CHARACTERIZATION AND EVALUATION OF *Pseudomonas* spp. FOR ABIOTIC STRESS TOLERANCE

THESIS

Submitted in partial fulfillment of
the requirement for the degree of

Master of Science in Agriculture

by

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(2017-11-129)

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DECLARATION

I, hereby declare that the thesis entitled **"Characterization and evaluation of *Pseudomonas* spp. for abiotic stress tolerance"** is a bonafide record of research done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Characterization and evaluation of *Pseudomonas* spp. for abiotic stress tolerance"** is a record of research work done independently by **Ms. Reshma, K. S. (2017-11-129)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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INTRODUCTION

1. INTRODUCTION

Plant health management is often jeopardized due to threats posed by soil borne fungal pathogens which may include *Pythium, Phytophthora, Rhizoctonia, Sclerotium* and Fusarium. These pathogens are generally considered as menace to agricultural production, due to their "masked" occurrence in the soil as dormant survival structures and sudden outbreaks under favourable conditions. Eventhough, chemical fungicides are found to be very effective in eliminating these plant pathogenic fungi, the imprudent use of fungicides lead to serious issues concerning environmental and human health.

Thus, the search for an environmentally safe green strategy lead to the development of bio control agents with dual role in plant health management and plant growth enhancement. Numerous attempts have been made by the scientists all over the world to isolate antagonistic microorganisms from the soil. Eventually, biocontrol research leads to discovery of a fascinating world of microbes inhabiting rhizosphere of plants, the rhizobiome. It is the presence of bountiful of nutrients in the root exudates which attract soil microbes to rhizosphere in comparison to the near starvation condition of bulk soil.

One of the important plant beneficial rhizobiome includes fluorescent pseudomonads which often play a pivotal role in plant growth fortification and plant protection. *Pseudomonas fluorescens*, *P. putida, P. chlororaphis, P. aureofacians* have already been established as efficient bio control agents against soil borne pathogens (Weller *et al*., 2007). These antagonistic bacteria with its arsenal of antimicrobial substances may antagonize the pathogen or outcompete them for nutrients and space.

The antifungal properties of *Pseudomonas* include antibiosis *i.e.,* the inhibition of microbial growth by a myriad of antibiotics, volatiles, toxins, and biosurfactants. It may also comprise competition, parasitism through cell wall-degrading mycolytic enzymes (chitinases and β-1, 3-glucanase) and induction of systemic resistance (Haas *et al*., 2005). Furthermore, pseudomonads are also known to promote plant growth and alleviate abiotic stresses on plants (Sandhya *et al*., 2009; Saravanakumar and Samiyappan, 2007).

Despite the immense antagonistic potential shown under*in vitro* studies, the biocontrol performance of pseudomonads is found to be variable under field conditions. The effective functioning of introduced *Pseudomonas* spp. depends on survival and proliferation in the plant rhizosphere which often gets affected by a range of abiotic stress factors including high temperature, salinity, acidity and drought (Amalraj *et al*., 2010). Although, several strains of *Pseudomonas* spp. have been isolated and screened for antagonistic potential, much importance has not been given to its abiotic stress tolerance. Thus, full potential of these bioinoculants could not be harnessed when applied in varied ecosystems.

Agricultural fields of Kerala are also affected by extreme weather and soil conditions as reported by many workers (Bastin *et al*., 2014; Koruth *et al*., 2014; Mohan and Sreelatha, 2016). However, research on biocontrol in Kerala has not yet focussed on abiotic stress tolerance of the biocontrol agents. Therefore, a study was undertaken for the selection of native *Pseudomonas* spp. from abiotically stressed soils having inherent stress tolerance and antagonistic activity for their consistent performance under field conditions of Kerala. The study encompasses the following objectives:

- Collection of soil samples and isolation of *Pseudomonas* spp. from abiotically stressed ecosystems
- Screening for *in vitro* stress tolerance and antagonistic potential
- Biochemical characterization of stress tolerant isolates
- *In vivo* evaluation of stress tolerant isolates for biocontrol efficacy and plant growth promotion
- Identification of stress tolerant and antagonistic isolates of *Pseudomonas* spp.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The genus *Pseudomonas* first named by Walter Migula in 1984, belongs to the subdivision gamma Proteobacteria, and encompasses enormous diversity of chemoorganotrophic Gram negative asporogenous aerobic motile rods (Palleroni *et al*., 1973). The etymology of the generic name is derived from two Greek words '*pseudes*' and '*monas*' meaning 'false units'. These ubiquitous bacteria live in diverse habitats as endophytes, epiphytes, saprophytes or pathogens to animals and plants. Plant rhizosphere also serves as a favourite niche for some of the *Pseudomonas* spp. which act as commensals to the plants by eliminating or suppressing plant pathogens and promoting plant defense activities and growth even under adverse climatic conditions. *Pseudomonas fluorescens*, *P. putida*, *P. chlororaphis, P. aureofaciens* and *P. aeruginosa* are few among them (Schippers *et al*., 1987). Competition for nutrients and space, antibiosis by production of various antibiotics and antibiotic like substances, lysis and induction of systemic resistance are some of the strategies adopted by pseudomonads to suppress plant pathogens and protect the plants from diseases (Chen *et al*., 2000).

Abiotic stresses like drought, higher temperature, soil salinity, acidity, metal toxicity etc. are some of the major agricultural problems limiting agricultural productivity. Bioinoculants applied in the agricultural soils to improve health and growth of the crop plants are also adversely affected by these abiotic stresses. Interestingly, some of the *Pseudomonas* strains have aggressive root colonizing ability, metabolic versatility and produce a wide range of secondary metabolites including enzymes, hormones and other organic compounds that favour the plant to combat various biotic and abiotic constraints (Ramamoorthy *et al*., 2001; Vivekanandan *et al*., 2004; Mayak *et al*., 2004). The

bio control research has therefore focussed on pseudomonads to the core and has harnessed colossal of data to develop efficient strains which can survive under such stressed ecosystems. Hence, an attempt was made to review some of the recently published work on abiotic stress tolerance of *Pseudomonas* spp. relevant to this study.

2.1 ISOLATION AND ENUMERATION OF *Pseudomonas* spp. FROM ABIOTIC STRESSED ECOSYSTEMS

Development of successful formulations of biocontrol agents primarily depends on efficiency of biocontrol strain to adapt, colonize and proliferate in the introduced environment. Thus isolation of native strains of *Pseudomonas* spp. with higher antagonistic potential and those which can tolerate adverse environmental conditions is a necessary step in biocontrol research.

2.1.1 Abiotic stressed ecosystems of Kerala

The beginning of 21st century has witnessed adverse consequences of climate change which is reflected in the field of agriculture also. The most important resource of agriculture, the soil is also deteriorating with increased soil salinity and acidity. In addition to this, occurrence of high temperature and drought contributes to decline in agricultural production.

A detailed soil analysis collecting one lakh samples in 23 agro ecological zones of Kerala showed that 91 per cent samples are acidic in reaction (Hamza *et al*., 2005). They attributed the acidic nature of Kerala soils mainly to leaching of basic cations like Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} and replacement by hydrogen. Chandramohan and Mohanan (2012) observed electrical conductivity of 10.9 to 19.9 dSm^{-1} in Kaipad during summer. Beena *et al*. (2014) conducted a

soil sampling survey across Kuttanadu region and observed that soils are extremely acidic showing a range of pH between 2.5 to 5.2. Koruth *et al*. (2014) stated that in general, pH of Kerala soils ranged from 3.0 to 6.8. Kavitha and Sujatha (2013) reported that pokkali and kole lands of Thrissur districts were extremely to strongly acidic in reaction.

Bastin *et al*. (2014) reported that soil samples from Palakkad district displayed a range of pH from 5.58 to 8.58 and 34.44 per cent of soil samples were acidic and in particular, 72.22 per cent of samples collected from Alathur taluk also revealed acidic nature. They also analyzed soil samples from Wayanad district and reported that although, pH of soil samples ranged from 5.27 to 6.98, majority of samples (80.56%) were acidic. When soil samples from Alappuzha district were analyzed, they observed that 97.22 per cent of samples were acidic with pH ranging from 3.91 to 7.50. Likewise, 92.19 per cent of soil samples collected from Thrissur district revealed acidic nature with a pH range of 3.5-7.4. Mohan and Sreelatha (2016) noticed a pH ranging from 3.9 to 4.26 in Vyttila soils.

A similar detailed soil survey was done by Doncy (2018) across various locations of Thrissur, Palakkad, Wayanad, Alappuzha, Ernakulam, and Kottayam districts. The soil samples from Alappuzha, Ernakulam and Kottayam districts were categorized as strongly acidic and pH values ranged from 2.80-3.50, 4.1- 4.3 and 3.9 to 4.70 respectively. Soil samples from Thrissur district showed strongly to moderately acidic nature (4.3- 5.4) whereas, samples from Palakkad and Wayanad districts revealed pH value of 5.20 to 6.20 (moderately to slightly acidic) and 5.4- 7.10 (moderately acidic to neutral) respectively. When these samples were analyzed for electrical conductivity (EC), it was shown that soil samples from Alappuzha, Ernakulam and Kottayam districts were found slightly saline with EC values ranging from 3.00-7.61, 2.05- 3.73 and 1.34- 2.35 dSm^{-1} respectively. However, soil samples from Thrissur, Palakkad and

Wayanad districts were found non saline.

From the above reviewed researches, it can be inferred that agricultural lands of Kerala are abiotically stressed and thus, may have adverse effect on bioinoculants applied on the field. However, reports showing poor performance of *Pseudomonas* spp. in Kerala are not available.

2.1.2. Isolation and enumeration of *Pseudomonas* spp.

Several attempts were made by the researchers to study the isolation of *Pseudomonas* spp. from various ecosystems as well as its enumeration, growth, cultural and morphological characters in different media.

Rhizobiome is a collective term used for the diverse population of rhizospheric microorganisms which are fueled by carbon-rich exudates of plant roots (Mommer *et al*., 2016). Root rhizosphere may harbor more number of microbial population than the bulk soil and hence, most of the researchers isolate native *Pseudomonas* spp. preferably from crop rhizosphere. Yeole and Dube (2001) observed that King's B medium yielded more population of *Pseudomonas fluorescens* than nutrient agar medium. A glimpse through the literature, points out that almost all the isolation and enumeration studies of *Pseudomonas* spp. has been carried out using King's B agar medium.

Tiwari and Thrimurthy (2007) performed serial dilution and plating technique to isolate 21 strains of *P. fluorescens* from rhizosphere of rice, maize, wheat, chickpea, mungbean, urdbean, soybean and sunflower using King's B media. Jayamma *et al*. (2009) collected 70 soil samples from rhizosphere of maize, sorghum, groundnut, chilli, castor, tomato and various forest plants across

different places of Telangana state for the isolation of Pseudomonads. Twenty seven isolates of *Pseudomonas* spp. were obtained by serial dilution and plating technique on King's B agar, out of which 18 were identified as fluorescent pseudomonads.

Siddiqui and Shakeel (2009) selected 21 isolates of *Pseudomonas* from rhizosphere of redgram crop in Aligarh district of Uttar Pradesh. Suresh *et al*. (2010) obtained 10 isolates of *Pseudomonas* spp.from the rhizosphere of maize, sorghum, paddy and pearl millet. Jayayprakashvel *et al*. (2010) isolated 32 *Pseudomonas* spp. from rhizosphere of coastal sand dune vegetation at Chennai Coast. Similarly, Saikia *et al*. (2011) isolated 25 fluorescent pseudomonads from tea growing tracts across four districts of upper Assam. Subramanian *et al*. (2014) surveyed various states of India to isolate 115 isolates of *Pseudomonas* spp. from various crop rhizosphere *viz*., jasmine (Gangtok, Sikkim), chilli (Mysore, Karnataka), rice (Patna, Bihar), cardamom, turmeric and paddy (Idukki, Kerala), tomato and corn (Bangalore, Karnataka).

For studying the plant growth promoting abilities of pseudomonads, Kumar *et al*. (2015) isolated 75 *Pseudomonas* spp. from various locations representing 13 states of India. Isolates showing fluorescence under UV light were purified and designated as P1-P75 and deposited in the culture collection of Central Research Institute for Dryland Agriculture (CRIDA), Hyderabad. All the isolates were preserved in glycerol solution (30%) at -20°C and revived periodically for subsequent studies. Seventeen bacterial isolates were isolated from the rhizosphere of vegetable crops from different regions of Jammu by Singh *et al*. (2017) and among which, seven isolates were identified as *Pseudomonas* spp. on the basis of their morpho-cultural and biochemical characters.

Siddiqui and Shakeel (2009) studied root colonization of pigeon pea

roots by various isolates of *Pseudomonas* spp. and noted maximum colonization of Pf 737 followed by Pf 736 and Pf 740 and they observed that the root colonization by different *Pseudomonas* isolates was between 2.3 x 10^4 to 1.2 x 10^4 cfu g^{-1} of soil. Soil samples collected from paddy fields at 10 locations in Cuddalore district of Tamil Nadu were subjected to serial dilution and plating by Sivasakthi *et al*. (2013). The location namely Sivayam recorded maximum population of 7.71x 10^6 cfu g^{-1} *Pseudomonas* spp. and Mangalam recorded the least of 0.21 x 10^6 cfu g^{-1} of soil. Ten isolates obtained by serial dilution were purified by streak plate method using King's B medium. In another study investigated by Subhashini *et al*. (2015), soil samples collected from tobacco growing soils of Andhra Pradesh, Karnataka, Tamil Nadu and West Bengal were used to enumerate different known PGPR strains and the result showed that the population of *Pseudomonas* was maximum in all the soil samples when compared to *Trichoderma*, *Bacillus* or *Streptomyces* spp. The highest count of 11.9 x 10^5 cfu g^{-1} was recorded from northern light soils of Andhra Pradesh and Karnataka.

A similar survey was undertaken by Shahira *et al*. (2015) to isolate *Pseudomonas*spp. from paddy fields of Tiruvannamalai district of Tamilnadu. Eventhough the total bacterial population ranged from 15.8 to 24.3 × 10^6 cfu g^{-1} of soil, the population of *Pseudomonas* ranged between 7.21 - 7.71 x 10^6 cfu g^{-1} with the highest count from the location Kattumalaiynur and the least from Mangalam. In another study by Manasa *et al*. (2017), soil samples were collected from the rhizosphere soils of groundnut, sunflower, maize, black gram, green gram, rice, soy bean and red gram fields of Telangana. In general, the *Pseudomonas* population ranged between 2- 6 x 10^6 cfu g^{-1} of the soil. Highest population was recorded from soil samples collected from paddy fields whereas lowest from maize rhizosphere.

Vithya *et al*. (2018) enumerated *Pseudomonas* spp. from soil samples collected from various locations of Thrissur district of Kerala and recorded

maximum population of fluorescent pseudomonads from Pazhayannur ($5.5x10^5$ cfu g^{-1}). Suman *et al*. (2018) attempted population of *Pseudomonas* spp. from soil samples of Rangareddy district, Telangana and it ranged between 1.2-6.7 $x10^7$ cfu g^{-1} soil with highest population in the black rice rhizospheric soils of Rangapur, Doma mandal (6.7). These studies substantiate the fact that rhizospheric microbial population is influenced by the quantity and composition of root exudates of different crop plants and also various environmental conditions prevailing in the locality.

2.2. PURIFICATION AND PRESERVATION OF *Pseudomonas* spp.

Maintenance and preservation of bacterial cultures has encountered as a serious issue in many microbiological studies. The cell viability and genetic stability have to be maintained during storage of microbial cultures. Many researchers have conducted studies on the purification and preservation of bacterial cultures using various methods.

Lyophilization or ultra-freezing are the best options for long-term storage as suggested by Gherna (1994). Although, cells remain viable for long time lyophilisation and ultra freezing are tedious and many laboratories may not have the facilities. Thus, other simpler methods such as storage of cultures under mineral oil, glycerol or distilled water are considered as suitable. Kelman (1956) observed that *Ralstonia solanacearum*retained virulence in tap water for several months. Heckly (1978) opined that for short term storage, bacterial cultures could be preserved in mineral oil. Likewise, Lacobellis and DeVay (1986) reported that *Pseudomonas syringae* remained viable for 20 years in sterile water.

Liao and Shollenberger (2003) compared the scope of sterile water and phosphate-buffered saline for storage of Gram positive bacteria and Gram

negative bacteria. They observed that a vast majority of the Gram-negative bacteria including *Pseudomonas fluorescens*, *P. viridiflava*, *Erwinia* spp. and *Xanthomonas campestris* survived equally well in water phosphate-buffered saline for at least 30 weeks. However, population of some of the Gram negative bacteria *viz*., *Lisseria monocytogenes* and *Staphylococcus aureus* dropped more rapidly in water than in phosphate-buffered saline.

2.3. CHARACTERIZATION AND IDENTIFICATION OF *Pseudomonas* spp.

Bacterial identification is generally based on phenotypic tests including morpho-cultural characteristics and biochemical pattern. A perusal of the literature revealed several studies on the cultural, morphological and biochemical characterization of *Pseudomonas* spp.

2.3.1 Cultural, morphological and biochemical characterization

Bacteria belonging to the genus *Pseudomonas* are characterized by some defining features such as rod shape, Gram negativity, polar flagellation, aerobic nature and incapability to form spore. According to Meyer and Abdallah (1978), most of the *Pseudomonas* spp. of PGPR are fluorescent and they owe their fluorescence to water soluble diffusible pigment called pyoverdin or pseudobactin which are essentially iron chelating siderophores.

Phenotypic characterization of *Pseudomonas aeruginosa* strain PUPa3 by Kumar *et al*. (2014) revealed that oxidase, gelatinase, nitrate reductase and arginine dihydrolase tests were positive for the isolate. The bacterium showed growth at 42^{o}C but not at 4^{o}C. Moreover, a wide range of carbon sources such as glucose, mannitol, citrate and trehalose, and nitrogen sources such as L-histidine

and L-tryptophan were utilized by the bacterium. However, these isolates were not able to utilize paraffin, arabinose, sorbitol, erythritol, maltose, adonitol, glycine, phenyl acetate, hippurate and nicotinate as carbon source and ornithine, leucine, alanine, valine, serine, phenylalanine, glutamic acid and cystine as nitrogen sources.

Pseudomonas putida Rs-198 rhizospheric isolate of cotton plants were characterized by Yao *et al*. (2010) and found to be dome shaped fluorescent yellowish green pigmented colonies which showed abundant growth at 4^{o}C and 39^{o}C and no growth at 42^{o}C. The isolate showed positive response to indole test, gelatin liquefaction, oxidase test, citrate test, catalase test, denitification and VP test. Negative response was observed with MR test and starch hydrolysis. It utilized dextrose, sucrose, xylose, ethanol and glycerol whereas left lactose unutilized.

Shivakumar *et al*. (2013) described *P.aerugonisa* FP6, the strain which was isolated from rhizospheric soil samples of Bengaluru as Gram-negative, motile rod and oxidase positive which hydrolyzed gelatin and produced diffusible pigment into the medium. Antibiotic susceptibility test revealed that FP6 isolate was resistant to cefepime, tetracycline, cefoxitin erythromycin, rifampicin, ampicillin, andcetrimoxazole. Also, it was moderately resistant to tetracycline, erythromycin, cotrimoxazole, rifampicin, ampicillin, cefepime and cefoxitin.

Kipgen and Bora (2017) characterized *P. fluorescens* as Gram negative short rods with a size varying between 0.6 to 0.8µm × 1.7 to 1.9µm. Colonies were observed to be circular shaped with convex, glistening surface and fluorescent appearance. Biochemically, all isolates showed similar results with regard to KOH test (+), catalase (+), arginine dehydrolase (+), oxidase (+), gelatin liquefaction (+), dextrose utilization (+), citrate utilization (+), fluorescent pigment production (+), growth at 4°C (+), growth at 27°C (+) and starch

hydrolysis (-). Nevertheless, other biochemical tests like levan formation, denitrification and H_2S gas production tests showed variation in response.

Chinthala *et al*. (2013) recorded four strains of *P. fluorescens* with positive results for arginine hydrolysis, gelatin hydrolysis and oxidase. All the isolates showed optimum growth at 4^{o}C. They also reported that all the strains were able to utilize glucose, galactose, glycerol, lactose, xylose fructose, trehalose, sucrose and ribose with the production of either gas or acid. However, two strains PSTPT1 and PSTPT2 were unable to utilize mannitol. Deshwal and Kumar (2013a) identified 140 *Pseudomonas* strains isolated from potato rhizosphere of Dehradun based on various biochemical tests and grouped into *Pseudomonas aeruginosa, P. cepacia, P. fluorescens* and *P. putida*.

Shahira *et al*. (2015) noticed all the 10 isolates of *Pseudomonas* spp. from paddy as small, round, irregular and fluorescent pigmented colonies on King's B medium. The colonies were found positive for gelatin hydrolysis, oxidase, catalase and citrate production and negative for methyl red test, Voges Proskauer test, indole, urease, nitrate reduction and starch hydrolysis.

Singh *et al.* (2017) observed cultural characters of individual colonies of 17 fluorescent isolates of *Pseudomonas* spp. on NA medium. The colonies were highly variable in terms of colour (light green, whitish, creamy and greenish) and margin (regular and irregular). However, only seven isolates showed positive response for starch hydrolysis and catalase test whereas negative for hydrogen sulphide and MR-VP test. All isolates were sensitive to ciprofloxcin, gentamicin and ceftriaxone.

Manasa *et al*. (2017) investigated the cultural, morphological and

biochemical characters of 15 *Pseudomonas* spp. isolated from rhizospheric soil. All the isolates developed small to medium, smooth, glistening colonies, with yellowish green or light green pigmentation. All the isolates expressed positive results for catalase and oxidase test whereas they were negative for Voges Prausker's test. Other tests showed variable reaction with different isolates. Most of the isolates hydrolysed starch and citrate and produced negative results for gelatin liquefaction, denitrification and H_2S test.

Prasad *et al*. (2017) studied carbohydrate utilization patterns of *Pseudomonas* isolates using biochemical kits. Most of the isolates were capable of using glucose, trehalose, melibiose, raffinose, saccharose and xylose as carbon source and lactose, cellobiose and adonitol were the least preferred carbohydrates by the isolates. The response to different source of nitrogen *viz*., phenylalanine, nitrite and urea were also variable among different strains.

Pseudomonas aeruginosa, an opportunistic human pathogen, often come across during the isolation of rhizobactera from soil. Eventhough, it has proved to be potent antagonistic and plant growth promoting bacteria, it is necessary to identify and eliminate from further *in vitro* or *in vivo* studies. Meagre cultural or biochemical characters could not be used for assured confirmation of *P. aeruginosa* and hence molecular identification is followed by many researchers.

According to Cox and Parker (1974) *P. aeruginosa* produces pearlescent colonies with characteristic grapelike odour of O-aminoacetophenone from tryptophan. Furthermore, isolates of *P. fluorescens* are reported to produce pyoverdin (fluorescein), yellow-green pigment whereas, *P. aeruginosa* strains are capable of producing pyoverdin, pyocyanin (blue-green pigment) and pyorubin

(red-brown pigment) (Meader *et al*., 1925: Yabuuchi and Ohyama, 1972: Meyer and Abdallah, 1978). King *et al.* (1954) suggested *Pseudomonas* Agar P (King's A media) for the production of pyocyanin and pyorubin production and *Pseudomonas* Agar F (King's B media) for fluorescein production. Hence, King's A medium could be used for distinguishing typical strains of *P. aeruginosa* from other fluorescent pseudomonads which turn into blue green colour upon incubation (Gilardi, 1991). However, there are reports affirming the chances of losing capability to produce pyocyanin by some of the strains.

Definitive clinical identification of *P.aeruginosa* often included its ability to grow at 42°C, the temperature which could not be survived by other species (Stanier *et al.,* 1966). A shortened gelatin test could also be used to differentiate *P. fluorescens* (positive) from *P. putida* (negative). Blazevic *et al*. (1973) reported that *P. aeruginosa* was resistant to antibiotic kanamycin and sensitive to carbenicillin, whereas, opposite pattern was observed with *P. fluorescens* and *P. putida*.

2.3.2 Molecular characterization

Eventhough, routinely practiced identification of bacteria through phenotypic characterization is not accurate at some instances, as it varies usually in the case of bacteria that show high catabolic versatility. Moreover, although some of the biochemical tests are performed within minutes, identification upto species level is a time-consuming process. Hence, rapid and accurate identification of bacterial species is therefore warranted, where molecular characterization offers hope.16S rRNA gene sequencing is a commonly used molecular method to identify bacteria that are not identified by conventional systems, and which can characterize previously undescribed species.

Wahyudi *et al*. (2011) isolated 27 strains of *Pseudomonas* spp. from soybean and were identified through molecular characterization by 16s rRNA sequencing as *Pseudomonas putida*, *P. stutzeri*, *P. monteilii*, *P. plecoglossicida*, *P.beteli*, *P.aeruginosa*, *P.mosselii* and *P. putida*.

Similarly, Sandeep and Jisha (2013) conducted soil sampling survey across vanilla growing areas in Kerala to isolate *Pseudomonas*spp. and identified two strains as *P. fluorescens* and *P. putida* based on molecular analysis. Another study was attempted by Saber *et al*. (2015) to isolate pseudomonads from rhizosphere of different plants including some conventional and medicinal plants such as mugwort, gazania, hypericum, goldenrod, sugar bush, cotton, corn, tomato, cucumber, bean, sesame and olive. Out of the 72 isolates obtained, OL2, SE8, OL4 and AR10 were screened out for antagonism and identified by 16S rRNA gene sequencing as *Pseudomonas otitidis* OL2, *P. otitidis* SE8, *P. otitidis* OL4 and *P. otitidis* AR10, with 99 per cent similarity.

Fathalla *et al.* (2015) identified *Pseudomonas* spp. isolated from root samples of various crops as *Pseudomonas plecoglossicida, P. corrugata, P. palleroniana, P. moraviensis* and *P. putida.* Similarly, Jun *et al.* (2015) characterized populas associated isolates of *Pseudomonas* spp. through molecular analysis and identified them to be belonging to *Pseudomonas fluorescens, P. chlororaphis* and *P. putida.* Kumar *et al.* (2015) isolated one strain of *Pseudomonas* spp. showing broad spectrum antagonistic activity from paddy rhizosphere and identified as *P. aeruginosa* ATCC 27853 with a similarity of 99 per cent by 16 srRNA sequencing. Similarly, Saini *et al*. (2016) isolated a bacterial strain BNJ-SS-45 from wheat rhizosphere and identified as *Pseudomonas protegens* (HQ905436) based on 16S rRNA gene sequence with query nucleotide sequence showing 99.0 per cent similarity.

2.4 SCREENING FOR ABIOTIC STRESS TOLERANCE OF *Pseudomonas* spp.

Pseudomonas spp. have been already established as the most commonly used bio control agent all over the world against a vast majority of plant pathogens. The antagonistic potential of biocontrol agent could not be harnessed when introduced in abiotically stressed ecosystems as the survival of these microorganisms is severely affected in these hostile environmental conditions. However, bacteria isolated from the stressed ecosystems itself, may possess inbuilt stress tolerance capacity, making it promising candidates for biocontrol of plant pathogens. Scarce number of researches has attempted to explore the untapped bio diversity from the stressed soils.

2.4.1 Abiotic stress tolerance of *Pseudomonas* spp.

Sandhya *et al*. (2009) reported 26 isolates of *Pseudomonas* spp. tolerated maximum level of drought stress of −0.73 MPa and further investigation revealed that strain GAP-P45, isolated from sunflower rhizosphere as an efficient EPS producer under drought. Tank and Saraf (2010) observed *P. fluorescens* and *P. aeruginosa* could tolerate salinity induced by NaCl (6%) and promoting the growth of tomato plants under salt stress. Gopalakrishnan *et al*. (2012) characterized seven isolates of rhizobacteria from paddy, showing potential bio control against sorghum charcoal rot. Among the isolates, *Pseudomonas* isolates (*P. plecoglossicida* and *P. monteilii*) showed good growth at 4 and 6 per cent NaCl, pH of 5 and temperature upto 40^{o}C.

Ali *et al*. (2013) demonstrated that nine isolates out of 17 fluorescent Pseudomonads tolerated a minimum water potential of -0.30MPa (15% PEG 6000) and stated that it is production of exopolysaccharides which is conferring drought tolerance. Far earlier, Chenu and Roberson (1996) had reported that exopolysaccharide (EPS), protects microorganisms from water stress by

enhancing water retention and regulating diffusion of substances.

Shivakumar *et al*. (2013) observed that *Pseudomonas aeruginosa* strain FP6 was able to grow at wide range of temperature (20 and 60°C), pH 5–10 and 1-4.5 M NaCl. Similarly, Manasa *et al*. (2016) also observed *Pseudomonas fluorescens* strain GGP-1 tolerating a broad range of *in vitro* abiotic stresses such as pH (4.5, 5.5, 6.5, 7.5 and 8.5), temperature (28, 37 and 45^{o}C), drought (15, 20, 25 and 32.6 per cent PEG) and salinity (1, 3, 5 and 7 per cent).

Mishra *et al*. (2017) isolated 51 strains of *Pseudomonas* spp. including several strains of *Pseudomonas guariconensis* and *P. stutzeri* from various locations of nearby volcanos in the Andaman and Nicobar islands and screened for tolerance to abiotic stresses. They observed that, 80, 34 and 38 per cent of bacterial isolates could tolerate upto 45^{o}C, 2 M salt concentration and 60 per cent PEG.

Likewise, Egamberdieva *et al.* (2017) reported that colonization of three isolates *viz.*, *Pseudomonas extremorientalis, P. aurantiaca* and *P. chlororaphis*, were partly affected under salt stress of 75 mM NaCl. Nevertheless, among the isolates, higher rhizosphere colonization was exhibited by *P. chlororaphis* and *P. extremorientalis* under saline and nonsaline soils.

Recently, Chari *et al*. (2018) screened 44 isolates of *Pseudomonas* spp. for abiotic constraints. They found that four isolates were showing growth at pH range from 4-10 whereas, five isolates tolerated salinity from 1.5 to 20 per cent of NaCl concentration. Moreover, three isolates displayed tolerance to temperature from 20°C-50°C and three isolates survived water potential from -0.05 Mpa to -0.73 Mpa. In the same year, Vithya *et al*. (2018) isolated 10 strains of rhizobacteria from different pepper growing areas of Thrissur district in Kerala. They reported that all the isolates of fluorescent pseudomonads were adaptive to

41°C temperature, pH 5.5 and -0.15M Pa osmotic stress (10 % PEG) and screened out PAP isolate (Pazhayannur) as the best abiotic stress tolerant *Pseudomonas* spp. among the isolates.

There are also reports evidencing growth promotion of plants under stressed conditions by bacteria isolated from stressed soil. Jha *et al*. (2011) reported that combined application of *Pseudomonas pseudoalcaligenes* and *Bacilllus pumilus* in paddy were able to protect plants from abiotic stress. Moreover, Yao *et al*. (2010) reported that germination and growth of cotton plants under salt stress was promoted by inoculation with *Pseudomonas putida*.

2.4.2 Biomolecules imparting stress tolerance

2.4.2.1 ACC deaminase production

Plants respond to both biotic and abiotic stresses by modulating the level of various growth hormones which in turn protect the plants from stress by inducing the production of stress related proteins (Singh *et al*., 2015). One of the most common stress mediating hormones is ethylene. However, when ethylene peak crosses its threshold level, it turns out as stress ethylene hindering plant growth and development by inducing leaf abscission, chlorosis and senescence (Glick, 2007).

Plant growth promoting rhizobacteria are equipped with ACC deaminase, an enzyme which blocks the production of stress ethylene. According to Honma and Shimomura (1978) ACC deaminase hydrolyzes 1-aminocyclopropane 1- carboxylate, the immediate precursor of ethylene in plants, into ammonia and α-ketobutyrate which are utilized for bacterial growth. Renuga (2005) screened *Pseudomonas putida* and *P. fluorescens* for ACC deaminase activity by growing on Dworkin Foster salt minimal medium amended with ACC

as a sole source of nitrogen and it was shown that only *P. putida* was capable of producing ACC deaminase. She also demonstrated that ACC deaminase activity is induced by low levels (l00nM) of ACC, and has a temperature optimum at 30^{o}C and optimum pH of 8.5.

Root associated bacteria may take up plant's internally produced ACC along with other molecules of root exudates and cleave into α-ketobutyrate and ammonia and subsequently act as sink for ACC. This will lower the production of growth inhibiting ethylene thereby promoting plant growth under abiotic or biotic stresses. Shaharoona (2006) made an observation on significant increases in plant height, root weight and total biomass of maize plant in response to inoculation with ACC deaminase producing strain of *P. fluorescens*.

Ali *et al*. (2013) reported that among the nine drought tolerant strains of *Pseudomonas* spp., only one isolate (SorgP4) grew well on DF salt minimal medium with ACC. They further continued the investigation by quantifying the amount of α-ketobutyrate produced during the deamination of ACC under both non-stress and drought stress conditions. And it was reported that the isolate SorgP4 showed maximum ACC deaminase activity (3.71±0.025 µM/mg protein/h of α-ketobutyrate) under non-stress and 1.42 ± 0.039 µM /mg protein/h of α-ketobutyrate under drought stress condition respectively. Chaubey and Archana (2015) also quantified ACC deaminase production among 25 isolates and observed to be in the range of 1-21 ug of α-ketobutyrate/mg of protein.

Naik *et al.* (2008) isolated fluorescent pseudomonads from banana rhizospheric soil and screened for the production of enzymes and hormones including ACC deaminase and reported 11 per cent of the isolates with ACC deaminase activity. Eventhough, isolates could be detected with ACC deaminase

production, their potential may vary in each isolate which was documented by Kushwah *et al*. (2015). Among the 11 ACCD producing isolates, maximum enzyme activity per hour was shown by P229 (43.3±0.60 µM a ketobutyrate/mg protein/h) while minimum in P5 (1.49±0.47 µM a ketobutyrate/mg protein/h).

Patil *et al*. (2016) developed an improved method of screening of *Pseudomonas* isolates for ACC deaminase activity by incorporating pH indicator dyes such as Bromothymol blue or Phenol red in ACC containing medium. They screened three bacteria for ACC activity *viz*., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophila* and *Bacillus subtilis* among which *P. aeruginosa* produced coloured zone around the colonies indicating the presence of ammonia as a result breakdown of ACC. Chari *et al*. (2018) screened 14 isolates of *Pseudomonas* spp., out of which four isolates showed strong ACCD production (PS 1, PS 11, PS 15, PS 30) and further plant growth promoting tests selected PS1 as best stress tolerant.

2.4.2.2 Exopolysaccharide Production

Sandhya *et al*. (2009) isolated EPS-producing fluorescent pseudomonads from various semiarid regions of India. The EPS production of drought tolerant isolates was higher under water stress than under normal conditions. They also observed a linear response of EPS production with increased level of stress. Moreover, *Pseudomonas putida* with the highest level of EPS production under water stress conditions was found to be alleviating drought stress in sunflower seedlings. Kumar *et al*. (2015) screened *Pseudomonas* spp. isolated from diverse agro-ecosystems of India and it was observed that the highest EPS production of 237 µg mLg^{-1} was seen in P12 strain.

2.5 ASSAY OF EXTRACELLULAR ENZYMES OF *Pseudomonas* spp.

One of the important antagonistic strategies of bacterial and fungal biocontrol agents is the production of lytic enzymes such as β- 1, 3-glucanase, chitinase and cellulase (Cabib, 1987). β- 1, 3-glucanase is a group of hydrolytic enzyme which degrade the polysaccharide in the growing environment in order to use the monomer sugars as a source of energy (Planas, 2000). Zhu *et al*. (2008) opined that β- 1, 3-glucanases are prevalent in bacteria and fungi, and hydrolyse glycosidic bonds between 1,3-linked glucopyranose residues found in β-glucans.

Vijayendra and Kashiwagi (2009) stated that exo-glucanases breakdown polysaccharides completely to release monosaccharide monomers, whereas endo-glucanases produce oligosaccharides by partial degradation of the exopolysaccharides. Lim and Kim (1995) determined the optimum pH and temperature for β -1, 3-glucanase activity as 5.5 and 40°C, respectively.

According to Paul *et al*. (2005) mycolytic enzymes such as β -1, 3-glucanases and β -1, 4-glucanases produced by *Pseudomonas fluorescens* was found to be effective in the lysis of *Phytophthora capsici* mycelium. Similar observations were made by Kitamura and Kamei (2006) stating that β-1, 3-glucanase activity as an important antagonistic trait responsible for degradation of *Pythium porphyrae* cell walls.

Saad (2006) observed mycolytic action of *Pseudomonas fluorescens* strains NRC1 and NRC3 against *Rhizoctonia solani* and *Phytophthora capsici* and attributed it to the production of β-1,3 and β-1,4-glucanases. Likewise, Arora *et al*. (2007) reported prominent role of β- 1,3-glucanase enzyme produced by fluorescent *Pseudomonas* isolates GRC3 and GRC4 in the growth suppression of

the phytopathogenic fungi *Rhizoctonia solani.* Dev and Dawande (2010) also found glucanase producing *Pseudomonas fluorescens* in the biocontrol of soil borne fungal plant pathogen, *Rhiozoctonia solani.*

Frindlender *et al*. (1991) correlated the antifungal activity of *Pseudomonas fluorescens* with enzyme production. Nagarajkumar *et al*. (2004) also observed a linear relationship between β-1,3-glucanase production of *Pseudomonas fluorescens* strains and its inhibitory potential towards *Rhizoctonia solani.* Singh *et al*. (2010) opined that β-1,3-glucanase produced by *Pseudomonas aeruginosa* PN1 is reported to cause mycelial lysis, vacuolation and granulation of cytoplasm, hyphal deformities and branching in polyphagous fungus *Macrophomina phaseolina*.

Cellulase is a hydrolytic enzyme which catalyzes cellulolysis, the decomposition of cellulose and some related polysaccharides. Cellulases break down the cellulose molecule into monosaccharides such as glucose, or shorter polysaccharides and oligosaccharides. Cellulase is produced chiefly by microorganisms such as fungi, bacteria, and protozoans.

Scanty literature are available with regard to cellulase production by *Pseudomonas* spp. Downer *et al*. (2001) and Rasmussen *et al*. (2002) noticed that cellulases are involved in the suppression of soil borne phytopathogens such as *Phytophthora cinnamomi* and *Fusarium culmorum.* Kumar *et al*. (2015) screened out potential antagonistic strains of *Pseudomonas* spp. for lytic enzymes and found that only five per cent of isolates were positive for cellulase production and 31 per cent produced protease.

2.6 BIO CONTROL POTENTIAL OF *Pseudomonas* spp. TOWARDS PLANT PATHOGENS

Soil borne fungal pathogens pose serious threat to horticultural and agricultural crops and are responsible for heavy yield losses annually as these pathogens often survive in the soil and plant debris for several years and cause disease during the following season (Punja, 1988). The predominant soil borne fungi comprise diverse species of *Fusarium, Rhizoctonia, Pythium, Phytophthora* and *Sclerotium* which cause diseases like damping off, wilt, root rot and seedling blight. Mayee and Datar (1988) observed 25 per cent yield losses in groundnut due to *Sclerotium rolfsii*. Likewise, *Rhizoctonia bataticola* causes plant loss upto 77 per cent and yield losses of 30-35per cent has been reported in soybean crop (Muthusamy and Mariappan, 1991). Chand and Khirbat (2009) reported 10-100 per cent yield losses annually in chick pea in India due to *Fusarium oxysporum* f. sp. c*iceri.*

Fluorescent pseudomonads suppress various soil borne diseases and their efficacy has been related both to their antagonistic activities and to their rhizosphere competitiveness. A perusal of literature revealed that *Pseudomonas* spp. have established as most promising group of bacteria for plant disease control and growth promotion.

2.6.1 *In vitro* antagonistic studies

In vitro antagonistic studies are used as a first filter to identify potential biocontrol strains. Attempts have been made by several workers to study the interaction of *Pseudomonas* spp. with various soil borne fungal pathogens.

Ahmadzadeh *et al*. (2006) screened 47 fluorescent Pseudomonads against *Macrophomina phaseolina, Rhizoctonia solani, Phytophthora nicotianae* var. *parasitica, Pythium* sp. and *Fusarium* sp. and documented 66, 40, 63, 48 and

27 per cent of isolates with prominent antagonistic activity against *R. solani, M. phaseolina, Pythium* sp*., P. nicotianae* and *Fusarium* sp., respectively.

Pseudomonas corrugata isolated from Indian Himalayan Region (IHR) was examined for its antagonistic activity against *Fusarium oxysporum* by Trivedi *et al*. (2008). Interestingly, although the bacterium did not inhibit *Fusarium* with diffusible antifungal metabolites, 49 per cent growth reduction was observed in sealed Petri plates after 120 h of incubation which indicated that the bacterium owe its higher degree of antagonism to volatile production.

Nandi *et al*. (2013) evaluated *in vitro* antagonistic efficacy of 21 rhizospheric isolates collected from okra, chilli ground nut, brinjal, cabbage and tomato fields in West Bengal. The results showed that two isolates of *P. fluorescens* PF-8 and PF-7 effectively inhibited *Rhizoctonia solani* with 75.8 and 75.2 per cent mycelial growth reductions respectively. Chennakesavulu *et al*., (2013) observed antagonistic activities of 36 isolates of *Pseudomonas fluorescens* against *Fusarium udum,* and they reported that only 15 isolates showed maximum inhibition. Highest percentage of inhibition (87.40%) was recorded in CPF4 isolate and the least in KPF12.

Shivakumar *et al*. (2013) observed immense antagonistic potential of *P. aeruginosa* FP6 against *Fusarium oxysporum* (99.2 %), *Rhizoctonia solani* (99.43 %) and *Phytophthora capsici* (68.77 %) in dual culture technique. They noticed that production of volatile compound (HCN) inhibited the mycelial growth to the extent of 68.62 to 99.93 per cent whereas, nonvolatile diffusible metabolites inhibited fungal growth ranging between 12.47 to 53.3 per cent.

Maurya *et al*. (2014) reported *Pseudomonas fluorescens* strain P.f 07 as the most efficient among other strains isolated from chilli fields of Meerut,

with the highest antagonistic activity against *Fusarium moniliforme* (65.45 %), *Rhizoctonia solani* (68.23 %). Ali (2014) compared the antagonistic efficiency of isolates of *Bacillus subtilis* and *Pseudomonas fluorescens* against *Rhioctonia solani* and *Fusarium oxysporum* by dual culture technique. They found five isolates of *P. fluorescens* to be the best followed by the isolate of *Bacillus subtilis*.

2.6.2 *In vivo* antagonistic studies

Besides, *in vitro* conditions, there are several reports where *Pseudomonas* spp. could significantly reduce the disease incidence both under greenhouse and field conditions. Fravel (1988) opined that antagonistic potential of biocontrol agents could not be correlated between and *in vitro* and *in vivo* conditions. Thus most of the biocontrol research uses combination of *in vitro* and *in vivo* methods to determine the antagonistic potential.

Pandey *et al*. (2001) reported that bioinoculation with two strains of *Pseudomonas corrugata* isolated from subtropical and temperate soils of Sikkim and Himalaya resulted in significant disease suppression in maize seedlings challenge inoculated with *Pythium ultimum*, *P.arrhenomanes* and *Fusarium graminearum* which cause damping-off of seedlings. Similarly, Anjaiah *et al.* (2003) evaluated biocontrol potential of *Pseudomonas aeruginosa* PNA1, isolated from chickpea rhizosphere against *Fusarium oxysporum* f. sp. *ciceris* and *Fusarium udum* under *in vivo* conditions. They observed significant reduction in incidence of *Fusarium* wilt in pigeonpea and chickpea and notably delayed wilting in susceptible plant genotype. Gravel *et al*. (2004) observed protection of tomato seeds from seed decay caused by *Pythium aphanidermatum* and *Pythium ultimum* by seed inoculation with *Pseudomonas marginalis*.

The efficacy of *Pseudomonas fluorescens* in suppressing sheath blight incidence in rice plants upto 81.03 per cent was reported by Heflish *et al.* (2017)

when applied as seedling root dip and foliar spray. Similarly, Madhavan *et al.* (2017) documented higher bio control potential of *Pseudomonas* isolates against *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *lycopersici* responsible for wilt complex disease in tomato plants. Minimum disease incidence of 10.41 and 12.50 per cent was observed against *R. solani* and *F. oxysporum* f.sp. *lycopersici*, when tomato seeds were bacterized with the isolate I-23.

Ravindran and Shaike (2013) evaluated potential of native *Trichoderma* spp. and *Pseudomonas* spp. against *Rhizoctonia* infection on vanilla plants. They observed more disease suppressive potential with the combined application of *Trichoderma harzianum* and *Pseudomonas fluorescens* followed by single inoculation of *Pseudomonas fluorescens, Trichoderma harzianum, Pseudomonas putida* and *Trichoderma virens* in decreasing order.

Afsharmanesh *et al*. (2010) assessed bio control activity of *Pseudomonas fluorescens* UTPF5 against *R. solani* in bean under *in vivo* conditions. The results showed that UTPF5 could suppress the disease by 33.34 per cent and 14.29 per cent by soil drenching and seed treatment, respectively.

2.7 PLANT GROWTH PROMOTION BY *Pseudomonas* spp.

Significant number of accumulating pieces of evidence substantiate the fact that *Pseudomonas* spp. are important group of commensals which provide fundamental support to the plants in acquiring nutrients, tolerating abiotic stresses and resisting phytopathogens (Meena *et al*., 2017).

Two strains of fluorescent pseudomonads, PS1 and PS2 isolated from

ground nut rhizosphere were found effective growth promoters when coated on ground nut seeds (Bhatia *et al*., 2008).Wahyudi *et al*. (2011) reported CRB44 and CRB 63 strains of *Pseudomonas* spp. isolated from soyabean rhizosphere as efficient plant growth enhancers which increased number of lateral roots, shoot and root length in cowpea seeds. Meera and Balabasker (2012) observed similar results of *Pseudomonas fluorescens* as it showed maximum germination per cent, increase in vigour index and shoot and root length in rice plants.

Gopalakrishnan *et al*. (2012) noted SRI- 360 strain of *Pseudomonas monteilii* isolated from paddy rhizosphere which significantly enhanced the tiller number, stover and grain yields, total dry matter, root length, volume and dry weight of rice plants over the un-inoculated control. Deshwal and Kumar (2013b) confirmed the efficacy of selected strains *viz*., PW-99, PW-136, PW-2, PW-56, PW-18, PW-43, PW-5 and PW-104 for superior PGPR abilities such as indole acetic acid (IAA), hydrogen cyanide (HCN), siderophore and phosphorous solubilisation based on *in vitro* studies. Furthermore, *in vivo* studies revealed that *P. fluorescen*s PW-5 produced maximum shoot, root and dry weight by 157.72, 408.06 and 233.84 per cent respectively as compared to control in rice plants.

Shivakumar *et al.* (2013) documented stimulatory effect of *P. aeruginosa* FP6 on all vegetative parameters of cowpea plant when applied as seed inoculant. They reported significant increase in number of pods and seeds per pod as compared to treatment with chemical fertilizers and attributed to the production of phytohormone. Bacterisation of tomato seeds with *Pseudomonas* isolate I-23 resulted in enhancement of plant growth characters such as seed germination, shoot and root as observed by Madhavan *et al.* (2017).

Suman *et al*. (2017) conducted standard roll towel method to study the

effect of antagonistic *Pseudomonas* strains (DBP, DMP1 and PVP2) isolated from paddy fields on germination of rice and recorded cent per cent germination with PVP2. However, under field study, the isolate DMP1 was found superior in promoting plant height, panicle number, number of tillers and chlorophyll content.

Adesemoye *et al*. (2008) compared some plant growth promoting rhizobacteria (PGPR) properties of *Bacillus subtilis* and *Pseudomonas aeruginosa* by inoculating on tomato, okra, and African spinach and observed no significant difference among those parameters. Conversely, Ali (2014) conducted a greenhouse study and observed *Pseudomonas fluorescens* out performed *Bacillus subtilis* in promoting the vegetative characters (plant height, number of branches, fresh weight and dry weight of plant) and yield attributes (number of fruits and fruit yield). In another study, increase in root length, shoot length, root and shoot fresh and dry weight of onion was observed when seed treated with both *Bacillus subtilis* and *Pseudomonas fluorescens* separately (Reetha *et al*., 2014).

From the reviewed literature, it is evident that in general, *Pseudomonas* spp. are excellent plant growth promoters and owe these stimulatory effect to wide array of plant hormones, aminoacids or siderophores.

MATERIALS & METHODS

3. MATERIALS AND METHODS

The present investigation on "Characterization and evaluation of *Pseudomonas* spp. from abiotic stressed ecosystems of Kerala" was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara during the period 2017-2019.The details of materials and methods employed in the study are outlined below.

3.1 SURVEY OF SOIL SAMPLES ACROSS STRESSED ECOSYSTEMS OF KERALA

Soil samples were collected from four locations of Kerala *viz*., Thrissur, Palakkad, Moncombu, and Vyttila representing stressed ecosystems *viz*., drought, high temperature, acidity and salinity. Rhizospheric soil samples upto a depth of 10 to 15 cm were collected from paddy fields. The rhizosphere soil particles closely adhering to the roots were gently teased out and mixed to provide composite soil sample. During sampling from each location, soil temperature was recorded by inserting soil thermometer to a depth of 5 cm. Thereafter, the collected samples were brought to the laboratory and stored under refrigerated conditions for further studies.

3.1.1 Estimation of pH, electrical conductivity and moisture content of soil samples

Soil microbial communities are particularly influenced by key edaphic factors like pH, electrical conductivity and soil moisture content. pH is a measure of hydrogen ion concentration in the soil, representing acidity or alkalinity and electrical conductivity is related to the total soluble cations and anions,

representing salinity. Water availability is one of the most important parameters regulating biological activities in soil. Soil moisture content will influence soil organisms through complex interactions with nutrient conditions, soil temperature and oxygen status of the soil (Schnurer *et al*., 1986). Changes in these soil parameters can alter population, distribution and biological activities of microorganisms. pH, EC and soil moisture were determined by standard procedures (Hesse, 1971).

3.1.1.1 pH

Ten gram of air dried soil sample was taken in a 50 ml beaker and 25 ml of distilled water was added to make 1: 2.5 soil-water suspension. This suspension was intermittently stirred for 30 min and was left for settling. Then the supernatant of the soil suspension was used for estimation of pH using a digital pH meter with glass and calomel reference electrodes calibrated to buffers pH 4 and 7.

3.1.1.2 Electrical conductivity (EC)

The electrical conductivity of the soil sample was determined by immersing glass electrode of digital conductivity meter in the soil suspension prepared in the same manner for pH measurement. The EC of the soil samples were expressed in dSm^{-1}.

3.1.1.3 Soil moisture

Soil moisture content is expressed by weight as the ratio of the mass of water present to the dry weight of the soil sample. For this, a known amount of soil sample (10 g) was air dried in hot air oven at 105^{o}C for 24 h. After drying, the weight of soil samples was determined. Soil samples were again oven dried at

105^{o}C and weighed again. The process was continued until two consecutive measurement of the weights were the same. The moisture content of soil sample on dry weight basis was calculated using the following formula:

$$\text{Soil moisture content (\%)} = \frac{\text{Weight of wet soil- Weight of dry soil x 100}}{\text{Weight of dry soil}}$$

3.2. ISOLATION AND ENUMERATION OF *Pseudomonas* spp. FROM SOIL

Soil sample weighing 10g from each location was added aseptically to 90 ml sterile water blank taken in 250 ml conical flask and agitated thoroughly using Orbitek shaker at 260 rpm. This standard soil suspension was subjected to serial dilution and plating technique for isolation of *Pseudomonas* spp. (Ashwitha *et al*., 2013). One ml of the soil suspension was added to 9 ml of sterile water to make a dilution of 10^{-2}. Serial dilution was continued to a final dilution of 10^{-6} and pour plate method was followed for isolation of *Pseudomonas* spp.

Media used for plating the soil suspension were King's B and *Pseudomonas* agar base medium, where, one ml of the soil suspension from aliquot dilutions of 10^{-4}, 10^{-5} and 10^{-6} was pipetted out into separate sterile Petri plates and 15 ml of sterile medium was poured. The plates were rotated in clockwise and anticlockwise directions in order to distribute the bacterial cells evenly on the medium. For each dilution, three replicates were maintained and kept for incubation at room temperature for 48h. After incubation, well separated individual bacterial colonies were enumerated and expressed as colony forming units per gram of soil (cfu g^{-1}).

3.2.1 Purification and preservation of *Pseudomonas* isolates

After enumeration, distinct bacterial colonies were picked up with a sterile loop and were purified by quadrant streaking on King's B plates. The pure cultures so obtained were preserved in both glycerol and sterile water for long term storage. For this, screw cap vials with glycerol (15%) were autoclaved for 20 min. The moisture from glycerol solution was removed by keeping vials in hot air oven at 65^0C for 2 h. Two loopful of each bacterial culture were inoculated into each of these vials and was kept at room temperature until further use. Bacterial cultures were also inoculated in the same way in screw cap vials with sterile water and kept at room temperature. For subsequent studies, isolates were revived from the vials by taking a loopful of bacterial suspension and streaking on King's B agar plates.

3.2.2 Screening and elimination of *Pseudomonas aeruginosa*

The purified cultures of *Pseudomonas* isolates were screened for the occurrence of *P. aeruginosa* by streaking on King's A medium as per the protocol of King *et al.* (1954). The isolates spot inoculated on King's B plates were also incubated at a temperature 42°C to screen out *P. aeruginosa* (Stanier *et al.,* 1966).

3.3 SCREENING OF *Pseudomonas* spp. FOR ABIOTIC STRESS TOLERANCE

All the bacterial isolates along with reference culture of *P. fluorescens* were tested for tolerance to abiotic stresses such as high temperature (28-50°C), salinity (0.5 M to 1.5 M NaCl), osmotic stress (10% and 30% PEG) and pH (3.5, 4.5 and 5.5). Three replications were maintained for each isolate for all the experiment and observations were recorded as mean of three replications.

3.3.1 *In vitro* screening of *Pseudomonas* spp. for temperature tolerance

Thermo tolerance of isolates of *Pseudomonas* spp. was assessed as per the protocol of Kumar *et al.* (2014). Test tubes containing 5 ml of sterilized Trypticase soya broth (TSB) was inoculated with loopful of bacterial culture followed by incubation for 48 h at various temperatures (28, 37, 45 and 50^0C). Bacterial growth was measured based on turbidity using spectrophotometer at 600 nm.

3.3.2 *In vitro* screening of *Pseudomonas* spp. for drought tolerance

The effect of osmotic stress on the growth of the isolates was studied by adopting the protocol of Kumar *et al*. (2014). A loopful of the bacteria was inoculated into sterile TSB amended with polyethylene glycol (PEG 6000 Da) at two different concentrations *viz*., 10 per cent (100 g PEG added in one litre of TSB contributes an osmotic pressure of -1.03 M Pa) and 30 per cent (contributes an osmotic pressure of -0.49 M Pa). Spectrophotometer reading was taken at 600 nm after two days of incubation.

3.3.3 *In vitro* screening of *Pseudomonas* spp. for salinity tolerance

Isolates of *Pseudomonas* spp. showing tolerance to salinity were evaluated by imposing salt stress in trypticase soya broth (TSB) as per the protocol reported by Manasa *et al.* (2017). A loopful of bacteria was inoculated to sterilized TSB at two different concentrations (5.8 and 8.5 percent) of NaCl. After an incubation period of two days, bacterial growth was measured at 600 nm using spectrophotometer.

3.3.4 *In vitro* screening of *Pseudomonas* spp. for acidity tolerance

Acid tolerance of *Pseudomonas* strains was assessed by following the procedure of Manasa (2015). The reaction TSB was adjusted to various pH levels

(3.5, 4.5 and 5.5) by using 0.1 N HCl and/ or 0.1 N NaOH. Loopful of all bacterial isolates was inoculated aseptically into tubes containing 5 ml of broth and kept for incubation. Spectrophotometer reading was taken at 600 nm and recorded.

3.4 *In vitro* SCREENING OF STRESS TOLERANT ISOLATES OF *Pseudomonas* spp. FOR THEIR ANTAGONISTIC POTENTIAL

In vitro antagonistic activity of the selected abiotic stress tolerant strains was tested against five major soil borne fungal pathogens *viz., Phytophthora capsici, Pythium aphanidermatum, Sclerotium rolfsii, Fusarium oxysporum,* and *Rhizoctonia solani* by bacterial ring inoculation technique (Adetuyi and Cartwright, 1985). Autoclaved caps of screw cap vials were dipped in bacterial lawn prepared with *Pseudomonas* isolates so that brim of the caps will be coated with the bacterial culture. These caps coated with the culture were slightly pressed in the centre of PDA plate in order to form a bacterial ring. Thereafter, a five mm mycelial disc of fungal pathogen was inoculated in the centre of the ring. PDA plates inoculated with pathogen alone served as control. Three replications were kept for each isolate and plates were incubated until the pathogen attained full growth in the control. Measurements on the radial growth of pathogen were taken and per cent inhibition of the growth of the pathogen over control was calculated using the formula by Vincent *et al*. (1947).

$$\text{Per cent inhibition (PI)} = \frac{C\text{-}T}{C} * 100$$

where, C- mycelial growth of pathogen in control

T- mycelial growth of pathogen in treatment

3.5 *In vitro* SCREENING FOR PRODUCTION OF BIOMOLECULES IMPARTING STRESS TOLERANCE AND ANTAGONISM

The selected abiotic stress tolerant isolates were further studied for production of enzymes like cellulase and *β*- 1, 3-glucanase which are involved in antifungal activity. Biomolecules imparting drought tolerance such as ACC (1-amino cyclopropane-1-carboxylate) deaminase and exopolysaccharide were also screened.

3.5.1 Screening for exopolysaccharide production

The exopolysaccharide producing cultures were screened on following medium mentioned in the protocol of Triveni (2000) Potassium nitrate (1.0 g/1) was also added to the medium as a nitrogen source. pH of the medium was adjusted to 7.0 followed by sterilization. The plates after inoculation were incubated at 30°C for 48 h and the colonies producing copious amount of exopolysaccharide were recorded.

3.5.2 Screening for ACC deaminase activity

ACC (1- amino cyclopropane-1-carboxylate) deaminase activity of drought tolerant *Pseudomonas* isolates were screened based on their ability to use ACC as a sole nitrogen source. Isolates were grown in five ml of TSB medium incubated at 28^{o}C at 120 rpm for 24 h. The cells were harvested by centrifugation at 3000 *g* for 5 min and washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and resuspended in one ml of 0.1 M Tris-HCl (pH 7.5). The bacterial suspensions in Tris-HCl were spot inoculated on Petri plates containing modified DF (Dworkin and Foster) salts minimal medium (Dworkin and Foster, 1958). Plates containing only DF salts minimal medium without ACC served as negative control and with Ammonium sulphate (0.2 % w/v) served as positive control. The plates were incubated at 28^{o}C for 72 h. Growth of isolates on ACC supplemented plates was compared to negative and positive controls and the isolates were selected based

on their growth by utilizing ACC as nitrogen source.

3.5.3 Screening for cellulase production

Cellulase activity of stress tolerant isolates of *Pseudomonas* spp. was determined by using the method described by Sadasivam and Manickam (2008). Isolates were grown in carboxy methyl cellulose (CMC)(1 %) medium for two days at room temperature. After incubation period, 0.5 ml of the enzyme extract was pipetted out into 32 mg of dry Whatmann No. 1 filter paper discs (substrate for the cellulase) in boiling tubes and incubated in water bath at 50^{o}C for one hour. After taking from water bath, 0.5 ml of DNS (dinitro salicylic acid) reagent was added immediately followed by boiling in water bath for five min. One ml of 40 per cent Rochelle salt solution was subsequently added and the mixture was cooled to room temperature. The volume of the mixture was made upto 5 ml with distilled water and the absorbance was measured at 540 nm using spectrophotometer. A standard graph was plotted with glucose in the range from 50 to 1000 µg ml^{-1}.

3.5.3.1 Calculation of cellulase activity

Glucose stock solution (1000 ppm) was prepared and from which 100, 300, 500, 600, 750 ppm solutions were made. Glucose solution and DNS reagent, 0.5 ml each, were boiled in a water bath for five min. The same procedure was continued as mentioned in 3.5.3. Standard graph was drawn by plotting absorbance against glucose concentration and a regression equation was constructed.

$$\text{Cellulase activity (U/ml)} = \frac{(\Delta E \times V_f)}{(\Delta t \times \sum \times V_s \times d)}$$

ΔE = Absorbance at 540 nm

V_f = Final volume including DNS

Vs = Volume of enzyme extract
Δt = Time of hydrolysis
∑ = Extinction coefficient = 0.001 X + 0.069, (where X = 0.18 mg for 1 µM glucose)
d = Diameter of cuvette

3.5.4 Screening for *β*-1, 3-glucanase production

The abiotic stress tolerant isolates of *Pseudomonas* spp. were screened for *β*- 1, 3-glucanase activity as per the protocol mentioned in Sadasivam and Manickam (2008). The isolates were grown in carboxy methyl cellulose (CMC) (1 %) medium for two days and enzyme extract was prepared. Enzyme extract (0.05 ml) was added to CMC solution (0.45 ml) in boiling tubes and incubated at 55^{o}C for 15 min. DNS reagent (0.5 ml) was added immediately after removing from water bath and thereafter kept in water bath again for 5 min. One ml of 40 per cent Rochelle salt solution was added while the tubes were still warm. Volume was made upto 5 ml after cooling the tubes to room temperature and the absorbance was read at 540 nm using spectrophotometer. A standard graph was plotted using glucose concentration in the range from 50 to 1000 µg ml^{-1}**.** Calculation of *β*- 1, 3-glucanase activity was carried out as detailed in 3.5.3.1.

3.6. *In vivo* BIOASSAY FOR TESTING THE EFFICACY OF ISOLATES

Roll paper method (ISTA, 1976) with slight modification was used for testing the bioefficacy of stress tolerant isolates. For this, cowpea seeds were taken and first treated with the antagonistic isolates by seed bacterization and thereafter followed by treatment with the pathogen (Shukla *et al.*, 2016).

3.6.1 Seed bacterization with bioagents

The method of Weller and Cook (1983) was followed for seed

bacterization. Bacterial suspensions were prepared from 48 h old cultures grown in NA. This suspension was mixed with carboxy methyl cellulose (CMC) (1%). Seeds of cowpea were surface sterilized with sodium hypochlorite solution (2%) followed by rinsing with sterile water. These surface disinfected seeds were then coated with slurry of bacterial culture and allowed to air dry overnight under aseptic conditions. Care was taken to avoid clumping of seeds.

3.6.2 Seed treatment with pathogen

Mycelial suspension of *Rhizoctonia solani* was prepared by scraping the mycelia from the culture grown in a Petriplate using a sterile scalpel and by adding 50 ml sterile water. Cowpea seeds earlier coated with bacterial suspension were then inoculated with the mycelial suspension of *Rhizoctonia solani* for 2 h. Seeds treated with the pathogen alone served as check. For each treatment three replications were maintained.

3.6.3 Roll towel method

Seeds treated as described earlier with each bioagent separately were placed on moist blotter sheets equi-spaced and covered with a moistened blotter and rolled. Ten seeds were placed on each blotter. Three such rolls were kept on a butter paper sheet and rolled as a single bundle and incubated in a growth chamber maintained at 25^{o}C and 80 percent relative humidity. Moisture in blotter sheets was maintained by applying sterile water whenever needed. Observations were taken 10 days after incubation. The efficacy of bioagents was rated as per a disease grading key based on seed rotting, infection occurring on roots and shoots The results are depicted in Table 3.1.

3.7 *In vivo* EVALUATION OF PROMISING STRESS TOLERANT ISOLATES OF *Pseudomonas* spp. FOR BIOCONTROL EFFICACY AND GROWTH PROMOTION

A pot culture experiment was laid out to test the antagonistic potential and plant growth promoting ability of the most promising isolates. Cowpea was selected as the test crop and *Rhizoctonia solani* as the test pathogen as it causes collar rot disease. The isolates were compared with a positive control which is the reference culture of *Pseudomonas fluorescens* of KAU and a negative control (without *Pseudomonas*). The experiment was carried out during April- June 2019 at College of Horticulture, Vellanikkara. The details of pot culture experiment are given below:

Design	: CRD
Treatment	: 6
Replications	: 3
Number of plants per replication	: 3
Crop	: Cowpea
Variety	**:** Bhagyalakshmi

3.7.1 Preparation of potting mixture and raising of crop

Readymade potting mixture consisting of sand, soil and cowdung in the ratio 1: 1: 1 was filled in grow bags of size 60 x 30 x 30 cm. Formalin (40 %) was diluted three times with water and used for chemical sterilization of potting mixtures. After drenching with formalin solution, the growbags were covered with polythene sheet so that the fumes of formalin will not escape from the bags.

Table 3.1 Disease Rating Scale

Disease incidence (%)	Description	Rating of bioefficacy of isolates
0	Germination >90%, no seed rotting, seedlings healthy, root and shoot portions well developed	Highly Efficient (HE)
1-15	Germination 80-90%, infection on main as well as lateral roots, seedlings are well developed	Efficient (E)
16-30	Germination 70-80%, development of roots restricted and growth is less compared to Score 1. Infection occurred on roots. Shoot portions developed but growth retarded compared to Score 1	Moderately Efficient (ME)
31-45	Germination 60-70%, length of roots and shoots short compared to Score 1. Germination of seeds inhibited.50% of root area infected. Shoot portions also showed infection.	Moderately Inefficient (MI)
46-60	Seed germination 50 to 60%. Development of roots and shoots greatly retarded. Shoot portions showed more infection.	Inefficient (I)
Above 60	Less than 50% germination and seed rotting	Highly Inefficient (HI)

Polythene sheet was removed 10 days prior to sowing. Afterwards seeds of cowpea var. Bhagyalakshmi which were treated with *Pseudomonas* isolates and reference culture were sown in grow bags with four seeds per grow bag. All the cultural operations except fungicide application were carried out as per the Package of Practices recommendations. Thirty days after sowing, the soil was drenched with suspension of *Pseudomonas* isolates prepared in sterile water containing a population of 10^7 cfu ml^{-1}at 50 ml per bag.

3.7.2 Challenge inoculation with *Rhizoctonia solani*

Antagonistic potential of *Pseudomonas* isolates were evaluated by challenge inoculation with the pathogen, *Rhizoctonia solani.* Rice grains were used for mass multiplication of the pathogen. The grains after thorough washing

with water were half boiled and 500 g of these grains were filled in 250 ml conical flask and plugged with nonabsorbent cotton. The flasks were autoclaved at 121^{o}C, 15 psi pressure for one hour. After cooling, two day old mycelial discs of the pathogen were inoculated aseptically into these flasks and were allowed to grow for 14 days at room temperature. The plants were challenge inoculated with mass multiplied *R. solani* @ 20 g (2 x 10^5 cfug^{-1}) after soil drenching with bacterial suspensions (Aswitha *et al.*, 2013).

3.7.3 Observations recorded

Observations on seed germination, plant biometric characters, yield and disease incidence were recorded at appropriate intervals.

3.7.3.1 Observations on germination

The number of seeds germinated, germination percentage, earliness in germination, and were noted.

3.7.3.2 Biometric observations

The biometric observations like plant height, number of leaves, days to flowering, number of pods and yield (weight and number of pods) were also recorded.

3.7.3.2.a Height of the plant

Height of each plant was measured and recorded at 30, 45 and 60 days after sowing.

3.7.3.2.b Number of leaves

Number of leaves in each plant were counted and recorded at 30, 45 and 60 days after sowing.

3.7.3.2.c Days to first flowering

Days to first flowering were noted and compared in each treatment.

3.7.3.2.d Yield

Weight and number of pods were recorded for each treatment and expressed in g/plant.

3.7.3.3 Assessment of disease incidence

Cowpea plants were observed for symptom appearance in the collar region of plants on week after challenge inoculation. Per cent disease incidence was calculated by adopting the formula given by Wheeler (1969).

$$\text{Per cent disease incidence (PDI)} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} * 100$$

3.8 CULTURAL, MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF PROMISING *Pseudomonas* ISOLATES

Pure cultures of selected stress tolerant and antagonistic isolates were studied for the morphological and biochemical characters as per Bergey's Manual of Systematic Bacteriology (Aneja, 2003).

3.8.1 Colony morphology

Cultural and morphological characteristics of each isolate were examined on King's B medium. Characters such as shape, size, elevation, surface, margin, colour, pigmentation,*etc*. of the colony of each isolate were recorded.

3.8.2 Gram's staining

A drop of sterile distilled water was placed in the center of glass slide. A loopful of inoculum from fresh bacterial culture was mixed with a drop of

water placed on slide to make a thin uniform bacterial smear. The smear was air dried and fixed through mild heating by passing the slide 3 to 4 times over the flame. The smear was then flooded with Crystal violet solution for 1 min and washed gently with flow of tap water. Then the slide was flooded with iodine solution and kept for 1 min, and thereafter drained out followed by washing with 95 per cent alcohol which acts as a decolorizer. After 15 to 30 seconds, the slide was washed with water and blotted carefully. The smear was then flooded with safranin solution for 1 min. The slide was washed gently with tap water and air dried, which was then examined under the microscope at 100X power with oil immersion and observations were recorded.

3.8.3 Gelatin liquefaction test

Gelatin is a protein which exists as a liquid above 25˚Cand solidifies when cooled below 25˚C. Gelatin hydrolysis was demonstrated by growing *Pseudomonas* isolates in nutrient gelatin slants and these were incubated at 28 ±2˚C for 48 h. Thereafter, the slants were kept under refrigerated condition at 4^0C. The isolates showing liquefaction were recorded as positive and the tubes that remained solid were recorded as negative for the test.

3.8.4 Indole production

Sterilized tryptophan broth tubes were inoculated with the overnight cultures of the isolates and incubated for 48 h at 28 ± 20℃. Following incubation, 10 drops of Kovac's indole reagent were added to each tube. The isolates showing production of red colour were recorded as positive for indole production.

3.8.5 Catalase test

This test was performed to study the presence of catalase enzyme in

bacterial colonies. Pure isolates (24 h old) were taken on glass slides and one drop of H_2O_2 (30%) was added. Observations on development of gas bubble were recorded which indicated the presence of catalase enzyme.

3.8.6 Methyl red Test

Sterilized glucose-phosphate broth tubes were inoculated with the test culture and incubated at 28 ± 20°C for 48 h. After incubation five drops of methyl red indicator was added to each tube and gently shaken. Observation on development of red colour was taken as positive and yellow colour production was taken as negative for the test.

3.8.7 Voges Prausker's Test

To the presterilized glucose-phosphate broth tubes, test cultures were inoculated and incubated at 37°C for 48 h. After incubation, ten drops of Baritt's reagent A was added and gently shaken followed by addition of ten drops of Baritt's reagent B. Development of pink colour in the broth was taken as positive for the test.

3.8.8 Citrate utilization

Isolates were streaked on Simmon's citrate agar slants and incubated at 28 ± 20°Cfor 24 h. Change in colour from green to blue indicated positive reaction for citrate utilization.

3.8.9 Hydrogen sulfide test

Sterilized hydrogen sulfide- indole-motility agar (SIM agar) stabs were inoculated along wall of the tubes with overnight cultures of the isolates and incubated for 48 h at 28 ± 20°C. The cultures were visualized for black colour

along the line of inoculation which indicated a positive reaction for the test.

3.8.10 Carbohydrate utilization

All the pure bacterial isolates were screened for carbohydrate fermentation abilities using four different carbohydrates *viz*., lactose, sucrose, dextrose and mannitol in peptone broth medium. Bacterial isolates were inoculated in the broth containing specific carbohydrate. The change in colour of peptone broth was observed for utilization of particular carbohydrate.

3.8.11 Oxidase test

Oxidase reaction was carried out by spreading an isolated colony on the oxidase disc. The reaction is observed within 5-10 seconds at 25-30°C. A change later than 10 seconds or no colour changes were considered as negative reaction.

3.8.12 Antibiotic sensitivity

Antibiotic tolerance of bacterial isolates was assessed by placing Hi Media antibiotic discs on bacterial lawn made from different isolates of *Pseudomonas* spp. on King's B plate. Observations were after 48 h after incubation period. The concentration of antibiotics which were used for the antibiotic sensitivity test is given below:

Table 3.2 Screening for antibiotic sensitivity

Sl. No.	Antibiotic	Concentration (ppm)
1.	Gentamicin	0.1
2.	Chloramphenicol	0.3
3.	Ampicillin	0.1
4.	Tetracycline	0.3

3.9 MOLECULAR CHARACTERIZATION OF PROMISING *Pseudomonas* ISOLATES

Apart from the cultural, morphological and biochemical characterization, the selected isolates of *Pseudomonas* spp. were also sent to at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram for species identification using 16S rRNA gene sequencing.

3.9.1 Genomic DNA Isolation and amplification

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.The genomic DNA was further amplified using PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA. The primer details are depicted in Table 3.3.Amplification was performed with an initial denaturation step at 95^{o}C for 5 min, followed by 35 cycles of 30 sec denaturation at 95^{o}C, 40 sec annealing at 60^{o}C, 1 min extension at 72^{o}C and a final extension step at 72^{o}C for 7 min.

Table 3.3 Primers used for PCR Analysis

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

3.9.1 Gel electrophoresis and sequencing of 16S rRNA gene

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 $\mu g ml^{-1}$ ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 h, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The PCR mix consisted of the following components:

PCR Product	(ExoSAP treated)	- 10-20 ng
Primer		- 3.2 pM (either forward or reverse)
Sequencing Mix		- 0.28 µl
Reaction buffer		- 1.86 µl
Sterile distilled water		- make up to 10µl

The sequencing PCR temperature profile consisted of 1st cycle at 96^{o}C for 2 min followed by 30 cycles at 96^{o}C for 30 sec, 50^{o}C for 40 sec and 60^{o}C for 4 min. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). The partial 16s rRNA gene sequences of the isolates were BLAST searched on NCBI GenBank website to identify the closest matches.

3.10 Statistical analysis

Analysis of variance was performed for the data collected in various experiments by employing Web Agri Stat Package (WASP2.0). Mean, level of

significance and standard error were obtained for various data sets. Multiple comparison between the treatment means where the F test was significant was done with Duncan's Multiple range Test (DMRT) (Gomez and Gomez, 1984).

RESULTS

4. RESULTS

The research on "Characterization and evaluation of *Pseudomonas* spp. for abiotic stress tolerance" was carried out in the department of Plant Pathology, College of Horticulture, Vellanikkara during the period 2017-2019. The results of experiments are presented below.

4.1 SURVEY, COLLECTION AND ANALYSIS OF SOIL SAMPLES FROM STRESSED ECOSYSTEMS OF KERALA

4.1.1 Soil sampling survey

A total of 26 representative soil samples were collected from abiotic stressed ecosystems of Kerala during the period February- April. Seven samples were collected from each Thrissur, Palakkad, Moncombu and five samples from Vyttila region (Plate 4.1). The samples were analyzed for soil reaction (pH), electrical conductivity (EC) and soil moisture content so as to ensure that they represent the abiotic stressed ecosystems. Soil properties such as texture and soil temperature were also noted at the time of collection. The details of soil sample collection and soil properties are depicted in Table 4.1. The samples were assigned with a sample code and were serially numbered with respect to the place of collection. Accordingly, the samples from Thrissur district were assigned with the code KTR, KRM and NDT representing the location *viz*., Kattoor, Karalam and Nadathara. Similarly, sample codes MCB and VYT represent Moncombu (Alappuzha district) and Vyttila (Ernakulam district) respectively. Samples from Palakkad district were designated with the code KMR, KLM, ORS and ORP representing the location Kuthiramulleri, Kallampatti, Orassery and Orappad.

A. Thrissur

B. Palakkad

C. Alappuzha

D. Ernakulam

Plate 4.1 Collection of soil samples ftrom different abiotically stressed locations of Kerala

Table 4.1 Details of soil samples collected from different locations of Kerala

Sl. No.	District	Location	Sample code	Soil texture	Soil temperature (°C)	pH	EC (dSm^{-1})	Soil moisture content (%)
1.	Thrissur	Kattoor-1	KTR1	Clay	42.0	5.40	0.02	1.2
2.		Kattoor-2	KTR2	Clay	40.0	5.20	0.02	3.9
3.		Kattoor-3	KTR3	Clay	43.0	5.00	0.02	2.9
4.		Karalam-1	KRM4	Clay	42.0	4.40	0.01	4.0
5.		Karalam-2	KRM5	Clay	41.0	4.40	0.01	2.2
6.		Karalam-3	KRM6	Clay	42.0	4.40	0.01	2.8
7.		Nadathara-1	NDT-1	Clay	40.0	6.00	0.02	1.2
8.	Palakkad	Kuthiramulleri1	KMR1	Sandy clay loam	43.0	5.80	0.02	2.3
9.		Kuthiramulleri2	KMR2	Sandy clay loam	44.0	6.00	0.02	2.7
10.		Kallampatti1	KLM1	Sandy clay loam	44.0	5.90	0.02	1.1
11.		Kallampatti2	KLM2	Sandy clay loam	46.0	5.60	0.02	2.0
12.		Orassery1	ORS1	Sandy clay loam	45.0	5.60	0.01	2.4
13.		Orassery2	ORS2	Sandy clay loam	44.0	5.60	0.02	1.88
14.		Orappad1	ORP1	Sandy clay loam	44.0	5.80	0.02	1.75

Sl. No	District	Location	Sample code	Soil texture	Soil temperature (°C)	pH	EC (dSm^{-1})	Soil moisture content (%)
15.	Alappuzha	Moncombu- 1	MCB1	Sandy clay	38.5	3.40	6.68	35.1
16.		Moncombu- 2	MCB2	Sandy clay	37.9	3.50	5.26	35.0
17.		Moncombu- 3	MCB3	Sandy clay	37.7	3.80	5.50	34.5
18.		Moncombu- 4	MCB4	Sandy clay	38.1	3.60	5.50	42.0
19.		Moncombu- 5	MCB5	Sandy clay	37.8	3.90	5.40	39.0
20.		Moncombu- 6	MCB6	Sandy clay	38.0	3.50	5.22	36.6
21.		Moncombu- 7	MCB7	Sandy clay	38.2	3.50	5.26	35.8
22.	Ernakkulam	Vyttila- 1	VYT1	Clay	37.6	4.30	4.10	23.3
23.		Vyttila- 2	VYT2	Clay	36.5	4.50	4.10	25.6
24.		Vyttila- 3	VYT3	Clay	36.0	4.20	4.30	24.8
25.		Vyttila- 4	VYT4	Clay	37.3	4.20	4.30	26.6
26.		Vyttila- 5	VYT5	Clay	37.1	4.60	4.20	28.1

4.1.2 Soil temperature and texture

Soil temperature and soil texture was recorded at the time of soil sampling itself and the observations are also tabulated in Table 4.1. The data revealed that the highest soil temperature of 46.0^{o}C was recorded from KLM2 of Palakkad district whereas, lowest temperature of 36.0^{o}C was observed from VYT3 of Ernakulam district. In general, higher soil temperature ranging from 43- 46° C were recorded from various locations of Palakkad district. Likewise, sampling sites of various locations of Thrissur district also recorded a higher temperature ranging from 40-43°C followed by Moncombu (37.7-38.5°C). Relatively a lower soil temperature was recorded from Vyttila, ranging from 36.0- 37.6°C. The data thus, indicates that at the time of soil sample collection, various locations of Palakkad and Thrissur district experienced the maximum temperature stress compared to Vyttila and Moncombu. While observing the soil texture, it was noticed that all the soil samples from Thrissur and Vyttila showed clayey texture, whereas samples from Alappuzha and Palakkad were observed to be sandy clay and sandy clay loam texture respectively.

4.1.3 Soil reaction

Soil samples collected from various stressed soils of Kerala were analyzed for pH under laboratory conditions. In general, soil samples from Alappuzha district showed a pH value in the range 3.4-3.9 rendering them extremely acidic. Among them, highest pH value of 3.9 was recorded with MCB5 whereas MCB1 showed lowest pH value of 3.4 which fall under ultra acidic category.

Soil samples collected from Vyttila region was also found to be extremely acidic since the samples showed pH value ranging from 4.2 to 4.5.

Similarly, analysis of soil samples collected from Thrissur district revealed that in general, soils are moderately acidic (4.4- 5.4). Soil samples KTR1, KTR2 and KTR3 seemed to be strongly acidic (5.0-5.4). In addition, the soil sample collected from Nadathara is having pH of 6.0 rendering it moderately acidic, whereas, soil samples collected from Karalam region (KRM4, KRM5 and KRM6) is found to have a pH of 4.4 making it extremely acidic. pH analysis of Palakkad soil samples revealed that soils are moderately acidic and fall in the range of 5.6 to 6.0 and among which, highest pH was observed with KMR2 and lowest with KLM2, ORS1 and ORS2. Thus, it can be inferred that all the collected soil samples are acidic which varied between moderately to ultra acidic.

4.1.3 Electrical conductivity and soil moisture content

Electrical conductivity (EC) of the soil samples collected from various locations was estimated using EC meter and was expressed in dSm^{-1}. The details are depicted in Table 4.1. In general, the highest range of EC value of 5.22 to 6.88 dSm^{-1} was observed with soil samples collected from Moncombu making them saline. This was followed by the soil samples collected from Vyttila which represented an EC ranging from 4.1 to 4.3 dSm^{-1}. Soil samples collected from other districts *viz*., Thrissur and Palakkad were found to be non saline since, the values ranged from 0.01dSm^{-1} to 0.02 dSm^{-1}. Among the saline soils, the highest EC was observed with MCB1 (6.68 dSm^{-1}) and lowest with VYT1 and VYT2 (4.1dSm^{-1}).

Soil moisture content of the samples were estimated by gravimetric method to ensure the drought affected regions. From the data in general, it can be observed that the moisture content ranged between 1.1 to 3.9 per cent where the soil samples collected from Palakkad and Thrissur district displayed the lowest moisture content which indicated that such soils are prone to drought. Whereas, the soil samples collected from Moncombu region was found to have soil moisture content in the range of 35.0- 42.0 per cent. It was followed by soil

samples from Vyttila ranging between 23.3-28.1 per cent. Among the drought affected soil samples, lowest soil moisture content was estimated from KLM1 collected from Kallampatti region of Palakkad district. Hence, after the physical characterization of soil samples, it was confirmed that the places from where the samples were collected are abiotic stressed locations and such soil samples were subjected to isolation of *Pseudomonas* spp. and used for further studies.

4.2 ISOLATION AND ENUMERATION OF *Pseudomonas* spp.

Soil samples were subjected to serial dilution and plating technique using two different isolation media, *viz*., King's B agar medium and *Pseudomonas* agar base (PAB) medium. Diluted soil suspension was plated at three different dilutions of 10^{-4}, 10^{-5} and 10^{-6}. However, colonies were better formed at the dilution of 10^{-5}. After an incubation period of 24 h, the bacterial colonies formed were enumerated and tentatively identified as *Pseudomonas* based on colony colour and appearance (Plate 4.2). The details of enumeration of *Pseudomonas* are presented in Table 4.2.

The data represented in the table clearly indicates that Kings' B agar medium is better suitable for isolation of *Pseudomonas* spp. than PAB medium since comparatively less number of colonies were formed on PAB medium from all the soil samples. It can also be inferred that in general, soil samples from Alappuzha yielded highest population of *Pseudomonas* spp. followed by soil samples from Palakkad, in both the medium. However, lowest population of *Pseudomonas*was recorded in soil samples procured from Vyttila Research Station, Ernakulum.

In general, soil samples from Palakkad district yielded a population of *Pseudomonas*spp. ranging from 0.24-0.74x10^{6} cfu g^{-1} in King's B agar medium. Also, when PAB medium was used for enumeration, population ranged from 0.2-

$4.5x10^6$ cfu g^{-1}. Among the soil samples collected from Palakkad district, highest population of 0.74×10^6 cfu g^{-1} was observed from ORP1 in King's B agar medium. Similar results were noticed in PAB medium with a population of 4.5×10^6 cfu g^{-1} whereas, lowest population was observed from the soil sample KLM2 with $0.24x10^6$ and $0.20x10^6$ in KB and PAB medium respectively. However, the soil samples KLM1, ORS1 and ORS2, yielded no colonies of *Pseudomonas* spp.

Soil samples from Thrissur district showed a population count of 2.3 – 9.12 x 10^5 cfu g^{-1} in KB medium and 1.01- 4.56 $x10^5$ cfu g^{-1}in PAB medium. No colonies of *Pseudomonas* spp. were obtained from the soil samples KTR1, KTR3 and NDT1. However, among the samples of Thrissur district, maximum population of *Pseudomonas* was recorded from one of the soil sample collected from kole lands of Kattoor region (9.12×10^5 cfu g^{-1}in KB agar and 4.56×10^5 cfu g^{-1}in PAB medium). The least number of colonies was observed in the sample from KRM6 on both the media when subjected to serial dilution.

Similarly, population of *Pseudomonas* spp. from soil samples of Ernakulam district was lowest compared to that of samples from other districts and it ranged from 3.14- 6.0 x 10^4 cfu g^{-1} in KB medium and 1.1- 2.29 x 10^4 cfu g^{-1} in PAB medium. Moreover, the soil samples VYT1 and VYT3 didnot yield colonies in both media. Likewise, when soil samples from Alappuzha district were enumerated for *Pseudomonas* spp., except for three samples (MCB1, MCB3 and MCB6), other samples yielded highest population of *Pseudomonas*. When KB agar medium was employed, population was attained in the range of 1.3- 7.81 x 10^6 cfu g^{-1}. However, in PAB medium, population ranged between 0.28- 2.64 x 10^6 cfu g^{-1}. Highest population was recorded from the soil sample MCB7 and lowest from MCB5.

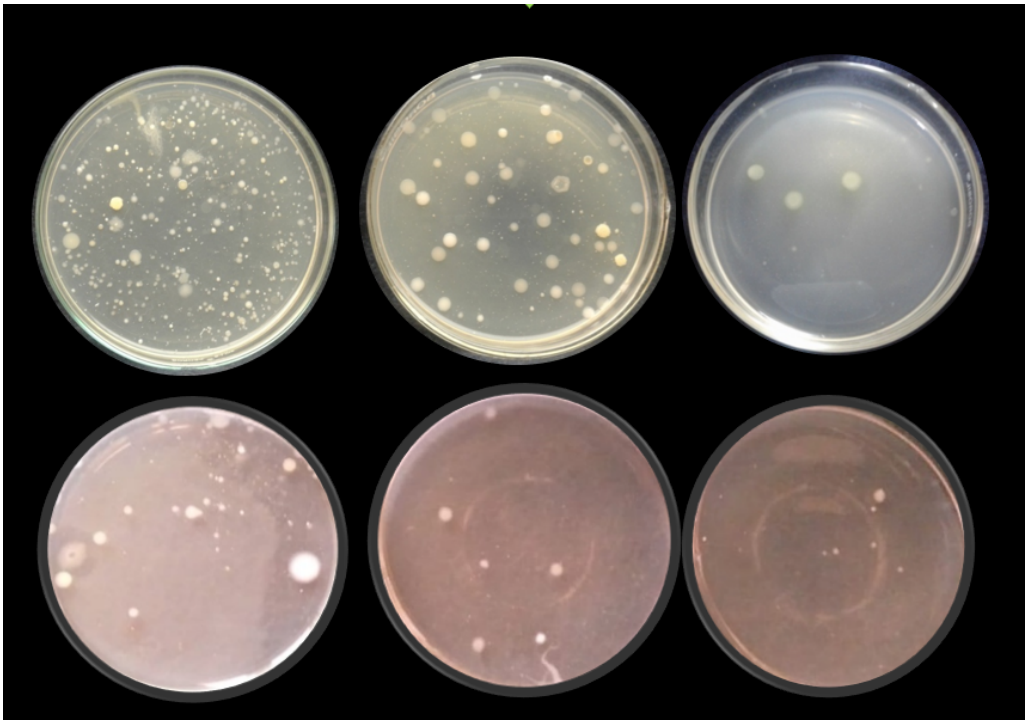


Plate 4.2 *Pseudomonas* colonies on King's B (A, B and C) and *Pseudomonas* agar base media (D, E and F) at three different dilutions *viz*., 10^{-4} (A and D), 10^{-5} (B and E) and 10^{-6} (C and F)

Table 4.2. Enumeration of *Pseudomonas* spp. from soil samples collected from abiotic stressed locations of various districts

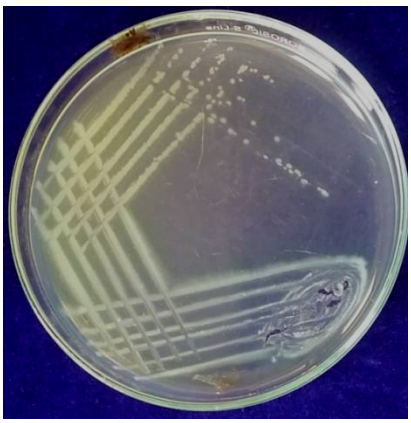
Sl. No.	District	Soil sample	Population (cfu g^{-1}) of *Pseudomonas* spp.		Isolate	Number of isolates
			Kings' B agar medium (KB)	*Pseudomonas* agar base medium (PAB)		
1.	Palakkad	KMR1	$(0.38 \times 10^6)^c$	$(0.25 \times 10^6)^b$	P1	4
2.		KMR2	$(0.45 \times 10^6)^b$	$(0.23 \times 10^6)^c$	P2	
3.		KLM1	0.00^e	0.00^e	Nil	
4.		KLM2	$(0.24 \times 10^6)^d$	$(0.20 \times 10^6)^d$	P4	
5.		ORS1	0.00^e	0.00^e	Nil	
6.		ORS2	0.00^e	0.00^e	Nil	
7.		ORP1	$(0.74 \times 10^6)^a$	$(0.45 \times 10^5)^a$	P7	
CD(0.05)			0.019	0.019		
8.	Alappuzha	MCB1	0.00^e	0.00^e	Nil	4
9.		MCB2	$(1.5 \times 10^6)^d$	$(0.28 \times 10^6)^d$	M2	
10.		MCB3	0.00^e	0.00^e	Nil	
11.		MCB4	$(4.5 \times 10^6)^b$	$(1.45 \times 10^6)^b$	M4	
12.		MCB5	$(1.3 \times 10^6)^c$	$(1.23 \times 10^6)^c$	M5	
13.		MCB6	0.00^e	0.00^e	Nil	
14.		MCB7	$(7.81 \times 10^6)^a$	$(2.64 \times 10^{6)a}$	M7	
CD(0.05)			0.096	0.019		
15.	Ernakkulam	VYT1	0.00^d	0.00^d	Nil	3
16.		VYT2	$(6.00 \times 10^4)^a$	$(2.29 \times 10^4)^a$	V2	
17.		VYT3	0.00^d	0.00^d	Nil	
18.		VYT4	$(3.14 \times 10^{4)c}$	$(1.10 \times 10^4)^c$	V4	
`19.		VYT5	$(3.57 \times 10^4)^b$	$(1.34 \times 10^4)^b$	V5	
CD(0.05)			0.06	0.019		
20.	Thrissur	KTR1	0.00^e	0.00^e	Nil	4
21.		KTR2	$(6.00 \times 10^5)^c$	$(3.23 \times 10^5)^b$	T2	
22.		KTR3	0.00^e	0.00^e	Nil	
23.		KRM4	$(9.12 \times 10^5)^a$	$4.56 \times 10^5)^a$	T4	
24.		KRM5	$(6.45 \times 10^5)^b$	$2.45 \times 10^5)^c$	T5	
25.		KRM6	$(2.30 \times 10^5)^d$	$1.01 \times 10^5)^d$	T6	
26.		NDT1	0.00^e	0.00^e	Nil	
CD(0.05)			0.06	0.019		

Hence, the data evidenced that Alappuzha soil samples recorded the highest population of *Pseudomonas* spp. followed by Palakkad whereas, lowest population was observed from soil samples collected from in and around areas of Vyttila Research Station.

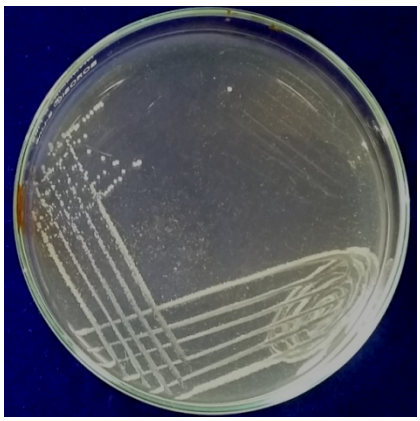
4.3 PURIFICATION AND PRESERVATION OF SELECTED ISOLATES OF *Pseudomonas* spp.

A total of 15 isolates of *Pseudomonas* spp. obtained from the soil samples collected from various districts were purified by quadrant streaking method (Plate 4.3). Least number of three isolates was obtained from Vyttila region of Ernakulum district, while four isolates each from the other three districts. The isolates obtained from each location were again abbreviated based on soil sample code. Accordingly, isolates P1, P2, P4 and P7 represent *Pseudomonas* spp. isolated from KMR1, KMR2, KLM2 and ORP1 of Palakkad district whereas T2, T4, T5 and T6 represent *Pseudomonas* spp. isolated from KTR2, KRM4, KRM5 and KRM6. Likewise, the isolates obtained from MCB2, MCB4, MCB5 and MCB7 were designated as M2, M4, M5 and M7. Similarly, isolates from the soil samples VYT2, VYT4 and VYT5 were represented with codes as V2, V4 and V5.

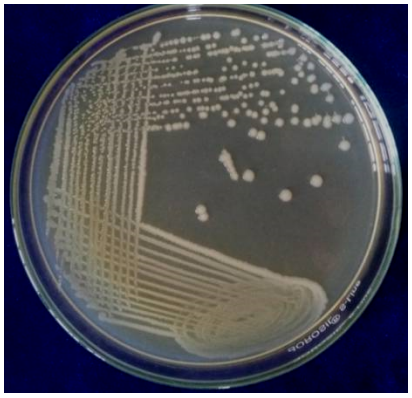
The purified *Pseudomonas* isolates as well as the reference culture of KAU collected from Department of Plant Pathology were preserved in sterile water and glycerol solution (15%) for further studies. Among 15 isolates, 10 showed fluorescence under UV transilluminator and such isolates were further screened for eliminating *P. aeruginosa* which is an opportunistic human pathogen.



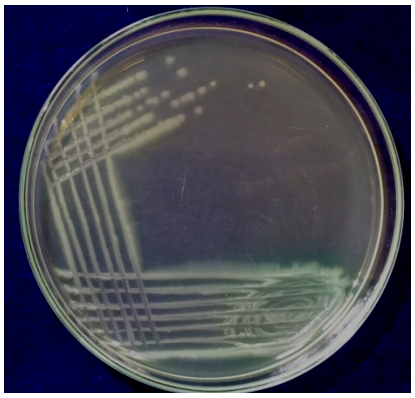
V2



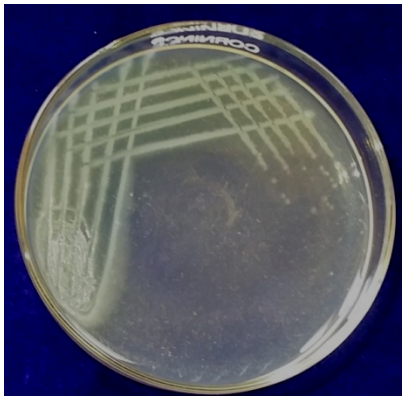
V4



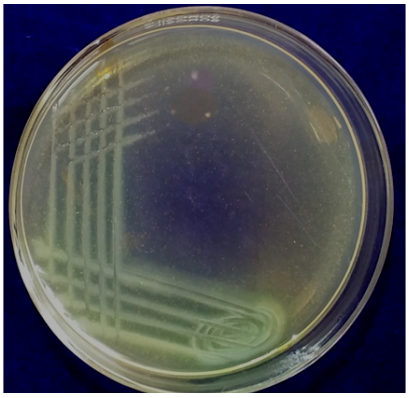
V5



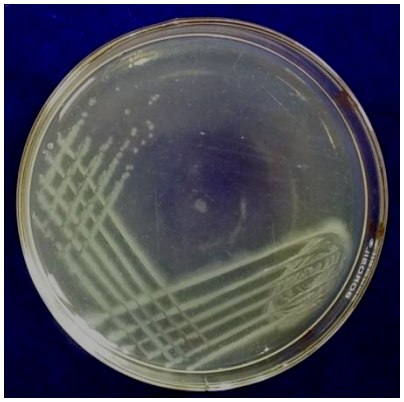
M2



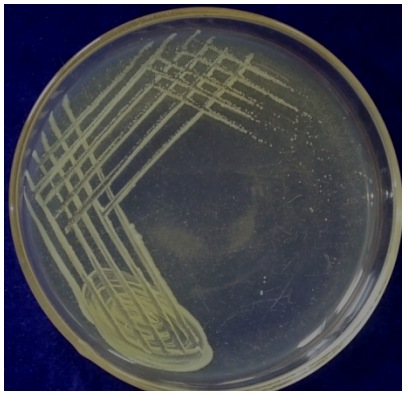
M4



M5

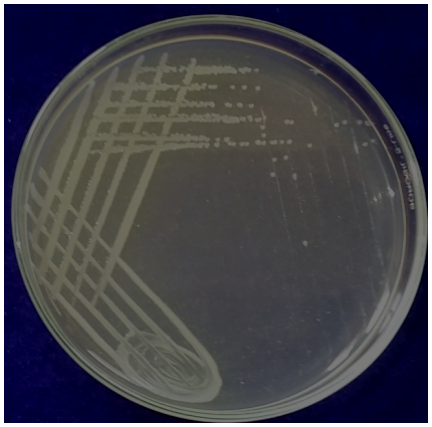


M7

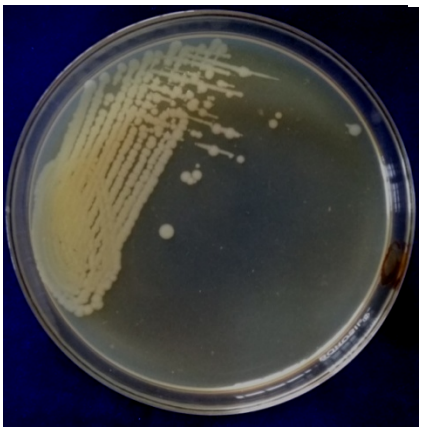


Reference culture of KAU

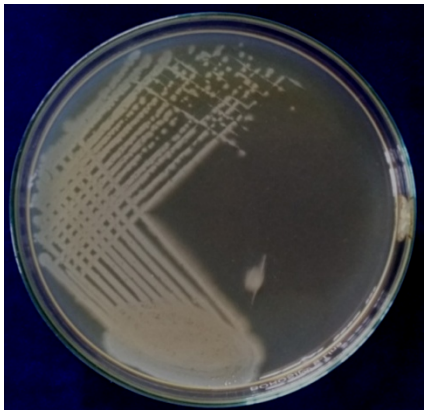
Plate 4.3 (i) *Pseudomonas* isolates obtained from different locations



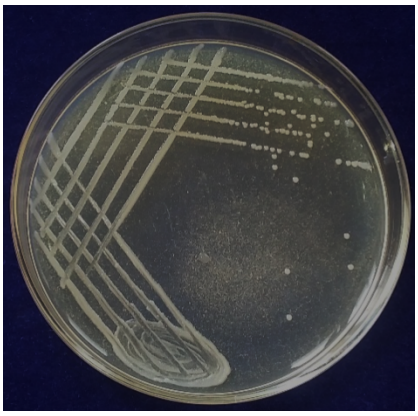
T2



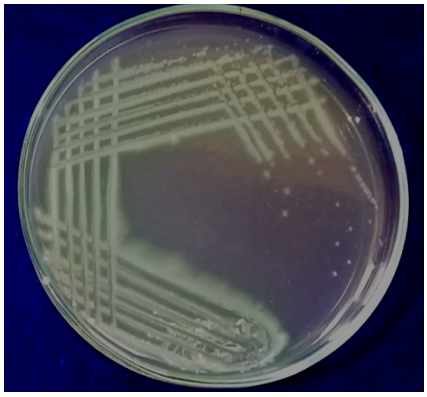
T4



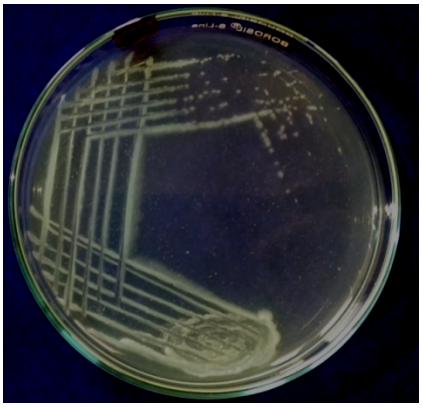
T5



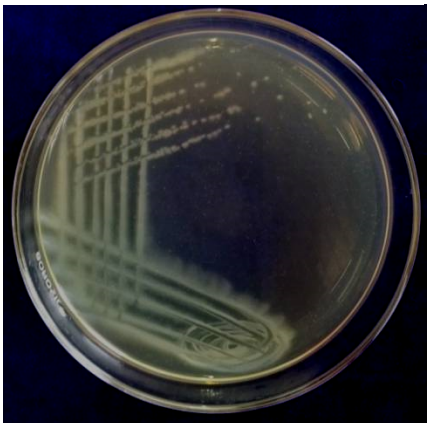
T6



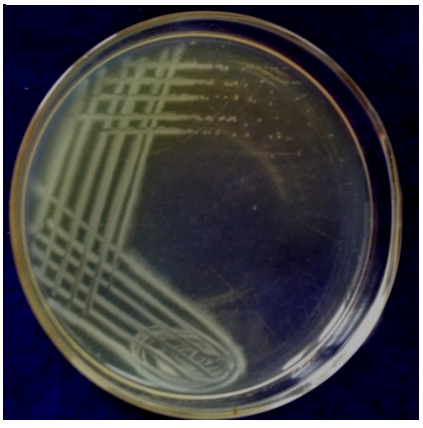
P1



P2



P4



P7

Plate 4.3 (ii) *Pseudomonas* isolates obtained from different locations

4.2.1 SCREENING AND ELIMINATION OF *Pseudomonas aeruginosa*

The nine fluorescent isolates from among the 15 along with the reference cultureof KAU were streaked on Kings' A agar medium and incubated for 48 h. The results of the experiment are furnished in Table 4.3. It was observed that the isolates P7 and M7 initially light green turned greenish blue colour after two days of incubation (Plate 4.4a). The other isolates such as M2, M4, M5, V2, P2 and T4 were also fluorescent green colour, but later turned into dull green colour after 48 h. The isolate P4 along with the reference culture turned into brownish cream colour after 48 h of incubation.

The isolates were also incubated at a temperature of 42°C to screen out *Pseudomonas aeruginosa* since the cultures of fluorescent pseudomonads in general will not grow at this temperature except for *P. aeruginosa.* From this study, it was observed that only two isolates *viz*., P7 and M7 showed abundant growth at 42°C (Plate 4.4b). Hence, from the above study, it was evident that P7 and M7 are cultures of *P. aeruginosa* and thus were eliminated.

4.3 SCREENING OF *Pseudomonas* spp. FOR ABIOTIC STRESS TOLERANCE

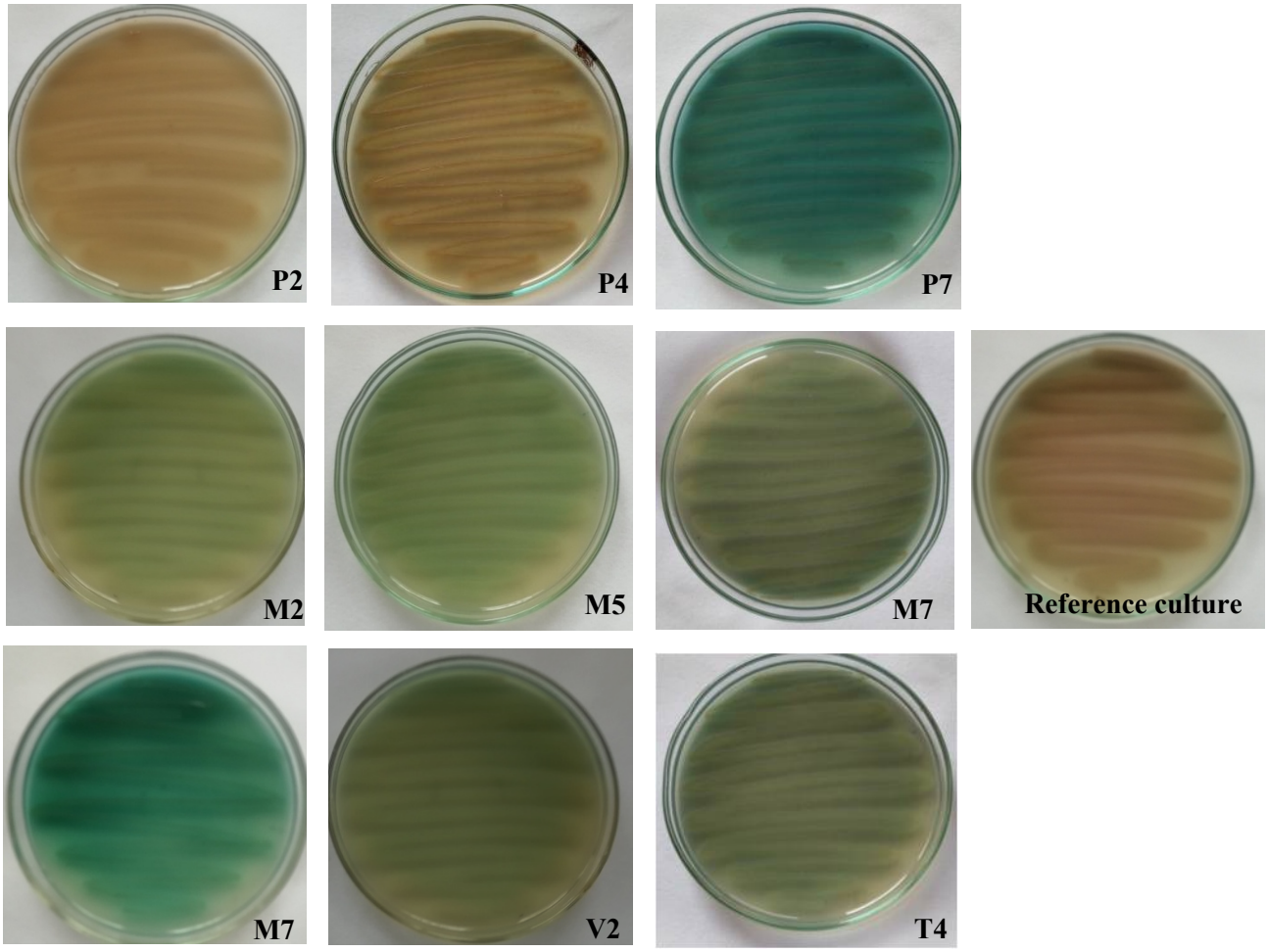
The native *Pseudomonas* spp. isolated from various locations were screened for abiotic stress tolerance by inducing temperature, drought, salinity and acidic stresses under *in vitro* conditions. The growth of isolates was measured in terms of optical density at 600 nm.

4.3.1 *In vitro* screening of *Pseudomonas* spp. for temperature tolerance

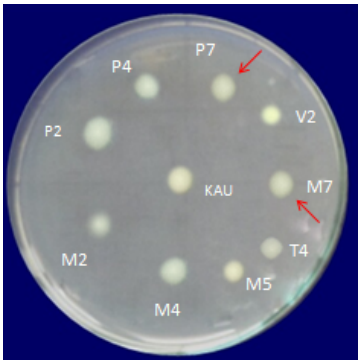
The isolates of *Pseudomonas* spp. were subjected to high temperature stress by exposing the isolates to varying levels of temperature *viz*. 25, 37, 45 and 50°C. The results of the experiment are tabulated in Table 4.4. A general pattern

Table 4.3 Screening of *Pseudomonas* isolates for elimination of *Pseudomonas aeruginosa*

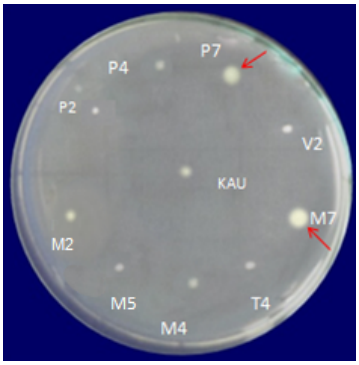
Sl. No.	Isolates	Color on Kings A medium	Growth at 42^{o} C
1.	P2	Dull green	+
2.	P4	Brownish cream	+
3.	P7	Greenish blue	++
4.	M2	Dull green	+
5.	M4	Dull green	+
6.	M5	Dull green	-
7.	M7	Greenish blue	++
8.	V2	Dull green	-
9.	T4	Dull green	-
10.	Ref.culture	Brownish cream	+



a) Growth of *Pseudomonas* isolates on King's A medium



b) Growth of *Pseudomonas* isolates at room temperature



c) Growth of *Pseudomonas* isolates at 42°C

Plate 4.4 Screening of *Pseudomonas* isolates for *P. aeruginosa*

Table 4.4 *In vitro* screening of *Pseudomonas* spp. for temperature tolerance

Sl. No.	Isolate	OD at 600 nm			
		25°C	**37 °C**	**45 °C**	**50 °C**
1.	P1	2.01^{d}	2.39^{f}	2.28^{a}	0.18^{jk}
2.	P2	2.09^{c}	2.52^{b}	2.24^{a}	0.62^{a}
3.	P4	2.18^{a}	2.42^{e}	1.01^{f}	0.34^{f}
4.	T2	1.89^{e}	2.21^{h}	1.86^{c}	0.38^{e}
5.	T4	2.11^{b}	2.48^{d}	1.83^{c}	0.34^{f}
6.	T5	2.12^{b}	2.50^{c}	1.45^{d}	0.62^{a}
7.	T6	1.65^{i}	2.07^{i}	1.94^{bc}	0.45^{d}
8.	M2	1.72^{g}	1.98^{j}	1.08^{e}	0.49^{c}
9.	M4	1.39^{k}	1.68^{k}	0.52^{h}	0.61^{b}
10.	M5	1.28^{l}	1.55^{l}	0.69^{gh}	0.31^{f}
11.	V2	1.01^{m}	1.42^{m}	0.85^{fg}	0.32^{g}
12.	V4	1.87^{f}	1.38^{n}	0.90^{efg}	0.22^{k}
13.	V5	1.43^{j}	2.49^{a}	2.10^{ab}	0.29^{h}
14.	Ref. culture	1.68^{h}	2.26^{g}	2.01^{bc}	0.23^{i}
CD (0.01)		0.017	0.017	0.029	0.017

* Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT

of growth was observed with all the isolates, when grown under various temperature levels. All the isolates showed an increased growth from 25 to 37°C and subsequently, as the temperature increased from 37 to 45 and 50°C theisolates subsided.

At 25°C, the optical density of the isolates ranged between1.01 and 2.18. Highest growth was exhibited by the isolate P4 and lowest by V2. When temperature was increased from 25 to 37°C, the growth of all the isolates showed a general rise which ranged from 1.75- 2.69. Thereafter, with increase in temperature to 45°C, the growth of all the isolates was found to declining.

The isolates P1 and P2 displayed maximum growth of 2.28 and 2.24 respectively. However at 50°C, only the isolates P2, T5 and M4 survived with an OD value ranging between 0.61-0.62 whereas, the other isolates showed comparatively poor growth of 0.18- 0.58.

4.3.2 *In vitro* screening of *Pseudomonas* spp. for salt tolerance

Salt stress was imposed to the isolates by amending 1.0 M and 1.5 M NaCl to the medium. In general, the OD was found to be very less even at 1.0 M concentration of sodium chloride implying sensitivity of isolates to salinity and it ranged between 0.22- 0.86 (Table 4.5). Highest growth of 0.86 OD at 1.0 M NaCl was displayed by T4 which was closely followed by V2 and V4 with OD 0.82 and 0.81 respectively. A significant decline in the reading was noticed at 1.5 M concentration compared to 1.0 M concentration whereas, M4 and V4 showed highest OD value of 0.5 and 0.48 at 1.5 M salt concentration.

Table 4.5 *In vitro* screening of *Pseudomonas* spp. for salt tolerance

Sl. No.	Isolate	OD at 600 nm	
		1 M	1.5 M
1.	P1	0.35^{f}	0.21^{f}
2.	P2	0.22^{i}	0.15^{h}
3.	P4	0.33^{g}	0.30^{c}
4.	T2	0.23^{i}	0.18^{g}
5.	T4	0.86^{a}	0.19^{g}
6.	T5	0.25^{h}	0.28^{d}
7.	T6	0.43^{e}	0.25^{e}
8.	M2	0.65^{c}	0.21^{f}
9.	M4	0.54^{d}	0.48^{b}
10.	M5	0.65^{c}	0.28^{d}
11.	V2	0.82^{b}	0.18^{g}
12.	V4	0.81^{b}	0.50^{a}
13.	V5	0.22^{i}	0.49^{ab}
14.	Ref. culture	0.23^{i}	0.24^{e}
CD (0.01)		0.017	0.017

* Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.6 *In vitro* screening of *Pseudomonas* spp. for drought tolerance

Sl. No.	Isolate	OD at 600 nm	
		10 % PEG	30 % PEG
1.	P1	1.94^{a}	0.04^{fg}
2.	P2	1.90^{bc}	0.12^{bc}
3.	P4	1.89^{c}	0.21^{a}
4.	T2	1.91^{b}	0.22^{a}
5.	T4	1.65^{d}	0.05^{ef}
6.	T5	1.53^{g}	0.11^{c}
7.	T6	1.58^{f}	0.09^{d}
8.	M2	1.63^{e}	0.09^{d}
9.	M4	1.01^{h}	0.05^{ef}
10.	M5	0.69^{i}	0.06^{e}
11.	V2	0.57^{j}	0.05^{ef}
12.	V4	0.45^{l}	0.02^{h}
13.	V5	0.38^{m}	0.13^{b}
14.	Ref. culture	0.49^{k}	0.03^{gh}
CD (0.01)		0.017	0.017

* Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.7 *In vitro* screening of *Pseudomonas* spp. for acid tolerance

Sl. No.	Isolate	OD at 600 nm		
		3.5	**4.5**	**5.5**
1.	P1	0.45^{g}	0.56^{g}	0.53^{i}
2.	P2	0.38^{h}	0.47^{i}	0.49^{j}
3.	P4	0.51^{e}	0.69^{e}	0.76^{g}
4.	T2	0.47^{f}	0.59^{f}	0.83^{f}
5.	T4	1.34^{b}	1.37^{b}	1.44^{c}
6.	T5	0.19^{m}	1.22^{c}	1.28^{e}
7.	T6	1.08^{c}	1.19^{d}	1.39^{d}
8.	M2	1.05^{d}	1.21^{cd}	1.20^{8}
9.	M4	1.49^{a}	1.38^{b}	1.56^{a}
10.	M5	1.48^{a}	1.46^{a}	1.52^{b}
11.	V2	0.21^{l}	0.27^{k}	0.25^{k}
12.	V4	0.35^{i}	0.69^{e}	0.61^{h}
13.	V5	0.32^{j}	0.54^{h}	0.52^{i}
14.	Ref. culture	0.29^{k}	0.38^{j}	0.76^{g}
CD (0.01)		0.016	0.020	0.017

* Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT

4.3.3 *In vitro* screening of *Pseudomonas* spp. for drought tolerance

Results furnished in Table 4.6 revealed that eventhough, drought stress was induced by adding 10 per cent PEG, most of the isolates showed good growth with an overall OD ranging between 0.38 and 1.94. Only five isolates including the reference culture displayed OD below one. Further, when drought stress was intensified to 30 per cent PEG the general range of OD dropped to 0.02- 0.22. From these results, it is evident that the higher level of osmotic stress was tolerated by the isolates P4 and T4 with optical densities of 0.21 and 0.22. Other isolates exhibited poor growth with OD ranging from 0.02- 0.12 with the least tolerance in the isolate V4.

M5 at all the tested pH of 3.5, 4.5 and 5.5 (Table 4.7). Among the 13 isolates, M4 and M5 showed consistent tolerance at three levels of pH by giving highest OD. At pH 5.5, maximum absorbance value were shown by M4 (1.56) closely followed by M5 (1.52). Whereas, at pH 4.5, M5 expressed highest optical density of 1.46 followed by M4 (1.38). When pH was decreased to 3.5, M4 and M5 were on par with each other with OD of 1.49.

Hence, based on the *in vitro* study for abiotic stress tolerance, P2, M4 and T5 were selected as temperature tolerant isolates whereas, M4 and V4 as salt tolerant. The isolates which exhibited maximum drought tolerance were P4 and T4, while, M4 and M5 were screened as the best acid tolerant strains. Thus, a total of seven isolates *viz*., M4, M5, P2, P4, V4, T4 and T5 were chosen as the abiotic stress tolerant strains and were evaluated further for their antagonistic potential.

4.4 *In vitro* EVALUATION OF STRESS TOLERANT ISOLATES OF *Pseudomonas* spp. FOR THEIR ANTAGONISTIC POTENTIAL

The antagonistic potential of stress tolerant isolates *viz*. P2, P4, M4, M5, T4, T5 and V4 were evaluated against five major soil borne pathogens *viz*., *Phytophthora capsici*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizoctonia solani*. T 63 es of plant pathogenic fungi and the reference culture of *Pseudomonas fluorescens* were collected from the Department of Plant Pathology, College of Horticulture, Vellanikkara. The biocontrol potential of these isolates were compared with the reference culture of KAU. The results of the *in vitro* screening of *Pseudomonas* isolates against mycelial growth of the above mentioned plant pathogens are recorded in Table 4.8.

The results revealed that the isolates P2, P4, M4 and M5 displayed strong antagonistic effect against all the tested fungal pathogens. The growth inhibition exhibited by these isolates ranged from 62.21 to 91.00 per cent. The highest percentage of growth inhibition against the pathogens *Pythium*, *Sclerotium* and *Fusarium* was shown by the isolate P4. The isolate P2 also exhibited maximum growth inhibition against *Pythium* (74.60 %) whereas M4 showed inhibition of 75.43 per cent of *Rhizoctonia* and 75.56 per cent of *Phytophthora capsici*.

In general, while comparing the susceptibility of fungal pathogens to these isolates, *Sclerotium rolfsii* was found to be suppressed by all the isolates except T4. Eventhough, T5 and V4 were not effective against other pathogens, these isolates could still reduce the growth of *Sclerotium rolfsii* by 21.0 and 54.0

per cent. Among all the fungal pathogens, the highest mycelial growth suppression was observed with *S. rolfsii* and it was noticed with the isolate P4 (91.0%). Data presented in table clearly indicated that the isolates P2, P4, M4 and M5 were highly antagonistic to all the tested fungal pathogens. It is evident from the data that the isolate T4 was not antagonistic to any of the pathogenic fungi as it showed zero per cent growth reduction. It is also clear that the isolates T5 and V4 also showed the least inhibitory activity against *Sclerotium* (21 and 50%) while no mycelial growth inhibition was recorded with other pathogenic fungi.

When isolates were screened for their growth inhibition against *Pythium aphanidermatum*, a per cent inhibition ranging from 0.00 to 74.61 was observed. The isolates P2 and P4 64 with each other, showing highest growth reduction of 74.61and 74.43 followed by KAU reference culture (72.88 %) and M4 (72.22 %). However, zero per cent inhibition in growth of *Pythium aphanidermatum* was observed with the isolates T4, T5 and V4.

The mycelial growth of *Phytophthora capsici* was found to be highly reduced by the isolate M5 with per cent inhibition of 75.56. This was closely followed by the isolates P4 and M4 with growth reduction of 74.42 per cent. The reference culture exhibited a per cent reduction of 72.0, while the isolates such as

T4, T5 and V4 didn't show any reduction in growth of *Phytophthora*.

The pathogen, *Rhizoctonia solani* was better suppressed by the isolate M4 with a growth inhibition of 75.43 followed by the reference culture of KAU (73.29 %). No inhibition in growth of *Rhizoctonia* was observed with the isolates T4, T5 and V4 whereas, comparatively less inhibitory activity was observed with the isolate P2 (65.54 %).

All the isolates showed highest antagonistic potential with per cent growth reduction against *Sclerotium rolfsii* except T4, which was not antagonistic

to any of the pathogens. Among which, maximum growth inhibition was observed with the isolate P4 (91.00 %) followed by P2. The least inhibition was recorded with the isolate T5 and no inhibition with T4.

The maximum inhibitory activity against *Fusarium oxysporum* was noted with the isolate P4 with a per cent growth reduction of 86.92. Isolates T4, T5 and V4 showed no inhibition in the growth of *Fusarium* which is similar to the case of *Pythium*, *Phytophthora* and *Rhizoctonia*. The other isolates displayed a growth inhibition ranging from 62.00 to 77.82 per cent.

The four isolates *viz*., P2, P4, M4 and M5 were selected as the potential antagonistic strains since they exhibited higher and consistent bio control activity against all the tested fungal pathogens. They were also found to be outperforming than the standard reference culture of KAU and thus these were selected as promising candidates with better stress tolerance and antagonism and therefore used for further studies.

4.5 *In vitro* SCREENING FOR PRODUCTION OF BIOMOLECULES IMPARTING STRESS TOLERANCE

The isolates showing prominent stress tolerance and antagonism *viz*., P2, P4, M4 and M5 were analysed for the production of biomolecules imparting these superior traits *viz*., exopolysaccharide and ACC deaminase for drought tolerance and cellulase and β-1,3 glucanase activity which are the mycolytic enzymes.

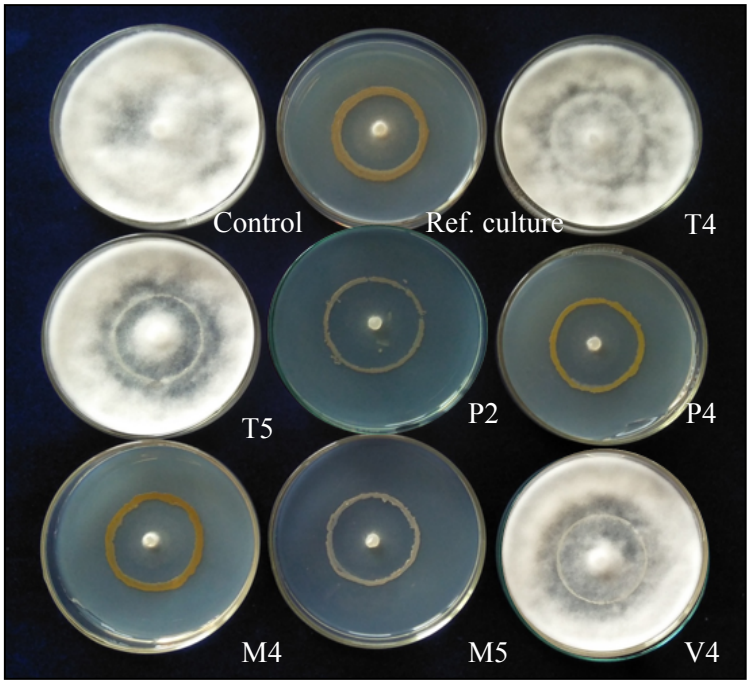
4.5.1 Screening for exopolysaccharide production

The isolates were screened for exopolysaccharide production by inoculating in the specific medium as per the procedure of Triveni (2000). The results showed that the isolates P4 and M4 were better producers of exopolysaccharide by abundant growth on the medium. The reference culture also produced higher exopolysaccharide (Table 4.9 and Plate 4.6).

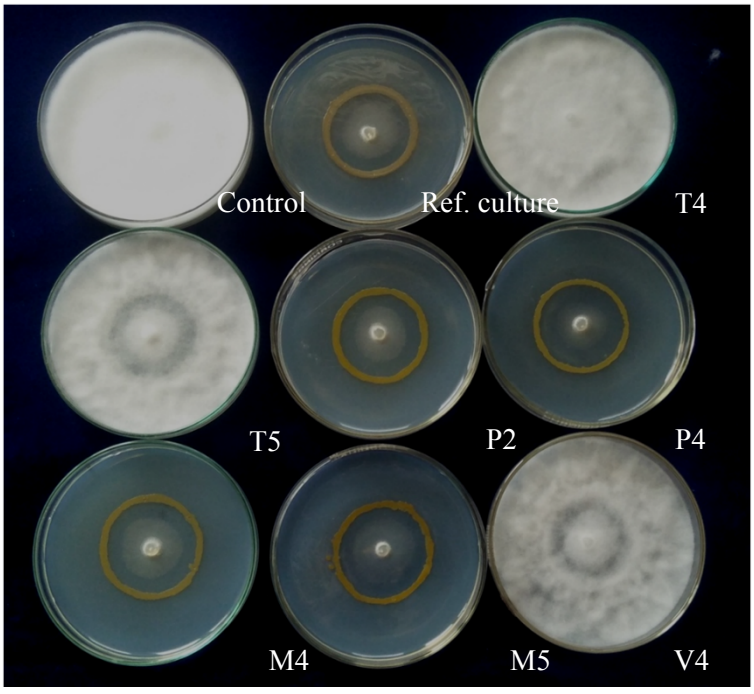
Table 4.8 *In vitro* evaluation of various isolates of *Pseudomonas* spp. against major soil borne fungal pathogens

Sl. No.	Isolates	Per cent inhibition over control				
		Pythium aphanidermatum	***Phytophthora capsici***	***Rhizoctonia solani***	***Sclerotium rolfsii***	***Fusarium oxysporum***
1.	T4	0.00 ^{e}	0.00^{e}	0.00(0.707)^{f}	0.00(0.707)^{g}	0.00(0.707)^{f}
2.	T5	0.00^{e}	0.00^{e}	0.00(0.707)^{f}	21.00(4.637)^{f}	0.00(0.707)^{f}
3.	P2	74.61(1.879)^{a}	73.66(1.870)^{c}	65.54(8.127)^{e}	88.84(9.452)^{b}	72.77(7.919)^{d}
4.	P4	74.43(1.878)^{a}	74.42(1.875)^{b}	71.21(8.469)^{c}	91.00(9.566)^{a}	86.92(9.350)^{a}
5.	M4	72.22(1.865)^{c}	74.42(1.875)^{b}	75.43(8.714)^{a}	85.62(9.280)^{c}	76.63(8.560)^{c}
6.	M5	67.76(1.837)^{d}	75.56(1.878)^{a}	71.10(8.462)^{d}	85.58(9.280)^{c}	62.21(7.919)^{e}
7.	V4	0.00^{e}	0.00^{e}	0.00(0.707)^{f}	51.04(7.179)^{e}	0.00(0.707)^{f}
8.	Ref. culture	72.88(1.877)^{b}	72.00(1.865)^{d}	73.29(1.865)^{b}	78.92(9.278)^{d}	77.82(8.782)^{b}
CD (0.01)		0.350	0.400	0.033	1.026	0.309

*Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT.
Log transformed values are given in parenthesis

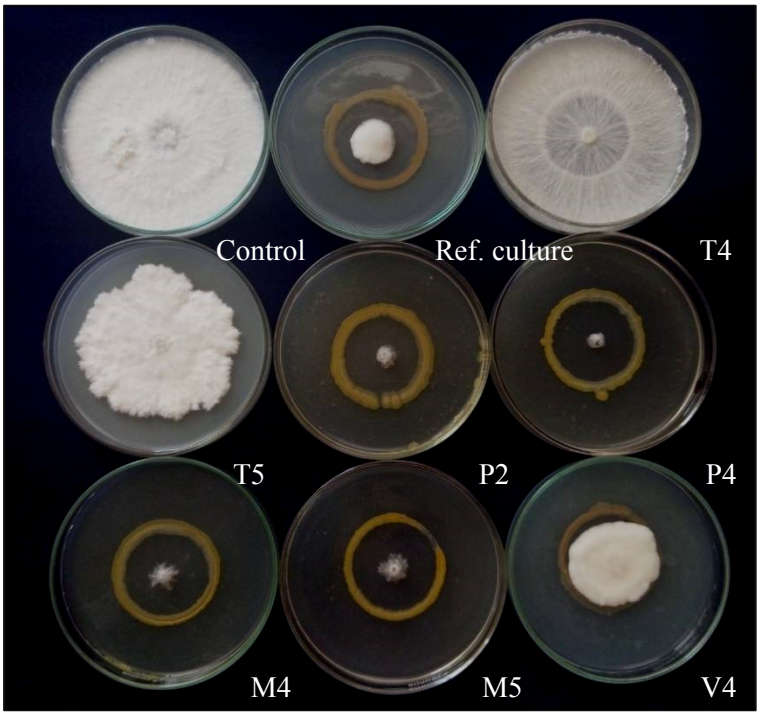


a) *Phytophthora capsici*



b) *Pythium aphanidermatum*

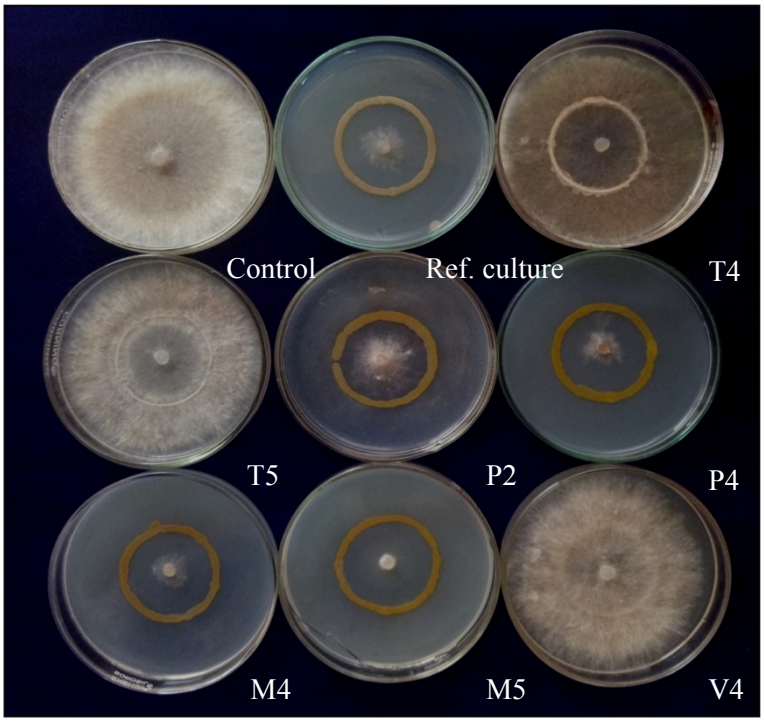
Plate 4.5(i) *In vitro* evaluation of various isolates of *Pseudomonas* spp. against major soil borne fungal pathogens



c) *Sclerotium rolfssi*

d) *Fusarium oxysporum*

Plate 4.5 (ii) *In vitro* evaluation of various isolates of *Pseudomonas* spp. against major soil borne fungal pathogens



e) *Rhizoctonia solani*

Plate 4.5 (iii) *In vitro* evaluation of various isolates of *Pseudomonas* spp. against major soil borne fungal pathogens

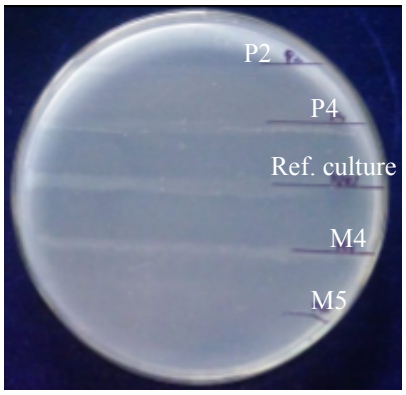


Plate 4.6 Exopolysaccharide production by *Pseudomonas* isolates

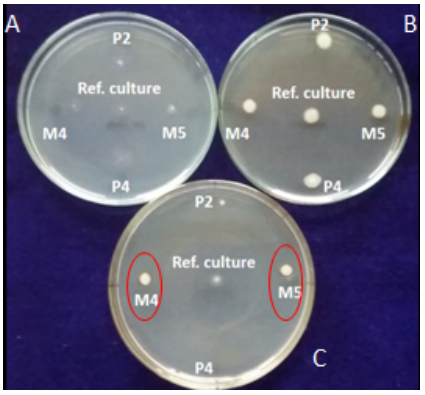


Plate 4.7 ACC deaminase production by *Pseudomonas* isolates
A-Negative control
B- Positive control
C- Treatment

4.5.2 Screening for ACC deaminase activity

The four isolates *viz*., P2, P4, M4 and M5 along with the reference culture were screened for the production of ACC deaminase activity by spotting on the DF salt minimal medium supplemented with ACC as sole nitrogen source. The results of the experiment are depicted in Table 4.10 and Plate 4.7. Apparently, very less growth was observed with all the isolates when grown on DF salts minimal medium without any nitrogen source, whereas, abundant growth with colonies of size 10 mm was observed on DF medium supplemented with ammonium sulphate as the nitrogen source. The isolate M4 and M5 were selected as the isolates with better ACC deaminase activity since these isolates produced comparatively abundant growth of 6 and 5 mm on DF medium with ACC respectively.

4.5.3 Screening for cellulase production

The cellulase activity of the isolates was determined as in 3.5.3 and the results of the experiment are presented in Table 4.11. Generally, the cellulase activity of the isolates ranged between 0.815 $Uml^{-1}$and 0.907 Uml^{-1} with the highest production by the isolate P2 and least by M5. The reference culture of KAU exhibited a cellulase activity of 0.888 Uml^{-1} which was comparable with the other isolates.

4.5.4 Screening for β-1, 3 glucanase production

When the isolates were analyzed for the production of β-1, 3 glucanase activity (Table 4.11), maximum activity was shown by the isolate M4 (1.010 Uml^{-1}) and minimum by P2 (0.81Uml^{-1}). Hence, it is evident from the above mentioned tests that M4 and P4 are better producers of exopolysaccharide

Table 4.9 Screening for exopolysaccharide production

Sl. No.	Isolate	Exopolysaccharide production
1.	**P2**	-
2.	**P4**	+
3.	**M4**	+
4.	**M5**	-
5.	**KAU ref. culture**	+

Table 4.10 Screening of promising *Pseudomonas* isolates for ACC deaminase activity

Medium	Diameter of spot (mm)				
	P2	**P4**	**M4**	**M5**	**KAU ref. culture**
Dworkin Foster salts (DF) medium without nitrogen source	Sparse growth	Sparse growth	Sparse growth	Sparse growth	Sparse growth
DF medium with ammonium sulphate as nitrogen source	10	10	10	10	10
DF medium with ACC as nitrogen source	3	4	6	5	3.2

*Mean of three replications

Table 4.11 Cellulase and β-1,3 – Glucanase production by promising *Pseudomonas* isolates

Sl. No.	Isolate	Cellulase production (Uml^{-1})	β-1,3 – glucanase production (Uml^{-1})
1.	P2	1.01	0.891
2.	P4	0.883	0.980
3.	M4	0.819	1.010
4.	M5	0.815	0.895
5.	Ref. culture	0.888	0.913

*Mean of three replications

whereas, M4 and M5 showed highest ACCD activity. Likewise, cellulase activity was significantly higher with the isolates P2 and P4 and β-1, 3 glucanase activity in the isolates P4 and M4. Thus, all the isolates when screened for the biomolecules imparting stress tolerance, they depicted a higher value, and henceforth, none of the isolates were eliminated after screening for enzyme analysis.

4.6 *In vivo* BIOASSAY FOR TESTING THE EFFICACY OF ISOLATES

The isolates were subjected to *in planta* analysis by modified roll towel method for determining the bioefficacy in terms of protection from disease incidence following the protocol of Srivastava *et al.* (2002). Cowpea seeds when treated with the pathogen, *Rhizoctonia solani* alone, per cent germination was reduced to 50 and moreover, the affected seedlings, showed rotting on roots and shoots (Table 4.12). However, the isolate M4 enhanced the germination percentage to 100 per cent with no symptoms of infection and showed well developed roots and shoots (Plate 4.8). Thus, the isolate M4 was rated as highly efficient bio control agent against the pathogen. The per cent germination exhibited by the reference culture was 87 per cent with 13 per cent disease incidence and hence, the culture was rated as efficient. The other isolates P2, P4 and M5 were rated as moderately efficient as the seeds treated with these isolates showed a germination of 80 per cent. Root and shoot growth was less pronounced compared to the seeds treated with M4 and moreover, the roots resulted in 26 per cent disease incidence.

Table 4.12 Bioefficacy of *Pseudomonas* isolates in terms of per cent germination and disease incidence

Sl. No.	Isolate	Germination (%)	Per cent disease incidence	Rating
1.	P2	80	26	Moderately efficient
2.	P4	80	26	Moderately efficient
3.	M4	100	0	Highly efficient
4.	M5	80	26	Moderately efficient
5.	Ref. culture	87	13	Efficient

Control

Reference culture

M4

M5

P2

P4

Plate 4.8 *In vivo* screening forbioefficacy of isolates by roll towel method

4.7 *In vivo* EVALUATION OF STRESS TOLERANT *Pseudomonas* spp. FOR DISEASE SUPPRESSION AND PLANT GROWTH PROMOTION

Promising strains of *Pseudomonas* spp. *viz*., P2, P4, M4 and M5 showing better stress tolerance and antagonistic activity under *in vivo* conditions were further evaluated for their *in vivo* bio control activity through a pot culture experiment. Cowpea- *Rhizoctonia solani* system was used as the model for the *invivo* experiment. The isolates were compared with a positive control (reference culture of *Pseudomonas fluorescens* of KAU) and a negative control, without the application of *Pseudomonas* spp. The pot culture experiment was carried out during May 2019 at College of Horticulture, Vellanikkara. Observations such as germination percentage and biometric characters like plant growth enhancement and per cent disease incidence were recorded at appropriate intervals (Plate 4.9).

4.7.1 Germination percentage

In the present pot culture experiment, cowpea seeds (var. Bhagyalakshmi) were bacterized with four selected isolates of *Pseudomonas* spp. and KAU reference culture of *Pseudomonas fluorescens* (positive control) and one set of seeds without any seed treatment (negative control) were sown in pots already inoculated with *Rhizoctonia solani*. Highest germination percentage was observed in T3 in which seeds were treated with *Pseudomonas* isolate M4 (96.29 %) (Table 4.13). It was followed by the isolate M5 which enhanced seed germination to 92.59 per cent. The lowest per cent of seed germination was observed in control (T6). When the seeds were treated with P2 and P4, germination percentage of 89.18 and 88.88 were recorded. From the data, it is inferred that M4 and M5 are the best isolates in enhancing seed germination.


a) View of the pot culture experiment



b) Challenge inoculation with *Rhizoctonia solani* **c) Symptom appearance**

Plate 4.9 *In vivo* evaluation of promising isolates of *Pseudomonas* spp. for disease suppression and plant growth promotion

Table 4.13 Effect of treatments on germination percentage of seeds

Sl. No.	Treatment	*Pseudomonas* isolate	Seed germination percentage (%)
1.	T1	P2	85.18
2.	T2	P4	88.88
3.	T3	M4	96.29
4.	T4	M5	92.59
5.	T5	Ref. culture	88.88
6.	T6	Control	66.66

Table 4.14 Effect of treatments on plant height at one WAS, 30, 30-45, 45-60 DAS

Sl. No.	Treatment	*Pseudomonas* isolate	Plant height (cm)		Relative per cent increase in height	
			One WAS*	30 DAS*	30-45 DAS*	45- 60 DAS*
1.	T1	P2	22.00	154.36^{b}	12.54^{d}	11.59^{c}
2.	T2	P4	88.88	154.75^{a}	13.88^{b}	12.08^{b}
3.	T3	M4	96.29	152.34^{c}	14.58^{a}	14.01^{a}
4.	T4	M5	92.59	155.67^{d}	13.56^{c}	11.56^{c}
5.	T5	Ref. culture	88.88	152.66^{d}	9.65^{e}	10.12^{d}
6.	T6	Control	66.66	145.68^{e}	7.89^{f}	6.56^{e}
CD(0.01)			NS	0.033	0.037	0.149

WAS- week after sowing, *DAS- days after sowing
*Mean of nine replications
In each column figures followed by same letter do not differ significantly according to DMRT

Biometric observation such as plant height, number of leaves, number of flowers, days to flowering, number of pods and yield were recorded.

4.7.2a Plant height

Height of cowpea plants were recorded at one week after sowing (WAS), 30 days after sowing (DAS), 45 DAS and 60 DAS and the data obtained is tabulated in Table 4.14. It was observed that there was no significant difference in plant height among the treatments at 1 WAS whereas, it differed significantly at and 30, 45 and 60 DAS. At 30 DAS, treatments 1 and 2 (isolates P2 and P4) recorded maximum height of 154.75 and 154.36 cm.

There was a significant difference in relative increase in plant height at 30- 45 DAS and 45- 60 DAS. At 30- 45 DAS, maximum height increase of 14.58 per cent was observed with P2 followed by P4 (13.88 %), whereas, minimum per cent increase was noted with control (7.89 %). Within the time interval of 45-60 DAS, treatment M4 recorded highest per cent increase of 14.01 followed by 12.08 (P4). Plants in control recorded lowest per cent increase as in the case of other time intervals.

4.7.2b Number of leaves

Observations on number of leaves were recorded at one WAS, 30, 45 and 60 DAS and the data is furnished in Table 4.15. No significant difference was observed among the treatments at one WAS and 30 DAS whereas, data differed significantly within the time interval of 45 and 60 DAS.

At 45 DAS, the plants treated with the isolate P4 recorded maximum

number of 25.55 leaves followed by P2 and M5 (23.89 and 23.44). Lowest number of leaves (19.0) was observed with control. In general, number of leaves produced by the plants ranged from 26.89 to 34.55 at 60 DAS with maximum number of leaves in M4 followed by P4 and M5 (34.55, 33.78 and 33.89). Minimum number of leaves of 26.89 was noticed with control.

4.7.2c Days to first flowering

Number of days taken for first flowering ranged from 40.33 to 44.67 (Table 4.16). It was noticed that the plants treated with M4 and M5 flowered earlier (40.33 and 40.78 DAS), whereas flowering was delayed upto 44.67 DAS in control.

4.7.2d Yield

Yield obtained after all pickings were observed and compared among the treatments and the data is recorded in Table 4.17 Generally, yield of cowpea plants ranged from 61.85 to 87.95 g with maximum yield with M4 whereas, minimum with control. Treatments with isolates, M5, P2, P4 and reference culture recorded a yield of 83.32, 79.24, 75.77 and 73.65g respectively.

After the pot culture experiment, observations on biometric parameters revealed that M4 was the best plant growth promoting *Pseudomonas* spp.

4.7.3 Per cent disease incidence

After 30 DAS, the plants were drenched with the respective isolates followed by challenge inoculation with *Rhizoctonia solani* at 45 DAS. Symptoms of water soaking and rotting were observed on the collar region within one week after challenge inoculation.

Table 4.15 Effect of treatments on relative per cent increase in number of leaves formed at one WAS, 30, 30-45, 45-60 DAS

Sl. No.	Treatments	*Pseudomonas* isolate	*Relative per cent increase in number of leaves formed			
			One WAS	30 DAS	30-45 DAS	45- 60 DAS
1.	T1	P2	4.44	12.00	23.89^{b}	30.67^{d}
2.	T2	P4	4.00	12.11	25.55^{a}	33.78^{b}
3.	T3	M4	4.00	11.78	22.00^{d}	34.55^{a}
4.	T4	M5	4.67	11.89	23.44^{bc}	33.89^{bc}
5.	T5	Ref. culture	4.00	12.00	23.00^{c}	32.11^{c}
6.	T6	Control	4.00	12.11	19.00^{e}	26.89^{e}
CD (0.01)					0.584	1.01

WAS- week after sowing, DAS- days after sowing
*Mean of nine replications
In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.16 Effect of treatments on days to first flowering

Sl. No.	Treatment	*Pseudomonas* isolate	*Days to first flowering
1.	T1	P2	42.11^{b}
2.	T2	P4	41.22^{c}
3.	T3	M4	40.33^{d}
4.	T4	M5	40.78^{cd}
5.	T5	Ref. culture	41.33^{c}
6.	T6	Control	44.67^{a}
CD (0.01)			0.642

*Mean of nine replications
In each column figures followed by same letter do not differ significantly according to DMRT

It is clear from the data (Table 4.18) that M4 showed the least disease incidence of 11.11 per cent followed by M5 (22.22%) whereas, the plants in control showed cent per cent disease incidence. In addition, P2, P4 and reference culture displayed per cent disease incidence of 33.33. Hence, it was concluded that M4 and M5 are the promising isolates which can reduce the disease incidence.

4.8 CULTURAL, MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF PROMISING *Pseudomonas* ISOLATES

The cultural, morphological and biochemical characters of promising stress tolerant isolates were studied along with the reference culture of *P. fluorescens* of KAU and the results are furnished in Table 4.19-4.20 and Plate 4.10.

Gram's reaction revealed that all the four isolates along with the reference culture were Gram negative short rods when observed under the compound microscope. Isolation of the bacteria on King's B agar medium yielded circular, small, round, opaque yellow green coloured colonies with round and entire margin after 24 h of incubation. The colonies had convex, smooth and shiny surfaces. The fluorescent yellow- green coloured pigmentation later diffused into the medium. All the isolates showed positive reaction for catalase and oxidase tests and utilized citrate as carbon source. None of the isolates produced urease, H_2S and indole. Similarly, all the isolates were negative for MR- VP test also. Gelatin hydrolysis was observed with the isolates M4 and M5. Of the four carbon compounds (glucose, mannitol, lactose and sucrose) tested, P2 and P4 utilized mannitol whereas, other isolates used none of the above mentioned sugars. All the four isolates including the reference culture were tested for sensitivity to the four antibiotics *viz*., ampicillin, tetracycline, gentamycin and

Table 4.17 Effect of treatments on yield

Sl. No.	Treatment	*Pseudomonas* isolate	Yield/ plant (g)
1.	T1	P2	79.24[c]
2.	T2	P4	75.77[d]
3.	T3	M4	87.95[a]
4.	T4	M5	83.32[b]
5.	T5	Ref. culture	73.65[e]
6.	T6	Control	61.85[f]
CD (0.01)			1.913

*Mean of nine replications
In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.18 Effect of treatments on days to per cent disease incidence

Sl. No.	Treatment	*Pseudomonas* isolate	Per cent disease incidence
1.	T1	P2	79.24[c]
2.	T2	P4	75.77[d]
3.	T3	M4	87.95[a]
4.	T4	M5	83.32[b]
5.	T5	Ref. culture	73.65[e]
6.	T6	Control	61.85[f]
CD (0.01)			1.913

*Mean of nine replications
In each column figures followed by same letter do not differ significantly according to DMRT

chloramphenicol which are usually used against *Pseudomonas* spp., and the diameter of inhibition zone formed was measured. The results are tabulated in Table 4.19 and were compared with the standard antibiotic sensitivity chart presented in Table 4.20, as to whether the isolates were sensitive or intermediate or resistant to the corresponding antibiotics. Antibiotic sensitivity test revealed that all the isolates including KAU reference culture were resistant to the antibiotic ampicillin (10 µg) as no inhibition zone was observed.

P2 and P4 showed similar response to all the tested antibiotics *viz*., resistance to gentamycin and ampicillin and susceptibility to tetracycline and chloramphenicol. The isolates M4 and the reference culture were found to be resistant to all the antibiotics whereas M5 was susceptible to only one of the tested antibiotic *i.e*., chloramphenicol. The antibiotic sensitivity test could be used as a marker for selection of *Pseudomonas* isolate from soil.

Based on the above mentioned cultural, morphological and functional characters, the identity of all the four isolates viz., P2, P4, M4 and M5 were confirmedupto genus *i.e Pseudomonas*. For further identification at species level, the isolates were sent to Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram for sequencing and molecular identification.

4.8 MOLECULAR CHARACTERIZATION OFPROMISING *Pseudomonas* ISOLATES

All the four isolates *viz*., M4, M5, P2 and P4 showing high abiotic stress tolerance like drought, temperature, acidity and salinity as well as better

Table 4.19 Cultural and morphological characters of *Pseudomonas* isolates

Sl. No.	Isolate	Shape	Size	Margin	Pigmentation	Elevation	Surface	Opacity	Gram's reaction
1.	M4	Round	Small	Entire, round	Fluorescent green	Convex	Smooth, shiny	Opaque	Negative, rod
2.	M5	Round	Small	Entire, round	Fluorescent green	Convex	Smooth, shiny	Opaque	Negative, rod
3.	P2	Round	Small	Entire, round	Fluorescent green	Convex	Smooth, shiny	Opaque	Negative, rod
4.	P4	Round	Small	Entire, round	Fluorescent green	Convex	Smooth, shiny	Opaque	Negative, rod
5.	Ref. culture	Round	Small	Entire, round	Fluorescent green	Convex	Smooth, shiny	Opaque	Negative, rod

Table 4.20 Biochemical characters of *Pseudomonas* isolates

Sl. No	Tests	Isolates of *Pseudomonas* spp.				
		P_2	P_4	M_4	M_5	Ref. culture
1.	Catalase	+	+	+	+	+
2.	Oxidase	+	+	+	+	+
3.	Urease	–	–	–	–	–
4.	Gelatin hydrolysis	–	–	+	+	–
5.	Citrate utilization	+	+	+	+	+
6.	H_2S production	–	–	–	–	–
7.	Indole production	–	–	–	–	–
8.	Glucose fermentation	–	–	–	–	–
9.	Lactose fermentation	–	–	–	–	–
10.	Sucrose fermentation	–	–	–	–	–
11.	Mannitol fermentation	+ (gas)	+ (gas)	–	–	–
12.	MR VP test	–	–	–	–	–

Table 4.21 Standard antibiotic sensitivity chart

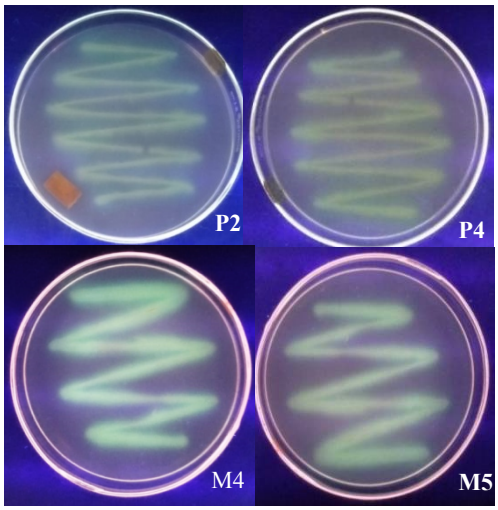
Antibiotic	Dose (µg)	Zone diameter (mm)		
		S	I	R
Ampicillin	10	≥17	14-16	≤13
Chloramphenicol	30	≥16	11-15	≤10
Tetracycline	30	≥19	15-18	≤14
Gentamycin	10	≥15	13-14	≤12

*s- sensitive, I- intermediate and R- resistant

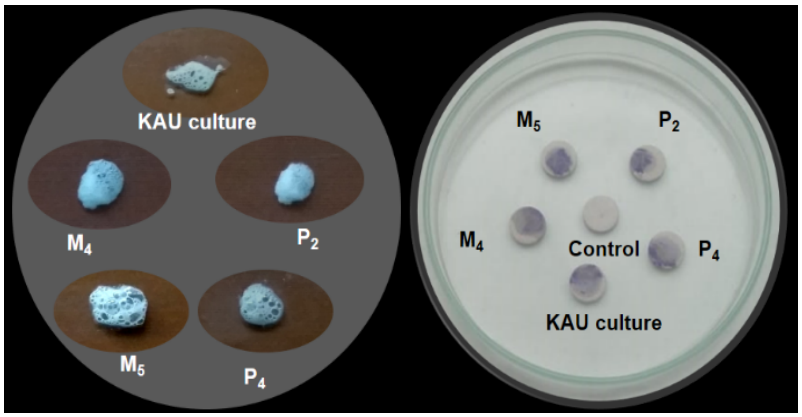
Table 4.22 Sensitivity of *Pseudomonas* isolates to antibiotics

Sl. No.	Antibiotic	P2	P4	M4	M5	Ref. culture
1.	Ampicillin	_	_	_	_	_
2.	Tetracycline	22 (S)	22 (S)	13 (R)	1.2 (R)	12 (R)
3.	Gentamycin	12 (R)	12 (R)	12 (R)	12.0 (R)	11 (R)
4.	Chloramphenicol	20 (S)	12 (I)	10 (R)	19 (S)	10 (R)

*s- sensitive, I- intermediate and R- resistant

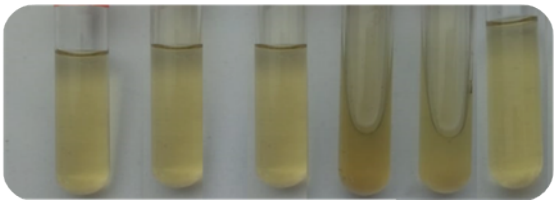


Fluorescence under UV light



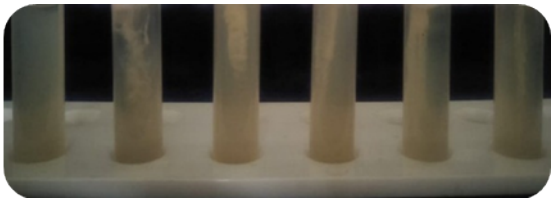
Catalase test

Oxidase test



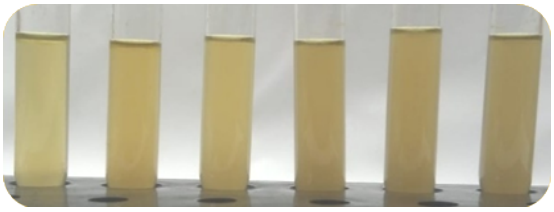
Control P2 P4 M4 M5 Ref. culture

Gelatin liquefaction test



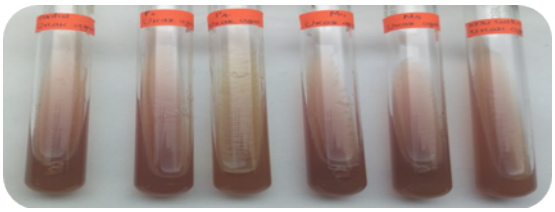
Control P2 P4 M4 M5 Ref. culture

H_2S test



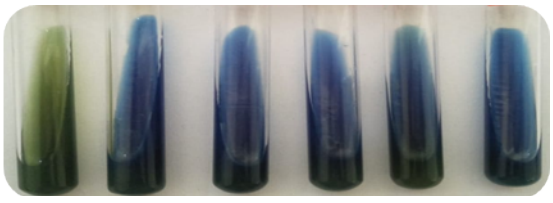
Control P2 P4 M4 M5 Ref. culture

Indole test



Control P2 P4 M4 M5 Ref. culture

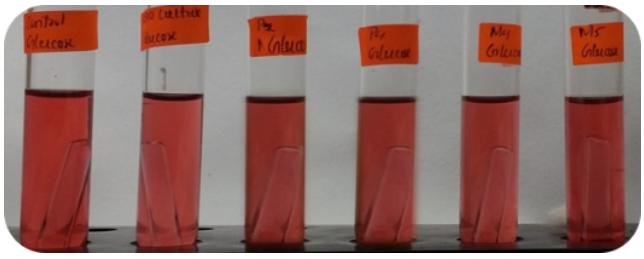
Urea hydrolysis test



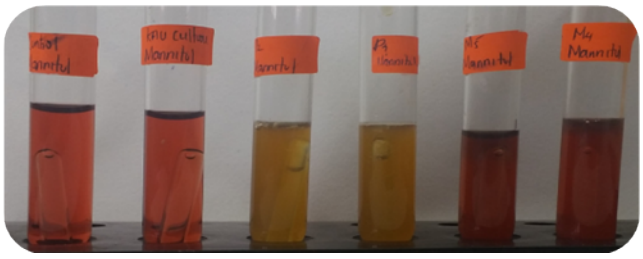
Control P2 P4 M4 M5 Ref. culture

Citrate utilization test

Plate 4.10 (i) Biochemical characters of *Pseudomonas* isolates

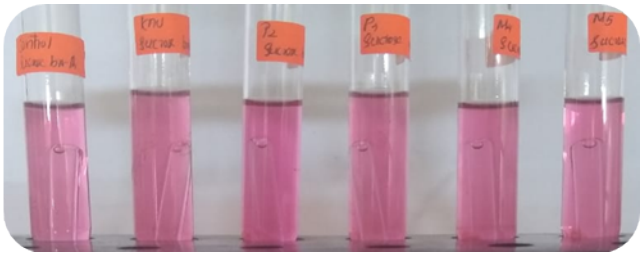


Control P2 P4 M4 M5 Ref. culture

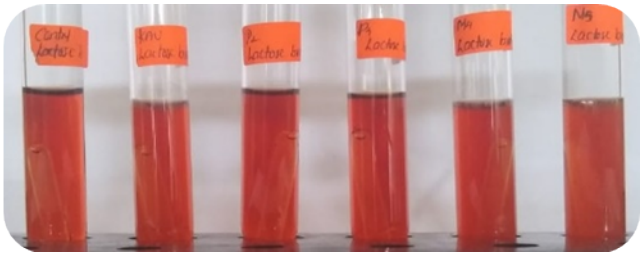


Control P2 P4 M4 M5 Ref. culture

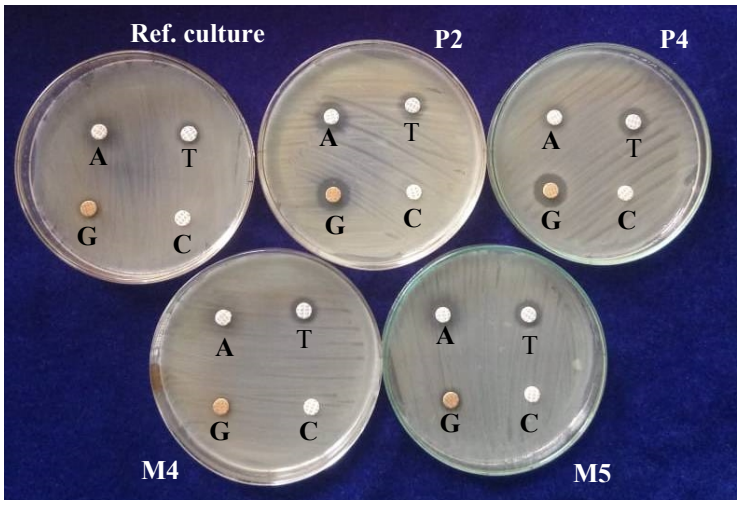
Sugar utilization test
A- Glucose B- Mannitol C- Sucrose D- Lactose



Control P2 P4 M4 M5 Ref. culture



Control P2 P4 M4 M5 Ref. culture



Antibiotic sensitivity test of isolates
C- Chloramphenicol T- Tetracycline G- Gentamycin A- Ampicillin

Plate 4.10 (ii) Biochemical characters of *Pseudomonas* isolates

exopolysaccharide, ACC deaminase and mycolytic enzyme activity along with their antagonistic potential towards major soil borne fungal pathogens were selected and finally subjected to molecular characterization. Molecular characterization was outsourced at RGCB, Thiruvananthapuram to identify the isolates upto species level. The isolated total genomic DNA was amplified using a thermocycler using 16s rRNA gene specific primers. The PCR products when run on 1.2 per cent agarose gel, showed an amplicon size of 1500 bp (Plate 4.11). Upon sequencing, the isolates P2, P4, M4 and M5 obtained nucleotides of 1240, 1244, 1259 and 606 bp (Table 4.23). These sequences were blasted in the online BLASTn programme of NCBI and thereafter, analyzed to find the homology of the isolate (Plate 4.12). The details of the results of sequence comparison of four isolates of fluorescent pseudomonads are given in Table 4.24 to 4.27.

4.81 Sequence comparison of *Pseudomonas* isolate P2 in BLASTn Analysis

Nucleotide sequence of 16s r RNA of the isolate P2 (Table 4.24) was compared with the database retrieved from NCBI. The results revealed that it showed homology to four strains of *Pseudomonas viz*., *Pseudomonas putida* B6-2 (Accession CP015202.1), *Pseudomonas putida* strain PC-2 (Accession CP011789.1),), *Pseudomonas putida* strain MN3078 (Accession CP006978.1), *Pseudomonas monteilii* SB3101 (Accession CP006979.1) and *Pseudomonas monteilii* SB3078 (Accession CP006978.1) with 100 per cent query coverage and 99.68 per cent identity.

Table 4.23 Genomic sequences of *Pseudomonas* isolates

***Pseudomonas* isolate**	**Sequence**
P2	TCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTC GAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTG CGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCG ACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGA CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCAT GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG TAAGCTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGC CAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCC AAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATG CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGA TGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGAC CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGC AGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGC TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTC CTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGG AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACA ATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGT AGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAA
P4	GCGGCGGACGAGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGA AACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACG CTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGAC GATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACT CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGC CGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTA AGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCA GCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGC GTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAA AACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCG TAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAG GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATG TCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCG CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCG GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTG AGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTG TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCT TAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATG GTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGT CCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCT

Pseudomonas isolate	Sequence
M4	GCGGCGGACGAGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGA AACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACG CTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGAC GATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACT CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGC CGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTA AGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCA GCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGC GTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAA AACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCG TAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAG GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATG TCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCG CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCG GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTG AGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTG TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCT TAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATG GTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGT CCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCT
M5	AATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGGATGTGAAAGCCCCGGG CTCAACTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATT TCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCAC CTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT GGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCG CAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA CCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACT CTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC GTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTCATGGTGGGCACTCTAAGGAGA CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGC CTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGC TAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCG GA

4.24 Sequence homology of *Pseudomonas* isolate P2 in BLASTn analysis

Sl. No.	Description	Maximum score	Query coverage	E value	Identity	Accession
1.	*Pseudomonas putida* B6-2	2305	100	0.0	99.68	CP015202.1
2.	*Pseudomonas putida* strain PC-2	2305	100	0.0	99.68	CP011789.1
3.	*Pseudomonas putida* strain MN3078	2305	100	0.0	99.68	CP006978.1
4.	*Pseudomonas monteilii* SB3101	2305	100	0.0	99.68	CP006979.1
5.	*Pseudomonas monteilii* SB3078	2305	100	0.0	99.68	CP006976.1

Table 4.25 Sequence homology of *Pseudomonas* isolate P4 in BLASTn analysis

Sl. No.	Description	Maximum score	Query coverage	E value	Identity	Accession
1.	*Pseudomonas fluorescens* Psf16	2320	100	0.0	99.92	MN256402.1
2.	*Pseudomonas fluorescens* Psf9	2320	100	0.0	99.92	MN56395.1
3.	*Pseudomonas fluorescens* Psf8	2320	100	0.0	99.92	MN56394.1
4.	*Pseudomonas fluorescens* Psf7	2320	100	0.0	99.92	MN56393.1
5.	*Pseudomonas fluorescens* Psf6	2320	100	0.0	99.92	MN56392.1
6.	*Pseudomonas fluorescens* Psf5	2320	100	0.0	99.92	MN56391.1

Table 4.26 Sequence homology of *Pseudomonas* isolate M4 in BLASTn analysis

Sl. No.	Description	Maximum score	Query coverage	E value	Identity	Accession
1.	*Pseudomonas aeruginosa* BAMCPA07.48	1548	93	0.0	94.08	CP015377.1
2.	*Pseudomonas aeruginosa* T52373	1548	93	0.0	94.08	CP008867.1
3.	*Pseudomonas aeruginosa* 12-4-4(59)	1548	93	0.0	94.08	CP013696.1
4.	*Pseudomonas aeruginosa* VA134	1548	93	0.0	94.08	CP013245.1
5.	*Pseudomonas aeruginosa* TL864	1548	93	0.0	94.08	CP013221.1
6.	*Pseudomonas aeruginosa* LM134	1548	93	0.0	94.08	CP013825.1

Table 4.27 Sequence homology of *Pseudomonas* isolate M5 in BLASTn analysis

Sl. No.	Description	Maximum score	Query coverage	E value	Identity	Accession
1.	*Pseudomonas aeruginosa* strain LES	91.38	100	0.0	99.84	CP012001.1
2.	*Pseudomonas aeruginosa* strain LES	91.43	100	0.0	99.84	CP011369.1
3.	*Pseudomonas aeruginosa* LES1365	91.49	100	0.0	99.84	CP006985.1
4.	*Pseudomonas aeruginosa* LES400	91.49	100	0.0	99.84	CP006964.1
5.	*Pseudomonas aeruginosa* TX152a	91.49	100	0.0	99.84	MK616102.1
6.	*Pseudomonas aeruginosa* AS580	91.49	100	0.0	99.84	PA616103.1

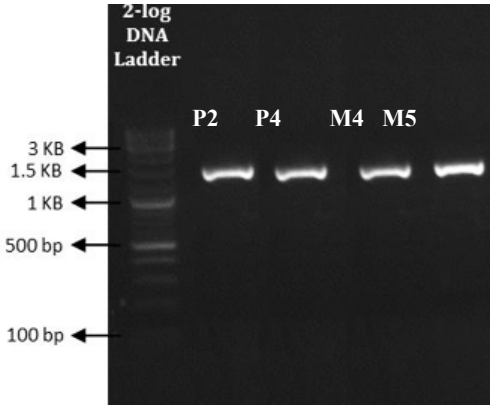
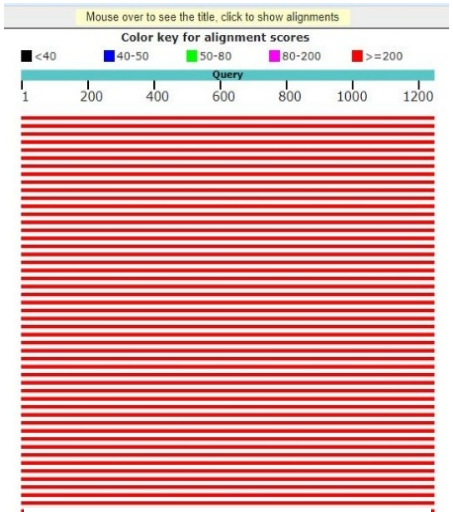
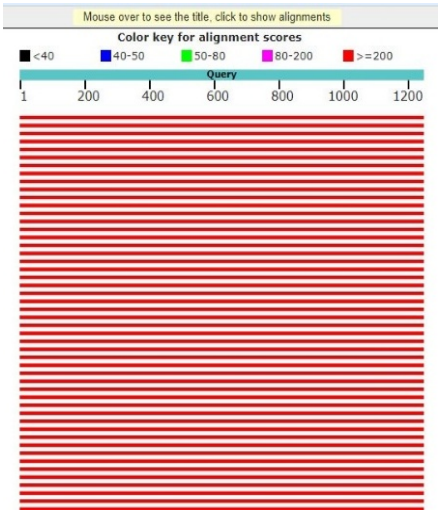


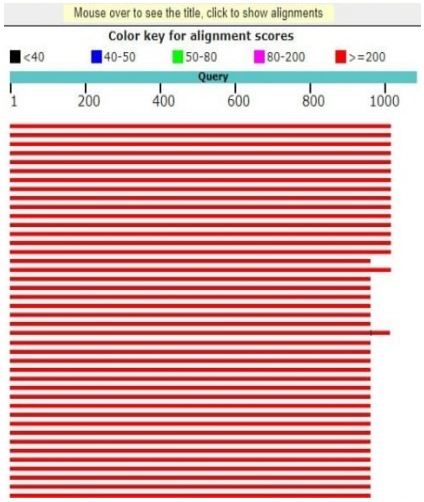
Plate 4.11 Gel image of PCR products of isolates



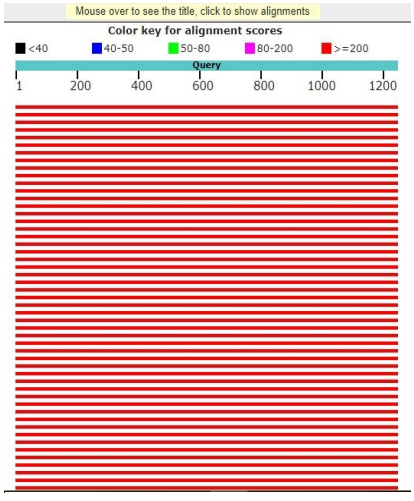
Sequence homology of *Pseudomonas* isolate P2



Sequence homology of *Pseudomonas* isolate P4



Sequence homology of *Pseudomonas* isolate M4



Sequence homology of *Pseudomonas* isolate M5

Plate 4.12 Sequence homology of isolates

4.82 Sequence comparison of *Pseudomonas* isolate P4 in BLASTn Analysis

Comparison of nucleotide sequence of the isolate P4 (Table 4.25) with other sequences in NCBI showed that the isolate is closely identical to *Pseudomonas fluorescens* Psf16 (Accession MN256402.1), *Pseudomonas fluorescens* strain Psf9 (Accession MN56395.1), *Pseudomonas fluorescens* Psf8 (Accession MN56394.1), *Pseudomonas fluorescens* Psf7 (Accession MN56393.1) and *Pseudomonas fluorescens* Psf6 (Accession MN56392.1) with 100 per cent query coverage and 99.92 per cent identity.

4.83 Sequence comparison of *Pseudomonas* isolate M4 in BLASTn Analysis

Sequence of the isolate M4 (Table 4.26) was compared with other sequences in NCBI database and the results evidenced that the isolate was homologous to *Pseudomonas aeruginosa* BAMCPA07.48 (Accession CP015377.1), *Pseudomonas aeruginosa* T52373 (Accession CP008867.1), *Pseudomonas aeruginosa* 12-4-4(59) (Accession CP013696.1), *Pseudomonas aeruginosa* VA134 (Accession CP013245.1), *Pseudomonas aeruginosa* TL864 (Accession CP013221.1) and *Pseudomonas aeruginosa* LM134 (Accession CP013825.1) with 93 per cent query coverage and 94.08 per cent identity.

4.84 Sequence comparison of *Pseudomonas* isolate M5 in BLASTn Analysis

When the nucleotide sequence of 16s r RNA of the isolate M5was compared with sequences deposited in NCBI database, 100 per cent query coverage and 99.84 per cent identity was obtained with four isolates of

Pseudomonas aeruginosa viz., Pseudomonas aeruginosa strain LES (Accession CP012001.1), *Pseudomonas aeruginosa* strain LES (Accession CP011369.1), *Pseudomonas aeruginosa* LES1365 (Accession CP006985.1) and *Pseudomonas aeruginosa* LES400 (Accession CP006964.1), *Pseudomonas aeruginosa* TX152a (MK61602.1) and *Pseudomonas aeruginosa* AS5804 (PA616103.1). The results of the sequence analysis are presented in Table 4.27.

After molecular characterization, the most promising stress tolerant and antagonistic isolates P2 and P4 were identified as *Pseudomonas putida* and *Pseudomonas fluorescens* respectively. The other isolates, M4 and M5 were also identified as *Pseudomonas aeruginosa* as they showed sequence homology towards these species.

DISCUSSION

5. DISCUSSION

A wide range of agriculturally important microorganisms have been exploited for crop health management. Among them, pseudomonads, a group of root associated antagonistic bacteria has gained much attention for its immense biocontrol and plant growth promoting potential. The antagonistic properties of *Pseudomonas* spp. could be endorsed with the production of antibiotics (phenazine-1-carboxylic acid, pyocyanin, pyrrolnitrin, and pyoluteorin and 2,4-diacetylphloroglucinol), siderophores, volatiles (HCN and ammonia) (Thomashow *et al*., 1990). Asthe crops are affected by abiotic stresses such as drought, high temperature, soil salinity, acidity, microbes are also known to be affected by these conditions. Likewise, fluorescent pseudomonads though have established as efficient biocontrol agents, its efficacy is observed to be declining under such adverse climatic conditions. The successful deployment of these organisms in stressed ecosystems depends on their ability to withstand and proliferate under adverse environments such as high temperature, salt stress, mineral deficiency, heavy metal toxicity and soforth. Such problems can be overcomed by sound screening program for efficient stress tolerant pseudomonads for effective deployment of these strains to draw one or more beneficial effects. Hence, the present investigation was proposed to isolate and identify native strains of pseudomonads, collected from different stressed ecosystems, for their ability to withstand adverse environments such as high temperature, salinity, drought and acidity along with antagonistic activity.

5.1 SURVEY, COLLECTION AND ESTIMATION OF ELECTRO CHEMICAL PROPERTIES OF STRESSED ECOSYSTEMS OF KERALA

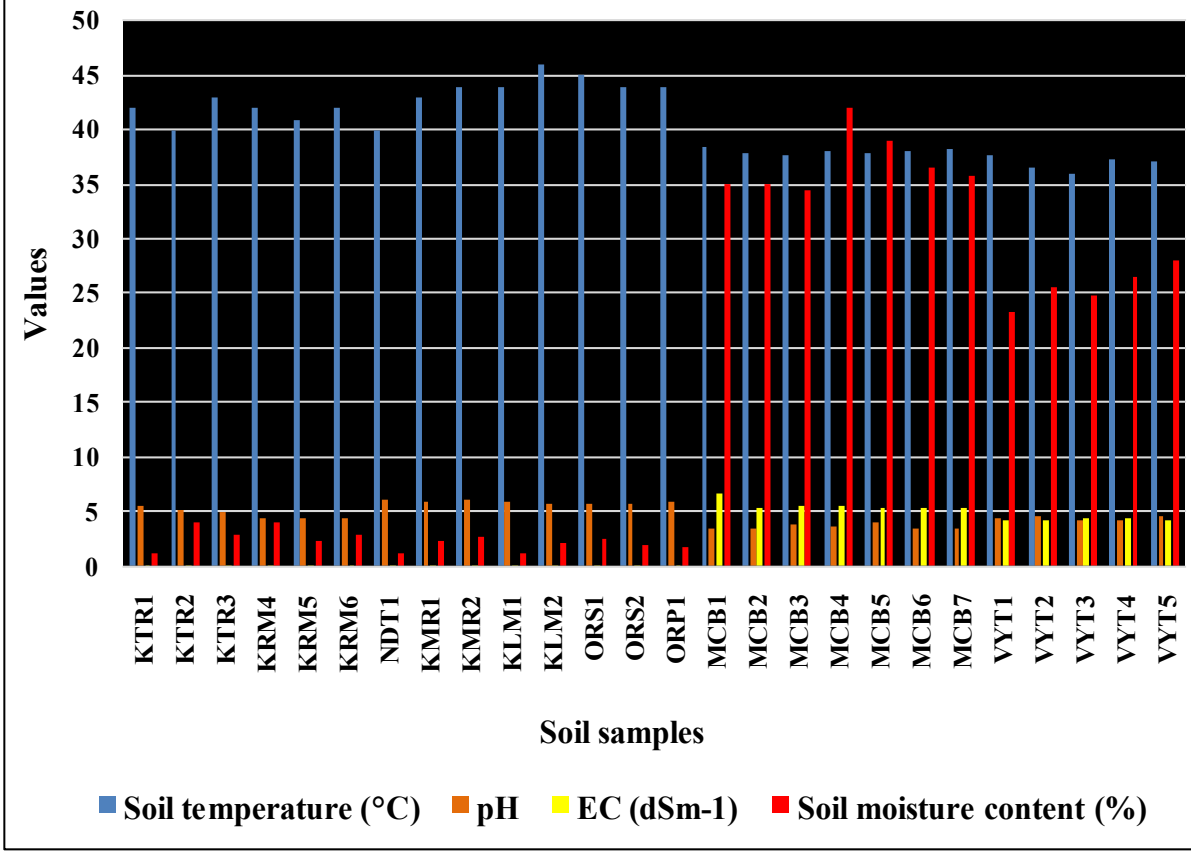
The research work was initiated with intensive soil sampling survey where, for all soil samples, various geographical and environmental characters

like agroecological region, climate, soil type, soil temperature and physical characteristics like pH and EC were determined following standard protocols. Accordingly, samples were collected from various stressed locations of Kerala *viz*., Palakkad, Thrissur, Alappuzha and Ernakulam representing drought, high temperature, acidity and salinity. The samples procured from Thrissur district were assigned with the code NDT, KRM and KRT whereas, samples from Palakkad district were designated with the code KMR, KLM, ORS and ORP. Similarly, codes MCB and VYT were given for samples from Alappuzha and Ernakulam district. Altogether, total of 26 representative soil samples were collected which were subsequently subjected to physico chemical analysis.

5.1.2 Soil temperature and texture

While collecting soil samples from different stressed ecosystems, soil temperature and texture was recorded at the time of sampling itself. In general, it was observed that, soil temperature ranged from 31- 46°C at various locations. The highest soil temperature was recorded from Kallampatti region of Palakkad whereas lowest temperature was observed from Vyttila of Ernakulam district. Soils of Thrissur district were also recorded with higher temperature ranging from 40- 43°C. Thus, from the data it is clear that from among the various locations, Palakkad and Thrissur district had experienced maximum temperature stress at the time of soil sampling. It was also observed that all the soil samples from Thrissur and Vyttila were clayey in texture, whereas samples from Alappuzha and Palakkad were observed to be sandy clay and sandy clay loam texture respectively. The results are in line with the findings of Doncy (2018) where she reported that the soil samples procured from Ernakulam and Thrissur had clayey texture whereas, samples from Palakkad and Alappuzha district were observed with sandy clay loam and sandy textures respectively.

Fig 5.1 Variation of pH, EC, temperature and moisture content of soil samples collected from different locations of Kerala



5.1.3 Soil reaction

When soil samples were analyzed for pH, it was observed that all the collected samples were acidic which varied from 3.4-6.0 rendering them moderately to ultra-acidic. In general, soil samples from Moncombu and Vyttila were observed to be extremely acidic with a pH value ranged from 3.4-3.9 and 4.2- 4.5 respectively. Likewise, analysis of soil samples collected from Thrissur and Palakkad district revealed that the samples were moderately acidic with a pH ranging from 4.4- 5.4 and 5.6-6.0 respectively.

The results are in conformity with Doncy (2018) where she reported that pH of soil samples from Alappuzha and Vyttila ranged from 2.8-3.5 and 4.1-4.3 respectively. She also reported that the pH of Kole lands of Thrissur varied from 4.3 to 5.4 and those of Palakkad soils from 5.2-6.2. Similar reports were also putforth by Bastin *et al*. (2014) where they reported that the soils of Palakkad, Alappuzha and Thrissur districts showed a pH ranging from 5.58 to 8.58, 3.91 to 7.5 and 3.5 to 7.4 respectively. The present findings are also in line with the report of Mohan and Sreelatha (2016) where they noticed a pH range of 3.9 to 4.26 at Vytilla. The results are also in congruence with the reports of Koruth *et al*. (2016) and Sujatha (2015).

5.1.4. Electrical conductivity and soil moisture content

Electrical conductivity of soil samples collected from Moncombu and Vyttila revealed that the soils were saline with an EC ranging between 5.22-6.68 and 4.1-4.3 dSm^{-1} respectively. However, the soil samples collected from Palakkad and Thrissur were found to be non saline since, EC ranged from 0.01 dSm^{-1} to 0.02 dSm^{-1}. Similarly, samples from Palakkad and Thrissur district displayed very low moisture content and in general, it ranged between 1.1 to 3.9 per cent indicating these areas as drought affected regions. Whereas, the soil samples collected from Moncombu region showed soil moisture content in the range of 35.3- 42.0 per cent which is followed by samples from Vyttila with a

moisture content of 23.3-28.1 per cent. The results of the present study were supported by Doncy (2018) as she also observed higher EC value in soil samples collected from Moncombu (3.00 to 7.61 dSm^{-1}) and Vyttila (2.05-3.73 dSm^{-1}) and the samples from Palakkad and Thrissur were found to be non saline with EC of 0.009- 0.24 dSm^{-1} and 0.088- 0.562 dSm^{-1} respectively. Similar findings were also documented by Bastin *et al.* (2014) where they noticed 0.03- 0.85 dSm^{-1} and 0.16 dSm^{-1} of electrical conductivity of soils from Palakkad and Alappuzha respectively.

5.2 ISOLATION AND ENUMERATION OF *Pseudomonas* spp.

A total of 15 isolates of *Pseudomonas* spp. were isolated from the soil samples collected from various locations using serial dilution and plating technique on King's B agar medium and *Pseudomonas* agar base medium. Results revealed that in Kings' B agar medium, more number of colonies were noticed in all the soil samples than PAB medium which is supported by Yeole and Dube (2001). Maximum population of *Pseudomonas* spp. was recorded in samples procured from Alappuzha in both the medium (1.3- 7.8 x 10^6 cfu g^{-1} in KB medium and 0.28- 2.64 x 10^6 $cfug^{-1}$ in PAB medium), whereas, minimum population of *Pseudomonas* was observed in soil samples collected from Vyttila Research Station, Ernakulum (3.14- 6.01 x 10^4 cfu g^{-1} in KB medium and 1.1- 2.9 x 10^4 cfu g^{-1} in PAB medium).

In addition, soil samples from Thrissur and Palakkad district exhibited a population count ranging from 0.24- 0.74 x 10^6 and 2.3 – 6.5x 10^5 cfu g^{-1} in King's B agar medium whereas, 0.2- 0.45 x 10^6 and 1.01-4.56 x10^5 cfu g^{-1} in PAB medium respectively. When soil samples *viz*., KLM1, ORS1 and ORS2 from Palakkad, KTR1, KTR3 and PTD1 from Thrissur, VYT1 and VYT3 from Ernakulam and MCB1, MCB3 and MCB6 from Alappuzha were subjected to serial dilution in KB and PAB medium, no *Pseudomonas* colonies were obtained.

This may be due to the fact that microbial population is highly affected by the adverse environmental conditions prevailing in those locations.

The variation in population of *Pseudomonas* spp. in various locations could be due to the difference in the soil type, environmental conditions and root exudates. Neumann and Romheld (2001) reported that plant roots release a wide range of organic compounds including sugars, amino acids, fatty acids, organic acids, enzymes, auxins and hydrogen cyanide. They also pointed out that quantity and quality of these root exudates may vary according to the stage of crop. Soils samples from Moncombu were collected at grain filling stage whereas from Vyttila, soil samples were collected from the rice stubbles after harvest. The findings of Suman *et al*. (2018) are in line with the present investigation where the microbial population varied from 1.2- 6.7x 10^7 cfu g^{-1} soil as the samples were collected from different stages of the rice crop and from different soil types. Similarly, Manasa *et al*. (2017) reported that *Pseudomonas* population was highest in soil samples collected from paddy fields whereas lowest from maize rhizosphere.

5.2.1 Purification and preservation of *Pseudomonas* spp.

The isolates obtained from each location were purified repeatedly by streaking on King's B agar plates. A total of 15 isolates were thus purified and preserved in sterile water and glycerol solution (15%) for further studies. Preservation and maintenance of *Pseudomonas* spp. in sterile water and glycerol solution is supported by several workers (Lacobellis and Deavay, 1986: Liao and Shollenberger, 2003) and could be attributed to decreased metabolic activity.

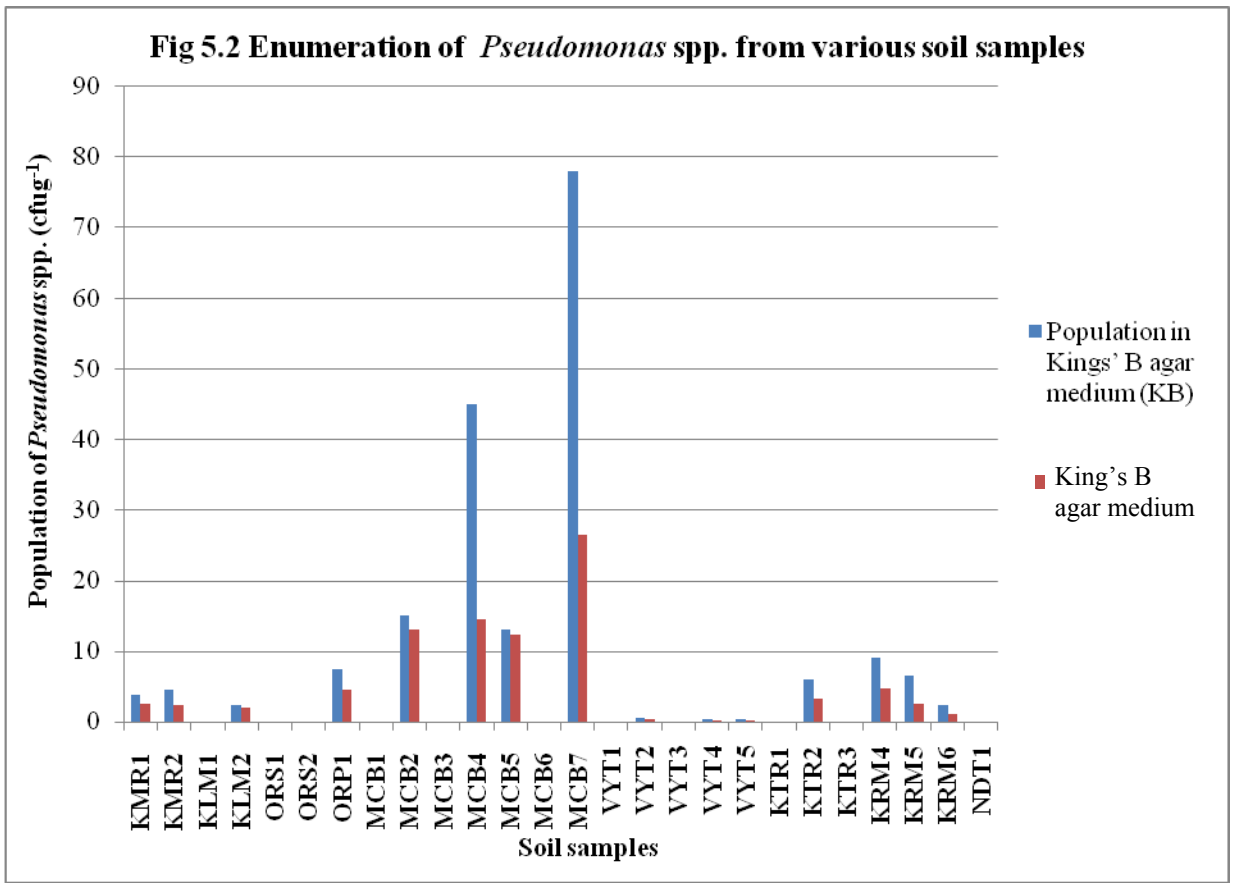


Fig 5.2 Enumeration of Pseudomonas spp. from various soil samples
Population of Pseudomonas spp. (cfug-1)
90
80
70
60
50
40
30
20
10
0
KMR1
KMR2
KLM1
KLM2
ORS1
ORS2
ORP1
MCB1
MCB2
MCB3
MCB4
MCB5
MCB6
MCB7
VYT1
VYT2
VYT3
VYT4
VYT5
KTR1
KTR2
KTR3
KRM4
KRM5
KRM6
NDT1
Soil samples
Population in Kings' B agar medium (KB)
King's B agar medium

5.2.2 Screening and elimination of *Pseudomonas aeruginosa*

A total of 15 isolates were obtained, out of which 10 isolates showed fluorescence under UV transilluminator and these were further screened for eliminating *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is an opportunistic human pathogen which is more ubiquitously found in agricultural soils compared to other fluorescent pseudomonads. Recent evidences suggest that *P. aeruginosa* dominates the *Pseudomonas* spp. closely followed by *P. putida* while very few isolates belonged to *P. fluorescens* (Shukla, 2016).

The isolates of pseudomonads were also incubated at 42°C to screen out *Pseudomonas aeruginosa,* since usually most of the fluorescent pseudomonads will not grow at this temperature except for *P. aeruginosa*. This is in accordance with the study conducted by Stanier *et al.* (1966). From the current study, it was observed that only two isolates *viz*., P7 and M7 did grow well at 42°C. Similar line of work was recorded by Hildebrand *et al.* (1988) where they also observed the optimum level of temperature of *P. aeruginosa* as 42°C.

Likewise, King *et al.* (1954) suggested Kings' A agar medium for differentiation of *P. aeruginosa* from other isolates of *Pseudomonas* spp. based on differences in pigment production. Henceforth, fluorescent isolates were streaked on Kings'A agar medium and after 48 h it was observed that the isolates P7 and M7 which were initially light green, turned greenish blue colour and thus were eliminated.

5.3. *In vitro* SCREENING OF *Pseudomonas* spp. FOR ABIOTIC STRESS TOLERANCE

Survival of an introduced strain of *Pseudomonas* spp. in the rhizosphere is affected by a number of abiotic constraints such as high soil temperature, salinity, drought and acidity. An understanding of the ability of *Pseudomonas* spp. to withstand different abiotic stress will enable effective

functioning of such strains within the particular conditions of the stressed ecosystems. Therefore, all the isolates in the current work were evaluated for their ability to tolerate abiotic stresses.

5.3.1 *In vitro* screening of *Pseudomonas* spp. for temperature tolerance

All the isolates showed an increased growth at a temperature ranging from 25 to 37 °C and thereafter, in general it was observed that with increase in temperature, growth of the isolates declined drastically.

The isolate P4 from Palakkad and V2 from Vyttila exhibited highest growth of 1.01 and 2.18 OD at 25^{o}C. Moreover, when the temperature was increased from 25 to 37^{o}C, the growth was found to rise with an OD ranging from 1.75 to 2.52. However, with increase in temperature to 45^{o}C, growth of all the isolates declined with P1 and P2 displaying comparatively better growth when compared to other isolates. At 50°C, the isolates P2, T5 and M4 alone survived with OD ranging between 0.61-0.62 whereas, the other isolates showed comparatively very less growth (0.18- 0.38).

According to Gopalakrishnan *et al*. (2012), *Pseudomonas* isolates *viz., P. plecoglossicida* and *P. monteilii* could survive at a temperature upto 40^{o}C. Tolerance of *Pseudomonas fluorescens* strain GGP-1 to temperature of 45^{o}C was observed by Manasa *et al*. (2016). Schinder and Keel (2001) opined that to combat with the adverse environmental conditions, bacteria like *Pseudomonas aeruginosa* often depend on regulatory mechanisms at genetic level which may include alternative sigma factors RpoS (s) and RpoE which confer tolerance towards hyperosmolarity and high temperature. Sandhya *et al.* (2009) reported a strain of *Pseudomonas* (AKM-P6) that improved tolerance of sorghum seedlings under high temperature conditions and according to them, the tolerance of the

reported strain was due to the production of heat shock proteins.

5.3.2. *In vitro* screening of *Pseudomonas* spp. for salt tolerance

Among the *Pseudomonas* isolates tested for stress tolerance, in general, it was observed that all the isolates were found to be very sensitive to salt stress as the OD was found very less even at 1.0 M NaCl (0.22- 0.86).When salinity was increased to 1.5 M, growth of all the isolates declined drastically. Highest growth at 1.0 M NaCl was displayed by T4 from Thrissur district, very closely followed by V2 and V4 from Vytilla, Ernakulam district with OD 0.82 and 0.81 respectively. However, M4 from Moncombu and V4 from Vyttila were found to survive even at 1.5 M salt concentration with optical density of 0.50 and 0.48 respectively.

Similar findings on salinity tolerance were putforth by Manasa *et al*. (2016) where they reported that *Pseudomonas fluorescens* strain GGP-1 could tolerate salinity upto 1.2 M. Conversely, much higher level of salt tolerance was observed by Shivakumar *et al*. (2013) in *Pseudomonas aeruginosa* strain FP6 which survived 1-4.5 M NaCl. Tank and Saraf (2010) reported that the strains *Pseudomonas fluorescens* and *P. aeruginosa* were able to survive at 6 per cent NaCl. Likewise, Johri *et al.* (1999) isolated and characterized salinity tolerant phosphate solubilizing bacteria that could survive at 5 per cent NaCl concentration.

According to Csonka (1989), salt tolerant microbes withstand the osmotic pressure by accumulation of organic ions like proline, glycine and betaine and inorganic ions like potassium cations at the cost of higher energy resulting in reduced growth rate. They observed that these organisms may also possess salt tolerant metabolic enzymes which withstand toxicity created by

accumulating inorganic ions. Likewise, Paul *et al*. (2008) noticed that *Pseudomonas fluorescens* strain MSP- 393 were able to produce a novel protein which counteracted detrimental effects of high osmolarity. Yap and Lim (1983) stated that salt stress tolerance is an important aspect of saprophytic ability and competitiveness among rhizobial isolates.

5.3.3 *In vitro* screening of *Pseudomonas* spp. for drought tolerance

Various isolates of *Pseudomonas* spp. were evaluated for their ability to tolerate drought and observed that when drought stress was induced by adding 10 % PEG, most of the isolates showed good growth with an overall OD between 1.01 and 1.94. However, when much higher drought was induced, only P4 from Palakkad and T4 from Thrissur tolerated 30 % PEG with optical densities of 0.21 and 0.22 respectively. The above conclusions are in agreement with Chari *et al*. (2018) where they evaluated 44 isolates of *Pseudomonas* spp. for drought tolerance and found that only three isolates could tolerate water potential from -0.05 Mpa to -0.73 Mpa. Timmusk and Wagner (1999) also contributed to the above findings and they reported that the presence of exopolysaccharide (EPS) around the bacterial coloniesexerted drought tolerance to them. The above findings were also closely supported by Manasa *et al*. (2016) as they observed that *Pseudomonas fluorescens* strain GGP-1 could tolerate all levels of drought stress (15, 20, 25 and 32.6 per cent PEG) and the drought tolerance of *Pseudomonas* spp. could be attributed to the production of exopolysaccharide (EPS) and accumulation of organic and inorganic compounds which increases the osmotic potential and thereby preventing water loss from cells.

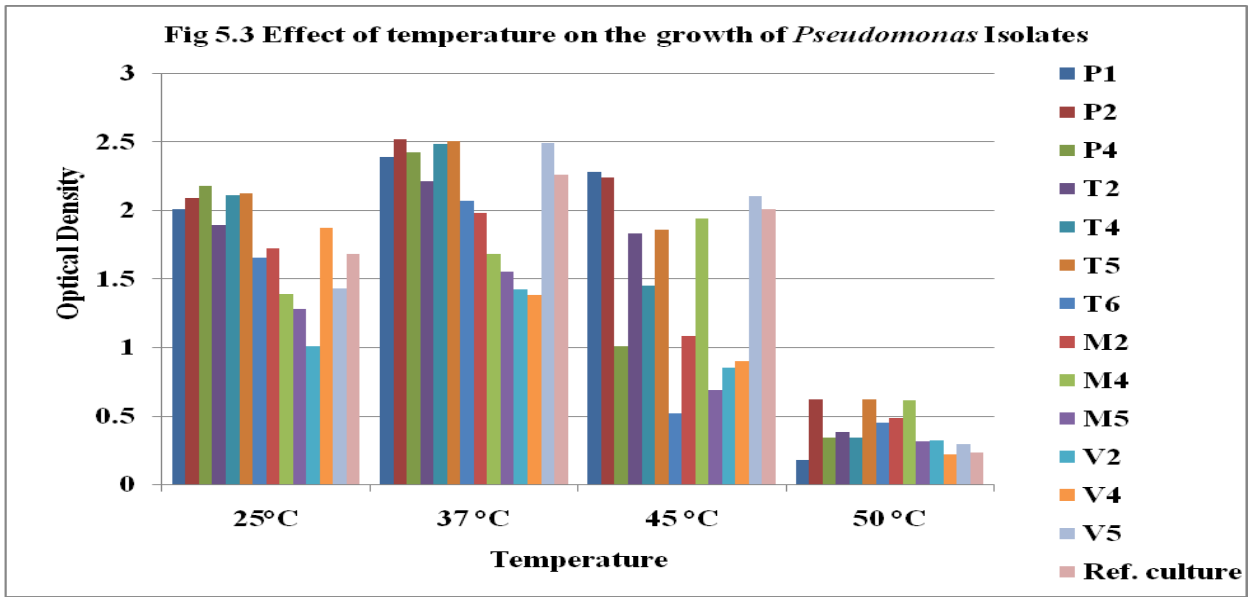


Fig 5.3 Effect of temperature on the growth of *Pseudomonas* Isolates
Optical Density
3
2.5
2
1.5
1
0.5
0
25°C
37 °C
45 °C
50 °C
Temperature
P1
P2
P4
T2
T4
T5
T6
M2
M4
M5
V2
V4
V5
Ref. culture

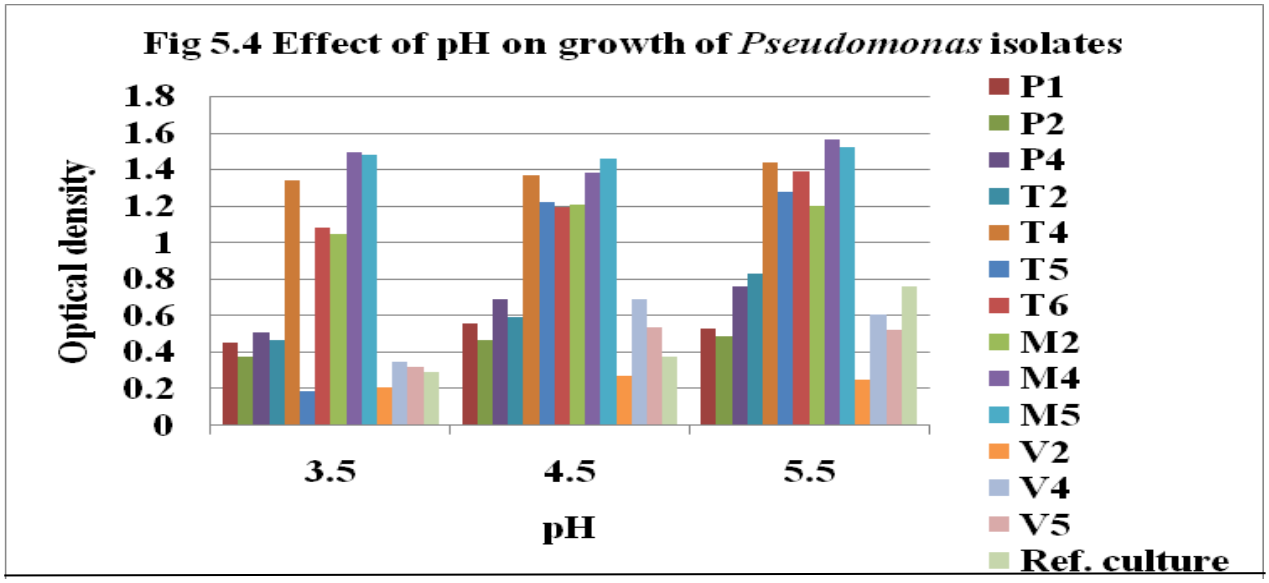


Fig 5.4 Effect of pH on growth of *Pseudomonas* isolates
Optical density
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3.5
4.5
5.5
pH
P1
P2
P4
T2
T4
T5
T6
M2
M4
M5
V2
V4
V5
Ref. culture

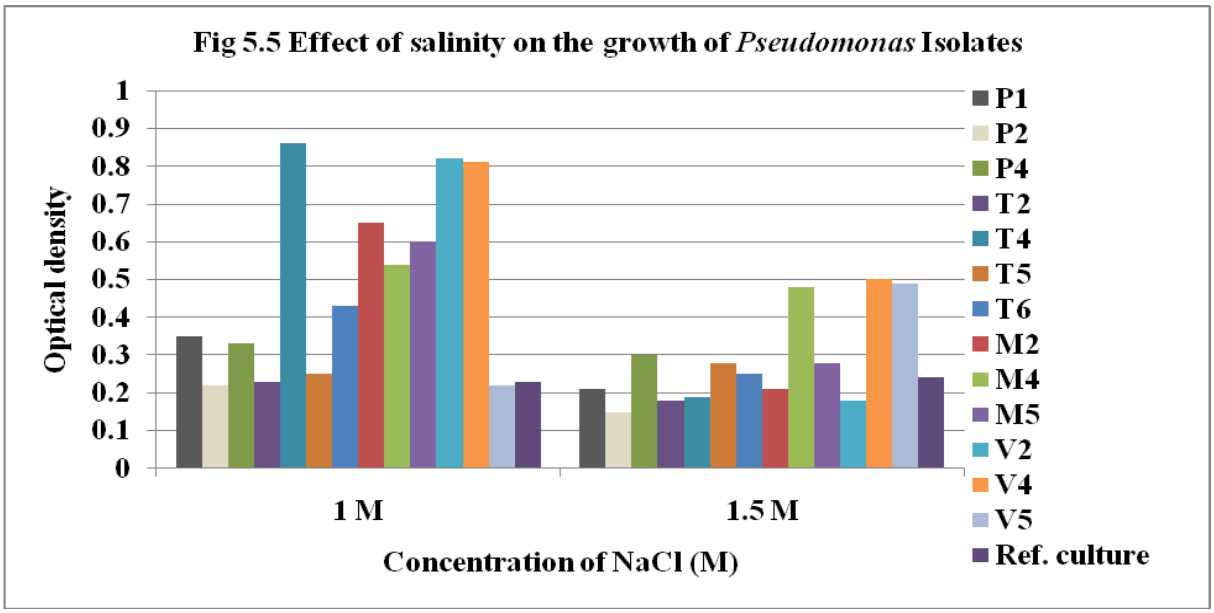


Fig 5.5 Effect of salinity on the growth of *Pseudomonas* Isolates
Optical density
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 M
1.5 M
Concentration of NaCl (M)
P1
P2
P4
T2
T4
T5
T6
M2
M4
M5
V2
V4
V5
Ref. culture

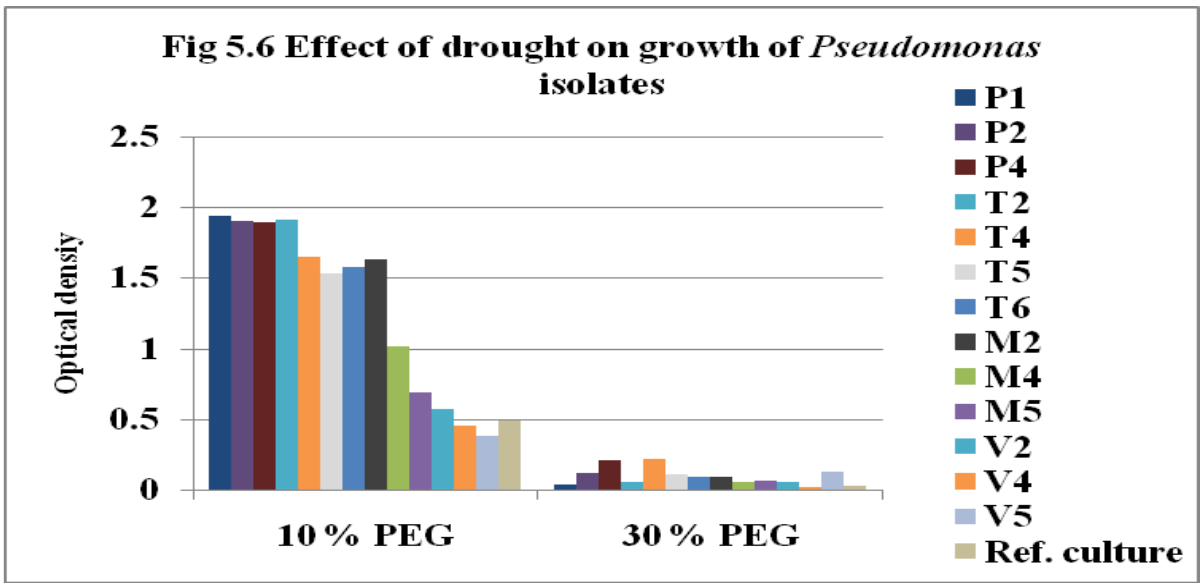


Fig 5.6 Effect of drought on growth of *Pseudomonas* isolates
Optical density
2.5
2
1.5
1
0.5
0
10 % PEG
30 % PEG
P1
P2
P4
T2
T4
T5
T6
M2
M4
M5
V2
V4
V5
Ref. culture

5.3.4 *In vitro* screening of *Pseudomonas* spp. for acid tolerance

Isolates were screened for their ability to tolerate acidity. It was observed that M4 and M5 showed consistent tolerance at three levels of pH (3.5, 4.5, 5.5) with highest OD. At pH 3.5, M4 and M5 from Moncombu were on par with each other with OD of 1.49, whereas, at pH 4.5, M5 expressed highest optical density of 1.46 followed by M4 (1.38). At pH 5.5, maximum spec reading was recorded by M4 (1.56), closely followed by M5 (1.52). These observations on acid tolerance of *Pseudomonas* spp. is in line with the findings of Gopalakrishnan *et al*. (2012) where they noticed good growth of *P. plecoglossicida* and *P. monteili* at pH 5. Similarly, Vithya *et al*. (2018) confirmed that an isolate of *Pseudomonas* from Pazhayannur of Thrissur district survived at pH 5.5.

Hence, in the current *in vitro* study for abiotic stress tolerance, *Pseudomonas* isolates *viz*., P2 from Palakkad, M4 from Moncombu and T5 from Thrissur were selected as temperature tolerant whereas, M4 and V4 from Vyttila as salt tolerant. Moreover, M4 and M5 were screened as best acid tolerant strains and highest drought tolerance was exhibited by the isolates P4 and T4. Thus, M4, M5 from Moncombu, P2 and P4 from Palakkad, T4 and T5 from Thrissur and V4 from Ernakulam district were chosen as abiotic stress tolerant strains and were further evaluated for their antagonistic potential to prove their efficacy against major soil borne plant pathogens.

5.4 *In vitro* EVALUATION OF STRESS TOLERANT STRAINS OF *Pseudomonas* spp. FOR THEIR ANTAGONISTIC POTENTIAL

In vitro tests are inevitable basic procedures for selecting effective antagonists in biocontrol research. Since the present study is mainly intended to select stress tolerant antagonistic strains of *Pseudomonas* spp., bacterial ring technique otherwise known as bangle method was carried out as delineated in the

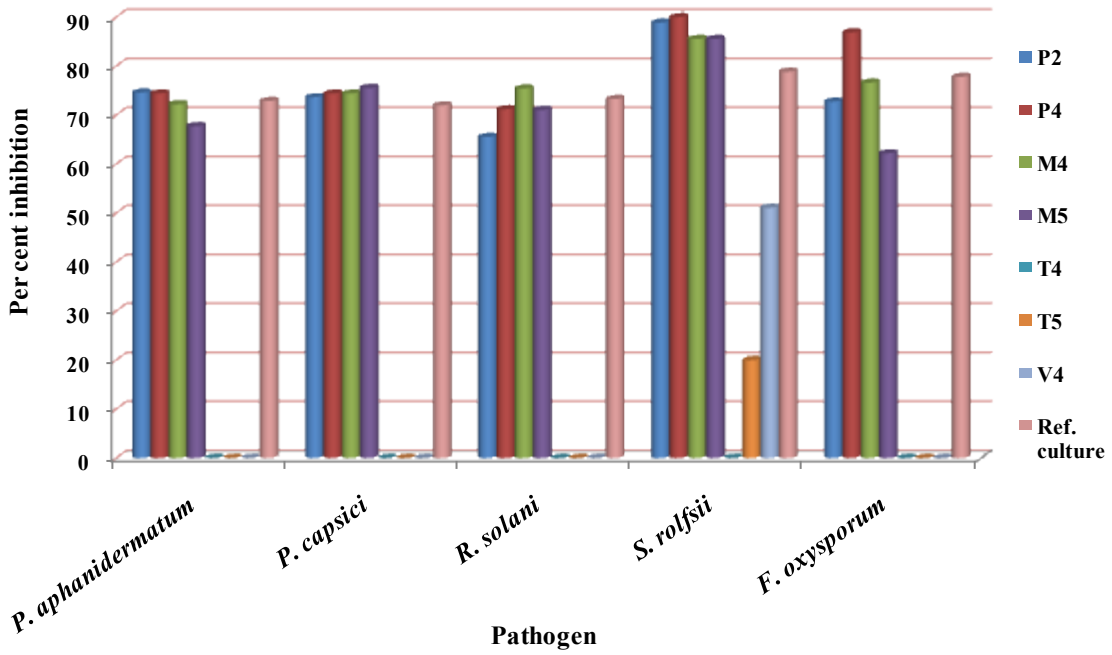
materials and methods, in order to have a clear idea of individual antagonistic strains against each phytopathogen. PDA medium was chosen for the assay, as it would provide suitable nutritional environment for bacterial secondary metabolism as well as for antibiotic production as suggested by Xu and Gross (1986).

In the present study, the antagonistic potential of stress tolerant isolates *viz*., P2, P4, M4, M5, T4, T5 and V4 was evaluated under *in vitro* conditions against five major soil borne pathogens *viz*., *Phytophthora capsici*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizoctonia solani* and compared with reference culture of *Pseudomonasfluorescens* of KAU. Out of the seven isolates screened, only four isolates *viz*., P2, P4, M4 and M5 were shortlisted based on the per cent growth inhibition of all the tested fungal pathogens which ranged from 62.21 to 91.00 per cent. The isolates T4, T5 and V4 showed very less and even no inhibitory action against the pathogens and hence eliminated from further studies. The higher antagonistic potential of M4, M5, P2 and P4 could be ascribed to comparatively higher production of antibiotic compounds or volatile gases or formation of siderophores in the medium, than the remaining isolates as suggested by Shukla (2016).

A comparison with reference culture of KAU revealed that the selected antagonistic isolates were found to be exhibiting higher antimycolytic activity than reference culture. Further, it is also pertinent to note that *Sclerotium rolfsii* was highly susceptible to *Pseudomonas* spp., as it was antagonized by the isolates which showed no such inhibition to other pathogens.

In the present study, it was observed that the isolates P2 and P4 from Palakkad showed highest growth reduction (74.61 and 74.43 per cent) of *Pythium*

Fig 5.7 Per cent inhibition of fungal pathogens by selected *Pseudomonas* isolates



aphanidermatum when grown in dual culture. The mycelial growth of *Rhizoctonia solani* was found to be highly suppressed by M4 with a per cent inhibition of 75.43. These findings are in congruence with the observations of Nandi *et al*. (2013) where they reported that two isolates of *P. fluorescens* PF-8 and PF-7 effectively inhibited *Rhizoctonia solani* with 75.8 and 75.2 per cent mycelial growth reduction respectively.

Among the isolates, P4 from Palakkad exhibited highest antagonism against *Sclerotium rolfsii* (91.0 %) whereas, the growth of *Phytophthora capsici* was highly reduced by the isolate M5 with per cent inhibition of 75.56. These findings are comparable with the studies of Bhatia *et al*. (2005) where they documented the antagonistic effect of 10 isolates of fluorescent pseudomonads against *S. rolfsii* under *in vitro* conditions with maximum growth inhibition of 73.0 and 70.0 per cent by isolates PS I and PS II respectively. The results are also found consistent with Shivakumar *et al*. (2013) where they also noticed the antagonistic potential of *P. aeruginosa* FP6 against *Phytophthora capsici*.

In this study, it was observed that *Fusarium oxysporum* was inhibited to the higher extent by the isolate P4 with a per cent growth reduction of 86.92. Similar line of work was recorded by Manjunatha *et al*. (2008) where they observed maximum inhibition of 54.46 per cent in mycelial growth of *Fusarium udum* by the bacterial antagonist *Pseudomonas fluorescens* isolate compared to control. The antagonistic effect of *Pseudomonas* against *Fusarium* is in concurrence with the study of Madhavi (2006) who documented 65.9 per cent suppression in the radial growth of the *F. solani* by *P. fluorescens.*

In general, it was noticed that the percentage growth inhibition of all the tested pathogens was higher which may be due to the fact that bacterial ring or bangle technique induce more antagonistic pressure on the pathogens from all

side as a ring, whereas in dual culture technique, only one or more streak is performed beside the pathogen disc placed in the centre or side of the Petri dish.

Moreover, *Pseudomonas* spp. are well known biocontrol agents used for the control of soil borne phytopathogenic fungi. Various mechanisms have been attributed to their antagonistic activity *viz*., hydrolytic enzymes like cellulase, glucanase, chitinases, volatile compounds, siderophores and antifungal antibiotics like phenazines, DAPG, pyrolnitrin, pyoluteorin and soforth which are confirmed by several researchers (Kloepper*et al*., 1980; Ramette*et al*., 2006; and Zadeh*et al*., 2008). Hence, in the current study, the isolates of *Pseudomonas* spp. possess both the characters *viz*., tolerance towards various abiotic stresses and antagonistic ability towards fungal pathogens which makes the selection an ideal one for their better performance under field conditions.

5.5 *In vitro* SCREENING FOR PRODUCTION OF BIOMOLECULES IMPARTING STRESS TOLERANCE

The most promising isolates showing higher stress tolerance and *in vitro* antagonism were screened for the production of biomolecules *viz*., exopolysaccharide and ACC deaminase for drought tolerance as well as cellulase and β-1, 3 glucanase for bio control potential.

5.5.1 Screening for exopolysaccharide production

One of the important mechanisms of rhizosphere bacteria in exerting drought tolerance is the formation of exopolysaccharide (EPS). According to Chenn and Guerif (1991) and Sandhya *et al*. (2010), EPS enhance soil water holding capacity and fertility by improving aggregation of soil particles. Many authors reported that bacteria survive under stress conditions due to the

production of EPS which protects microorganisms from water stress by enhancing water relation and also by regulating the difference of organic carbon sources (Roberson and Firestone, 1992; Chenn, 1993; Chenn and Roberson, 1996). Bashan *et al*. (2004) later confirmed that EPS consist of a network of fibrillar material which aid in the attachment of bacterial cell to the plant roots.

In this study, when the selected isolates were screened for exopolysaccharide production, it was observed that P4 and M4 produced higher EPS. Similar studies were conducted by Kumar *et al*. (2015) where they observed highest EPS production of 237 µg mLg^{-1} with P12 strain. Likewise, Sandhya *et al*. (2009) also reported EPS-producing fluorescent pseudomonads isolated from various semiarid regions of India and EPS production of the isolate was linearly correlated with drought stress. This suggests that exopolysaccharide production could have protected the isolates from osmotic stress, which accounted for the higher tolerance of M4 and P4. It is very well evident that P4, isolated from Palakkad district experienced higher soil temperature with very high soil water deficit or soil moisture stress. Although, M4 was isolated from samples with higher moisture content, the soil temperature of that location was 36.1^{o}C.

5.5.2 Screening for ACC deaminase production

Drought stress is one of the major agricultural problems limiting productivity in most of the arid and semiarid regions. Interestingly, it has been observed that certain PGPR strains possess the enzyme ACC deaminase where this enzyme can cleave the plant ethylene precursor ACC to ammonia and α-ketobutyrate thereby, lower the level of ethylene under various biotic and abiotic stresses especially, the drought. (Mayak *et al*., 2004b). Rhizobacteria with ACC deaminase activity couldlower the level of ACC in the stressed plants and thereby reduce stress ethylene synthesis and hence prevent damage to the plants. Among the various PGPR strains, soil borne fluorescent pseudomonads have excellent

root colonizing ability, catabolic versatility and produce a wide range of enzymes and metabolites that favour the plant to withstand under varied biotic and abiotic stress conditions (Ramammorthy *et al*., 2001; Vivekanandan *et al*., 2004). In the present study, the isolates P2 and M5 showed good growth on Dworkin foster medium with ACC as sole nitrogen source and were selected as the isolates with better ACC deaminase activity. This higher ACCD activity could have accounted for higher temperature tolerance of P2 isolate. Similar results were obtained by Aswitha *et al.* (2013) as they noticed higher ACCD production in *Pseudomonas* spp. isolated from drought prone areas such as Tamil Nadu, Karnataka, Maharashtra, Gujarat, Rajasthan, Haryana, Uttar Pradesh and Orissa. There are several reports that the ACC deaminase gene encoding ACC deaminase enzyme has been isolated from soil bacteria under non stress and under abiotic stress. (Klee *et al*. 1991; Campbell and Thompson, 1996; Hontzeas *et al*., 2005; Onofre Lemus *et al*., 2009). The ACC deaminase producing bacteria are therefore, known to facilitate the growth of plants especially under stressful conditions, so the ACDS gene coding for enzyme ACC deaminase can be a very useful candidate gene for the development of bioinoculants for abiotic stress management in plants.

5.5.3 Screening for cellulase production

Cellulase, a hydrolytic enzyme catalyzes cellulolysis, the decomposition of cellulose and some related polysaccharides. These enzymes are involved in the suppression of soil borne pathogens. The current study on cellulase producing pseudomonads revealed that enzyme activity ranged between 0.815 $Uml^{-1}$and 0.907 $Uml^{-1}$with highest production by the isolate P4 and the least by M5. Kumar *et al*. (2015) similarly screened *Pseudomonas* spp. for cellulase enzymes and found only 5 per cent of isolates were positive for cellulase production.

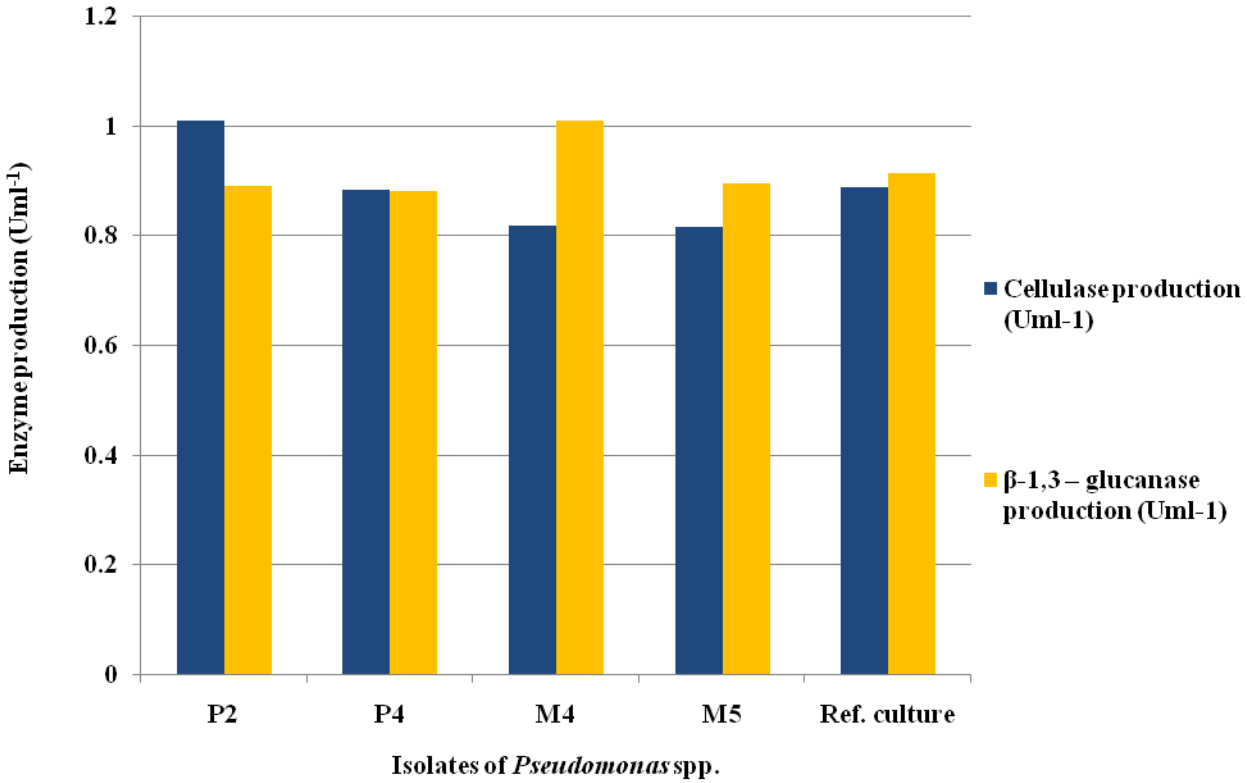
5.5.4 Screening for β- 1, 3- glucanase production

β- 1, 3- glucanases are hydrolytic enzymes prevalent in bacteria and fungi which degrade the polysaccharide to release monosaccharide monomers. Frindlender *et al.* (1991) has correlated the antifungal activity of *Pseudomonas fluorescens* with enzyme production. In the present study, highest β- 1, 3- glucanase activity was noticed in the isolate M4 (1.010 Uml^{-1}). A linear relationship between β- 1, 3- glucanase activity and antagonistic potential against *Rhizoctoniasolani* was observed by Nagarajan *et al*. (2004). According to Diby *et al*. (2005), mycolytic enzymes such as β -1, 3-glucanases and β -1, 4-glucanases produced by *Pseudomonas fluorescens* are involved in the mycelial lysis of *Phytophthora capsici*. The present findings are in accordance with Kitamura and Kamei (2006) who attributed cell wall lysis of *Pythium porphyrae* to β-1, 3-glucanase activity. Recently, Singh *et al*. (2010) observed mycelial lysis, vacuolation and granulation of cytoplasm, hyphal deformities and branching in *Macrophomina phaseolina* during dual culture with β-1,3-glucanase producing *Pseudomonas aeruginosa* PN1.

5.6 *In vivo* BIOASSAY FOR TESTING EFFICACY OF ISOLATES

It is pertinent to state that before employing pseudomonads or any other antagonists as BCAs, it is an essential prerequisite to test their bioefficacy against target pathogen for selecting potential isolates. Hence, a rapid bioassay was chosen for selecting highly antagonistic organisms. Thus*, in planta* analysis of *Pseudomonas* isolates for its plant protection and growth promoting ability was determined as per the protocol of Srivastava *et al*. (2002) with cowpea as test

Fig 5.8 Cellulase and β- 1,3-glucanase production by promising *Pseudomonas* isolates



crop and *Rhizoctonia solani* as test pathogen. It was noticed that the isolate M4 enhanced the germination percentage to 100 per cent with higher plant growth promotion and thus it was rated as highly efficient bio control agent against *Rhizoctonia*. Similarly, the reference culture of KAU, *Pseudomonas fluorescens* was rated as efficient whereas, the other isolates P2, P4 and M5 as moderately efficient.

The plant growth promotion as observed in this study is in accordance with Shivakumar *et al.* (2013) where they studied the effect of inoculation of *Pseudomonas aeruginosa* on cowpea seeds and observed higher germination percentage and seed vigour as compared to uninoculated control.

Meera and Balabaskar (2012) documented similar findings of increased germination, shoot length, root length and vigour index of rice seedlings under *in vivo* conditions when bacterized with culture filtrates of *Pseudomonas* isolates. The enhancement in plant growth by *Pseudomonas* spp. might be due to the production of amino acids, salicylic acid and IAA as reported by several workers (O'Sullivan and O'Gara, 1992; Dowling and O' Gara, 1994).

5.7 *In vivo* EVALUATION OF STRESS TOLERANT *Pseudomonas* spp. FOR DISEASE SUPPRESSION AND PLANT GROWTH PROMOTION

Several researchers pointed out that the isolates which proved best under *in vitro* antagonistic studies may perform poor under field conditions due to several interfering factors such as environmental conditions and availability of nutrients. Hence, the present study was shifted from *in vitro* to *in vivo* with a pot culture experiment to evaluate the actual antagonistic ability of the four selected isolates *viz*., M4, M5, P2 and P4 against *Rhizoctonia solani* using cowpea variety 'Bhagyalakshmi'.

In the present study, though all the *Pseudomonas* isolates exhibited

higher yield and biometric characters such as enhanced seed germination, plant height, number of leaves, earliness in flowering as compared to uninoculated control, the isolates differed among themselves in the degree of plant growth promoting potential. The isolate M4 outperformed all other isolates in terms of seed germination (96.29 %), plant height (14.11 % increase within 45-60 DAS), number of leaves produced (34.55 numbers at 60 DAS), earliness in flowering (40.33 days) and yield (87.95 g).

Similar findings of enhanced germination, yield and yield attributes were reported by several workers. Wahyudi *et al*. (2011) observed increased number of lateral roots and root and shoot length in cowpea plants treated with CRB44 and CRB 63 strains of *Pseudomonas* spp. This is in line with the findings of Gopalakrishnan *et al*. (2012) who reported that *Pseudomonas monteilii* induced higher tiller number, stover and grain yields, total dry matter, root length, volume and dry weight of rice plants over the un-inoculated control in rice plants. According to Meera and Balabasker (2012), rice plants exhibited maximum germination per cent, increase in vigour index and shoot and root length when treated with *Pseudomonas fluorescens*. Similar increase in seed germination and plant vigour was observed in tomato seeds bacterized with *Pseudomonas* isolate I-23 (Madhavan *et al.*, 2017).

The enhancement in plant growth characters could be attributed to the production of plant growth promoting hormones and aminoacids or inactivation of plant inhibiting hormones such as ACC as observed by several workers (O' Sullivan and Gara, 1992, Khalid, *et al.,* 2004, Blaha, *et al.,* 2006).

The study was continued further to evaluate *in vivo* antagonistic potential by drenching the plants with four isolates, along with reference culture at 30 DAS followed by challenge inoculation with *Rhizoctonia solani* at 45 DAS. It was observed that the isolate M4, protected the plants from the pathogen and thereby reduced disease incidence to 11.11 per cent which was closely followed by M5 (22.22%). However, plants in control showed cent per cent disease incidence indicating the vulnerability of plants to *Rhizoctonia* in the absence of antagonist. It was observed that P2 and P4 could protect the plants from disease whereas, the reference culture showed the least protection with a per cent disease incidence of 33.33. Hence, it was concluded that M4 and M5 are identified as the promising isolates with highest disease protection.

Similar *in vivo* antagonistic studies were carried out by several workers. Heflish *et al.* (2017) reported increased resistance to sheath blight pathogen in rice plants when *Pseudomonas* spp. was applied as seedling root dip and foliar spray. Likewise, Madhavan *et al.* (2017) documented minimum per cent disease incidence of 10.41 in tomato plants when challenge inoculated with *Rhizoctonia.*

Pandey *et al*. (2001) reported the efficacy of *Pseudomonas corrugata* in suppression of damping off of maize seedlings, challenge inoculated with *Pythium ultimum, P. arrhenomanes* and *Fusarium graminearum*. This is in agreement with Anjaiah *et al*. (2003) where they reported higher antagonistic ability of *Pseudomonas aeruginosa* PNA1 against *Fusarium oxysporum* f. sp. *ciceris* and *Fusarium udum* under *in vivo* conditions even in susceptible genotypes.

The difference in the prophylactic action of the isolates against the pathogen could be attributed to the differences in competitive ability with the pathogen, production of antibiotic and antibiotic like substances or lytic enzymes.

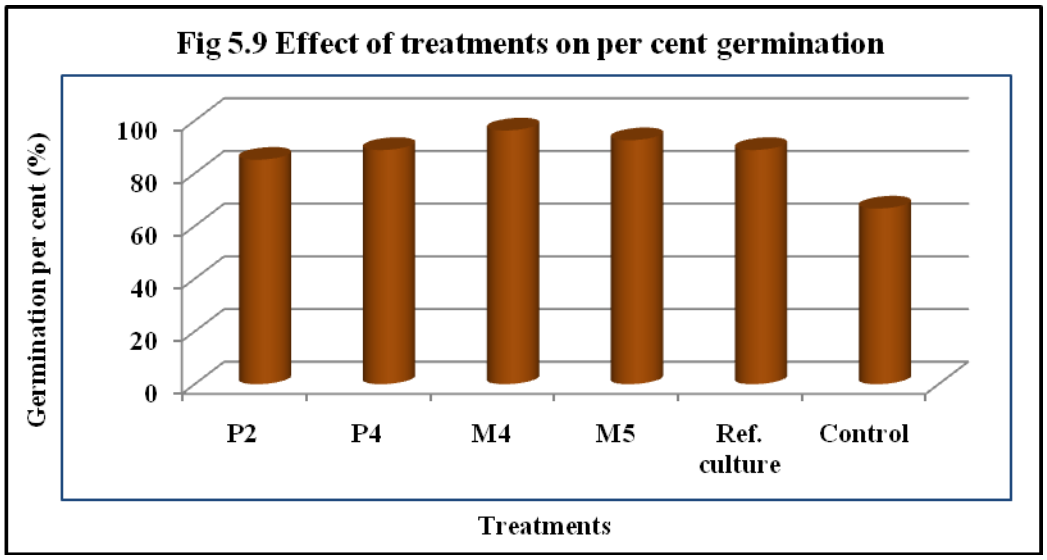


Fig 5.9 Effect of treatments on per cent germination
Germination per cent (%)
100
80
60
40
20
0
P2
P4
M4
M5
Ref. culture
Control
Treatments

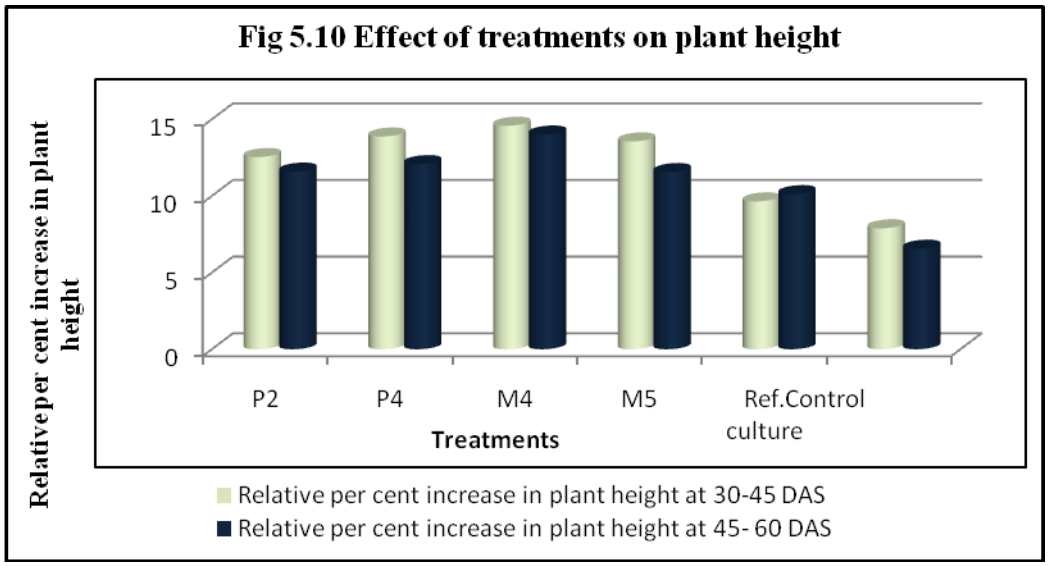


Fig 5.10 Effect of treatments on plant height
Relative per cent increase in plant height
15
10
5
0
P2
P4
M4
M5
Ref.Control culture
Treatments
Relative per cent increase in plant height at 30-45 DAS
Relative per cent increase in plant height at 45- 60 DAS

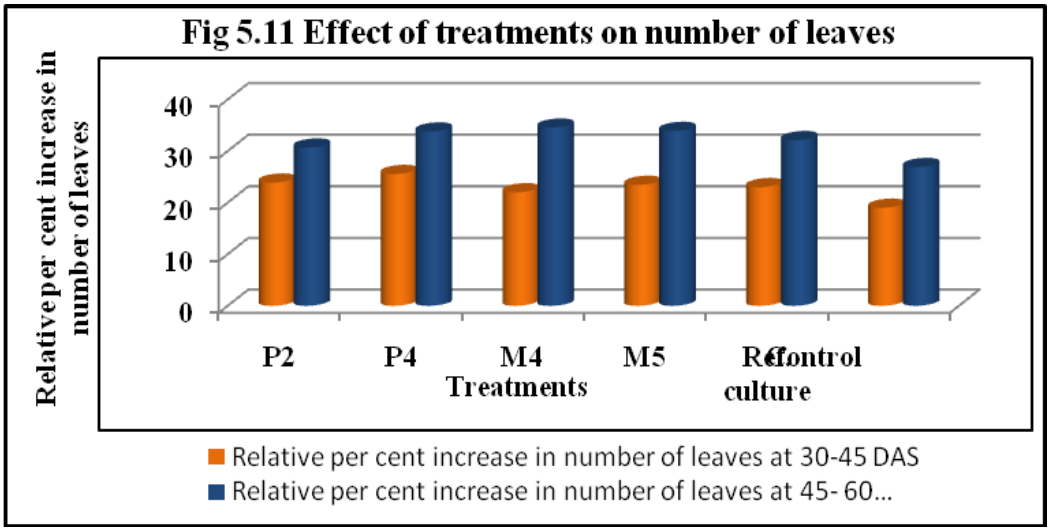


Fig 5.11 Effect of treatments on number of leaves
Relative per cent increase in number of leaves
40
30
20
10
0
P2
P4
M4
M5
Ref.control culture
Treatments
Relative per cent increase in number of leaves at 30-45 DAS
Relative per cent increase in number of leaves at 45- 60...

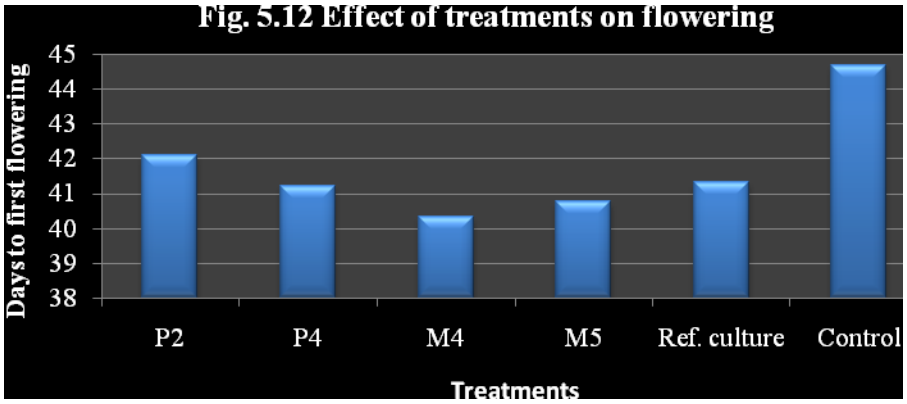


Fig. 5.12 Effect of treatments on flowering
Days to first flowering
45
44
43
42
41
40
39
38
P2
P4
M4
M5
Ref. culture
Control
Treatments

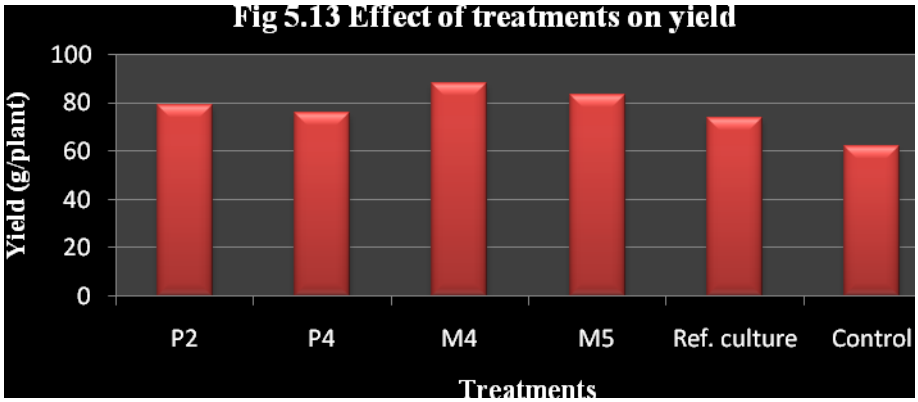


Fig 5.13 Effect of treatments on yield
Yield (g/plant)
100
80
60
40
20
0
P2
P4
M4
M5
Ref. culture
Control
Treatments

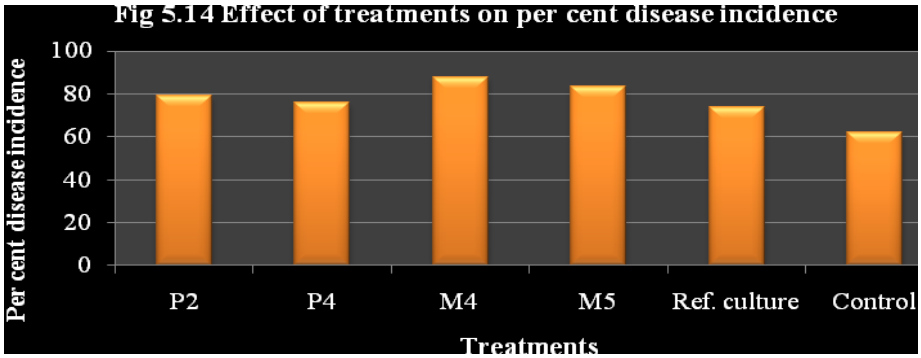


Fig 5.14 Effect of treatments on per cent disease incidence
Per cent disease incidence
100
80
60
40
20
0
P2
P4
M4
M5
Ref. culture
Control
Treatments

plant growth promotion, the other isolates *viz*., M5, P2 and P4 were found to be superior or equally efficient as reference culture of KAU compared to that of control. Hence, all the isolates *viz*., P2, P4, M4 and M5 were subjected to cultural, morphological and biochemical characterization for the tentative identification of the isolates.

5.8 CULTURAL, MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF PROMISING *Pseudomonas* ISOLATES

The cultural, morphological and biochemical characters of all the four isolates along with the reference culture of KAU were studied in detail. Microscopic observations revealed that all the isolates were Gram negative, non-spore rods. In addition to this, the isolates were confirmed as fluorescent pseudomonads as they exhibited fluorescence under UV light. Many researchers suggest that fluorescent pseudomonads could produce fluorescent yellow green water soluble diffusible pigment on King's B agar (Reddy and Rao, 2009). Fluorescent green pigmentation could be attributed to the production of siderophores (Shahira *et al*., 2015).

The individual colony characters of these isolates were further studied by streaking on King's B agar. All the isolates took 24 h for establishing single colonies on the medium which was also reported by Vlassak *et al*. (1992). It was observed that all the isolates yielded circular, small, round, opaque, yellow green coloured colonies with round and entire margin and convex, smooth and shiny surfaces. These findings are confirmed by the reports of Hagedoron (2001) who characterized *P. fluorescens* as strictly aerobic, Gram negative, straight or curved rods, without resting spore and colonies were observed as yellow to creamish white and green pigmented and many species produced extra cellular fluorescence pigment also. In the current study, when *Pseudomonas* isolates were

subjected to antibiotic sensitivity test, it was observed that all the isolates were resistant to the antibiotic ampicillin (10 µg). Moreover, the isolates, M4 and the reference culture were resistant to all the antibiotics whereas M5 was susceptible to only one of the tested antibiotic *i.e.* chloramphenicol. Likewise, P2 and P4 were resistant to gentamycin and ampicillin and susceptible to tetracycline and chloramphenicol.

Similar antibiotic resistance profiling was performed by several workers. Kieboom and de Bont, (2001) reported that several species of *Pseudomonas* such as *P. putida* exhibited resistance to multiple antibiotics. Similarly, Jagadish (2006) confirmed that native strain of *Pseudomonas* sp. B-25 showed resistance to ampicillin at all concentrations tested *i.e*. 25-250 ppm. Gopalrao *et al*. (2014) documented intrinsic antibiotic resistance profiles of selected *Pseudomonas* isolates and was found highly resistant to ampicillin (10 µg), rifampicin (5 µg), chloramphenicol (10 µg), streptomycin (10 µg), tetracyclin (30 µg), vancomycin (30 µg), spectinomycin (100 µg), carbenicillin (100 µg) and polymyxin-B (300 µg) and medium resistance to gentamycin (10 µg) and kanamycin (30 µg).

When the isolates were characterized for its biochemical properties, it was noticed that all the isolates produced positive results for catalase, oxidase and citrate utilization test whereas, negative for urease, H_2S, MR-VP and indole tests. Similar observations were documented by Shahira *et al*. (2015), Manasa *et al*. (2017), Singh et al. (2017) and Vadnerker *et al.* (2018). Among the tested sugars (glucose, lactose, mannitol and sucrose), none of the isolates utilized these sugars except P2 and P4 which utilized mannitol. According to Prasad *et al*. (2017) and Shukla (2016), *Pseudomonas* spp. shows catabolic versatility to various sugars tested. The ability of the bacteria to utilize different substrates containing carbon, hydrogen and oxygen varies with the battery of enzymes present in the bacteria (Cowan, 1974).

Shukla (2016) reported that pseudomonads never produce indole from tryptophan and a positive indole reaction or a positive MRVP reaction is a proof that the culture under investigation does not belong to the genus *Pseudomonas.* The isolates M4 and M5 hydrolysed gelatin indicating the production of the enzyme, gelatinase which hydrolyses protein to polypeptide, peptide, and fatty acid so that they could be utilized for its growth. The results of the current study are in conformity with several workers (Shivakumar *et al*., 2013: Shukla, 2016).

Thus, based on cultural, morphological and functional characters, isolates were identified as *Pseudomonas* spp. only. However, Pseudomonas spp. being a group of bacteria with high catabolic versatility should not be identified by meagre cultural, morphological or biochemical characterization. Hence, all the isolates were sent to RGCB for sequencing and molecular identification.

5.9 MOLECULAR CHARACTERIZATION OF PROMISING *Pseudomonas* ISOLATES

16S ribosomal RNA or 16S rRNA) is the component of the 30S small subunit of a prokaryotic ribosome. The genes coding for it are referred to as 16S rRNA gene and are used molecular identification, due to the slow rates of evolution of this region of the gene.16S rRNA gene sequencing is a widely used bioinformatics tool in identifying biochemically unidentified bacteria or for providing conformity to biochemically identified bacteria. It has been the most common housekeeping genetic marker used for a number of reasons.16S rRNA gene is found in almost all bacteria as a conserved region and often exist as a multigene family, or operons. And also, the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001).

In this study the isolates were outsourced at RGCB, Thiruvananthapuram for idenfication using 16S rRNA gene sequencing. When the nucleotide sequences of the isolates obtained from RGCB were compared with database of NCBI, it was observed that the isolates M4 and M5 showed homology towards *Pseudomonas aeruginosa* whereas, P2 and P4 were closely identical to *Pseudomonas putida*.

Kumar *et al.* (2015) isolated antagonistic *Pseudomonas* strain from paddy rhizosphere and identified by 16 srRNA sequencing as *P. aeruginosa* ATCC 27853 with a similarity of 99 per cent. Similarly, Saini *et al*. (2016) isolated a *Pseudomonas* strain BNJ-SS-45 from wheat rhizosphere and identified as *Pseudomonas protegens* (HQ905436) with 99.0 per cent similarity.

Hence, while recalling the results of the current study, it is clear that a total of 15 isolates were obtained from 26 soil samples collected from various stressed locations of Kerala with highest population of *Pseudomonas* spp. recorded from soil samples of Alappuzha district and the least from Vyttila region of Ernakulum district. From the 10 fluorescent pseudomonads, two isolates were identified as *Pseudomonas aeruginosa* and hence eliminated from further studies. Thus, 13 isolates as well as the reference culture of KAU were screened for abiotic stress tolerance and six isolates were selected *viz*., P2, P4, M4, M5, T4, T5 where V4, P2, M4 and T5 were selected as temperature tolerant; whereas, M4 and V4 as salt tolerant strains; P4 and T4 as drought tolerant while, M4 and M5 from Alappuzha district as acid tolerant strains.

Thereafter, six stress tolerant strains were evaluated for antagonistic potential under *in vitro* conditions against major soil borne fungal pathogens *viz*., *Phytophthora capsici, Pythium aphanidermatum, Sclerotium rolfsii, Fusarium oxysporum,* and *Rhizoctonia solani* and four isolates (P2, P4, M4 and M5) were selected. These four isolates were further analyzed for biomolecules imparting

stress tolerance and antagonism. Thus, M4 and P4 with higher exopolysaccharide production and P2 and M5 with highest ACCD activity were selected. In addition, P2 and P4 also showed maximum cellulase activity and P4 and M4 with highest β- 1,3 glucanase activity.

Hence, all the four isolates were evaluated for their antagonistic potential through pot culture experiment against *Rhizoctonia solani* using cowpea plants. Among the various isolates, M4 was selected as the best strain as it showed highest plant growth promoting potential as well as disease suppressing ability. Later, all the four isolates were subjected to cultural, morphological and biochemical characterization as all of them performed equally or was found superior to the reference culture of KAU. From the results, it was observed that only the isolates P2 and P4 were identified as *Pseudomonas putida* due to its inability to hydrolyze gelatin. Thereafter, all the isolates were subjected to molecular characterization through 16s rRNA sequencing. Based on nucleotide homology to the sequences of NCBI database, the isolates P2 and P4 were confirmed as *Pseudomonas putida* and M4 and M5 identified as *Pseudomonas aeruginosa*.

Since, *Pseudomonas aeruginosa* is an opportunistic human pathogen the isolates M4 and M5 should not be used as a biocontrol agent. Hence, the present study resulted in identification of two isolates of *Pseudomonas* spp. from paddy fields of Palakkad district *viz., Pseudomonas putida* strain P2 and P. *fluorescens* strain showing tolerance to drought and temperature respectively.

In the current study, the most well studied phenomenon is the abiotic stress tolerance of various isolates of *Pseudomonas* spp. along with their antagonistic activity towards plant pathogens with a result suppression of plant disease. However, a major constraint with application of such microbial inoculant is the inconsistent field performance, which could be attributed to their poor

survival and competence in the adverse environmental conditions; thereafter a careful choice of different conditions is necessary to generate a meaningful data. This study therefore paves way for the ideal selection of bioagents having abiotic stress tolerance and proven antagonistic activity for their effective functioning under field conditions. Further, strains of *Pseudomonas viz*., P2 and P4 possessing tolerance to abiotic stress and antagonistic activity would enlist them as candidate strains for further characterization and application. Moreover, in view of the present climate change scenario, application of these stress tolerant isolates would significantly help the farming community by overcoming such drastic climate changes. Further, application of such isolates is also known to overcome the deleterious effect of chemical fertilizers and pesticides. The study should be further complemented with multilocational field trials to prove the effectiveness in the management of various fungal diseases as the *in vitro* and pot culture studies alone do not always reflect what happens in the field.

Our results therefore suggest that, the selection and use of these abiotic stress tolerant strains with multiple activities for the facilitation of plant growth in stressed environments, will be a highly important area for future research. Hence, further evaluation of these strains is needed to uncover their efficiency as plant growth promoting bacteria in soil plant systems.

SUMMARY

6. SUMMARY

Bacteria belonging to the genus *Pseudomonas* are one among the most commonly used biocontrol agent against a number of plant pathogens. Pseudomonads are also known to promote plant growth and ameliorate the drastic effects of abiotic stress on plants. Eventhough, isolates of *Pseudomonas* spp. show immense antagonistic potential under *in vitro* conditions, the field performance is often variable. It could be attributed to the poor survival and rhizospheric competence of pseudomonads under adverse environmental conditions *viz*., salinity, acidity, high temperature and drought. And also, the strains available for the farmers are not suitable for abiotically stressed soils of Kerala. Hence, the present research on "Characterization and evaluation of abiotic stress tolerant strains of *Pseudomonas* spp." was conducted with an objective to isolate native *Pseudomonas* spp. from abiotic stressed ecosystems of Kerala having inherent stress tolerance and antagonistic potential.

1. A total of 16 soil samples were collected fromvarious abiotically stressed locations of four districts of Kerala *viz*., Thrissur, Palakkad, Ernakulam and Alappuzha
    - At the time soil sampling, the locations of Palakkad and Thrissur experienced high soil temperature (40-46^{o}C)
    - Soil samples collected from Thrissur and Palakkad districts were having very low soil moisture content (1.1- 3.9%)
    - Soil samples procured from Alappuzha and Ernakulam districts were saline (EC 4.1-6.68 dSm^{-1}) and extremely acidic (pH 3.4- 4.5)

2. Soil samples were subjected to serial dilution and plating technique for enumeration and isolation of *Pseudomonas* spp.
    - Kings' B agar medium was found suitable for isolation of

- *Pseudomonas* spp. than *Pseudomonas* agar base (PAB) medium since comparatively less number of colonies were formed on PAB medium from all the soil samples
- Kings' B agar medium at 10^{-5} dilution was standardized for isolation of *Pseudomonas* spp.
- Maximum population of *Pseudomonas* spp. were recorded from Moncombu (1.3-7.8x10^{6} cfu g^{-1}), whereas, minimum in soil samples collected from Vyttila (3.14- 6.01 x 10^{4} cfu g^{-1})
- Soil samples from Thrissur district showed a population count of 2.3 – 9.12 x 10^{5} cfu g^{-1}
- Palakkad district yielded a population of *Pseudomonas* spp. ranging from 0.24- 0.74 x 10^{6} cfu g^{-1}
- After enumeration, a total of 15 isolates were obtained and thereafter,purified by quadrant streaking and designated with sample codes based on locations of collection *viz*., P for Palakkad, T for Thrissur, M for Moncombu and V for Vyttila
- Among the 15 isolates, nine were fluorescent which were subsequently screened for *P. aeruginosa,* an opportunistic human pathogen
- Two isolates M7 and P7 were eliminated as these were found as *P. aeruginosa* based on their growth at 42^{0}C and King's A medium

3. Thereafter, 13 isolates were subjected to *in vitro* screening for abiotic stress tolerance and compared with the reference culture of *P. fluorescens*
    - The isolates P2, M4 and T5 were selected as thermotolerant (50^{o}C), M4 and V4 as salt tolerant (1.5 M NaCl), P4 and T4 as drought tolerant (30% PEG) and M4 and M5 as acid tolerant (pH 3.5) strains

4. These seven abiotic stress tolerant isolates *viz*., M4, M5, P2, P4, V4, T4 and

T5 were evaluated further for their *in vitro* antagonistic potential against five major soil borne fungal pathogens (*Phytophthora capsici, Pythium aphanidermatum, Sclerotium rolfsii, Rhizoctonia solani* and*Fusarium oxysporum*)

- Among these, four isolates *viz*., P2, P4, M4 and M5 were selected as potential biocontrol strains which showed higher antagonistic activity showing a per cent growth inhibition ranging from 62.21 to 91.00 per cent against all the test pathogens
- Against *Pythium aphanidermatum*, the isolates P2 and P4 showed the highest growth reduction of 74.61 and 74.43 respectively
- The maximum inhibitory activity against *Fusarium oxysporum* was noted with the isolate P4 with a per cent growth reduction of 86.92
- Growth of *Phytophthora capsici* was found to be highly reduced by the isolate M5 with per cent inhibition of 75.56
- *Rhizoctonia solani* was suppressed by the isolate M4 with highest growth inhibition of 75.43
- The isolate P4 was found highlyantagonistic against *Sclerotium rolfsii* (91.00 %)

5. The four isolates *viz*., P2, P4, M4 and M5 were analysed for ACC deaminase, exopolysaccharide, cellulase and β-1, 3 glucanase
    - M4 and M5 showed comparatively higher ACC deaminase enzyme activity
    - Exopolysaccharide production was found best with M4 and P4
    - Highest cellulase was recorded by the isolate P2
    - Highest β-1, 3 glucanase production was recorded by M4

6. A preliminary screening of the four isolates for testing their bioefficacy against *Rhizoctonia solani* on cowpea seeds were carried out using roll towel method
    - The isolates were rated based on germination per cent and per cent disease incidence

- Isolate M4 was rated as highly efficient, reference culture as efficient and the other isolates P2, P4 and M5 as moderately efficient

7. All the four isolates were further evaluated against *R. Solani* under pot culture experiment using cowpea as test crop
    - M4 showed significantly highest seed germination, biometric characters and yield as well as with lowest per cent disease incidence
    - Other three isolates were also found superior or equally efficient as reference culture of KAU.

8. The best performing isolate with promising traits of stress tolerance, antagonism and plant growth promotion *viz*., P2, P4, M4 and M5 were subjected to cultural, morphological, biochemical and molecular characterization and the isolate P2 was identified as *Pseudomonas putida*, P4 as *P. fluorescens* and M4 and M5 as *P. aeruginosa.*

9. *Pseudomonas aeruginosa*, being an opportunistic human pathogen could not be used for further studies and hence, the present study identified two promising strains of *Pseudomonas* spp. *viz*., *P. putida* and *P. fluorescens* from Palakkad district with higher antagonistic potential and abiotic stress tolerance than the reference culture of KAU
    - *Pseudomonas putida* strain P2 with tolerance to high temperature
    - *Pseudomonas fluorescens* strain P4 with drought tolerance

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APPENDICES

APPENDIX-I

Composition of media used for serial dilution

1.King's B media(pH 7.2)

Peptone	:	20.0 g
Glycerol	:	10.0 ml
$MgSO_4.7H_2O$	:	1.5.0 g
K_2HPO_4	:	K_2HPO_4 : 1.5 g
Agar	:	20.0 g
Distilled water	:	1000 ml

2.King's A media (pH 7.2)

Peptone	:	20.0 g
Glycerol	:	10.0 ml
$MgSO_4.7H_2O$	:	1.5.0 g
K_2HPO_4	:	K_2HPO_4 : 1.5 g
Agar	:	20.0 g
Distilled water	:	1000 mL

APPENDIX-II

Composition of media used for bacterial ring method

Potato dextrose agar

Potato	:	200.0 g
Dextrose	:	20.0 g
Agar	:	20.0 g
Distilled water	:	1000 mL

APPENDIX-III

1.Composition of medium used for exopolysaccharide production

Sucrose	:	3.0 g
Na2HPO4	:	0.5 g
K2SO4	:	0.1 g
KH2PO4	:	0.3 g
MgSO4.7H2O	:	0.025 g
CaCb,2H2O	:	0.001 g
Distilled water	:	1000 ml

2.Composition of media used ACC deaminase assay

Dworkin and Foster salts minimal medium

Glucose: 2.0 g	2.0 g
Gluconic acid: 2.0 g	2.0 g
Citric acid : 2.0 g	Citric acid : 2.0 g
KH_2PO_4 : 4.0 g	KH_2PO_4 : 4.0 g
Na_2HPO_4: 6.0 g	Na_2HPO_4: 6.0 g
$MgSO_4.7H_2O$: 0.2 g	$MgSO_4.7H_2O$: 0.2 g

Micronutrient solution (10 ml)

$CaCl_2$	:	2.0 g
$FeSO_4.7H_2O$	:	2.0 g
H_3BO_3	:	2.0 g
$ZnSO_4.7H_2O$	:	4.0 g
Na_2MoO_4	:	6.0 g
KI	:	0.2 g
NaBr	:	10 mg
$MnCl_2$	:	10 mg
$COCl_2$	:	5 mg
$CuCl_2$	:	5 mg
$AlCl_3$	:	2 mg
$NiSO_4$	:	2 mg
Distilled Water	:	1000 ml
ACC	:	3 mM

3.**Composition of media used for enzyme assay**

a. Carboxy methyl cellulose medium

Sodium nitrate	:	2.0 g
Dipotassium phosphate	:	1.0 g
Magnesium sulphate	:	0.5 g
Potassium chloride	:	0.5 g
Carboxy methyl cellulose	:	2.0 g
Peptone	:	0.02 g
Agar	:	17.0 g
Distilled water	:	1000 ml

Appendix IV

Composition of media used for biochemical characterization of isolates

1. Nutrient gelatin agar medium (pH 6.8)

Peptone	:	5 g
Beef extract	:	3 g
Gelatin	:	120 g
Distilled Water	:	1000 ml

2. Urea agar medium(pH : 6.8)

Peptone	:	1 g
NaCl	:	5 g
KH_2PO_4	:	10 g
Agar	:	20 ml
Distilled Water	:	1000 ml

3. 1 % tryptone broth (Indole)

Peptone	:	10g
Distilled Water	:	1000 ml

4. SIM agar medium(H_2S) (pH 7.3)

Peptone	:	30g
Beef extract	:	3 g
Ferrous Ammonium Sulphate	:	0.2 g
Sodium thiosulphate		0.025 g
Agar	:	3 g
Distilled Water	:	1000 ml

5.MRVP broth (5 ml) (pH 6.9)

Peptone	:	7 g
Dextrose	:	5 g
Potassium phosphate	:	5 g
Distilled Water	:	1000 ml

6.Simmond's Citrate agar (pH 6)

Ammonium dihydrogen phosphate	:	1 g
Dipotassium phosphate	:	1 g
Sodium citrate	:	2 g
$MgSO_4$	:	0.2 g
Agar	:	15 g
Bromothymol blue	:	0.8 g
Distilled Water	:	1000 ml

7. Fermentation medium (carbohydrates) (pH : 7.3)

Peptone	:	10 g
Carbohydrate (lactose/ mannitol/ glucose/ dextrose/ sucrose)	:	5 g
NaCl	:	15 g
Phenol red	:	0.018 g
Distilled Water	:	1000 ml

CHARACTERIZATION AND EVALUATION OF *Pseudomonas* spp. FOR ABIOTIC STRESS TOLERANCE

Abstract of a Thesis

Submitted in partial fulfillment of
the requirement for the degree of

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by

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Characterization and evaluation of abiotic stress tolerant strains of *Pseudomonas* spp.

Abstract

Pseudomonas spp. are one among the most extensively used biocontrol agent in plant disease management to control foliar, soil borne or seed borne pathogens. However, prevalence of abiotic stresses such as drought, high temperature, salinity and acidity may affect the field performance due to poor survival under adverse conditions. To date, very little effort has been taken to tap microbial diversity from stressed ecosystems of Kerala. Thus, a study was undertaken to isolate native strains of *Pseudomonas* spp. having inherent stress tolerance.

Purposive soil sampling survey was conducted during the period February- April 2018 and a total of 26 representative soil samples were collected from four districts *viz.*, Ernakulam, Palakkad, Thrissur and Alappuzha. It was revealed that various locations of Palakkad and Thrissur district experienced high temperature (40-46^{o}C) and low moisture of 1.1-3.9 per cent. Samples procured from Alappuzha and Ernakulam districts were extremely acidic (pH 3.4- 4.5) andsaline (EC 4.1-6.68 dSm^{-1}). The results of soil analysis confirmed that the collected soil samples were abiotically stressed and there could be chances of obtaining stress tolerant native isolates of *Pseudomonas* spp. For isolation of *Pseudomonas* spp., soil samples weresubjected to serial dilution and plating technique in King's B agar and Pseudomonas agar base, among which King's B agar yielded optimum number of *Pseudomonas* colonies at 10^{5} dilution.Maximum population of *Pseudomonas* spp. was recorded in samples procured from Moncombu (1.3-7.8x10^{6} cfu g^{-1}), whereas, minimum population was observed in soil samples collected from Vyttila (3.14- 6.01 x 10^{4} cfu g^{-1}). A total of 15 isolateswere purified and designated with sample codes based on the place of collection. Among the nine fluorescent isolates, M7 and P7 were identified as *Pseudomonas aeruginosa*, an opportunistic human pathogen based on its growth at 42^{o}C and on King's A agar medium and thus, were eliminated.

Isolates of *Pseudomonas* spp. were subjected to *in vitro* screening for abiotic stress tolerance and compared with reference culture of KAU. The isolates P2, M4 and T5 were selected as temperature tolerant (50^{o}C), M4 and V4 as salt tolerant (1.5 M NaCl), P4 and T4 as

drought tolerant (30% PEG) and M4 and M5 as acid tolerant (pH 3.5) strains. Subsequently, a total of seven abioticstress tolerant isolates *viz*., M4, M5, P2, P4, V4, T4 and T5 were evaluated further for their *in vitro* antagonistic potential against five major soil borne fungal pathogens using Bangle method.The four isolates*viz*., P2, P4, M4 and M5 were selected as the potential antagonistic strains since they outperformed reference culture of KAU and showed higher and consistent biocontrol activity with per cent growth inhibition ranging from 62.21 to 91.00 per cent against *Phytophthora capsici, Pythium aphanidermatum, Sclerotium rolfsii, Rhizoctonia solani* and *Fusarium oxysporum.* Further studies revealed M4, M5 as better producers of ACC deaminase enzyme and M4, P4 as better producers of exopolysaccharide. Highest cellulase and β-1, 3 glucanase production was recorded by the isolates P2 and M4 respectively.

A preliminary screening of the isolates were carried out for testing the bioefficacy on cowpea seeds against *Rhizoctonia solani* using roll towel method. The results revealed that the isolate M4 was highly efficient, reference culture as efficient and the other isolates P2, P4 and M5 as moderately efficient.All the four isolates were further evaluated for their *in vivo* activity through a pot culture experiment using cowpea- *R. solani* system. Eventhough, M4 outperformed all other isolates with highest seed germination, biometric characters and yield as well as with lowest per cent disease incidence, other three isolates were also found superior or equally efficient as reference culture of KAU. The best performing isolates with promising traits of stress tolerance, antagonism and plant growth promotion *viz*., P2, P4, M4 and M5 were identified based on cultural, morphological, biochemical and molecular characterization. The isolate P2 was identified as *Pseudomonas putida*, P4 as *P. fluorescens* and M4 and M5 as *P. aeruginosa.*

Thus, the current investigation has thrown light on the prevalence of abiotic stress tolerant strains of *Pseudomonas* spp. in stressed ecosystems which would significantly help the farming community to overcome such drastic climate changes. However, the study should further be complemented with large scale multilocational trials to prove their efficacy under field conditions.