaCl	– 5g
Distilled water	– 1000 ml

(i) Reagent B

1.56 of ascorbic acid is dissolved in 200 ml of Reagent A.

(j) Reagent A

12 g of Ammonium molybdate is dissolved in 250 ml of distilled water. 0.291 g of antimony potassium tartarate is dissolved in 100 ml of distilled water. Both these solutions are added to 1000 ml of approx. 5N H_2SO_4. This solution is mixed thoroughly and made up to 2 L with distilled water.

APPENDIX - II

Weather data during crop growth (February-April, 2013)

Month	Weeks	Temperature		Mean RH	Mean SS hr.	Rain fall	Rainy days	Mean Evp.
		Max.	Min.					
February	1st week	35	23.6	0.61	8.5	0	0	4.5
	2nd week	34.6	24.4	0.61	6.9	17	1	5.9
	3rd week	33.9	23	0.58	8.8	67.4	1	4.4
	4th week	36.3	21.8	0.42	10.1	0	0	5.6
March	1st week	35.1	25.4	0.61	4.9	7.8	1	5.5
	2nd week	35.1	24.3	0.69	6.9	6.8	1	4.3
	3rd week	36.2	24.7	0.71	8.3	0	0	4.8
	4th week	34.6	24.9	0.72	7.3	0	0	4.5
April	1st week	34.2	25.3	0.75	5	0	0	4
	2nd week	35.4	25.7	0.73	6.9	0	0	4.8
	3rd week	34.5	24.5	0.68	7.6	0	0	4.8
	4th week	35.2	25.1	0.69	6.5	0	0	4.6
	5th week	34.7	26	0.75	3.9	0	0	3.4

APPENDIX - III

Nucleotide sequences of POL-1 isolate

CACACGGCCACTCGTTGATTCGACTTCACCCCAATCATCTGTCCCA
CCTTAGGCGGCTAGCTCCTTACGGTTACTCCACCGACTTCGGGTGTT
ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA
CGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGC
TTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTT
TATGGGATTGGCTTGACCTCGCGGTCTTGCAGCCCTTTGTACCATCC
ATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGA
GTGCCCAACTAAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA
TGCACCACCTGTCACTCTGTCCCCCGAAGGGGAACGCTCTATCTCT
AGAGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGC
TTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAA
TTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCT
TAATGCGTTAGCTGCAGCACTAAAGGGCGGAAAACCCTCTAACACT
TATCACTCATCGTTTACGGCGTGGACTACCCAGGGTATCTAATCCT
GTTTGCTCCCCACGCTTTCTCGCCTCAGCGTCAGTTCAGACCAAAA
AGCCGCCCTTCGCCAACTGGTGTTCCTCCACATCTTCTACGCATTTC
ACCGCTACCCGTGGAATTCCGCTTTTCTCTTCTGACCTCAAGTTTCC
CAAGTTTCCA

Nucleotide sequences of PMD-7 isolate

AAATTGGTTGCGCAGTCTTACCATGCAGTCGAGCGGGGGAAGGTA
GCTTGCTACCGGACCTAGCGGCGGACGGGTGAGTAATGCTTAGGA
ATCTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCTAATAC
CGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCG
CTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAG
GCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGC
CGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAG
GAGGAGGCTACTTTAGTTAATACCTAGAGATAGTGGACGTTACTCG
CAAAATAAGCACCGGCTAACTCTGTGCCAGCATCCGCGGTAATACA
GAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAATGCGTGCGTA
TGCGGCTTATT

APPENDIX – IV

MPN table for 3 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs per gram and 95 percent confidence intervals.

Positive tubes				Conf. lim.	
0.1	*0.01*	*0.001*	*MPN/g*	*Low*	*High*
0	0	0	<3.0	–	9.5
0	0	1	3	0.15	9.6
0	1	0	3	0.15	11
0	1	1	6.1	1.2	18
0	2	0	6.2	1.2	18
0	3	0	9.4	3.6	38
1	0	0	3.6	0.17	18
1	0	1	7.2	1.3	18
1	0	2	11	3.6	38
1	1	0	7.4	1.3	20
1	1	1	11	3.6	38
1	2	0	11	3.6	42
1	2	1	15	4.5	42
1	3	0	16	4.5	42
2	0	0	9.2	1.4	38
2	0	1	14	3.6	42
2	0	2	20	4.5	42
2	1	0	15	3.7	42
2	1	1	20	4.5	42
2	1	2	27	8.7	94
2	2	0	21	4.5	42
2	2	1	28	8.7	94
2	2	2	35	8.7	94
2	3	0	29	8.7	94
2	3	1	36	8.7	94
3	0	0	23	4.6	94
3	0	1	38	8.7	110
3	0	2	64	17	180
3	1	0	43	9	180
3	1	1	75	17	200
3	1	2	120	37	420
3	1	3	160	40	420
3	2	0	93	18	420
3	2	1	150	37	420
3	2	2	210	40	430
3	2	3	290	90	1,000
3	3	0	240	42	1,000
3	3	1	460	90	2,000
3	3	2	1100	180	4,100
3	3	3	>1100	420	–

SCREENING OF ACID-TOLERANT CONSORTIA OF *AZOSPIRILLUM* AND PHOSPHATE SOLUBILIZING BACTERIA FROM LATERITIC SOILS

By

ATHULYA, M. M.

(2011-11-141)

ABSTRACT OF THE THESIS

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Department of Agricultural Microbiology

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA

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ABSTRACT

A study was undertaken on "Screening of acid-tolerant consortia of *Azospirillum* and phosphate solubilizing bacteria from lateritic soils". The main objective was to develop an acid-tolerant and efficient consortium of *Azospirillum* and phosphate solubilizing bacteria (PSB) for acidic soils of Kerala.

Lateritic soils with acidic pH were collected from ten different locations of Thrissur district for the isolation of *Azospirillum* and phosphate solubilizing bacteria. The pH of the soil ranged from 5.22 (Vellanikkara) to 6.51 (Madakkathara).

The highest population of *Azospirillum* was recorded in soils of Madakkathara (MD) and Mulayam (MU) ($1.1x10^5$ MPN g^{-1}). However, Chelakkara (CH), Elanad (EL) and Perumpilavu (PV) recorded least population ($0.9x10^4$ MPN g^{-1}). Highest population of PSB was obtained from Mulayam ($12.7x10^6$ cfu g^{-1}) and least PSB population was obtained from Perumpilavu (PV) ($1.0x10^5$ cfu g^{-1}). The population of both *Azospirillum* and phosphate solubilizing bacteria were higher in rhizosphere soil than non-rhizosphere soil. A total of six isolates of *Azospirillum* and 35 isolates of PSB were obtained.

The isolates AND-4 (*Azospirillum* from Nadavarambu) and AMU-2 (*Azospirillum* from Mulayam) were acid-tolerant upto pH 4.5. Similarly, PMD-7 (PSB from Madakkathara) and POL-1 (PSB from Ollur) were also tolerant upto pH 4.5.

The AND-4 isolate of *Azospirillum* sp. fixed highest amount of nitrogen (17.94 mg of N g^{-1} of malate) followed by AMU-2 isolate (14.58 mg of N g^{-1} of malate). The isolate AMU-2 showed maximum IAA production (51.95 µg ml^{-1}) followed by AND-4 (30.53 µg ml^{-1}). The PMD-7 was the most efficient P-solubilizer (207.22 µg ml^{-1}) followed by POL-1(187.78 µg ml^{-1}). The PMD-7 isolate produced maximum IAA (33.07 µg ml^{-1}).

Sixteen combinations of *Azospirillum* and PSB were compatible. The three consortia selected based on acid tolerance, efficiency and compatibility were

AND-4 (*Azospirillum* sp.) + PMD-7 (*Acinetobacter* sp.), AMU-2 (*Azospirillum* sp.) + PMD-7 (*Acinetobacter* sp.) and AND-4 (*Azospirillum* sp.) + POL-1 (*Bacillus megaterium*).

Among the consortia, *Azospirillum* sp. + *Bacillus megaterium* (T_4) was found to be the most efficient in enhancing the growth of okra under acidic pH based on biometric and yield parameters. However, the POP recommendation (T_1) recorded maximum plant height, maximum number of fruits, fruit yield and minimum days for flowering.

The population of *Azospirillum* and PSB increased at the time of harvest in all the treatments except in T_7 (Absolute control). The population of *Azospirillum* was highest in T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) (23.38×10^3 MPN g^{-1}) and minimum in T_7 (Absolute control) (7.73×10^3 MPN g^{-1}). The population of PSB was highest in T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) (6.43×10^5 cfu g^{-1}) and minimum in T_7 (Absolute control) (2.08×10^5 cfu g^{-1}).

The maximum nitrogen uptake was recorded in T_1 (KAU POP recommendation) (0.99 g $plant^{-1}$). Among the consortia, T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (0.81 g $plant^{-1}$) and T_3 (*Azospirillum* sp. + *Acinetobacter* sp.) (0.80 g $plant^{-1}$) recorded highest nitrogen uptake. The maximum uptake of phosphorus was noticed in T_1 (KAU POP recommendation) (0.41 g $plant^{-1}$) followed by T_4 (*Azospirillum* sp. + *Bacillus megaterium*) (0.36 g $plant^{-1}$).

The most efficient isolates AND-4 and AMU-2 were identified as *Azospirillum* sp. The most efficient PSB isolates POL-1 and PMD-7 were identified as *Bacillus megaterium* and *Acinetobacter* sp. respectively.

The present studies clearly showed that the acid-tolerant and efficient consortium of *Azospirillum* + *Bacillus megaterium* (T_4 treatment) performed better under pot culture studies, which was on par with chemical fertilizer. However, further studies are needed to evaluate its efficiency under field conditions.