Rajesh Kannan Velu
Editor

Microbiological Research in Agroecosystem Management

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Preface

It is my great pleasure to bring out this edited volume of the articles presented in the "National Conference on Microbiological Research in twenty first Century" (NCOMRIT-12), conducted at Bharathidasan University, Tiruchirappalli, India, during 27–28 February 2012. This conference focused on four interesting and vital fields in Microbiology such as: agriculture and biodiversity, bioprospecting and drug discovery, environment and bioremediation and medical and infectious diseases. We have already published the findings on bioprospecting and drug discovery and medical and infectious diseases in the special issue "International Journal of Medicobiological Research" (Volume 1: Issue 6: August 2012), a peer reviewed refereed journal. The remaining selected articles in the fields of agriculture and biodiversity and environment and bioremediation are edited and published in this volume which the editor hopes are a valuable contribution to the field of agroecosystem management by microbial facilitations.

The core aim of this volume is to provide a broad spectrum of agroecosystems structure, function and maintenance involved in microbial research. I assume as stated above, that agriculture and biodiversity and environment and bioremediation might fulfill this objective. This book consists of 20 research articles focusing on the emerging problems in the field and the positive findings. The findings are that of the authors concerned. These articles are arranged progressively linking themselves thematically. The editor hopes that these would prompt the budding scholars to further their research which in turn would certainly help the agriculturists.

At this juncture, I would like to convey my deep appreciation to all contributors including the accepted manuscript and other authors, who submitted their manuscript that could not be accommodated in this edition due to time and space constraints. I am indebted to my Professor Dr. M. Murugesan, Professor of English for his constant encouragement and constructive suggestions. My special thanks are due to Ms. Richa Sharma, Dr. Mamta Kapila and Ms. Deepshikha Chauhan from Springer (India), and all my research scholars for their great effort in publishing the book. In addition, I record my sense of gratitude to Springer (India) private limited and Bharathidasan University for their strong co-operation and encouragements.

Rajesh Kannan Velu

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Dr. Rajesh Kannan Velu is currently working as Assistant Professor of Microbiology, Bharathidasan University, Tamil Nadu, India. He extends his service as member of board of studies in Microbiology, Biotechnology, Botany and Life Sciences and member reviewer of more than 25 international reputed scientific journals in regular and also a member of editorial board in several scientific publications. His field of specialization is "Rhizosphere Biology".

He has published 75 research papers and several review articles in international refereed journals. Further, he has contributed 55 microbial strains to NCBI Genbank. He has organized two scientific conferences at the national level and delivered several talks in Tiruchirappalli All India Radio. He is an active member of the Indian Science Congress Association, International Mycorrhizae Research Society, International Plant–Microbes Interactions Association, International Ecological Association, Indian Association of Biomedical Scientists and Association of Microbiologists of India.

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A Probe on the Status of Microorganisms in the Air, Soil and Solid Waste Samples of Ariyamangalam Dumping Site at Tiruchirappalli District, South India

A. Chandramohan, V. Sivasankar, C. Ravichandran, and R. Sakthivel

Abstract

This research contribution is concerned with the microbial occurrence in air, groundwater, soil and solid wastes around a dumping site with an area of about 48 acres located at Ariyamangalam of Tiruchirappalli District, South India. The groundwater and soil samples were collected within a radius of 2 and 0.5 kms respectively. Severe microbial dominance in the groundwater samples was inferred from the MPN per 100 ml of total coliforms. The Karl Pearson correlation was carried out for the total coli forms with the physico-chemical properties of the water samples. The correlation study ascertained the influence of nitrogen and its oxy anions on the growth of microorganisms in operating the biogeochemical cycle. The aero-microbial study conducted at the dumping site registered the survival of six genera each of bacterial and fungal organisms which alleviate the possibility of hazards to the environment and residents' health. Various harmful and infectious diseases that spread through the microorganisms originated from the dumping site are also discussed in this chapter.

Keywords

Microbial contamination · Air · Water · Soil · Solid wastes · Ariyamangalam

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Introduction

Microorganisms are generally highly sensitive to the surrounding environments. In addition, water quality is strongly influenced by the dynamics of microbial community and ecosystem functions (organic matter contents and nutrient recycling). Coliforms, a group of common bacteria, live in soil, water, and in the digestive tracts of humans and animals. Most common coliforms are harmless and serve as significant components in

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the digestive system. However, some coliforms may cause illness in humans. *Escherichia coli*, faecal coliforms and other types of harmful bacteria are indicators of contamination in water. Ingestion of water containing coliform bacteria increases the risk of water-borne illness. The Centre for Disease Control (CDC) estimates that 900–1,000 people die each year as a result of microbial contamination of drinking water (Borkovich 2008). The World Health Organisation has estimated that diarrhoea kills annually 2.2 million people worldwide (WHO 2006). Diarrhoeal illness remains a major killer in children and it is estimated that 80 % of all illnesses in developing countries is related to water and sanitation; and that 15 % of all child deaths under the age of 5 years in developing countries result from diarrhoeal diseases (Thompson and Khan 2003; WHO/UNICEF 2000, 2004). Lang et al. (2001) have estimated that 35 % of the total reported gastroenteritis is due to water-related microbial contamination.

Total coliforms can also survive and grow in water distribution systems, particularly in the presence of biofilms (WHO 2004). The use of bacteria as water quality indicators can be viewed in two ways: first, the presence of such bacteria can be taken as an indication of faecal contamination of water and thus as a signal to determine why such contamination is present, how serious it is and what steps can be taken to eliminate it; second, their presence can be taken as an indication of the potential danger of health risks that faecal contamination poses (Papini et al. 2005; Ryu et al. 2005; McQuaig et al. 2006). Water-borne transmission of pathogenic *E. coli* has been well documented for recreational and contaminated drinking waters. A well publicised water-borne outbreak of illness caused by *E. coli* O157:H7 (and *Campylobacter jejuni*) occurred in the farming community of Walkerton in Ontario, Canada (WHO 2004).

The presence of microorganisms in groundwater is heavily dependent upon geologic conditions such as flow pathways and mechanisms, sunlight, temperature, pH and soil properties. The type, size and activity of the microbial community are also important factors that influence the transport of microorganisms. These organisms can cause intestinal infections, dysentery, hepatitis, typhoid fever, cholera, gastroenteritis and other problems.

Microorganisms abound in the soil and are critical to decomposing organic residues and recycling soil nutrients. There are more microbes in a teaspoon of soil than the people on the earth. Soils contain about 8–15 tonnes of bacteria, fungi, protozoa, nematodes, earthworms and arthropods. Bacteria are the smallest and most hardy microbes in the soil and can survive under harsh conditions like tillage. Bacteria are only 20–30 % efficient at recycling carbon, have high nitrogen content (3–10 carbon atoms to 1 nitrogen atom or 10–30 % nitrogen), lower carbon content and a short life span. Soils that are biologically active and have higher amounts of active carbon recycle release more nutrients for plant growth than soils that are biologically inactive and contain less active organic matter.

Micro-organism populations change rapidly in the soil as soil organic matter (SOM) products are added, consumed and recycled. The amount, the type and availability of the organic matter will determine the microbial population and how it evolves. Each individual organism has certain enzymes and complex chemical reactions that help the organism to assimilate carbon. As waste products are generated and the original organic residues decompose, new micro-organisms may take over, feeding on the waste products, which is the new flourishing microbial community, or the more resistant SOM. The early decomposers generally attack the easily digested sugars and proteins followed by micro-organisms that attack the more resistant residues. Bacteria are generally less efficient at converting organic carbon into new cells. Aerobic bacteria assimilate about 5–10 % of the carbon while anaerobic bacteria only assimilate 2–5 %, leaving behind many waste carbon compounds and inefficiently using energy stored in the SOM. Soil organic matter is composed of mostly carbon, but associated with the carbon it has high amounts of nitrogen and sulphur from proteins, phosphorus and potassium. Factors such as moisture, pH,

soil depth and particle size affect SOM decomposition. Hot, humid regions store less organic carbon in the soil than dry, cold regions due to increased microbial decomposition.

Keeping in mind the various beneficial and harmful effects of microbial organisms, this contribution is focused on the microbial contamination of air, soil and water due to the influence of solid wastes at the proximity of the dumping yard located at Ariyamangalam of Tiruchirappalli District in South India.

Materials and Methods

The present study area is a dumping site located at Ariyamangalam of Tiruchirappalli District, South India (Fig. 1). Ariyamangalam dumping site of about 48 acres is situated on the eastern side of the Tiruchirappalli–Thanjavur National Highway.

Sampling

Microbial Examination

Groundwater samples were collected from the north, south, east and west of the dumping site at a radius of 2 km during the summer and winter seasons. A total of 144 samples (72 each in summer and winter) from 12 stations were collected in both the seasons. Microbial examination (TC per 100 ml) was carried out on all the six samples at each station and an average of these values is given.

Groundwater Samples

Four replicate tubes of lauryl sulphate tryptose (LST) broth were inoculated with 10 mL (double strength), then with 1 mL of sample homogenate decimal dilutions (1:1, 1:10, 1:100, 1:1000 or higher dilutions). Tubes were incubated for 48 h at 35 ± 0.5 °C. Tubes were read for gas production after 48 h. The LST tubes that showed positive gas formation within 48 h were recorded. Results were tabulated from MPN tables and reported as presumptive coliform bacteria in MPN per ml.

Air, Soil and Solid Waste Samples

The Air samples were collected by open plate method in and around the dumping site. Microorganisms were identified based on their biochemical properties (Benson 2002).The soil and solid waste samples were serially diluted (10^{-4}, 10^{-5}) and sub-cultured on nutrient agar using pour plate techniques and the total bacterial counts determined after 36 h of incubation at 37 °C using methods as described by Olutiola et al. (2000). Slant agar was prepared in Bijou bottles using nutrient agar. After 24 h of incubation, the plates were examined for growth and distinct colonies were picked on the incubated plates and subcultured on freshly prepared nutrient agar to obtain pure strains, which were kept in the sterile slant agar. Pure cultures of isolates were kept on nutrient agar slants at 12 °C until used. The isolates were identified on the basis of cellular morphology following Gram stain and results of biochemical testing, including catalase production, growth in 6.5 % NaCl broth, haemolytic activity and motility (Devriese et al. 1992).

Results

Total Coliforms in Groundwater

The MPN test revealed total coliforms per 100 ml of water samples indicating the poor quality of water in the bore well sources. The maximum value of 30.5 TC per 100 ml in summer and 16.4 TC per 100 ml in winter was examined for the water from bore well sources (Fig. 2). The Karl Pearson correlation values revealed that TC was moderately correlated with the physico-chemical parameters, viz., pH

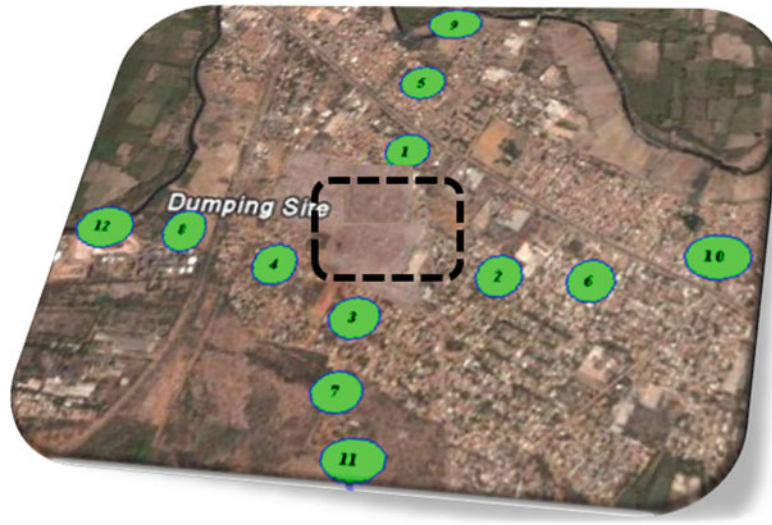

Fig. 1 Aerial view of the dumping site at Ariyamangalam, Tiruchirappalli District, South India

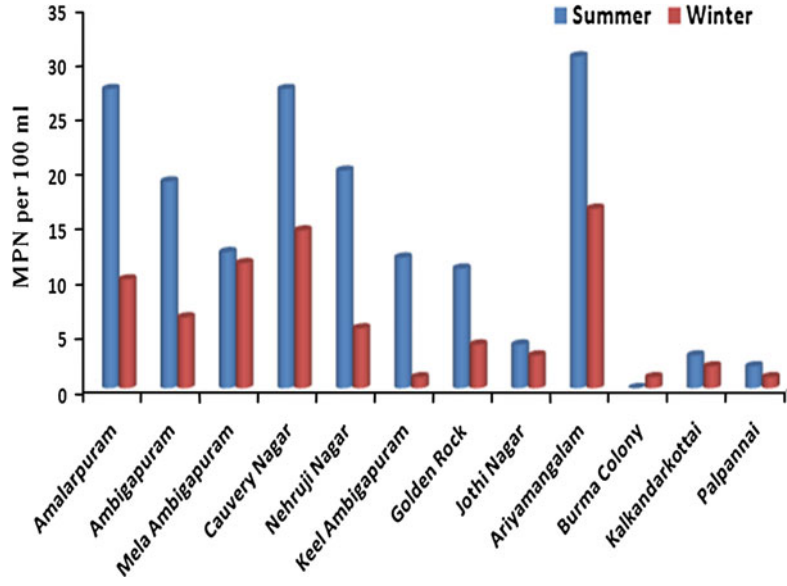

Fig. 2 MPN per 100 ml in the groundwater samples at the dumping yard at Ariyamangalam of Tiruchirappalli District, South India

(0.422), chemical oxygen demand (0.455), Kjeldahl Nitrogen (0.650), nitrate (0.411) and Fe (0.441) in the winter season. The physico-chemical parameters with moderate correlation with TC in summer were electrical conductivity (0.416), chemical oxygen demand (0.690), total hardness (0.451), Kjeldahl Nitrogen (0.631), nitrite (0.420), Fe (0.487) and Na (0.405). The correlation values between total coliforms and other physico-chemical parameters are given in Table 1.

Microbiological Population in Air

The aero-microbial study conducted at the dumping yard at Ariyamangalam identified five genera of bacteria and six genera of fungi. The northern side of the dumping yard was examined with only one type of bacteria, *Bacillus* sp., whereas the southern side was studied with four types of bacterial and fungal species. The eastern side was observed with four bacterial species but one genus of bacteria and three genera of

Table 1 Karl Pearson correlation values for total coliforms with the other physico-chemical parameters in groundwater

Physico-chemical parameters	Correlation value	
	Summer	Winter
pH	−0.132	0.422
Electrical conductivity	0.416	−0.091
Chloride	0.175	0.054
Sulphate	0.184	0.012
Total hardness	0.451	0.097
Dissolved oxygen (DO)	−0.377	−0.328
Biochemical oxygen demand (BOD)	0.668	0.258
Chemical oxygen demand (COD)	0.690	0.455
Kjeldahl Nitrogen	0.631	0.650
Nitrate	0.320	0.411
Nitrite	0.420	0.381
Fe	0.487	0.441
Na	0.445	0.098
K	0.253	0.346

fungi dominated on the western side. The bacterial population on the northern and western sides were found with only one genera, i.e. *Bacillus* sp. but the eastern and southern sides were examined with four genera of bacterial organisms. The population of fungal species was absent in the northern and eastern sides but they were almost equally populated in the western and southern sides.

Bacteriological Contamination in Soil and Solid Waste

The bacterial and fungal organisms were isolated from the soil samples within a radius of 0.5 km in north, south, east and west directions. Six genera of bacteria and two genera of fungi were examined in all the directions of the dumping yard at Ariyamangalam. The bacterial organism of *Pseudomonas* sp. and fungal organism, *Rhizopus* sp. were also isolated in the soil samples in all directions of the dumping site. The bacteriological examination of the solid waste at the dumping yard showed the presence of five genera of bacterial organisms. The organisms, *Enterobacter* sp. and *Klebsiella pneumonia,* were found in addition to the organisms that were present in the soil and air samples. The identification of certain micro-organisms in soil samples is shown in Figs. 3 and 4.

Discussion

The various microorganisms that were isolated from the air, soil and solid waste samples are listed in Table 2. The most probable number is a suitable and most widely used method to determine the microbial quality of water. The excessive bacterial population indicates that the water is highly contaminated with microorganisms and is hazardous for drinking purposes, as its consumption leads to various water borne diseases. Current investigations have rendered values that exceed the permissible limit of WHO (0 coliforms/100 ml). The presence of >10 coliforms/dl in water is designated as polluted or unhealthy for drinking purpose (APHA 1998). The examined total coliform in all the bore well samples exceeded the limit of WHO and The Bureau of Indian Standards. This was evident from the bacteriological contamination of the bore well sources located in the north, south, east and west of the dumping yard at Ariyamangalam in Tiruchirappalli District.

The level of total coliform contamination of all water samples in the four directions may be associated with the anthropogenic activities and defecation by domestic animals. The presence of total coliform bacteria in drinking water causes various water-borne diseases like nausea, vomiting, diarrhoea, gastroenteritis, etc. (WHO 1980; Daniels et al. 2000; Paul 2003).

The movement of sewage and leachate in the ground may also be attributed to the ultimate deterioration of the water table and hence the groundwater sources (Ferguson et al. 1996). It may also be corroborated that the use of contaminated drawers/containers to draw water from some wells is another source of contamination. In addition to the above facts, most of the pathogens from faecal matter remain near the

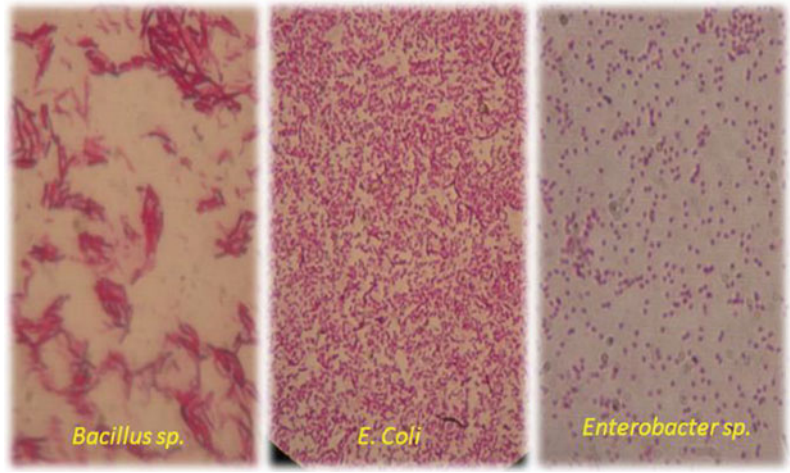

Fig. 3 Some identified bacterial organisms isolated from soil at the dumping site

Fig. 4 Microbial population of soil sample in nutrient and blood agar media

point of origin or source and may travel along with the water flow through pores in the surrounding soil and may enter the well through cracked drums/casing. It is more appropriate to account the inter-dependence of total coliforms, Kjeldahl Nitrogen, nitrate, nitrite and chemical oxygen demand from the correlation values both in the summer and winter. Based on the correlation values, the oxygen demand in water as a consequence of organic matter enhances the growth of bacteria and fungi in the aquatic system. The moderate correlation values between Fe and TC in both summer and winter indicate the consumption of iron species for the growth of microorganisms. The inter-dependence between pH and total coliform (0.422) during winter suggests the growth of microorganisms as a function of temperature. Poor correlation between chloride and total coliforms (0.175 in summer and 0.054 in winter) may be attributed to the inhibiting action of chlorine on the growth of microorganisms (Abdulrahman and Eltahir 2011).

The aero-microbial investigation of the present study is almost similar to earlier reports (Rylander et al. 1964; Crook et al. 1986; Rahkonen et al. 1990; Markanday et al. 2004) at the proximity of sanitary landfills and waste water treatment plants. Most of the bacterial organisms identified were opportunistic pathogens, which may cause infections, and can commonly occur in the air, soil, plants, food and water. The improper and careless handling of solid wastes during ultimate disposal by municipal workers in the field may be considered as the risk causing factors to the environment and health of the populace. Also, the main risk for the residents may be deemed as a result of

Table 2 List of microorganisms in air, soil and solid waste samples from the dumping site at Ariyamangalam in Tiruchirappalli District, South India

Microorganisms	Air				Soil				Solid waste
Bacteria	N	E	S	W	N	E	S	W	
Bacillus subtilis	✔	✔	✔	✔					
Bacillus megaterium					✔	✔	✔	✔	
Bacillus sp.	✔	✔	✔	✔					✔
Clostridium sp.		✔	✔						
Enterobacter sp.									✔
Escherichia coli					✔	✔	✔	✔	✔
Flavobacterium sp.					✔	✔	✔	✔	
Klebsiella pneumonia									✔
Micrococcus sp.		✔	✔		✔	✔	✔	✔	
Pseudomonas sp.	×	×	×	×	✔	✔	✔	✔	✔
Serratia sp.			✔						
Staphylococcus sp.		✔			✔	✔	✔	✔	
Streptococcus faecalis					✔	✔	✔	✔	
Streptococcus sp.									✔
Fungus									
Alternaria sp.			✔						
Aspergillus fumigates					✔	✔	✔	✔	
Aspergillus niger			✔						
Cladosporium sp.				✔					
Fusarium sp.			✔						
Mucor sp.				✔					
Penicillium sp.			✔	✔	✔	✔	✔	✔	
Rhizopus sp.					✔	✔	✔	✔	✔

breeding—disease vectors (such as flies, mosquitoes, etc.), stray animals and birds (Fig. 5). The spread of solid waste by stray animals like dogs, cows, etc., is a nuisance to a wider area (Markanday et al. 2004). Workers who dwell at the proximity of the disposal site were found infected with various pathogens which cause diseases (Kejding 1964; Watt and Lindsay 1984).

Aspergillus that originated from the dumping yard is well known for the spoilage of varieties of food materials and also produce aflatoxin and mycotoxin which are responsible for the biodegradation of foods and vegetables. A research report by Kannan et al. (1994) has stated that high amounts of aflatoxins present in contaminated food exert their toxicological effect on animals and man. *Mucor* sp. in the air environment is the causal organism of fruit and vegetable rot, besides being responsible for the mycoses of the lungs in human beings. *Aspergillus fumigates* is known to be associated with dust and their endotoxins in landfills and compost plants (Clark et al. 1983). The isolated spores of the bacterial organisms, *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp., reflected the contamination of pesticides and heavy metals in these soils (Abou et al. 2008). The abundance of these bacteria was typical of soil environment with high species richness and functional diversity. The presence of *Enterobacter* sp. in solid wastes has been noted in intravascular device-related infections, surgical site infections and extra-intestinal infections (Farmer et al. 2007; Russo and Johnson 2008). *Enterobacter* sp. is also known for nitrogen fixation in soils. It is a free-living nitrogen fixer that is distributed to the soil matrix via animal

Fig. 5 Status of dumping site at Ariyamangalam, Tiruchirappalli, South India

faeces. These N_2-fixing microbes grow in humid environments on leaf surfaces or in leaf sheaths, the soil and root surfaces (Akpor et al. 2006). *Flavobacterium* in soils are concentrated around the rhizosphere.

Klebsiella pneumonia is found in the respiratory, intestinal and urinogenital tracts of animals and man. When it gets outside of the gut, serious infection can occur. It tends to affect people with underlying illnesses such as alcoholism, diabetes and chronic lung disease. Infectious diseases are caused by the presence of microorganisms in the soil.

Conclusion

This study cautions that the open dumping of garbage serves as a breeding ground for disease vectors such as flies, mosquitoes and cockroaches which leads to detrimental effects on the environment and human health. Improper dumping and poor monitoring of solid wastes lead to high risk of spreading diseases like typhoid, cholera, dysentery, yellow fever, encephalitis, plague, malaria and dengue fever through vectors carrying harmful microbes. The correlation values of total coliform with chemical oxygen demand, nitrogen, nitrate and nitrite revealed the execution of the biogeochemical cycle in the environment and the dependence of microorganisms on soil nutrients for their growth. The groundwater samples around the dumping site were severely contaminated with various biotic and abiotic factors such as anthropogenic activities, stray animals and leachate from the solid waste dumping site. The government should come forward with strict norms and regulations to monitor the dumping site and also to take care of the laborers who work in the field without proper safety measures. Awareness of infectious diseases through air and soil need to be conducted for the workers and residents of this Ariyamangalam area in Tiruchirappalli district.

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Antifouling Activity of Prodigiosin from Estuarine Isolate of *Serratia marcescens* CMST 07

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Abstract

Microbial biofilms on the surfaces of man–made structures in the marine environment cause serious problems for marine industries. Currently used heavy metal-based toxic antifoulants has created environmental problem, which mandates the necessity of "eco-friendly" antifoulants. Marine-based microbial secondary metabolites are promising potential sources of nontoxic antifouling compounds. In the present study, we have investigated the antifouling potentials of bacterial red pigment prodigiosin extracted from *Serratia marcescens* CMST 07. Prodigiosin was showed high antifouling activity against marine fouling bacteria like *Alteromonas* sp. and *Gallionella* sp. minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the pigment was about 6.75 and 12.5 μg/ml respectively against *Alteromonas* sp. LD_{50} of prodigiosin against artemia (artemia toxicity study) was about 50 μg/ml. Prodigiosin significantly ($P < 0.01$) inhibits cyanobacterial adhesion on glass surface, which augments the possibility of using bacterial pigments as the source of antifouling compounds for controlling the fouling problem in the marine environments.

Keywords

Antifouling · Bactericidal · Prodigiosin · *Serratia marcescens*

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Introduction

Marine biofouling is a serious problem caused by the accumulation and settlement of microbial slimes, diatoms, barnacles, tunicates, bryozoans etc., on the hulls of seafaring vessels (Clare 1996; Abarzua et al. 1999; Bhosale et al. 2002; Railkin 2003; Rasmussen and Ostgaard 2003). Biofouling on ship results in an increase in roughness of the hull that increases frictional resistance leads

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to increased fuel consumption and associated environmental compliances (Depree 2006). Globally, billions of dollars are spent annually to control fouling on a variety of objects that are placed in marine environment. Currently available marine antifoulants are heavy metal-based that possess hazardous environmental problems (Bellas 2006; IMO 2007; Qian 2010; Thomas and Brooks 2010). Extension of this research area is essential to identify novel effective non-toxic compound having potent anti-micro and macro fouling properties. These biogenic compounds could also be used effectively for future development of antifouling paints (Hellio et al. 2001; Fusetani 2004; Greer et al., 2003). Naturally, marine environments harbor highly diverse microbial communities, which possess functionally undesirable and unexplored potentials (Whitman et al. 1998; Rappe and Giovannoni 2003), and they produce a variety of chemical deterrents for their defense purposes (Ren et al. 2001; Kubanek et al. 2002; Paul and Puglisi 2004). These bioactive compounds of marine microbial origin exhibit antifouling activity against variety of micro and macro foulants (Hellio et al. 2001).

Prodigiosin is a red-pigment produced as a secondary metabolite by *Serratia*, *Streptomyces*, *Pseudomonas*, *Pseudoalteromonas*, and few other bacteria, which share a common pyrrole dipyrromethene core structure and have a wide variety of biological properties, including antibacterial, antifungal, immunosuppressive, and anticancer activities (Bennett and Bentley 2000; Montaner and Perez-Thomas 2003). The present investigation reports the potential of red pigment prodigiosin extracted from estuarine bacteria *Serratia marcescens* CMST 07 for controlling the growth of marine micro and macro foulants.

Materials and Methods

Bacterial Strains

Red pigment prodigiosin producing *Serratia marcescens* CMST 07 (estuarine isolate) was obtained from Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, and the marine fouling bacteria such as *Bacillus* sp., *Pseudomonas* sp., *Alteromonas* sp. and *Gallionella* sp. were obtained from Department of Zoology, Scott Christian College, Nagercoil. All bacterial cultures were stored in ZoBell marine agar (Himedia, India) at 4 °C.

Production, Extraction, and Characterization of Prodigiosin

Serratia marcescens was grown in nutrient broth (Giri et al. 2004) with 1.5 % NaCl at room temperature in shaking condition (150 rpm) for 24 h and further incubated at 28 °C for 72 h in static condition under dark. After the incubation, cells were harvested by centrifugation at 8,000 rpm for 15 min (Remi, India). Prodigiosin was extracted using chloroform: Methanol mixtures of increasing polarity (2:1 and 1:2 v/v), until the solution remains colorless (Nakashima et al. 2005). The crude extract was evaporated to dryness and the amount of pigment obtained on a dry weight basis was calculated. The resulting product was identified as prodigiosin by UV-visible spectrophotometry in the range 200–700 nm in 95 % ethanol and further subjected to thin layer chromatography (TLC) for further purification using the mixture of chloroform and methanol (9:1) as the solvent system (Casullo de Araujo et al. 2010). RF value of the extract was compared with standard prodigiosin (Sigma).

Antibacterial Assay

Antibiotic assays against four fouling bacteria were carried out using standard disk diffusion method (Bauer et al. 1966). The extracted prodigiosin pigment was filter sterilized by passing through Syringe driven filter (0.25 μm pore size; Himedia, India). The four different fouling bacteria were subcultured with marine broth (Himedia, India) for 12 h at 30 °C. Each bacterial strain was inoculated onto marine agar plates and then

dried Himedia sterile disks (6 mm) impregnated with 20 μg of crude prodigiosin were positioned on them. Disk impregnated with DMSO was maintained as controls to determine possible inhibitory activity of the solvent. The diameter of the inhibition zone around each disk was measured after the incubation at 30 °C for 24 h.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The pigmented prodigiosin obtained from the *S. marcescens* was diluted with DMSO at the standard concentration (100 μg/ml) and then two fold serially diluted up to the final concentration of 3.275 μg/ml in marine broth of 32 well microtiter plates. Thereafter, 100 μl of inoculum at a concentration of 1×10^7 CFU/ml was added to each well (Basri and Fan 2005). The microtiter plates were incubated at room temperature for 24 h. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms. Following MIC determination, MBC was determined by subculturing an aliquot of 50 μl from each well showing no apparent growth. Least concentration of extract showing no visible growth on subculturing was taken as MBC.

Antifouling Assay

Biofilm Inhibition Assay

Different concentration of prodigiosin was used to evaluate the inhibition of biofilm formation. The overnight grown culture of biofilm bacteria was transferred to the microtitre plate (2×10^8 CFU/ml), and different concentrations of prodigiosin were added to each well to the final volume of 200 μl. The plates were incubated for 24 h at 37 °C, and then bacterial biofilm was evaluated using crystal violet staining method (Maldonado 2007). Bacteria without prodigiosin treatment were used as control.

Artemia Bioassay

Brine shrimp *Artemia parthenogenetica* (KKT1) was collected from Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam. Brine shrimps were cultured in seawater at 25 ± 2 °C for 2 days before bioassay. Different concentration of prodigiosin coated Petri dishes were filled with 15 ml filtered seawater. Approximately 15 larvae were transferred to each plate and prodigiosin free plates were used as control. The plates were incubated at 25 ± 2 °C for 72 h with a light–dark cycle of 13:11 h. Dead and immobile larvae were counted after 24 and 48 h, respectively.

Cyanobacteria Adhesion Assay

Pure culture of the marine cyanobacteria *Synechococcus* sp. was cultured in sterile ASN III medium. The influence of prodigiosin on the growth of *Synechococcus* sp. was investigated by the direct contact test. Sterile microscopic slide was coated with prodigiosin as the contact surface. Uncoated slides were used as control. Slides were immersed in sterile 100 ml seawater adjusted with nutrients (seawater enrichment media) and 8×10^7 cells/ml *Synechococcus* sp. was added and incubated for 48 h under a light source of 1,000 X with a light–dark cycle of 13:11 h at 28 °C. Attached cells were counted by microscopically at 450 X magnification.

Barnacle Settling Assay

Balanus amphitrite larvae were obtained from adult barnacles collected from the Colachal coast (April, 2010). They were continuously fed with the brine shrimp *Artemia salina*. Newly settled barnacles were kept in aquaria with flowing, filtered seawater. Larvae were collected

by filtration (450–490 mm mesh size filter). Larvae were transferred to a 1.5 L flask with aerated seawater (27 °C) and fed with microalgae (Clare 1996). The cyprid stage barnacles were used for settling study. Settling test was performed using sterile Petri dishes and different concentrations of prodigiosin were coated (from 50 to 200 μg/ml). After drying, the experimental Petri dishes were filled with (5 ml) filtered seawater for 3 days and the dishes were replenished with 5 ml of fresh filtered seawater and then 20 cyprids transferred to each and assayed for settlement after 3 days.

Statistical Analysis

All the experiments were performed in triplicates to ensure probability and reproducibility of the results. One-way ANOVA analysis was used to test for significant differences between the concentration of prodigiosin on antifouling activity against fouling bacteria, artemia survival, attachment of cyanobacteria, and barnacle bioassay.

Results

The crude red pigment extracted from *S. marcescens* was purified by TLC and had the same *Rf* value (0.89) as compared with prodigiosin reference material (Fig. 1). The maximum absorption of the pigment was analyzed using UV–visible spectrophotometer at 531 nm.

Antibacterial Activity of Prodigiosin

The extracted prodigiosin was assessed for the antibacterial activity against different fouling bacteria (Figs. 2, 3). Prodigiosin exhibited a broad range of antibacterial activity as it inhibited both Gram-positive bacteria (*Bacillus* sp. 8.3 ± 1.52) as well as Gram-negative bacteria (*Alteromonas* sp. 16.3 ± 2.08; *Gallionella* sp. 9.3 ± 1.15; *Pseudomonas* sp. 6.3 ± 1.52). Further, the MIC of red pigment from *S. marcescens* against biofouling bacteria was examined to determine the lowest concentration of antibacterial material require to inhibit cell growth completely (Table 1). Red pigment at a concentration of 12.5 < mg/l had showed antibacterial activity against *Alteromonas* sp., while *Gallionella* sp. was completely inhibited at a concentration of 25 < mg/L. *Bacillus* sp. and *Pseudomonas* sp. were completely inhibited at higher concentration (100 mg/l). The MBC values of prodigiosin against different fouling bacteria were examined and shown in Table 1. Prodigiosin exhibited the low value of MBC as no viable cell growth was observed for *Alteromonas* sp. and *Gallionella* sp. at concentrations of 25 μg/ml and 50 μg/ml respectively on the solid medium. For *Bacillus* sp. no viable cell growth was observed at a concentration of 100 μg/ml, whereas viable growth was observed for *Pseudomonas* sp. even at the higher concentration tested in this study.

Antifouling Against Fouling Bacteria

Antifouling activity of prodigiosin was examined for its ability to inhibit fouling potential of fouling bacteria and shown in Fig. 4. Prodigiosin inhibits the biofilm formation at the concentration of 100 μg/ml against *Alteromonas* sp. and 200 μg/ml against *Gallionella* sp. For *Bacillus* sp. more than 90 % of the biofilm formation was inhibited at a concentration of 200 μg/ml, while only 40 % inhibition was observed against *Pseudomonas* sp. even at the higher concentration.

Artemia Survival Study

Brine shrimp lethality test has been previously used to evaluate bioactivity of the new metabolite (Meyer et al. 1982). Similarly, the inhibitory effect of prodigiosin on the survival of brine shrimp *Artemia* was studied at different concentrations of extracted red pigment (Fig. 5). Compare to control low concentration of prodigiosin also induces mortality of *Artemia* larvae. Prodigiosin at 50 μg/ml concentrations have shown 50 % (46.67 %) lethal effects after

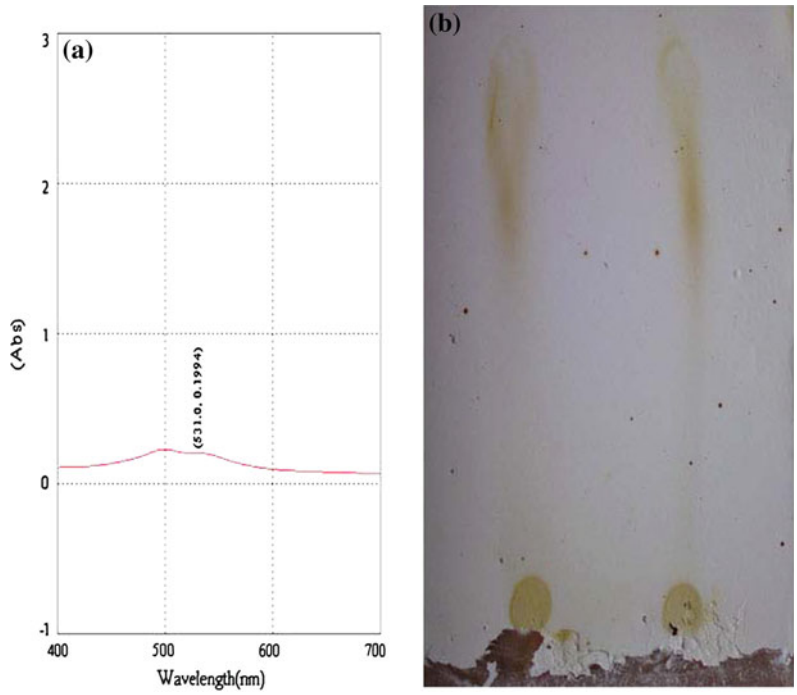

Fig. 1 Characterization of red pigment prodigiosin. **a** UV Spectrophotometric characterization of prodigiosin. **b** TLC analysis of prodigiosin. *M* standard prodigiosin (*Sigma*); *S* extract from *S. marcescens* cmst 07

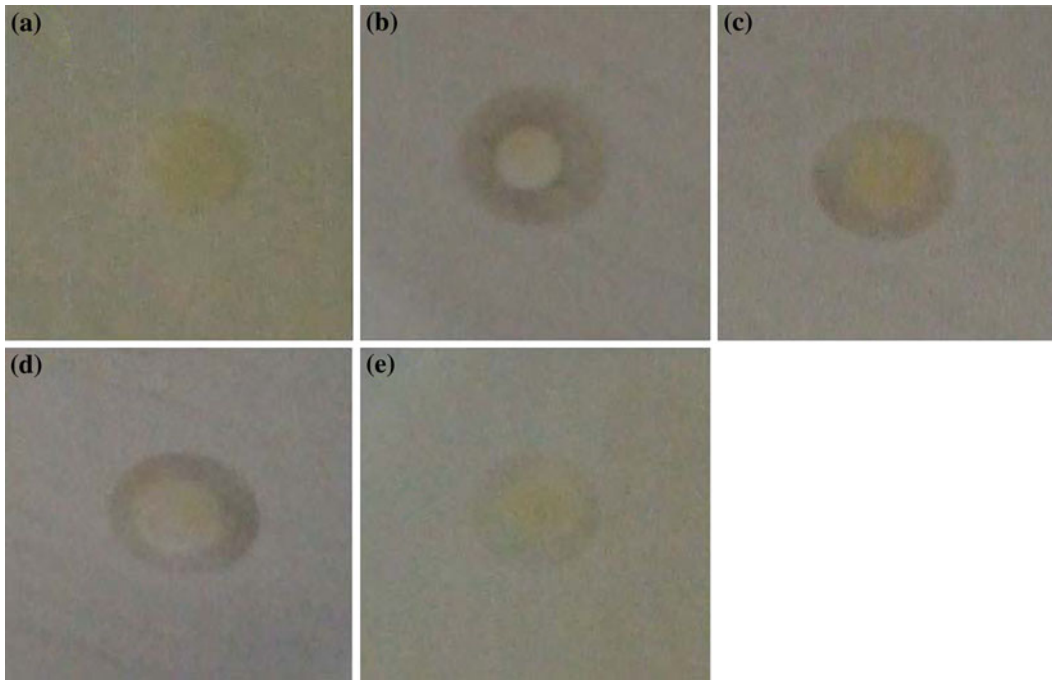

Fig. 2 Antibacterial activity of prodigiosin against marine fouling bacteria (10 µg/disk). **a** control, **b** *Alteromonas* sp., **c** *Gallionella* sp., **d** *Bacillus* sp. **e** *Pseudomonas* sp

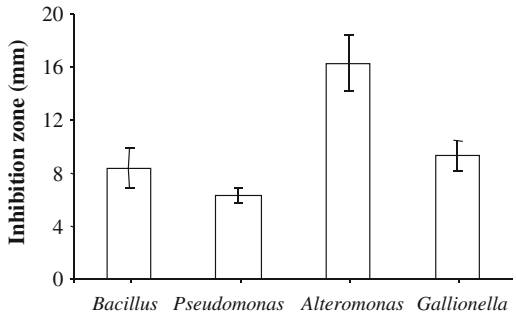

Fig. 3 Antibacterial activity of prodigiosin. Sterile antibiotic disk with 20 μg of prodigiosin was placed on agar with respective bacteria. All values are mean of three individual replicates with ±SE

24 h of exposure, which can be considered as LD_{50}. Exposure to potassium dichromate was treated as positive control that exhibited significant lethality (LC_{50} value < 1.0 mg/ml) against the brine shrimp (data were not shown). With the comparison to the positive control, prodigiosin had shown higher lethality against *Artemia* survival.

Anti-algal Assays

The inhibitory effect of prodigiosin against fouling prokaryotic algae was assayed using the cyanobacteria *Synechococcus* sp. and the results have demonstrated that the concentration-dependent inhibition of cyanobacterial attachment on glass surfaces by prodigiosin (Table 2, Fig. 6). Red pigment obtained from estuarine bacterium.

S. marcescens had showed anti-cyanobacterial activity against *Synechococcus* sp. at 25–50 μg/ml concentrations (ID_{50}).

Barnacle Settling Assay

Inhibition of larval settlement on solid surfaces by marine bacteria is commonly found in seawater (Wieczorek and Todd 1998). Indeed, settlement inhibition assays using barnacle cyprids have been used routinely to examine the antifouling properties of synthetic and natural compounds. Effect of red pigment on barnacle setting was performed with *Balanus amphitrite* larvae and the results exhibited significant inhibition of the settlement of *B. amphitrite* cyprids (Fig. 7). When the cyprids were exposed to various concentrations of prodigiosin ranging from 50 to 200 μg/cm^2 larval settlement was inhibited in a dose-dependent manner. EC_{50} was between 100 and 200 μg/cm^2.

Discussion

The present investigation has aimed at finding alternative solution to the problem of marine biofouling, a serious problem faced by maritimes industries (Abarzua et al. 1999) and leads to enormous economic losses worldwide. Red pigment prodigiosin producing *S. marcescens* was used in this antifouling study, which was obtained from Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam. The results obtained using prodigiosin of *S. marcescens* against biofouling validate the broad antibacterial potentials of the red pigment and are in agreement with the previous literature revealed the inhibitory effect of prodigiosin against both Gram-positive and Gram-negative bacteria (Mekhael and Yousif 2009; Samrot et al. 2011). Mekhael and Yousif (2009) have shown higher inhibitory effect of prodigiosins against Gram-positive bacteria than Gram-negative bacteria, whereas in the present study prodigiosin has higher activity against Gram-negative *Alteromonas* sp. and *Gallionella* sp. than Gram-positive *Bacillus* sp. Samrot et al. (2011) have reported that ethanol: HCl extract of *Serratia* has antibacterial activity and its zone of inhibition was higher against both Gram-negative (*E. coli* and *Pseudomonas* sp.) and Gram-positive (*S. aureus*) bacteria.

The results of MIC and MBC assays were clearly demonstrated the potentiality of red pigment prodigiosin as an effective antibacterial compound against fouling marine bacteria. It is known that the antibacterial activity of prodigiosin is the result of their potential to pass through the outer membrane and to their

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of prodigiosin against biofilm bacteria

Organism	Prodigiosin concentration (μg/ml)					
	100	50	25	12.5	6.75	3.275
MIC						
Bacillus sp	−	+	+	+	+	+
Pseudomonas sp	−	+	+	+	+	+
Alteromonas sp	−	−	−	−	+	+
Gallionella sp	−	−	−	+	+	+
MBC						
Bacillus sp	−	+	+	+	+	+
Pseudomonas sp	+	+	+	+	+	+
Alteromonas sp	−	−	−	+	+	+
Gallionella sp	−	−	+	+	+	+

+ presence of bacterial growth, − absence of bacterial growth

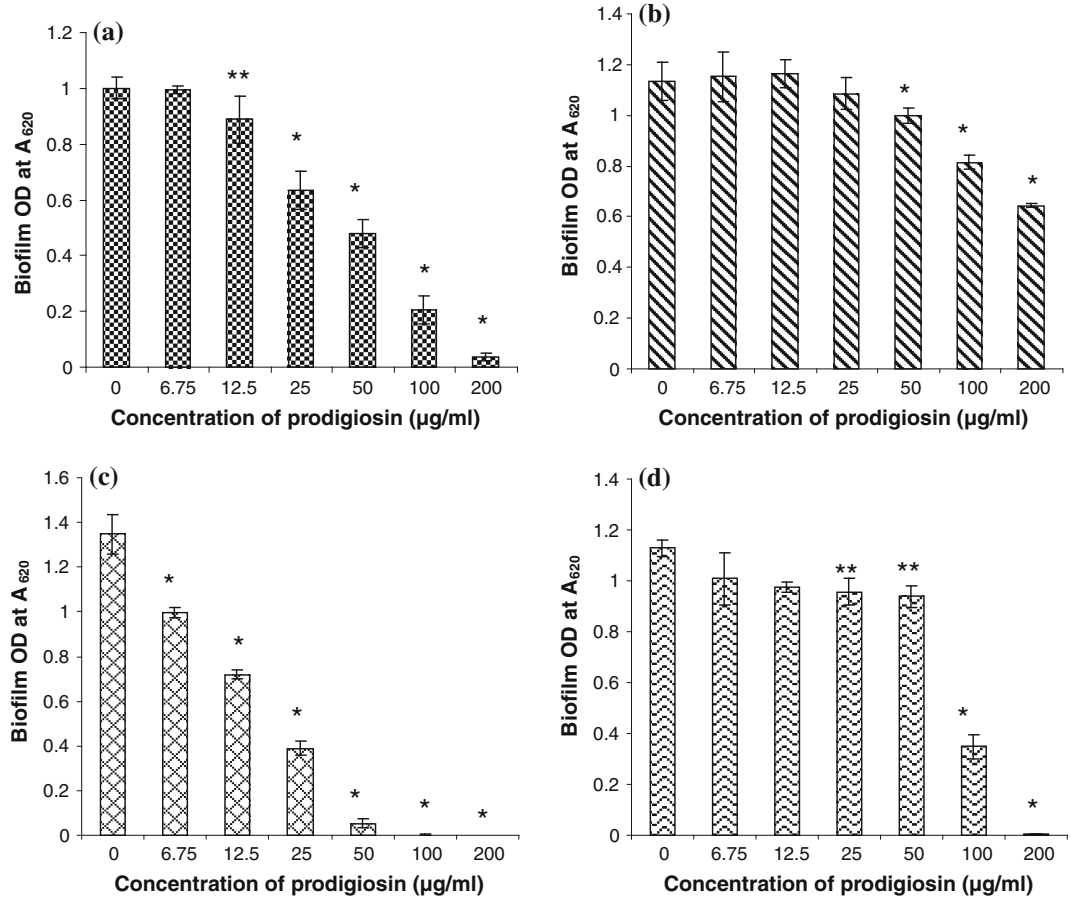

Fig. 4 Antifouling activity of prodigiosin against fouling bacteria. All values are mean values of three individual experiments with ± SE. **a** *Bacillus* sp., **b** *Pseudomonas* sp., **c** *Alteromonas* sp., **d** *Gallionella* sp. $P < 0.0001$ in ANOVA at different concentrations. * $P < 0.01$ significant, ** $P < 0.05$ significant in Tukey HSD test

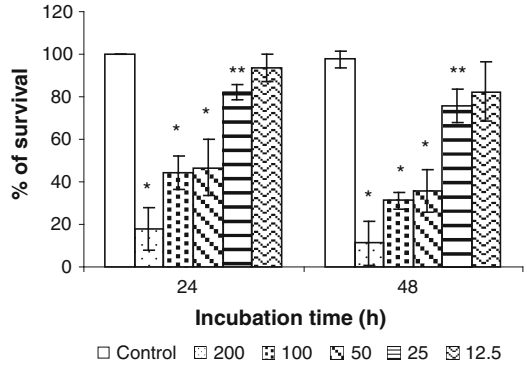

Fig. 5 Survival of artemia against concentration of prodigiosin. All values are mean of three individual replicates with ±SD. One way ANOVA $P < 0.0001$ significant was observed. *Asterisk* $P < 0.01$, *double asterisk* $P < 0.05$ significant in Tukey HSD test

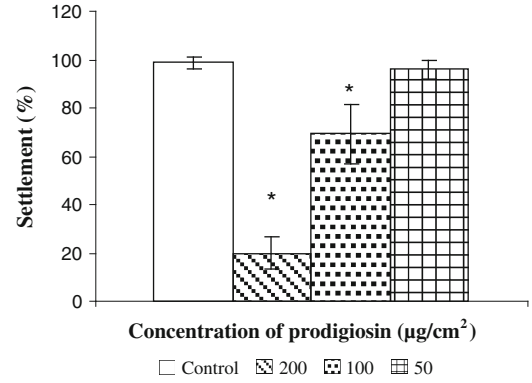

Fig. 7 Effect of prodigiosin against Barnacle settlement [after 3 days of incubation (n = 25)]. One way ANOVA $P = 0.913233$; Tukey HSD test *Asterisk* $P < 0.01$ significant

Table 2 Effect of prodigiosin on cyanobacterial adhesion on glass surface

Time (h)	Number of cyanobacterial (cells/cm^2)					
	Control	200	100	50	25	12.5
24	66.25 ± 14.40	7.75* ± 2.21	11.5* ± 2.64	56.75 ± 17.87	59.75 ± 19.37	62 ±14.30
48	189.75 ±38.24	16.5* ±4.12	61.25* ±9.74	106.25* ±10.04	142.25 ±34.86	191.5 ±34.81

All values are mean of triplicates with ± standard deviation. One way ANOVA P < 0.0001; Tukey HSD test * $P < 0.01$ significant

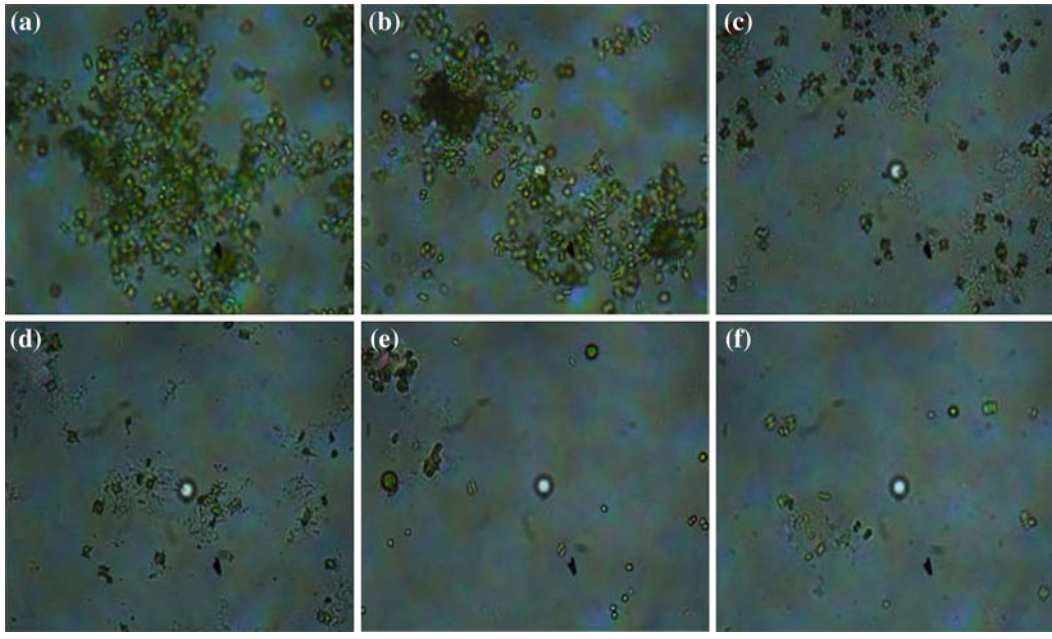

Fig. 6 Effect of prodigiosin against cyanobacterial adhesion (slide adhesion assay (400 X magnification). **a** control, **b** 12.5 µg/cm^2, **c** 25 µg/cm^2, **d** 50 µg/cm^2, **e** 100 µg/cm^2, **f** 200 µg/cm^2

capacity for inhibiting target DNA modulating enzymes, such as DNA gyrase and topoisomerase IV, which inhibit the cell growth (Berlanaga and Vinas 2000). Since, the antibacterial activity of a compound may depend on the destruction of the physical structure or the inhibition of any metabolic reaction in a microorganism, it seems that the presence and the level of the antibacterial activity of the red pigment varied significantly with the type of fouling bacteria used. Furthermore, the red pigment caused growth inhibition along with bactericidal activity, it suggests that the red pigment is an effective antibacterial agent.

Marine-based microorganisms have lot of unexplored potentials; however, the exploration of marine resources for antifouling compounds is very limited (Dobretsov et al. 2006; Paul et al. 2006). The present investigation reveals the biological activity of red pigment prodigiosin against marine fouling bacteria that were exposed into the same ecological conditions. Interestingly, prodigiosin extracted from the *S. marcescens* had showed broad spectrum of antifouling activity against biofoulants of marine environment like *Alteromonas* sp., *Gallionella* sp. and *Pseudomonas* sp. by means of significant decrease in the adherence and biofilm formation of fouling bacteria compared with control. Similarly, antibacterial activity of marine organisms against biofilm forming bacteria is reported in earlier (Wilsanand et al. 1999; Marechal et al. 2004). A new yellow pigment has been isolated from the marine bacterium *Pseudoalteromonas tunicata* and identified as a new member of the tambjamine class of compounds has antifouling activity against marine fouling (Egan et al. 2002; Frank et al. 2005). Considering the results of this study may give immense values of marine-based natural products and the needs for the collection of novel marine resources for discovery and development of effective natural products.

Assaying anti-algal activities by prodigiosin against fouling cyanobacteria *Synechococcus* sp. have been demonstrated concentration-dependent inhibition of cyanobacterial attachment on glass surfaces. Similarly, Egan et al. (2002) have been identified a compound from the marine bacterium *P. tunicata* exhibited anti-algal activity that inhibits settlement of spores of *Ulva lactuca*. Another marine bacterium *Alteromonas* sp. produced 2-n-Pentyl-4-quinolinol, which inhibited the growth of diatoms even at nanomolar concentrations (Long Richard et al. 2003). Kang et al. (2005) have reported anti-cyanobacterial effects *Pseudomonas putida* against *Microcystis aeruginosa*. Furthermore, effect of red pigment on barnacle setting was performed with *Balanus amphitrite* larvae and the results were exhibited significant inhibition of the settlement of *B. amphitrite* cyprids. Thus, results of these bioassay studies clearly indicate the positive response of prodigiosin as an antifouling natural metabolite, which are in agreement with previously reported studies of *B. amphitrite* settlements (Rittschof et al. 1984; Oclarit et al. 1994; Maki et al. 1998; Lau et al. 2003).

Based on this study, it seems that bacterial red pigment prodigiosin has the potential to inhibit the growth of marine fouling bacteria, cyanobacteria, and invertebrates. The findings of the study proved that, the red pigmented prodigiosin has an alternative to chemical antifoulants against marine micro and macro foulants, which encourage to developing a novel broad spectrum antifouling formulation in future. Furthermore, molecular studies require proving the mechanism of the compound act as an effective antifouling.

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Application of Plackett–Burman Design to Optimize Bioprocess Variables for Decolorization of Reactive Red 195 by a Termite Associated Bacterial Consortium BUTC7

K. Nanthakumar, K. Karthikeyan, S. Suriyanarayanan, and P. Lakshmanaperumalsamy

Abstract

In the present study, an attempt has been made to understand about the dye decolorization capacity of termite based microbial consortia. Initial decolorization experiments were carried out to know about the decolorization ability of the consortia and in continuation, its ability of decolorizing reactive red 195 by applying response surface methodology (RSM) was assessed. Plackett–Burman design was applied for this study to understand the most influencing variables out of all the variables and central composite design was applied in order to find out the most suitable concentration range of the selected variables. The quadratic model having R^2 of 0.8950 was generated to describe the combined effect and the interactions. Statistical analysis indicates that the interactions between the variables are found to be significant. The optimum conditions responsible for maximum decolorization efficiency (97.23 %) were found to be pH 7.0, 0 rpm (static mode), 500 mg/L of dye concentration, and 0.25 % yeast extract. All these factors had a significant cumulative positive effect for maximum decolorization performance in the medium. The experimental and predicted values derived during the present study supported the reliability of this design. Hence from the promising results observed in the present study, it is

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confirmed that the microbial consortium isolated from termite can be employed as potential candidate for the treatment of reactive dyes containing effluents.

Keywords
Termite · Microbial consortium · Decolorization · Reactive red 195 · Plackett–Burman design

Introduction

The textile industry is one of the greatest generators of liquid effluent, due to the high quantities of water used in the dyeing processes and more than 800,000 t of dyes are annually produced worldwide, of which 60–70 % are azo dyes (Moutaouakki et al. 2000; Jin et al. 2007). Reactive azo group of dyes is widely used as textile colorants, typically for cotton dyeing, due to their variety of brilliant colors shades, high wet fastness profiles, ease of application, and minimal energy consumption (Carliell et al. 1995; Hao et al. 2000).

Discharge of wastewater from dye manufacturing units and textile processing industries results in pollution of aquatic systems (Keharia and Madamwar 2003). Hence, treatment of such dye containing effluent is essential to prevent deterioration of ecosystem. Bioremediation of textile dyes is gaining significance as it is cost-effective, environment friendly, and produces less sludge (Robinson et al. 2000; Chen et al. 2003). Many microorganisms belonging to different taxonomic groups of bacteria, actinomycetes, fungi, and algae have been reported for their ability to decolorize azo dyes (Chang et al. 2001; Khehra et al. 2005). Microorganisms are capable of utilizing a variety of complex chemicals including dyes as their sole source of carbon but only few researchers have been successful in isolating such culture (Sarnaik and Kanekar 1999). Microorganisms, being highly versatile, have developed enzyme systems for the decolorization and mineralization of dyes under certain environmental conditions. Microorganisms have the ability not only to decolorize dyes but also to detoxify it (Adedayo et al. 2004; Kumar et al. 2007).

In termites, this study has been selected as source for potent dye degrading bacteria, because they are assumed to have harbor microorganisms responsible for digestion of complex substances as the gut flora consists of bacteria, archaea, and archaezoa (Konig and Varma 2006). When a biological treatment system for dye containing wastewaters is concerned, several considerations have to be made in which environmental conditions of a biotreatment system greatly influence its efficiency.

Keeping the above background information and our preliminary results on decolorization, we made an attempt to assess the efficiency of a novel termite associated bacterial consortium on decolorization of reactive red 195 by Plackett–Burman design and central composite design.

Materials and Methods

Dye Stuff and Chemicals

Reactive red 195 was procured from a local textile industry situated in Coimbatore, Tamilnadu, India and used for the study. One gram of dye was dissolved in de-ionized water and made up to 1,000 ml to give 1,000 mg/L. From the stock solution, required concentrations of dye solutions were prepared and filter sterilized using 0.45μ membrane filter and used. Chemicals used in the study were of highest purity and are of analytical grade.

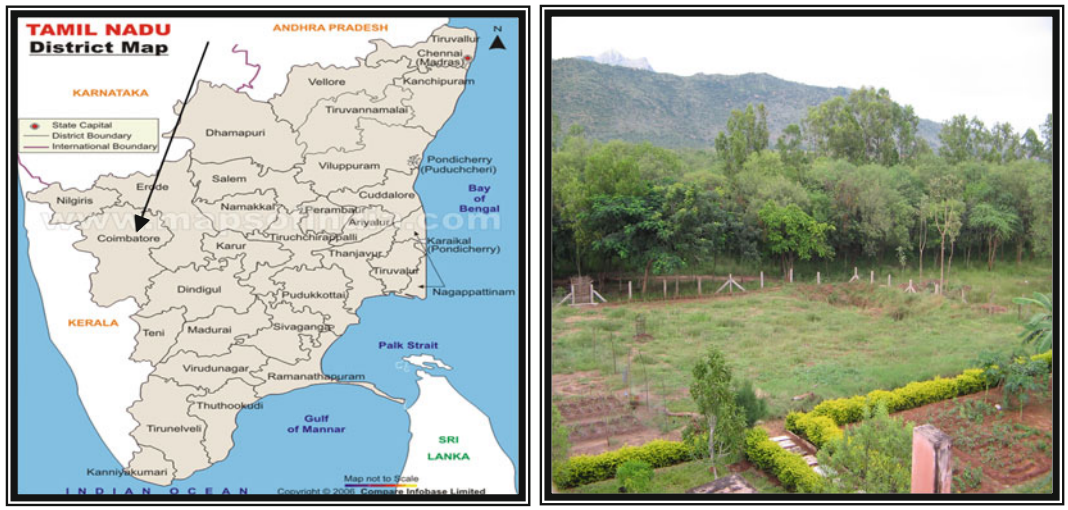

Fig. 1 **a** The study area (Coimbatore district) and **b** environment termatorium habitat

Preparation of Termite Homogenate

Termites were collected from a termatorium located in Bharathiar University campus (Fig. 1a, b), which was washed with sterile distilled water and surface sterilized with diluted ethanol (70 %). 10 g of termites were homogenized in a sterile mortar with sterile distilled water and the homogenate was transferred to flasks containing 50 ml of sterile saline blank. The flask was kept in a shaker at 120 rpm for 15 min, for uniform distribution and this homogenate was used for decolorization studies.

Isolation of Axenic Bacterial Strains from Termites by Pour Plate Technique

Individual bacterial strains present in the homogenate were isolated by pour plating technique (Azambuja et al. 2004). After incubation, the bacterial colonies were isolated and further purified for its usage in dye decolorization experiment.

Selection of Dyes and Dye Decolorizers

The dye decolorization capacity of the individual isolates was assessed and the potent strains were grown in the same medium as consortium in nutrient broth for up to 18 h and selected as potent consortia (BUTC7) and this was used for further experiments.

Decolorization Measurement

After incubation, the samples were centrifuged at 10,000 rpm for 15 min and the suspended biomass was separated. The absorption spectra (545 nm) of the clear supernatant were recorded at λ_{max} of the dyes using a spectrophotometer (UV-Vis 3210, Hitachi, Japan). Medium containing dyes without the inoculum was taken as control. The initial and final absorbance values obtained were then used to calculate percentage decolorization of the dye.

$$\% \text{ decolorization} = \frac{\text{Initial absorbance value} - \text{Final absorbance value}}{\text{Initial absorbance value}} \times 100$$

Preparation of Bacterial Consortium Inoculum

One ml of grown culture of potent dye decolorizer was taken from each flask and inoculated in 50 ml sterile nutrient broth and incubated up to 18 h. After 18 h of incubation, 10 ml of the broth was drawn from each flask, mixed thoroughly and adjusted to 1.0 OD at 600 nm using sterile medium in a spectrophotometer (UV–Vis Hitachi, 3210). One ml of the above bacterial consortium was used as inoculum for all the experiments. The potent dye decolorizing bacterial strains were used as consortia to check the efficiency in decolorizing reactive red 195 by statistical analysis by applying response surface methodology and central composite design.

Response Surface Methodology and Central Composite Design for Decolorization of Reactive Red 195

The main goal of response surface is to hunt efficiently for the optimum values of the variables, so that the response is maximized (Dey et al. 2001). Media components used in any bioprocesses such as pH, temperature, and nutrients in general are considered to be the most important parameters which affect process of any industrial system. This is mainly achieved by optimizing the different variables by changing one parameter at a time and maintaining others at a constant level. This method is found to be time consuming and also often does not yield reliable results. In this regard, statistical method such as RSM is found to be a useful model for studying the effect of copious factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. This methodology consists of Plackett–Burman design as first optimization step and CCD as a second step to optimize the factors that have significant effects on the process, and hence this methodology was adopted for the study. The influence of pH, temperature, agitation, incubation time, dye concentration, glucose, yeast extract, KH_2PO_4, chromium, phenol, and sodium salts in the dye decolorization was investigated in 12 runs using Plackett–Burman design.

Plackett–Burman Design for Screening of Nutrients

Plackett–Burman design was applied for screening of nutrients with respect to their main

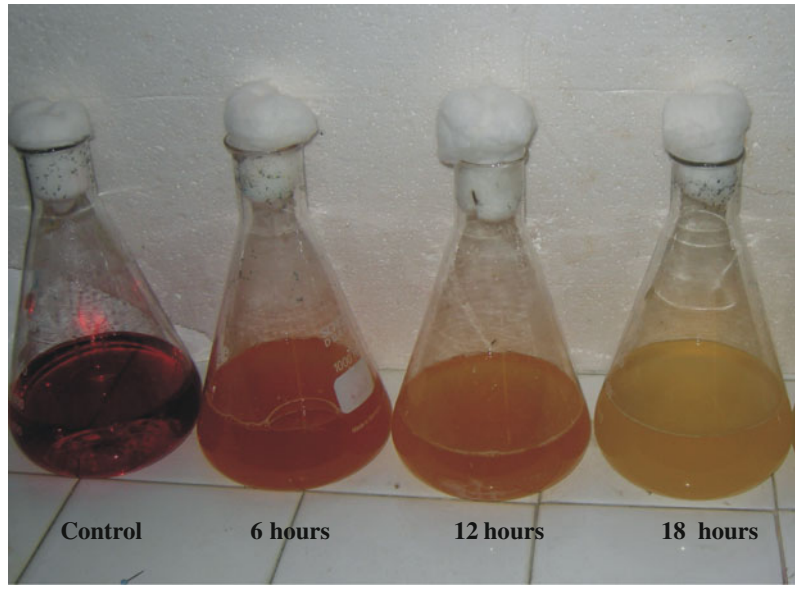

Fig. 2 Reactive red 195 decolorization by the bacterial consortium BUTC17 at various time intervals

effects and not their interaction effects. The medium components were screened for 11 variables at two levels of maximum (+) and minimum (−). According to this design, the number of positive signs (+) is equal to $(N + 1)/2$ and the number of negative signs (−) is equal to $(N-1)/2$ in a row (Plackett and Burman 1946).

This design was used to formulate the media components and the flask culture experiments on decolorization were performed and the response was calculated as percentage of dye decolorization. In general, all the experiments were performed in triplicate and the average percentage dye decolorization was considered. The effect of each variable was calculated using the following equation (Plackett and Burman 1946).

$$E = (\sum M_+ - \sum M_-/N)$$

where, E is the effect of tested variable, M_+ and M_- are responses (dye decolorization) of trials at which the parameter was at its higher and lower levels respectively, and N is the number of experiments was carried out. The standard error (SE) of the variables was calculated by using Student's t.

Optimization of Medium Components Using RSM

RSM is an efficient experimental methodology to determine the ideal conditions for a system containing multiple variables and is found to be useful rather than optimizing by the conventional method where variables are changed in consecutive steps while keeping one factor (Liu and Tzeng 1998). The screened medium components affecting dye decolorization were optimized using CCD (Box and Wilson 1951; Box and Hunter 1957).

According to this design, the treatment of combinations is represented as $2^k + 2k\ n_0$ where 'k' is the number of independent variables and n_0 being the number of times the experiment was performed. For statistical analysis, the variables X_i have been coded as x_i according to the following transformation (Plackett and Burman 1946):

$$x_i = X_i - X_0/\delta X$$

where, x_i is dimensionless coded value of the variable X_i, X_0 the value of the X_i at the center point, and δX is the step change. A total number of 30 experiments were employed for optimizing the medium components and this system behavior was explained by the following quadratic equation (Plackett and Burman 1946):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where, Y is the predicted response, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect, and β_{ij} is the interaction effect. The statistical model was validated for decolorization of reactive red 195 under different environmental conditions.

Results

Development of Bacterial Consortium

The decolorization pattern of reactive red 195 by the bacterial consortium at various time intervals.

Optimization of Process Using RSM for Decolorization of Reactive Red 195

The decolorization response in the 12 run experimental set up is represented in Table1 and Fig. 2. The Pareto chart depicts the order of significance of the variables involved in decolorization of reactive red 195 (Fig. 3). Among the variables screened for this experiment, the most effective factors with high level of significance were in the order as follows: dye concentration, yeast extract, static condition, and pH and consequently these factors were further investigated with CCD to find the optimal range of these variables. Statistical analysis of this design demonstrates that the model F value of 0.89 is significant and values of $p < 0.05$ indicate

Table 1 Plackett–Burman design to evaluate factors influencing reactive red 195 decolorization by bacterial consortium

Run	A	B	C	D	E	F	G	H	J	K	L	Decolorization (%)
1	4	20	0	0	10	0.25	0.25	0.1	5.0	2.0	1.0	9.6
2	4	20	0	72	10	1.0	1.0	0.1	50.0	10.0	5.0	75.7
3	4	60	0	72	1000	0.25	1.0	0.5	50.0	2.0	1.0	54.6
4	11	20	300	72	10	1.0	1.0	0.5	5.0	2.0	1.0	25.6
5	4	60	300	0	1000	1.0	1.0	0.1	5.0	2.0	5.0	19.8
6	4	60	300	72	10	0.25	0.25	0.5	5.0	10.0	5.0	23.4
7	4	20	300	0	1000	1.0	0.25	0.5	50.0	10.0	1.0	5.8
8	11	20	0	0	1000	0.25	1.0	0.5	5.0	10.0	5.0	12
9	11	60	0	72	1000	1.0	0.25	0.1	5.0	10.0	1.0	45.6
10	11	20	300	72	1000	0.25	0.25	0.1	50.0	2.0	5.0	66.8
11	11	60	0	0	10	1.0	0.25	0.5	50.0	2.0	5.0	12.7
12	11	60	300	0	10	0.25	1.0	0.1	50.0	10.0	1.0	12.5

A pH *B* Temperature (° C) *C* Agitation (rpm)
D Incubation time (hrs) *E* Dye concentration (mg/L) *F* Yeast extract (%)
G Glucose (%) *H* KH_2PO_4 (%) *J* Chromium (g/L)
K Phenol (mg/L) *L* Sodium chloride (%)

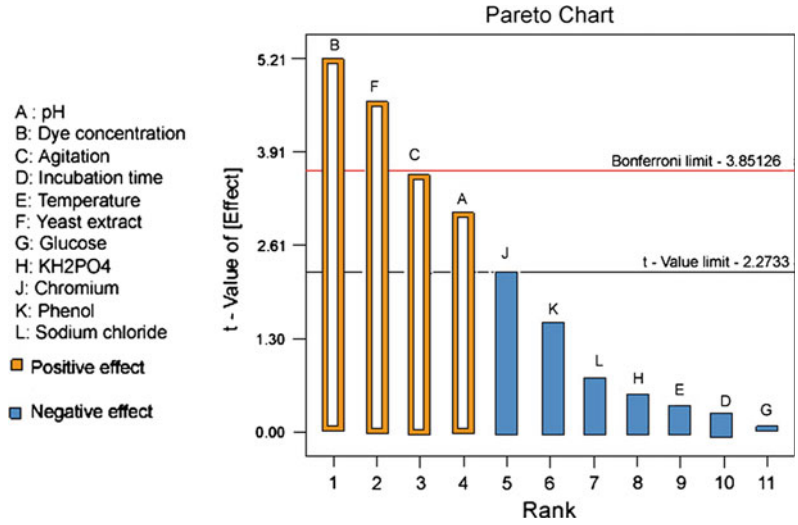

Fig. 3 Pareto chart for reactive red 195 decolorization decolorization

model terms are significant. Regression analysis was also performed on the results and first-order polynomial equation was derived in order to notify the independent variable which showed the decolorization of reactive red 195 and the equation is as follows:

$$\text{Reactive red 195 (\% decolorization)} = +34.09 + 14.53A + 7.51C + 11.43E + 3.01F$$

Central Composite Design

The result of 30 run CCD of four variables with + effects such as pH, agitation, dye concentration, and yeast extract were chosen for optimization of decolorization of reactive red 195 by the bacterial consortium BUCT17 is shown in Table 2, which is presented as decolorization corresponding to combined effect of four components in their specified ranges.

Table 2 Experimental plan for optimization of variables involved in reactive red 195 decolorization using CCD

Run	pH	Agitation (rpm)	Dye concentration (mg/L)	Yeast extract (%)	Decolorization (%)	
					Experimental	Predicted
1	0	2	0	0	39.42	33.00
2	0	0	0	2	32.06	36.17
3	0	0	0	−2	22.95	22.17
4	1	1	−1	−1	21.91	22.17
5	1	1	−1	1	46.09	39.17
6	−1	1	1	−1	46.23	42.50
7	0	0	2	0	64.17	58.67
8	1	1	1	1	21.9	27.67
9	0	0	0	0	87.07	94.17
10	0	0	0	0	**97.23**	94.17
11	−1	1	1	1	24.54	31.50
12	0	0	0	0	96.83	94.17
13	0	0	0	0	94.15	94.17
14	1	−1	1	−1	40.04	42.83
15	1	−1	1	1	41.12	33.83
16	−1	−1	−1	−1	31.63	31.67
17	−1	1	−1	−1	31.79	31.50
18	0	0	0	0	96.03	94.17
19	−1	−1	−1	1	25.22	22.67
20	2	0	0	0	11.01	5.33
21	1	−1	−1	1	15.01	19.83
22	1	−1	−1	−1	11.34	14.83
23	1	1	1	−1	22.22	28.67
24	−1	−1	1	−1	23.09	24.17
25	0	0	−2	0	54.99	53.67
26	0	0	0	0	95.01	94.17
27	−2	0	0	0	**2.01**	2.00
28	−1	−1	1	1	21.65	21.17
29	0	−2	0	0	15.43	15.33
30	−1	1	−1	1	54.55	54.50

Decolorizations varied markedly with the condition were tested, in the range of 2.01–97.23 %. Lowest decolorization was observed at run 27, where lower pH with optimum concentrations of yeast extract, dye concentration, and static condition, whereas maximum decolorization of 97.23 % was observed at pH 7, 0 rpm, 500 mg/L of the dye with 0.25 % yeast extract (run 10), which strongly suggests that these variables had played a significant role in decolorization pattern of reactive red 195.

The results obtained were subjected to the analysis of variance with the regression model given as; $Y = +94.17 + 0.83A + 4.42\ B + 1.25\ C + 3.50\ D - 3.13\ A\ B + 3.88\ A\ C - 1.50\ A\ D - 5.37\ BC + 3.00\ BD - 3.50\ C\ D - 22.62\ A2 - 17.50\ B2 - 9.50\ C2 - 16.25\ D2$.

Where Y is the response value (% decolorization of reactive red 195) with coded levels of pH, agitation, dye concentration, and yeast extract as A,B,C, and D respectively. Next to pH, another most influencing variable for

Table 3 Analysis of variance for reactive red 195 decolorization

Source	Sum of square	Degree of freedom	Mean square	*F*-value	*p*-value	
Model	4,774.58	3	1,591.53	0.89	0.0043	Significant
A-pH	2,531.71	1	2,531.71	16.0607	0.0039	–
C-agitation	676.501	1	676.501	4.29159	0.0720	–
E-dye concentration	1,566.37	1	1,566.37	9.93673	0.0136	–
F-yeast extract	22.1408	1	22.1408	1.40243	0.3019	–
Residual	1,261.07	8	157.634	–	–	–
Cor total	6,035.65	11	–	–	–	–

Table 4 ANOVA for CCD (quadratic model) for reactive red 195 decolorizationdecolorization

Source	Sum of square	Degree of freedom	Mean square	*F*-value	*p*-value	
Model	25,683.1	14	1834.51	9.12893	<0.0001	Significant
A-pH	16.6667	1	16.6667	0.08294	0.7773	–
B-agitation	468.167	1	468.167	2.3297	0.1477	–
C-dye concentration	37.5	1	37.5	0.18661	0.6719	–
D-yeast extract	294	1	294	1.46301	0.2452	–
AB	156.25	1	156.25	0.77754	0.3918	–
AC	240.25	1	240.25	1.19554	0.2915	–
AD	36	1	36	0.17914	0.6781	–
BC	462.25	1	462.25	2.30026	0.1501	–
BD	144	1	144	0.71658	0.4106	–
CD	196	1	196	0.97534	0.3390	–
A^2	14,040.4	1	14,040.4	69.8683	<0.0001	–
B^2	8,400	1	8,400	41.8003	<0.0001	–
C^2	2,475.43	1	2,475.43	12.3183	0.0032	–
D^2	7,242.86	1	7,242.86	36.0421	<0.0001	–
Residual	3,014.33	15	200.956			–
Lack of fit	2,947.5	10	294.75	22.0511	0.0016	Significant
Pure error	66.8333	5	13.3667	–	–	–
Cor Total	28,697.5	29	–	–	–	–

CV 4.19; R^2 0.8950

effective decolorization of reactive red 195 was found to be yeast extract at a concentration of 0.25 %. In this study, dye concentration has an influence on decolorization of reactive red 195 and a optimum dye concentration of 500 mg/L was observed, whereas 1,000 mg/L was the maximum concentration used in the present study. The next influencing variable for decolorization of reactive red 195 is static condition (no agitation was employed), which influence decolorization on effectively, whereas aeration (increased dissolved O_2 level) strongly inhibited decolorization of reactive red 195.

The ANOVA results are presented in Tables 3 and 4, the quadratic regression model suggested that it is very significant with a model F value of 9.12 and R^2 value (multiple correlation coefficient) of 0.89 (near 1) denotes better correlation between the experimental and predicted values. In the present case, a low value (4.19) denotes that the experiments performed are highly reliable. The *p* value denotes the

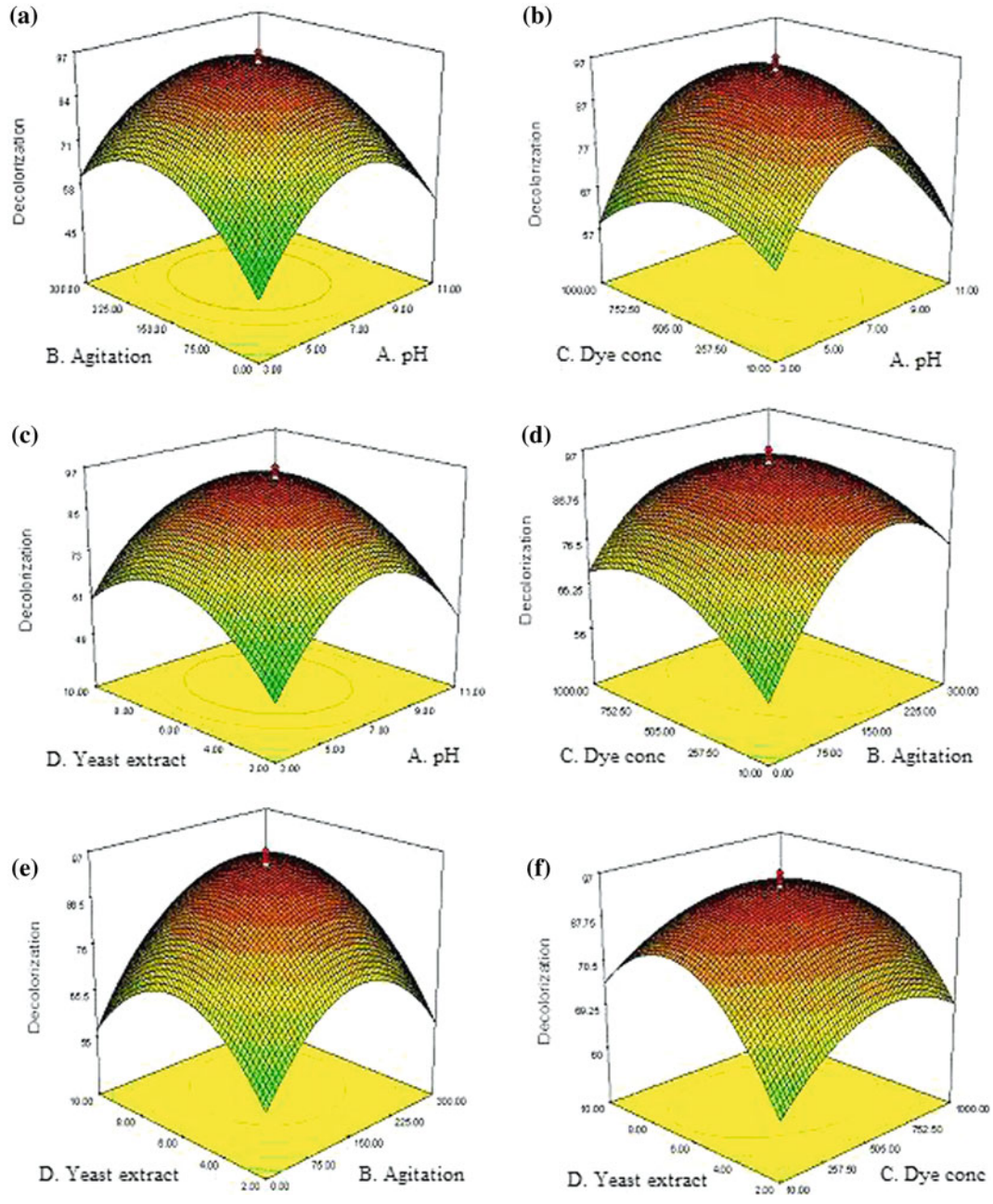

Fig. 4 The effect of various factors on reactive red 195 decolorization by bacterial consortium BUTC17

significance of coefficients and also important in understanding the pattern of mutual interactions between the variables.

The interaction effects and optimal levels of the variables affecting decolorization were determined by plotting the response surface curves (Fig. 4), which is showcase the behavior of response (percentage decolorization) with respect to simultaneous change in two variables.

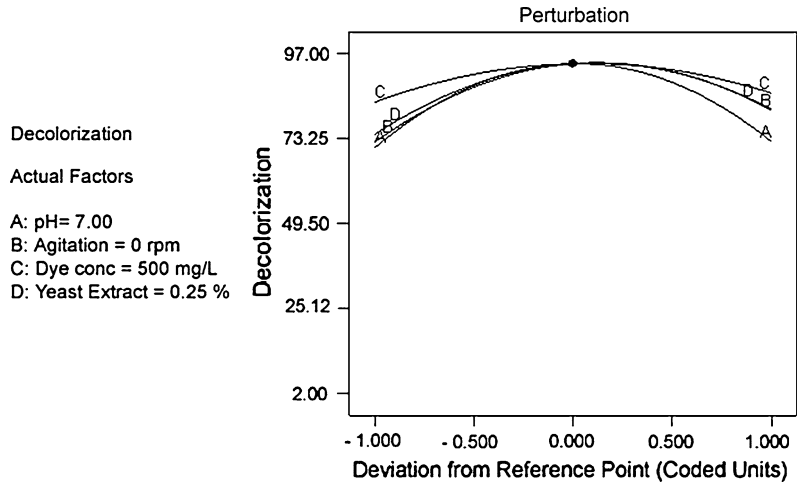

Fig. 5 Optimum values of the tested variables for reactive red 195 decolorization decolorization

Model Validation

The maximum experimental decolorization of reactive red 195 was 97.23 %, whereas the predicted value was 94.17 %, which established a strong agreement between them. The optimum values of the tested bioprocess variables are pH 7.0, agitation 0 rpm, dye concentration 500 mg/L, and yeast extract of 0.25 % as shown in perturbation graph (Fig 5). Confirmation of the decolorization pattern was done to validate the optimum combination of the process variables. The results were obtained from these experiments employed RSM was found to be encouraging for optimizing similar dye decolorization studies under pilot or industrial scale conditions.

Discussion

Development of Bacterial Consortium

Based on the initial decolorization experimental results (data not shown), the study was planned to optimize the operational variables by applying RSM and CCD. The main objective of RSM is to determine the optimum operational conditions for the system or to determine a region that satisfies operating specifications (Ravikumar et al. 2006).

Optimization of Process Using RSM for Decolorization of Reactive Red 195

Microbial decolorization process which uses several variables (carbon and nitrogen source, dye concentration, inoculum size, pH, and temperature) is considered to be the most important parameters which affect the process (Wong and Yuen 1996; Chen et al. 2003; Nachiyar and Rajkumar 2003; Khehra et al. 2005), and hence it is required to investigate the influencing nature of these variables on the decolorization process.

In line with our studies, decolorization (80 %) of a textile azo dye, Disperse Yellow 211 (DY 211) in simulated aqueous solution by *Bacillus subtilis* involving Box-Behnken design matrix and found temperature, pH, and initial dye concentration as the most influencing variables (Sharma et al. 2009). Similar report on decolorization of 91.0 % of Direct Black 22, a textile dye, by a novel microbial consortium by RSM (Mohana et al. 2008).

Central Composite Design

Bacterial culture generally exhibits maximum decolorization at a neutral pH value or a slightly alkaline pH value and the rate of color removal tends to decrease rapidly at highly acidic or

alkaline pH conditions (Bhatt et al. 2005; Handayani et al. 2007). Bacterial consortium employed in the present study also exhibited a similar better performance at pH 7. Studies reported glucose were utilized during decolorization of dye acid red 27 and reactive red 2 by *Enterococcus faecalis* under a batch system and concluded that it might be due to the role of glucose as a cosubstrate (Handayani et al. 2007) i.e. the source of electron donors, which are needed for azo bond cleavage (Sponza and Isik 2004; Mendez et al. 2005) and it is also established that glucose acts as an effective electron donors for dye reduction (Tan 2001).

Decolorization percentage of the dye decreases with increase in the initial dye concentrations, which might be due to inhibitory effects caused due to toxicity of dye (Korbahti and Rauf 2008). Decolorization and degradation of Navy blue HER by *Trichosporon beigelii* NCIM-3326 within 24 h of incubation under static condition and concluded that it may be due to enzyme activity by the bacterial consortium got induced under static conditions with maximum decolorization (Saratale et al. 2009).

Similarly, R^2 values of 0.993, 0.987 and 0.968 had been derived for decolorization and degradation of Ponceau 2R, malachite green and anthraquinone blue and suggests that the fitted linear plus interactions models could explain 99.3, 98.7, and 96.8 % respectively of the total variation (Levin et al. 2005). In general, the larger magnitude of t and smaller the value of p, more significant is the corresponding coefficient term (Montgomery 1991).

Conclusion

It can be concluded that the present study, that the bacterial consortium obtained from termites possess higher color removal efficiency and interestingly the experimental values were very much concurrent with the predicted values, which reflected the suitability of RSM. By applying these statistical methods for optimization of experiments, we could observe the process variables completely achieving decolorization values up to 97.23 %. From this study, the bacterial consortium has the ability to decolorize dyes which indicates its potential application for decolorizing textile dyeing effluents which are reactive in nature, it was strong evidence. Further it is planned to design a bioreactor for treating similar dyes in large-scale system with the aid of this novel consortia and application of this design as well for future large-scale applications. To our knowledge, decolorization of reactive red 195 by termite associated bacterial consortium is reported for the first time.

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Arbuscular Mycorrhizal Fungal Strains and Soil Type Influence Growth, Nodulation, and Nutrient Uptake of *Casuarina equisetifolia*

T. Muthukumar, E. Uma, and P. Priyadharsini

Abstract

The effects of arbuscular mycorrhizal (AM) fungal species and strains on seedling growth and uptake of nutrients were determined for *Casuarina equisetifolia* under nursery conditions. Seedlings of *C. equisetifolia* were inoculated individually with four strains each of *Acaulospora scrobiculata* and *Glomus aggregatum* in two soil types (alfisol and vertisol). Seedling height, root collar diameter, nodulation, dry weights, nutrient contents, nutrient uptake efficiencies, mycorrhizal inoculation effect (MIE), and seedling quality were determined at harvest. Seedlings inoculated with different AM fungal strains invariably had significantly higher plant growth, and nutrient parameters measured. Nevertheless, the response was higher for seedlings inoculated with strains of *G. aggregatum* compared to those inoculated with strains of *A. scrobiculata*. The mycorrhizal response as measured by MIE was significantly affected by soil types. These results suggest the importance of selecting a specific AM fungal strain suited for a soil type in forest nurseries for the production of high-quality seedlings.

Keywords

Acaulospora scrobiculata · Arbuscular mycorrhizal fungi · *Casuarina* · *Glomus aggregatum* · Seedling quality · Soil types · Strains

Introduction

Actinorhizal plants can grow and improve fertility of disturbed and infertile soils owing to their capacity to fix atmospheric nitrogen. The nitrogen-fixing capacity of actinorhizal plants results from their symbiosis with root nodulating actinomycetes, *Frankia*. In addition to the association with *Frankia*, many of the actinorhizal plants are also capable of associating with

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either ectomycorrhizal or arbuscular mycorrhizal (AM) fungi or both, resulting in a tripartite or multipartite symbiosis (He and Critchley 2008). Mycorrhizal fungi improve the nutrition and water status of actinorhizal plants by their ability to take up water and nutrients from the soil and transfer it to plant roots. Further, *C. equisetifolia* can also enhance its nutrient uptake by developing cluster roots under nutrient-deficient conditions (Zaïd et al. 2003).

Studies on the influence of soil types on AM fungal colonization and function are limited. Santiago et al. (2002) reported that the effect of AM fungi and *Bradyrhizobium* sp. inoculation on *Dalbergia nigra* growth and nitrogen content were influenced by soil types. In a nursery study assessing the influence of bioinoculants (*Glomus aggregatum*, *Bacillus polymyxa*, *Azospirillum brasilense*) on seedling growth promotion of bamboo (*Dendrocalamus strictus*) in the presence or absence of fertilizer application, it was found that the extent of AM colonization, growth, and nutrient uptake parameters were significantly influenced by soil types (Muthukumar and Udaiyan 2006).

The genus *Casuarina* is one of the four genera in the family Casuarinaceae (the others being *Allocasuarina*, *Ceuthostoma*, and *Gymnostoma*) (He and Critchley 2008). Of the 18 species of *Casuarina*, *Casuarina equisetifolia* is introduced and planted worldwide including India. *Casuarina equisetifolia* L. is one of the most valued multipurpose trees with wide applications in agroforestry, land restoration or reclamation, and silviculture (He and Critchley 2008). This tree has been successfully intercropped with cashew and coconut along the coastal belt of India (Kumar 1981).

The response of *C. equisetifolia* to ectomycorrhizal and AM inoculation has been well documented both under nursery and field conditions (Vasanthakrishna et al. 1994; Rajendaran et al. 2003; Rajendaran and Devaraj 2004). We (Muthukumar and Udaiyan 2010) have recently shown that growth, nutrient uptake, and accumulation and seedling quality of *C. equisetifolia* were enhanced significantly by inoculation with AM fungi and plant growth promoting rhizobacteria individually or in combinations. Previous studies have clearly shown a varied growth response of *C. equisetifolia* to different strains of *Frankia* (Reddell and Bowen 1985; Rosbrook and Bowen 1987; Miettinen and Smolander 1989). Similarly, Thoen et al. (1990) have shown that mycorrhizal forming ability in *Casuarina* and *Allocasuarina* species under in vitro conditions tended to vary with the isolates of the ectomycorrhizal fungus *Pisolithus*. However, the varied effects of different AM fungal genera and species on plant growth and acquisition are well documented (Graham and Abbott 2000; Shukla et al. 2009, 2012; Muthukumar et al. 2012); a few data are available concerning the variability of different strains of an AM fungus. Strains of *Glomus mosseae* and *Glomus intraradices* have been shown to influence plant growth variedly in barley (*Hordeum vulgare*) and maize (*Zea mays*) (Leart et al. 2003; Bidondo et al. 2012). Nevertheless, the influence of different strains of AM fungus on tree seedling growth or nutrient acquisition is lacking. Till date, studies assessing the response of *C. equisetifolia* to AM fungi have involved either a single or multiple species, but the effects of different strains of AM fungus on the growth and nutrient uptake of *C. equisetifolia* have not been investigated. Therefore, the purpose of the present investigation is to determine for *C. equisetifolia* grown under nursery conditions, the effect of inoculation with different strains of AM fungi on growth, nutrient uptake, and quality of the seedlings under different soil types.

Materials and Methods

Nursery Site and Soil Preparation

This study was conducted at the plant nursery of the Botany Department, Bharathiar University, Coimbatore (11°10′N and 96°93′E, altitude 410 m a.s.l), Tamil Nadu, India. The climate is monsoonal with an annual precipitation of 640 mm and a dry season between January and April. The maximum

and minimum monthly temperatures are 31 and 21 °C respectively. Alfisol and vertisol soils were used in this study. Topsoil (0–30 cm) was collected from fallow fields that had remained uncultivated for more than 5 years and from represented areas. In these sites plantations are to be raised in the future. The vegetation in both fields was very sparse and was dominated by grasses. Chemical analysis of the soils prior to experiment using standard procedures is presented in Table 1. Spores of indigenous AM fungi in the field soils were isolated by wet-sieving and decanting techniques. Spores were identified based on spore morphology and subcellular characters and compared to the original descriptions in Schüβler's web site (www.lrzmuenchen.de/~schuessler/amphylo/amphylo_species.html) and the culture database established by INVAM (http://invam.cag.wvu.edu).

Seed Source and Germination

Seeds of *C. equisetifolia* were collected from a 12-year-old tree growing in the Bharathiar University Campus, Coimbatore, Tamil Nadu, India. Seeds scarified with 95 % Conc. H_2SO_4 for 2 min., rinsed in several changes of distilled water, and soaked overnight in distilled water were germinated in trays (30 × 22 × 6 cm, L × W × H) of heat sterilized (121 °C for 3 h) sand. Seedlings were used in experiment after 6 weeks when they were 2 cm tall.

AM Fungal Inoculum and Inoculation

Strains of *Acaulospora scrobiculata* Trappe and *Glomus aggregatum* Schenck & Smith emend. Koske maintained on roots of sorghum [*Sorghum bicolor* (L.) Moench.] in open pot cultures containing native sterilized soils. The number of infective propagules per gram of the inoculum was ascertained by most probable number (MPN) technique (Feldmann and Idczak 1992). Five grams of the AM fungal inoculum consisting of finely chopped mycorrhizal roots and soil containing extraradical hyphae and spores was placed in the planting hole at the time of seedling transplantation (Table 1, Fig. 1). A sterilized composite AM fungal inoculum was added to the control treatment. Inoculum of AM fungi raised in open pot cultures can be colonized by microorganisms that are known to influence plant growth (Ames et al. 1987; Secilia

Table 1 Chemical and arbuscular mycorrhizal (AM) properties of the soils used in the study

Characteristics	Soil Type	
	Alfisol	Vertisol
pH (H_2O)[a]	7.4	8.3
Total nitrogen (mg kg^{-1})[b]	8.7	8.1
Available phosphorus (mg kg^{-1})[c]	0.3	0.4
Exchangeable potassium (mg kg^{-1})[d]	18.2	17.9
Organic matter (%)[e]	0.62	0.71
Indigenous AM fungal propagules (g^{-1}soil)[f]	5.2	4.8
Indigenous AM fungi	*Gigaspora gigantea*, *Glomus geosporum*, *Glomus mosseae*	*Glomus viscosum*, *Glomus geosporum*, *Sclerocystis sinuosa*, *Scutellospora calospora*

[a] Soil–water mixture ratio 1:2
[b] Jackson (1971)
[c] Olsen et al. (1954)
[d] Davis (1962)
[e] Piper (1950)
[f] Feldmann and Idczak (1992)

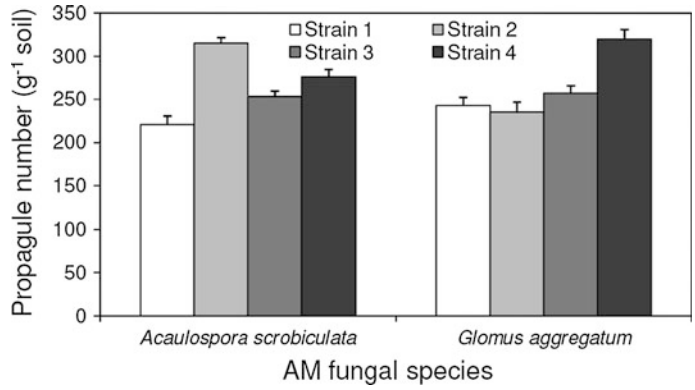

Fig. 1 Arbuscular mycorrhizal fungal propagules in the inoculum of different strains of *Acaulospora scrobiculata* and *Glomus aggregatum* used in the study. Error bars indicate ± 1 S.E

and Bagyaraj 1987). So the non-mycorrhizal soil microbes in AM fungal inocula were equalized across treatments by applying 10 ml microbial cocktail to each plant. The microbial cocktail was prepared by blending 10 g of the inoculum soil from the eight inoculants fungi in 2 l deionized water and filtering it through a 38-µm sieve. Mycorrhizal treatments also received a similar composite microbial cocktail of the eight AM fungi (Table 2).

Frankia Inoculation

At transplantation 5 ml of the nodule suspension (equivalent to 5 mg of nodule f. wt.) was pipetted around the roots of all the seedlings including control. *Frankia* inoculum suspension was prepared from ca.1.0 g of fresh nodule collected from *C. equisetifolia* trees growing in Bharathiar University Campus. The young nodules were separated, surface sterilized (30 % H_2O_2, 30 min), rinsed in sterile distilled water, and ground in a sterile mortar and pestle. The nodule suspension was made up to 1 l with 2 % sucrose solution (Reddell et al. 1988) prior to inoculation.

Seedling Transplantation and Experimental Setup

One healthy and vigorously growing seedling was transplanted into each black polythene bag (45 cm deep, 25 cm wide) containing 7.5 kg field soil. There were nine treatments involving four strains of *A. scrobiculata* and *G. aggregatum* each and an uninoculated control. Each treatment had ten replicates and the whole experiment involved 180 bags (9 × 2 × 10) set up in the nursery in a completely randomized design. The bags were rearranged every 15 days to ensure uniform growth conditions. No nutrients were added and the seedlings were watered to maintain field capacity. The average maximum and minimum temperatures during the experimental period was 34 and 23 °C, rainfall was 345 mm, and relative humidity was between 55 and 75 %.

Analyses

At harvest, the soil was washed from the roots and the number of nodules counted visually. A weighed portion of the root sample was preserved in formalin–acetic acid–alcohol (FAA) solution for the assessment of AM fungal colonization. Shoot, root, and nodules were separated and oven dried at 70 °C to a constant weight for the determination of dry mass. Fixed root samples were washed, cleared in 2.5 % KOH solution at 100 °C for 90 min, bleached in alkaline H_2O_2, acidified with 1.0 % HCl, and stained with 0.05 % trypan blue in lactoglycerol (Koske and Gemma 1989). The extent of AMF colonization was quantified according to the magnified intersection method (McGonigle et al. 1990).

Shoot and root tissue nitrogen (N) was determined by the micro Kjeldahl digestion method using concentrated H_2SO_4 digest and selenium as

Table 2 Vegetation, host species, and soil characters from which *Acaulospora scrobiculata* and *Glomus aggregatum* strains used in this study originated

Species	Abbreviation	Vegetation	Host species	Soil characters*
Acaulospora scrobiculata	AS1	Forest	*Tectona grandis*	pH, 7.6; 7.6 mg kg^{-1} N; 0.35 mg kg^{-1}P; 10.2 mg kg^{-1}K; 0.95 % OM
Glomus aggregatum	GA1			
Acaulospora scrobiculata	AS2	Scrub jungle	*Acacia* sp.	pH, 8.1; 8.2 mg kg^{-1}N; 0.23 mg kg^{-1}P; 15.7 mg kg^{-1} K; 0.54 % OM
Glomus aggregatum	GA2			
Acaulospora scrobiculata	AS3	Grassland	*Cymbopogon caesius*	pH, 7.4; 8.6 mg kg^{-1}N; 0.42 mg kg^{-1} P; 13.5 mg kg^{-1} K; 0.4 % OM
Glomus aggregatum	GA3			
Acaulospora scrobiculata	AS4	Agricultural field	*Glycine max*	pH, 7.8; 9.8 mg kg^{-1} N; 0.52 mg kg^{-1} P; 18.6 mg kg^{-1} K; 0.3 % OM
Glomus aggregatum	GA4			

**N* total nitrogen, *P* available phosphorus, *K* exchangeable potassium, *OM* organic matter, For procedures see Table 1

catalyst. Total N was estimated using a Technicon Auto Analyzer (Gedko International, UK). Tissue P concentration in seedling tissues was determined by the molybdenum blue method (Jackson 1971) using a Spectronic 20 electro photocolorimeter after wet-ashing the plant samples in a nitric–sulfuric–perchloric acid mixture. Tissue potassium (K) was estimated by flamephotometry (Davis 1962).

The efficiency of nutrient uptake (ENuU) defined as the amount of nutrients absorbed per unit root mass was calculated according to Gray and Schlesinger (1983):

$$\mathrm{ENuU} = \frac{\mathrm{Plantnutrientcontent}(\mu g)}{\mathrm{Rootbiomass(mg)}}\,\mu g\ \mathrm{mg}^{-1}$$

Mycorrhizal inoculation effect (MIE) was calculated according to Bagyaraj et al. (1988):

$$\mathrm{MIE} = \frac{\text{DW of inoculated seedling} - \text{DW of uninoculated seedling}}{\text{DW of inoculated seedling}} \times 100$$

Seedling quality index (SQI) was calculated according to Dickson et al. (1960):

$$\mathrm{SQI} = \mathrm{TDW} \,/\, [(\mathrm{H} \,/\mathrm{RCD}) + (\mathrm{SDW}/\mathrm{RDW})]$$

where TDW is total plant dry weight in g $plant^{-1}$, H is height in cm, RCD is root collar diameter in cm, SDW is shoot dry weight in g, and RDW is root dry weight in g.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed on all data to compare the overall treatment effects. Two-way and three-way ANOVA was used to assess the influence of AM fungal strains and soil types on treatment. Differences between means were determined using the Duncan's multiple range test (DMRT). Percentage data on mycorrhizal colonization were arcsine square root transformed prior to analysis. Regression analysis was used to assess the relationship between various plant and fungal parameters.

Results

Seedling Growth

Inoculation of *A. scrobiculata* and *G. aggregatum* strains significantly increased plant growth parameters like seedling height, root collar diameter, nodule numbers, and plant dry weights (Tables 3, 6). The plant growth parameters were significantly influenced by soil type, AM fungi, as well as their strains. Generally, the increase in growth was higher in vertisol soil than in alfisol for strains for both AM fungal species. Seedlings inoculated with *G. aggregatum* strains exhibited a higher growth compared to those inoculated with *A. scrobiculata*. However, the influence of soil and the interaction between soil and fungi were not significant for root collar diameter. Although seedlings inoculated with *A. scrobiculata* strains were 16 % taller than control in alfisol soil, the seedlings inoculated with *A. scrobiculata* strains were slightly shorter (2 %) in vertisol soil compared to uninoculated seedlings. The significant reduction in height of seedlings inoculated with strains AS2 and AS3 accounted for the marginal reduction in the average seedling height of *A. scrobiculata* inoculated seedlings in vertisol soils. However, seedlings inoculated with *G. aggregatum* strains were 34 and 18 % taller compared to control in alfisol and vertisol respectively. *Glomus aggregatum* inoculated seedlings were, respectively, 15 and 22 % taller than *A. scrobiculata* strains inoculated seedlings in alfisol and vertisol soils.

Root collar diameter of *A. scrobiculata* strains inoculated seedlings were 18 and 11 % higher than control in alfisol and vertisol soils. Similarly, seedling inoculated with *G. aggregatum* had root collar diameter that was 29 and 21 % higher than control in alfisol and vertisol respectively. In spite of the variations in root collar diameters between *G. aggregatum* and *A. scrobiculata* strain inoculated seedlings, the difference was <10 % in both soil types.

Shoot dry weights of *A. scrobiculata* strain inoculated seedlings were 22 % higher in both the soil types. The shoot dry weight of *G. aggregatum* inoculated seedlings was 73 and 74 % higher than control, respectively, in alfisol and vertisol soils. Shoot dry weight of seedlings inoculated with *G. aggregatum* strains was 42–43 % higher compared to seedlings inoculated with *A. scrobiculata* in both soil types. Root dry weights when compared to control were 25 and 21 % higher for seedlings inoculated with *A. scrobiculata* strains and 100 and 107 % higher for seedlings inoculated with *G. aggregatum* in alfisol and vertisol soils respectively. The root dry weight of seedlings inoculated with *G. aggregatum* strains was 60–69 % higher compared to seedlings inoculated with *A. scrobiculata*.

Nodulation

Nodulation and nodular mass were significantly increased by AM fungal inoculation (Table 3) and significantly varied between AM fungal strains and soil types (Tables 3, 6). Inoculation of *A. scrobiculata* increased average nodulation and nodular mass by 22 and 21 % in alfisol and 25 and 48 % in vertisol soils. Likewise, nodulation and nodular mass of *G. aggregatum* inoculated seedlings were 79 and 132 % higher in alfisol and 96 and 216 % in vertisol soils compared to uninoculated seedlings. Compared to *A. scrobiculata*, the nodule numbers and nodular mass of *G. aggregatum* inoculated seedlings were 46 and 91 % higher in alfisol and 49 and 69 % higher in vertisol soils.

Mycorrhizal Colonization

Root samples of all the *C. equisetifolia* seedlings examined were invariably colonized by AM fungi. Colonization was characterized by intracellular hyphal coils or arbusculate coils, intercellular hyphae, with inter or intracellular vesicles. Inoculation of different AM fungal species and their strains significantly enhanced the colonization levels (Fig. 2, Table 6).

Table 3 Influence of *Acaulospora scrobiculata* (AS) and *Glomus aggregatum* (GA) strains on growth and nodulation of *Casuarina equisetifolia* as assessed after 120 days after transplantation

Soil type/ treatments	Seedling height (cm)	Root collar diameter (mm)	Nodule number (Plant $^{-1}$)	Dry weight (Plant $^{-1}$)		
				Shoot (g)	Nodule (mg)	Root (g)
Alfisol						
Control	24.40 j	9.12 k	28 hi	1.47 m	18.35 l	0.23jk
AS1	26.50 i	10.61 ghi	33 g	1.65 k	22.34 i	0.28 g
AS2	27.10 i	10.45 hi	30 h	1.60 l	20.12 k	0.25 hi
AS3	28.80 gh	10.85 efg	35 g	1.85 i	22.56 i	0.29 g
AS4	30.50 e	11.05 def	39 f	2.08 g	24.03 h	0.33 f
GA1	31.60 d	11.96 bc	48 c	2.27 f	42.31 c	0.42 cd
GA2	31.40 d	10.87 efg	42 e	2.58 c	32.14 g	0.44 bc
GA3	34.20 b	12.18 b	58 a	2.75 b	50.16 a	0.50 a
GA4	33.10 c	11.98 bc	52 b	2.56 c	45.39 b	0.48 a
Vertisol						
Control	29.30 fg	9.73 j	20 k	1.46 m	11.25 p	0.21 k
AS1	29.30 fgh	10.72 fgh	30 h	1.67 k	20.18 k	0.28 g
AS2	27.00 i	10.31 i	27 i	1.56 l	17.38 m	0.24 ij
AS3	28.40 h	10.86 efg	20 k	1.78 j	13.58 o	0.24 hij
AS4	29.90 ef	11.12 de	23 j	1.90 h	15.29 n	0.26 h
GA1	35.20 a	11.31 d	45 d	2.35 e	41.52 d	0.40 e
GA2	35.50 a	12.58 a	40 ef	2.86 a	40.17 e	0.49 a
GA3	34.00 bc	11.73 c	38 f	2.55 c	38.11 f	0.44 b
GA4	33.21 c	11.36 d	30 h	2.41 d	21.32 j	0.41 de

Means in a column followed by different alphabet(s) are significantly ($P < 0.05$) different according to DMRT

Nevertheless, the extent of colonization in both the soils was not influenced by the propagule number in the inoculum ($R^2 = 0.263$ and $R^2 = 0.011$; $P > 0.05$). Inoculation with *A. scrobiculata* increased colonization levels by 61 and 75 % in alfisol and vertisol compared to control. Similarly, inoculation with *G. aggregatum* increased colonization levels by 235 and 213 % than control in alfisol and vertisol respectively. Arbuscular mycorrhizal colonization was 109 and 74 % higher in *G. aggregatum* than in *A. scrobiculata* inoculated seedlings. *Glomus aggregatum* inoculated seedlings recorded a 16 % higher colonization levels in vertisol soil than alfisol soil indicating the significant influence of soil type. Arbuscular mycorrhizal colonization was significantly and linearly related to seedling biomass, nodule numbers, and nodular mass (Figs. 3, 4).

Nutrient Contents

Nutrient concentration in shoots and roots of *C. equisetifolia* seedlings was significantly enhanced in response to inoculation with strains of *A. scrobiculata* and *G. aggregatum* (Tables 4, 6). Inoculation of *A. scrobiculata* strains increased shoot N contents by 9 and 14 % in alfisol and 5 and 4 % in vertisol soils. In contrast, inoculation of *G. aggregatum* strains increased shoot and root N content by 81 and 74 % in alfisol and 77 and 57 % in vertisol soils compared to their respective controls. A significant influence of soil type on nutrient uptake was evident with AM fungal inoculated seedlings accumulating higher tissue N in alfisol than in vertisol soils.

Like N, shoot and root P concentrations were also significantly influenced by the fungal strains

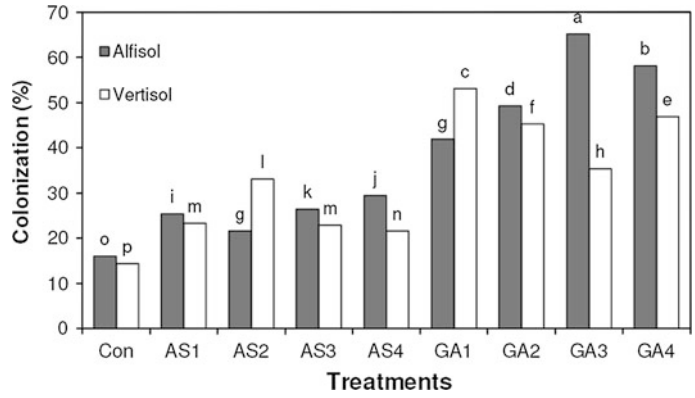

Fig. 2 Arbuscular mycorrhizal colonization of *C. equisetifolia* roots as assessed after 120 days after transplanting. Treatments include inoculation with different strains of *Acaulospora scrobiculata* (AS1, AS2, AS3, AS4) and *Glomus aggregatum* (GA1, GA2, GA3, GA4) or uninoculated control (Con). Bars bearing different letters are significantly different according to DMRT ($P < 0.05$)

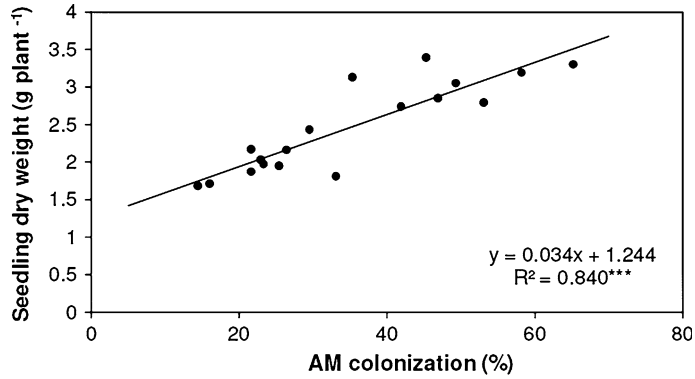

Fig. 3 Relation between arbuscular mycorrhizal (AM) colonization and dry weight of the seedlings. (*** Significant at $P < 0.001$)

and soil types (Tables 4, 6). Seedlings inoculated with *A. scrobiculata* strains had 18 and 44 % higher P in their shoots and roots compared to uninoculated seedlings in alfisol soil. In vertisol, seedlings inoculated with *A. scrobiculata* had 11 and 39 % higher P in their shoots and roots compared to control seedlings. Inoculation of *G. aggregatum* strains increased shoot and root P contents by 81 and 132 % in alfisol and 78 and 114 % over control in vertisol soils. The three-way ANOVA indicated that the soil and strain effects as well as the soil and fungal interaction were not significant for root P. Nodule number was significantly and linearly related to plant P (Fig 5).

Casuarina equisetifolia seedlings inoculated with *A. scrobiculata* and *G. aggregatum* strains accumulated more K in their tissues and were influenced by soil type (Tables 4, 6). Inoculation of *A. scrobiculata* increased shoot and root K by 10 and 8 % in alfisol and 9 and 3 % in vertisol soils. Nevertheless, compared to control, *G. aggregatum* strains inoculated seedlings had 45 and 27 % higher K in shoots and roots in alfisol. In vertisol soil, seedlings inoculated with *G. aggregatum* had 59 and 46 % higher K in their shoots and roots compared to uninoculated seedlings.

Efficiency of Nutrient Uptake

The N, P, and K uptake efficiencies were significantly influenced by AM fungi, their strains and soil types (Tables 5, 6). Average N uptake efficiency was 6 and 3 % higher than control for

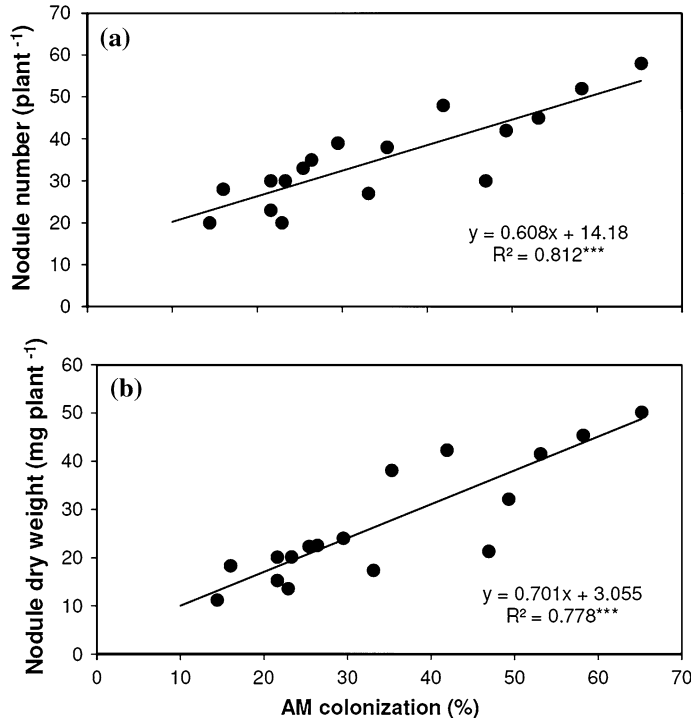

Fig. 4 Relation of arbuscular mycorrhizal (AM) colonization to nodule number (**a**) and nodule dry weight (**b**). (*** Significant at $P < 0.001$)

seedlings inoculated with *A. scrobiculata* in alfisol and vertisol soils. Whereas, N uptake efficiency of *G. aggregatum* inoculated seedlings was, respectively, 54 and 48 % higher than uninoculated seedlings in alfisol and vertisol soils. The efficiency of P uptake was 16 and 11 % higher than control for seedlings inoculated with *A. scrobiculata*. Whereas the efficiency of P uptake was 57 and 52 % higher than control for *G. aggregatum* strains inoculated seedlings in alfisol and vertisol soils respectively. Phosphorus uptake efficiency of *G. aggregatum* inoculated seedlings were 45–80 % higher than seedlings inoculated with *A. scrobiculata* strains. The efficiency of K uptake was 6 % higher for seedlings inoculated with *A. scrobiculata* strains in both the soil types. However, the efficiency of K uptake in alfisol and vertisol soils were 23 and 34 % higher for *G. aggregatum* inoculated seedlings than uninoculated seedlings. Nutrient uptake efficiencies were significantly related to the extent of AM fungal colonization (Fig. 6).

Mycorrhizal Inoculation Effect

Soil type had no significant influence on mycorrhizal inoculation effect (MIE) (Fig. 7 and Table 6). The MIE of seedlings inoculated with strains of *G. aggregatum* was 148 % higher than *A. scrobiculata* inoculated seedlings in alfisol soil; whereas the variations in the MIE of seedlings inoculated with the two fungi were only marginal (1 %) in vertisol. Change in MIE for soil types was higher for *A. scrobiculata* inoculated seedlings (12 %) compared to *G. aggregatum* (1.25 %) inoculated seedlings. The MIE was linearly related to the extent of AM colonization (Fig. 9a).

Seedling Quality Index (SQI)

Seedling quality was significantly influenced by soil types, AM fungi, and their strains (Fig. 8 and Table 6). The SQI of seedlings inoculated with *A. scrobiculata* was 26 and 31 % higher

Table 4 Influence of *Acaulospora scrobiculata* (AS) and *Glomus aggregatum* (GA) strains on tissue nutrient content of *Casuarina equisetifolia* as assessed after 120 days after transplantation

Soil type/Treatments	Nitrogen (%)		Phosphorus (%)		Potassium (%)	
	Shoot	Root	Shoot	Root	Shoot	Root
Alfisol						
Control	0.651 m	0.530 jk	0.120 m	0.059 e	0.270 ij	0.260 e
AS1	0.696 j	0.598 p	0.141 i	0.078 de	0.297 efg	0.278 de
AS2	0.673 kl	0.585 i	0.138 ijk	0.069 e	0.283 ghi	0.270 e
AS3	0.723 i	0.599 i	0.135 k	0.090 cde	0.299 efg	0.289 cde
AS4	0.755 h	0.632 h	0.152 h	0.103 b–e	0.310 e	0.290 bc
GA1	1.018 e	0.863 d	0.206 d	0.126 a–d	0.378 d	0.393 a
GA2	0.953 g	0.719 g	0.179 g	0.113 b–e	0.362 cd	0.370 b
GA3	1.432 a	1.101 a	0.259 a	0.173 a	0.428 a	0.149 f
GA4	1.306 c	1.013 b	0.223 c	0.135 abc	0.401 bc	0.412 ab
Vertisol						
Control	0.658 lm	0.519 kl	0.128 l	0.060 e	0.263 j	0.258 e
AS1	0.660 lm	0.540 j	0.136 jk	0.071 e	0.275 hij	0.263 e
AS2	0.742 h	0.588 i	0.153 h	0.099 cde	0.306 ef	0.289 cde
AS3	0.691 j	0.520 kl	0.139 ijk	0.083 ab	0.272 ij	0.260 e
AS4	0.680 jk	0.512 l	0.140 ij	0.080 de	0.290 fgh	0.250 e
GA1	1.001 f	0.760 f	0.198 f	0.138 abc	0.415 ab	0.350 bcd
GA2	1.391 b	0.973 c	0.260 a	0.097 cde	0.426 a	0.420 ab
GA3	1.231 d	0.716 g	0.250 b	0.152 ab	0.420 a	0.396 a
GA4	1.031 e	0.813 e	0.202 e	0.126 a–d	0.415 ab	0.342 bcd

Means in a column followed by different alphabet(s) are significantly ($P < 0.05$) different according to DMRT

compared to control in alfisol and vertisol soils respectively. Likewise, the SQI of seedlings inoculated with *G. aggregatum* strains were 78 and 90 % higher compared to uninoculated seedlings in alfisol and vertisol soils respectively. The seedling quality of *G. aggregatum* strain inoculated seedlings were higher compared to *A. scrobiculata* strain inoculated seedlings in both the soil types. A significant linear relationship existed between SQI and AM colonization (Fig. 9b).

Discussion

The growth of *C. equisetifolia* seedlings in different soil types was enhanced significantly by inoculation with strains of *A. scrobiculata* and *G. aggregatum* under nursery conditions. The results agree with earlier studies where inoculation of AM fungi has been shown to enhance growth of nursery raised *C. equisetifolia* seedlings (Vasanthakrishna et al. 1994, 1995; Rajendran et al. 2003; Muthukumar and Udaiyan 2010). Several investigators have shown the existence of genus and species-specific variations in the ability of AM fungi to promote plant growth and nutrient acquisition of actinorhizal plants (Yamanaka et al. 2005; He and Critchley 2008). The present study involving four strains each of *A. scrobiculata* and *G. aggregatum* confirms that the differences in plant benefits may also occur at intraspecific level. Of the two fungi studied, *G. aggregatum* improved seedling growth and nutrient acquisition to a greater extent than *A. scrobiculata*. Although a previous comparison of the growth promoting ability of these two fungi for a plant species is unavailable, existing information does indicate plant growth promotion by these fungi in different plant species (Ba and Guissou 1996; Ba et al. 2000; Gemma et al. 2002; Muthukumar and

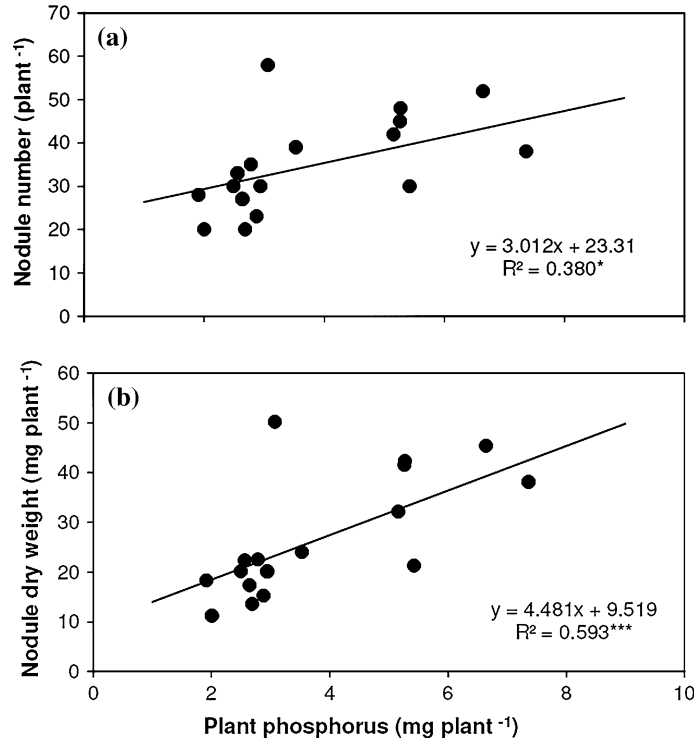

Fig. 5 Relation of plant phosphorus (shoot + root) to nodule number (**a**) and nodule dry weight (**b**). (*, ***Significant at $P < 0.05$ and $P < 0.001$)

Table 5 Influence of *Acaulospora scrobiculata* (AS) and *Glomus aggregatum* (GA) strains on efficiency of nutrient uptake in *Casuarina equisetifolia* at 120 days after transplantation

Soil type treatments	Efficiency of nutrient uptake (μg mg^{-1})		
	Nitrogen	Phosphorus	Potassium
Alfisol			
Control	45.01 jk	7.91 h	19.07 j
AS1	44.84 jk	8.65 fg	19.36 j
AS2	46.22 ij	8.98 f	19.70 ij
AS3	48.92 ghi	8.91 fg	20.63 hi
AS4	51.43 fgh	10.10 e	21.38 gh
GA1	58.38 e	11.33 c	22.45 efg
GA2	59.87 de	11.03 cd	23.71 cd
GA3	83.03 a	14.75 a	23.01 de
GA4	75.34 b	12.65 b	24.44 c
Vertisol			
Control	48.58 hi	9.05 f	19.93 ij
AS1	42.30 k	8.31 gh	18.00 k
AS2	51.79 fg	10.46 de	21.83 fg
AS3	53.75 f	10.60 de	21.71 gh
AS4	52.83 f	10.63 de	22.87 def
GA1	61.54 d	12.05 b	25.84 b
GA2	84.84 a	15.01 a	27.20 a
GA3	75.13 b	15.33 a	27.16 a
GA4	65.58 c	12.51 b	26.53 ab

Means in a column followed by different alphabet(s) are significantly ($P < 0.05$) different according to DMRT

Table 6 Results of ANOVA with corresponding *F*-ratio for the various seedling growth, nodulation, nutrient, and arbuscular mycorrhizal parameters assessed

Parameters	Two way (with control)			Three way (without control)						
	Soil (S1)	Treatments (T)	S1 × T	Soil (S2)	Fungi (F)	Strains (st)	S2 × F	S2 × St	F × St	S2 × F × St
	(df = 1,162) [#]*(df = 1,159)*	*(df = 8,162)* [#]*(df = 7,159)*	*(df = 8,162)* [#]*(df = 7,159)*	*(df = 1,144)*	*(df = 1,144)*	*(df = 3,144)*	*(df = 1,144)*	*(df = 3,144)*	*(df = 3,144)*	*(df = 3,144)*
SH[a]	113.62***	194.99***	26.64***	51.60***	112.67***	19.87***	23.09***	31.46***	23.02***	7.99***
RCD	1.86 ns	104.91***	21.25***	<1 ns	313.37***	9.41***	<1 ns	21.36***	8.02***	30.59***
NN	739.57***	243.48***	51.07***	723.28***	1,379.32***	23.10***	10.25***	132.03***	33.08***	5.06**
SDW	16.09***	2,086.86***	47.68***	26.38***	11,895.30***	260.36***	29.30***	95.96***	357.24***	37.23***
NDW	5,416.34***	7,245.75***	1,113.88***	4,628.98***	42,299.71***	730.25***	68.64***	2,077.90***	768.46***	820.49***
RDW	90.16***	576.72***	22.58***	90.58***	3,435.11***	16.48***	2.27 ns	45.48***	47.18***	11.90***
SN	23.51***	416.39***	588.43***	8.30**	526.74***	251.16***	1.64 ns	424.11***	180.70***	197.14***
RN	1,004.40***	2,089.40***	556.47***	1,061.28***	12,902.84***	82.38***	69.08***	886.79***	113.81***	477.61***
SP	77.55***	2,176.81***	227.00***	62.98***	12,857.78***	222.20***	52.28***	414.35***	314.50***	173.94***
RP	42.78***	1,036.13***	68.35***	1.29 ns	25.59***	1.11 ns	1.59 ns	3.36*	1.01 ns	1.13 ns
SK	55.65***	2,812.00***	163.00***	76.15***	17,058.03***	61.08***	479.04***	221.68***	93.72***	22.56***
RK	59.92***	442.06***	299.73***	4.32*	310.43***	39.69***	29.12***	53.69***	37.95***	57.95***
ENU	22.90***	258.54***	38.16***	20.20***	1,759.99***	122.56***	<1 ns	74.77***	23.37***	57.51***
EPU	110.40***	213.54***	17.66***	98.21***	1,073.35***	79.16***	4.27*	31.27***	36.03***	15.12***
EKU	114.13***	94.57***	9.51***	121.68***	546.42***	29.91***	42.86***	4.88***	6.00***	6.14***
AM	672.26***	3,433.20***	654.05***	210.61***	6,783.44***	19.91***	210.11***	333.29***	20.77***	218.77***
MIE[#]	3.15 ns	633.10***	13.13***	3.15 ns	3,848.48***	92.24***	9.09***	23.60***	102.17***	4.00**
SQI	146.88***	600.72***	30.33***	127.11***	3,103.79***	51.52***	<1 ns	52.83***	68.93***	28.64***

[a] *SH* seedling height, *RCD* root collar diameter, *NN* nodule number, *SDW* shoot dry weight, *NDW* nodule dry weight, *RDW* root dry weight, *SN* shoot nitrogen, *RN* root nitrogen, *SP* shoot phosphorus, *RP* root phosphorus, *SK* shoot potassium, *RK* root potassium, *ENU* efficiency of nitrogen uptake, *EPU* efficiency of phosphorus uptake, *EKU* efficiency of potassium uptake, *AM* Arbuscular mycorrhizal colonization, *MIE* mycorrhizal inoculation effect, *SQI* seedling quality

*,**,***Significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. *ns* Not significant

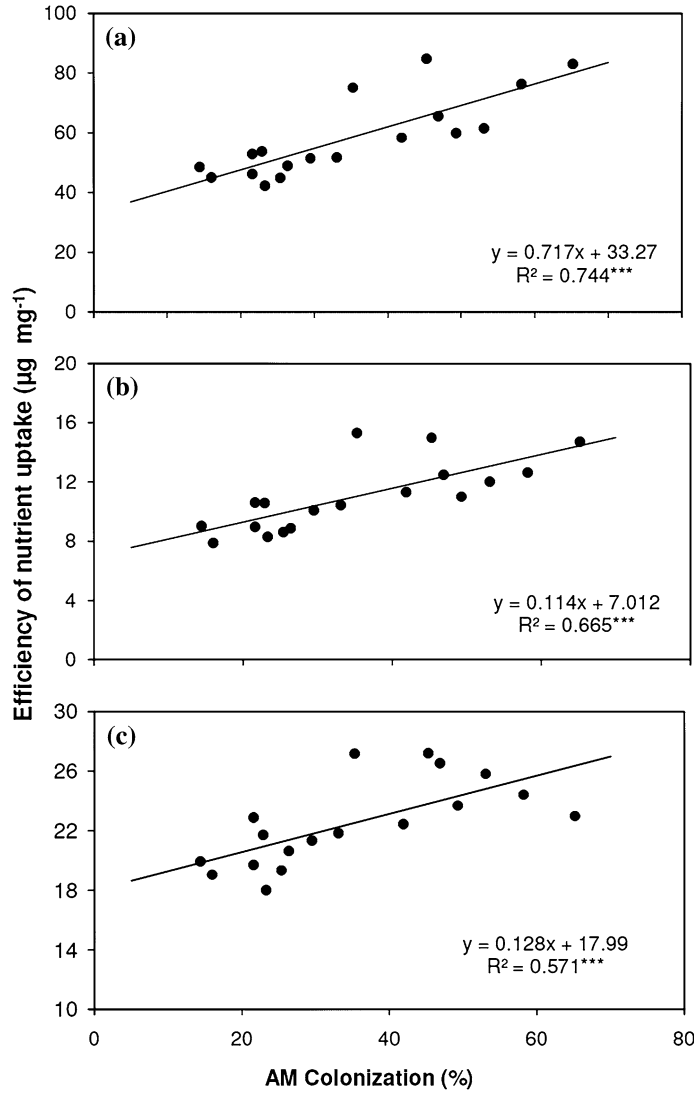

Fig. 6 Relationship of arbuscular mycorrhizal (AM) colonization to uptake efficiencies of nitrogen (**a**), phosphorus (**b**), and potassium (**c**). *** Significant at $P < 0.001$

Udaiyan 2006; Tian et al. 2002; Shukla et al. 2009, 2012). The differences in the benefits incurred by the fungi on *C. equisetifolia* could be caused by two possible factors: variation in the carbon-sink strength and the amount of extraradical hyphae produced. Although these factors are not studied here, previous studies do clearly indicate the role of these factors in intraspecific AM fungal variabilities. Lerat et al. (2003) showed that the differences in *G. mosseae* strains to influence plant growth in *H. vulgare* was related to their variations in carbon-sink capacity. Graham et al. (1982) found that the ability of *Glomus fasiculatum* strains to confer growth enhancement in citrus was related to the amount of external hyphal production by the strains. Recently, it was found that strains of *G. intraradices* could differ in their mycelia architecture and their ability to support mycorrhizospheric microorganisms (Bidondo et al. 2012).

Maximum AM colonization occurred in seedlings inoculated with the strain GA3 in alfisol and strain GA2 in vertisol. Such variations in the extent of colonization have been reported in wheat colonized by different strains of *Acaulospora laevis* and *Scutellospora calospora* (Graham and Abbott 2000). Wilson and

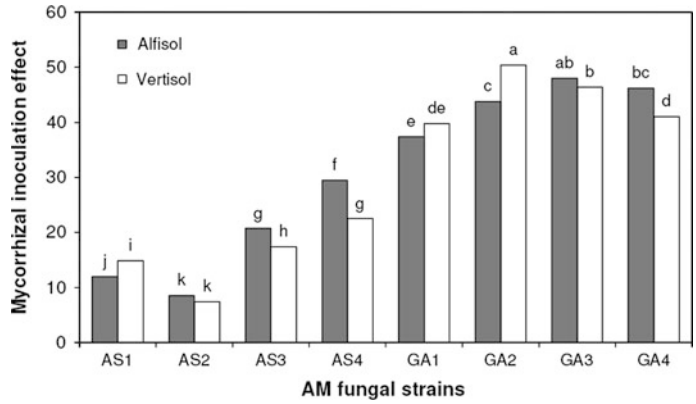

Fig. 7 Mycorrhizal inoculation effect of *Casuarina equisetifolia* as assessed after 120 days after transplanting. Treatments include inoculation with different strains of *Acaulospora scrobiculata* (AS1, AS2, AS3, AS4) and *Glomus aggregatum* (GA1, GA2, GA3, GA4). Bars bearing different letters are significantly different according to DMRT ($P < 0.05$)

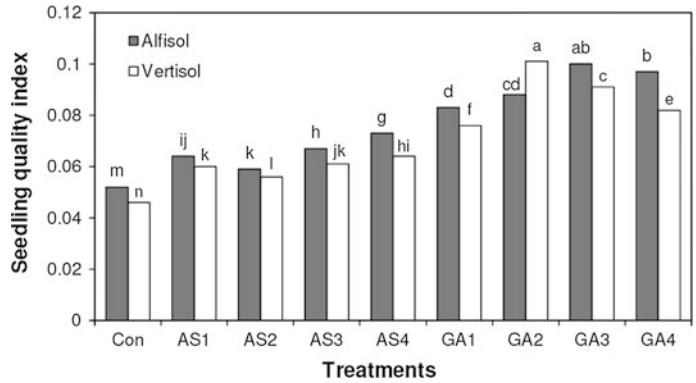

Fig. 8 Seedling quality of *Casuarina equisetifolia* as assessed after 120 days after transplanting. Treatments include inoculation with different strains of *Acaulospora scrobiculata* (AS1, AS2, AS3, AS4) and *Glomus aggregatum* (GA1, GA2, GA3, GA4) or uninoculated control (Con). Bars bearing different letters are significantly different according to DMRT ($P < 0.05$)

Trinick (1983) used the term aggressiveness to indicate the ability of AM fungi to compete with other AM fungi for colonization space within roots. In this context, strains of *G. aggregatum* studied here could be termed as more aggressive than strains of *A. scrobiculata*. Aggressive root colonizing AM fungi are essential for inoculation programs since rapid colonization determines the effectiveness of the inoculant fungi over the aggressive resident fungi that commonly occur in native soils.

In accordance with Muthukumar and Udaiyan (2010), inoculation of AM fungal strains increased seedling root dry weights. It has been shown that AM fungi could alter plant root system architecture and root morphologies (Berta et al. 1990; Bouma et al. 2001). In *Allium cepa* (Toro et al. 1997) and *Prunus cerasifera* (Berta et al. 1995) AM fungi has been shown to increase root dry weights through increasing the root diameter. The possession of a larger root system may be advantageous for seedlings when transplanted onto soil that is nutrient deficient.

In this study, the significant variation in nodulation by *Frankia* inoculated with different AM fungal strains and soil types is in accordance with Masuka and Makoni (1995) where soil types are found to affect nodulation in

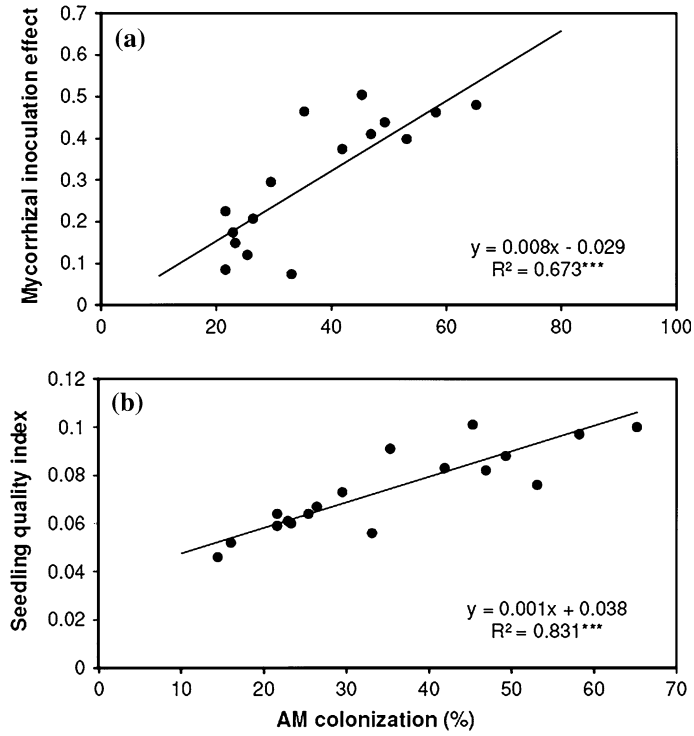

Fig. 9 Relation of arbuscular mycorrhizal (AM) colonization to mycorrhizal inoculation effect (**a**) and seedling quality (**b**). (*** Significant at $P < 0.001$)

Casuarina cunninghamiana. It has been shown that co-inoculation of AM fungi and *Frankia* significantly increased nodulation of actinorhizal plants (Sempavalan et al. 1995; Wheeler et al. 2000; Tian et al. 2002; Muthukumar and Udaiyan 2010). This is also confirmed in the present study by the existence of a significant linear relation between AM colonization and nodulation variables. The number of nodules per plant and the nodular mass was stimulated by increasing plant P as evidenced by the linear relation between plant P and nodular variables. Although P stimulation of nodulation in actinorhizal plants like legumes has been ascribed to general stimulation of plant growth (Yang 1995; Reddell et al. 1997), the existence of an exponential relation between plant P and nodular variables in this study suggests the influence could be specific for nodulation. Such stimulation of nodulation specifically by plant P has been reported earlier (Valverde et al. 2000; Gentili and Huss-Danell 2002).

In this work, nutrient concentrations in the shoots and roots of the seedlings were increased by AM inoculation. The increased nutrient concentrations could have resulted from the increased uptake of nutrients facilitated by AM fungi. The higher amount of nutrient uptake per unit of the root for AM inoculated seedlings is in line with previous studies where such a response has been reported (Muthukumar et al. 2001; Muthukumar and Udaiyan 2006, 2010). The existence of a significant linear relation between AM colonization levels and the efficiency of nutrient uptake confirms this. Among the various nutrients, the higher N content of the inoculated seedlings is of interest as 15–80 % of the plant nitrogen in *Casuarina* is derived from nitrogen fixation (He and Critchley 2008). It has been suggested that P may not only play an important role in nodulation, but can also have a significant role in nitrogen fixation of actinorhizal plants (Gentili and Huss-Danell 2003). It is conceivable that the improved nitrogen content of AM inoculated seedlings may be due to the improvement in P nutrition which would be followed by improved colonization by AM fungi. This fact is supported by the existence of a significant ($P < 0.01$) relation between plant N, plant P, and AM variables (N × P:

$R^2 = 0.691$; N × AM: $R^2 = 0.736$; P × AM: $R^2 = 0.407$).

The mycorrhizal dependency or response as evidenced by MIE suggests that MIE could be influenced by AM fungal species, their strains and soil types. Variations in mycorrhizal response to different strains of *A. laevis* and *S. calospora* have also been reported for wheat (Graham and Abbott 2000). By contrast to what was found by Graham and Abbott (2000), the aggressive strains of *G. aggregatum* in this study increased nutrient uptake and seedling growth significantly. Further, the existence of a linear relation between AM colonization and MIE suggests that the rate of colonization could be used as a predictor for the plant growth response to AM inoculation in low nutrient soils. The quality of AM fungi inoculated seedlings is significantly higher, which is in line with studies (Muthukumar et al. 2001; Rajendaran et al. 2003; Muthukumar and Udaiyan 2006, 2010) where such a response has been reported for *C. equisetifolia* and other species. This improvement in seedling quality of inoculated seedlings could have been the result of enhanced nutrient status brought about by improved nutrient uptake and nitrogen fixation.

Conclusion

In conclusion, *C. equisetifolia* seedlings inoculated with different AM fungal strains exhibited varied growth response. In this work, we have shown that the plant response to AM fungi can vary with the strains selected as well as the soil type used. Thus, selection of a compatible AM fungi for a plant species should not only include strains that are compatible with the host but also should be compatible with the soil type that is essential for successful inoculation programs.

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Biodegradation of Direct Red-28 by *Bacillus* sp. Strain DRS-1 Under Aerobic Conditions and Analysis of Phytotoxicity Levels

J. Hemapriya, S. Vijay Anand, and V. Rajeshkannan

Abstract

Discharge of Azo dyes into the environment that generates toxicity and mutagenesis is a major concern in society; alternate ecofriendly approaches are needed to remediate the dye-contaminated wastewaters discharged from a range of textile industries. The only way to make the environment pollution free is by means of microorganisms. In this study, a potential bacterium capable of degrading the Red-28 azo dye was isolated from the dye-contaminated effluents by decolorization of Red-28 and with various culture conditions for bacterial biomass production as temperature, pH, and agitation rates with concentration of dye, carbon sources, various nitrogen sources, and various metal ions. The strain was identified as *Bacillus* sp. DRS-1 is based on the 16S rDNA gene sequence. The identified *Bacillus* consortium was shown to decolorize which was observed through biomass activity and decolorization with batch assay, optimum at 45 °C and pH 8.0. The degraded sample was further monitored through HPLC, FTIR, and GC–MS; it was cross-checked with the processed water quality profiles by the Ministry of Text tiles, Government of India. The decolorization was studied by various immobilization matrices and phytotoxicity experiments with bacterial consortium of *Bacillus* sp DRS-1. The results showed that maximum decolorization was with Ca-alginate matrix. The present study was suggested as a direction for the development of successful large-scale biotechnological progression for the removal of dyes and by-products in textile effluents.

Keywords

Biodegradation · *Bacillus* sp · Aerobic condition · Phytotoxicity

Introduction

Textile industries utilize enormous amounts of water and chemicals for the wet processing of textiles and also use various types of synthetic

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dyes to impart attractive colors of commercial importance (Easton 1995). The major classes of synthetic dyes that are used for textile dyeing and other industrial applications include acidic, basic, azo, reactive, anthraquinone, and triaryl methane dyes (Padmavathy et al. 2003; Garcia-Montano et al. 2007). Azo dyes, which are aromatic compounds with one or more –N=N– groups, constitute the largest class of synthetic dyes used in commercial applications (Akhtar et al. 2005; Kumar et al. 2007). In 1994, the world's productions of dyes was around one million ton, of which more than 50 % was azo dyes (Stolz 2001). Azo dyes are widely used in a number of industries such as textiles, dyeing, food, cosmetics, paper printing, with the textile industry being the largest consumer (Frank et al. 2002). There are more than 100,000 commercially available dyes with over 7×10^7 tons of dyestuff produced annually worldwide (Asad et al. 2007).

In general, all dyes do not bind to the fabric; depending on the class of the dye, its loss in wastewaters could vary from 2 % for basic dyes to as high as 50 % for reactive dyes, leading to severe contamination of surface and groundwaters in the vicinity of dyeing industries (Ganesh et al. 1994; O' Neill et al. 1999). Further, their discharge into surface water leads to aesthetic problems and obstructs light penetration and oxygen transfer into water bodies, hence affecting hydrophytes and aquatic life (Rajaguru et al. 2002; Umbuzeiro et al. 2005). Textile effluents may contain dyes of various intense colors, such as dyes having the functional groups of alkenes, aromatic, C–N, and S–O bonds of red color. Some are made by inorganic molecules such as Al–O, Si–O, K–O, and N=N bonds which are responsible for color development in wastewater (Manikandan et al. 2009). Color is the first contaminant in the wastewater. In addition to their visual effect and their adverse impact in terms of COD, many dyes are toxic, mutagenic, and carcinogenic (Isik and Sponza 2003; Acemioglu 2004). The toxicity of industrial effluents may be attributed to the presence of metals, chlorides, etc., and the breakdown products of dyes (O'Neill et al. 1999; Hao et al. 2000).

Irrigation with untreated industrial effluent at different concentrations drastically reduced the germination and vigor index of crops like rice, cow-pea, and maize and decreased the nitrogen fixation ability in green gram. But the diluted effluent improved the germination rate and increased the chlorophyll, carbohydrate, and protein content. However, the biologically treated effluent enhanced the yield and quality of many cereals and pulses (Manikandan et al. 2009). Dyes are identified as the most problematic compounds in textile effluents due to their high water solubility and low degradability (Balan and Monteiro 2001; Toh et al. 2003). Moreover, the high volumetric rate of industrial effluent discharge in combination with the increasingly stringent legislation makes the search for appropriate treatment technologies an important priority (Romero et al. 2006; Khelifi et al. 2009). With the increased use of a variety of dyes and synthesis of new ones to satisfy the ever-growing and changing needs of man, pollution by dye wastewaters is currently recognized as a serious environmental issue (Moreira et al. 2004; Mendez-Paz et al. 2005).

There are several reports on the use of physicochemical methods for color removal from dye-containing effluents (Coro and Laha 2001; Jethva et al. 2001; Dominguez et al. 2005). Extensively used coagulation/flocculation techniques produce large amounts of sludge, which require safe disposal. Adsorption, and to a certain extent, the membrane filtration techniques lead to secondary waste streams which need further treatment. These constraints have led to the consideration of advanced oxidation processes (AOP) and biological methods as attractive options for the treatment of dye-containing wastewaters (Muthukumar et al. 2005). AOP are defined as those processes that employ strong oxidizing agents (H_2O_2, Fenton's reagent) or heterogeneous photocatalysts such as TiO_2, ZnO_2, Mn, and Fe in the presence or absence of an irradiation source. These involve mainly the generation of (OH) radical for the destruction of refractory and hazardous pollutants (Kdasi et al. 2004). However, the AOP are energy consuming

and cost intensive. Physical and chemical methods are not widely used for the treatment of industrial wastewater due to the high cost and secondary pollution that are generated by the excessive usage of the chemicals (Jadhav et al. 2008). Alternative biodegradation systems through the use of bacteria have been shown to be highly effective (Pearce et al. 2003).

Environmental biotechnology is constantly expanding its efforts in the biological treatment of dye-contaminated wastewaters (Junghanns et al. 2008). Synthetic dyes are recalcitrant to microbial degradation because they contain substitutions such as azo, nitro, or sulfo groups (Pagga and Brown 1986; Shaul et al. 1991). Microbial decolorization is an environment-friendly and cost competitive alternate to physicochemical treatment decomposition processes (Umbuzeiro et al. 2005; Dafale et al. 2008). It is known that several microorganisms, including bacteria, yeasts, fungi, and algae, can decolorize and even completely mineralize many azo dyes under certain environmental conditions. Many researchers have reported on the microbial decolorization of azo dyes (Diniz et al. 2002; Savelieva et al. 2004; Carvalho et al. 2008; Ponraj et al. 2011). The decolorization process is achieved by the reductive cleavage of azo bond in anaerobic conditions, but the end products of this reaction are often more dangerous than the parent compounds (Idaka et al. 1987; Talarposhti et al. 2001). Moreover, their complete mineralization occurs only in the presence of molecular oxygen, so that a further step is required (McMullan et al. 2001; Xu et al. 2006). However, decolorization of azo dyes can be achieved by lignolytic fungi and aerobic bacteria in aerobic conditions (Wesenberg et al. 2003; Tan et al. 2005). In this case, one-step degradation process can be carried out, resulting in azo dye decolorization and at the same time aerobic degradation of the metabolites formed (Steffan et al. 2005; Barragan et al. 2007).

This study deals with the biodegradation of Direct Red-28 by a newly isolated bacterial strain *Bacillus* sp. DRS-1 under aerobic conditions. The optimal culture conditions for maximizing the bacterial biomass and decolorization ability of *Bacillus* sp. strain DRS-1 were investigated. The degradation of Direct Red-28 by the isolate was monitored by HPLC, FTIR, and GC–MS. Furthermore, immobilization studies were carried out with various immobilization matrices. Finally, phytotoxicity assay was performed to assess the toxicity of treated and untreated dye sample on selected plants.

Materials and Methods

Azo Dye Used

Synthetic textile azo dye, Direct Red-28 used for this study was procured from a local textile dyeing unit. Stock solution was prepared by dissolving 1 g of Direct Red-28 in 100 ml distilled water. The dye solution was sterilized by membrane filtration (Millipore Millex®-GS, 0.22 mm filter unit), since azo dyes may be unstable to moist heat sterilization. All chemicals used were of highest purity available and of analytical grade.

Isolation and Screening of Bacterial Strains Decolorizing Direct Red-28

Approximately, 100 effluent samples were collected from the three different sites (S_1, S_2, and S_3). The collected samples were serially diluted and spread over basal nutrient agar medium (composition gl^{-1}: peptone, 5.0; beef extract, 3.0; and NaCl, 5.0) containing 50 ppm of Direct Red-28. The pH was adjusted to 7.0 before autoclaving and incubated at 37 °C for 5 days (Senan and Abraham 2004). Colonies surrounded by halo (decolorized) zones were picked and streaked on nutrient agar plates containing Direct Red-28 (Wong and Yuen 1996). The plates were reincubated at 37 °C for 3 days to confirm their abilities to decolorize Direct Red-28. Different colonies of dye decolorizing bacteria were picked and restreaked several times to obtain pure cultures. The pure cultures were maintained on dye-containing nutrient agar slants at 4 °C.

Decolorization Assay

A loopful of bacterial culture was inoculated in Erlenmeyer flask containing 100 ml of nutrient broth and incubated at 150 rpm at 37 °C for 24 h. Then, 1 ml of 24-h-old culture of DRS-1 strain was inoculated in 100 ml of nutrient broth containing 50 ppm of Direct Red-28 and reincubated at 37 °C till complete decolorization occurs. Suitable control without any inoculum was also run along with experimental flasks. 1.0 ml of sample was withdrawn every 12 h and centrifuged at 10,000 rpm for 15 min. Decolorization extent was determined by measuring the absorbance of the culture supernatant at 500 nm using UV–visible spectrophotometer (Hitachi U 2,800), according to Chen et al. (2003).

$$\text{Decolorization efficiency}\,(\%) = \frac{\text{Dye}\,(i) - \text{Dye}\,(r)}{\text{Dye}\,(i)} \times 100$$

where, Dye (i) refers to the initial dye concentration and Dye (r) refers to the residual dye concentration. Decolorization experiments were performed in triplicates.

Bacterial Strain and Culture Conditions

Bacterial strain that showed maximum decolorization percentage on Direct Red-28 was aerobically cultured in nutrient broth containing 50 ppm of Direct Red-28. The pH was adjusted to 7.0. For frequent use, the culture was maintained by transfer to a fresh medium at 24 h intervals. When required for prolonged periods, it was maintained by subculturing once every 7 days on slants, prepared by solidifying the above medium with 2.0 (w/v) agar.

16S rDNA Analysis of DRS-1 Isolate

The DRS-1 cells were inoculated into basal nutrient broth medium and incubated at 35 °C for 24 h. The culture was centrifuged at 8,000 rpm for 4 min to separate cell pellet. Then the total genomic DNA was extracted using bacterial genomic DNA isolation kit (RKT09), purchased from Chromous Biotech Pvt. Ltd., Bangalore, India. The 16S rDNA sequence of the isolate DRS-1 was amplified via the polymerase chain reaction (PCR), using two universal primers: the 16S forward primer 5′-AGAGTRTGATCMTYGCTWAC-3′ and the 16S reverse primer 5′-CGYTAMCTT WTTACGRCT-3′, which yielded a product of approximately 1484 bp (~1.5 kb). The purified PCR product was directly sequenced using Big Dye Terminator version 3.1 Cycle sequencing kit according to procedure of Pidiyar et al. (2004). The sequencing reactions were run on AB-PRISM automated sequencer (ABI-3730 genetic analyzer).

The nucleotide sequence analysis was done at BLAST-n site at NCBI server www.ncbi.nlm.nih.gov/BLAST. The alignment of the sequences was done using CLUSTAL W program VI.82 at European Bioinformatics site (www.ebi.ac.uk/clustalw). The analysis of 16S rDNA gene sequence was done at ribosomal data base project (RDP) II (http://rdp.cme.msu.edu). The sequence was refined manually after crosschecking with the raw data to remove ambiguities. The phylogenetic tree was constructed using the aligned sequences by the neighbor joining method using kimura-2 parameter distances in MEGA 2.1 software (Kumar et al. 2001).

Optimization of Various Culture Conditions for Bacterial Biomass and Direct Red-28 Decolorization by DRS-1 Strain

Effect of Temperature, pH, Agitation Rates, and Dye Concentrations

The effect of temperature, pH, agitation rates, and dye concentration on both bacterial biomass and dye decolorizing ability of *Bacillus* sp. strain DRS-1 was studied. This was carried out by incubating the bacterial strain at different temperatures (20–60 °C), different initial pH

values of the medium (pH 4.0–10.0), different agitation speeds (0–200 rpm), and various dye concentrations (50–1,000 ppm). Bacterial biomass with decolorization percentage was measured at optimum growth (24 h).

Effect of Carbon Sources

The effect of various soluble carbon sources (1 % w/v) such as glucose, sucrose, lactose, maltose, and starch on bacterial growth and dye decolorization extent of Direct Red-28 by *Bacillus* sp. strain DRS-1 was investigated after 24 h of incubation at 45 °C.

Effect of Various Nitrogen Sources

The effect of two different categories of nitrogen sources (1 % w/v), viz, organic nitrogen sources (tryptone, beef extract, peptone, yeast extract, and meat extract) and inorganic nitrogen sources [$(NH_4)_2SO_4$, KNO_3, NH_4Cl, NH_4NO_3, and $NaNO_3$] were investigated on the bacterial growth and dye decolorization ability of *Bacillus* sp. strain DRS-1, after 24 h of incubation at 45 °C.

Effect of Various Metal Ions

The effect of various metal ions on bacterial growth and dye decolorization percentage by *Bacillus* sp. strain DRS-1 was investigated by cultivating the bacteria in basal nutrient broth media containing 50 ppm Direct Red-28, in the presence of various metal ions (5 mM) such as $MnCl_2$, $MgCl_2$, $HgCl_2$, $ZnSO_4$, $CoCl_2$, and $FeSO_4$, incubated at 45 °C for 24 h.

Analysis of Biodegraded Samples by HPLC, FTIR, and GC–MS

Biodegradation of Direct Red-28 was monitored by high performance liquid chromatography (HPLC), Fourier transform infrared (FTIR) spectroscopy, and Gas chromatography–mass spectroscopy (GC–MS).

HPLC Analysis of Decolorized Sample

10 ml of decolorized sample was taken after 24 h of incubation, centrifuged at 12,000 g for 30 min, and filtered through 0.45 μm membrane filter (Millipore). The filtrate was then extracted with diethyl ether and flash evaporated in rotary vacuum evaporator in temperature controlled water bath (50 °C) and residue was dissolved in 2 ml of HPLC grade methanol and used for analysis. This extracted sample was analyzed by HPLC having a mobile phase of 50:49.6:0.4 % (methanol: water: disodium hydrogen phosphate). HPLC conditions: HPLC analysis was carried out using 18 columns with a flow rate of ml min^{-1}, chart speed of 1 cm min^{-1}, and UV detector at 280 nm.

FTIR Analysis of Decolorized Sample

The biodegraded Direct Red-28 was characterized by FTIR spectroscopy (Perkin-Elmer, Spectrum one). The analysis results were compared with the control dye. The FTIR analysis was done in the mid IR region (400–4,000 cm^{-1}) with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio (5:95). The pellets were fixed in sample holder and then analyzed (Saratale et al. 2009).

GC–MS Analysis of Biodegraded Sample

Biodegraded sample was centrifuged and filtered through 0.45 μm membrane filter. The filtrate was then extracted with diethyl ether and flash evaporated in rotary vacuum evaporator in temperature controlled water bath (50 °C) and residue was dissolved in HPLC grade methanol for GC–MS analysis. GC–MS conditions: The GC–MS analysis of metabolite(s) was carried out using a QP5050 gas chromatography

coupled with mass spectrophotometer (Shimadzu double beam spectrophotometer, UV1601). The analysis was performed in the temperature programming mode at an ionization voltage of 70 eV. The temperature of the restex column (0.25 mm, 60 m; XT1-5) was kept at 60 °C for initial 2 min, then raised up to 220 °C with rate of 8 °C/min, and then raised up to 220 °C with rate of 2 °C/min and held at 260 °C for 15 min. The temperature of the injection port and the GC–MS interface was maintained at 270 and 280 °C respectively. The flow rate of helium as a carrier gas was 0.1 ml/min. The compounds were identified using WILEY and NIST library on the basis of mass spectra and retention time.

Influence of Various Immobilization Matrices on Decolorization of Direct Red-28 by *Bacillus* sp. Strain DRS-1

Whole Cell Immobilization in Ca-Alginate

Sodium alginate solution (4 %, w/v) was prepared by dissolving sodium alginate in 100 ml boiling water and autoclaved at 121 °C for 20 min. The cell suspensions of *Bacillus* sp. strain DRS-1 (4 g wet weight) was added to the alginate slurry, mixed, and stirred well for 10 min to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into 0.2 M $CaCl_2$ solution from 5 cm height and kept for curing at 4 °C for 1 h. The cured biocatalysts were washed twice with 50 mM Tris–HCl buffer (pH 7.0) and placed in the same buffer (Johnsen and Flink 1986).

Immobilization of Whole Cells in Agar–Agar

A definite quantity of agar–agar was dissolved in 18 ml of 0.09 % sodium chloride solution to get final concentration of 2 % and sterilized by autoclaving. The cell suspension (2 ml) was added to the molten agar–agar maintained at 40 °C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4-inch-diameter petriplates, and allowed to solidify. The solidified agar blocks were cut into equal sized cubes (4 mm^3), added to sterile 0.1 M phosphate buffer (pH 7.0), and kept in the refrigerator (1 h) for curing. The cured biocatalysts were washed twice with 50 mM Tris–HCl (pH 7.0) and placed in the same buffer (Adhinarayana et al. 2005).

Immobilization of Whole Cells in Polyacrylamide

The cell suspension was prepared by adding 0.03 g cells to 10 ml chilled sterile distilled water. To another 10 ml of 0.2 M sterile phosphate buffer (pH 7.0), the following chemicals were added: 2.85 g acrylamide, 0.15 g bisacrylamide, 10 mg ammonium persulfate, and 1 ml NNN^1N^1 tetramethylethylenediamine (TEMED). The cell suspension and the above phosphate buffer mixture were mixed well and poured into sterile flat bottomed 10 cm-diameter petri plates. After polymerization, the acrylamide gel was cut into equal size cubes (4 mm^3), transferred to 0.2 M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 h for curing. The cured biocatalysts were washed twice with 50 mM Tris–HCl (pH 7.0) and placed in the same buffer (Adhinarayana et al. 2005).

Immobilization of Whole Cells in Gelatin

2 ml of cell suspension was added to 15 ml of 20 % sterile gelatin maintained at 45 °C, and poured into a sterile petridish. The gel was over layered with 10 ml of 5 % glutaraldehyde for hardening at 30 °C. Then the blocks were cut into small-sized cubes (4 mm^3). The cured

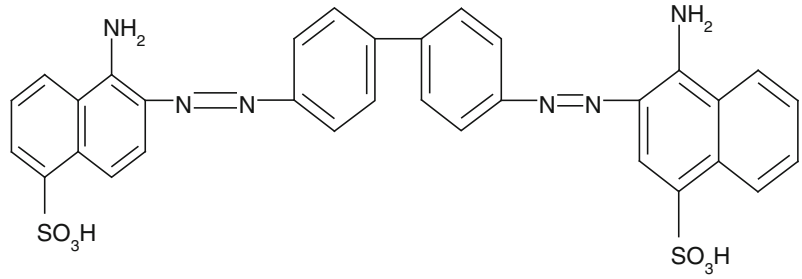


Fig. 1 Chemical structure of Direct Red-28

biocatalysts were washed twice with 50 mM Tris–HCl (pH 7.0) and placed in the same buffers (Veelken and Pape 1982).

Phytotoxicity Studies

Phytotoxicity tests were performed to assess the toxicity of the untreated and treated dye. The ethyl acetate extracted products of Direct Red-28 degradation were dried and dissolved in 5 ml sterile distilled water to make a final concentration of 100 ppm for phytotoxicity studies. The phytotoxicity tests were carried out on two kinds of seeds, one from grains, *Sorghum vulgare* Pers. (monocot) and the other from pulses *Phaseolus mungo* L. (dicot) commonly practiced in Indian agriculture (Parshetti et al. 2006). The study was carried out at normal room temperature. Ten healthy plant seeds of each variety were treated separately with 5 ml of control dye, Direct Red-28, and its degraded products (100 ppm) per day. Control set was carried out using distilled water at the same time. Germination percentage as well as the length of plumule and radical was recorded after 7 days (Saratale et al. 2009).

Statistical Analysis

For statistical analysis, standard deviation for each experimental result was calculated using Excel Spread Sheets available in Microsoft Excel. The results presented in this study is the mean of three independent determinations. Bars correspond to standard deviation.

Results

Dyestuff Used

The dyestuff used in this study was Direct Red-28 with color index number 22120 (www.sigmaaldrich.com) and molecular formula of $C_{32}H_{22}N_6S_2O_6Na_2$. The absorption maximum of this dye was 500 nm. They are widely used in textile and leather industries. The structure of Direct Red-28 is shown in Fig. 1.

Isolation and Screening of Bacterial Strains Decolorizing Direct Red-28

Fifteen different bacterial isolates (DRS-1–DRS-15) that were capable of decolorizing Direct Red-28, a synthetic textile azo dye were isolated from the textile effluent samples including S_1, S_2, and S_3 in and around Tiruvettipuram, Tiruvannamalai district, Tamil Nadu, India. Of them, DRS-1 isolate (Figs. 2 and 3) was found to be the most promising strain that showed the maximum decolorization percentage of 92.25 %, within 24 h of incubation. In contrast, DRS-6 and DRS-7 strains showed poor decolorization efficiency of 53.06 and 50.32 % respectively (Table 1). Morphological, cultural, and biochemical characteristics of DRS-1 strain are tabulated in Table 2.

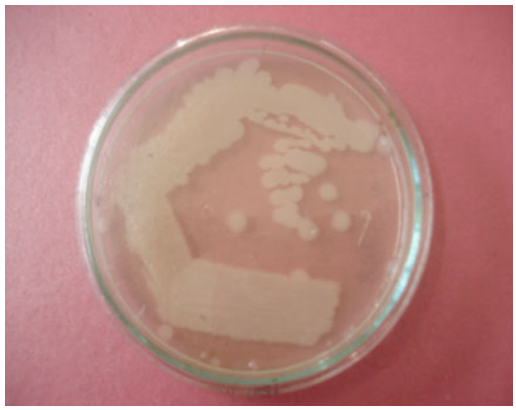


Fig. 2 *Bacillus* sp. strain DRS-1 grown in nutrient agar plate

Fig. 3 Decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1

16S rDNA Analysis of DRS-1 Isolate

A total of 1,484 base sequences of PCR amplified 16S rDNA gene was determined from the isolate DRS-1 (Fig. 4), which corresponds to more than 99 % of the gene sequence. In the analysis at NCBI and RDP sites it showed homology to 16S rDNA sequences from *Bacillus* species. In the phylogenetic analysis, the sequence formed a cluster within *Bacillus* species with 100 % bootstrap value, confirming the identity of the isolate as a strain of this species (Fig. 5). The highest similarity value exists with *B. anthracis*. JH16 (gene bank entry: DQ232744). The bacterial strain was identified as *Bacillus* sp. strain DRS-1 (Fig. 6).

Optimization of Culture Conditions for Maximizing Bacterial Biomass and Dye Decolorizing Ability of *Bacillus* sp. Strain DRS-1

Effect of Incubation Time

Of the various parameters, incubation time played a significant role in maximizing both bacterial growth and dye decolorizing ability of *Bacillus* sp. strain DRS-1. The results of this study revealed that the dye decolorizing ability of the isolate was dependent on the bacterial growth. The bacterial cells started multiplying within 4 h and reached their maximum growth within 24 h and thereafter started to decline, due to the depletion of nutrients and accumulation of toxic metabolites. Similarly, dye decolorizing ability of DRS-1 strain on Direct Red-28 was found to be maximized after 24 h of incubation (Figs. 7 and 8).

Effect of Temperature

The effect of incubation temperature on both bacterial growth and dye decolorizing ability of *Bacillus* sp. strain DRS-1 was analyzed at temperatures ranging from 25 to 60 °C. The results shown in Figs. 9 and 10 reveal that *Bacillus* sp. strain DRS-1 showed strong decolorizing activity and highest bacterial growth from 40 to 50 °C, with optimum being 45 °C after 24 h of incubation. The incubation at 30, 50, and 60 °C was found to decrease both bacterial biomass and dye decolorizing ability of the bacterial strain; however, the decolorization percentage of the isolate was found to be greatly inhibited at temperature below 30 °C.

Table 1 Bacterial strains decolorizing Direct Red-28 under aerobic conditions

Sl. No	Isolates	Sample collection site	Time taken for maximum decolorization (h)	Decolorization efficiency (%)
1	DRS-1	S1	24	92.25
2	DRS-2	S1	24	41.93
3	DRS-3	S3	60	70.48
4	DRS-4	S2	60	81.77
5	DRS-5	S3	60	64.51
6	DRS-6	S2	36	53.06
7	DRS-7	S1	72	50.32
8	DRS-8	S1	24	64.80
9	DRS-9	S3	24	59.19
10	DRS-10	S2	24	70.96
11	DRS-11	S1	36	65.32
12	DRS-12	S1	24	56.12
13	DRS-13	S2	48	67.82
14	DRS-14	S2	48	75.14
15	DRS-15	S3	60	65.96
16	DRS-16	S3	60	62.74

Note The isolates were considered for the table that showed above 50 % decolorization ability

Effect of pH

Comparison of decolorization efficiency and bacterial biomass of *Bacillus* sp. strain DRS-1 at various pH is presented in Figs. 11 and 12. The bacterial isolate grew well in a broad range of pH (5.0–10.0); its decolorizing ability does not have strict pH requirement. Bacterial biomass (5.39) and dye decolorizing ability (95.64 %) were found to be optimized at slightly alkaline pH (8.0). However, bacterial growth and dye decolorizing ability (47.74 %) were found to be greatly repressed at pH 4.0. The decolorization efficiency of the isolate at pH 5.0 and 10.0 was found to be 69.67 and 64.51 % respectively

Effect of Dye Concentrations

The influence of different dye concentrations (0–1,000 ppm) were analyzed on bacterial biomass and decolorization ability of *Bacillus* sp. strain DRS-1. The results shown in Figs. 13 and 14 reveal that the decolorization rate increased linearly with increase in initial dye concentration up to 100 ppm. As the dye concentration increased in the culture medium, a decline in color removal was attained. At high concentration (1,000 ppm), Direct Red-28 greatly suppressed both bacterial biomass (2.26) and decolorization ability (21.77 %).

Effect of Agitation Speeds

Microorganisms vary in their oxygen requirement. The effect of various agitation speeds (0–250 rpm) on the bacterial growth and color removal capacity of *Bacillus* sp. strain DRS-1 was studied at 45 °C after 24 h of incubation. The decolorization ability of the isolate was found to be maximized at static conditions. Shaking conditions highly repressed the decolorizing ability of *Bacillus* sp. strain DRS-1. In contrast, the bacterial biomass was found to be maximized when incubated at 200 rpm (Figs. 15 and 16).

Table 2 Morphological, physiological, and biochemical characteristics of strain DRS-01

Sl. No	Test	Observations
1	Morphology	
	Grams staining	Positive
	Cell shape	Pleomorphic rod
	Motility	Motile
	Cell arrangement	Single, paired, and short chains
2	Colony characters on nutrient agar	
	Colony morphology	Irregularly round
	Colony size	2–3 mm
	Colony elevation	Raised
	Colony density	Dull, opaque
	Colony edge	Entire
	Pigmentation	Nonchromogenic (greyish white)
3	Sugar fermentation	
	Lactose	Negative
	Maltose	Positive-acid
	Glucose	Positive-acid
	Sucrose	Positive-acid
4	IMVIC	
	Indole	Positive
	Methyl red	Positive
	Voges Proskauer	Positive
	Citrate	Negative
5	Enzyme reaction	
	Urease production	Negative
	Catalase activity	Positive
	Oxidase	Positive
	Coagulase	Negative
6	H_2S production	Positive
7	Strain type	*Bacillus* sp.

Effect of Carbon Sources

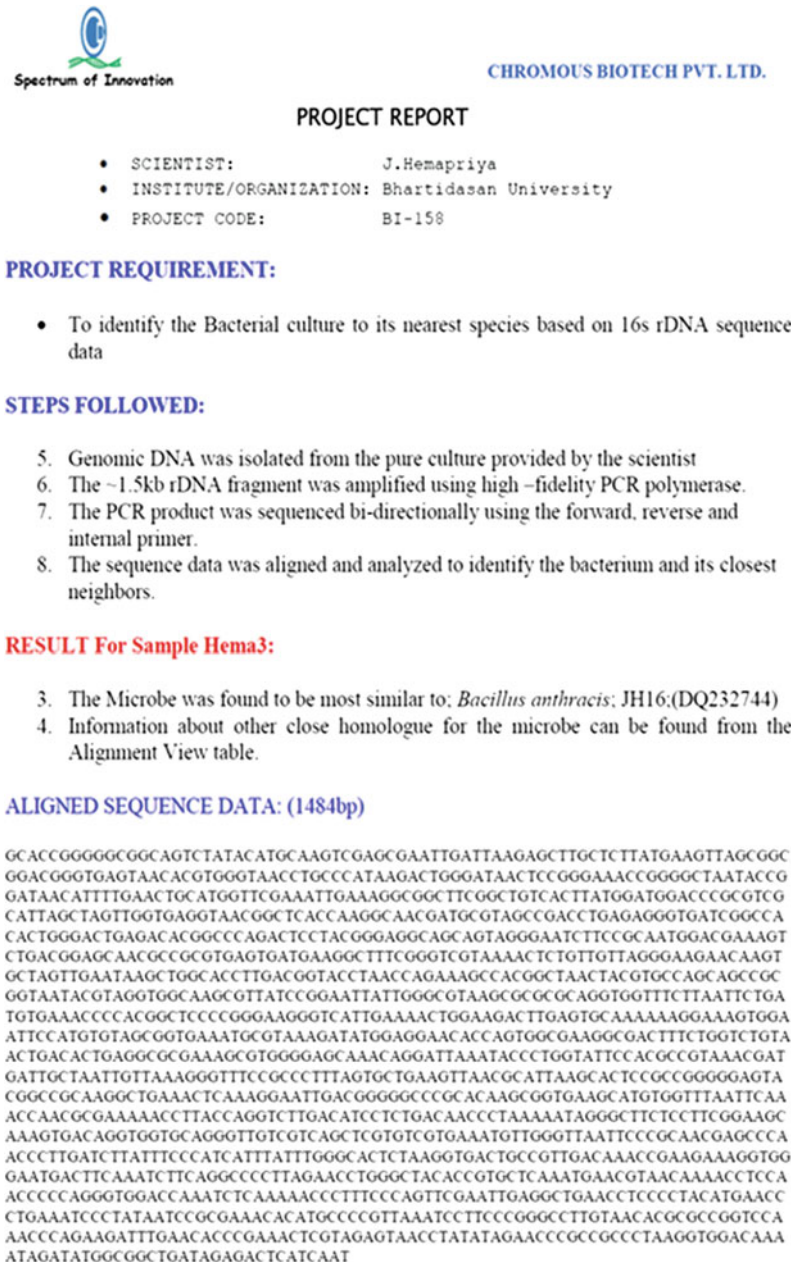
The influence of different soluble carbon sources including glucose, sucrose, lactose, maltose, and starch was studied on bacterial growth and decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1. The bacterial isolate utilized most of the carbon sources tested. Of them, glucose was found to be the most efficient carbon source, showing highest biomass (6.32) and dye decolorization capability (98.06 %) of *Bacillus* sp. strain DRS-1. However, the incorporation of maltose and starch drastically reduced the decolorization capability of the bacterial strain (Table 3).

Effect of Nitrogen Sources

The effects of different organic and inorganic nitrogen sources were analyzed after 24 h of incubation. Of the various organic nitrogen sources tested, peptone and yeast extract showed a significant effect on both bacterial biomass (5.80 and 6.36 respectively) and dye decolorizing ability (94.19 and 97.74 % respectively) of *Bacillus* sp. strain DRS-1. However, the addition of tryptone, beef extract, and meat extract slightly downregulated both bacterial growth and decolorization efficiency of the isolate (Table 4). Of the various inorganic nitrogen sources tested, $NaNO_3$ was found to be effective in enhancing the decolorization process (93.87 %) and bacterial biomass (5.41). In contrast, $(NH_4)_2SO_4$, NH_4Cl, and NH_4NO_3 negatively regulated the bacterial biomass and dye decolorization efficiency of *Bacillus* sp. strain DRS-1 (Table 5).

Effect of Metal Ions

The influence of various metal ions (Mn^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+}, and Fe^{2+}) on the bacterial biomass and dye decolorization by *Bacillus* sp. strain DRS-1 was examined after 24 h incubation. Of the various metal ions tested, Mg^{2+} and Mn^{2+} showed good bacterial growth with high decolorization efficiency. The most effective metal ion for the elevated decolorization efficiency was found to be Mn^{2+} (Table 6), whereas incorporation of Co^{2+} and Zn^{2+} greatly reduced both bacterial growth and color removal capacity of the *Bacillus* sp. strain DRS-1.



Spectrum of Innovation

CHROMOUS BIOTECH PVT. LTD.

PROJECT REPORT

- SCIENTIST: J.Hemapriya
- INSTITUTE/ORGANIZATION: Bhartidasan University
- PROJECT CODE: BI-158

PROJECT REQUIREMENT:

- To identify the Bacterial culture to its nearest species based on 16s rDNA sequence data

STEPS FOLLOWED:

5. Genomic DNA was isolated from the pure culture provided by the scientist
6. The ~1.5kb rDNA fragment was amplified using high –fidelity PCR polymerase.
7. The PCR product was sequenced bi-directionally using the forward, reverse and internal primer.
8. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors.

RESULT For Sample Hema3:

3. The Microbe was found to be most similar to; *Bacillus anthracis*; JH16;(DQ232744)
4. Information about other close homologue for the microbe can be found from the Alignment View table.

ALIGNED SEQUENCE DATA: (1484bp)

GCACCGGGGGCGGCAGTCTATACATGCAAGTCGAGCGAATTGATTAAGAGCTTGCTCTTATGAAGTTAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCG
GATAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCG
CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGT
GCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAGCGCGCGCAGGTGGTTTCTTAATTCTGA
TGTGAAACCCCACGGCTCCCCGGGAAGGGTCATTGAAAACTGGAAGACTTGAGTGCAAAAAAGGAAAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGTAAAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAAATACCCTGGTATTCCACGCCGTAAACGAT
GATTGCTAATTGTTAAAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCGGGGGAGTA
CGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGAAGCATGTGGTTTAATTCAA
ACCAACGCGAAAAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAAAAATAGGGCTTCTCCTTCGGAAGC
AAAGTGACAGGTGGTGCAGGGTTGTCGTCAGCTCGTGTCGTGAAATGTTGGGTTAATTCCCGCAACGAGCCCA
ACCCTTGATCTTATTTCCCATCATTTATTTGGGCACTCTAAGGTGACTGCCGTTGACAAACCGAAGAAAGGTGG
GAATGACTTCAAATCTTCAGGCCCCTTAGAACCTGGGCTACACCGTGCTCAAATGAACGTAACAAAACCTCCA
ACCCCCAGGGTGGACCAAATCTCAAAAACCCTTTCCCAGTTCGAATTGAGGCTGAACCTCCCCTACATGAACC
CTGAAATCCCTATAATCCGCGAAACACATGCCCCGTTAAATCCTTCCCGGGCCTTGTAACACGCGCCGGTCCA
AACCCAGAAGATTTGAACACCCGAAACTCGTAGAGTAACCTATATAGAACCCGCCGCCCTAAGGTGGACAAA
ATAGATATGGCGGCTGATAGAGACTCATCAAT

Fig. 4 PCR amplified 16s rDNA sequence of DRS-1 strain

Analysis of Biodegraded Samples by HPLC, FTIR, and GC–MS

HPLC Analysis of Decolorized Sample

The HPLC analysis of dye sample collected at 0 h incubation showed one major peak with retention time of 19.300 min (Fig. 17). As the decolorization progressed, the biodegradation of the parent compound was observed with 16 detectable peaks (retention time 1.308, 1.475, 1.667, 2.858, 3.358, 4.208, 4.617, 5.108, 6.025, 8.167, 12.767, 14.275, 16.083, 23.125, and 28.767) at 24 h extracted metabolites; however, major peak was not observed at 19.300 min, clearly indicating the biodegradation of Direct Red-28 by *Bacillus* sp. strain DRS-1 (Fig. 18).

Fig. 5 Alignment view and distance matrix table for DRS-1 isolate

CHROMOUS BIOTECH PVT. LTD.

PROJECT REPORT

- SCIENTIST: J.Hemapriya
- INSTITUTE/ORGANIZATION: Bhartidasan University
- PROJECT CODE: BI-158

Hierarchy View:

Domain: Bacteria
Phylum: "Firmicutes"
Class: "Bacilli"
Order: Bacillales
Family: Bacillaceae
genus: Bacillus

ALIGNMENT VIEW and DISTANCE MATRIX TABLE:
(With Sample Hema3 sequence taken as reference sequence)

S_ab score	Organism Name	NCBI Accession No
0.954	Bacillus cereus; Tim-r01;	AB050630
0.955	Bacillus sp. B-1;	AY189746
0.954	Bacillus anthracis; JH14;	DQ232742
0.958	Bacillus anthracis; JH16;	DQ232744
0.954	Bacillus sp. SDB21A;	DQ323748
0.942	bacterium SEHEP2;	EU723266
0.941	bacterium BREP13;	EU723269
0.942	bacterium SEHEN2;	EU723272
0.945	Bacillus sp. DET9;	EU851974
0.946	Bacillus thuringiensis; EI-17;	FJ613545
0.950	Bacillus thuringiensis; EI-18;	FJ613546
0.938	Bacillus sp. B17(2009);	FJ494897
0.947	Bacillus sp. LP1MB;	GU272341
0.948	Bacillus sp. LP1ME;	GU272344
0.948	Bacillus sp. LP2MK;	GU272360
0.951	Bacillus sp. TP1MA;	GU272363
0.949	uncultured Bacillus sp.; A8DMCS05;	GU936826
0.942	Bacillus sp. SC116(2010);	HM566469
0.943	Bacillus sp. SC84(2010);	HM566616
0.954	Bacillus sp. DU171(2010);	HM567053

S_ab: Sequence Match Score

FTIR Analysis of Decolorized Sample

The FTIR spectrum of the control dye sample compared with the decolorized sample after 24 h was shown in Fig. 19. The spectrum of the control dye represented stretching between C–Cl at 668 cm^{-1}, C–N stretching vibrations at 1,170 cm^{-1}, –OH stretching vibration at 1043 cm^{-1}, stretching at S = O at 1,048 cm, and stretching vibration at 2,926 cm^{-1} for C–N, 3,434 cm^{-1} represented the presence of free NH group from parent dye structure. The FTIR spectrum of 24 h extracted metabolites showed significant changes in the positions of peaks when compared to control dye. Stretching vibrations at 1,112 cm^{-1} showed C–OH stretching. Peaks at 2,837 and 2,950 cm^{-1} represented C–H deformation. Stretching vibration at 2,155 cm^{-1} showed C–N stretching, clearly expressing the breakdown of Direct Red-28.

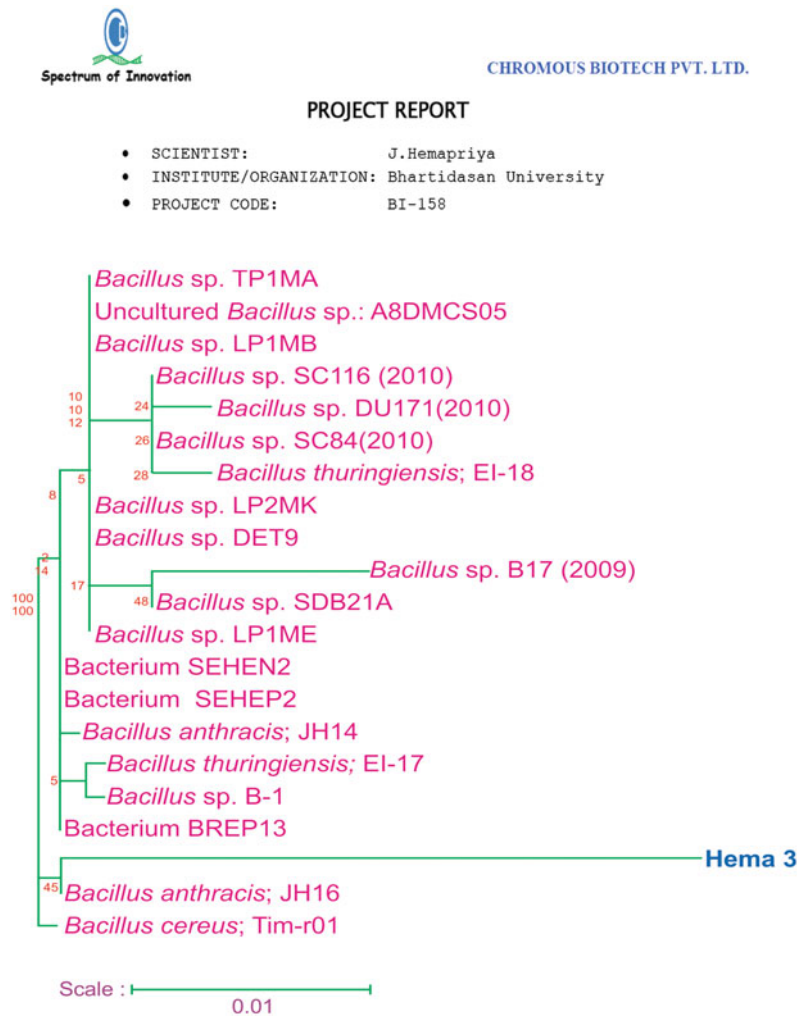


Fig. 6 Phylogenetic tree of DRS-1 isolate and related organisms, aligned based on 16S rDNA (neighbor joining tree). Scale bar number corresponds to nucleotide changes per sequence position

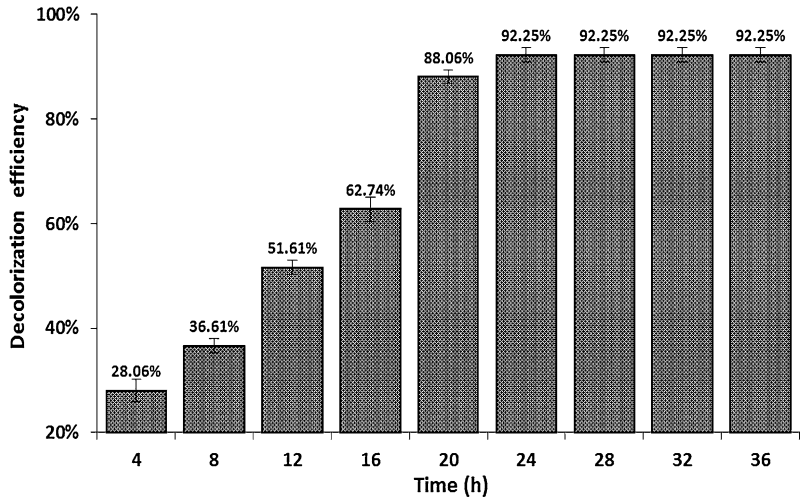


Fig. 7 Effect of incubation time on decolorization efficiency of *Bacillus* sp. strain DRS-1

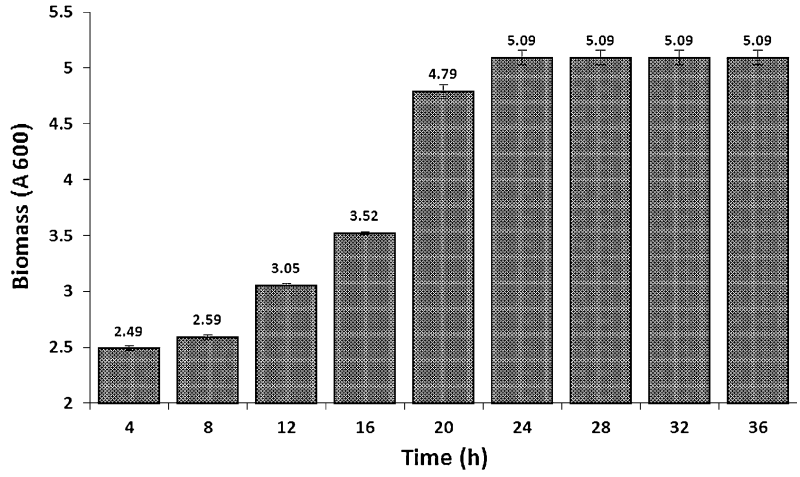


Fig. 8 Effect of incubation time on biomass of *Bacillus* sp. strain DRS-1

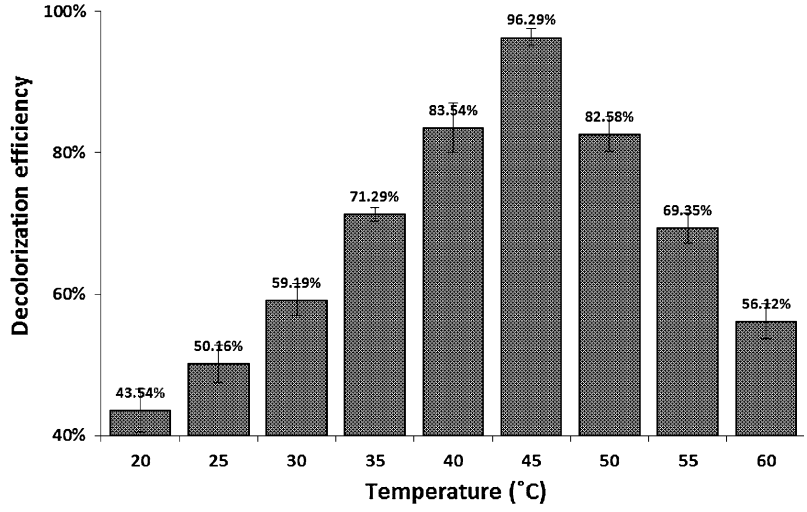


Fig. 9 Effect of incubation temperature on decolorization efficiency of *Bacillus* sp. strain DRS-1

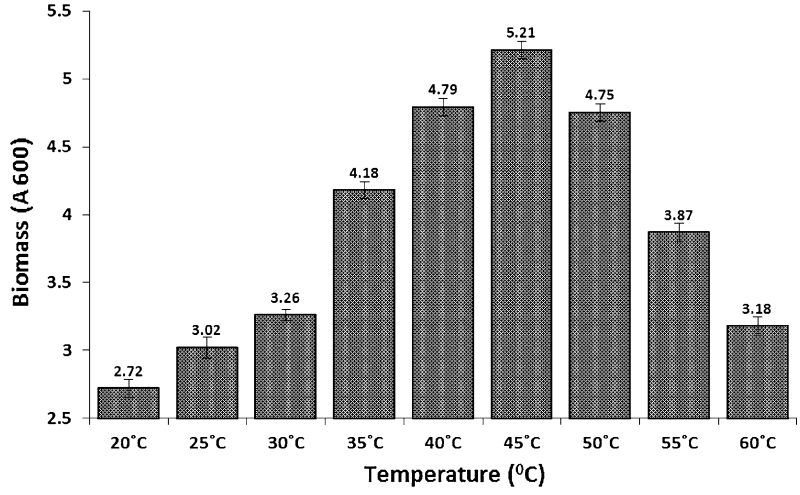


Fig. 10 Effect of incubation temperature on biomass of *Bacillus* sp. strain DRS-1

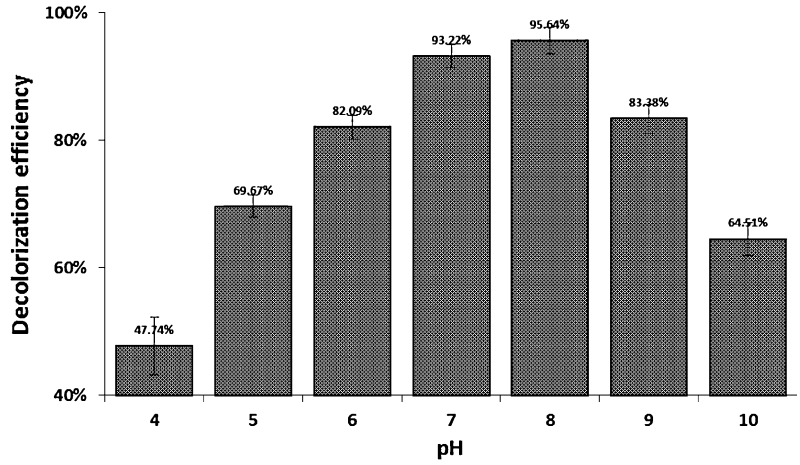


Fig. 11 Effect of pH on decolorization efficiency of *Bacillus* sp. strain DRS-1

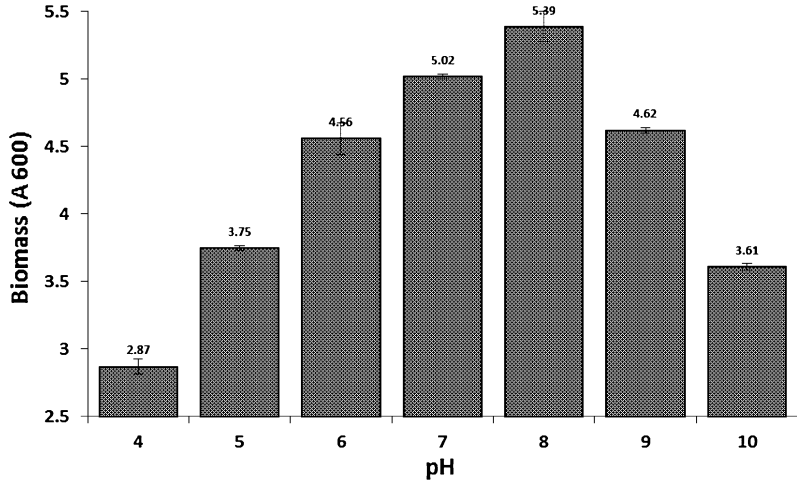


Fig. 12 Effect of pH on biomass of *Bacillus* sp. strain DRS-1

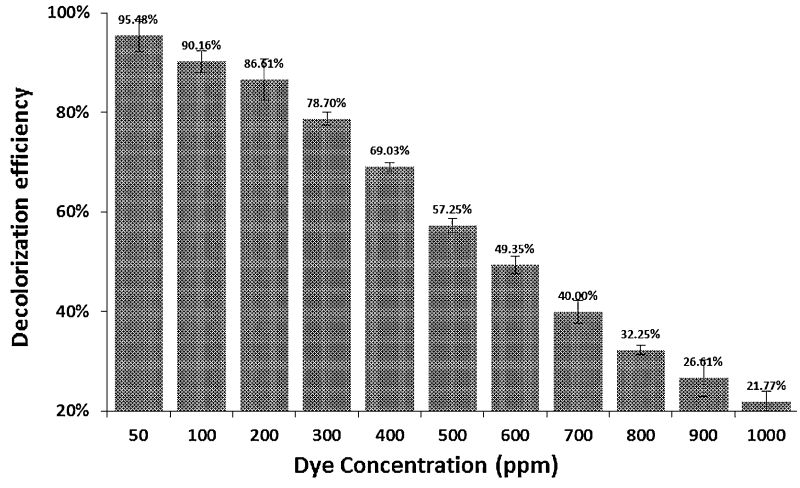


Fig. 13 Effect of dye concentration on decolorization efficiency of *Bacillus* sp. strain DRS-1

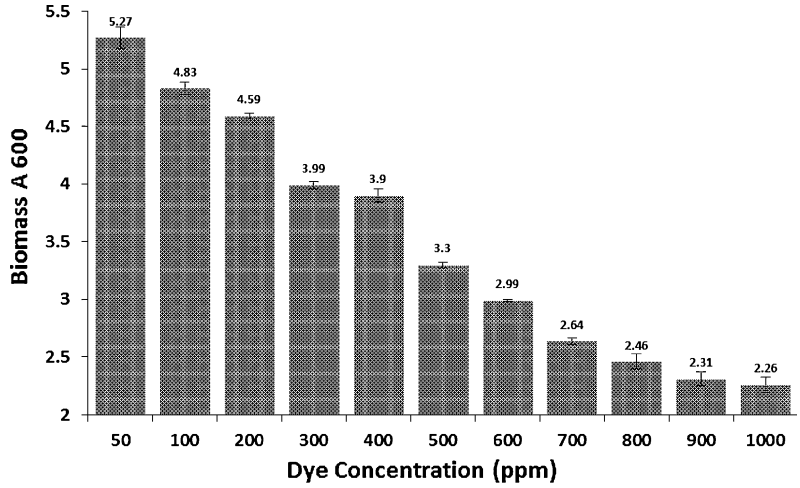


Fig. 14 Effect of dye concentration on biomass of *Bacillus* sp. strain DRS-1

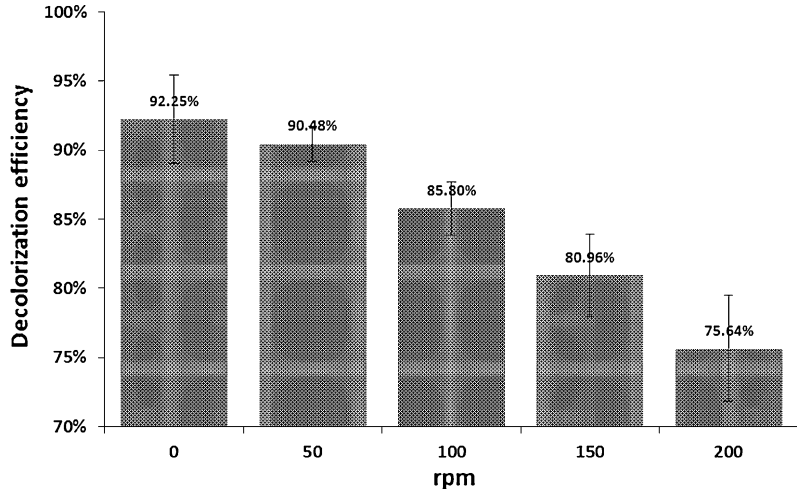


Fig. 15 Effect of agitation on decolorization efficiency of *Bacillus* sp. strain DRS-1

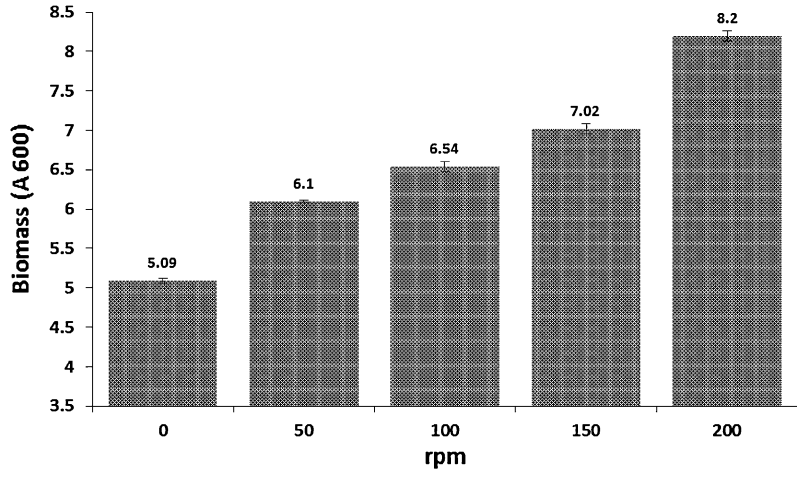


Fig. 16 Effect of agitation on biomass of *Bacillus* sp. strain DRS-1

Table 3 Effect of carbon sources on decolorization efficiency of *Bacillus* sp. strain DRS-1 and its biomass

Sl.No	Carbon sources (gl^{-1})	Decolorization efficiency	Biomass A_{600}
1	Control	92.25 % ± 0.05	5.83 ± 0.02
2	Glucose	98.06 % ± 0.01	6.32 ± 0.02
3	Sucrose	96.61 % ± 0.01	6.21 ± 0.03
4	Lactose	93.22 % ± 0.01	5.92 ± 0.02
5	Maltose	89.35 % ± 0.01	5.79 ± 0.02
6	Starch	87.41 % ± 0.01	5.83 ± 0.01

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Table 4 Effect of organic nitrogen sources on decolorization efficiency of *Bacillus* sp. strain DRS-1 and its Biomass

Sl.No	Organic nitrogen sources (gl^{-1})	Decolorization efficiency	Biomass A_{600}
1	Control	92.25 % ± 0.05	5.83 ± 0.02
2	Tryptone	84.51 % ± 0.01	5.66 ± 0.03
3	Beef extract	88.06 % ± 0.004	5.96 ± 0.03
4	Peptone	94.19 % ± 0.003	5.80 ± 0.06
5	Yeast extract	97.74 % ± 0.008	6.36 ± 0.05
6	Meat extract	83.54 % ± 0.005	5.43 ± 0.16

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Table 5 Effect of inorganic nitrogen sources on decolorization efficiency of *Bacillus* sp. strain DRS-1 and its biomass

Sl. No	Inorganic nitrogen sources (gl^{-1})	Decolorization efficiency	Biomass A_{600}
1	Control	92.25 % ± 0.05	5.93 ± 0.05
2	$(NH_4)_2SO_4$	80.48 % ± 0.02	4.01 ± 0.03
3	KNO_3	90.16 % ± 0.01	4.98 ± 0.06
4	NH_4Cl	82.41 % ± 0.009	4.60 ± 0.06
5	NH_4NO_3	87.25 % ± 0.01	4.53 ± 0.02
6	$NaNO_3$	93.87 % ± 0.01	5.41 ± 0.02

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

GC–MS Analysis of Biodegraded Sample

GC–MS analysis of biodegraded sample (after 24 h) was carried out to study the degraded products obtained during the decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1. The GC–MS chromatogram (Fig. 20) showed the presence of three different peaks with retention times of 13.737, 25.852, and 26.521 respectively. Moreover, it was concluded that the decolorization sample completely lacked the presence of banned carcinogenic amines, which are prohibited in accordance with the Consumer Goods Ordinance (Textiles committee, Ministry of Textiles, Govt. of India) (Table 7).

Table 6 Effect of various metal ions on decolorization efficiency of *Bacillus* sp. strain DRS-1 and its biomass

Sl. No	Metal ions (5 mM)	Decolorization efficiency	Biomass A_{600}
1	Control	92.25 % ± 0.05	5.83 ± 0.02
2	$MnCl_2$	97.52 % ± 0.003	6.24 ± 0.1
3	$MgCl_2$	95.78 % ± 0.003	6.13 ± 0.05
4	$HgCl_2$	90.84 % ± 0.01	5.07 ± 0.1
5	$ZnSO_4$	74.27 % ± 0.002	3.68 ± 0.04
6	$CoCl_2$	70.63 % ± 0.008	3.51 ± 0.06
7	$FeSO_4$	87.20 % ± 0.001	4.65 ± 0.1

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Influence of Various Immobilization Matrices on Decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1

Decolorization of Direct Red-28 by Ca-alginate Immobilized Cells

Decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1 cells entrapped in Ca-alginate linearly increased with increase in the incubation periods, and reached the highest level (99.03 %) after 24 h of incubation, whereas the free cells of *Bacillus* sp. showed a maximum decolorization efficiency of 92.25 % after 24 h of incubation (Fig. 21).

Decolorization of Direct Red-28 by Agar–Agar Immobilized Cells

Agar–agar entrapped *Bacillus* sp. strain DRS-1 cells started decolorizing Direct Red-28 after 4 h

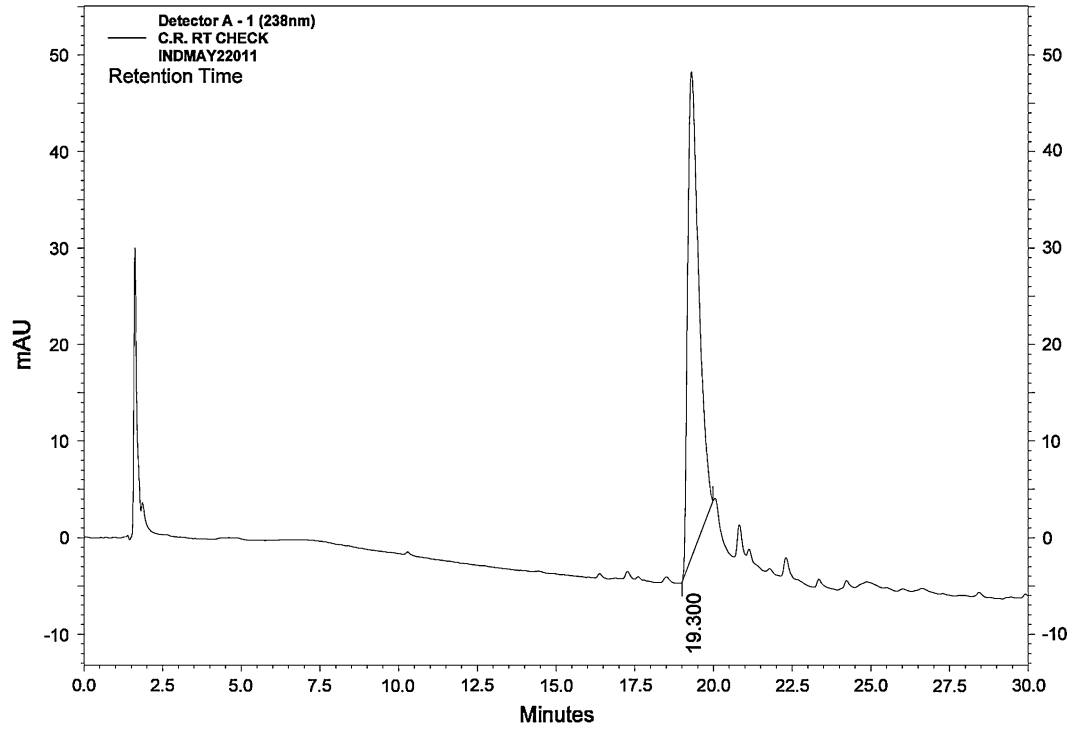


Fig. 17 HPLC-chromatogram of control Direct Red-28 sample

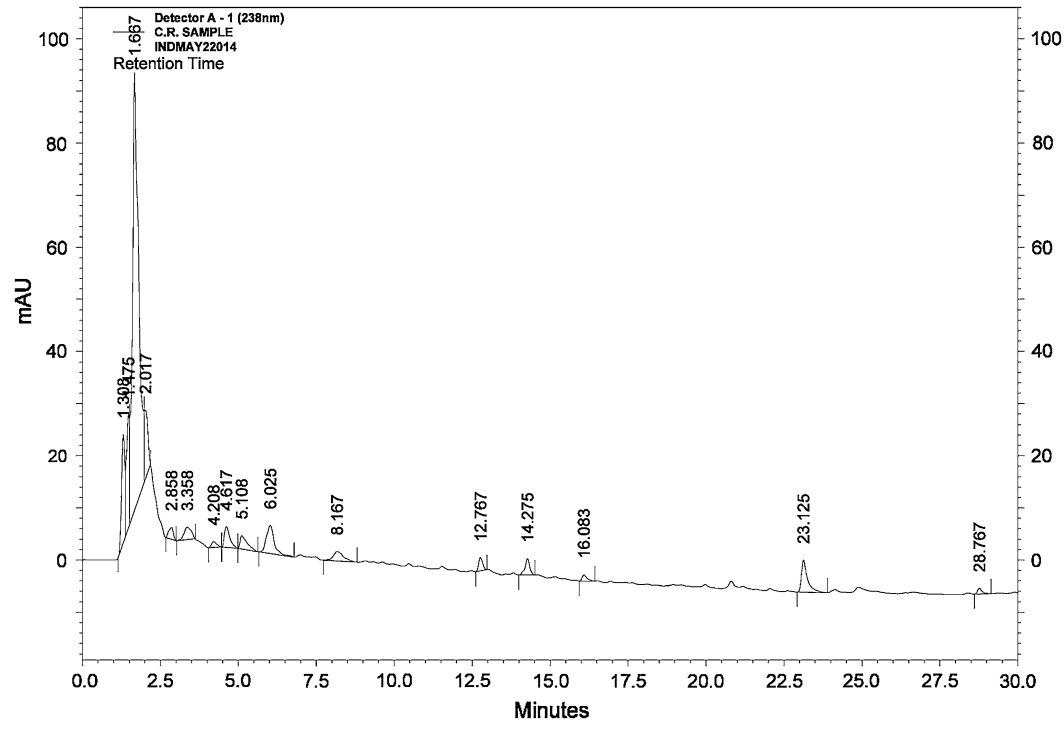


Fig. 18 HPLC-chromatogram of decolorized Direct Red-28 Sample

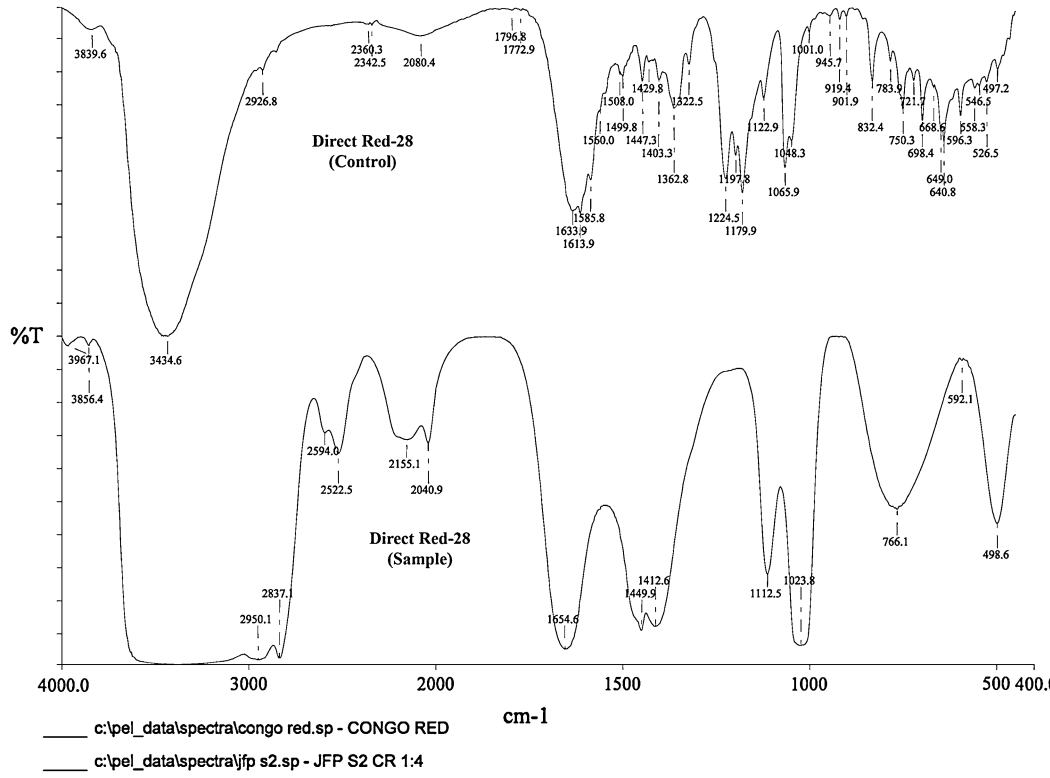


Fig. 19 FTIR analysis of control and decolorized samples of Direct Red-28

of incubation under static conditions. However, the decolorizing efficiency of *Bacillus* sp. strain DRS-1 cells reached a maximum level (96.77 %) after 24 h of incubation (Fig. 22). Interestingly, the decolorizing efficiency of agar–agar immobilized cells was found to be greater than the free cells, but comparatively lower than the Ca-alginate immobilized *Bacillus* sp. strain DRS-1 cells.

Decolorization of Direct Red-28 by Polyacrylamide Immobilized Cells

Polyacrylamide immobilized *Bacillus* sp. strain DRS-1 cells showed maximum decolorization efficiency of 93.22 % after 24 h of incubation (Fig. 23). Decolorization percentage of polyacrylamide immobilized *Bacillus* sp. strain DRS-1 cells was found to be almost the same as that of free cells, but lower than the cells entrapped in the above-mentioned matrices.

Decolorization of Direct Red-28 by Gelatin Immobilized Cells

Decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1 cells immobilized in gelatin started after 4 h of incubation, gradually increased and reached the maximum level (87.58 %) after 24 h of incubation. The results shown in Fig. 24 reveal that the decolorization efficiency of gelatin entrapped *Bacillus* sp. strain DRS-1 cells was

TEXTILES COMMITTEE

Sample Information

Analyzed by	: Admin
Analyzed	: 9/2/2010 10:17:28 AM
Sample Type	: Unknown
Level #	: 1
Sample Name	: CEQCU10-3022
Sample ID	: DIRECT RED 28
IS Amount	: [1]=1.000
Sample Amount	: 1.000
Dilution Factor	: 1.000
Vial #	: 1
Injection Volume	: 1.000
Data File	: C:\GCMSsolution\Data\Project1\ceqcu10-3022.qgd
Org Data File	: C:\GCMSsolution\Data\Project1\CEQCU10-3022.QGD
Method File	: C:\GCMSsolution\Data\Project1\AMINEPR_MET.qgm
Org Method File	: C:\GCMSsolution\Data\Project1\AMINEPR_MET.qgm
Report File	:
Tuning File	: C:\GCMSsolution\System\Tune1\JAN\TUNE_020910.qgt
Modified by	: Admin
Modified	: 9/2/2010 3:46:10 PM

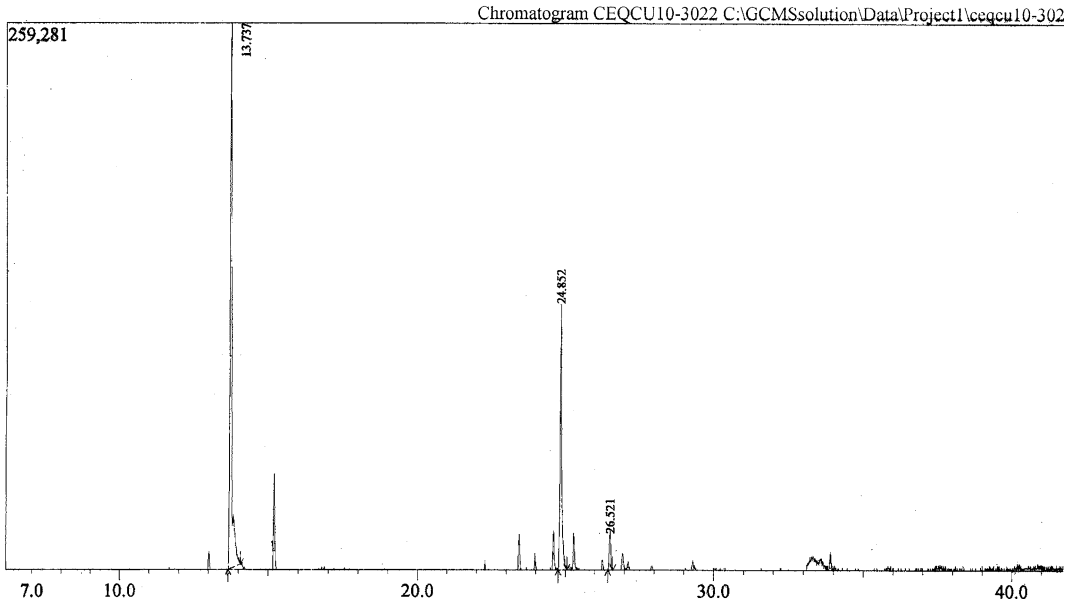


Fig. 20 GC–MS analysis of Direct Red-28 sample degraded by *Bacillus* sp. DRS-1 after 24 h of Incubation

lower compared with free cells and cells immobilized with Ca-alginate, agar–agar, and polyacrylamide.

Phytotoxicity Assay

Phytotoxicity tests were performed to assess the toxicity of the untreated and treated Direct Red-28 dye samples (Fig. 25). *S. vulgare* seeds treated with tap water showed 100 % germination, the mean plumule length of 27.68 ± 0.10 cm, and the mean radical length of 6.88 ± 0.21 cm. In contrast, the seeds treated with untreated sample showed only 90 % germination, the mean plumule length of 7.93 ± 0.65 cm, and the radical length of 2.95 ± 0.48 cm. Whereas the seeds treated with degraded dye sample showed 100 % germination, the mean plumule length of 20.72 ± 0.87 cm, and the radical length of 4.86 ± 0.54 cm. *P. mungo* seeds treated with tap water showed 100 % germination, the mean

Table 7 GC–MS analysis report of biodegraded Direct Red-28 sample showing the absence of banned aromatic amines

क्षेत्रीय प्रयोगशाला
वस्त्र समिति
(भारत सरकार, वस्त्र मंत्रालय)
एफ. के. सि. सि. आए - डब्लू. टि. सि. बिल्डिंग,
केंपेगौडा रोड, बेंगलूर - 560 009.
फोन : 2220 8010 टेलीफ्याक्स : 080-2226 1401
ई मेल : tcblr@dataone.in

Regional Laboratory
TEXTILES COMMITTEE
(Ministry of Textiles, Govt. of India)
Textile Testing Services-Eco Cell
FKCCI-WTC Building, 1st Floor
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Ph. : 2220 8010 Tel./Fax : 080-2226 1401
Email : tcblr@dataone.in

TEST REPORT

Test Report No. TC/LB/ST/3258/2010-11 **Date:** 2.9.2010

TEST RESULTS

Lab. Sample No. **CEO - 3022**

Details of release of individual arylamines (mg/kg) on reductive cleavage with sodium di-thionite.

Sr. No.	Name of the amines	Contents in mg/kg
i	4-Aminobiphenyl	Not Detected
ii	Benzidine	Not Detected
iii	4-Chloro-o-toludine	Not Detected
iv	2-Naphthylamine	Not Detected
v	p-Chloroaniline	Not Detected
vi	2-4-Diaminoanisole	Not Detected
vii	4-4-Diaminodiphenylmethane	Not Detected
viii	3,3-Dichlorobenzidine	Not Detected
ix	3,3-Dimethoxybenzidine	Not Detected
x	3,3-Dimethylbenzidine	Not Detected
xi	3,3-Dimethyl-4,4-diaminodiphenylmethane	Not Detected
xii	p-Cresidine	Not Detected
xiii	4,4-Methylene-bis-(2-choroaniline)	Not Detected
xiv	4,4-Oxydianiline	Not Detected
xv	4,4-Thiodianiline	Not Detected
xvi	o-Toluidine	Not Detected
xvii	2-4-Toluylenediamine	Not Detected
xviii	2,4,5-Trimethylaniline	Not Detected
xix	O-Aminoazotoluene	Not Detected
xx	2-Amino-4-nitro-toluene	Not Detected
xxi	p-Aminoazobenzene	Not Detected
xxii	2-Methoxyaniline	Not Detected
xxiii	2,4-Xylidine	Not Detected
xxiv	2,6-Xylidine	Not Detected
	Sum Parameter	Nil

Signature and seal of the Officer
के. नारायण
K. NARAYANA
उप निदेशक (प्रयोगशालाएँ)
Deputy Director (Labs)

Complaints, if any, are to be received within 45 days of date of issue of the test report.

Sample is not drawn by Textiles Committee. Results relate only to the samples tested.
This test report is not to be published in any form without the explicit written consent of the Textiles Committee.

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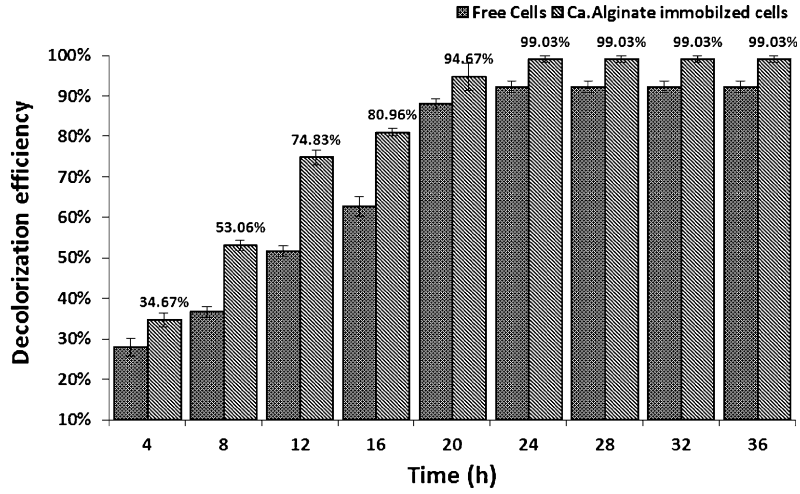

Fig. 21 Decolorization ability of calcium alginate immobilized cells and free cells of *Bacillus* sp. strain DRS-1

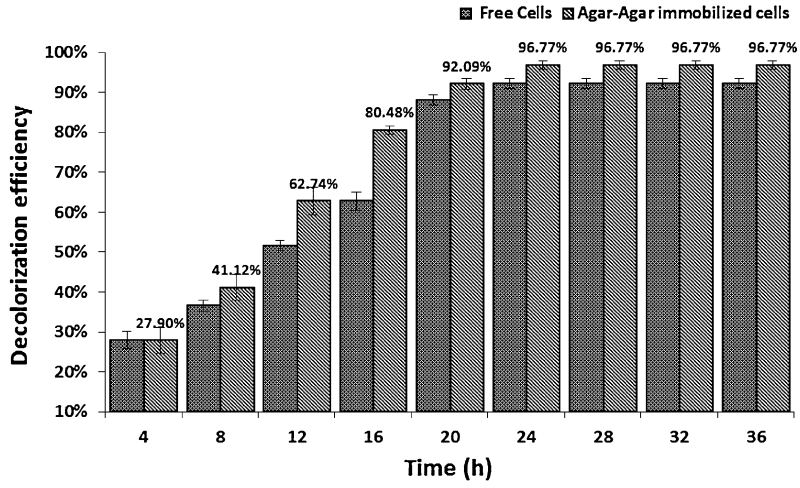

Fig. 22 Decolorization ability of agar–agar immobilized cells and free cells of *Bacillus* sp. strain DRS-1

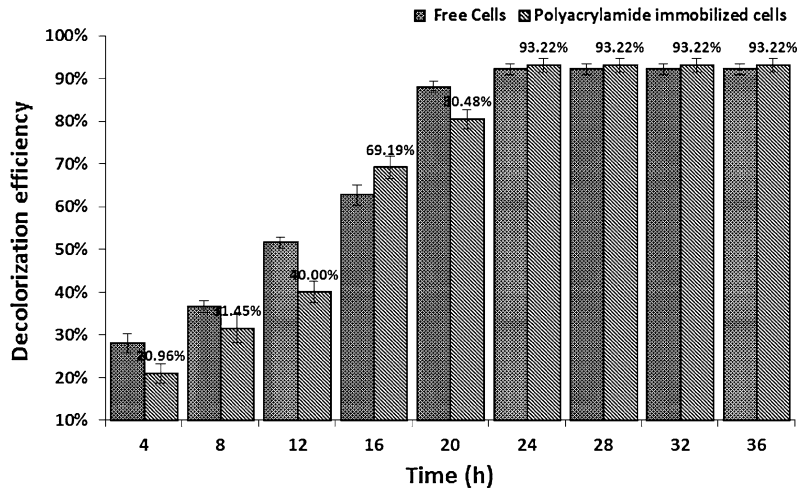

Fig. 23 Decolorization ability of polyacrylamide immobilized cells and free cells of *Bacillus* sp. strain DRS-1

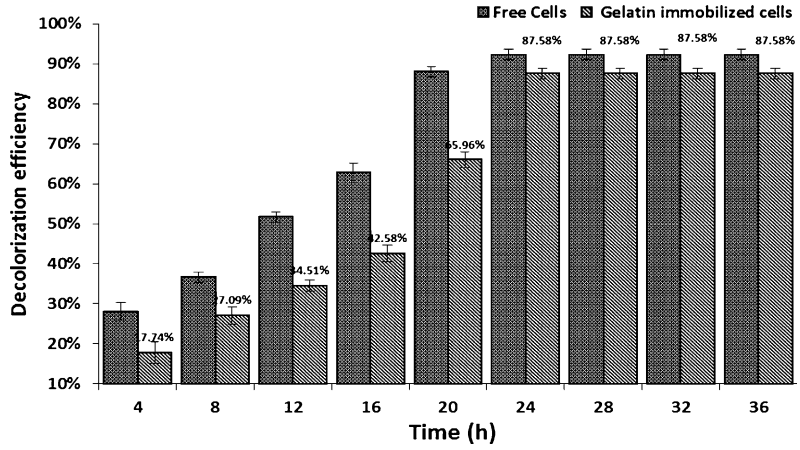

Fig. 24 Decolorization ability of gelatin immobilized cells and free cells of *Bacillus* sp. strain DRS-1

plumule length of 22.26 ± 0.17 cm, and the mean radical length of 5.87 ± 0.15 cm. In contrast, seeds treated with biodegraded dye sample showed only 90 % germination, the mean plumule length of 8.51 ± 0.70 cm, and the mean radical length 2.40 ± 0.11 cm, whereas seeds treated with degraded dye sample showed 100 % germination, the mean plumule length of 18.74 ± 1.05 cm, and the radical length of 4.53 ± 0.37 cm. The result indicated that the degraded dye sample contains nontoxic metabolites, resulting in good germination rate as well as significant root and shoot length of *S. vulgare* and *P. mungo* L., when compared to the untreated dye sample, where inhibition in all these parameters was observed (Tables 8 and 9).

Discussion

Wastewater from textile industries pose a threat to the environment as large amounts of chemically different dyes are used for various industrial applications such as textile dyeing and a significant proportion of these dyes enter the environment via wastewater (Dayaram and Dasgupta 2008). The presence of dyes imparts an intense color to effluents, which leads to environmental as well as aesthetic problems (Singh and Singh 2006). The treatment of azo dye-containing wastewaters still presents an arduous task and a technical challenge (Pandey et al. 2007). As regulations are becoming even more stringent, there is an urgent need for technically feasible and cost-effective methods. Economical removal of color from effluents remains an important problem although a number of successful systems have evolved employing various physicochemical and biological processes. Regulatory agencies are increasingly interested in decolorization technologies (Anjaneyulu et al. 2005; Khadijah et al. 2009). The overwhelming majority of the current research works in the field of textile effluent decolorization has been dealing with the various aspects of the applications of microbiological techniques, with the search for new microorganisms providing higher decomposition rates and with the elucidation of the principal biochemical and biophysical processes underlying the decolorization of dyes.

The incubation time holds the key in maximizing the bacterial growth and dye decolorization ability of *Bacillus* sp. strain DRS-1. The bacterial growth and decolorizing capability of the isolate indicated that there was a distinct growth associated nature of the color removing efficiency. Maximum decolorization activity coincided with maximum biomass and both were observed at the late exponential phase and stationary phase, suggesting that the factors involved in the decolorization process were produced as the primary metabolites. Both bacterial biomass and percentage decolorization of *Bacillus* sp. strain DRS-1 maximized after 24 h

Fig. 25 Phytotoxicity study of Direct Red-28 and its degradation products

of incubation. This result is in complete accordance with the decolorization of Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112 (Kalme et al. 2007), Direct Blue-15 by a bacterial consortium (Kumar et al. 2007), and Navy Blue RX by *Streptomyces krainskii* SUK-5

Table 8 Phytotoxicity study of Direct Red-28 and its degradation products on *Sorghum vulgare* Pers

Sl. No	Parameters studied	Tap water	Direct Red-28 (100 ppm)	Treated sample (100 ppm)
1	Germination (%)	100	90	100
2	Plumule (cm)	27.68 ± 0.10	7.93 ± 0.65	20.72 ± 0.87
3	Radical (cm)	6.88 ± 0.01	2.95 ± 0.48	4.86 ± 0.54

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Table 9 Phytotoxicity study of Direct Red-28 and its degradation products on *Phaseolus mungo* L

Sl. No	Parameters studied	Tap water	Direct Red-28 (100 ppm)	Treated sample (100 ppm)
1	Germination (%)	100	90	100
2	Plumule (cm)	22.26 ± 0.17	8.51 ± 0.70	18.74 ± 1.05
3	Radical (cm)	5.87 ± 0.15	2.40 ± 0.11	4.53 ± 0.31

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

(Mane et al. 2008). In contrast, decolorization of 1-amino-4-bromo anthraquinone-2-sulfonic acid by *Sphingomonas herbicidovorans* was achieved within 14 h of incubation (Fan et al. 2009).

Temperature variation had a significant effect on the bacterial biomass and decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1. Dye decolorization by the bacterial isolate was found to be maximized at 45 °C after 24 h of incubation. In contrast, 37 °C has been proved to be the ideal incubation temperature for maximizing the decolorization of Acid Orange-10 by *B. fusiformis* KMK5 (Kolekar et al. 2008), Anthraquinone dye AB25 by *B. cereus* DC11 (Deng et al. 2008), and Congo Red by *Bacillus* sp. (Gopinath et al. 2009). In general, the increase in temperature in the reaction has a linear relation with the reaction rate, whereas in the present investigation the reactions are biological, which depend on the living organisms. Since living organisms are more sensitive to temperature, such reactions are also more sensitive for variations in temperatures (Husseiny 2008). The inhibition of dye decolorization by *Bacillus* sp. strain DRS-1 at high temperature was presumably due to the growth suppression of the bacterial cell. Decolorization of Congo Red by *Bacillus* sp. VT-II was maximized at 40 °C (Sawhney and Kumar 2011). Optimum decolorization of Reactive Red-22 by *E. coli* strain NO_3 and *P. luteola* was achieved at 42 °C (Chang et al. 2000, 2001).

The initial pH of the fermentation medium required for obtaining maximum decolorization of Direct Red-28 by *Bacillus* sp. strain DRS-1 depends not only upon the bacterium, but also upon the ingredients of the fermentation medium. The best decolorization was achieved at a broad range of pH (6.0–10.0), with optimum being pH 8.0. In contrast, neutral pH was found to be effective in maximizing both bacterial growth and dye decolorization efficiency of many bacterial strains (Kilic et al. 2007; Wang et al. 2009). The pH tolerance of the decolorizing bacteria is important because the reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions (Aksu et al. 2007). The fact that *Bacillus* sp. strain DRS-1 could decolorize Direct Red-28 in a relatively wide range of pH makes it suitable for practical biotreatment of dyeing mill or textile effluents (Wang et al. 2009). In contrast, decolorization of Methyl Red by *Klebsiella pneumoniae* RS-13 (Wong and Yuen 1996), Reactive Brilliant Blue (KN-R) by *Rhodocyclus gelatinosus* XL-1 (Dong et al. 2003), and Reactive azo dye by a microbial consortium (Kodam et al. 2005) was found to be achieved in a narrow pH range.

In general, bacterial community is sensitive to the variation in the oxygen requirement. Agitation was another important parameter that affected the biomass and dye decolorizing ability of *Bacillus* sp. strain DRS-1. Bacterial growth

was found to be maximized under shaking conditions at 200 rpm. According to Nascimento and Martins (2004), oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for all the cellular activities. The variation in the agitation speed has been found to influence the extent of mixing in the shaking flasks and also affect the nutrient availability. Similarly, the biomass of many bacterial strains including *Teredinibacter turnerae* (Elibola and Moreira 2005), *Chromohalobacter* sp. TVSP-101 (Vidyasagar et al. 2007), and *Halobacterium* sp. strain JS_1 (Vijayanand et al. 2010) was found to be maximized under shaking conditions. Reduced agitation rates (<200 rpm) significantly repressed the growth of *Bacillus* sp. strain DRS-1, due to the insufficient aeration and nutrient availability. In contrast, *Bacillus* sp. strain DRS-1 efficiently decolorized Direct Red-28 under static conditions, whereas the shaking conditions greatly repressed the decolorization efficiency of the isolate. Similar results were reported by many researchers (Jadhav et al. 2008; Kalme et al. 2007; Mabrouk and Yusef 2008). In contrast, decolorizing ability of some microorganisms was found to be elevated at shaking conditions (Mane et al. 2008; Fan et al. 2009). According to Chang et al. (2004), the microbial growth and bacterial decolorization processes are independent as decolorization via azoreductases is repressed under aerobic conditions.

Synthetic dyes are deficient in carbon content and biodegradation of dyes without any added carbon sources is found to be difficult (Padmavathy et al. 2003). Of the various carbon sources, glucose was found to be an ideal candidate in optimizing both bacterial biomass and decolorization efficiency of *Bacillus* sp. strain DRS-1. This result was in concurrence with the findings of many researchers (Swamy and Ramsay 1999; Kapdan et al. 2000; Khelifi et al. 2009). Two options have been argued for many years regarding the use of glucose on decolorization of azo dyes (Wang et al. 2009); one deems that dyes are not a carbon source since the anaerobic bacteria obtain energy from the glucose instead of dyes and thereby glucose enhances the decolorizing performance of the biological systems (Sarioglu et al. 2007), while another deems that glucose can inhibit the decolorizing activity (Chen et al. 2003). The incorporation of sucrose as the carbon source negatively regulated the bacterial growth and decolorizing ability of *Bacillus* sp. strain DRS-1. In contrast, sucrose elevated the decolorization efficiency (97 %) of a heterocyclic monoazo disperse dye by *B. firmus* (Arora et al. 2007).

Dye decolorization percentage and bacterial biomass of *Bacillus* sp. strain DRS-1 heavily depend upon the availability of a suitable nitrogen source in the fermentation medium, which has regulatory effects on bacterial growth (Patel et al. 2005). In microorganisms, N_2 (organic and inorganic) is metabolized into proteins, nucleic acids, amino acids, and cell wall components (Ibrahim and Al-Salamah 2009). Of the various organic N_2 sources tested, yeast extract was found to be the most superior in maximizing the bacterial growth and decolorizing ability of *Bacillus* sp. strain DRS-1. Similarly, yeast extract maximized the decolorization of many azo dyes (Arora et al. 2007; Khelifi et al. 2009). Of the various inorganic nitrogen sources, $NaNO_3$ was found to be effective in maximizing bacterial biomass and color removal efficiency of *Bacillus* sp. strain DRS-1, whereas ammonium salts including $(NH_4)_2SO_4$, NH_4Cl, and NH_4NO_3 slightly suppressed the bacterial growth and decolorization ability of the isolate. The repression might be attributed due to the fast release of ammonia from the inorganic nitrogen sources. These reports strongly suggested that the ammonium-specific repression was likely to be the factor involved. Similarly, the presence of ammonium salts was shown to repress the growth of many bacterial strains (Shafee et al. 2005; Ibrahim and Al-salamah 2009). In contrast, $(NH_4)_2SO_4$ and NH_4Cl were found to be effective in maximizing the decolorization of an azo dye and Indigo (Arora et al. 2007; Khelifi et al. 2009).

Supplementation of the culture medium with suitable metal cations improved substantially the growth and decolorization ability of *Bacillus* sp. strain DRS-1. Of the various metal ions tested, Mn^{2+} and Mg^{2+} ions positively regulated the growth and decolorizing percentage of the

isolate. Similar results were reported by Park et al. (2007) and Silveira et al. (2009). According to Morihara (1974), the stimulating effect of the metal ions can be attributed either to the stabilization of the outer membrane or to the interaction of metal ions directly with the enzymes. On the other hand, the inhibitory effect of certain metal ions like Co^{2+}, Zn^{2+}, and Fe^{2+} may be due to their oligodynamic effect. To accurately appraise the decolorizing ability of *Bacillus* sp. strain DRS-1, the isolate was tested against different concentrations of the azo dye, Direct Red-28. Decolorization percentage increased linearly with the increase in dye concentration up to 100 ppm. In addition, a substrate inhibition effect was observed with the further increase in dye concentration. This result was in complete accordance with the findings of many researchers (Kodam et al. 2005; Parshetti et al. 2006; Asad et al. 2007; Kalyani et al. 2009). Reduction in color removal may result from the toxicity of dyes to bacteria through the inhibition of metabolic activities. Azo dyes generally contain one or more sulfonic acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms (Chen et al. 2003; Shrivastava et al. 2005).

The exact mechanism of azo dye reduction is not clearly understood yet. Therefore, the term azo dye reduction may involve different mechanisms like enzymatic (Shrivatsava et al. 2005) and meditated (Field and Brady 2003) locations like intracellular (Mechsner and Wuhrmann 1982) and extracellular processes in which reducing equivalents from either biological or chemical sources are transferred to the dye (Stolz 2001; Sanghi et al. 2006). Oxidative biodegradation takes place upon the action of enzymes such as peroxides and laccases (Kandelbauer et al. 2004). Bacterial extracellular azo dye-oxidizing-peroxidases have been characterized in *S. chromofuscus* (Pasti-Grigsby et al. 1996). To disclose the possible mechanism of the dye decolorization, the products of biotransformation of Direct Red-28 were analyzed by HPLC, FTIR, and GC–MS. HPLC results of the control sample showed a major peak with retention time of 19.300 min, whereas the decolorized sample showed 16 peaks with different retention times indicating the breakdown of Direct Red-28. The FTIR spectrum of 24 h extracted metabolites showed a significant change in position of peaks when compared to the control dye spectrum. Similar work was carried out by Deng et al. (2008); Kalyani et al. (2009). The capability of *Bacillus* sp. strain DRS-1 in the detoxification of harmful carcinogenic amines generated by the biodegradation of Direct Red-28 was studied by GC–MS analysis, which revealed the complete absence of carcinogenic compounds, which are prohibited in accordance with the Consumer Goods Ordinance (Textile Committee, Ministry of Textiles, Govt. of India). Similar results were reported by Jadhav et al. (2008) and Phugare et al. (2011).

According to Lange et al. (1995), many sulfonated aromatic amines accumulate in the environment as evidenced by their occurrence in the surface water, where they are considered to be the substantial polluting factor. Other dye metabolites such as unsulfonated aromatic amines are relatively stable in aquatic conditions and are poorly degraded under anaerobic or aerobic wastewater treatment conditions (Pinheiro et al. 2004). Thus, both sulfonated and unsulfonated aromatic amines are important groups of environmental pollutants formed during the reduction of azo dyes that can potentially be of big concern to assess the toxicity of the dye before and after biodegradation. The plant seeds germination percentage and the length of the plumule and radical of both *S. vulgare* and *P. mungo* seeds were less with control dye, Direct Red-28 treatments as compared to its extracted degradation products and water. This study has revealed that the metabolites generated during the biodegradation of Direct Red-28 are less toxic compared to the untreated dye sample. Similar observations were noticed in the decolorization of Malachite Green (Parshetti et al. 2006), Direct Blue-15 (Kumar et al. 2007), Reactive Red-2 (Kalyani et al. 2008), and Navy Blue RX (Mane et al. 2008).

Apparently, there is still a need to develop novel biological processes leading to a more effective cleanup of azo dyes (Chung and Stevans 1993). In order to protect microbial cells

from possible toxic effects due to pollutant metabolites or changes in the environment conditions, immobilization of the biocatalyst was applied (Steffan et al. 2005). Of the various immobilization matrices studied, Ca-alginate as a matrix instigated maximum decolorizing ability of *Bacillus* sp. strain DRS-1.

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Biological Decolorization of Sulfonated Azo Dye C.I. Acid Blue 193 by *Bacillus cereus* KTSMD-03 and Its Azoreductase Characterization

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Abstract

Wastewater effluents from textile and other dyestuff industries contain significant amounts of synthetic dyes, which adversely affect water resources, soil fertility, aquatic organisms, and ecosystem integrity. Thus, prior treatment is required to prevent groundwater contamination. Among the multitudinal dyes, azo dyes are the predominant class of colorants that are characterized by one or more azo bonds, used in tattooing, cosmetics, foods, and consumer products. In this study, bacteria were subjected to acclimatization with C.I. Acid Blue 193 (AB 193) in minimal basal medium. The most promising bacterial isolate was used for further dye degradation studies. The 16S rRNA gene sequencing and biochemical characteristics revealed the isolated organism as *Bacillus cereus* KTSMD-03. Optimization of parameters for dye decolorization was studied under static anoxic condition. The optimum pH and temperature for the decolorization was 7.0 and 35 °C, respectively, at static conditions. Additional carbon and nitrogen sources namely, glucose, sucrose, starch, peptone, and yeast extract were added in different combinations to enhance the percentage of decolorization. Of these, peptone with glucose and sucrose showed the maximum decolorization of 91.07 %. In bacteria, the dyes are mainly metabolized by azoreductase that catalyzes an NADH-dependent reduction. Hence in this investigation, kinetic study was carried out for purified enzymes from *B. cereus* KTSMD-03. Biodegradation of dye was monitored by UV–VIS spectrophotometer and HPLC. Facile conditions and high decolorization potential of the bacterial strain showed it to be as an effective tool for biological treatment of dyeing effluents.

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Acid blue 193 · Azo dyes · Azoreductase · *Bacillus cereus* · Decolorization · Kinetics

Introduction

Rapid growth of industrialization, urbanization, and man's urge for color has led to the increased usage of dyes. Dyes are widely used in textile, dyeing, cosmetics, paper, leather, color photography, pharmaceutical, food, and other industries (Mohan et al. 2002; Sathiya Moorthi et al. 2007). These compounds absorb light with wavelengths in the visible range, i.e., 400–700 nm (Van der Zee et al. 2003). They are composed of a group of atoms called chromophores which imparts color. The most important chromophores are azo (–N=N–), carbonyl (–C=O), methane (–CH=), nitro ($–NO_2$), and quinoid ($O=(C_6H_4)=O$) groups. Several varieties of dyes are available such as acidic, reactive, basic, disperse, azo, diazo, anthraquinone-based, and metal-complex dyes (Welham 2000). Among these, azo dyes comprise the largest chemical class of synthetic dyes which are more versatile. They play a significant role in almost every type of application and are widely used as colorants (Wong and Yuen 1996). More than 60–70 % of 10,000 dyes predominately used in the textile industry are azo dyes (Carliell et al. 1995). Almost 70 % of dye production in the world represents azo dyes (Zollinger 1987; Dos Santos et al. 2003).

The release of dye effluents into the environment is undesirable, not only because of their color, but also because dyes from wastewater and their breakdown products are toxic and/or mutagenic to life. Most dyes are visible in water and their concentrations are as low as 1 mg l^{-1}. Textile-processing wastewaters contain dye concentrations in the range of 10–200 mg l^{-1} and are usually regarded as highly colored. Although dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments, thereby affecting the ecosystem through the food chain (Weisburger 2002). Moreover, dyes also serve as one of the major sources of heavy metals (Wagner 1993) in water and soil (Zehra et al. 2009) and their persistent nature causes misbalance in the ecosystem. In developing nations, dyes are responsible for environmental pollution since the effluents from textiles are often untreated and discharged into rivers and open fields (Bakshi et al. 1999). Disposal of these dyes into the environment causes serious damage, since they may significantly affect the photosynthetic activity of hydrophytes by reducing light penetration and also they may be toxic to some aquatic organisms due to their breakdown products. As a result, color in wastewater has been considered as a pollutant that has to be treated before its release into aquatic bodies (Anjaneyulu et al. 2005; Parshetti et al. 2006).

Dyes can be removed from wastewater by physical, chemical, biological, or combinations of these methods. Application of physical/chemical methods generates significant amount of sludge and also easily causes secondary pollution due to excess chemical usage. It is also expensive and has limited applicability (Vandevivere et al. 1998). Hence, it is necessary to develop a biological method (aerobic and anaerobic) for treatment of a wide range of dyes in wastewater.

Biological treatment is the most popular and efficient method for remediation of industrial effluents. The treatment of textile effluent has been studied extensively and much research has been focused on fungal aerobic systems, which are quite difficult commercially for upscale due to the sensitivity in culturing conditions (Stolz 2001; Wesenberg et al. 2003). On the other hand, bacteria has shown to be

ideal since it can withstand extreme environmental conditions (Kalyani et al. 2009) and hence it is necessary to investigate the potential bacteria to decolorize the dyes and also novel enzymes which can bioremediate textile effluent for an overall effective treatment of dye wastewaters. Azoreductase is a specialized azo dye reducing enzyme produced by microbes to degrade dyes under aerobic conditions (Coughlin et al. 1999; Quezada et al. 2000). However, the use of whole cells rather than isolated enzymes is advantageous, because purification processes are extremely high and the cell offers protection to the enzymes from environmental stress.

The objective of this study is to evaluate the decolorization of AB 193 by the isolated bacterial strain at different batch conditions and also to examine the influence of various cosubstrates to achieve maximum color removal. Furthermore, characterization of azoreductase from the isolated strain is also carried out.

Materials and Methods

Sample Collection

Tannery effluent sample was collected from common effluent treatment plant (CETP), Chrompet, an industrial area situated in the south-western part of Chennai, Tamil Nadu, India. The effluent was stored at 4 °C to avoid changes in its characteristics.

Characterization of Tannery Effluent

Physico-chemical parameters such as pH, total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), biological oxygen demand (BOD), chemical oxygen demand (COD), color, odor, nitrate, sulfate, sulfide, and phenolic compounds were determined. Heavy metals such as Cr, Fe, Zn, and Mn in tannery effluents were also estimated (APHA, AWWA, and WEF 1998).

Chemicals and Media

C.I Acid Blue 193 (AB 193), a reddish navy 1:2 premetalized dye was obtained from Rajasthan Dye Chemicals Ltd., Chennai, India. The majority of chemical compounds and media components were purchased from Himedia Labs, Mumbai, India. Sephadex G-75 and DEAE cellulose were purchased from Sigma-Aldrich, USA. All the media, buffers, microcentrifuge tubes, tips, reagents, etc., used in this study were sterilized at 15 lbs/inch^2 for 20 min unless otherwise specified. Heat-labile chemicals were filtered in membrane with porosities of 0.22 μm (Millipore).

Screening of Dye Decolorizing Bacteria

Dye decolorizing bacteria were isolated from the sample (CETP effluent) by enrichment culture technique. Tannery effluent was added to minimal basal medium (MBM) supplemented with peptone and yeast extract containing 0.025 g l^{-1} of AB 193 and incubated at 37 °C in orbital shaker at 150 rpm (Orbitek, Scigenics Biotech). After 24 h of incubation, the inoculum was transferred to a fresh MBM medium along with dye and five successive transfers were made. Around 10 % of the inoculum from the fifth enrichment was transferred to MBM containing dye and incubated at 37 °C in a shaker (150 rpm) for 24 h.

Identification of Dye Decolorizing Bacteria

Colonies isolated from MBM agar plates which tolerate high concentration of AB 193 were then subsequently transferred to Luria-bertani broth medium. The bacterial strain with the highest dye tolerance capacity was first identified by morphological and physiological characteristics to generic level according to Bergey's Manual of Systematic Bacteriology (Holt et al. 1994). Further, bacterial identification was confirmed by 16S rRNA sequence analysis.

Effect of Incubation Time on Decolorization of AB 193 at Static and Shaking Conditions by *B. cereus* KTSMD-03

Bacterial culture (OD 0.1 at 600 nm) was inoculated separately into Erlenmeyer flask containing MBM medium amended with 50 mg l^{-1} of AB 193. Experiments were carried out under static condition (i.e., neither aeration nor agitation was employed) and shaking conditions at 150 rpm (35 °C) in an orbital shaker. Uninoculated controls were also incubated under the same conditions to check the abiotic decolorization of dye. Samples were withdrawn at 12 h intervals up to 144 h, and centrifuged at 10,000 rpm for 15 min. Dye decolorization and growth of bacteria were measured spectrophotometrically (Shimadzu) at 586 and 600 nm respectively. From the difference in initial and final OD values, the percent of dye removed was calculated.

Effect of pH on the Decolorization of AB 193 by *B. cereus* KTSMD-03 at Static Condition

The pH was varied from 4 to 10 using dilute HCl or NaOH in MBM amended with 50 mg l^{-1} of AB 193. The culture (OD 0.1 at 600 nm) was inoculated and incubated for 108 h under static condition at 35 °C. Dye decolorization and microbial growth were determined spectrophotometrically. Based upon the color removal, optimum pH was determined.

Effect of Temperature on the Decolorization of AB 193 by *B. cereus* KTSMD-03 at Static Condition

The temperature was varied from 25 to 50 °C in MBM amended with 50 mg l^{-1} of AB 193 and pH of the medium was adjusted to 7.0 as determined from the above experiment. Precultured cells (OD 0.1 at 600 nm) were inoculated and incubated for 108 h under static condition. Dye decolorization and microbial growth were measured and the optimum temperature was determined.

Effect of Carbon Sources on the Decolorization of AB 193 by *B. cereus* KTSMD-03

Glucose, sucrose, and starch at different concentrations (0.5–3 %) under optimized conditions of pH and temperature were individually investigated for decolorization of AB 193. Dye concentration of 50 mg l^{-1} was chosen and decolorization study was conducted under static condition at 35 °C. Aliquots of spent medium were withdrawn at 12 h intervals for 108 h. Dye decolorization and microbial growth were measured and its optimum carbon concentration was determined.

Effect of Nitrogen Sources on the Decolorization of AB 193 by *B. cereus* KTSMD-03

The effect of nitrogen sources (peptone and yeast extract) on dye decolorization was investigated at different concentrations (0.5–3 %). Dye concentration and experimental condition were maintained as given in the previous experiment. Optimum nitrogen source was determined by analyzing the dye decolorization and microbial population of *B. cereus* KTSMD-03.

Effect of the Combination of Carbon and Nitrogen Sources on Decolorization of AB 193 by *B. cereus* KTSMD-03

Different combinations of carbon and nitrogen sources on dye decolorization were investigated with optimal concentration of glucose, sucrose, starch, peptone, and yeast extract in the media. Dye concentration and experimental condition were maintained as in previous experiments. Dye decolorization and microbial population would decide the suitable combinations of carbon and nitrogen concentrations.

Effect of Initial Dye Concentration on Decolorization of AB 193 by *B. cereus* KTSMD-03

Different concentrations of AB 193 (50–700 mg l^{-1}) in MBM medium supplemented with 2 % glucose, 0.5 % sucrose, and 1 % peptone inoculated with *B. cereus* KTSMD-03 were incubated under static conditions at 35 °C for 108 h. Dye decolorization and microbial growth were measured.

HPLC Analysis. Dye degradation was analyzed using HPLC (Shimazdu) system. Samples were centrifuged and filtered through 0.45 μm membrane filter (Millipore). The filtrates were extracted thrice with diethyl ether and flash evaporated in a rotary vacuum evaporator at 45–50 °C in a water bath. The residue was dissolved in methanol. Samples were then analyzed in HPLC using a C-18 reverse phase column (length—250 mm, diameter—4.6 mm, particle size—10 μm) (Biosystems). Solvent system consisted of methanol and water (50:50) at a flow rate of 1 ml min^{-1}. The separated components were detected using UV–VIS detector at 254, 226, and 280 nm. Solvents used in mobile phase were filtered using 0.2 μm nylon filters. A control without the bacterium or azoreductase containing 50 mg l^{-1} of AB 193 was prepared and analyzed under the same conditions. Standards namely, 1 amino-2-naphthol and sodium 4-amino-3-hydroxy-naphthalene-1 sulfonate (Sigma-Aldrich Chemicals, USA) were injected for comparison and confirmation (Bafana et al. 2008b).

Extraction and Purification of Azoreductase from *B. cereus* KTSMD-03

Bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min and washed thrice with 50 mM sodium phosphate buffer (pH 7.0). The washed pellet was resuspended in the same buffer containing 0.5 mM EDTA and 0.1 % (v/v) mercaptoethanol. Cells were disrupted by freezing and thawing followed by 5 min sonication at 4 °C. Sonicated solution containing cellular debris and unbroken cells were removed by centrifugation at 15,000 rpm for 15 min at 4 °C. The supernatant containing the crude enzyme extract (soluble protein fraction) was used for purification process (ammonium sulfate precipitation, dialysis, freeze drying, ion-exchange chromatography, and molecular exclusion chromatography). Protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin as a standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on one-dimensional 12 % polyacrylamide slab gel containing 0.1 % SDS (Laemmli 1970).

Assay of Azoreductase from *B. cereus* KTSMD-03

The reaction mixture (AB 193, NADH, and enzyme) was dissolved in 50 mM sodium phosphate buffer at 35 °C. Initially, the reaction mixture without NADH was preincubated for 4 min, followed by the addition of NADH. Azoreductase activity was determined spectrophotometrically at 586 nm. Dye decolorization was monitored by the decrease in color intensity. The slope of the initial linear decrease of absorption (ΔA min^{-1}) was used to calculate the azoreductase activity based on the molar absorption coefficient of azo dye. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH min^{-1}.

Effect of pH on Activity of Purified Azoreductase from *B. cereus* KTSMD-03

The reaction mixture (sample, dye, and NADH) at different pH (4–10) using various buffers [acetate (4–6), phosphate (6–7), tris (8–9), and carbonate (10)] were incubated at 35 °C for 30 min. pH stability of purified azoreductase was determined by measuring the residual activity of the enzyme spectrophotometrically at 586 nm.

Effect of Temperature on Activity of Purified Azoreductase from *B. cereus* KTSMD-03

Assay was conducted for different temperature ranges (25–70 °C) in 50 mM sodium phosphate buffer at pH 7.0 for 30 min. The residual activity of azoreductase was determined spectrophotometrically at 586 nm.

Kinetics of Azoreductase from *B. cereus* KTSMD-03

Kinetic values of the Michaelis–Menten constants (K_m), maximal velocity (V_{max}) for the reduction of AB 193, and oxidation of NADH by the purified azoreductase were determined by varying the concentration of one substrate at a time. The concentrations of AB 193 and NADH were 0.005–0.040 mM and 0.037–0.300 mM respectively. K_m and V_{max} values were calculated from Lineweaver–Burk double-reciprocal plots. The data were analyzed using GraphPad Prism version 3.0.

Experiments were conducted in triplicates and statistical analysis was carried out in SPSS package (version 16.0). All data were analyzed by standard deviation to assure the reproducibility of results and represented as error bars in each figure presentations.

Results

Physico-chemical Characterization of Tannery Effluent

The effluent was dark brownish black in color and had an unpleasant odor which might be due to the decomposition of the skin and hides of animals. Characterization of tannery effluent (Table 1).

Table 1 Physico-chemical characteristics of tannery effluent

Sl. No.	Parameters	Values
1	Color	Dark brownish black
2	Odor	Unpleasant
3	pH	7.6
4	BOD, mg l^{-1}	1,200
5	COD, mg l^{-1}	4,227
6	Nitrate, mg l^{-1}	1,619.68
7	Sulfate, mg l^{-1}	1,417.9
8	Sulfide, mg l^{-1}	905.98
9	Total solids, mg l^{-1}	9,098
10	Total dissolved solids, mg l^{-1}	7,538
11	Total suspended solids, mg l^{-1}	1,560
12	Phenolic compounds, mg l^{-1}	38.6
13	Chromium, mg l^{-1}	9.37
14	Iron, mg l^{-1}	3.28
15	Manganese, mg l^{-1}	2.62
16	Zinc, mg l^{-1}	3.34

Isolation of Dye Decolorizing Bacteria

Ten pure cultures were isolated after enrichment culture technique, of which only four strains showed decolorization of AB 193, i.e., initially partial and as incubation time proceeded, complete clearing of the dye occurred. Among these only one isolate produced the largest decolorization zone around the colony on agar plate and tolerated up to 1,500 mg l^{-1} of AB 193 (Fig. 1).

Identification of Dye Decolorizing Bacteria

In the biochemical tests, the strain gave positive results for Gram's reaction, voges proskauer, citrate utilization, nitrate reduction, endospore, oxidase, catalase, motility, starch hydrolysis, casein hydrolysis, gelatin liquefaction, and negative results for indole, methyl red, and urease activity. The promising isolate which decolorized AB 193 was identified as *Bacillus* sp. according to Bergey's Manual (Holt et al. 1994). Furthermore, the partial sequencing resulted in 799 nucleotide of *B. cereus* KTSMD-03 which was submitted to the Gen Bank under the accession number HM543567.

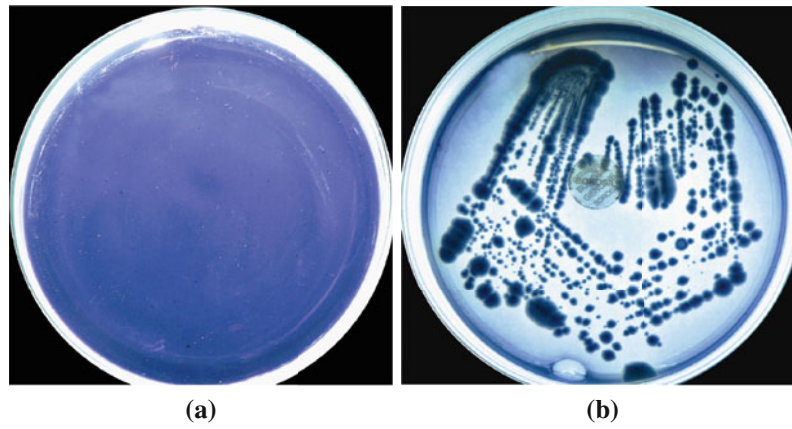

Fig. 1 Decolorization zone on AB 193 amended plate. **a** Control AB 193. **b** Culture amended with AB 193

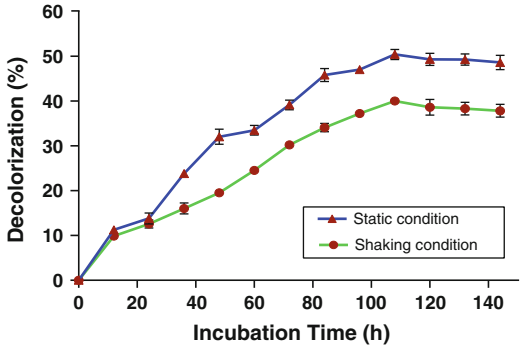

Fig. 2 Effect of incubation time on decolorization of AB 193 by *B. cereus* KTSMD-03 at static and shaking conditions

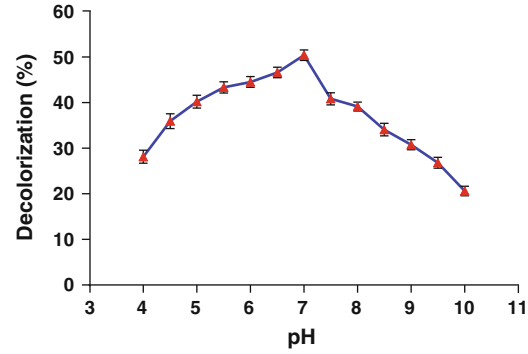

Fig. 3 Effect of pH on decolorization of AB 193 by *B. cereus* KTSMD-03

Effect of Incubation Time on Decolorization of AB 193 at Static and Shaking Conditions by *B. cereus* KTSMD-03

At static and shaking conditions, 50.41 and 40.02 % decolorization were observed for AB 193 by *B. cereus* KTSMD-03 (Fig. 2). In static condition, dye decolorization was steadily increased up to 108 h. With further increase in incubation time, the dye decolorization remained constant. Compared to static conditions, agitated cultures grew well but showed decreased decolorization efficiency. Uninoculated controls (shaking and static) showed no color removal.

Effect of pH on Decolorization of AB 193 by *B. cereus* KTSMD-03 at Static Condition

With increase in pH from 4 to 7, the decolorization of AB 193 also increased from 28 to 50 %. At alkaline conditions, i.e., from 7 to 10, the decolorization of dye decreased drastically. The optimum pH for decolorization of AB 193 by *B.cereus* KTSMD-03 was at 7 (Fig. 3).

Effect of Temperature on Decolorization of AB 193 by *B. cereus* KTSMD-03 at Static Conditions

Dye decolorization increased steadily from 25 to 35 °C and the maximum was observed at 35 °C

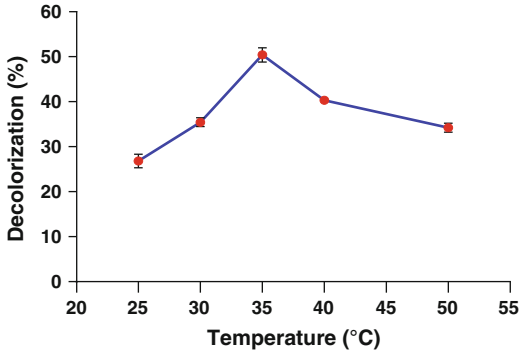

Fig. 4 Effect of temperature on decolorization of AB 193 by *B. cereus* KTSMD-03

(Fig. 4). With further increase in temperature to 50 °C, decolorization decreased.

Effect of Carbon Sources on Decolorization of AB 193 by *B. cereus* KTSMD-03

The decolorization profile is for a fixed concentration (50 mg l^{-1}) of AB 193 at varying initial concentrations of different cosubstrates (Fig. 5). The initial color removal was significantly enhanced when the concentration of glucose was increased from 0.5 to 2 %. Color removal was 71.58, 73, 75.81, and 65.56 % in MBM supplemented with 0.5, 1, 2, and 3 % of glucose, respectively, at 72 h of incubation. Optimum concentration of 2 % glucose was needed for decolorization of AB 193. Only around 50.41 % color removal of AB 193 was observed in plain MBM, but decolorization performance was enhanced to about 75 % when MBM was supplemented with carbon source (2 % glucose).

At a concentration of 0.5 % sucrose, AB 193 was decolorized to 69.12 %, but further increase in concentration to 1, 2, and 3 % of sucrose; decolorization was 67.49, 65.38, and 62.58 % respectively. When glucose was substituted with starch at concentrations of 0.5, 1, 2, and 3 %, AB 193 was decolorized to 8.42, 9.17, 6.62, and 5.06 %, respectively, within 72 h of incubation. In the presence of carbon sources, viz., glucose, sucrose, and starch, glucose was effective for the decolorization of AB 193.

Effect of Nitrogen Sources on Decolorization of AB 193 by *B. cereus* KTSMD-03

In our studies, medium supplemented with 0.5,1, 2, and 3 % peptone showed decolorization of 47.28, 61.85, 51.63, and 50.04 %, respectively, at 72 h of incubation (Fig. 6). In the presence of 1 % peptone, maximum (61.85 %) decolorization of AB 193 was observed. Above this concentration decreased color removal was seen. The decolorization of AB 193 by *B. cereus* KTSMD-03 decreased from 55.42 to 42.34 % as the concentration of yeast extract was increased from 0.5 to 3 %. Compared with yeast extract,

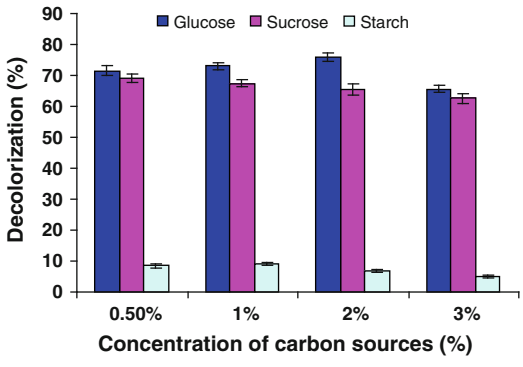

Fig. 5 Effect of carbon sources on decolorization of AB193 by *B. cereus* KTSMD-03

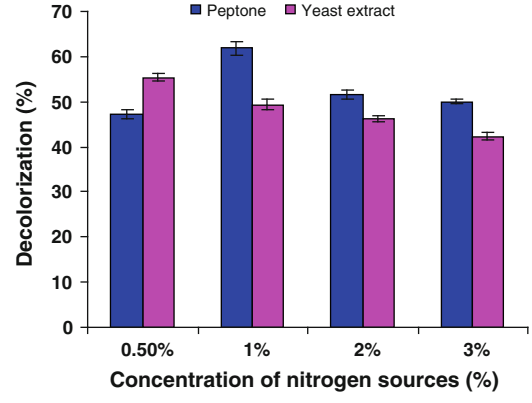

Fig. 6 Effect of nitrogen sources on decolorization of AB 193 *B. cereus* KTSMD-03

Table 2 Effect of various co-substrates on decolorization of AB 193 by *B. cereus* KTSMD-03 at static condition

Sl. No.	Co-substrates	Incubation time (pH)	Decolorization
1	MBM	108	50.41 ± 1.12
2	MBM + glucose + sucrose	96	65.92 ± 1.90
3	MBM + glucose + starch	96	58.42 ± 1.27
4	MBM + sucrose + starch	96	55.40 ± 1.16
5	MBM + glucose + sucrose + starch	96	62.23 ± 1.32
6	MBM + glucose + peptone	72	71.35 ± 1.20
7	MBM + sucrose + peptone	72	82.60 ± 1.31
8	MBM + starch + peptone	72	67.18 ± 1.13
9	MBM + glucose + sucrose +peptone	72	91.07 ± 1.19
10	MBM + glucose + starch +peptone	72	77.45 ± 1.56
11	MBM + sucrose + starch +peptone	72	71.89 ± 1.46
12	MBM + glucose + sucrose + starch + peptone	72	84.48 ± 1.70
13	MBM + glucose + yeast extract	48	75.77 ± 1.31
14	MBM + sucrose + yeast extract	48	72.58 ± 1.51
15	MBM + starch + yeast extract	48	58.83 ± 1.63
16	MBM + glucose + sucrose + yeast extract	72	85.82 ± 1.64
17	MBM + glucose + starch + yeast extract	72	72.43 ± 1.17
18	MBM + sucrose + starch + yeast extract	72	70.14 ± 1.77
19	MBM + glucose + sucrose + starch + yeast extract	72	79.74 ± 1.63
20	MBM + peptone + yeast extract	72	63.23 ± 1.49
21	MBM + glucose + peptone + yeast extract	72	84.90 ± 1.74
22	MBM + sucrose + peptone + yeast extract	72	85.94 ± 1.81
23	MBM + starch + peptone + yeast extract	72	82.78 ± 1.61
24	MBM + glucose + sucrose +peptone + yeast extract	72	83.35 ± 1.52
25	MBM + glucose + starch +peptone + yeast extract	72	79.86 ± 1.57
26	MBM + sucrose + starch +peptone + yeast extract	72	75.14 ± 1.40
27	MBM + glucose + sucrose + starch +peptone + yeast extract	72	85.89 ± 1.37

peptone served as better nitrogen source for removal of AB 193.

Effect of Combinations of Carbon and Nitrogen Sources on Decolorization of AB 193 by *B. cereus* KTSMD-03

Decolorization of AB 193 in the presence of carbon and nitrogen sources at different combinations are summarized in Table 2. Of various carbon and nitrogen source combinations, glucose, sucrose, and peptone in MBM achieved maximum color removal of AB 193–91.07 %, but lower decolorization (55 %) was seen when MBM was supplemented with sucrose and starch.

Effect of Initial Dye Concentration on Decolorization by *B. cereus* KTSMD-03

AB 193 was decolorized to 91.07 % and 85.81 % under static conditions at 50 and 100 mg l^{-1} concentrations (Fig. 7). With further increase in dye concentration to 200 mg l^{-1}, decolorization was reduced. Complete decolorization of AB 193

Table 3 Purification of azoreductase of *B. cereus* KTSMD-03 using AB 193 as a substrate

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Cell extract	584.2	27.90	0.047	100	1
$(NH_4)_2$ SO_4 precipitation	196.3	21.45	0.109	76.9	2.3
DEAE cellulose	18	17.58	1.020	63	21.7
Sephadex G-75	7.3	16.93	2.31	60.7	49.17

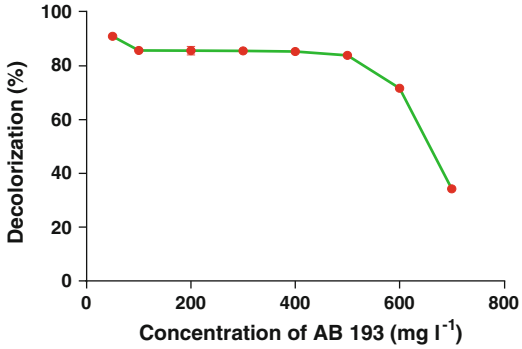

Fig. 7 Effect of initial dye concentration on decolorization of AB 193 by *B. cereus* KTSMD-03

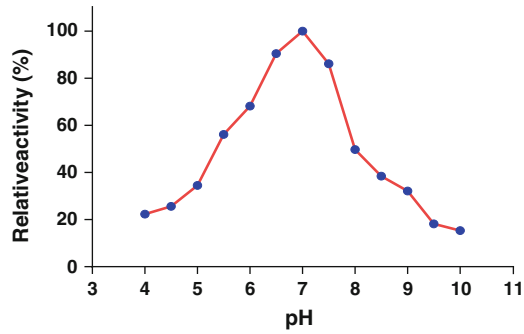

Fig. 8 Effect of pH on azoreductase activity using AB 193 as a substrate

was achieved at 50 mg l^{-1} and around 85 % removal of AB 193 was observed up to 500 mg l^{-1}. Hence, these results indicate that the decolorization was inversely related to the concentration of dye in the medium.

Assay of Azoreductase

The crude extract of disrupted cells of *B. cereus* KTSMD-03 contained 584 mg of protein, corresponding to approximately 28 units of azoreductase. More than 80 % of contaminated proteins were removed by purification steps and recovery was 17.6 units in 18 mg of protein. Purification of azoreductase from *B. cereus* KTSMD-03 using AB 193 as substrate is given in Table 3. Around 49-fold purification of the enzyme with a yield of approximately 61 % was achieved. The specific activity of the final purified enzyme was 2.3 U/mg of protein and its molecular weight was found to be 28 kDa.

Effect of pH and Temperature on Activity of Purified Azoreductase from *B. cereus* KTSMD-03

The purified enzyme showed maximum activity at a broad pH range (6–8) and exhibited bell-shaped profile in the presence of AB 193. At pH 6.5 and 10 when AB 193 was used as a substrate, azoreductase activity was retained to about 90 and 15 % respectively. Maximum azoreductase activity was achieved for AB 193 at pH 7.0, which confirmed that the enzyme from *B. cereus* KTSMD-03 is stable (Fig. 8).

Increased azoreductase activity was observed linearly with increase in temperature range of 20–35 °C and its maximum activity was at 35 °C. Optimum temperature for azoreductase activity for AB 193 was observed at 35 °C (Fig. 9). Protein was not denatured when *B. cereus* azoreductase was preincubated at temperature ranges from 10 to 35 °C, and it did not affect the enzyme activity. However, thermal inactivation of the enzyme occurred above

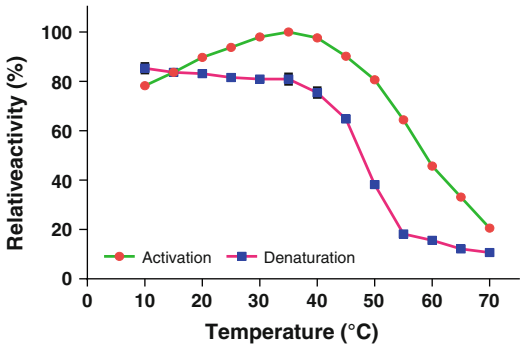

Fig. 9 Effect of temperature on azoreductase activity using AB 193 as a substrate

40 % which resulted in total activity loss at 70 °C.

HPLC Analysis

HPLC profile of the control without the bacterium or azoreductase exhibited two peaks which correspond to AB 193 at a retention time of 6.25 and 11.93 min (Fig. 10a). In case of the dye with *B. cereus* KTSMD-03 or with the purified azoreductase showed the appearance of two major peaks (Fig. 10b) at different times (1.54 and 5.69 min). Further, the metabolites when compared with the standard indicates the breakdown of AB 193 and formation of 1-amino-2-naphthol and sodium 4-amino-3-hydroxy-naphthalene-1-sulfonate which confirms the degradation of dye.

Kinetics of Purified Azoreductase Using AB 193 as a Substrate

Double reciprocal enzymatic reactions by varying the concentration of one substrate (AB 193 or NADH) and fixing the other substrate concentration (AB 193 or NADH) resulted in a sequence of lines (Fig. 11a, b) with a common intersection on the left of the 1/V axis. Azoreductase activity corresponds to sequential pattern rather than ping-pong mechanism. From secondary plots of 1/V intersections against the corresponding reciprocals of substrate concentration, a V_{max} value of 5.68 and 3.32 μmol $min^{-1}$and K_m values of 51.12 and 22.38 μM for NADH and AB 193, respectively, were obtained (Table 4).

Discussion

C.I. Acid Blue 193 was selected as a model sulfonated azo dye for the optimization of decolorization process. Only few studies have been successful in isolating microorganisms capable of utilizing dye as their sole carbon source (Sarnaik and Kanekar 1999). The obligate necessity of co-substrates for growth of dye-decolorizing bacteria is necessary; therefore, in our study isolation was attempted by employing peptone and yeast extract as co-substrates (Vijaya et al. 2003; Maier et al. 2004). In the decolorization medium, peptone (nitrogen source) or yeast extract (growth factor) is necessary to activate the coenzyme-producing metabolic pathways to induce the azoreductase activity for dye decolorization (Chang et al. 2001).

The 16S rRNA sequence analysis is employed as a framework for the modern classification of bacteria (Ki et al. 2009). Partial sequencing of the 16S rRNA resulted in 799-nucleotide length. The 16S rRNA sequence of this strain showed 98 % sequence similarity with *B. cereus* strain CMST-SP1 (HM101153) and other *Bacillus* groups namely, *B. cereus*, *B. anthracis,* and *B. thuringiensis.*

Reports are available on the decolorization by *Bacillus* sp. ADR isolated from soil for C.I. Reactive Orange 16 (Telke et al. 2009) and the bacterial strain *Bacillus odysseyi* SUK3 for Reactive Blue 59 (Patil et al. 2008). Mabrouk and Yusef (2008) reported the decolorization of Fast Red by *Bacillus subtilis* HM and the degradation product was *p*-aminoazobenzene (Zissi et al. 1997).

In comparison to the shaking cultures, the decolorization performance of *B.cereus* KTSMD-03 was better at static anoxic condition where depletion in oxygen content was observed. The reason for decreased decolorization at shaking condition could be due to the competition in oxidation of reduced electron

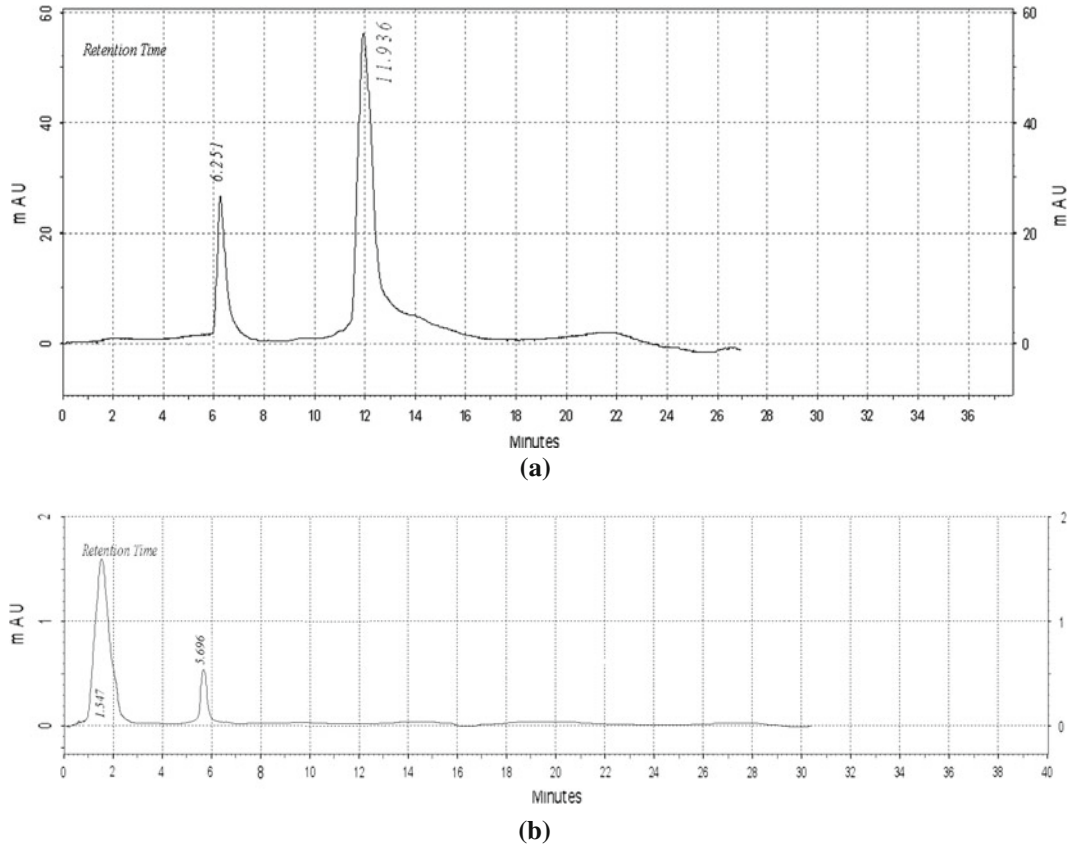

Fig. 10 HPLC elution profiles of AB 193 and its degraded metabolites by *B. cereus* KTSMD-03. **a** Chromatogram of AB 193. **b** Chromatogram of extracted metabolites

carriers with either oxygen or azo groups as electron acceptor (Mabrouk and Yusef 2008; Dawkar et al. 2010). Under shaking conditions, the aerobic respiration of the strain might dominate the utilization of NADH and inhibit azoreductase for obtaining electrons from NADH to decolorize azo dyes (Stolz 2001; Chang et al. 2004). Decolorization of azo dyes cannot take place in extreme anaerobic conditions that is under oxygen-free nitrogen sparging. This clearly implies that bacteria needs small amount of oxygen to maintain basic cellular activity for decolorization (Chen 2002). Similar results were observed in case of the following bacterial strains: *Shewanella putrefaciens* AS96, *Comamonas* sp. UVS, *B. subtilis*, *Vibrio harveyi* TEMS1, and *Pseudomonas* sp. SUK1. All these strains have shown promising results for dye degradation under static conditions (Khalid et al. 2008; Jadhav et al. 2008; Gurulakshmi et al. 2008; Ozdemir et al. 2008; Kalyani et al. 2009).

Decolorization of AB 193 was optimum at pH 7 and it was inhibited at acidic and alkaline pH values. At optimum pH, the surface of biomass gets negatively charged, which enhances the binding of positively charged dye. Binding occurs through electrostatic force of attraction and it results in a considerable increase in color removal (Daneshvar et al. 2007). Below the optimum pH, H^+ ions compete effectively with dye cations, causing a decrease in color removal efficiency. At alkaline pH, the azo bonds will be deprotonated to negatively charged compounds and it results in obstruction of azo dye decolorization. Similarly, azo dye decolorization was high at pH 7 in case of *E. coli* and *P. luteola* (Chang and Lin 2001). Dye decolorization of Scarlet R, Direct Fast Scarlet 4BS, and RB-5

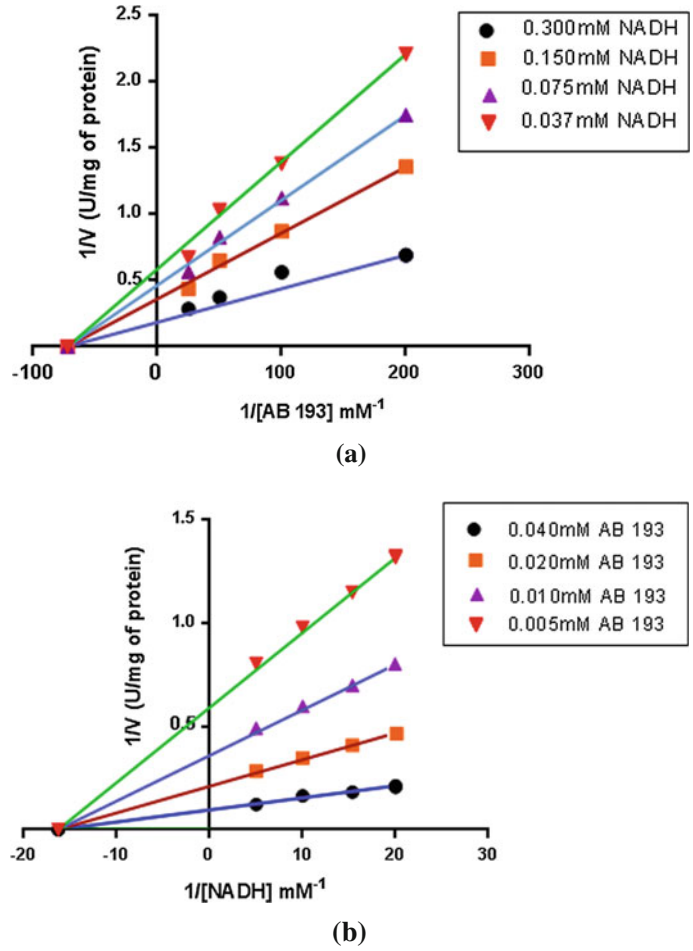

Fig. 11 Kinetics of the azoreductase activity purified from *B. cereus* KTSMD-03 using AB 193 as a substrate. **a** At varying NADH concentrations. **b** At varying AB 193 concentrations

Table 4 Michaelis constants (K_m) and maximal velocities (V_{max}) for azoreductase purified from *B. cereus* KTSMD-03

Substrate	K_m, μM	V_{max}, U mg^{-1} of protein
NADH	51.52	5.68
AB 193	22.38	3.32

was maximum at pH 7 (He et al. 2004; Dafale et al. 2008). Most of the azo dye reducing species of *Pseudomonas luteola*, *Bacillus,* and *Enterobacter* sp. EC3 (Chang et al. 2001; Kalme et al. 2007; Wang et al. 2009) were able to reduce the dye at neutral pH.

Dye decolorization (AB 193) was enhanced at 35 °C but it drastically decreased with increase in temperature (40 °C). Reduced color removal beyond 35 °C may be due to the loss of cell viability or thermal deactivation of decolorizing enzyme (Panswad and Luangdilok 2000; Cetin and Donmez 2006). It implies that the bacterium is mesophilic and the enzyme responsible for decolorization has its activity between 30 and 40 °C. Results obtained are also correlated with earlier studies (Khalid et al. 2008), where the decolorization of Methyl Red and RBR X-3B by *Vibrio* sp. and *Rhodopseudomonas palustris* was the maximum around 30–35 °C (Adedayo et al. 2004; Liu et al. 2006). Reports also show that *Klebsiella pneumoniae* RS-13 and *Acetobacter liquefaciens* S-1 had no decolorization of methyl red at 45 °C (Wong and Yuen 1998).

Dyes are usually deficient in carbon content and biodegradation without any carbon source is found to be very difficult (Vijaya et al. 2003).

Decolorizations of azo dyes are dependent on carbohydrate metabolism. Glucose as a co-substrate, acts as a source of electron donors, which are needed for cleavage of azo bond (Mendez-Paz et al. 2005; Khan and Husain 2007; Dafale et al. 2008). As a result, decolorization of AB 193 was higher if glucose was present in the culture medium when compared with starch. Highest and lowest decolorization of AB 193 was observed with 2 % glucose (75.81 %) and 1 % starch (9.17 %) by *B. cereus* KTSMD-03. Sucrose at 0.5 % concentration increases the color removal of AB 193; with further increase in concentration, decreased color removal was observed. Mohana et al. (2008) have reported that the addition of sucrose as co-substrate enhanced poly azo dye Direct Black 22 decolorization. Ozdemir et al. (2008) have reported the decolorization of AB 210 to about 91.7 % when sucrose was used as co-substrate. Utilization of starch as a co-substrate by microbes could be encouraging from the commercial point of view (Moosvi et al. 2005). Starch at 1 % concentration was not effective to decolorize AB 193. In contrast to our study, earlier reports showed improved decolorization of dye in the presence of starch (Ozdemir et al. 2008). The effect of carbohydrate on dye removal indicates that the sugar might have synergistic as well as antagonistic effect on dye removal by microbes (Aksu and Donmez 2000). In the presence of carbon sources, viz., glucose and sucrose, glucose was effective for the decolorization of AB 193.

In the decolorization medium, peptone (nitrogen source) or yeast extract (growth factor) is necessary to activate the coenzyme-producing metabolic pathways to induce the azoreductase activity for dye decolorization (Chang et al. 2001). Peptone is considered to be one of the best co-substrates for bacterial growth and decolorization (Liu et al. 2006). The presence of peptone regenerates NADH and this acts as an electron donor for the azo dye reduction. In this study, peptone concentration of 1 % showed maximum decolorization of AB 193 (61.85 %). In addition, peptone significantly enhances the strain's activity of azo dye decomposition. Similar studies on decolorization of dye with peptone have also been reported (Ramya et al. 2008; Saratale et al. 2009). Bhatti et al. (2008) reported Cibacron Red decolorization in the presence of various nitrogen sources including peptone.

Yeast extract in the medium activates the necessary coenzyme for the metabolic pathway of azoreductase and serve as key components for decolorization (Chang et al. 2001). Substitution of yeast extract for peptone gave poor cell growth and low color reduction efficiency. Yeast extract is generally selected for improvement of decolorization because it is cheaper than tryptone or peptone (Chang et al. 2000; Chen et al. 2003). Bhatt et al. (2005) demonstrated decreased decolorization of dye at high concentration of yeast extract.

Optimization of medium components is important to predict nutrient supplementation for effective dye removal (Mohana et al. 2008). Decolorization of dyes was enhanced when carbon and nitrogen sources are available in the growth medium (Sheth and Dave 2009). The decolorization rate of azo dyes is increased by using various combinations of co-substrates which generate redox mediators that catalyze the reaction rate by shuttling electrons from the biological oxidation of primary electron donors or from bulk donors to the azo dyes as electron acceptors (Rau et al. 2002; Khan and Husain 2007).

The dye at high concentration has inhibitory effect on azo bond reduction and causes reduced decolorization. In various carbon and nitrogen source combinations, glucose, sucrose, and peptone present in the medium completely decolorized AB 193. It was noticed that at low concentration (50 mg l^{-1}), the rate of decolorization is efficient and reduced up to 91 %, while with increase in dye concentration decreased color removal was noticed. This is due to the toxicity of dye to bacterial cells by inhibiting the metabolic activity or saturation of cells with dye products or the electrons fail to reach the azo bond chromophores or inactivation of transport system or the blockage of active sites in azoreductase enzymes by the dye molecules (Sponza and Isik 2002; Pearce et al. 2006; Vijaykumar et al. 2007). The effluent from textile industries has variations in dye concentration

and thus, the ability of the organism to degrade the dye at wide range of concentration is an important factor for effective biodegradation (Gopinath et al. 2009).

HPLC is an effective technique for the analysis of metabolites for the degraded azo dyes due to its accuracy, high separation efficiency, and simplicity of procedure. HPLC analyses were performed to confirm the presence of degraded products. The products of AB 193 were identified by the retention times as compared with the standards. In our study, during degradation of AB 193, the presence of 1-amino-2-naphthol as one of the metabolites proved the involvement of a reductive process in the initial decolorization step. Similar to our report, 1-amino-2-naphthol has been identified as a degraded product on reduction of orange II (Zimmerman et al. 1982). Degradation of DR 28 to benzidine and 4-ABP by *Bacillus velezensis* was reported by Bafana et al. (2008a). The degradation of methyl red, resulted in the appearance of two major peaks with retention times of 5.8 and 7.1 min, respectively, and was identified as 2-aminobenzoic acid (ABA) and N, N'-dimethyl-*p*-phenylenediamine (DMPD) by HPLC analysis (Chen et al. 2005; Ooi et al. 2007). The metabolites formed after decolorization of AB 193 showed peak with different retention time than the parent dye which confirms degradation.

Azo reductase localized in the cytoplasmic fraction requires NADH as a cofactor for the decolorization activity. These enzymes isolated from several bacteria are shown to be inducible flavoproteins which use both NADH and NADPH as electron donors (Moutaouakkil et al. 2003). Azoreductase from *B. cereus* decolorized AB 193 using NADH as electron donor. It is not necessary to remove oxygen or preincubate with NADH as in the case of previous report (Maier et al. 2004) and this confirms that the azoreductase from the strain KTSMD-03 is oxygen insensitive.

Azoreductase showed an optimum at pH 7, as reported for the same enzyme from *Pseudomonas* sp. (Nachiyar and Rajakumar 2005; Pricelius et al. 2007). pH dependence of azoreductase reaction is the implication of the varying degrees of ionization of functional group in the enzyme as well as in the substrate. This is due to the reversible redox reactions performed by the azoreductase enzyme (Zehender 1988). Maier et al. (2004) reported an optimum pH of 7.0 for azoreductase in the decolorization of Mordant Black 9, Mordant Brown 96, and Reactive Black 5. The maximum azoreductase activity observed at 35 °C is similar to the growth temperature of mesophilic bacteria. Similarly, previous reports showed optimum azoreductase activity at 35 °C (Nachiyar and Rajakumar 2005; Ooi et al. 2007; Pricelius et al. 2007). At lower temperature, the activity is slow but steadily increases as temperature increases. This is related to enzyme kinetics, where the rate of enzyme reaction increases as temperature increases and it is achieved by the maximum rate at which enzyme and substrate collide with each other (Clark 1977). Thermal inactivation of azoreductase was noticed above 40 °C and total activity loss at 70 °C. The apparent loss in activity is observed due to the loss of protective matrices, which normally protect the azoreductase enzyme during its purification process (Walker et al. 2000).

Azoreductase utilized NADH as an electron donor and the rate of reaction increased with increase in concentration of NADH, which indicates that NADH plays an important role in dye degradation. These results suggest that the reaction mechanism is in a sequential pattern rather than a ping-pong type. A similar report was observed where the enzyme reaction was sequential rather than in a ping-pong manner (Moutaouakkil et al. 2003; Nachiyar and Rajakumar 2005).

Conclusion

Adaptation of a microbial community toward toxic or recalcitrant compounds is very useful to improve the rate of the decolorization process. It was confirmed that *B. cereus* KTSMD-03 could tolerate AB 193 up to 1,500 mg l^{-1}. This study confirms the ability of isolated bacterial culture *B. cereus* KTSMD-03 to decolorize the textile and leather dye AB 193 with decolorization efficiency

of above 90 %. The presence of co-substrates is considered essential for attaining maximum decolorization of sulfonated azo dye AB 193.

The purification and characterization of azoreductase from *B. cereus* KTSMD-03 contribute to our understanding of azo dye degradation and make it possible for the biotechnological application of treating dye containing industrial wastewater. Thus, a biological process could be adopted as a cost-effective pretreatment combined with conventional biological treatment system or a tertiary step for decolorization of dye wastewater effluents. This would increase the applicability of using the strain in practical dye wastewater decolorization. In order to enhance process efficiency the search for cheaper supplementary carbon and nitrogen sources would be essential in the future work.

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Bioremediation of Tannery and Textile Effluent by Plasmid Curing Heavy Metal Resistance Bacteria

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Abstract

The heavy metal resistant bacteria were isolated from tannery to textile effluents. The isolates were identified as *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. and the results showed that the isolated strains were able to grow a wide range of pH (5–10) at temperatures (28–45 °C). The isolates strains were tested for salt tolerance, heavy metals and antibiotics. Plasmid curing agents were used to cure the resistant plasmid if any. The growth of the isolates in the plates containing heavy metals showed that their heavy metal resistance was not plasmid mediated. On the other hand, the isolates which were resistant to several antibiotics became sensitive to some of them indicating antibiotic resistance to the plasmid.

Keywords

Bioremediation · Textile · Tannery · Heavy metal · Bacteria

Introduction

Heavy metals include some toxic chemical elements and their deviated chemical compounds. Most of these compounds do not functionally involve in any activity for life. On the other hand, these heavy metals frequently generate strong reactive oxygen species (ROS) and directly/indirectly cause gene mutations, and therefore their presence is hazardous to cells. Although their toxicities might be different, the protein damages and their competitions for the entry of certain essential elements might be the most likely effects on the cell. Some of these heavy metals are necessary for life, namely copper, iron and zinc. These metal ions are essential trace elements that are required for a number of enzymes. At certain concentration levels, these elements participate in some enzyme activities. In excess concentrations, the toxic effects of these dual functional ions are revealed. The extensive industrial usage of chromium and other heavy metal compounds and subsequent release of effluents in the environment contaminate the ecosystem. The heavy metals discharge from industries like the metal finishing industry, petroleum refining, leather tanning, iron and steel industries, textile manufacturing and paper industry elevated its concentration in aquifers and groundwater (Sultan and Hasnain 2005).

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Contamination of the aquatic environment by toxic metal ions is a serious pollution problem. Unlike organic pollutants, chemical or biological processes cannot degrade toxic metal ions. To remediate the aquatic environment, the toxic metal ions should be concentrated in a form that can be extracted conveniently, possible for reuse or at least for proper disposal. Natural resources including plants and micro-organisms are extensively explored to combat metal ion pollution. Endophytic bacteria are described as non-pathogenic bacteria found within the tissues of healthy or symptomless plants. These bacteria are found in most, if not all, plant species, span a wide range of bacterial phyla and are known to have plant growth promoting and pathogen control activities.

Recent research suggests that these beneficial impacts may, in the case of plants growing at contaminated sites, extend to the degradation of xenobiotic compounds. Although endogenous bacterial endophytes capable of degrading hydrocarbons are likely to be widespread, to date there are only a few studies on heavy metal accumulator plants in phytoremediation. Rashed and Soltan (2002) found that bacterial strains were isolated from root and stem tissues of different heavy metal accumulates. The distribution of these bacteria was spatially limited both within and between the individual accumulator cultivars. Water hyacinth (*Eichhornia crassipes*) is one of the plant species that attracted considerable attention because of its ability to grow in heavily polluted water together with its capacity for metal ion accumulation though other studies have clearly manifested that hyacinths are efficient in the phytoremediation of heavy metals. The main aim of this research is to use the plasmid curing heavy metal resistant bacteria for the bioremediation of heavy metal contaminated soils.

Materials and Methods

Collection of Tannery and Textile Samples

Effluent samples were collected in the tannery and textile industries. Samples collected in sterilised glass containers were brought to the laboratory for microbiological analysis.

Isolation of Heavy Metal Resistant Bacteria

Heavy metal resistant bacteria were isolated on nutrient agar (peptone 5 g, sodium chloride 5 g, yeast extract 3 g, beef extract 3 g, agar 18 g, distilled water 1,000 ml and pH 7), nutrient agar with single heavy metal (10 μg/ml of potassium chromate) and nutrient agar with multi heavy metals (10 μg/ml of potassium chromate, zinc chloride, mercuric sulphate, magnesium sulphate and barium chloride). The nutrient agar plates were prepared and sterilised at 121 °C for 15 min. After sterilisation, the nutrient agar was cooled 45–50 °C. The heavy metals were added into the nutrient agar. Then nutrient agar that contained heavy metal was poured into the sterile petri plates.

The samples serially diluted in the ten-fold dilution method (9 ml of sterilised distilled water was taken in the six test tubes. One ml of sample was transferred to the first test tube. The dilution rate was 10^{-1}. One ml of 10^{-1} dilution sample was transferred to the second test tube. The dilution rate of the second test tube was 10^{-2}. The samples were serially diluted until the 10^{-6} dilution). 0.1 ml of serially diluted sample was spread on the nutrient agar containing heavy metals. The plates were incubated at 37 °C for 48 h. The microorganisms were isolated and stored at 4 °C.

Identification of Heavy Metal Resistant Bacteria

Gram Staining

The smear was prepared in clean glass slides. It was dried and heat fixed. It was stained with the basic dye crystal violet for 45 s (Solution A: crystal violet (90 % dye content) 2.0 g dissolved in ethyl alcohol (95 %) 20.0 ml and Solution B: Ammonium oxalate 0.8 g dissolved in distilled

water 80.0 ml. solutions A and B were mixed). The stain was washed off with distilled water. The smear was followed by treatment with an iodine solution (iodine 1.0 g, potassium iodide 2.0 g and distilled water 300.0 ml). Iodine solution was removed by gently washing with distilled water. The smear was next decolourised by washing with ethanol at 95 %. The smear was counterstained with safranin 30 s (Safranin (2.5 % solution in 95 % ethyl alcohol) 10.0 ml and distilled water 100.0 ml). Then it was dried and observed under the microscope.

Motility Test

Peptone water (peptone 5 g and distilled water 1,000 ml) was prepared in six test tubes. Bacterial isolates were inoculated into the peptone water and incubated at 37 °C for 24 h. After incubation, hanging drop technique was used to detect the motility of the tannery textile isolates.

IMViC Test

Indole Test

Tryptophanase enzyme production of tannery and textile isolates was detected by using peptone water tubes. The isolates were inoculated into the peptone water tubes. The tubes were kept for incubation at 37 °C for 24 h. After the incubation period, 2–3 drops of KOVAC'S reagent (P-Dimethylaminobenzaldehyde 5.0 g dissolved in amyl alcohol 75.0 ml, then added the hydrochloric acid 25.0 ml) were added and the results were observed.

Methyl Red Test

Acid production of tannery and textile isolates were detected by using MR-VP broth. MR-VP broth (peptone 7.0 g, potassium phosphate 5.0 g, dextrose 5.0 g, distilled water 1,000.0 ml and pH 6.9) was prepared and sterilised at 121 °C for 15 min. The isolates were inoculated into sterilised MR-VP broth tubes at 37 °C for 48 h. After the incubation period, 2–3 drops of methyl red reagent (methyl red 0.1 g, ethyl alcohol (95 %) 300.0 ml and distilled water 200 ml) were added and the results were observed.

Voges Proskauer Test

Non-acidic end product was detected in the tannery and textile isolates by using MR-VP broth. MR-VP broth was prepared and sterilised at 121 °C for 15 min. The isolates were incubated into MR-VP broth at 37 °C for 48 h. After the incubation period, 2–3 drops of Barrit's reagent A and B were added into the culture tubes and the results were observed.

Citrate Utilisation Test

Simmons citrate agar was used to detect the ability of citrate utilisation by the tannery and textile isolates. Simmons citrate agar (SCA) medium (Ammonium dihydrogen phosphate 1.0 g, dipotassium hydrogen phosphate 1.0 g, sodium chloride 5.0 g, sodium citrate 2.0 g, magnesium sulphate 0.2 g, bromothymol blue 0.08 g, agar 18.0 g, distilled water 1,000.0 ml and pH 6.9) was prepared and sterilised at 121 °C for 15 min. The isolates were streaked on the agar slants and incubated for 24 h at 37 °C.

Urease Test

Christenson urea agar was used to detect the urease enzyme production by the tannery and textile isolates. Christenson urea agar medium (peptone from meat 1.0 g, D (+) glucose 1.0 g, sodium chloride 5.0 g, potassium dihydrogen phosphate 2.0 g, phenol red 0.012 g, urea 20.0 g, agar 12.0 g, distilled water 1,000 ml and pH 6.8) was prepared and sterilised at 121 °C for 15 min. The isolates were streaked on the Christenson urea agar slants and incubated for 24 h at 37 °C.

Starch Hydrolysis Test

Starch agar was used to detect the amylase production by the tannery and textile isolates. Starch agar medium [starch (soluble) 20.0 g, peptone 5.0 g, beef extract −3.0 g, agar 18.0 g, distilled water 1,000.0 ml and pH 7] was prepared and sterilised 121 °C for 15 min. The isolates were streaked as a single line on starch agar medium. Inoculated plates were incubated at 37 °C for 48 h. After incubation, Iodine solution (iodine 1.0 g, potassium iodide 2.9 g and distilled water 300.0 ml) was flooded on the surface of plates for 30 s and the results were observed.

Screening of Tannery and Textile Isolates by Using Selective Media

The three selective media were used for the identification of the tannery and textile isolates. Mannitol salt agar (MSA) (D-mannitol 10.0 g, sodium chloride 75 g, peptone 10 g, beef extract 1 g, phenol red 0.025 g, agar 16 g, distilled water 1,000 ml and pH 7.5), *Bacillus cereus* medium (D-mannitol 10.0 g, sodium chloride 10 g, peptone 10 g, beef extract 1 g, phenol red 0.025 g, agar 16 g and distilled water 1,000 ml) and fluorescence agar (peptone 20 g, dipotassium phosphate 1.5 g, magnesium sulphate 5 g, agar 12 g and distilled water 100 ml) selective media were prepared. The isolates were inoculated on the selective media plates. The plates were kept for incubation at 37 °C for 48 h.

Determination of pH Tolerance of Tannery and Textile Isolates

pH tolerance was determined by the method described by Tippannavar et al. (1989). Plates of nutrient agar with different pH (2, 4, 5, 7, 8, 9 and 10) were prepared. The cultures of isolates already grown in their respective broths were then spot inoculated on the plates with approximately 3×10^6 organisms. The plates were incubated at 37 °C for 48 h.

Determination of Temperature Tolerance of Tannery and Textile Isolates

The cultures of isolates already grown in their respective broths were then spot inoculated on the plates (nutrient agar) with approximately 3×10^6 organisms. The plates were incubated at different temperatures (28, 37, 40, 45 and 55 °C) for 48 h.

Determination of Salt Tolerance of Tannery and Textile Isolates

Microtitre plate wells from each column in row 1 were marked and 100 µl of sodium chloride stock (50 %) and blank without sodium chloride stock solution was added. 50 µl of saline (0.75 % of sodium chloride) was added to rows 2–12. Two-fold serial dilutions were performed by transferring 50 µl of solution from row 1 to row 2, using a multichannel pipette. This was repeated down row 2 to row 12. 40 µl of double strength nutrient broth (peptone 10 g, sodium chloride 10 g, yeast extract 6 g, beef extract 6 g, distilled water 1,000 ml) and 10 µl of different bacterial culture was added to all the wells in separate columns, so that the final concentrations of the inoculums in all the wells were 5×10^5 cfu/ml. To prevent dehydration, the plates were covered with a plastic cover and then incubated at 37 °C overnight. The bacterial growth was determined after addition of 40 µl of P-iodonitrotetrazolium violet (0.2 mg/ml). The minimum inhibitory concentrations (MIC) of the isolates were taken as the lowest concentration of the NaCl.

Determination of Heavy Metal Tolerance of Tannery and Textile Isolates

Plates of nutrient agar containing in the six concentrations (10, 100, 250, 500, 750 and 1,000 µg) of five heavy metals (potassium chromate, zinc chloride, mercuric sulphate,

magnesium sulphate and barium chloride) were prepared. Already grown in their respective broths, they were then spot inoculated in the plates with approximately 3×10^6 organisms. The plates were incubated at 37 °C for 48 h.

Determination of Minimum Inhibitory Concentration of Heavy Metals of Tannery and Textile Isolates

Microtitre plate wells from each column in row 1 were marked and 100 µl of heavy metals stock 10 mg/ml (potassium chromate, zinc chloride, mercuric sulphate, magnesium sulphate, barium chloride) and blank without heavy metals stock solution was added. 50 µl of saline (0.75 % of sodium chloride) was added to rows 2–12. Two-fold serial dilutions were performed by transferring 50 µl of solution from row 1 to row 2, using a multichannel pipette. This was repeated down row 2 to row 12. 40 µl of double strength nutrient broth and 10 µl of different bacterial culture was added to all the wells in separate columns, so that the final concentrations of the inoculums in all the wells were 5×10^5 cfu/ml. To prevent dehydration, the plates were covered with a plastic cover and then incubated at 37 °C overnight. The bacterial growth was determined after addition of 40 µl of P-iodonitrotetrazolium violet (0.2 mg/ml). The MIC of the isolates were taken as the lowest concentration of the heavy metal.

Determination of Antibiotic Resistance of Tannery and Textile Isolates

Antibiotic sensitivity test was performed by Bauer et al. (1966) disc diffusion method using Muller-Hinton agar (MHA) plates (starch 1.5 g, casamino acids 17.5 g, beef infusion 300 g, agar 17.0 g, distilled water 1,000 ml and pH 7.4). The isolates were inoculated into the nutrient broth and allowed to grow at 37 °C for 24 h. 100 µl of bacterial cultures were added on MHA plates and the following antibiotic discs (ampicillin, chloramphenicol, kanamycin, gentamycin, tetracycline Himedia, Mumbai) were applied over the agar and incubated the plates for 24 h at 37 °C. The inhibition spectrum was measured by using the comparison of standard chart.

Curing of Plasmids in Heavy Metals Resistance Tannery and Textile Isolates

Fresh overnight culture broths were prepared in several tubes. Plasmid curing agents (ethidium bromide 10, 25 and 50 µg/ml, sodium dodecyl sulphate (SDS) 10 % and acridine orange 20, 25 and 30 µg/ml) were added at various concentrations. The tubes were incubated on rotary shaker at 37 °C for about 24 h. The cultures were plated onto the nutrient agar plates.

Determination of Heavy Metals Tolerance by Using the Plasmid Cured Derivatives

Three different types of media plates were prepared. In the first type of media, the heavy metal (potassium chromate 80 µg/ml) were added in nutrient agar. In the second type of media, the heavy metals (potassium chromate 80 µg/ml, zinc chloride 40 µg/ml and magnesium sulphate 5 mg/ml) were added in nutrient agar. In the third type of media, the nutrient agar was prepared. This was used as control plates. The culture of plasmid-cured derivatives were spot inoculated on the heavy metal supplemented plates and control plates. The plates were incubated at 37 °C for 48 h.

Determination of Antibiotics by Using the Plasmid Cured Derivatives

Antibiotic sensitivity test was performed by Bauer et al. (1966) disc diffusion method using Mueller-Hinton agar (MHA) plates. The plasmid cured derivatives were inoculated into the nutrient broth and allowed to grow at 37 °C for

Table 1 Isolation of heavy metal resistant bacteria from tannery and textile samples

Sl. No.	Sampling sources	Nutrient agar	Nutrient agar + A		Nutrient agar + B	
			No. of colonies (CFU)	Frequency of colonies (%)	No. of Colonies (CFU)	Frequency of colonies (%)
1	Tannery	413	392	95	364	88
2	Textile	387	375	96	347	89.6

A—10 µg/ml of potassium chromate, B—10 µg/ml of potassium chromate, zinc chloride, mercuric sulphate, magnesium sulphate and barium chloride

24 h. 100 µl of bacterial isolates were added on MHA plates and the following antibiotics discs (ampicillin, chloramphenicol, kanamycin, gentamycin and tetracycline Himedia, Mumbai) were applied over the agar and incubated the plates for 24 h at 37 °C. The inhibition spectrum was measured by using the comparison of standard chart.

Determination of Heavy Metal Resistant CFU After Plasmid Curing

The control isolates and plasmid cured derivatives were serially diluted in the sterilised distilled water until 10^{-5} dilution. Two types of nutrient agar were prepared. In the first type of media, the heavy metals were added in the nutrient agar. In the second type of media, the nutrient agar were prepared. This was used as control plates. The serially diluted cultures were spread on the nutrient agar and nutrient agar and heavy metal incorporated plates. The plates were incubated at 37 °C for 48 h.

Results

Effluent samples were collected in the tannery and textile industries. Heavy metal resistant bacteria were isolated on nutrient agar, nutrient agar with single heavy metal (10 µg/ml of potassium chromate) and multi heavy metals (10 µg/ml of potassium chromate, zinc chloride, mercuric sulphate, magnesium sulphate and barium chloride). The results of colony forming unit differed in the nutrient agar, nutrient agar with single and multiheavy metals plates. The colony forming units of tannery and textile samples are tabulated in Table 1. As per the table, the frequency of the CFU in the potassium chromate containing plate was 95–96 % and in the plate containing heavy metals (potassium chromate, zinc chloride, mercuric sulphate, magnesium sulphate and barium chloride) it was 88–89.6 %.

Six bacterial strains TnS-1, TnS-2, TnS-3, TxS-1, TxS-2 and TxS-3 were isolated from the heavy metals containing plates. The isolates were identified as *Bacillus* sp., (TnS-1 and TnS-2), *Staphylococcus* sp. (TnS-3 and TxS-1) and *Pseudomonas* sp. (TxS-2 and TxS-3). These strains were aerobic, motile (TnS-1, TxS-2 and TxS-3), Gram-positive rod (TnS-1 and TnS-2), Gram-positive cocci (TnS-3 and TxS-1) and Gram-negative rod (TxS-2 and TxS-3). Other biochemical characteristic and selective media of heavy metal resistant isolates are shown in Table 2.

Three types of selective media were used for the confirmation of the species. *B. cereus* medium were used for the identification of *Bacillus* sp. and *B. cereus* formed pink colour colonies on the medium. Other *Bacillus* sp. formed yellow colour colonies. *Staphylococcus* sp. formed yellow colour colonies on the mannitol salt agar. *Pseudomonas* sp. formed grey fluorescent colour on the fluorescent agar medium.

The heavy metal resistant isolates were able to grow over a wide pH range 5–10 (TnS-1, TnS-2 and TnS-3) and 7–10 (TxS-1, TxS-2 and TxS-3). The strains were alkaliphilic and neutrophilic. The pH tolerances of the heavy metal resistant isolates are shown in Table 3. The temperature tolerance of the tannery and textile isolates are shown in Table 3. The isolates were grown in the range of 45 °C. TnS-1 was grown at 55 °C.

Table 2 Morphological and biochemical characteristics of tannery and textile isolates

Sl. No	Character/Test	Isolates					
		TnS-1	TnS-2	TnS-3	TxS-1	TxS-2	TxS-3
1	Gram Staining	+	+	+	+	−	−
2	Shape	Rod	Rod	Cocci	Cocci	Rod	Rod
3	Motility	+	−	−	−	+	+
4	Indole	−	−	−	−	−	−
5	Methyl red	−	−	+	+	−	−
6	Voges proskaeur	+	+	+	+	−	−
7	Citrate utilisation	−	−	−	−	+	+
8	Urease	−	−	−	−	−	−
9	Starch Hydrolysis	+	+	−	−	−	−
10	*B. cereus* medium	Pink	Yellow	Yellow	Yellow	−	−
11	Mannitol salt Agar	−	−	Yellow	Yellow	−	−
12	Fluorescence agar	−	−	−	−	Crey fluorescent	Crey fluorescent
13	Result	*Bacillus*	*Bacillus*	*Staphylococcus*	*Staphylococcus*	*Pseudomonas*	*Pseudomonas*

+ Positive,− negative

Table 3 pH and temperature tolerance of tannery and textile isolates

Sl. No.	Isolates	pH							Temperature (°C)				
		2	4	5	7	8	9	10	28	37	40	45	55
1	TnS-1	–	–	+	+	+	+	+	+	+	+	+	+
2	TnS-2	–	–	+	+	+	+	+	+	+	+	+	–
3	TnS-3	–	–	+	+	+	+	+	+	+	+	+	–
4	TxS-1	–	–	–	+	+	+	+	+	+	+	+	–
5	TxS-2	–	–	–	+	+	+	+	+	+	+	+	–
6	TxS-3	–	–	–	+	+	+	+	+	+	+	+	–

– Nil growth, + growth

Table 4 MIC of sodium chloride for the tannery and textile isolates

Sl. No	Isolates	Salt concentrations (%)											
		50	45	40	35	30	25	20	15	10	7.5	5	2.5	1.25
1	TnS-1	–	–	–	–	–	–	+	++	++	++	++	++	++
2	TnS-2	–	–	–	–	–	–	+	++	++	++	++	++	++
3	TnS-3	–	–	–	–	–	–	+	++	++	++	++	++	++
4	TxS-1	–	–	–	–	–	–	+	++	++	++	++	++	++
5	TxS-2	–	–	–	–	–	–	+	++	++	++	++	++	++
6	TxS-3	–	–	–	–	–	–	+	++	++	++	++	++	++

– Nil growth, + Moderate growth, ++ Heavy growth

When compared to the other temperatures, the best growth of all isolates was exhibited at 37 °C. The MIC of salt was determined for heavy metal resistant bacterial isolates from tannery and textile samples are shown in Tables 4 and 5. All the isolates exhibited high salt tolerance up to 20 % of NaCl. The high growths of all isolates were exhibited at 15 % of NaCl. One possible reason for the high salt tolerance is the use of large amounts of salts in the tanning industry.

Table 5 Sodium chloride tolerance in the tannery and textile isolates

Sl. No.	Salt concentrations (%)	Isolates	Growth behaviour	Total (%)	Result
1	50	No growth	–	0	–
2	45	No growth	–	0	–
3	40	No growth	–	0	–
4	35	No growth	–	0	–
5	30	No growth	–	0	–
6	25	No growth	–	0	–
7	20	TnS-1, TnS-2, TnS-3 TxS-1, TxS-2, TxS-3	+	100	Tolerance
8	15	TnS-1, TnS-2, TnS-3 TxS-1, TxS-2, TxS-3	++	100	Tolerance
9	10	TnS-1, TnS-2, TnS-3 TxS-1, TxS-2, TxS-3	++	100	Tolerance
10	7.5	TnS-1, TnS-2, TnS-3 TxS-1, TxS-2, TxS-3	++	100	Tolerance
11	5.0	TnS-1, TnS-2, TnS-3 TxS-1, TxS-2, TxS-3	++	100	Tolerance
12	2.5	TnS-1, TnS-2, TnS-3 TxS-1, TxS-2, TxS-3	++	100	Tolerance
13	1.25	TnS-1, TnS-2, TnS-3 TxS-1, TxS-2, TxS-3	++	100	Tolerance

– Nil growth, + Moderate growth, ++ Heavy growth

Heavy metals were supplemented in the nutrient agar plates in five concentrations (10, 100, 250, 500, 750 and 1,000 μg per ml) of heavy metals (potassium chromate, zinc chloride, mercuric sulphate, and magnesium sulphate and barium chloride). The isolates were resistant to 100–250 μg/ml of chromium and zinc. All the isolates except two isolates (TnS-3 and TxS-3) were sensitive to the mercuric sulphate. The two isolates (TnS-3 and TxS-3) were tolerated only with low level 10 μg/ml of mercuric sulphate. All the isolates showed resistance to high (1,000 μg/ml) concentration of magnesium and barium. The heavy metal tolerances of tannery and textile isolates are shown in Tables 6 and 7. The MIC of various heavy metals was determined for metal resistant bacteria (Tables 8 and 9). All the isolates exhibited high resistance to magnesium and barium and high sensitivity to mercury. The isolates were inhibited by chromium and zinc in the concentration range of 156–625 μg/ml. Heavy metals tolerance pattern and heavy metal resistant profile are shown in Tables 10 and 11.

Table 12 shows the result of the antibiogram. The heavy metal resistance isolates were resistant to ampicillin, kanamycin and tetracycline. TnS-1, TxS-1 and TxS-2 were intermediate resistant to chloramphenicol. TnS-2, TnS-3 and TxS-3 were resistant to chloramphenicol. The strain TxS-2 was sensitive to gentamycin. The antibiotic resistant pattern of the tannery and textile isolates are shown in Table 13. For the heavy metal and antibiotic resistance, plasmid curing agents (acridine orange, SDS, ethidium bromide) were used. From the results, it is evident that the concentration of more than 10 μg/ml of ethidium bromide destroyed the heavy metal resistance isolates. In fact the concentration of ethidium bromide more than 10 μg/ml was neglected for the further work.

After curing, there was no loss in heavy metal resistance in all the isolates. The results are shown in Table 14. However, the isolates became sensitive to some antibiotics. Plasmid cured derivatives appeared to be sensitive to antibiotics such as ampicillin (all strains), gentamycin (all strains), kanamycin (TnS-2 and TxS-2) and chloramphenicol (TnS-1, TxS-1 and TxS-2).

Table 6 Determination of heavy metals tolerance bacteria from tannery samples

Sl. No.	Concentrations of Heavy metals in media (μg/ml)	TnS-1					TnS-2					TnS-3				
		Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba
1	10	+	+	–	+	+	+	+	–	+	+	+	+	+	+	+
2	100	+	+	–	+	+	+	+	–	+	+	+	+	–	+	+
3	250	+	+	–	+	+	+	–	–	+	+	–	+	–	+	+
4	500	–	–	–	+	+	–	–	–	+	+	–	–	–	+	+
5	750	–	–	–	+	+	–	–	–	+	+	–	–	–	+	+
6	1,000	–	–	–	+	+	–	–	–	+	+	–	–	–	+	+

Mg magnesium, *Ba* barium, *Cr* chromium, *Zn* zinc, *Hg* mercury, – Nil growth, + growth

Table 7 Determination of heavy metals tolerance bacteria from textile samples

Sl. No.	Conc. of Heavy metals in media (μg/ml)	TxS-1					TxS-2					TxS-3				
		Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba
1	10	+	+	−	+	+	+	+	−	+	+	+	+	+	+	+
2	100	+	+	−	+	+	+	−	−	+	+	+	+	−	+	+
3	250	−	−	−	+	+	−	−	−	+	+	−	−	−	+	+
4	500	−	−	−	+	+	−	−	−	+	+	−	−	−	+	+
5	750	−	−	−	+	+	−	−	−	+	+	−	−	−	+	+
6	1,000	−	−	−	+	+	−	−	−	+	+	−	−	−	+	+

Mg magnesium, *Ba* barium, *Cr* chromium, *Zn* zinc, *Hg* mercury, − Nil growth, + growth

Table 8 MIC of different heavy metals against tannery isolates

Sl. No.	Conc. of heavy metal (mg/ml)	TnS-1					TnS-2					TnS-3				
		Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba
1	10	−	−	−	+	+	−	−	−	+	−	−	−	−	++	+
2	5	−	−	−	++	++	−	−	−	++	−	−	−	−	++	+
3	2.5	−	−	−	++	++	−	−	−	++	−	−	−	−	++	++
4	1.25	−	−	−	++	++	−	−	−	++	+	−	−	−	++	++
5	625	−	−	−	++	++	−	−	−	++	++	−	−	−	++	++
6	312	−	+	−	++	++	+	−	−	++	++	−	−	−	++	++
7	156	+	++	−	++	++	++	+	−	++	++	+	++	−	++	++
8	78	++	++	−	++	++	++	++	−	++	++	++	++	−	++	++
9	39	++	++	−	++	++	++	++	−	++	++	++	++	−	++	++
10	19.5	++	++	−	++	++	++	++	−	++	++	++	++	−	++	++
11	9.75	++	++	−	++	++	++	++	−	++	++	++	++	+	++	++
12	4.80	++	++	−	++	++	++	++	−	++	++	++	++	+	++	++

Mg magnesium, *Ba* barium, *Cr* chromium, *Zn* zinc, *Hg* mercury, − nil growth, + growth

Table 9 MIC of different heavy metals

Sl. No.	Concentration of heavy metal (mg/ml)	TxS-1					TxS-2					TxS-3				
		Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba	Cr	Zn	Hg	Mg	Ba
1	10	−	−	−	+	−	−	−	−	++	+	−	−	−	−	−
2	5	−	−	−	++	+	−	−	−	++	++	−	−	−	++	+
3	2.5	−	−	−	++	++	−	−	−	++	++	−	−	−	++	++
4	1.25	−	−	−	++	++	−	−	−	++	++	−	−	−	++	++
5	625	−	−	−	++	++	−	−	−	++	++	−	−	−	++	++
6	312	−	−	−	++	++	−	−	−	++	++	−	−	−	++	++
7	156	−	−	−	++	++	−	−	−	++	++	−	−	−	++	++
8	78	+	+	−	++	++	++	+	−	++	++	+	+	−	++	++
9	39	++	++	−	++	++	++	++	−	++	++	+	++	−	++	++
10	19.5	++	++	−	++	++	++	++	−	++	++	++	++	−	++	++
11	9.75	++	++	−	++	++	++	++	−	++	++	++	++	+	++	++
12	4.87	++	++	−	++	++	++	++	++	++	++	++	++	+	++	++

Mg magnesium, *Ba* barium, *Cr* chromium, *Zn* zinc, *Hg* mercury, − nil growth, + growth

Table 10 Heavy metals tolerance pattern of tannery and textile isolates

Sl. No.	Isolates	Concentration of heavy metals (μg/ml)											
		10,000	5,000	2,500	1,250	620	312	156	78.0	39.0	19.5	9.75	4.875
1	TnS-1	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba, Zn	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr
2	TnS-2	Mg	Mg	Mg	Mg, Ba	Mg, Ba	Mg, Ba, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr
3	TnS-3	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr, Hg	Mg, Ba, Zn, Cr, Hg
4	TxS-1	Mg	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr
5	TxS-2	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr, Hg
6	TxS-3	–	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr	Mg, Ba, Zn, Cr, Hg	Mg, Ba, Zn, Cr, Hg

Mg magnesium, *Ba* barium, *Cr* chromium, *Zn* zinc, *Hg* mercury

Table 11 Heavy metal resistance profile of tannery and textile isolates

Sl. No.	Isolates	Concentrations of heavy metals (μg/ml)				
		Cr	Zn	Hg	Mg	Ba
1	TnS-1	156	312	–	10,000	10,000
2	TnS-2	312	156	–	10,000	1,250
3	TnS-3	156	156	9.75	10,000	10,000
4	TxS-1	78	78	–	10,000	5,000
5	TxS-2	78	78	4.87	10,000	10,000
6	TxS-3	78	78	9.75	5,000	5,000

Mg magnesium, *Ba* barium, *Cr* chromium, *Zn* zinc, *Hg* mercury

Table 12 Antibiotic susceptibility test for tannery and textile isolates

Sl. No.	Isolates	Antibiotics				
		A	C	K	G	T
1	TnS-1	R (Nil)	I (13 mm)	R (11 mm)	R (12 mm)	R (11 mm)
2	TnS-2	R (8 mm)	R (Nil)	R (9 mm)	R (11 mm)	R (11 mm)
3	TnS-3	R (Nil)	R (Nil)	R (10.5 mm)	R (11 mm)	R (7.5 mm)
4	TxS-1	R (Nil)	I (17.5 mm)	R (9 mm)	R (10 mm)	R (12 mm)
5	TxS-2	R (10 mm)	I (17 mm)	R (12 mm)	S (15 mm)	R (12 mm)
6	TxS-3	R (11.5 mm)	R (Nil)	R (15 mm)	R (Nil)	R (12.5 mm)

A Ampicillin, *C* Chloramphenicol, *K* Kanamycin, *G* Gentamycin, *T* Tetracycline, *R* Resistance, *I* Intermediate, *S* Susceptible

Table 13 Antibiotic resistance pattern of tannery and textile isolates

Sl. No.	Isolates	Antibiotics profile
1	TnS-1	A^r, C^i, K^r, G^r, T^r
2	TnS-2	A^r, C^r, K^r, G^r, T^r
3	TnS-3	A^r, C^r, K^r, G^r, T^r
4	TxS-1	A^r, C^i, K^r, G^r, T^r
5	TxS-2	A^r, C^i, K^r, G^s, T^r
6	TxS-3	A^r, C^r, K^r, G^r, T^r

A Ampicillin, *C* Chloramphenicol, *K* Kanamycin, *G* Gentamycin, *T* Tetracycline, *R* Resistance, *I* Intermediate, *S* Susceptible

There was no sensitive strain for the tetracycline antibiotic. Table 16 shows that the resistant isolates became sensitive. This means that the resistance was carried out by plasmid. Table 16 shows the comparison of metal and antibiotic resistance pattern of chromate resistant parental strains and their plasmid cured derivatives. From the plasmid cured derivatives, the colony forming unit was observed, which is shown in Table 17. Loss of heavy metal resistance occurred at low frequency (0–20 %). Acridine orange did not cure the heavy metal resistant plasmid in the concentration of 20 μg/ml and plasmid was cured at the low frequency rate (0–20 %) at the concentration of acridine orange of 25 and 30 μg/ml. SDS showed 0–10 % plasmid curing frequency at a concentration of 10 %. Ethidium bromide showed 10–20 % curing frequency at a concentration of 10 μg/ml (Table 15).

Discussion

Tanneries are one of the major industries that are blamed for environmental pollution (Khan and Khan 1998; Mir and Hai 1999). The most important component of pollutants from tanneries is Cr and Khalil et al. (1991) report that the effluents only from Kasul Pakistan, includes about 300 kg day^{-1} of Cr which leads to the deterioration of aquifers and irrigated land. Hence it is imperative to extract detoxify Cr before the discharge of effluents. Bacterial interaction with metals offer an alternative candidate for detoxification of heavy metals and

Table 14 Presence of bacterial growth after plasmid curing in heavy metal enriched media

Sl. No.	Isolates	Concentration of plasmid curing agents						
		Ethidium Bromide (μg/ml)	SDS (%)	Acridine orange (μg/ml)				
		10	25	50	10	20	25	30
Medium- 1								
1	TnS-1	+	−	−	+	+	+	+
2.	TnS-2	+	+	−	+	+	+	+
3.	TnS-3	+	+	−	+	+	+	+
4.	TxS-1	+	+	+	+	+	+	+
5.	TxS-2	+	+	+	+	+	+	+
6.	TxS-3	+	+	+	+	+	+	+
Medium-2								
1.	TnS-1	+	−	−	+	+	+	+
2.	TnS-2	+	+	−	+	+	+	+
3.	TnS-3	+	+	−	+	+	+	+
4.	TxS-1	+	+	+	+	+	+	+
5.	TxS-2	+	+	+	+	+	+	+
6.	TxS-3	+	+	+	+	+	+	+
Medium-3								
1.	TnS-1	+	−	−	+	+	+	+
2.	TnS-2	+	+	−	+	+	+	+
3.	TnS-3	+	+	−	+	+	+	+
4.	TxS-1	+	+	+	+	+	+	+
5.	TxS-2	+	+	+	+	+	+	+
6.	TxS-3	+	+	+	+	+	+	+

Medium 1 Nutrient agar + Heavy metal (potassium chromate-80 μg/ml), *Medium 2* Nutrient agar + Heavy metals (Potassium chromate −80 μg/ml, magnesium sulphate −5 mg/ml and zinc chloride), *Medium 3* Nutrient agar, − Nil growth, + Moderate growth

Table 15 Antibiotic susceptibility test for plasmid cured derivatives

Sl. No.	Isolates	Antibiotics				
		A	C	K	G	T
1.	TnS-1	S (17.5 mm)	S (18.5 mm)	R (11 mm)	S (15 mm)	R (11 mm)
2.	TnS-2	I (15 mm)	R (nil)	I (16.5 mm)	S (16 mm)	R (11 mm)
3.	TnS-3	S (17 mm)	R (nil)	R (10.5 mm)	S (17 mm)	R (7.5 mm)
4.	TxS-1	I (16 mm)	S (19 mm)	R (9 mm)	S (16 mm)	R (12 mm)
5.	TxS-2	I (16.5 mm)	S (19.5 mm)	S (18 mm)	S (15.5 mm)	R (12 mm)
6.	TxS-3	S (18.5 mm)	R (nil)	R (15 mm)	S (16.5 mm)	R (12.5 mm)

A Ampicillin, *C* Chloramphenicol, *K* Kanamycin, *G* Gentamycin, *Te* Tetracycline, *R* Resistance, *I* Intermediate, *S* Susceptible

metal resistant bacteria that can be utilised for this purpose. Basu et al. (1997) reported that the bacterial population (CFU) in three different sites did not show any significant variation and was of the order of 2.15–2.77 $\times$ 10^6 cfu/g of sediment.

The bacterial count on PYE agar containing Cr^{6+} decreased with increasing concentrations of Cr. This might have been due to the inability of sensitive organisms to grow on Cr supplemented plates. Bacterial population of the Cr

Table 16 Comparison of metal and antibiotic resistance pattern of chromate resistant parental strains and their cured derivatives

Sl. No	Isolates	Resistance of parental strains	Resistance of cured derivatives	Resistances lost with curing
1	TnS-1	Cr, Zn, Mg, Ba, A, C, K, G, T	Cr, Zn, Mg, Ba, K, T	A, C, G
2	TnS-2	Cr, Zn, Mg, Ba, A, C, K, G, T	Cr, Zn, Mg, Ba, C, T	A, K, G
3	TnS-3	Cr, Zn, Mg, Ba, A, C, K, G, T	Cr, Zn, Mg, Ba, C, K, T	A, G
4	TxS-1	Cr, Zn, Mg, Ba, A, C, K, G, T	Cr, Zn, Mg, Ba, K, T	A, C, G
5	TxS-2	Cr, Zn, Mg, Ba, A, C, K, G, T	Cr, Zn, Mg, Ba, T	A, C, K, G
6	TxS-3	Cr, Zn, Mg, Ba, A, C, K, G, T	Cr, Zn, Mg, Ba, C, K, T	A, G

Cr chromium, *Zn* zinc, *Mg* magnesium, *Ba* barium, *A* Ampicillin, *C* chloramphenicol, *G* Gentamycin, *K* Kanamycin, *T* Tetracycline

contaminated sediments were also reported by Luli et al. (1983) and Losi and Frankenberger (1994). This study reports the variation observed in nutrient agar with single and multiheavy metals. Kamalakannan and Lee (2008) reported that heavy metal resistant organisms were isolated from the Sunchon Bay sediments. All the isolates were identified as *Bacillus* sp, which is similar to the results of the previous work reporting the presence of *Bacillus* sp in metal-contaminated environments (Kamalakannan et al. 2007). These results are also supported by Sultan and Hasnain (2000), Nair and Krishnamoorthi (1991). Similarly, in this study, tannery and textile isolates were identified based on Gram stain, morphology, biochemical tests and selective media. The isolates were identified as *Bacillus* sp., *Staphylococcus* sp. and *Pseudomonas* sp. Sultan and Hasnain (2000) have reported that temperature is an important physical factor that affects microbial cells by influencing the rates of biochemical reactions and enzyme synthesis (Chaloupka 1985) and at extreme temperature, the process becomes more rate limiting (Patterson and Gillespie 1972). The optimum temperature for growth of these isolates was 37 °C both in absence and presence of chromate (10 μg ml^{-1}) in the medium.

Some bacteria have shown the best growth at 28 °C. The growth was seriously hampered by high temperature especially in the presence of chromate salt. An increased temperature causes changes in membrane composition (Benschoter and Ingram 1986), imbalance between synthesis and degradation of cellular proteins and ultimately cell death (Strnadova et al. 1991). Adverse effects of high temperature appear to have become aggravated in the presence of chromate. This is correlated to this work. In this study, the heavy metal bacterial isolates were grown in the range of temperatures (28–45 °C). The *Bacillus* sp (TnS-1) was found to be tolerating temperature up to 55 °C.

The bacterial isolates were tolerated in the range of pH 5–10. Similarly, Sultan and Hasnain (2000) reported that the pH is another important environmental factor, which controls the growth of micro-organisms. The Cr-resistant isolates were able to grow over a wide pH range of 5–9, but in the presence of chromate salt they exhibited poor growth at acidic pH levels. According to Francis (1990), pH affects the ionic state of metal that in turn affects the microbial growth. Solubility and availability of metallic salt is more at acidic pH which results in poor bacterial growth at acidic pH. All the isolates showed confluent growth up to a salt concentration of 2 %. In this study, the isolates tolerated a 20 % NaCl concentration. The isolates showed high salt tolerance as compared to the isolates reported by Tippannaver et al. (1989). One possible reason for the high salt tolerance is the use of large amounts of salt in the tanning industry.

Several reasons could be given for this: first, the difference might be due to degrees of polymetallic pollution. Second, the type of organic constituents and presence of negatively charged ions like chloride in the medium may bind with the metal and alters the bioavailability and toxicity of metals resulting in differences in MIC of

Table 17 Elimination of heavy metals resistance factors of tannery and textile isolates by treatment with various plasmid curing agents in various concentrations

Sl. No.	Isolates	Inoculum size (cells/ml)	Concentration of plasmid curing agent (per ml)	Time of incubation (hrs)	Viable count (cells/ml)	No. of colonies showing heavy metal susceptibility/ no of colonies tested	Frequency of heavy metal susceptible colonies (%)
1.	TnS-1	2.8×10^3	A.o 20 µg	24	5.6×10^6	0/20	0
			A.o 25 µg	24	1.4×10^6	1/20	5
			A.o 30 µg	24	2.3×10^4	3/20	15
			SDS 10 %	24	8×10^5	0/20	0
			E.b 10 µg	24	6.3×10^5	4/20	20
2.	TnS-2	3.2×10^3	A.o 20 µg	24	5.2×10^6	0/20	0
			A.o 25 µg	24	2.4×10^6	0/20	0
			A.o 30 µg	24	4.8×10^4	4/20	20
			SDS 10 %	24	6.8×10^6	2/20	10
			E.b 10 µg	24	2.1×10^6	4/20	20
3.	TnS-3	3.1×10^3	A.o 20 µg	24	2.4×10^7	0/20	0
			A.o 25 µg	24	3.8×10^6	3/20	15
			A.o 30 µg	24	8.7×10^5	4/20	20
			SDS 10 %	24	6.7×10^6	1/20	5
			E.b10 µg	24	3.2×10^6	2/20	10
4.	TxS-1	3.4×10^3	A.o 20 µg	24	4.8×10^6	0/20	0
			A.o 25 µg	24	2.3×10^6	3/20	15
			A.o 30 µg	24	7.8×10^5	4/20	20
			SDS 10 %	24	2.3×10^5	1/20	5
			E.b 10 µg	24	3.8×10^6	2/20	10
5.	TxS-2	2.9×10^3	A.o 20 µg	24	2.40×10^6	0/20	0
			A.o 25 µg	24	5.6×10^5	0/20	0
			A.o 30 µg	24	8.7×10^4	2/20	10
			SDS10 %	24	6.7×10^6	0/20	0
			E.b 10 µg	24	3.2×10^6	4/20	20
6.	TxS-3	3.6×10^3	A.o 20 µg	24	4.13×10^6	0/20	0
			A.o 25 µg	24	3.8×10^5	0/20	0
			A.o 30 µg	24	6.8×10^4	3/20	15
			SDS 10 %	24	7.4×10^6	2/20	10
			E.b 10 µg	24	3.2×10^7	4/20	20

A.o Acridine orange, *SDS* Sodium dodecyl sulphate, *Eb* Ethidium bromide

metal (Bezverbnaya et al. 2005). Sultan and Hasnain (2005) have shown that four bacterial strains exhibiting a very high level of resistance to chromate, i.e. 40 mg/ml of potassium chromate in nutrient agar, were isolated from the effluents of Shafiq Tannery, Kasur, Pakistan. Several reports indicate that the resistance level was much higher than for the strains (Mckean et al. 2000; Cheung and Gu 2003; Pal and Paul 2004 and Comargo et al. 2005). The chromium resistance level of the tannery and textile isolates as per the present study is lower than the previous report.

Aoukaty and Appana (1990) reported that the decreased growth of these strains at higher concentration of chromate might be attributed to

increase in generation time and decrease in cell division/cellular multiplication. Nair and Krishnamoorthi (1991) have demonstrated in *P. aeruginosa* a decrease in protein DNA, RNA, sugar and lipid contents, at higher concentration of Cr. Sultan and Hasnain (2000) have screened the strain's resistance to the salts of other metals. The Cr-resistant strains exhibited resistance to 100 μg ml^{-1} each of Ba, Fe, Co, Mn, Ni, Pb and Zn. The resistance level of their strains is much lower than the strains in this study, but all the strains were sensitive to Hg. Faisal and Hasnain (2000) have also reported that all strains showed very high level resistance against potassium chromate both in nutrient broth (up to 25 mg ml^{-1}) and on nutrient agar (40 mg ml^{-1}). Basu et al. (1997) have also reported that chromium resistant bacterial isolates from effluent of tanneries could resist up to 250 μg ml^{-1} of Cr in the medium.

The chromium resistant bacteria isolated from polluted soil could resist up to 100 μg ml^{-1} of Cr(VI) (Megharaj et al. 2003). Faisal and Hasnain (2004) have reported that the chromium strains have a broad range of heavy metals (Mn, Ni, Zn, Pb, Cu, and Co) and antibiotics resistance. Pleiotrophic metal resistances may be due to common mechanisms required for tolerance and resistance to these metals, a regulatory factor or common operon. Schneider and Schwers Furth (1991) have also reported that the occurrence of pleiotrophic metal resistances in bacteria were isolated from polluted water. The heavy metal resistant strains were checked for resistances against various antibiotics (ampicillin, chloramphenicol, kanamycin, gentamycin and tetracycline). The increased usage of antibiotic and disinfectants in health care, heavy metals in industries creates the selective pressure for survival of bacteria in a contaminated environment. Thus, in a multiple stressed environment, bacterial cells acquire these resistances by a change in the genetic makeup, either by mutation or by transfer of resistant genes between bacteria. Many investigators have also reported a similar result regarding the association between heavy metal and antibiotic resistance (Hasnain and Sabri 1992; Lawrence 2000; Verma et al. 2001).

Aeromonas isolates recovered by Miranda and Castillo (1998) from different polluted water sources exhibited their resistance to many antibiotics. They concluded that highly polluted water had shown higher antibiotic multiresistances than moderately polluted water. Numerous studies have reported that bacterial flora present in the stressed environmental condition may carry plasmids not only in terms of the frequency but also in size (Baya et al. 1986). Tanaka et al. (1977) have reported the presence of large plasmids in *Bacillus* species. In order to check the heavy metals and antibiotic resistance mediated by plasmids, plasmid curing was performed for all the isolates. After the curing, the isolates showed that resistance to all the heavy metals tested. However, results showed that the plasmid did not mediate the heavy metal resistance. Plasmid and Chromosome encoded and chromosomally controlled chromium resistances have been described by Cervantes and Silver (1992). For antibiotics, heavy metal resistant bacterial strains became sensitive to ampicillin, chloramphenicol, kanamycin and gentamycin, whereas it indicates the presence of resistance genes. All the isolates exhibited resistance to tetracycline and the possibility of resistance gene in chromosomal DNA. Plasmid-encoded multiple metal and antibiotic resistances have been reported by other workers (Nies and Silver 1989; Alonso et al. 2000; Mahapatra et al. 2002). These results clearly show that these heavy metal resistant strains have great potential for heavy metal detoxification and this can be utilised for developing a bioremediation process for contaminated environments.

Conclusion

Heavy metals include some toxic chemical elements and their deviated chemical compounds. Most of these compounds are not functionally involved in any activity for life. Some of these heavy metals are necessary for life, namely copper, iron and zinc. The other heavy metals polluting the environments, which are discharged from industries tannery and textile

manufacturing and paper industry, elevated its concentration in aquifers and groundwater.

Heavy metal resistant bacterial isolates were identified as *Bacillus* sp, *Pseudomonas* sp and *Staphylococcus* sp. The isolates checked for antibiotic resistances exhibited resistance. Further plasmid curing agents were used to cure a resistant plasmid; these indicated clearly the various tolerance and antibiotic resistance mediated by plasmids. It is concluded that, heavy metal resistant bacteria may be used for bioremediation of metal-contaminated environments, and these micro-organisms can be used, or their enzymes, to return the natural environment altered by contaminants to its original condition.

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Characterization of Groundnut Rhizosphere *Pseudomonas* sp. VSMKU 2013 for Control of Phytopathogens

R. Charulatha, H. Harikrishnan, P. T. Manoharan, and V. Shanmugaiah

Abstract

A total of 140 Fluorescent *pseudomonads* spp. were isolated from rhizosphere soil of groundnut. All isolates were evaluated in vitro for selection of potential antagonistic fluorescent pseudomonads (FPs) against fungal pathogens. Among 140 FPs, 82 FPs isolates showed antagonistic activity against *Macrophomina phaseolina* for primary screening with zone of inhibition range 0.9–2.9 cm. For secondary screening of other fungal pathogens such as *Rhizoctonia solani, Fusarium oxysporum, Fusarium udum*, and *Alternaria alternata* the zone of inhibition range was 0.7–1.9 cm, 0.7–2.9 cm, 0.9–2.9 cm, and 0.2–3.0 cm. All antagonistic FPs were screened for the production of indole acetic acid (59/82), hydrogen cyanide (61/82), siderophore (40/82), phosphate solubilization (66/82), hydrolytic enzymes such as protease (51/82), amylase (4/82), gelatinase (51/82), cellulase (48/82), and pectinase (3/82). Among 82 antagonistic bacteria, the strain designates as VSMKU-2013 were selected for further studies based on the antagonistic potential. The strain VSMKU-2013 was identified as *Pseudomonas* sp. based on the biochemical and morphological characters. The germination, growth promotion of groundnut seeds, and identification of IAA genes are in progress.

Keywords

Pseudomonas · Groundnut rhizosphere · IAA · Plant pathogens

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Introduction

Fluorescent pseudomonads (FPs) are colonizing plant roots and compete with native soil microflora better than other inoculated strains. In recent years, substantial awareness has been compensated to plant growth promoting rhizobacteria (PGPR). Generally FPs are aggressive

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root colonizers and play an important role in the biological control of plant diseases caused by soil-borne fungal pathogens. The mechanism of disease reduction may involve antagonism by the production of siderophore-mediated competition, which results in the elimination of fungal pathogens in the rhizosphere by a reduction in the availability of iron for the survival of pathogens (Schippers et al. 1987).

In recent years, other mechanisms based on the production of antibiotics, hydrogen cyanide, and IAA have been concerned in the reduction of plant pathogenic fungi and adverse rhizobacteria by PGPR (Dowling and O'Gara 1994; Shanmugaiah 2007; Shanmugaiah et al. 2010). FPs protect peanut and rice seedlings from *Rhizoctonia solani* and *Sclerotium rolfsii* (Dube and Podile 1989; Shanmugaiah et al. 2010). It is well known that FPs have been known to excrete hydrolytic enzymes such as chitinase, protease, amylase, and β-1, 3-glucanases to digest the fungal cell wall chitin, glucan, and other substrates, respectively, and use these as carbon and energy sources (Leah et al. 1991) and are also reported to produce a wide range of antifungal metabolites (Shanmugaiah et al. 2005; Haas and Défago 2005; Ajit et al. 2006). These attributes make FPs candidates for biocontrol agents (Garbeva et al. 2004).

Materials and Methods

Collection of Rhizosphere Soil Samples

Samples were randomly collected from groundnut rhizosphere in Madurai and Dindigul districts. One whole groundnut plant, after chopping off the shoots, was carefully uprooted (along with the adhering soil, without breaking the secondary and tertiary roots), placed in a polythene bag, labeled, and tied. Then the polythene bag was transported to the lab where the roots were shaken to dislodge and separate loosely adhering soil aggregates around primary, secondary, and tertiary roots, and the adhering soils were collected and stored in a refrigerator at 8 °C for further studies.

Isolation of Bacterial Strains

Ten grams of soil from each sample was separately suspended in 90 ml of distilled water in a flask and placed on an orbital shaker (at 100 rpm) at room temperature (28 ± 2 °C) for 30 min. At the end of shaking, the soil samples were serially diluted up to 10^{-10}. Diluted suspensions were plated on Kings B agar by spread plate technique and placed at 28 °C for 48 h. At the end of the incubation, the plates were visualized under UV Illuminator at 365 nm for production of fluorescent pigment.

Fluorescence Production

Bacteria were streaked with King's B (King et al. 1954) agar and incubated at 28 ± 2 °C for 48 h. At the end of the incubation, the plates were observed under UV light for production of fluorescence. Distinct single colonies of fluorescent bacteria were picked, subcultured to purity, and maintained in KBA slants and also in 40 % glycerol vials.

Antifungal Activity

Totally 140 bacterial isolates were evaluated for primary screening against *M. phaseolina* by dual culture assay. For this, a fungal disk of 6 mm diameter was placed on one edge of the PDA plate (1 cm from the corner) and bacterial isolate was streaked on the other edge of the plate (1 cm from the corner), followed by incubation at 28 ± 2 °C for 96 h or till the pathogen covered the entire plate in control. Inhibition of fungal mycelium (halo zone) around the bacterial colony was scored positive and inhibition zone measured. Antagonistic strains 82 were subjected to secondary screening against other fungal pathogens such as *R. solani, F. udum, A. alternata, F. oxysporum* and were tested by the above said method. Among the 82 strains VSMKU-2013 had shown prominent antagonistic activity compare to other FPs and control. Zone of clearance (ZOC) was measured using the following formula:

$$\text{ZOC} = [(C - T)/C]100$$

where C = radial growth of fungus in control and T = radial growth of fungus in dual culture.

Fungal hyphae surrounding zone of inhibition and from control plates were observed under the microscope by standard procedure.

IAA Production

It was done as per the protocols of Patten and Glick (1996). The bacteria were grown in Luria broth supplemented with L-tryptophan (1 μg ml^{-1}) for 72 h. At the end of the incubation, cultures were centrifuged at 10,000 g for 10 min and the supernatants collected. One ml of this culture filtrate was allowed to react with 2 ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$) at 28 ± 2 °C for 30 min. At the end of the incubation, pink color was developed, which indicates the presence of IAA. Quantification of IAA was done by measuring the absorbance in a spectrophotometer at 530 nm.

HCN Production

HCN was estimated qualitatively by Lorck (1948). The bacteria were grown in Kings B agar amended with glycine (4.4 g/L). One sheet of Whatman filter paper (8 cm diameter) was soaked in 1 % picric acid (10 % sodium carbonate; filter paper, and picric acid were sterilized separately) for a minute and struck underneath the Petri dish lids. The plates were sealed with Parafilm and incubated at 28 ± 2 °C for 48 h. Development of reddish brown color on the filter paper indicated positive reaction for HCN production.

Phosphate Solubilization

All the isolates were screened for their phosphate-solubilizing ability on Pikovskaya agar (Pikovskaya 1948) which contains (g/L): 0.5 g yeast extract, 10 g dextrose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4$. $7H_2O$, 0.0001 g $MnSO_4.H_2O$, 0.0001 g $FeSO_4$. $7H_2O$, and 15 g agar. All antagonistic FPs were streaked on Pikovskaya agar and incubated for 72 h at 28 ± 2 °C.

Hydrolytic Enzyme Production

Production of protease was determined using skim milk agar on the basis of proteolytic activity. Cellulase, gelatinase, amylase and pectinase were determined on nutrient agar medium amended with appropriate substrates. After 24 h, cellulose production was observed by flooding 0.5 % Congo red solution over the cellulose agar plates. Similarly, Gram's iodine was flooded over the starch agar plate and tested for amylase activity. Mercuric chloride was flooded over the gelatin and casein agar plates to check the zone of clearance for protease (Shanmugaiah et al. 2008).

Identification of Selected Strain

Effective fluorescent pseudomonad designated as VSMKU 2013 was selected for further studies due to its broad spectral antagonistic performance. Different physiological tests suggested in Bergey's Manual of Determinative Bacteriology (Williams 1994) were carried out to identify the isolate as VSMKU 2013.

Results

Isolation and Screening of Antagonistic Fluorescent Pseudomonads

In this study, a total of 140 FPs was isolated from groundnut rhizosphere soils and screened for antifungal activity against *M. phaseolina.* Among them, 82 FPs showed inhibitory activity against *M. phaseolina.* The zone of inhibition of mycelial growth of the above fungal pathogens ranged from 0.9 to 2.9 cm. Further secondary screening of 82 isolates highlights fungal pathogens such as *R. solani, F. oxysporum,*

Table 1 Antagonistic activity of groundnut rhizosphere FPs against fungal pathogen

Fungal pathogens	Number of antagonistic FPs	Zone of inhibition (cm)
Macrophomina phaseolina	82	0.9–2.9
Rhizoctonia solani	48	0.7–1.9
Fusarium oxysporum	67	0.7–2.9
Fusarium udum	59	0.9–2.9
Alternaria alternata	74	0.2–3.0

F. udum, and *A. alternata.* The zone of inhibition of mycelial growth of the above fungal pathogens ranged from 0.7 to 1.9 cm, 0.7 to 2.9 cm, 0.9 to 2.9 cm, and 0.2 to 3.0 cm (Table 1, Fig. 1).

Production of Lytic Enzymes

Productions of fungal cell wall degrading enzymes were analyzed, because lytic enzymes are an important mechanism for fungal inhibition. Among 82 FPs, protease, gelatinase, and cellulose production was more than other enzymes such as amylase and pectinase (Table 3).

Production of Phosphate Solubilization, HCN, and Siderophore

Among the 82 FPs, 66 exhibited the production of phosphate solubilization, whereas hydrogen cyanide and siderophore production obtained 61 and 40 FPs compared to other isolates of FPs (Table 3).

Production of IAA

Out of 82 FPs, 59 showed the production of IAA compared to other isolates (Table 3).

Identification of VSMKU 2013

The broad spectral antifungal potential of isolates, isolate VSMKU-2013, was identified as *Pseudomonas* sp. based on morphological and physiological tests (Table 3). The bacterial antagonist VSMKU 2013 was Gram-negative, rod shaped, and produced yellowish green pigment on King's B medium. It also showed gelatin liquefaction, arginine dihydrolase, oxidase, catalase, citrate utilization, protease, and sucrose-positive and hence the strain VSMKU 2013 was identified as *Pseudomonas* sp.

Discussion

Plant pathologists are facing major challenges for the management of soil-borne plant pathogens. The management of plant pathogens with pesticides has resulted in environmental pollution and resistance among pathogens. In this studyof a total of 140 FPs of groundnut rhizosphere, 82 have shown antifungal activity against. *M. phaseolina.* Owing to the antagonistic activity of 82 FPs, another test for other fungal pathogens such as *R. solani, F. oxysporum*, and *A. alternata* in dual plate assay, as evident from the zone of inhibition ranges 0.7–1.9 cm, 0.7–2.9 cm, 0.9–2.9 cm, and 0.2–3.0 cm, was observed (Table 1 and Fig. 1). FPs are extensively used for the control of charcoal and root rot of groundnut and rice sheath blight and at the same time they can enhance plant growth and yield apart from suppressing the growth of pathogens (Rabindran and Vidhyasekaran 1996; Vidhyasekaran and Muthamilan 1999).

Furthermore, this study provides new evidence for a close association of gluconic acid metabolism with antagonistic activity against plant pathogens. In the processes of plant growth, phytohormones, e.g., production of IAA, ethylene, cytokines, and gibberellins, hormones can be synthesized by the plant themselves and also by their associated microorganisms. Furthermore, plant-associated bacteria can influence the hormonal balance of the plant.

The production of lytic enzymes, secondary metabolites, and plant growth hormones is a common phenomenon among FPs such as protease, amylase, cellulase, gelatinase, pectinase,

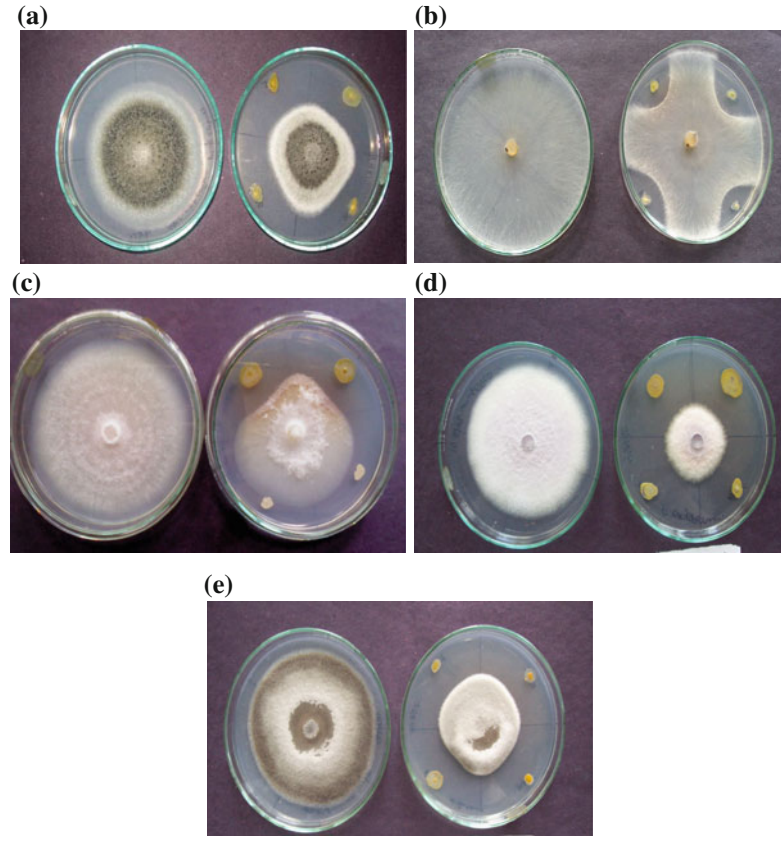

Fig. 1 Screening of fluorescent pseudomonads against fungal pathogens **a** *M. phaseolina* + FPs, **b** *R. solani* + FPs, **c** *F. udum* + FPs, **d** *F. oxysporum* + FPs, **e** *A. alternate* + FPs

phosphate solubilization, hydrogen cyanide, siderophore, and IAA (Table 2). Earlier studies have reported that the level of IAA in vitro vary from 3.6 to 11.8 ppm in case of FPs of pea rhizosphere and 2.0–21.6 ppm in rice rhizospheres (Thakuria et al. 2004). Similarly, in previous research, range of soluble P was found to vary from 1.38 to 15.7 ppm in culture filtrates due to inoculation with FPs from different crop rhizosphere. Parasitism is one of the major mechanisms involved in the biological control of plant pathogens by *P. fluorescens*. Several cell wall degrading enzymes such as chitinase and β-1, 3-glucanase are involved in this process. Biocontrol agents are *Serratia marcescens* (Lee et al. 1992), *P. cepacia* (Fridlender et al. 1993), *P. stutzeri* (Lim et al. 1991), *P. fluorescens* (Velazhahan et al. 1999; Meena et al. 2000), and *P. aeruginosa* MML2212 (Shanmugaiah 2007; Shanmugaiah et al. 2010). The ability of antagonistic bacteria to produce proteolytic enzyme, chitinase and cellulase was at a high percentage and the composition of antagonistic mechanisms was specific for each isolate. Some of the tested isolates could exhibit more than plant trails, which may promote plant growth directly or indirectly or synergistically.

Phosphate solubilizing microorganisms convert insoluble phosphates into soluble forms through the process of acidification, chelation, exchange reactions and production of gluconic acid reported that free-living P solubilizing bacteria release phosphate irons from sparing soluble inorganic and organic P compounds in soil and thereby contribute to an increased soil phosphate pool available for the plants. Significant relation between the antagonistic potential of FPs strains and their level of production of lytic enzymes, secondary metabolites, and IAA was observed.

The selected strain was identified as *Pseudomonas* sp. based on the physiological and morphological tests (Table 3). The selected strain

Table 2 Lytic enzymes, secondary metabolites and IAA production by groundnut rhizosphere FPs

Lytic enzymes, secondary metabolites and IAA	Number of strains positive
Protease	51
Cellulase	48
Gelatinase	51
Amylase	04
Pectinase	03
Phosphate solubilization	66
HCN	61
Siderophore	40
IAA	59

Table 3 Biochemical characterization of VSMKU 2013

Test	Result
Gram staining	Gram negative, rod shape
Motility	Motile
Oxidase	+
Catalase	+
Citrate utilization	+
Indole production	–
Nitrate reduction	–
Gelatinliquification	+
Arginine dihydrolase	+
Glucose	+
Arabinose	–
Sucrose	+
Chitinase	–
Cellulase	–
Protease	+
Amylase	–
Pectinase	–
Strain	*Pseudomonas* sp.

has broad spectral antagonistic performance with more antagonistic activity. This study has indicated that, further study needs to be warranted for investigation of the exact mechanism involved in controlling plant pathogens.

Conclusion

In this study is explored the diverse antagonistic potential, fungal cell wall degrading enzymes, volatile substance, secondary metabolites, and phytohormones production obtained from FPs. Further need to study the purification and characterization of secondary metabolites from FPs is warranted.

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Dynamics of AM Fungi Association and Spore Density in Black Pepper (*Piper nigrum* L.)

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Abstract

The study on the relations between environmental factors and AM fungal variation was carried out in the rhizosphere of *P. nigrum.* The root colonization of AM fungi was observed from 21.10 to 88.33 %. It was recorded as maximum during September 2001 and minimum during March 2000. The total AMF spore number varied from 12.00 to 90.30 g^{-1} soil in *P. nigrum*. The maximum and minimum spore numbers were recorded during the study periods of March and August respectively. AMF spores belonging to five species were recorded and identified as *Acaulospora* sp., *Gigaspora* sp., *Glomus aggregatum*, *G. fasciculatum*, and *G. geosporum*. Temperature was significantly and positively correlated to EC but negatively to RH. Likewise, rainfall was significantly correlated to soil nitrogen, %RLC, %RLH, and %RLV. Rainfall had significantly negative correlation with AM fungal spore density. Soil nitrogen was observed to have a significantly positive correlation with %RLA and %RLV. Spore population had significantly negative correlation to %RLC, %RLH, and %RLA.

Keywords

Piper nigrum · *Glomus fasciculatum* · *Acaulospora* sp. · *Gigaspora* sp. · *Glomus aggregatum* · *G. geosporum*

Introduction

Organic matter plays a vital role in the productivity of soil quality. Soil is a substrate for the growth and multiplication of microbes. It serves as a source of food for microbes, which are responsible for converting complex organic materials into simple substances readily used by plants. The increased microbial activity in the

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rhizosphere leads to competition between the component species for nutrient and space, and these may affect the plants either adversely or favorably depending on the type of microbes inhabiting the rhizosphere, which may either directly or indirectly influence the growth of the plants. Roots of the plants are known to be intimately associated with mycorrhizal fungi.

The mycorrhizal fungi are important in sustainable agriculture due to their role in plant and soil nutrition acting as agent in transporting mineral nutrients to the plant and C compounds to the soil and its biota (Reide 1990). These fungi are known to occur in all soil types and to enhance the uptake of diffusion-limited nutrient such as phosphorus (P) in soil with low availability in spite of their high P retention capacity. Arbuscular mycorrhiza (AM) fungi are not host specific. They differ in their ability to enhance nutrient uptake and plant growth. Differences in host plants and soil fertility stimulate differential sporulation by AM fungi species in the field (Hayman 1975) and spore production is seasonal in several habitats (Brundrett 1991). However, studies on distribution of AM fungi, the difference in edaphic and climatic factors, and host plants have been so great that it is difficult to explain what factors determine the presence and absence of a particular species (Weller et al. 1988).

The purpose of this study is to find the influence of edaphic and climatic factors on the degree of AM fungal association in the host of black pepper; and to identify the occurrence and dominance of microbial spores, particularly by AM fungi present in the soil at a particular time.

Materials and Methods

Study Area

A survey was carried out to assess the seasonality of AMF in 15-year-old plantations of black pepper (*Piper nigrum)* in the Western Ghats ecosystem, Southern India. The root and rhizosphere soil samples were collected at planters' field, Thandikudi located in lower Pulney hills in Kodaikanal, Tamil Nadu at an elevation of 1,400 m a.s.l.

Climatic Data

Climatological data [minimum and maximum temperature, relative humidity (RH%), and rainfall] were obtained from Regional Coffee Research Station, Thandikudi, Kodaikanal, Tamil Nadu, India.

Sampling

Root and rhizosphere (up to 30 cm soil depth) soil samples were collected from pepper plantations. The samples were collected at monthly intervals for a period of 12 months from November 2000 to October 2001. During each sampling five individuals were selected. The roots were dug out, washed free of soil, and fixed in formalin-acetic acid-alcohol. The rhizosphere soils (1 kg) from the individuals were mixed to form a composite soil sample; air-dried, packed in polythene bags, and kept at room temperature until further analysis.

pH

10 gram of dry soil was taken in a beaker and 100 ml of water added to make a suspension of 1:10 (w/v) dilution and the pH was determined with a digital pH meter.

Electrical Conductivity

10 gram of dry soil was taken in a beaker and 100 ml of water was added to make a suspension of 1:10 (W/V) dilution and the electrical conductivity was measured with a digital electric conductivity meter.

Analysis of Soil Nutrient Content

The total nitrogen (N) and available phosphorus (P) were determined respectively by micro-Kjeldahl and molybdenum blue methods (Jackson 1973). Exchangeable K was extracted from the soil in ammonium acetate solution (pH 7) and measured with a digital flame photometer (Jackson 1973). Soil organic carbon was determined according to Piper (1966).

Preparation of Roots and AMF Assessment

Fixed roots were washed free of FAA, and observed under a dissection microscope (X 20) for AM fungal spores attached to them. After examination, the roots were cut into 1 cm bits, cleaned with 2.5 % KOH (Koske and Gemma 1989), acidified with 5 N HCl, and stained with trypan blue (0.5 % in lacto glycerol). The roots were kept overnight immersed in stain for staining. The stained roots were examined using a compound microscope (X 200–400) for AM fungal structures and the percentage of root length colonization was estimated according to magnified intersection method (McGonigle et al. 1990).

Enumeration and Isolation of AM Fungal Spores

100 gram of soil was dispersed in 1L water and the suspension was decanted through 710–38 μm sieves. The residues in the sieves were washed in the beakers. The sieves were dispersed in water and filtered through grided filter papers. Each filter paper was spread on a petri dish and scanned under a dissection microscope X 40 magnification and all intact spores were counted. Sporocarps and spore clusters were considered as one unit. Intact AM fungal spores were transferred using a wet needle to polyvinyl alcohol-lacto glycerol with or without Melzers reagent on a glass slide for identification. Spores were identified based on spore morphology and subcellular characters and compared with original descriptions (Schenck and Perez 1990). Spore morphology was also compared with the culture database established by INVAM (http://invam.cag.wvu.edu).

Statistical Analysis

All data were subjected to Analysis of Variance (ANOVA) and the means were separated using Duncan's multiple range test (DMRT). Data on AM colonization and spore numbers were arcsine and log transformed [In (1 + X)], respectively, prior to statistical analysis. Pearson's correlation analysis was used to assess the relationships between edaphic-climatic factors, spore number, and root colonization (Zar 1984).

Results

This study on relations between environmental factors and AM fungal variables were carried out in the rhizosphere of *P. nigrum.* According to the climatic factors, it was shown that high temperatures in the month of April and May of nearly 25 °C with 100 % of RH and 30 °C with also 100 % of RH, respectively (Fig. 1). The edaphic factors show that the soil moisture content percentage was high in March with more than 22 %, at the same time January and February had very low moisture content at around 2–3 %; soil pH was neutral in April and in the rest of the months it was acidic in nature; EC level of the soil was fluctuated throughout the year, but it maintained the stable level from March to July at 60 mS cm^{-1} (Fig. 2). Soil nitrogen content showed a high level in April and May from 0.6 to 0.7 mg Kg^{-1} (Fig. 3); Phosphorous content in the soil was high in October at 0.014 mg Kg^{-1} (Fig. 4); Potassium

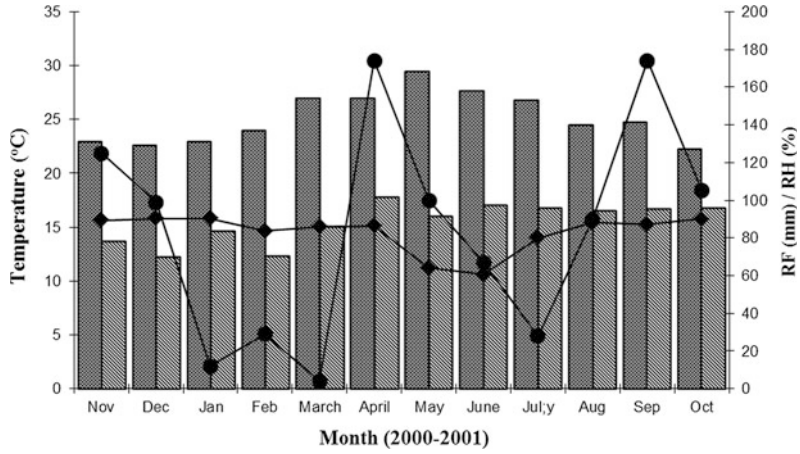

Fig. 1 Climatic data of the study sites in *Piper nigrum* plantation during the period studied

level was high in November at more than 1.2 mg Kg^{-1} than other months (Fig. 5). The organic carbon ranged between 1.6 and 4.3 in the soil of *P. nigrum* and in the month of December had a high percentage throughout the plantation period during November 2000 to October 2001 (Fig. 6).

The AMF variation study showed that the total AMF spore number varied from 12.00 to 90.30 g^{-1} in this host rhizosphere soil. A number of spores were presented in the period of March; more than 85 in 25 g $soil^{-1}$ (Fig. 7) which belonged to five species were recorded and identified as *Acaulospora* sp., *Gigaspora* sp., *Glomus aggregatum*, *G. fasciculatum*, and *G. geosporum*. Arbuscules root length was high in the months of December and April which was more than 2.5 % (Fig. 8), vesicles root length was high in April, May, and September at the same level of 80 % (Fig. 9), hypal length was minimum in January, February, March, July, and August, and the rest of the months in the study period showed large of almost 65 % (Fig. 10), and colonization of root length percentage was more in the months of May and September, above 80 % (Fig. 11).

The statistical relation between the environmental factors and mycorrhizal variables in *piper nigrum* was studied (Table 1). Temperature was positively correlated to EC significantly at 0.05 levels, but insignificantly on negative correlation to RH at 0.01 levels. Likewise, rainfall was significantly and positively correlated with RLC, RLH, RLV, RLV although negatively correlated with AMF at 0.001 levels. Simultaneously, the soil nitrogen content was positively correlated to RLV and RLA at level of 0.05 significantly. AMF was significantly and negatively correlated at 0.01 with RLC and 0.05 levels with RLH, RLV and RLA. Similarly, the RLA was correlated with RLC, RLH, and RLV significantly at the positive level of 0.01.

Discussion

The variations in abundance of AM fungi as assessed by spore number and root length colonization throughout the year have attracted considerable attention (Abbot and Rabson 1991). Many such studies on the difference in mycorrhizal formation and spore number have concentrated on short-lived annuals, but the result for long-lived may be considerably different (Hayman 1974; Smith 1980). The present investigation clearly indicates the periods and factors that favor root colonization and AMF associated with black pepper. During the study period low spore count was recorded in the rhizosphere of the crop. Moreover, the low spore density of AMF is due to the acidic nature of the soil. The above finding is supported by Udaiyan et al. (1996). The other reason for low spore number can be the presence of AMF propagules like the intraradical structures persisting in root which are the main source of inoculum for perennial plants (Baylis 1969).

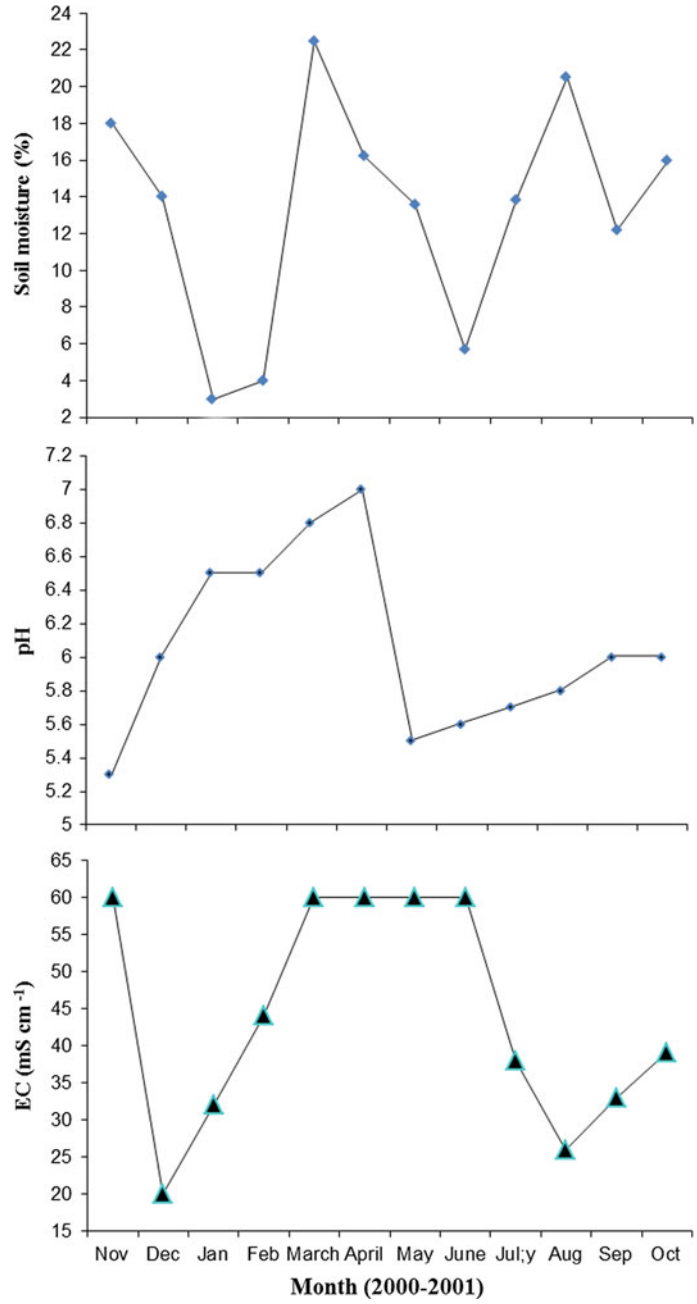

Fig. 2 Edaphic factors of the study site of *Piper nigrum* plantation during the period studied

During the study period the variation in AMF colonization and spore population in black pepper may also be due to seasonal influence. Higher AMF colonization and low spore number observed in host species, in this study is in agreement with those noted by Udaiyan et al. (1996) in *Acacia farnesiana* and *A. planifrons* and Rajesh Kannan (2002) in *Casuarina equisetifolia* and *Dalbergia latifolia.* The reduction in spore numbers may result from spore germination and limited spore life span on the activity of antagonistic soil microorganisms, which may coincide with root growth (Mosse and Brown 1968; Sutton and Barron 1972). Sporulation has also been

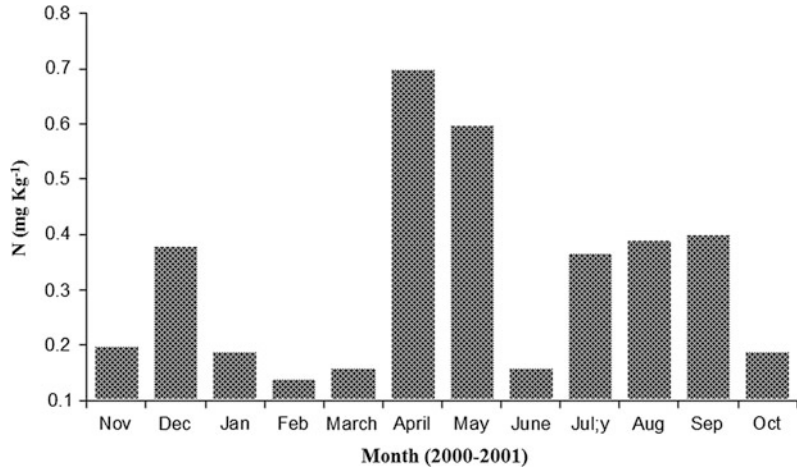

Fig. 3 Soil nitrogen content of the study site of *Piper nigrum* plantation during the period studied

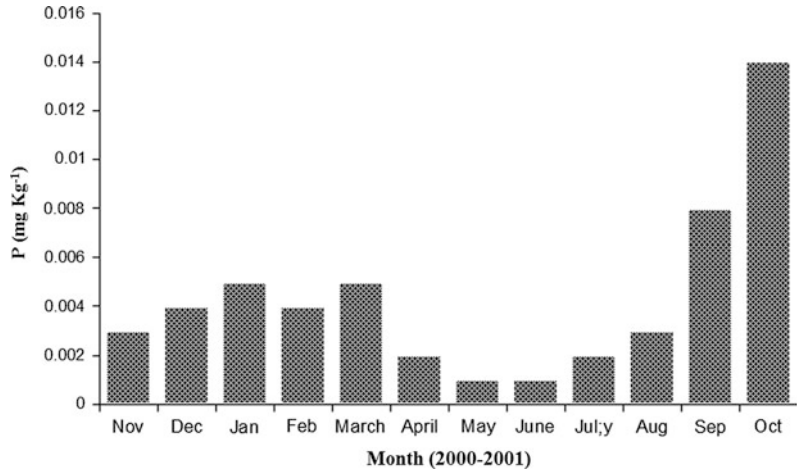

Fig. 4 Soil phosphorus content of the study site of *Piper nigrum* plantation during the period studied

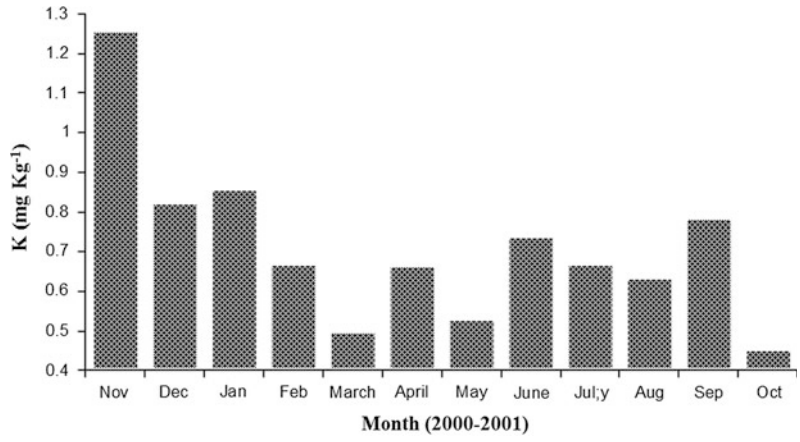

Fig. 5 Soil potassium content of the study site of *Piper nigrum* plantation during the period studied

reported to be depressed by hyperparasitic fungi (Schenck and Nicolson 1977). A seasonal variation in mycorrhizal spore number was studied previously (Ebbers et al. 1987; Dhillion et al. 1988; Koske and Gemma 1989). In most cases, spores were less abundant during the period of

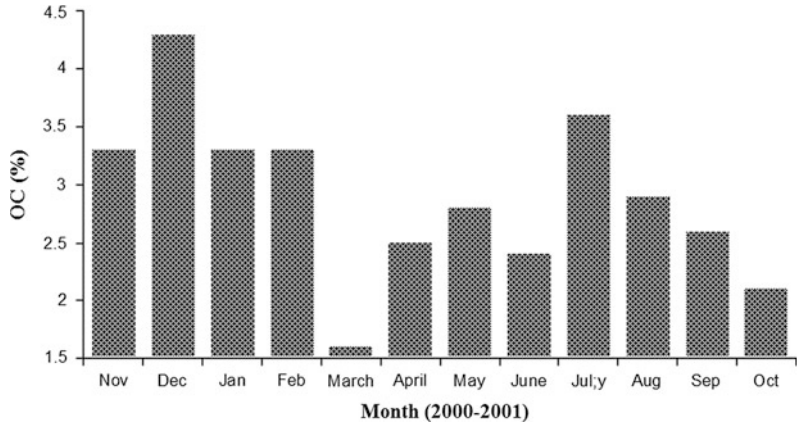

Fig. 6 Soil organic carbon content of the study site of *Piper nigrum* plantation during the period studied

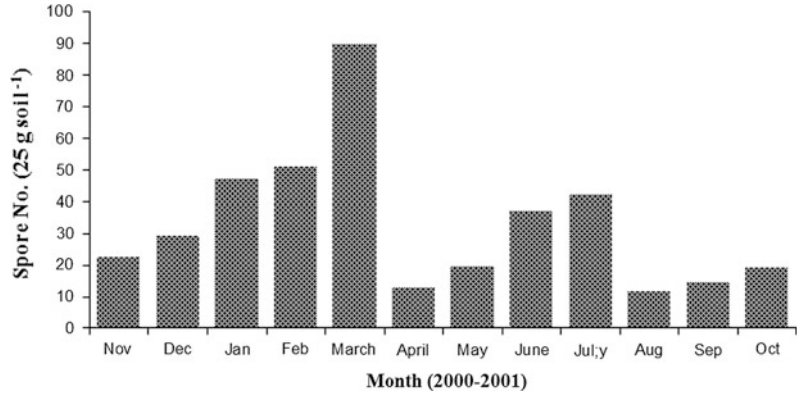

Fig. 7 Incidence of AM fungal spore numbers in *Piper nigrum* plantation during the period studied

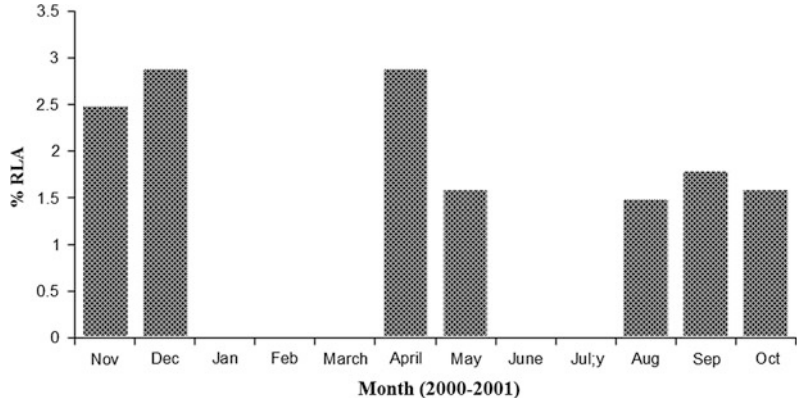

Fig. 8 Percentage of root length arbuscular in *Piper nigrum* plantation during the period studied

mycorrhizal formation and became more numerous during the period of roots senescence and/or of the growing season (Brundrett 1991). Spore density has significantly negative correlation to %RLC, %RLH, and %RLA, which is in contrast with the observations of Hetrick and Bloom (1986) as wide range of host, fungal, and environmental factors are known to influence AMF formation and subsequent spore production. Under environmental conditions where root growth is continuous, the vegetative phase of AM fungi may be actively involved in initiating infection and spreading onto new roots. Further, the mycorrhizal dependency of the actively-growing host may delay the fungal reproductive phase to a certain extent.

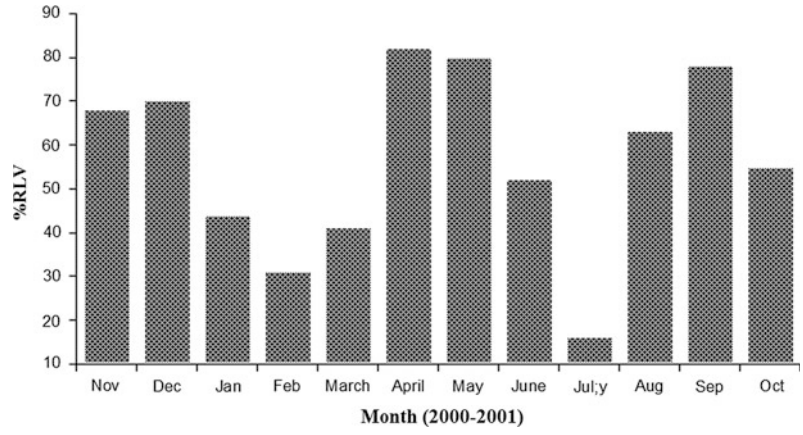

Fig. 9 Percentage of root length vesicles in *Piper nigrum* plantation during the period studied

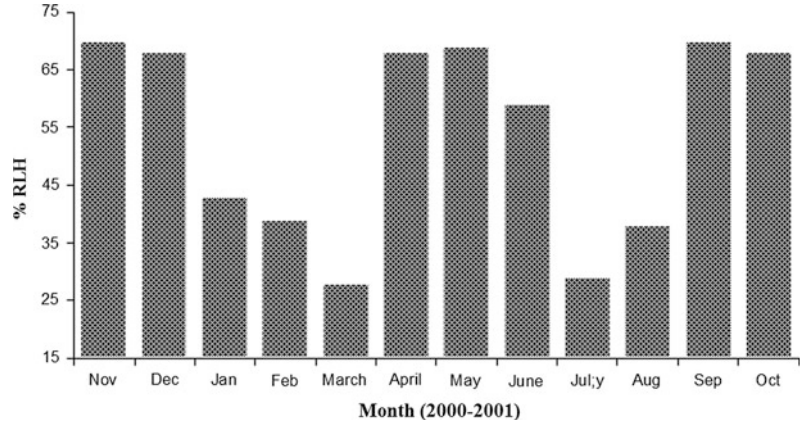

Fig. 10 Percentage of root length hyphae in *Piper nigrum* plantation during the period studied

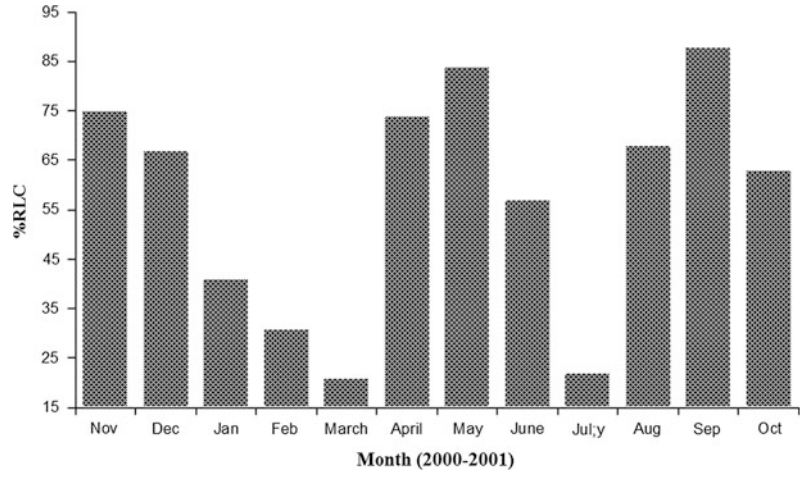

Fig. 11 Percentage of root length colonization in *Piper nigrum* plantation during the period studied

Root colonization was highest during September in black pepper when the soil moisture was moderate in these plantations. The hosts' dependence on AM fungi for nutrients might have enhanced mycorrization with decreasing soil moisture. The reduction in soil moisture level

Table 1 Correlation coefficients of climatic, edaphic, and mycorrhizal variables in *Piper nigrum*

Climatic factors[a]						Soil factors[b]						Arbuscular mycorrhizae[c]				
		Max temp	Mini temp	RF	RH	SM	pH	EC	SN	SP	SK	OC	AMF	%RLA	%RLV	%RLH
Arbuscular mycorrhizae[c]	%RLC	−0.017	0.231	0.890**	−0.065	0.168	−0.372	0.002	0.543	0.060	0.237	0.017	−0.844**	0.788**	0.833**	0.871
	%RLH	−0.111	0.087	0.823**	−0.077	−0.002	−0.296	0.130	0.374	0.188	0.285	0.048	−0.696*	0.764**	0.819**	
	%RLV	0.081	0.176	0.848**	−0.001	0.285	−0.103	0.108	0.607*	−0.003	0.183	−0.093	−0.667*	0.828**		
	%RLA	−0.230	0.006	0.848**	0.330	0.443	−0.117	−0.088	0.591*	0.078	0.294	0.217	−0.703*			
	AMF	0.133	−0.348	−0.819**	−0.017	−0.068	0.419	0.214	−0.570	0.067	−0.189	−0.225				
	OC	−0.375	−0.572	−0.047	0.185	−0.319	−0.309	0.546	0.092	−0.321	0.515					
Soil factors[b]	SK	−0.408	−0.408	0.209	0.238	−0.149	−0.353	−0.003	−0.180	−0.262						
	SP	−0.590*	0.058	0.133	0.515	0.100	0.154	−0.343	−0.334							
	SN	0.461	0.427	0.611*	−0.128	0.279	0.068	0.060								
	EC	0.619*	0.246	0.048	−0.517	0.121	0.027									
	pH	−0.029	−0.023	−0.152	0.397	−0.042										
	SM	0.088	0.225	0.274	0.295											
Climatic factors[a]	RH	−0.788**	−0.311	0.108												
	RF	−0.034	0.344													
	Min. Tem	0.506														

[a] Max temp, Maximum temperature; Mini temp, Minimum temperature; RH, Relative humidity; RF, Rainfall

[b] SM, Soil Moisture; pH; EC, Electrical conductivity; N, Nitrogen; P, Phosphorous; K, Potassium; OC, Organic carbon

[c] Spore, AMF spore No; %RLC., Root length colonization; %RLH, Root length hyphae; %RLV, Root length vesicles %RLA, Root length arbuscules

**, * Significantly at 0.01, 0.05 levels respectively

adversely affected root colonization but enhanced sporulation. However, spore numbers also tended to reduce at very low soil moisture.

Similarly, increase in percentage of root colonization as well as spore population at moderate soil moisture level has been observed by Sangeeta Kaushal (2000).

The presence of arbuscules during these periods further confirms an active growth of host and its dependence on mycorrhizal fungi (Jasper et al. 1989). Arbuscules are the site of nutrient transfer from fungus to host and their presence is an indication of mutualism (Koske et al. 1992). Reinhardt and Mille (1990) also reported that higher arbuscular infections during the host's active growth phase. Studies by Sparling and Tinker (1978) have clearly demonstrated that the development of arbuscules in plant is controlled by host nutrient demand. In black pepper, soil moisture was significantly correlated with percentage of root length arbuscules (%RLA). Soil moisture conditions have also been proposed to influence root growth and AMF colonization (Allen 1984).

The extrametrical phase of the AM fungi in the soil, its growth, and development is influenced by the edaphic factors. A study on the effect of pH on AM fungi has shown that germination of AMF spores is sensitive to pH. However, the effect of pH on root colonization tends to vary with strains of AMF species (Medeiros et al. 1994).

Plant survival in the natural ecosystems depends upon the ability to take up water under fluctuating soil moisture conditions (Sala and Lauanroth 1982). Information about the effect of soil moisture on mycorrhizal formation and spore abundance is very limited (Smith and Gianinazzi-Pearson 1988), although the role of AM fungi enhancing the water uptake by plants under fluctuating soil moisture conditions is well documented. Douds and Schenck (1991) found that water availability is an important determinant of AMF spore germination. However, either the rapid root growth could be greater than the infective capacity of AM fungi (Abbott and Robson 1991) or the higher flush of nutrient released from accumulated soil organic matter following moisture-induced decomposition (Swift et al. 1981) may affect variations in mycorrhizal formation.

The RLC% was positively correlated with soil moisture, indicating the response of AMF colonization in roots of fluctuating soil moisture levels (Allen and Allen 1983). This study has revealed that soil N was correlated positively with spore density and negatively to RLC% in this plant. Soil N plays an important role in influencing the mycorrhizal formation and function mainly through changes in soil pH. Soil N was positively correlated with spore number in *Accacia planiferons* (Udaiyan et al. 1996), however, the effect of N on AM fungal spore abundance is related to other soil factors and to the host with which they are associated (Hayman 1982). But soil N was correlated negatively with EC and temperature, positively with spore population, and RLC% in *D.latifolia* (Rajesh Kannan, 2002). A similar observation was found in *A. farnesiana* and spore number of *A. planifrons* (Udayan et al., 1996). The stimulatory effect of soil N on root colonization has also been reported.

The spore density of pepper soil was negatively correlated with rainfall. These observations agreed with those of Udaiyan et al.(1996) and Rajesh Kannan (2002) and emphasize that climatic factors can strongly influence AMF colonization (Furlan and Forti 1973; Hayman 1974) The influence of soil P on AMF structures is in accordance with the observations of Udaiyan et al. (1996) where soil P tended to influence root colonization in *A. planifrons*. It is well established that increasing soil P can reduce AMF formation and the inhibition may be due to the direct effect of P on the external hyphal network development or indirectly associated with host P status (Sanders 1975). However, the effect of P on spore density tends to vary with host species.

Native soil often contains AMF spores of more than one species and spore belonging to three AMF genera were isolated from the two host species of rhizosphere soil. Generally, more than one AMF species are quite common in the rhizosphere of perennial hosts (Thapar and Khan 1985), and this is substantiated by the recovery of spores belonging to different species in each

host. The possibility of environmental factors in addition to host factors is important in influencing AMF distribution (Khalil et al. 1992). The rhizosphere is the natural reservoir of a myriad of microorganisms, which are activated to grow around developing plant roots. Factors such as soil type, pH, temperature, age, and conditions of plants are known to influence rhizosphere (Rovira 1965).

Conclusions

The mycorrhizal fungi are important in sustainable agriculture due to their role in plant and soil nutrition acting as agent in transporting mineral nutrients to the plant. The fungi also provide drought tolerance and disease resistance to various crops. This study concludes that the influence of edaphic and climatic factors on the degree of AM fungal association in the host may help in systematic field application of AM fungi for enhancing the plant health, sustainable yield, and also for reducing the incidence of various soilborne diseases.

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Effects of Bioinoculants on Quality Seedling Production and Nutrient Uptake of *Casuarina equisetifolia* Forst. Grown in Decomposed Coir Pith

T. S. Saravanan, K. Rajendran, M. Uma, and P. Chezhian

Abstract

Nursery experiments were conducted to select suitable bioinoculants and their combinations to improve the quality of seedling production of *Casuarina equisetifolia* forst using locally available bioresources. *Casuarina* seeds were germinated in nursery mother bed and transplanted to 300 cc root trainer with a potting mixture of unsterilized decomposed coir pith and inoculated individually and in combinations of *Azospirillum brasilense, Pseudomonas fluorescens*, and *Trichoderma viride*. Uninoculated seedlings (control plants) were also maintained for comparing growth performance. Root length, shoot height, basal diameter, and biomass were recorded. Concentration of nutrient in plant tissue and nutrient uptake was also estimated for six months after inoculation. Results showed that the bioinoculants increase the growth and biomass of *Casuarina equisetifolia* seedlings. Of all the treatments, *Azospirillum* alone and along with other bioinoculants of *Azospirillum* + *Pseudomonas* + *Trichoderma* were found to be the most effective microorganism on increasing the growth, biomass, and quality of seedlings.

Keywords

Azospirillum · *Casuarina equisetifolia* · Decomposed coir pith · *Pseudomonas* · Root trainer · Seedlings quality · *Trichoderma*

Introduction

The substantial socioeconomic importance of casuarinas (several members of the family Casuarinaceae) has ensured ongoing global interest in research and development of this group of nitrogen-fixing trees. *Casuarina* is being cultivated by farmers approximately in 62,373 ha of land in the coastal as well as inland districts of Tamil Nadu, India (Annual report on

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non-food crops 2008–2009, Department of Economics and Statistics, Govt. of Tamil Nadu, India). *Casuarina equisetifolia* has been introduced and cultivated successfully in India and is increasingly being planted as farm forestry plantation. It yields 120–140 ton/ha^{-1} in 3 years duration. The wood is highly suitable for firewood and charcoal production, and also as pulp wood for paper making. The energy from the charcoal is 3,450 kJ kg^{-1}, hence it is considered as a good firewood species (Orwa et al. 2009). It is a good shelter species and windbreaker, hence it is commonly called a bio-shield plant.

Due to these advantages, farmers, governmental, and non-governmental organizations have planted farm forestry, agroforestry, and plantation forestry in private and public lands in rural and urban areas. At present the pinch on fertilizer consumption is being felt more in India, since the country cannot afford to either import the required fertilizer at high cost and subsidize the sale to the farmers or build new fertilizer plants at formidable cost. Hence the farmers are prepared for organic farming practices by using bioinoculants. Bioinoculants are cost-effective and ecofriendly inputs providing alternative source of plant nutrients, thus increasing farm income by providing extra yields and reducing input cost. Bioinoculants can increase crop yield by 20–30 %, in replacing chemical N and P by 25 %, stimulate plant growth, activate soil biologically, restore natural fertility, and provide protection against drought and some soil-borne diseases. Several bioinoculants are widely used in agriculture crops. *Azospirillum* is an important nonsymbiotic associative, nitrogen-fixing rhizosphere bacteria and fixes atmospheric nitrogen in soil (Krishnamoorthy 2002; Vijayakumari and Janardhanan 2003).

Casuarina responds well to *Azospirillum* inoculation (Rajendran et al. 2003; Rajendran and Devaraj 2004). These inoculants need more attention in view of their triple action of nitrogen fixation, biocontrol, and production of plant growth regulators. Phosphobacterium also produces auxin and gibberellin, which may have favorable effects on plant growth (Somani et al. 1990). The stimulative effect of phosphobacterial inoculation on plant growth in phosphorus-deficient soil has been reported by Asea et al. (1988). Inoculation of *Eucalyptus camaldulensis* with phosphobacteria in an unsterilized soil enhanced collar diameter, fresh weight, and dry weight compared to uninoculated control (Mohammad and Prasad 1988). In *Leucaena leucocephala*, an increase of 33.2 % in plant height was observed following inoculation with phosphobacteria (Young 1990). Similarly, the *Trichoderma* strains also solubilize a number of poorly soluble nutrients (Altomarne et al. 1991; Harman 2000). However, in tree crops it is still at an experimental stage.

The potting medium plays an important role in determining the growth of a healthy fibrous root system. It physically supports growing seedlings and stores as well as supplies the nutrients, water, and air to the root systems. The better media will be the development of a healthy, fibrous root system and subsequently a better seedling is produced which will survive after outplanting and commence quick growth. Lately, root trainers are increasingly being used to produce sturdy seedlings with more fibrous root formation. These containers have open bottoms and vertical ribs, which help to avoid root coiling. The free flow of air constantly sloughing up the drainage hole results in proliferation of lateral roots (Gera et al. 1996). Decomposed coir pith can also substitute soil or sand in the conventional nursery mixture for raising seedlings. Coir wastes after biodegradation can be effectively used as manure for increasing yield of crops (Ramasamy 1986). Coir pith alone used as potting medium has shown a spectacular increase in water holding capacity of potting mixture, when tomato plants were grown on coir pith-based potting mixture (Baskaran and Saravanan 1997).

Decomposed coir pith-based substrate used for the production of planting stock in root trainer in forest nurseries, Institute of Forest Genetics and Tree Breeding, Coimbatore and Tamil Nadu Newsprint and Papers Limited, Karur, Tamil Nadu, India, is very low in beneficial microbial population. The quality of

seedling is very poor due to insufficiency of desired microorganisms (many of the microorganisms are host specific) and the rate of mineralization and nitrogen fixation is very low; as a result the quality of the seedling is very poor. It is difficult to establish such seedlings in the initial stage in the field . This problem can be overcome by providing suitable bioinoculants. It has been already reported that the use of bioinoculants results in better growth and nutrient uptake of tree seedlings. Plants colonized by nitrogen-fixing bacteria of genus *Azospirillum* have promoted tree growth (Wong and Sternberg 1979). Similarly, *Azospirillum* along with other bioinoculants improves the quality of *C. equisetifolia* tree seedlings (Rajendran et al. 2003). However, the efficiency of individual and combined application of bioinoculants on the growth and quality of *C. equisetifolia* seedlings grown in root trainer with potting medium of decomposed coir pith needs to be studied. Hence, the present study was undertaken to find the compatibility of different bioinoculants and their augmentation effect of quality seedling production of *C. equisetifolia*.

Materials and Methods

Seeds

Casuarina equisetifolia forst fruits were collected from a single tree, located at the semi-arid region of Cuddalore district of Tamil Nadu, India. Seeds were graded and uniform size was used for raising seedlings. Seedlings were raised in unsterilized sand in nursery mother bed (Plate 1) and transplanted in 300 cc root trainer with a potting mixture of unsterilized decomposed coir pith (Table 1) and inoculated individually and in combinations with *Azospirillum, Pseudomonas*, and *Trichoderma*. Uninoculated seedlings (control plants) also were maintained for comparing growth performance. In order to find out suitable bioinoculants and their combinations to achieve maximum growth in overall and minimize the cost of seedling production, the following treatments were given 7 days after germination.

Isolation and Mass Multiplication of Microorganisms

Azospirillum

N-free semi-solid malate medium was used as an enrichment culture for *Azospirillum* (Dobereiner et al. 1976). Roots were washed in sterile distilled water and in 25 mM phosphate buffer, followed by three more washings (Baldani and Dobereiner 1980). The root samples were cut into pieces 5 to 8 mm long and placed in 10 ml serum vials containing 5 ml of NFB medium. Other vials containing NFB medium were inoculated with rhizosphere soil. These cultures were incubated at 32 °C for 72 h. Later, two different tests were conducted to verify typical *Azospirillum* colonies: (1) white, dense, undulating pellicle 1–3 mm below the surface from vials were streaked on Congo red plates which were incubated at 32 °C. After 72 h small scarlet colonies were observed, indicating the presence of *Azospirillum* sp. (Rodriguez Caceres 1982). *Azospirillum* was counted by the most probable number (MPN) method, using McCrady tables.

Pseudomonas

King's B medium, a selective one (King et al. 1954) was used for the isolation of *Pseudomonas fluorescens*. One ml of soil suspension from aliquot dilutions (10^5 to 10^8) was aseptically added. Twenty ml of sterilized, melted, and cooled medium was added and poured in each petri plate, and incubated at 28 ± 2 °C for 24 h. Well-separated individual colonies with yellow-green and blue-white pigments were marked and detected by viewing under UV light. The individual colonies were picked up with sterilized loop and transferred onto fresh King's B medium. The plates were incubated at 28 ± 2 °C for

Plate 1 Various stages of seedling production in root trainers using decomposed coir pith compost

24 h. The single colonies were developed and transferred to King's B medium for mass multiplication.

Trichoderma

Conidial suspensions of fungal antagonist *Trichoderma* were prepared by flooding 10-day-old cultures with sterile distilled water. Scraping the surface with a bent glass rod, and filtering the suspension through four layers of cheese cloth, conidial spores were washed with sterile distilled water thrice by centrifugation at 2000 rpm for 20 min followed by resuspension of the pellet in sterile 1 % CMC solution. 10 ml of solution was inoculated by making holes in the root zone.

Treatment

Seven days after transplantation in root trainer, 10 ml culture of *Azospirillum*, *Pseudomonas*, and *Trichoderma* were inoculated by making holes in the rhizosphere of seedlings.

Table 1 Chemical and physical properties of decomposed coir pith used for potting media for nursery experiments

Chemical composition	Decomposed coir pith	Physical properties[a]	Decomposed coir pith
Lignin (%)	4.80	Bulk density (g/cc)	0.1025
Cellulose (%)	10.10	Particle density g/cc)	0.3936
Organic carbon (%)	24.90	Porosity (%)	56.76
Nitrogen (%)	1.05	Maximum water holding capacity (%)	526.31
Phosphorous (%)	0.06	Volume expansion of 100 ml (%)	22.92
Potassium (%)	1.20	pH	6.80
Calcium (%)	0.50	EC (mhos/cm)	1.4
Magnesium (%)	0.48		
Iron (ppm)	0.09		
Manganese (ppm)	25.0		
Zinc (ppm)	15.8		
Copper (ppm)	6.20		
C:N ratio	24.1		
Volume (mm)	0.58		

Chemical properties of raw and decomposed coir pith (Subramanian et al. 1998)
[a] Physical properties of decomposed coir pith in present study

T_1 *Azospirillum* (*Azospirillum brasilense*)
T_2 *Pseudomonas* (*Pseudomonas fluorescens*)
T_3 *Trichoderma* (*Trichoderma viride*)
T_4 *Azospirillum* + *Pseudomonas*
T_5 *Azospirillum* + *Trichoderma*
T_6 *Pseudomonas* + *Trichoderma*
T_7 *Azospirillum*+ *Pseudomonas* + *Trichoderma*
T_8 Control (Decomposed coir pith alone)

Experimental Design

Nursery experiment was conducted at the plantation division, Tamil Nadu Newsprint and Papers Ltd, Kagithapuram, Karur, Tamil Nadu, India. The experiment was set up in a completely randomized block design (CRBD) with eight treatments and five replicates. All the plants were kept under identical nursery condition up to 180 days.

Seedling survival percentage was calculated using the following formula:

$$\text{Seedling survival percentage} = \frac{\text{Number of seedlings present in each treatment}}{\text{Total number of seedlings transplanted in each treatment}} \times 100$$

Harvesting and Measurement

After 180 days of transplanting from each treatment, a total of 24 seedlings were randomly selected and height and basal diameter were recorded. Seedlings were carefully uprooted without disturbing the root system and washed in running tap water. Excess water was wiped out by placing them between folds of blotting paper. The seedlings were cut at the collar region, dried separately at 70 °C in paper bags in hot air oven, and biomass estimation (root and shoot dry weight) was carried out using top pan electronic balance.

Seedlings Quality Index

Seedlings quality index (SQI) was calculated using the formula of Dickson et al. (1960).

$$\text{SQI} = \frac{\text{Total dry weight (g/ plant)}}{\frac{\text{Height (cm)}}{\text{Collar diameter (mm)}} + \frac{\text{Shoot dry weight (g/plant)}}{\text{Root dry weight (g/plant)}}}$$

Percentage of Nodulated Seedlings

Percentage of nodulated seedlings by *Frankia* was assessed 6 months after inoculation. 24 seedlings were examined and the number of nodules bearing seedlings was counted. The percentage of nodules bearing seedlings was calculated by the following formula:

$$\text{Percentage of nodulated seedlings} = \frac{\text{Number of seedlings with nodules}}{\text{Total number of seedlings observed}} \times 100$$

Nutrient Analysis

Plant samples were taken for biochemical analysis. The oven-dried plant samples were ground to pass through a 0.5 mm plastic sieve before digestion.

Estimation of Nitrogen and Phosphorous

The dried plant material was ground in a mortar and pestle and the total nitrogen content was estimated by the conventional micro-Kjeldahl method Umbriet et al. (1972). Total phosphorus was estimated by the method of Fiski-Subba-Rao as modified by Bartlett (1959).

Estimation of Total Potassium, Calcium, and Magnesium

One gram of plant sample was digested with tri-acid mixture with HNO_3: H_2 SO_4: $HClO_4$ in the ratio of 9:2:1 until it became colorless. After digestion it was filtered and the volume was made up to 100 ml. Potassium in the extract was determined using a flame photometer (Jackson 1973). Calcium and magnesium were determined by the Versenate method as described by Jackson (1973).

Statistical Analysis

The data were statistically analyzed by analysis of variance (ANOVA) with SPSS and treatment means were separated using Duncan's Multiple Range Test ($P < 0.05$) (Duncan 1955). Pearson's correlation analysis was used to assess the relation between the seedling growth, biomass, and nutrient uptake.

Results

Shoot Height, Root Length, and Basal Diameter

Analysis of growth data revealed that the significant differences in shoot height, root length, and basal diameter were recorded in *C. equisetifolia* seedlings inoculated with the different microbial inoculants compared to the uninoculated control (Table 2).

Shoot Height

From the analysis of growth data the combined inoculation with *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7) had shown significantly higher shoot height and recorded 48.56 % increase over control. This was statistically on par with individual inoculation of *Azospirillum* (T_1) followed by combined inoculation of *Azospirillum* + *Pseudomonas* (T_4) and recorded a 47.42 % and 34.78 % increase over control at 180 days after inoculation.

Root Length

Significant differences in root length were recorded in *C. equisetifolia* seedlings inoculated with different microbial inoculants compared to the uninoculated control (Table 2). From the analysis of growth data, the combined inoculation of *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7) was found to be the most effective

Table 2 Effect of microbial inoculants on the growth of *C. equisetifolia* seedlings (180 days after inoculation)

Treatments	Collar diameter (mm)	Shoot length (cm)	Root length (cm)	Total length (cm)	No of needles	No of nodules
T_1	0.17^{abc} (17.64)	22.05^{bc} (34.78)	17.61^{bc} (24.19)	39.66^{c} (17.70)	29.00^{bc} (41.68)	2.33^{bc} (64.37)
T_2	0.17^{abc} (17.64)	21.84^{bc} (34.15)	14.36^{ab} (7.03)	36.20^{b} (13.24)	22.08^{ab} (23.41)	1.83^{abc} (54.64)
T_3	0.15^{ab} (6.66)	21.75^{bc} (33.88)	14.55^{ab} (8.24)	36.30^{b} (13.38)	22.58^{ab} (25.11)	1.50^{ab} (44.66)
T_4	0.21^{cd} (33.33)	27.35^{d} (47.42)	15.93^{ab} (16.19)	43.28^{d} (21.89)	34.00^{cd} (50.26)	2.66^{c} (68.79)
T_5	0.19^{bcd} (26.31)	25.25^{cd} (43.04)	17.60^{bc} (24.14)	42.85^{d} (21.42)	37.75^{d} (55.20)	2.83^{c} (70.67)
T_6	0.16^{ab} (12.5)	17.96^{ab} (19.93)	20.15^{c} (33.74)	38.11^{bc} (15.76)	23.66^{ab} (28.52)	1.58^{ab} (47.46)
T_7	0.23^{d} (39.13)	27.96^{d} (48.56)	16.27^{ab} (15.85)	44.23^{d} (22.92)	39.41^{d} (57.09)	2.16^{bc} (61.57)
T_8	0.14^{a}	14.38^{a}	13.35^{a}	27.73^{a}	16.91^{a}	0.83^{a}

Means followed by a common letter(s) in the same column are not significantly different at the 5 % level by Duncan's Multiple Range Test (DMRT). Values in parenthesis represent % increase over control

Treatments: T_1—*Azospirillum*; T_2—*Pseudomonas*; T_3—*Trichoderma*; T_4—*Azospirillum* + *Pseudomonas*; T_5—*Azospirillum* + *Trichoderma*; T_6—*Pseudomonas* + *Trichoderma*; T_7—*Azospirillum* + *Pseudomonas* + *Trichoderma*; T_8—Control

in increasing the root length of seedlings. Of all the treatments, individual inoculation with *Azospirillum* (T_1) had shown maximum root length of 17.61 cm which was a 24.19 % increase over the control. This was statistically on par with the combined inoculation of *Pseudomonas* + *Trichoderma* (T_6).

Total Length

Seedling treated with combined inoculation of *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7) showed significantly higher total length and recorded 22.92 % increase over control. This was statistically on par with individual inoculation of *Azospirillum* (T_1) followed by combined inoculation of *Azospirillum* + Pseudomonas (T_4) which recorded a 17.70 and 21.89 % increase over the control at 180 days after inoculation.

Basal Diameter

The combined inoculation of *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7) showed significantly higher basal diameter. It recorded a 39.13 % increase over control. The double inoculation of *Azospirillum* + *Pseudomonas* (T_4), *Azospirillum* + *Trichoderma* (T_5) inoculated seedlings showed similar growth.

Shoot Biomass

The data pertaining to dry matter accumulation of shoot, root, and total biomass are presented in Table 3. Significant differences observed among the treatments are evaluated at 180 days after inoculation. The highest biomass in the shoot was recorded in seedlings inoculated with *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7), which registered 67.49 % increase over control.

Root Biomass

Statistically highly significant difference was found in different types of microbial inoculation on root biomass of *C. equisetifolia* seedlings. Inoculation of *Azospirillum* (T_1) alone and in combination with other inoculants was found to significantly increase root biomass, when

Table 3 Effect of microbial inoculants on the biomass of *C. equisetifolia* seedlings (180 days after inoculation)

Treatments	Needle dry wt (g/plant)	Stem dry wt (g/plant)	Root dry wt (g/plant)	Nodule dry wt (g/plant)	Total dry wt (g/plant)
T_1	0.8667^{abc} (53.07)	0.5567^{bc} (48.52)	0.5600^{bc} (46.42)	0.0300^{ab} (66.66)	2.0133^{bcd} (50.16)
T_2	0.4300^{a} (5.41)	0.3800^{ab} (24.55)	0.3233^{a} (7.20)	0.0200^{a} (50.00)	1.1533^{ab} (12.99)
T_3	0.5833^{ab} (30.27)	0.4767^{abc} (39.85)	0.4500^{ab} (33.33)	0.0333^{ab} (69.96)	1.5433^{abc} (34.98)
T_4	1.0200^{bc} (60.12)	0.6667^{cd} (56.99)	0.5733^{bc} (47.67)	0.0467^{abc} (78.58)	2.3067^{cde} (56.50)
T_5	1.3267^{c} (69.34)	0.8067^{d} (64.46)	0.6767^{c} (55.66)	0.0733^{bc} (86.35)	2.8833^{de} (65.19)
T_6	0.6767^{ab} (39.89)	0.4433^{abc} (35.32)	0.4533^{ab} (33.81)	0.0400^{abc} (75.00)	1.6133^{abc} (37.80)
T_7	1.3733^{c} (70.38)	0.8933^{d} (67.90)	0.7333^{c} (59.08)	0.0867^{c} (88.46)	3.0867^{e} (67.49)
T_8	0.4067^{a}	0.2867^{a}	0.3000^{a}	0.0100^{a}	1.0034^{a}

Means followed by a common letter(s) in the same column are not significantly different at the 5 % level by DMRT (Duncan's Multiple Range Test). Values in parenthesis represent % increase over control
Treatments: T_1 —*Azospirillum*; T_2—*Pseudomonas*; T_3—*Trichoderma*; T_4—*Azospirillum* + *Pseudomonas*; T_5—*Azospirillum* + *Trichoderma*; T_6—*Pseudomonas* + *Trichoderma*; T_7—*Azospirillum* + *Pseudomonas* + *Trichoderma*; T_8—Control

compared to other treatments. Root biomass was highest in *Azospirillum* + *Pseudomonas* + *Trichoderma* (Table 3).

Total Biomass of Seedling

Seedling biomass was highest in seedlings treated with *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7), Azospirillum + *Trichoderma* (T_5) and showed a 67.49 and 65.19 % increase over the control. Single inoculation of seedling treated with *Azospirillum* (T_1) showed higher biomass which recorded 50.16 % more than that of the control and it was statistically on par with *Azospirillum* + *Pseudomonas* (T_4) and *Azospirillum* + *Trichoderma* (T_5) inoculated seedlings (Table 3).

Percentage of Nodulated Seedlings

Nodules were found in all the seedlings without inoculation of *Frankia*. Percentage of nodulated seedlings was higher in the seedlings treated with phosphate solubilizing microorganism of *Pseudomonas* (T_2) individually and in combinations with other bioinoculants.

Nutrient Concentration Percentage

Nitrogen, Phosphorus, Potassium, Calcium, and Magnesium

Nutrient concentration of seedling is in the order of nitrogen > calcium > potassium > magnesium > phosphorus (Table 4).

Nutrient Uptake

Nitrogen and Phosphorus

Total nitrogen and phosphorus content of *C. equisetifolia* seedlings inoculated with bioinoculants had significantly increased over control (Table 5). The highest nitrogen content was observed in seedlings inoculated with *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7) followed by *Azospirillum* + *Trichoderma* (T_5) which recorded at 0.069 and 0.065 per plant

Table 4 Biomass and nutrient concentration of *C. equisetifolia* seedlings grown in coir pith compost (180 days after inoculation)

Treatments	Biomass (g/plant)	Nutrient % concentration in plant tissue				
		N	P	K	Ca	Mg
T_1	2.0133^{bcd}	2.193	0.133	1.49	1.9	0.43
T_2	1.1533^{ab}	1.803	0.103	1.383	1.53	0.33
T_3	1.5433^{abc}	1.853	0.127	1.373	1.447	0.4
T_4	2.3067^{cde}	2.233	0.13	1.417	1.947	0.4
T_5	2.8833^{de}	2.257	0.137	1.553	2.047	0.45
T_6	1.6133^{abc}	1.777	0.090	1.530	1.540	0.42
T_7	3.0867^{e}	2.24	0.13	1.517	1.963	0.46
T_8	1.0034^{a}	1.643	0.083	1.473	1.423	0.41

Means followed by a common letter(s) in the same column are not significantly different at the 5 % level by Duncan's Multiple Range Test (DMRT)

Treatments: T_1— *Azospirillum*; T_2—*Pseudomonas*; T_3—*Trichoderma*; T_4—*Azospirillum* + *Pseudomonas*; T_5—*Azospirillum* + *Trichoderma*; T_6—*Pseudomonas* + *Trichoderma*; T_7—*Azospirillum* + *Pseudomonas* + *Trichoderma*; T_8 —Control

Table 5 Biomass and nutrient uptake of *C. equisetifolia* seedlings (180 days after inoculation)

Treatments	Biomass (g/plant)	Nutrient uptake (g/plant)				
		N	P	K	Ca	Mg
T_1	2.0133^{bcd}	0.044145	0.002677	0.029994	0.038247	0.008656
T_2	1.1533^{ab}	0.020794	0.001188	0.01595	0.017645	0.003806
T_3	1.5433^{abc}	0.028597	0.00196	0.02119	0.022332	0.006173
T_4	2.3067^{cde}	0.051509	0.002999	0.032686	0.044911	0.009227
T_5	2.8833^{de}	0.065076	0.00395	0.044778	0.059021	0.012975
T_6	1.6133^{abc}	0.028668	0.001452	0.024683	0.024845	0.006776
T_7	3.0867^{e}	0.069142	0.004013	0.046825	0.060592	0.014199
T_8	1.0034^{a}	0.016486	0.000833	0.01478	0.014278	0.004114

Means followed by a common letter(s) in the same column are not significantly different at the 5 % level by DMRT (Duncan's Multiple Range Test)

Treatments: T_1— *Azospirillum*; T_2—*Pseudomonas*; T_3—*Trichoderma*; T_4—*Azospirillum* + *Pseudomonas*; T_5—*Azospirillum* + *Trichoderma*; T_6—*Pseudomonas* + *Trichoderma*; T_7—*Azospirillum* + *Pseudomonas* + *Trichoderma*; T_8—Control

respectively. Similarly, phosphorus content was recorded 0.004 and 0.003 per plant.

Potassium, Calcium, and Magnesium

K, Ca, and Mg content in the seedlings showed the highest amount in combination of *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7) followed by *Azospirillum* + *Trichoderma* (T_5). It recorded 0.046, 0.060, and 0.014 plant of K, Ca, and Mg uptake in seedlings treated with *Azospirillum* + *Pseudomonas* + *Trichoderma* (Table 5).

Microbial Inoculation Effect

Microbial inoculations significantly altered the nutrient use efficiencies of *C. equisetifolia* seedlings. Microbial inoculation effect of seedling

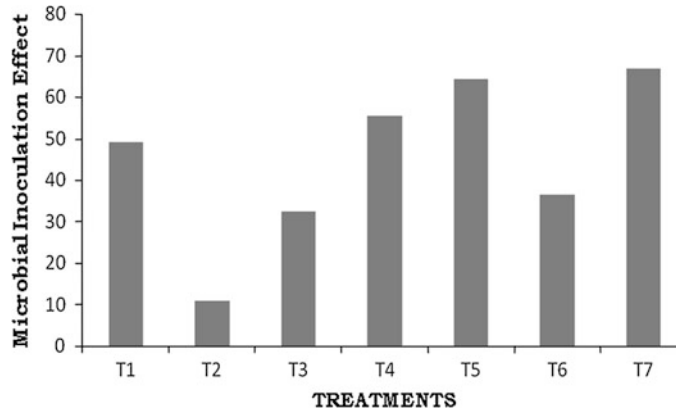

Fig. 1 Microbial inoculation effect of *C. equisetifolia* seedlings (180 days after inoculation)

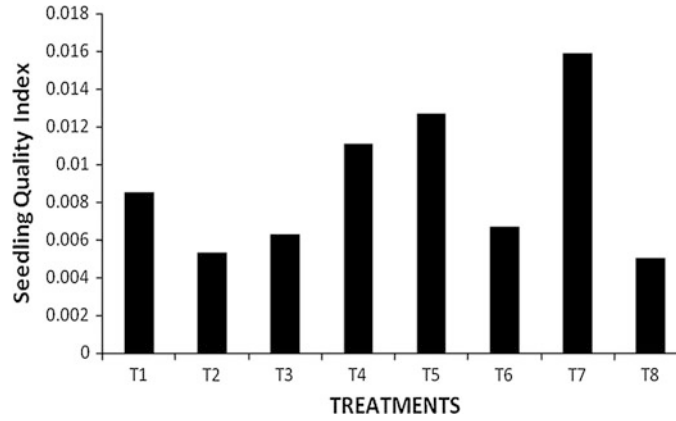

Fig. 2 Seedling quality index of *Casuarina equisetifolia* seedlings (180 days after inoculation)

was higher in seedling treated with *Azospirillum* and its combination with *Trichoderma* and *Pseudomonas* (Fig. 1).

Seedling Quality Index

Good quality seedlings were obtained from seedlings inoculated with *Azospirillum* + *Pseudomonas* + *Trichoderma* (T_7) followed by *Azospirillum* + *Trichoderma* (T_5) (Fig. 2).

Discussion

The comparison of seedlings with various treatments is more authentic on the basis of quality parameters, rather than on actual values of height on collar diameter. Seedling quality specifications have been traditionally based on certain morphological characters such as sturdiness (height/diameter ratio), root/shoot ratio and some other features (Cleary 1978) form the quality parameters of the seedlings. It appears that seedlings grow in coir pith compost with a combination of different bioinoculant sources significantly and maintain superiority in comparison to other treatments. In this study, the height, diameter, dry matter, and quality seedlings were higher in the *C. equisetifolia* seedlings inoculated with bioinoculants. The increase in growth may be attributed to high accumulation of nutrients in the plant tissue.

Biologically active products, more appropriately called microbial inoculations, containing active strains of a selective microorganism like *Azospirillum*, Phosphobacterium, *Trichoderma*, and *Pseudomonas* alone or in combination, help

plant growth through different mechanisms, including biological nitrogen fixation and solubilization of insoluble phosphate fertilizer. Nitrogen-fixing bacteria of the genus *Azospirillum* have promoted plant growth of agronomically important field crops by 10–30 % in the field experiment (Okon 1985; Sumner 1990; Rajendran and Devaraj 2004) . Increased crop yield in germination rate, plant height, and leaf size (Saikia et al. 2001) has enhanced minerals and water uptake, increased dry matter accumulation, root surface area, root diameter density, and root hair (Okon and Kapulnik 1986) to support the earlier reports. In this study, *Azospirillum* inoculated seedlings showed better growth and root biomass when compared to control. The better seedling growth may be attributed to the increased root biomass and accumulation of nitrogen (Wong Sternberg 1979; Rajendran et al. 2003) and the production of gibberellins and cytokinin-like substances (Tien et al. 1979). The above results corroborate with earlier studies on quality seedling production of *C. equisetifolia* (Rodriguez-Barrueco et al. 1991; Rajendran et al. 2003; Muthukumar and Udaiyan 2010), *Moringa oleifera* (Kasthuri Rengamani et al. 2006), *Acacia nilotica* (Rajendran and Jayashree 2007), and *Delonix regia* (Meenakshisundaram et al. 2011).

Growth promoting effect of inoculation with *Azospirillum* and phosphobacterium alone or dual inoculation with both non-symbiotic bioinoculants was found in several tree species such as Casuarina (Swaminath and Vadiraj 1988; Rajendran et al. 2003). *Casuarina* trees were treated with bioinoculants in farm forestry (Rajendran and Devaraj 2004) and *Moringa oleifera* by Kasthuri Rengamani et al. (2006). In this study, phosphobacterium inoculated seedlings produced better plant height, stem girth, and total biomass. This may be due to the inoculation of phosphate solubilizing microorganism of *Pseudomonas*, which has shown stable and consistent capacity to solubilize insoluble phosphorus and thus making it available to plants.

Trichoderma are present in substantial quantities in nearly all agricultural soils and in other environments such as decaying wood and their use is only now being recognized worldwide as an alternative in plant disease control (Harman et al. 2004). Different species of *Trichoderma* have the potential to control soilborne plant pathogens more effectively than chemicals (Papavizas 1985) and they also exhibit plant-growth promoting activity (Kleifeld and Chet 1992; Duffy et al. 1996; Harman and Bjorkman 1998). Rice plants grown in soil amended with 50 g of *Trichoderma harzianum* per square meter of soil had significantly higher plant height (75.6 cm) than that of controls (Kharakrang et al. 2002). In this study, single inoculation of *Trichoderma* and *Pseudomonas*, and dual Inoculation of *Trichoderma* with *Pseudomonas* have shown that there was no significant influence in the growth and biomass of *C. equisetifolia* seedlings grown in decomposed coir pith. However, it is relevant to mention here that phosphobacterium by virtue of its capacity to multiply certain growth promoting substances like IAA and GA might induce the growth of *C. equisetifolia* seedlings (Ramamoorthy 1982; Gaur 1990). Of all the treatments, combined inoculations of *Azospirillum* + *Pseudomonas* + *Trichoderma* produced excellent growth, biomass, and tissue nutrient concentration. The greater height, diameter, and dry matter of the *C. equisetifolia* seedlings due to co-inoculation of all the bioinoculants might strongly improve accumulation of nitrogen due to *Azospirillum* (Rajendran et al. 2003) and more phosphorus uptake by phosphobacterium (Kuccy 1987).

Nutrient management has been well recognized since early times, which has become highly relevant with the advent of commercial forestry, where there is always a thrust to increase the production and biomass removal besides maintaining the site fertility. Estimation of the essential mineral elements in plants is an important aspect in the study of plant growth and ecosystem structure. In the case of fast-growing species, it is essential to study the geochemical cycle of the essential elements in support of their survival in future (Faizmohsin et al., 2005). In this estimation nutrient uptake was higher in seedlings treated with bioinoculation. Nutrient accumulation of seedling is in the order of

Table 6 Pearson's correlation coefficients for collar diameter, total length, total dry weight, and nutrient uptake

Parameters	Collar diameter	Total length	Total dry weight	N	P	K	Ca	Mg
Collar diameter	–	0.792*	0.860**	0.868**	0.833*	0.832*	0.863**	0.820*
Total length		–	0.684**	0.701**	0.689**	0.634**	0.690**	0.604**
Total dry weight			–	0.996**	0.985**	0.995**	0.993**	0.993**
N				–	0.991**	0.989**	0.998**	0.985**
P					–	0.974**	0.986**	0.972**
K						–	0.991**	0.997**
Ca							–	0.984**

n = 24 * and ** Correlation is significant at the 0.05 and 0.01 level (2-tailed)

nitrogen > calcium > potassium > magnesium> phosphorus.

Frankia inoculated seedlings showed better growth, nodulation, and nutrient concentration as reported by Reddell (1990), indicating that the artificial application of nodule crush increased dry matter yield of *Casuarina*. Similarly, Rajendran et al. (2003) obtained increased overall growth, nodulation, shoot and root dry weight, and nitrogen content by inoculation of *Frankia* in *C. equisetifolia*. However, in the present assessment of seedlings grown in decomposed coir pith nodules were found in all the seedlings without inoculation of *Frankia*. The percentage of nodulated seedlings was higher in seedlings treated with phosphate solubilizing microorganisms individually and in combinations with other bioinoculants. This shows that coir pith compost support *Frankia* infection in seedlings naturally and phosphate solubilizing microorganisms help to improve the nodule number and nodular biomass in the seedlings as shown in Table 6.

Conclusion

Increasing dry land farming and development technologies for arid lands with soil-related constraints now acquire new importance and emerge as new frontiers for agricultural and farm forestry development. Increased agro and farm forestry production has to come through with the adoption of better management technology. Long-term sustainability in agriculture and forestry is possible only through the use of low cost farm grown inputs, which work in harmony with nature. Bioinoculants act as perpetually renewable inputs helping in better tree crop nutrient management and maintenance of soil health, better soil, and water management leading to improved forestry practices. It is inferred that with appropriate technology, the use of efficient microbial inoculants yield increased growth and biomass of *C. equisetifolia* seedlings. This study clearly shows that the application of *Azospirillum* along with *Trichoderma* and *Pseudomonas* plays a significant role in increasing the growth response of *C. equisetifolia* seedlings in a stipulated period, thereby producing good quality planting stock. These treated seedlings may also perform better in nutrient impoverished soil.

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Efficacy of AMF and PGPR Inoculants on Maize (*Zea mays* L.) Plant Growth and Their Rhizosphere Soil Properties

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Abstract

Field trial was carried out using microbial inoculants through arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria. The inoculants were applied individually and also different combination in *Zea mays*. L (Maize), and plants were grown in the Department of Microbiology experimental garden, Bharathidasan University, Tiruchirappalli, Tamilnadu, India. In this context, the microbial equilibrium influence on the growth and development of maize plants is studied for every 15-day period of harvest up to 75 days. The dual inoculation treatment (T_4 and T_5) significantly increased microbial biomass and NPK accumulation in soil, and these dually inoculated plants displayed higher specific activity than their comparable to control. From all the treatments, comparatively the combined treatment of (T_6) showed an active maize growth through the periodic analysis of chlorophyll, carbohydrate, protein, metabolite synthesize like phenol and flavonoids. It is concluded that T_6 has a stimulatory effect on mycorrhizal root colonization and also improve the maize plant growth and soil properties.

Keywords

Maize · PGPR · AMF colonization · Microbial biomass · Combined inoculation

Introduction

The rhizosphere is a zone of influence on plant roots by the soil microbiota with physical, chemical and biological properties different from those of the root-free bulk soil. A characteristic of the rhizosphere is that microbial diversity is altered and that the activity and number of microorganisms increased and the supply of photosynthetic and decaying plant

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material to the root-associated microbiota a key issue for rhizosphere formation and functioning (Kennedy 1998). There are abundant microorganisms thriving in soil, especially in the rhizosphere of plants. A considerable number of bacterial and fungal species possess a functional relationship and constitute a holistic system with plants. They were able to exert beneficial effects on plant growth (Vessey 2003). Application of beneficial microbes in agricultural practices started several decades ago, and now increasing evidence that these beneficial microbial populations can also enhance plant resistance to adverse environmental stresses such as water and nutrient deficiency and heavy metal contamination (Shen 1997). A group of bacteria were now referred to as plant growth-promoting rhizobacteria (PGPR), which participate in many key ecosystem processes such as those involved in the biological control of plant pathogens, nutrient cycling and seedling establishment, and therefore deserve particular attention for agricultural or forestry purposes (Weller and Thomashow 1993; Elo et al. 2000).

The use of fertilizers, including chemical fertilizers and manures, to enhance soil fertility and crop productivity has often negatively affected the complex system of the biogeochemical cycles (Steinshamn et al. 2004). Research activities aimed at achieving better use efficiency of fertilizers, including the use of PGPR and AMF as supplements to fertilizers have steadily increased in the last two decades, as indicated from a search of a scientific literature database. Historically, microbial inoculants have been used to achieve biological control or plant growth promotion. However, plants can interact with several soil microorganisms, including PGPR and AMF that make the plant more tolerant to such stresses (Vessey 2003). Thus, plants inoculated with PGPR or AMF usually grow better than non-inoculated plants under conditions of nutrient limitation (Canbolat et al. 2006). Beneficial effects are usually enhanced when both microorganisms are co-inoculated, although this depends on the bacterium–fungus pair (Valdenegro et al. 2001). There have been several mechanisms described by which PGPR can increase plant growth.

In this present study, we examined the effects of AMF and PGPR inoculants on the growth of maize under nursery conditions and analysed the importance of soil microorganisms and their interactions with roots. The impact of AMF colonization, morphology and biochemical was also analysed periodically for the promotion of maize crop.

Materials and Methods

Experimental Design

This study was conducted in the Department of Microbiology experimental garden, Bharathidasan University, Tiruchirappalli, Tamilnadu, India. The standard size of polythene bags 35 × 8 cm was used for plant cultivation. The bag media that comprise of red soil, sand and organic manure (cow dung) in the ratio of 2:1:1 were used. After package of soil mixture into polythene bags, maize seeds were sown along with the inoculum of 7 different combinations. The media-filled polythene bags were arranged in complete randomized block design (CRBD). Water was sprayed gently over the growing plants.

Microbial Population

The total number of cultivable microorganisms was analysed in the polybag medium using the standard serial dilution and pour plate method on a specific nutrient media. Randomly collected soil samples were sieved to remove large particles, and 1 g of soil was weighed. It was taken in a conical flask having 10 ml of sterilized distilled water and mixed well for 3 min. This helps in the removal of microbes, which adhere in soil particles. Then, it was serially diluted to various dilutions up to 10^{-4} dilution. From that, 1 ml was poured into sterilized petri plates and nutrient agar medium for isolating total bacteria,

Pseudomonas agar for *Pseudomonas* sp., Luria-Bertani agar for *Bacillus* sp., Sabouraud dextrose agar for fungi and actinomycetes isolation agar for actinomycetes isolation were used. Antibiotic streptomycin sulphate was added to the medium to inhibit bacterial growth on fungal plate. Ketaconazole and streptomycin were added to inhibit both bacterial and fungal growth on actinomycetes isolation agar plate. After 48 h of incubation, the number of bacterial colonies grown on the plate was counted; in the same way, actinomycetes were counted on 7^{th} day.

AM Fungal Colonization

Fixed roots were washed free of FAA and observed under a dissection microscope for the presence of AM fungi. After examinations, the roots were cleared in 2.5 % HCL and stained with trypan blue (0.5 % in lacto glycerol). The roots were kept overnight immersed in stain. The stained roots were examined under compound microscope for AM fungal colonization structures, and the percentage of root length colonization was estimated according to magnified intersection method (McGonigle and Fitter 1990).

AM Fungal Spore Population

AM fungal spore population in each soil sample was enumerated after isolation by a modified wet-sieving and decanting technique, and the spores were identified according to Schenck and Perez (1990). Twenty-five gram of soil samples was dispersed in 300 ml of water, and the suspension was decanted through 710- to 38-μm sieves. The residues in the sieves were washed in the beakers. The sievates were dispersed in water and filtered through gridded filer papers. Each filter paper was spread on a petri plate and scanned under a dissection microscope × 40 magnification, and all intact spores were counted. Sporocarps and spore clusters were considered as one unit. Intact AM fungal spores were transferred using wet needle to polyvinyl alcohol-lactoglycerol with or without Melzer's reagent on a glass slide for identification. Spores were identified based on spore morphology and subcellular characters and compared with original descriptions.

In vitro Spore Germination and Hyphal Growth

The effect of PGPR in spore germination and hyphal growth of AMF was explored under in vitro conditions and was assessed using the bioassay procedure of Walley and Germida (1997). Using the sterile cotton swab, SAB cells from a stock culture were streaked on triplicate 1.5 % TSA plates to obtain a uniform lawn of bacterial growth. Plates were incubated at 27 °C for 24 h.

Direct Assay Technique

The clean, decontaminated AMF spores ($n = 50$) were pipetted onto a sterile 13 mm dia. 0.22 μm pore size filter and placed on a sterile 25 mm dia. 0.22 μm pore size filter that was laid on 24-h bacterial lawn. Plates were sealed with parafilm and incubated at 27 °C for 15 days, after which the plates were examined for spore germination and hyphal growth under stereomicroscope at 10X and 40X magnification. Control treatments were set up in an identical manner but without bacteria. All treatments were replicated three times.

Analysis of Soil Macronutrients

Initially, polybag media of soil profile were analysed for its physical and chemical properties, that is, total nitrogen (N), available phosphorus (P), exchangeable potassium (K) in soil testing laboratory, Tamilnadu Agricultural Department, Mannarpuram, Tiruchirapalli, Tamil Nadu, India.

Estimation of Chlorophyll

The estimation of total chlorophyll in leaves was done following the methods of Witham et al. (1971). Chlorophyll was extracted from maize sheath using the 80 % acetone as solvent. One gram of fresh sheath sample was taken in a clean mortar and pestle, and it was ground using the 80 % acetone. Then, it was centrifuged to sediment the residues. The supernatant was stored in clean bottles, and the pellet was again ground with the same volume of acetone. Until the pellet became white, the extraction procedure continued. Then, the final volume of the supernatant was making up to 100 ml using the 80 % acetone. Then, it was read in the visible spectrometer at 663 and 645 nm.

Determination of Total Carbohydrate

One hundred milligram of plant sample was taken in a boiling tube, and it was hydrolysed by keeping in boiling water bath for 3 h with 5 ml of 2.5 N HCL and cooled to room temperature. It was neutralized with solid sodium carbonate until the effervescence ceases. Make up the volume to 100 ml with distilled water and centrifuged. Supernatant was collected, and 0.5 ml was taken for the analysis. Then, 4 ml of anthrone reagent was added. Tubes were heated for 8 min in a boiling water bath. It was allowed to cool rapidly, and green to dark green colour was read at 630 nm. From the standard graph of glucose, the amount of carbohydrate present in the sample tubes was calculated (Hedge and Hofreiter 1962).

Estimation of Protein

The protein concentration was determined by the method of Lowry et al. (1951); 100 mg of plant sample was taken in a mortar and pestle, and it was ground with phosphate buffer (pH 7.2) and centrifuged to sediment the residues, and the supernatant of 0.1 ml was taken in a test tube and made up to 1 ml; 5 ml of alkaline copper reagent was added to all the tubes including the blank. Mix well the contents and allowed to stand for 10 min. Then, 0.5 ml of Folin–Ciocalteu reagent was added, mixed well and incubated at room temperature in dark for 30 min, blue colour was developed, and it was read at 660 nm. Standard graph of BSA was drawn, and the amount of protein was calculated.

Estimation of Phenol

The concentration of phenol content present in the tissues was analysed according to the method of Malick and Singh (1980); 100 mg of plant sample was ground with mortar and pestle in 10 volumes of 80 % ethanol and centrifuged at 10,000 rpm for 20 min. Supernatant was collected, and the residue was re-extracted with 5 times the volume of 80 % ethanol, centrifuged and pooled the supernatant. Supernatant was allowed to evaporate and dissolve the residue in a 5 ml of distilled water. Pipette out 0.2 ml into test tubes, and the volume was made up to 3 ml using distilled water. Then, 0.5 ml of Folin–Ciocalteu reagent was added. Mixed well and kept in boiling water bath for exactly 1 min, cooled and measured the absorbance at 650 nm against blank.

Estimation of Flavonoid Content

The concentration of flavonoid content present in the tissues was analysed; 100 mg of plant sample was ground with mortar and pestle in 10 volumes of 95 % ethanol and centrifuged at 10,000 rpm for 20 min. Supernatant was collected, and the residue was re-extracted with 5 times the volume of 80 % ethanol, centrifuged and pooled the supernatant; 0.5 ml of the supernatant was mixed with 95 % ethanol. After thorough mixing, 0.1 ml of 10 % aluminium chloride and 0.1 ml of 1 M potassium acetate were added to the supernatant, and finally, the

volume was made up to 5 ml using distilled water. Then, the mixture was incubated for 30 min at room temperature. Mixed well and measure the absorbance at 415 nm against blank was measured.

Acid Phosphatase Assay

The activity of acid phosphatase was determined according to the method of Fiske and Subba Rao (1925) using p-nitrophenyl phosphate; 1 g of soil sample was dispensed in 10 ml of ice-cold 50 mM citrate buffer (pH 5.3). Filtered through four layers of cheese cloth and used the filtrate as an enzyme source. Para-nitrophenyl phosphate hydrolysing activity was estimated by incubating 0.5 ml of enzyme with 3 ml of substrate for 15 min at 37 °C. Drawn 0.5 ml of incubated sample and mixed it with 9.5 ml of sodium hydroxide. The p-nitrophenol released was estimated spectrometrically by measuring the absorbance at 405 nm. The released p-nitrophenol is yellow in colour in alkaline medium. The specific activity expressed in moles of p-nitrophenol released per minute for per milligram of protein. The assay procedure was similar to that of acid phosphatase for alkaline phosphatase except for substrate solution (375 mg of glycine, 10 mg of magnesium chloride and 165 mg of p-nitrophenyl phosphate dissolved in 42 ml of 0.1 N NaOH and dilute to 100 ml at pH of 10.5).

Amylase Assay

The activity of amylase was determined according to the method of Kruger (1972) using starch as the substrate; 1 g of soil sample was dispensed in 5 volumes of ice-cold 0.1 M sodium acetate buffer solution overnight at 4 °C, and the supernatant used as an enzyme source. The starch hydrolysing activity was estimated by incubating 1 ml of enzyme with 1 ml of starch at 27 °C for 15 min. The reaction was stopped by the addition of 2 ml of dinitrosalicylic acid. The mixture was incubated in boiling water bath for 5 min. While the tubes were warm, 1 ml of potassium sodium tartarate was added. After cooling it in running tap water, it was made up to the volume of 10 ml using distilled water. The maltose released was estimated spectrometrically by measuring the absorbance at 560 nm. One unit of amylase expressed as milligram of maltose produced during 5 min incubation with 1 % starch.

Cellulase Assay

The activity of cellulase was determined according to the method of Denison and Koehn (1977) using the carboxymethylcellulose (CMC) as substrate; 1 g of soil sample was dispensed in 10 ml of ice-cold 0.1 M sodium citrate buffer (pH 5.0). Filtered it through four layers of cheesecloth and used the filtrate as an enzyme source. The enzyme cellulase hydrolyses the cellulose into reducing sugar due to its cellulolytic activity. This was estimated by incubating 0.1 ml of enzyme with 0.4 ml of 1 % CMC solution at 55 °C for 15 min. The reaction was stopped by the addition of 0.5 ml of dinitrosalicylic acid reagent. The mixture was incubated in a boiling water bath for 5 min. While the tubes were warm, 1 ml of potassium sodium tartarate was added and allowed to cool to room temperature. The volume was made up to 5 ml. The released reduced sugar was estimated spectrometrically by taking absorbance at 540 nm. The enzyme activity as the milligram glucose released per minute for per milligram of protein was calculated.

Results

Morphometric Analysis

The maize plants morphometric parameter include shoot fresh weight, number of leaves, shoot height and root length, which was analysed in all the treated and control plants on

every interval. Among all the treatments, T_6 treatment was found to be maximum performance on 15th day, of analysis, whereas it increased further in all the intervals and in the final harvest. These data showed the higher improvement in the T_6-treated maize plant growth, when compared to other treatments including control (Table 1).

Soil Microbial Population

The total soil microbial population was analysed on every 15 days of interval up to 75 days. Random soil samples were collected and examined before and after the treatments. Before the treatment, the total microbial population was found about 216×10^4/g. After inoculation, all the treatments showed higher microbial population, especially the combined inoculations of T_6 increasing microbial population from 15 to 45th day (Table 2). But during the periods of 60 and 75th day examination, the microbial population starts suppressed, and their number got decreased.

Percentage of AMF Colonization and Spore Number

Percentage of AMF colonization in maize roots was examined under the microscope for their presence of internal hyphae, arbuscules and vesicles on every 15 days of intervals. Among all the treatments, higher AMF colonization was recorded in T_3 treatment. Initially, the AMF colonization was found to be 56 % with internal hyphae but without any arbuscules and vesicle structure; in 75th day interval, 70 % colonization was observed. This showed an increased colonization over every periodic interval. The AM fungal spore number was found to be increased in T_4 treatment; 42 spores were present initially in 25 g of soil, which increased to 47 numbers on 75th day. Thus, the spore number and AMF colonization were found to be increased in all AMF-inoculated soil (Tables 3 and 4).

Table 1 Morphometric analysis of maize plant after 15–75 days of inoculation

Treatments	Shoot length (cm)/days					Root length (cm)/days					No. of leaves/days					Plant fresh weight (g)/days				
	15	30	45	60	75	15	30	45	60	75	15	30	45	60	75	15	30	45	60	75
Control	7.5	7.6	10.2	13	20	4.4	5	5.9	6	6	3	3	4	5	7	0.14	0.35	0.59	1.83	2.36
T_1 (*Bacillus*)	7.6	8.2	11	15	26	5.8	8	6.2	7.5	6.2	3	5	5	5	6	0.20	0.40	0.97	2.02	2.66
T_2 (*Pseudomonas*)	7.5	8	12.6	16	33	8.5	8	9	4.5	8	3	4	5	6	7	0.20	0.36	1.11	2.27	3.16
T_3 (AMF)	7.5	7	13.2	20	19	5.0	11	10	6	8	3	4	5	6	7	0.19	0.34	0.90	2.37	2.96
T_4 (AMF + *Pseudomonas*)	7.5	8.5	13.9	21	27	6.5	4.5	6.5	5.5	8	3	4	5	6	7	0.34	0.42	1.08	2.58	3.38
T_5 (AMF + *Bacillus*)	7.6	7.8	13.8	20	20	6.9	6	6.5	5	7.3	3	5	5	6	7	0.19	0.44	0.95	1.98	2.70
T_6 (AMF + *Bacillus* + *Pseudomonas*)	8.5	8.5	14.1	23	25	8.5	4	5	9	7	3	4	5	6	7	0.28	0.35	0.98	4.12	4.40

Table 2 Soil microbial population for initial and after 15-day intervals up to 75 days

Periods (Days)	Treatments	Microbial population				
		Total bacterial count (10^{-4}/g)	*Bacillus* (10^{-4}/g)	*Pseudomonas* (10^{-4}/g)	Actinomycete (10^{-4}/g)	Fungi (10^{-2}/g)
Initial	Random soil	216	74	109	2	135
15	Control	83	80	39	7	82
	T_1	176	98	90	2	95
	T_2	117	85	80	3	98
	T_3	155	65	105	4	129
	T_4	179	80	120	4	128
	T_5	124	78	28	4	120
	T_6	208	75	95	5	135
30	Control	299	249	98	30	102
	T_1	332	256	129	5	150
	T_2	382	315	200	9	143
	T_3	324	328	89	45	139
	T_4	308	320	430	10	210
	T_5	293	203	196	15	156
	T_6	379	300	322	12	150
45	Control	300	240	289	32	120
	T_1	370	252	290	20	129
	T_2	377	250	290	22	110
	T_3	396	259	298	40	132
	T_4	400	290	300	46	135
	T_5	410	389	389	49	130
	T_6	450	300	490	53	14
60	Control	189	280	136	15	132
	T_1	200	296	148	10	136
	T_2	271	291	190	12	135
	T_3	189	290	158	15	140
	T_4	189	298	150	12	192
	T_5	198	289	192	10	198
	T_6	209	250	200	10	201
75	Control	153	253	149	13	31
	T_1	198	260	157	43	48
	T_2	217	267	183	26	63
	T_3	153	227	165	16	60
	T_4	190	290	155	28	59
	T_5	190	300	198	58	38
	T_6	220	282	250	70	68

Treatments details: Control; T1 (*Bacillus*); T2 (*Pseudomonas*); T3 (AMF); T4 (AMF + *Pseudomonas*); T5 (AMF + *Bacillus*); T6 (AMF + *Bacillus* + *Pseudomonas*)

Table 3 Percentage of AMF colonization in maize root from 15 to 75th day of inoculation

Treatments	Days after inoculation				
	15	30	45	60	75
Control	3	14	12	16	10
T_1 (*Bacillus*)	11	22	28	38	28
T_2 (*Pseudomonas*)	19	30	26	64	36
T_3 (AMF)	40	56	76	70	70
T_4 (AMF + *Pseudomonas*)	49	44	70	68	64
T_5 (AMF + *Bacillus)*	52	38	72	64	60
T_6 (AMF + *Bacillus* + *Pseudomonas*)	59	52	80	66	48

Table 4 Spore population in 25 g of maize soil from 15 to 75th days of inoculation

Treatments	Days after inoculation				
	15	30	45	60	75
Control	5	13	13	16	16
T_1 (*Bacillus*)	8	20	17	34	21
T_2 (*Pseudomonas*)	10	27	14	33	36
T_3 (AMF)	29	34	63	62	51
T_4 (AMF + *Pseudomonas*)	35	42	52	46	47
T_5 (AMF + *Bacillus)*	40	54	45	36	51
T_6 (AMF + *Bacillus* + *Pseudomonas*)	43	37	63	81	42

Effects of PGPR on AMF Spore Germination and Hyphal Growth in In vitro Condition

Germination rate of spores and hyphal length of AMF in the absence of bacteria (PGPR) said to be control were nil. *P. fluorescens* promoted the spore germination by producing germ tube length to a very small extent. Out of fifty spores, two spores promote the germination that emerged out with 6 mm length of germ tube. In *B.subtilis*, there was no indication for AMF spore germination. It not only inhibited the spore germination and hyphal growth of AMF, but also decreased the root colonization. In the combined treatment, germination of spores was not yet observed. But prolonged incubation might have promoted the spore germination to certain extent (Table 5).

Soil Macronutrient Analysis

The soil macronutrient such as N, P and K was analysed over every periodic interval, in order to analyse their content pattern. Initially, the soil NPK content was found to be 121.8, 1 and 324 kg/acre, respectively. Among the three nutrients, phosphorus content was too low in the soil. But during every interval after inoculation, all the nutrients (NPK) increased to certain extent. In addition, the dual inoculation with AMF (T_4, T_5 and T_6) showed an increased concentration of NPK. In combined treatments, the NPK found to be 92.4, 6 and 101.5 kg/acre, respectively, in 15th day of analysis, whereas the NPK of 60th day found with 154, 25.5 and 201 kg/acre, respectively, this was increased 50 times more than the initial volume. But the NPK of 75th day analysis showed the decreased patterns (Table 6).

Table 5 Effects of PGPR on AMF spore germination and hyphal growth in in vitro condition

AMF	Control	*P.fluorescens* + AMF (T_4)	*B.subtilis* + AMF (T_5)	*P.fluorescens* + *B.subtilis* + AMF (T_6)
Spore germination (%)		2/50	0/50	0/50
Hyphal elongation (mm)	–	0.08	–	–
Germ tube emergence	No	Yes	No	No
Hyphal length about the dm of spore	–	1	–	–
Hyphal growth greater than dm of spore	–	1	–	–

Biochemical Profiles

Total Chlorophyll Content

The maize plant was taken for the analysis of total chlorophyll content present in 0.1 g of plant tissue sample. The analysis was done over every 15 days of interval. Among all the treatments, the dual inoculation of T_4 showed increasing total chlorophyll content from 3.75 to 6.46 mg/0.1 g of plant tissue samples from 15 to 75th day. The chlorophyll content of T_2 treatment also increased slightly of 2.45 mg/0.1 g of plant tissue sample during 15th day, which increased significantly day by day and finally on 75th day of 6.65 mg/0.1 g (Fig. 1).

Total Carbohydrate Content

The total carbohydrate content present in 0.1 g of plant tissue sample was analysed on every 15 days of interval up to 75 days. In overall analysis, T_3 treatment showed an increase in carbohydrate content from 8 to 28.64 mg/0.1 g of plant tissue sample in 15–75th day, respectively. This showed a threefold increase in carbohydrate content from initial to final analysis. In addition, T_1 treatment showed fourfold increase in carbohydrate content from 7.92 to 28.92 mg/0.1 g of plant tissue samples (Fig. 2).

Total Protein Content

The plant tissue sample was examined for total protein content in every 15-day interval up to 75 days. T_1 treatment showed a higher protein content; their concentration increased from 59.6 to 128.47 mg/0.1 g of plant tissue sample. The protein content of T_3 treatment was found to be 53.6 mg/0.1 g of plant tissue sample initially, which increased to 180.5 mg/0.1 g of plant tissue sample on the 75th day of analysis. Similar result was also observed in T_6 treatment. Initially, it was found to be 52.4 mg of protein, which increased further to 136.36 mg/0.1 g of plant tissue sample on 75th day analysis (Fig. 3).

Total Phenol Content

An aromatic compound of total phenol present in the treated maize plant was examined on every 15 days of intervals present in 0.1 g of plant tissue sample. In overall period, the phenol content of T_2 treatment was initially found to be 16.86 mg/0.1 g increased to 60.36 mg/0.1 g of plant tissue sample on 75th day. When compared to other treatments, the T_6 treatment was found with 12 mg/0.1 g of plant tissue sample of the phenolic content in 15th day of analysis, which was increased to 66.31 mg/0.1 g of plant tissue sample observed in 75th day (Fig. 4).

Total Flavonoid Content

An aromatic compound of flavonoid found in 0.1 g of plant tissue sample was examined on every 15 days of interval to 75th day. Among all the treatments, T_6 treatment showed an increasing flavonoid content from 28 to 532.8 mg/0.1 g of plant tissue sample from 15 to 75th day, respectively. Also, the dual treatment of T_4 showed a significant increase in flavonoid

Table 6 Maize rhizosphere soil macronutrients pattern for initial and after 15-day intervals up to 75 days

Periods (Days)	Treatments	Soil Macronutrients		
		Nitrogen	Phosphorus	Potassium
Initial	Random soil	121.8	1.0	324
15	Control	116.2	6	118
	T_1	123.2	7	93.5
	T_2	61.6	8.5	106
	T_3	119	8.5	106
	T_4	102.2	12	98.5
	T_5	124.6	6	85.5
	T_6	92.4	6	101.5
30	Control	204.4	34.4	174.5
	T_1	54.6	14.5	171.5
	T_2	98	11	126
	T_3	84	12	116.5
	T_4	112	6	114.5
	T_5	95.2	12	119.5
	T_6	100.8	6	85.5
45	Control	185	12	185
	T_1	155	14	164
	T_2	146	15	157
	T_3	147	16	184
	T_4	157	13	159
	T_5	156	15	162
	T_6	184	18	163
60	Control	127	30.5	225
	T_1	132	23	205
	T_2	148	39.8	199
	T_3	92	28	214
	T_4	129	19.5	165
	T_5	140	18.5	209
	T_6	154	25.5	201
75	Control	94	31.5	206
	T_1	161	23	217
	T_2	80	30.5	247
	T_3	125	31.5	237
	T_4	113	25.5	206
	T_5	111	34	253
	T_6	94	22	198

Treatments details: Control; T1 (*Bacillus*); T2 (*Pseudomonas*); T3 (AMF); T4 (AMF + *Pseudomonas*); T5 (AMF + *Bacillus*); T6 (AMF + *Bacillus* + *Pseudomonas*)

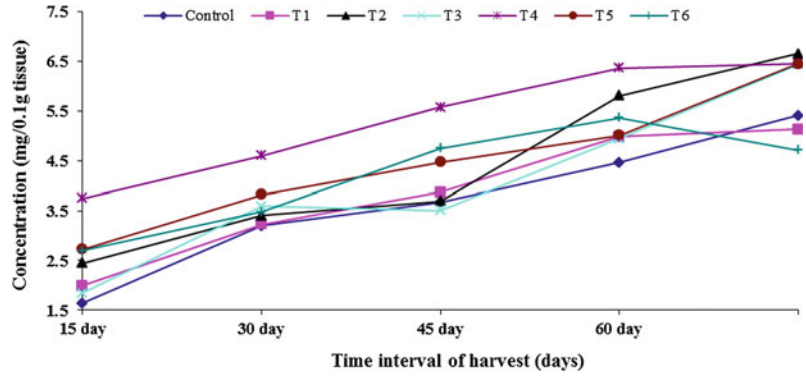

Fig. 1 Total chlorophyll in leaves of maize plants from 15 to 75th day of inoculation

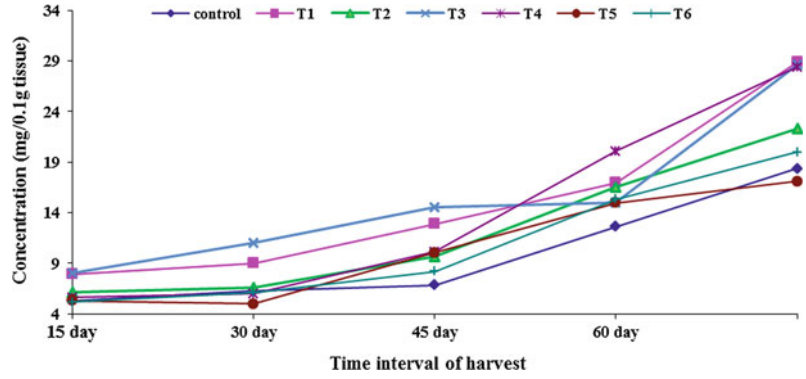

Fig. 2 Total carbohydrate in leaves of maize plants from 15 to 75th day of inoculation

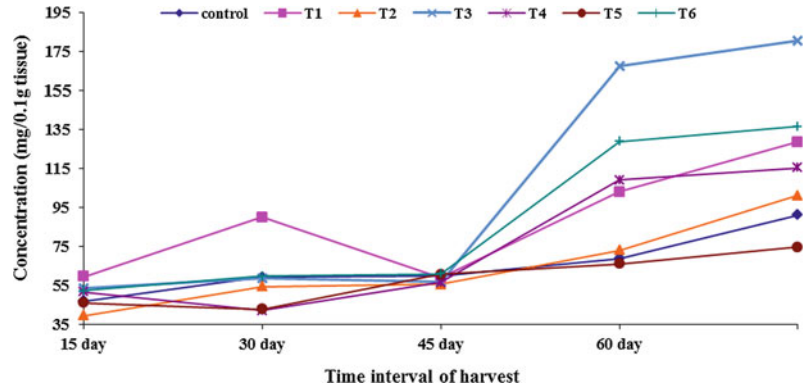

Fig. 3 Total protein in leaves of maize plants from 15 to 75th day of inoculation

concentration from 50 to 465.2 mg/0.1 g of plant tissue samples (Fig. 5).

Enzyme Activity

Phosphatase Activity

The enzyme phosphatase hydrolyses the p-nitrophenol phosphate as substrate and thereby released the end product of p-nitrophenol compound. Hence, the activity of both alkaline and acid phosphatase was assayed on every 15-day intervals to 75 days in the maize grown soil. Initially, both of its activity was found to be 0.2 mg/g/24 h and 0.01 mg/g/24 h, respectively, which were found to be lower in its activity. After inoculation, among all other treatments, T_4, T_5 and T_6 showed maximum activity throughout the periodic intervals. In the treatment of T_6, the activity of alkaline phosphatase was found to be 10 mg/g/24 h in 15th day,

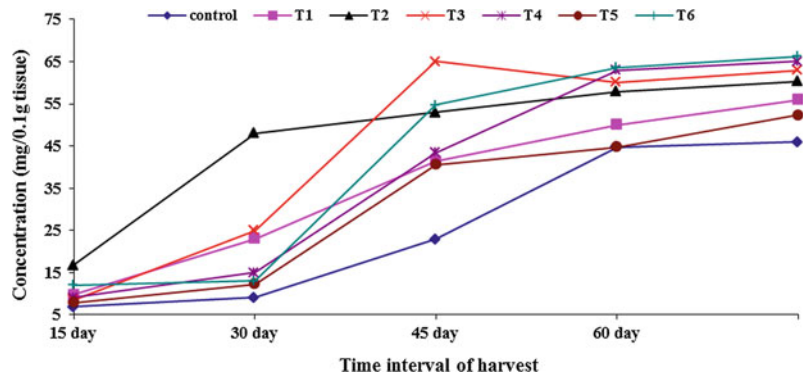

Fig. 4 Total phenol in leaves of maize plants from 15 to 75th day of inoculation

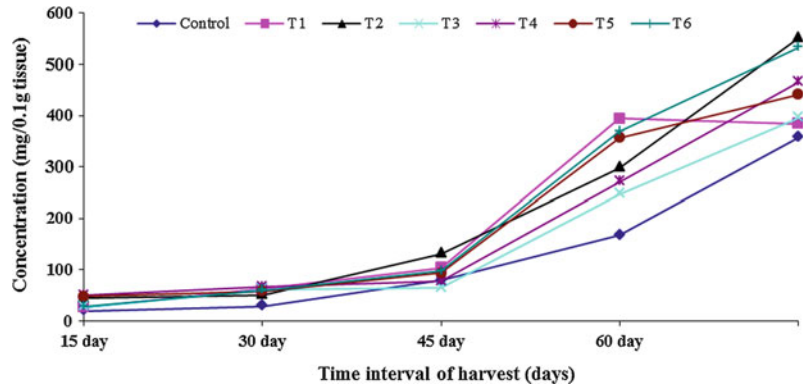

Fig. 5 Total flavonoid in leaves of maize plants from 15 to 75th day of inoculation

simultaneously, the activity increased and reached up to 32.9 mg/g/24 h in 75th day. The activity of acid phosphatase was the highest in T_6 treatment when compared to all other treatments including control. The T_6 treatment showed 0.1 mg/g/24 h during 15th day, whereas during the 75th day, the activity increased to 3.2 mg/g/24 h (Figs. 6 and 7).

Amylase Assay

Soil enzyme levels were examined on every 15 days of interval up to 75 days. Initially, the soil amylase activity was 26 µg/g/15 min of incubation found in the soil. But after inoculation, the maximum soil enzyme level was noticed in the treatment T_6 that was observed throughout the period. Though the T_6 treatment showed 37 µg/g/15 min of amylase enzyme activity during initial analysis, but in the final analysis, 170 µg/g/15 min of activity was observed. This result showed that the enzyme activity expressed by the combination of *Pseudomonas*, *Bacillus* and AMF bioinoculants was found to be supported (Fig. 8).

Cellulase Assay

The cellulase levels in the soil were analysed by assaying the amount of carboxymethyl cellulose as substrate that was reduced. This was regularly assayed on every 15 days of periodic interval up to 75 days. Initially, the cellulase enzyme level was found with 12 µg/g/15 min. But after inoculation, especially the treatment T_6 showed a higher levels of 41 and 160 µg/g/15 min initially on 15 and 30th day, but from 45 to 75th day the enzyme level declined, this result was noticed in all the other treatments also. The T_4 treatment showed a slight increased activity with 28.5 and 66.5 µg/g/15 min during 15 and 30th day (Fig. 9).

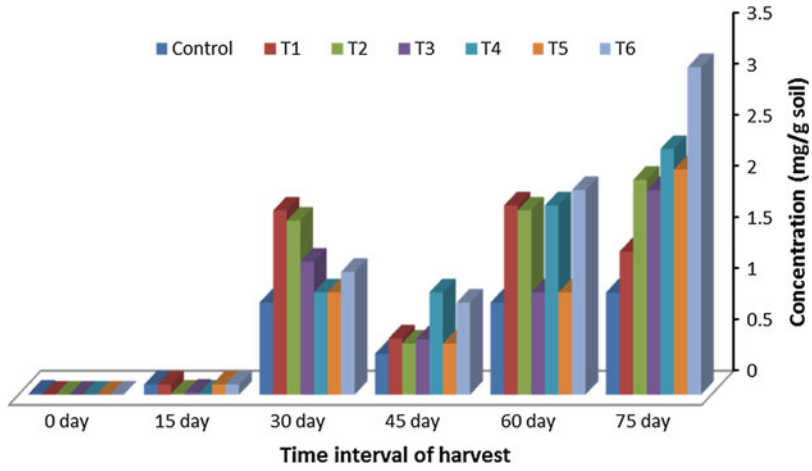

Fig. 6 Acid phosphatase activity in maize grown soil from initial and after 15 to 75th day of inoculation

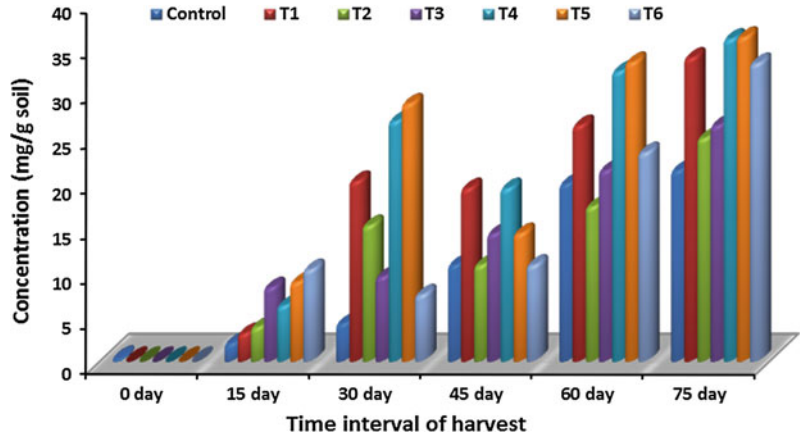

Fig. 7 Alkaline phosphatase activity in maize grown soil from initial and after 15 to 75th day of inoculation

Discussion

Morphometric Analysis

In general, growth of plants was assessed by plant fresh weight and quantifying the total length of plant described by Gamalero et al. (2004). Burd et al. (2000) had reported that PGPR might enhance the plant height and productivity by synthesizing phytohormones, increasing the local availability of nutrients by the plants. The inoculation by the combined treatments (T_6) improved the total shoot fresh weight, shoot height, number of leaves and root length during every 15 days of maize plant harvest. Also, the improved growth status caused by dual inoculation could have alleviate and also promoted the maize yield and its component when compared to the control plant. Initially, maize plant shoot fresh weight was higher in T_6 treatment compared with control that indicates the secretion of efficient growth factors induces the plant growth and nourishes it. The host plant shoot and root biomass was affected by microbial inoculum, water stress and carbon dioxide level.

The enhancing effect of seed inoculation with rhizobacteria on shoot weight and yield of maize was reported by many researchers (Shaharoona et al. 2006), such as improvement might be attributed to nitrogen fixation and phosphate-solubilizing

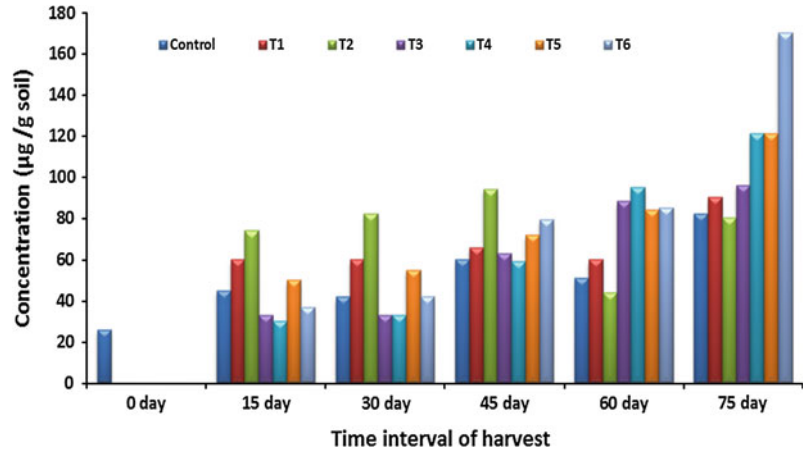

Fig. 8 Amylase activity in maize grown soil from initial and after 15 to 75th day of inoculation

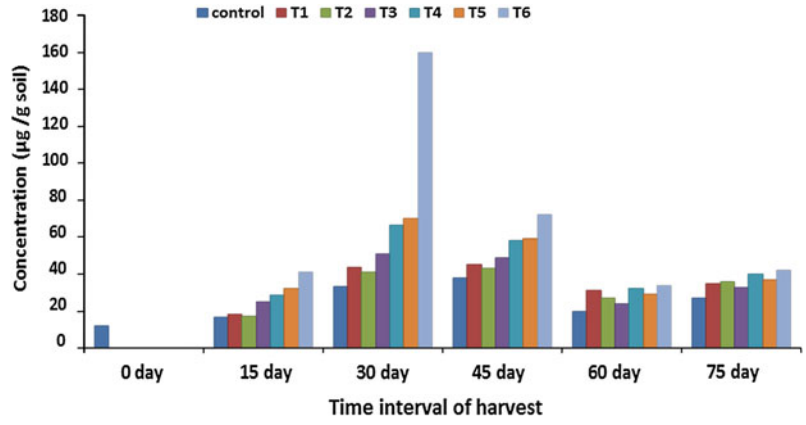

Fig. 9 Cellulase activity in maize grown soil from initial and after 15 to 75th day of inoculation

capacity of bacteria as well as the ability of these microorganisms to produce growth-promoting substances (Salantur et al. 2006). The present experiment revealed that the seed inoculation with *B. subtilis* and *P. fluorescens* (T_6) results in the increase of plant height, leaves number and fresh weight. Similar findings were reported by Siddiqui and Shaukat (2002). However, rhizobacteria-producing auxins can influence plant growth, including root development, which improves the uptake of essential nutrients, thus increasing plant growth (Vikram 2007). This may imply that rhizobacteria had more competitive ability to survive and affect the growth of inoculated plants in the presence of indigenous microflora (Khalid et al. 2004).

According to previous studies, plant growth-stimulating bacteria released some chemotaxis to root exudates, which help the plant for better growth. Regardless of PGPR mode of action, plant growth promotion depends on efficient plant root colonization. Several bacterial properties are believed to contribute to this colonization capability such as chemotaxis towards root exudates, metabolism of root exudates component, suppression of competitive microorganisms and most importantly the ability to bind with plant root surface (Motavalli et al. 2004). Munir et al. (2003) had also reported the increase in fresh weight, plant height and leaf size of plants with bacterial inoculation.

Soil Bacterial Population

Apart from effective changes in the chemistry of soil, certain factors affected the bacterial population and the activities of various enzymes present in the soil. The soil microorganisms

have an important factor on soil fertility and plant health (Gianinazzi and Schuepp 1994). Before inoculation of plant seeds with bioinoculum, the soil microbial load was as usual. But after inoculation, the microbial population was significantly increased on every 15-day examination. But their suppression has been noticed from 60th day onwards. This may be due to depleted nutrient content; hence, nutrient competition occurred between the microbial populations. Further, the changes recorded in the macronutrient content of soil may also be responsible for the reduction recorded in the bacterial population. Giller et al. (1998) have reported that the death of cells due to disruption of essential functions and to move gradual changes in population sizes for their viability or competitive ability. Chemical signalling between compatible host and microorganisms like PGPR and AMF triggers a chain of reaction preceding successful root colonization (Buee et al. 2000). Chemotaxis is responsible for competitive colonization by extracellular PGPR. Root exudates such as carbohydrates and amino acid act are chemoattractant and thereby stimulate PGPR to root surfaces (Somers et al. 2004). The observed results showed that the combined treatment of T_6 an attractive and efficient biological system to augment the macronutrient and water availability to the plants.

AMF Colonization and Spore Population

Mycorrhizae are fungi that form mutually beneficial relationship commonly found with plant roots. AMF colonization was greatly affected by a change in the microbial population. Mycorrhizae helper bacteria have been described for the ectomycorrhizal symbiosis (Garbaye 1994). Plants with highest root AMF colonization showed maximum growth. In contrast, the lowest growth was found in the plants that had the lowest AMF colonization (Marulanda et al. 2003). Comparatively, the percentage of hyphal colonization was greater in treatment of T_3 and in combined treatment of T_6, when compared with dually inoculated plants, T_3 and T_6 was greater than T_4 and T_5 treatments. While the decrease recorded in total mycorrhizal percentage in all treatment was due to the general reduction recorded in percentage of vesicle and arbuscule. Among the structures, arbuscules are the most important one for the nutrient transfer from fungus to the roots, and vesicle is the storage organ for the fungus.

The reduction recorded in percentage of vesicle in all the treatments is the clear indication of reduced supply of nutrients from the plant source to the fungi. The reduction recorded in the percentage of the arbuscule and its total absence in all the treatments may be due to the inability of mycorrhizae to re-establish arbuscule, the structure that undergo degeneration and regeneration within a short lifespan (Bonfante-Fasolo 1984). The result of which would be a decrease or absence of nutrient transfer from soil to the plant. Since maize is a highly mycorrhizal dependent plant, the reduction in plant biomass might be due to the decrease in colonization. It is possible that under field condition where interaction with other microorganisms takes place, a negative effect on plant growth could occur. Extra radical mycelium helps the plant to take up water and nutrients and increases the defences against soil pathogenic microorganisms.

Effects of PGPR on AMF Spore Germination and Hyphal Growth in In vitro condition

Offre et al. (2007) have suggested that the bacteria preferentially associated with mycorrhizal roots were at least not deleterious or even beneficial towards AMF and the symbiosis. Among both bacterial effects always, the reference species of *P. fluorescens* gave the positive effect on AMF spore germination. Frey-klett et al. (2007) have reported that mycorrhizal helper bacteria could have evolved selective mechanism of interaction with their microbial surroundings having neutral or positive effect on their host mycorrhizal association, but negative effects on

root pathogens that might threaten their habitat. There were no spores germinated in the presence of *B.subtilis*. The spore germination was inhibited intensively with the vigorous growth of *B. subtilis*. This indicates that some volatile compounds derived from *Bacillus* might be generated to not only inhibit the spore germination but also inhibit hyphal extension. Therefore, *B.subtilis* had an inhibitory effect on spore germination and hyphal extension. In combined treatment, the germination rate was found to be nil, and this might be due to the presence of *Bacillus* sp., which will inhibit the germination.

Soil Macronutrients Analysis

The acquisition of nutrients from soil is governed by the root growth and its interaction with abiotic and biotic components of soil. This interaction is largely due to the physical, chemical and biological properties of the rhizosphere (Hinsinger et al. 2009). The dual inoculation of rhizobacteria and mycorrhizae resulted in an increase of soil nutrients. However, the results imply that the increase was not directly induced by the activity of soil microorganisms (Wu et al. 2005). Barea et al. (2002) have suggested that microbial inoculation improved biomass and phosphorus accumulation in plants with dual inoculation (T_4 and T_5) being most effective. Phosphorus is the major nutrient for plants and microorganisms. Initially, the soil was fairly poor in soil available phosphorus. But available phosphorus in soil was increased with inoculation of AMF (T_3) and in combination with PGPR (T_4 and T_5). Native soil phosphorus is mostly unavailable to crops because of its low solubility. Therefore, the AMF colonization and P-solubilizing bacteria play an important role in improving phosphorus bioavailability.

The beneficial effect of AMF-associated plants for nutrient acquisition has been well documented (Duponnois and Plenchett 2003). Harrison (2005) has suggested that in AM symbiosis, plants receive all their phosphorus via fungal symbionts. There was no difference of available potassium in soil between the treatments. However, the inoculation of beneficial microbes exerted a stimulating effect on potassium uptake by the plants. Combined inoculation of T_6 treatments seemed to be the most effective to improve the plant nutrient uptake. Nitrogen concentration in plants under different treatments ranged from 121.8 (control) to 154 kg/acre (T_6). Although the combined inoculation (T_6) showed unexpectedly low N content in 30th day, the fungi still assisted the host to assimilate maximum total N and resulted in a higher biomass. The maximum NPK assimilation was obtained with the combined inoculation of T_6 treatment.

Biochemical Profiles

Plant leaves are metabolically the most active parts, wherein the atmospheric carbon is fixed as carbon skeletons part of which are subsequently modified by the introduction of N from soil source, thereby the growth and metabolic activities are ensured and sustained (Griffith 1975). The total chlorophyll content present in different treatments was analysed periodically, and this study reveals that in all treatments, T_4 treatment showed higher chlorophyll content which increased significantly on every harvest. The dual inoculation of *Pseudomonas* and AMF (T_4) enhanced the growth of host plant. The observed reduction in the chlorophyll content of maize leaves on the *Bacillus*-treated plant might be due to the interference of certain factors in the biosynthesis of chlorophyll and possibly due to the reduction recorded in N content. Plants provide a framework for chloroplast. The protein content in leaves showed an increasing trend mostly in AMF-treated plants (T_3) and in combined treatments (T_6). Therefore, the observed reduction in the protein content of various other treatments may also be considered as a reason for the decrease in chlorophyll content recorded.

The carbohydrate content is increased in maize leaves on *Pseudomonas* treatment along with AMF (T_4), therefore may be attributed to

increased chlorophyll content of leaves. From this, it is known that translocation of sugars from leaves and synthesis of proteins is greatly increased. Thus, the findings further confirm that the effect of maize growth enhanced well by means of *Pseudomonas* along with AMF (T_4), when compared to the *Bacillus* + PGPR (T_5) as well as in the combined treatment T_6. Plants secrete certain metabolic compounds like phenol, flavonoids, aromatic compounds that are produced in order to overcome the pests and insect attack. Mostly the *P. fluorescens* individually as well as in dual t and in combined treatment showed an efficient synthesis of phenolic compounds. Particularly, *Pseudomonas* played a beneficial activity in soil by inducing plant defence mechanism against plant pathogen. Deepti et al. (2009) have reported that antifungal metabolites producing PGPR did not necessarily interfere with AMF symbiosis and may even promote it, thus carefully chosen the combinations of such bioinoculants could lead to better plant growth.

Enzymes Activities

Amylase is mainly considered as an extracellular enzyme of microorganisms (Hoffman and Hoffman 1955). Phosphatase exists as both an extracellular and an intracellular enzymes (Brookes et al. 1984) but cellulase an extracellular enzyme in soil (Stevenson 1968). The activities of all the three enzymes were higher in combined treatments as well as in dual treatments. The degree of activity in single treatments is found to be comparatively lower than the combined treatments. Further, the changes recorded in the macronutrient contents in soil may also be responsible for the changes observed in the activities of soil enzymes. Reduction in the level of enzyme activities in soil may be due to (1) masking the active groups; (2) by protein denaturation; (3) by other effects on enzyme configuration; (4) decreased level of contribution from microorganisms; and (5) failure of the resistant organism to elaborate the enzyme. In view of the observed reduction in the bacterial population, a reduction in the level of contribution of enzymes from microorganisms could be envisaged. Of the various enzyme studied, amylase and cellulase are the enzymes involved in the hydrolysis of polysaccharides. Phosphates brought about the hydrolysis of organically bound phosphorus. Microbial biomass and activity are generally closely related, because it is through the biomass that the transformations of the important organic element occur (Frankenberger and Dick 1983). The observed decrease in NPK values in soil may be the result of failure to compensate the reduction caused by the plant uptake through microbial activities of the soil.

To conclude it is identified that inoculation of PGPRs like *P. fluorescens* and *B. subtilis* in single, dual and combined treatments might adversely affect the formation of AMF associations in soil. Under in vitro condition, *P. fluorescens* stimulated the germination and hyphal growth of AMF spores, which suggests that certain stimulatory compounds produced by the *Pseudomonas* sp. diffused into the agar, thereby promotes the AMF germination, whereas *B. subtilis* inhibits spore germination and hyphal growth under in vitro conditions. The combined treatment showed a little emergence of germ tube, and the prolonged incubation may enhance the spore germination. From this, the application of *B. subtilis* acting as a biocontrol agents inhibits the growth of fungal plant pathogens. *Pseudomonas fluorescens* is thought to represent a valid alternative for the use of chemical pesticide to protect plants against soil-borne diseases and act as a bioinoculants. AMF can reduce root diseases caused by soilborne pathogens. Antimycorrhizal metabolites released by PGPR in rhizosphere do not seem to affect the growth and symbiotic establishment of AMF.

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Enhancement of Soil Fertility Through Agro Inputs on Response to Cover Crop of *Crotalaria juncea* L.

M. Devi, E. King Solomon, D. Nivas, and S. Chandru

Abstract

Soil is a fundamental natural resource on sustainable agricultural and economic development, to retain the soil fertility by only through various agro inputs. The green-manure crop in agro ecosystem is *Crotalaria juncea* (Sunn hemp), because it has the ability to produce large biomass, potential to build organic matter levels, to involve in carbon sequestration, to fix large amounts of nitrogen, to reduce soil erosion and to recycle plant nutrients. Knowledge about changes of soil nutrient status in rhizosphere soil and phytochemical characteristics in *C. juncea* crop is important to understand the soil fertility management. In this present study, a field experiment of *C. juncea* is performed with compost, chemical fertilizer (Urea and superphosphate) and bioinoculants (AMF, phosphobacteria, *Trichoderma viride* and *Azospirillum* sp.) are used individually and combined to investigate the soil nutrient, morphological growth, soil enzymes and phytochemical status are compared. Combined usage of chemical and biofertilizers (T_6) proves to be more effective in the soil fertility than the other combinations. In the order of these agro inputs, the importance of green manure for tropical organic cropping for soil fertility is highlighted.

Keywords

Bioinoculants · Chemical fertilizers · Crotalaria juncea · Phytochemical status · Soil enzymes

Introduction

Soil is a natural media for plant growth which is made up of four basic components such as minerals, air, water and organic matter. The majority of soil minerals are represented around 45 % of total volume, water and air around

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25 % each and organic matter from 2 % to 5 % and arbitrates numerous ecological processes by which interacts closely with water, air and plants (Doran and Parkin 1994). Soil quality is the capacity to function within natural or managed ecosystem limits, to sustain plant and animal productivity, maintain or enhance water, air quality and support human health and habitation (Karlen et al. 1997). Soil quality can be determined only with a soil indicators, that is, physical, chemical and biological categories depending upon soil function. An interaction between soil physicochemical properties and biotic factors is the outcome of a complex environment, which is directly proportional to each other (Griffin 1969).

Soil fertility will decline by several reasons as farming practices, including burning crop residues, leaving soil as empty, unprotected from the sun and wind. Climate change is expected to bring more extreme weather events such as flooding, drought and more unpredictable weather. Excessive or insufficient use of fertilizers and improper crop rotations also lead to declining soil fertility. Reason for decline of soil organic matter levels is poor on-farm management of soil organic matter along with suboptimal use of organic and biological nutrient sources, combined with the concurrent soil nutrient mining and poor water management. Over usage and inefficient utilization of mineral fertilizers result in deterioration of environmental quality; many areas in the region witnessed both the conditions of inadequate availability and affordability of key agricultural inputs like mineral fertilizers.

There is a serious concern about the overdose of fertilizers as shown by the general trend of some farmers to misuse and apply excessive amounts of inorganic fertilizers especially urea, which will likely to add deeper problems for soil fertility. In addition to that soil fertility it can also often be enhanced through mulching process by covering the soil surface with a layer of organic material. Mulches are particularly useful to prevent soil damage, because they reduce the destructive effect of raindrop impact on soil aggregation and soil erosion. It also improves soil and water status by increasing infiltration rates and also stimulating growth of soil biota (Gershuny and Smillie 1986).

Intercropping also helps to prevent erosion and soil surface damage. Organic wastes through the process of composting and soil microbes also involve in many beneficial roles to maintain soil fertility (Francis 1986). Crop productivity may be enhanced by adding some beneficial microorganisms into the soil. Those species that fix nitrogen, enhance phosphorus availability nutrition, dissolve minerals and increase mineralization of soil organic matter. The widely used strains for the nitrogen-fixing bacterium as *Rhizobium*, *Azotobacter*, *Azospirillum* and the mycorrhiza fungi which enhance plant phosphorus nutrition (McGuinnes 1993).

Crop rotation can also decrease the need for inorganic supplements such as grains, legumes, nutrients added to retain the soil structure and fertility. Some crops are grown solely for the purpose of enhancing soil fertility. These crops are not harvested, but rather they are plowed into the soil, while they are still growing are referred to as green manures. Typically, green manures or cover crops are grown in rotation or as "improved fallow" and are mixed into the soil before seeding the subsequent crop, allowing enough time for the decomposition of residues and the mineralization of nutrients. Green manures serve many different purposes in soil fertility: improve soil physical structure, to prevent erosion, and improve soil nutrient status (Sarrontino 1997).

Crotalaria juncea L. (Sunn hemp) is an annual legume crop that produces over 5,000 pounds of biomass and over 100 pounds of nitrogen per acre, resistant to nematodes and can grow on droughty soil with low fertility; moreover, it is a green-manure crop used to conserve the soil ecosystem owing to rapid growth and requirement of short growing seasons. The present study is to investigate the soil fertility improvement through morphology of crop, phytochemical and physicochemical nature of the soil before and after treatment, activity of enzymes and enumerate the rhizosphere microbial communities with various

Table 1 Treatment combinations used in this study

Treatments	Infertile soil	Compost	Chemical fertilizer* (each 2 grams)	Bioinoculants (each 2 grams)
T_1	✓	–	–	–
T_2	✓	✓		
T_3	✓	–	✓	–
T_4	✓	–	–	✓
T_5	✓	✓	–	✓
T_6	✓	–	✓	✓
T_7	✓	✓	✓	✓

Infertile soil (T_0), control (T_1), compost (T_2), chemical fertilizer (T_3), biofertilizer (T_4), compost and biofertilizer (T_5), chemical and biofertilizer (T_6), compost, chemical and biofertilizer (T_7) chemical fertilizers (Urea and superphosphate); bioinoculants (AMF, phosphobacteria, *Trichoderma viride* and *Azospirillum* sp.)

chemical, organic and microbial inputs to *C. juncea* soils.

Materials and Methods

Study Site, Soil Sampling and Processing

The cover crop of Sunn hemp (*Crotalaria juncea*) seed was sown in polybags and the entire experiment was carried out in complete randomized block design under nursery conditions with each treatments having five replicates for compost soil, chemical fertilizers (Urea and superphosphate), Bioinoculants (AMF, phosphobacteria, *Trichoderma viride* and *Azospirillum* sp.) were applied in different combinations (Table 1) and allowed to grow for 30 days. *Crotalaria juncea* rhizosphere soils were collected directly from the plant under different treatments and sieved through a 2-mm sieve to remove litter and unwanted pebbles, dried and stored at 4 °C for further analysis.

Determination of Plant Growth Parameters and Phytochemical Characters

The *C. juncea* shoot height, root length and number of leaves were measured in each treatment after 30 days. The shoot height was recorded from soil ground level to tip of the shoot height, and root length was recorded from below the soil level to the root cap, expressed in centimeters. The number of leaves present in the plant after harvesting stage was counted and recorded.

Estimation of Chlorophyll Content

One gram of fresh *C. juncea* leaves were crushed with 80 % ethanol using mortar and pestle, till the disappearance of color, and the volume was adjusted up to 100 ml with 80 % ethanol and transferred to the conical flask wrapped with an aluminum foil to prevent photo oxidation of pigments. The absorbance was measured at 645 and 663 nm on spectrophotometer.

Estimation of Carbohydrate

Carbohydrate was estimated through Anthrone's method; extract was prepared by grinding of 0.1 g of fresh *C. juncea* leaves and hydrolyzed with 5 ml of 2.5 N HCl condensed on a water bath for 3 h, and it was neutralized by the addition of sodium carbonate (to be added till the effervescence ceases); then, it was made up to 100 ml and centrifuged. The supernatant was taken and 4 ml of Anthrone's reagent was added and kept in a boiling water bath for 10 min. Then, the tubes were cooled with running tap water and the absorbance was read at 630 nm.

Protein Estimation

Fresh *C. juncea* leaves were homogenized in 10 ml of cold phosphate buffer. The extract was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was used as the further source for protein estimation. One ml supernatant was added to 5 ml of alkaline copper solution and incubated for 10 min and after incubation 0.5 ml of Folin–Ciocalteu reagent was added to the mixture, the peptide linkages are broken down and give a violet color, was measured at 660 nm.

Enumeration of Microbial Population

The serial dilution plate technique was employed to count the rhizosphere soil microbes; One gram of sieved rhizosphere soil was dissolved in 10 ml of distilled water, serially diluted till 10^{-7} dilution and inoculated in nutrient agar for bacteria, Sabouraud's dextrose agar for fungi and starch casein agar for actinomycetes, respectively. The inoculated bacteria and fungi plates were incubated at 37 °C for 24 h, and actinomycetes were incubated for 5 days. After incubation period, the colonies were counted and expressed as colony forming units.

Analysis of Soil Nutrients

The soil nutrient status was analyzed in soil testing laboratory, and following procedure was adopted for analysis. Organic carbon in the soil was estimated by the modified Walkley–Black method (Walkley and Black 1934); and total nitrogen (Subbiah and Asija 1956), available phosphorus, exchangeable potassium by flame photometry (Knudsen et al. 1982); calcium and magnesium combinations were estimated by direct titration with EDTA and sulfur by using heat-stable sulfur method (Williams and Steinbergs 1959). Micro nutrients such as Zn, Cu, Mn and Fe were estimated by a method using DTPA (Diethylene Triamine Penta Acetic Acid) developed by Lindsay and Norwell (1978).

Soil Enzyme Activity

Amylase Assay

To determine the amylase activity, 1 g of soil with 10 ml of phosphate buffer (pH 6.9) was centrifuged at 5,000 rpm for 10 min and 1 ml of supernatant was added with 1 ml of substrate, incubate for 15 min, and the reaction was stopped with 2 ml of DNS (Di nitro salicylic acid) reagent and incubated on boiling water bath for 5 min, and then, 1 ml of potassium sodium tartarate (40 %) was added and made up to 10 ml through distilled water and read the absorbance at 540 nm (Tabatabai 1994).

Cellulase Assay

One gram of soil suspended with sodium citrate buffer (pH 5.0) was centrifuged at 5,000 rpm for 10 min, and the filtrate 0.05 ml supernatant was mixed with 0.45 ml of substrate 1 % carboxy methyl cellulose and allowed to react at 50 °C on boiling water bath for 15 min, and the reaction was stopped with 0.5 ml of DNS and reincubated for 5 min on boiling water bath. After incubation, 1 ml of potassium sodium tartarate (40 %) was added and read the absorbance at 540 nm (Tabatabai 1994).

Total Phosphatase Assay

In 0.5 ml of enzyme extract filtrate from 1 g of soil with buffer solution and 3 ml of substrate was added and incubated for 15 min, 0.5 ml of incubated solution was taken and 9.5 ml of NaOH was added to stop the reaction and read the absorbance at 410 nm (Kuperman and Carreiro 1979).

Statistical Analysis

All obtained results were statistically calculated using the one-way Analysis of Variance

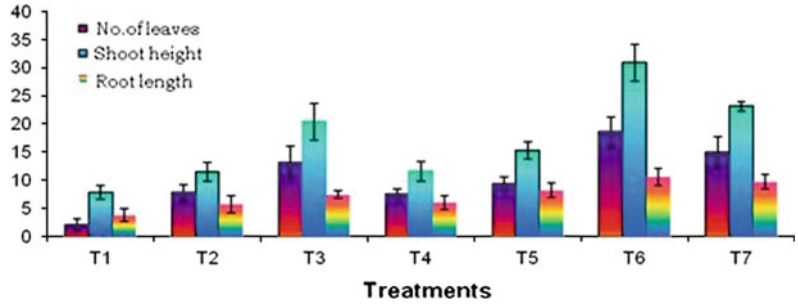

Fig. 1 Plant morphology characters of agro inputs treated *C. juncea*. Infertile soil (T_0), control (T_1), compost (T_2), chemical fertilizer (T_3), biofertilizer (T_4), compost and biofertilizer (T_5), chemical and biofertilizer (T_6), compost, chemical and biofertilizer (T_7)

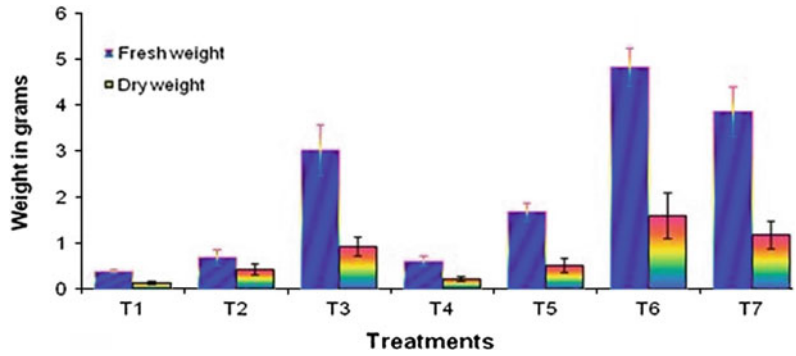

Fig. 2 Plant biomass of agro inputs treated *C. juncea*. Infertile soil (T_0), control (T_1), compost (T_2), chemical fertilizer (T_3), biofertilizer (T_4), compost and biofertilizer (T_5), chemical and biofertilizer (T_6), compost, chemical and biofertilizer (T_7)

(ANOVA) and Pearson's correlation, and the results of each treatment were compared with one another to assess the relationships between each parameter. All the calculations were performed using SPSS.

Results

Plant Growth Parameters

C. juncea plant morphometric characters showed tremendous changes in soil after it was treated with various agro inputs in the case of plant biomass and plant growth parameters such as number of leaves, shoot height and root length, T_6 (chemical fertilizer and bioinoculants) showed highest rate than all other treatments. Subsequently, T_7 and T_3 showed higher rate, but T_7 was higher than T_3. Addition to that, treatments of T_5, T_4 and T_2 showed good response than control (Figs. 1 and 2). Shoot height was significantly ($p < 0.01$) correlated with chlorophyll *a* ($r = 0.90$), carbohydrate ($r = 0.96$), protein ($r = 0.97$). Number of host plant leaves were significantly ($p < 0.01$) correlated with chlorophyll *a* ($r = 0.96$) and chlorophyll *b* ($r = 0.83$) at the significance level of $p < 0.05$. Carbohydrate ($r = 0.99$), protein ($r = 0.99$), shoot height ($r = 0.98$), root length ($r = 0.95$) were positively correlated at the significant level of $p < 0.001$; and root length was positively correlated with chlorophyll *a* ($r = 0.96$) at the significant level of $p < 0.01$, chlorophyll *b* ($r = 0.76$) at significant level of $p < 0.05$, and carbohydrate ($r = 0.92$), protein ($r = 0.94$), shoot height ($r = 0.93$) were significantly correlated at 0.01 level. Fresh weight

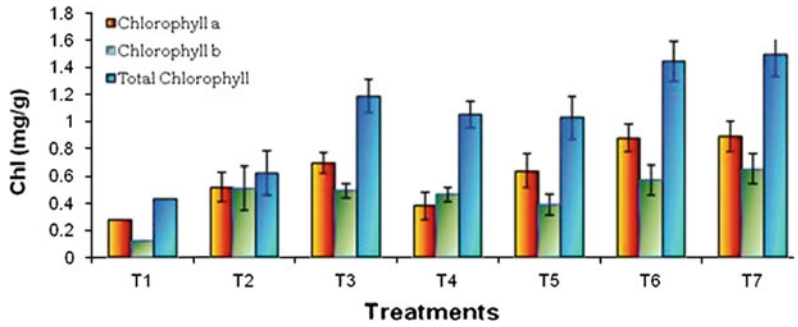

Fig. 3 Agro inputs treated C. *juncea* plants chlorophyll level. Infertile soil (T_0), control (T_1), compost (T_2), chemical fertilizer (T_3), biofertilizer (T_4), compost and biofertilizer (T_5), chemical and biofertilizer (T_6), compost, chemical and biofertilizer (T_7)

was positively correlated with chlorophyll *a* ($r = 0.90$) at $p < 0.01$ level and significantly ($p < 0.05$) correlated with total chlorophyll ($r = 0.78$) also positively correlated with carbohydrate ($r = 0.95$), protein ($r = 0.96$), shoot height ($r = 0.99$), root length ($r = 0.92$) and number of leaves ($r = 0.95$) at the significant level of $p < 0.01$. Dry weight was positively correlated with chlorophyll *a* ($r = 0.90$) and *b* ($r = 0.76$) at the level of significance $p < 0.001$ and $p < 0.005$, respectively. Moreover, that the dry weight was significantly ($p < 0.01$) correlated with carbohydrate ($r = 0.95$), protein ($r = 0.96$), shoot length (0.99), root length ($r = 0.91$), number of leaves ($r = 0.96$) and fresh weight ($r = 0.99$).

All the plant morphometric characters have been correlated with bacterial population, such as shoot height ($r = 0.83$), root length ($r = 0.82$), number of leaves ($r = 0.76$), fresh weight ($r = 0.76$), and dry weight ($r = 0.78$) was significant level at $p < 0.05$.

Phytochemical Characters

The highest amount of chlorophyll present in T_7 (compost, chemical fertilizer and bioinoculants) (1.48 mg/g) and T_6 (1.4 mg/g), both has minor differences. Next to that T_3 had showed higher amount of chlorophyll (1.185 mg/g) and T_4 (1.04 mg/g), T5 (1.02 mg/g) and had similar amount of chlorophyll, which was compared with control (T_1 − 0.043 mg/g) (Fig. 3). The highest amount of carbohydrate and protein were found in T_6 (chemical fertilizer and bioinoculants treated soil) with 137.5 and 108.7 mg/g, respectively (Fig. 4). Chlorophyll *b* was positively correlated with chlorophyll *a* ($r = 0.85$) significantly at $p < 0.05$ level, and carbohydrate was positively correlated with chlorophyll *a* ($r = 0.95$), chlorophyll *b* ($r = 0.85$) at $p < 0.01$ and $p < 0.05$ levels, respectively, and protein was positively correlated with chlorophyll *a* ($r = 0.96$) which was significant at the level of $p < 0.01$, and chlorophyll *b* ($r = 0.82$) was significant at $p < 0.05$. Simultaneously, carbohydrate ($r = 0.64$) significant level was at $p < 0.01$.

Microbial Populations

Bacterial population in the treated soil has drastically increased than infertile soil (T_0). Treated plants of T_6 (chemical fertilizer and bioinoculants) have the highest number of bacterial population, when compared with others. Next to that, T_7 and T_5 plants showed high bacterial population. Treatments of T_2, T_3 and T_4 exhibited significant difference among themselves, but it was higher than control (T_1). According to fungal population, the treated soils showed high numbers in T_7, T_6, T_4 and T_2, when compared with an infertile soil. Whereas, T_5 showed equal number of population with infertile soil. T_1 and T_3 had less number of fungal populations than infertile soil (T_0). In the case of

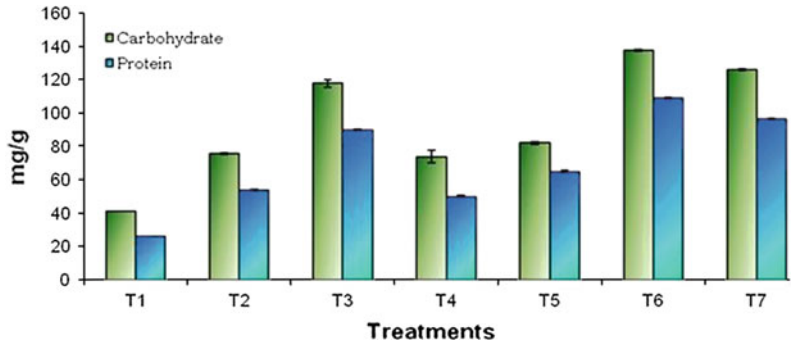

Fig. 4 Agro inputs treated C. *juncea* plants carbohydrate and total protein content. Infertile soil (T_0), control (T_1), compost (T_2), chemical fertilizer (T_3), biofertilizer (T_4), compost and biofertilizer (T_5), chemical and biofertilizer (T_6), compost, chemical and biofertilizer (T_7)

Table 2 Microbial populations between untreated and treated soils

Microbial populations	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7
Bacteria (cfu/g soil)	18	36	46	55	60	100	185	80
Actinomycetes (cfu/g soil)	30	4	2.8	3.6	3.6	6.4	3.8	4
Fungi (cfu/g soil)	2	1.6	2.4	1	2.4	2	2.8	3.2

Infertile soil (T_0), Control (T_1), Compost (T_2), Chemical fertilizer (T_3), Biofertilizer (T_4), Compost and Biofertilizer (T_5), Chemical and Biofertilizer (T_6), Compost, Chemical and Biofertilizer (T_7)

actinomycetes population, all the treatments showed less infertile soil. Compared with treated soils, T_5 has high number of actinomycetes than others. The T_6, T_3 and T_4 treatments showed negligible number of difference between them. Moreover, T_1 and T_7 showed equal number of actinomycete (Table 2).

Soil Nutrient Analysis

The soil nutrient levels have been increased considerably with level of pH that greatly increased in treated soil (7.15–8.06) than in infertile soil (pH 5.68). Treated plants of T_7 and T_6 soils showed alkaline nature. The pH was positively correlated with chlorophyll *b* ($r = 0.77$), carbohydrate ($r = 0.77$), shoot length ($r = 0.82$), number of leaves ($r = 0.77$), fresh ($r = 0.84$), and dry weight ($r = 0.86$) was significant at the level of $p < 0.05$. In the case of electrical conductivity (EC), it has been decreased in all the treated soils, while compared with infertile soil (0.37 dSm^{-1}). Treatments of T_2 (0.31 dSm^{-1}) and T_4 (0.35 dSm^{-1}) have small changes in EC content, but lower than infertile soil. Organic carbon and organic matter were increased in the treated soil than in the infertile soil (0.25 % and 0.50). Organic matter was correlated with organic carbon ($r = 0.99$) and at significant level of $p < 0.01$. The total nitrogen was high (131.28 kg/acre) in T_6 (chemical fertilizer and bioinoculants). Soil nitrogen was positively correlated with chlorophyll *a* (0.79), protein ($p < 0.77$), shoot height ($r = 0.79$), root length ($r = 0.80$), fresh weight ($r = 0.83$) and dry weight (0.83) at the significance of $p < 0.05$.

The amount of available phosphorus was reduced in the treated soil than in the infertile soil (5.5 kg/acre). And high amount of available phosphorus (4.33 kg/acre) was found in T6 (chemical fertilizer and bioinoculants)-added soil than the other treated soils 3.94, 3.46, 4.01, 3.47, 3.32, 3.56 kg/acre for T_1, T_2, T_3, T_4, T_5 and T_7, respectively. The amount of exchangeable potassium was increased in the treated soil than in the infertile soil (85 kg/acre). Potassium was negatively correlated with chlorophyll *a* ($r = 0.90$), carbohydrate ($r = 0.79$), protein

($r = 0.81$), root length ($r = 0.83$) and number of leaves ($r = 0.83$) at the significant level of $p < 0.05$. Soil zinc level was increased in treated soil than in the infertile soil (0.84 ppm). Treated soil copper and iron also increased in amount than the infertile soil (0.20 ppm and 2.15 ppm). The copper level showed high in T_1 control soil (1.27 ppm and 8.87 ppm) than other treated soils. The soil manganese was low in treated soil than in the infertile soil (4.01 ppm). Soil calcium and magnesium have been increased in the treated soil than in the infertile soil (19.60, 9.50 and 5.80 C. Mole $Proton^+$/kg, respectively). T_7 (the compost, chemical fertilizer and bioinoculants)-treated soil showed the high amount (25.42, 13.46 and 11.70 Mole $Proton^+$/kg) of CEC, calcium and magnesium, respectively. CEC was positively correlated with total chlorophyll ($r = 0.93$) and copper ($r = 0.76$) at the significant levels of $p < 0.01$ and $p < 0.05$. Calcium was positively correlated with total chlorophyll ($r = 0.77$), copper ($r = 0.82$) and manganese ($r = 0.90$) significantly at $p < 0.01$. In addition, calcium was negatively correlated with EC ($r = 0.76$) and showed significant level at $p < 0.05$. Magnesium was positively correlated with total chlorophyll ($r = 0.92$), at the significant level of $p < 0.01$. Moreover, it was significantly correlated with fresh weight ($r = 0.78$), pH ($r = 0.76$) and calcium ($r = 0.83$). Sodium was positively correlated with copper ($r = 0.79$), manganese ($r = 0.85$) and magnesium ($r = 0.81$) at significance level of $p < 0.05$ and calcium at $p < 0.01$, and it was also negatively correlated with EC ($r = 0.77$) at the significant level of $p < 0.05$. Exchangeable potassium has significantly ($p < 0.05$) correlated with total chlorophyll ($r = 0.77$). The amount of sodium and potassium has been increased in the treated soil than in the infertile soil (0.15 and 0.04) (Table 3).

Soil Enzymes

The amylase activity was decreased in all treatments than in infertile soil (T_0 – 3.075 units/ml). The quantity of amylase present in T_1 (8.29 units/ml) was found to be high than other treatments; T_7 (7.65 units/ml) and T_4 (7.59 units/ml) was next high amount of amylase in the treatments. The amylase activity was significantly ($p < 0.05$) correlated with manganese ($r = 0.85$). In soil cellulase enzyme activity, the high amount of enzyme was found in T_4 (8.76 units/ml) and it was drastically reduced T_6 (1.02 units/ml) (Fig. 5). In acid phosphatase, all the treated soil showed less amount of enzyme activity, when compared with infertile soil (2.2 units/ml). Acid phosphatase activity was negatively correlated with bacterial population ($r = 0.92$) at the significant level of $p < 0.01$. In alkaline phosphatase, all the treated soil showed less amount of enzyme activity than infertile soil (1.9 units/ml). Moreover, alkaline phosphatase activity was negatively correlated with dry weight ($r = 0.77$) and pH ($r = 0.87$) at significance level of $p < 0.05$.

Discussion

Soil fertility research in recent years has been focused as to shift toward the combination of organic and inorganic nutrient sources to reverse the negative nutrient balances with cropping system of agriculture (Vanlauwe et al. 2001). The similar studies also tried to improve the nitrogen content in crops by the incorporation of some tropical green-manure legumes into a range of cropping systems (Yano et al. 1994; Ohdan et al. 1995; Daimon and Kotoura 2000). Plants grown from inoculated seeds displayed higher total fresh and dry weights and biomass partition to the plant (Barassi et al. 2006), as our present study with the cover crop of *C. juncea* improved the soil fertility.

According to the chlorophyll in phytochemical analysis, if the rate of chlorophyll content increase, it will lead to an increase in the amount of carbohydrate and protein of the plant. Previous studies showed that the high Co_2 exposure on various plant species correlates in decrease the photosynthetic capacity with total insoluble carbohydrates and starch content. Carbohydrates are the energy sources for most plant

Table 3 Soil nutrient status before and after treatment

Parameters	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7
pH	5.68	7.32	7.35	7.37	7.26	7.15	8.05	8.06
EC ($dSm^{-}1$)	0.37	0.21	0.31	0.29	0.35	0.22	0.25	0.25
Organic carbon (%)	0.25	0.29	0.36	0.26	0.30	0.26	0.31	0.32
Organic matter	0.50	0.59	0.73	0.53	0.61	0.51	0.62	0.65
Available nitrogen (kg/acre)	82.6	106.28	109.32	116.7	89.36	121.54	131.2	127.12
Available phosphorus (kg/acre)	5.5	3.94	3.46	4.01	3.47	3.32	4.33	3.56
Available potassium (kg/acre)	85.0	151.2	131.4	134.0	140.8	130	126.6	131.6
Zinc (ppm)	0.84	1.24	0.78	1.08	1.14	1.08	0.96	1.00
Copper (ppm)	0.20	1.27	0.48	0.68	0.96	1.17	1.00	1.11
Iron (ppm)	2.15	8.87	4.32	5.42	4.42	5.40	5.19	5.25
Manganese (ppm)	4.01	3.4	2.23	2.51	2.56	2.40	2.80	3.29
Cation exchange capacity (C. mole $proton^{+}$/kg)	19.60	22.68	18.72	21.92	21.94	23.18	24.04	25.42
Calcium (C. mole $proton^{+}$/kg)	9.50	13.38	9.58	10.62	10.36	11.64	12.64	13.46
Magnesium (C. mole $proton^{+}$/kg)	5.80	10.64	8.52	10.06	9.68	10.60	12.46	11.70
Sodium (C. mole $proton^{+}$/kg)	0.15	2.36	1.34	1.60	1.51	1.78	2.20	2.04
Potassium (C. mole $proton^{+}$/kg)	0.04	0.25	0.22	0.28	0.27	0.24	0.28	0.26

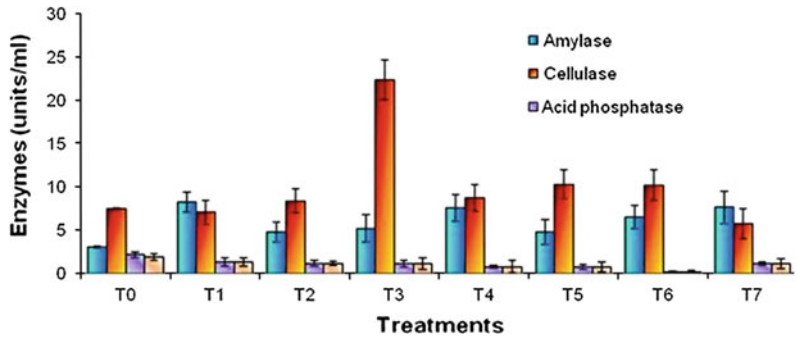

Fig. 5 Agro input treated *C. juncea* plant rhizosphere soil enzyme levels. Infertile soil (T_0), control (T_1), compost (T_2), chemical fertilizer (T_3), biofertilizer (T_4), compost and biofertilizer (T_5), chemical and biofertilizer (T_6), compost, chemical and biofertilizer (T_7)

physiological processes such as respiration and cell growth, and sugars have important hormone-like functions as primary messengers, due to their essential role in plant growth, development and metabolic links with primary physiological processes (Rolland et al. 2002). On hypothesis that increased carbohydrate contents in plant tissue affect repression of genes; encoding expression of RuBisCO and other photosynthetic proteins under elevated Co_2 conditions has been proved by number of previous studies (Nie et al. 1995; Moore et al. 1999).

The microbial population of bacteria and fungi showed high level in the treated soil than in the infertile soil. In the case of actinomycetes, population showed very less in all treatment than in infertile soil. The same type of result has been

reported for soil bacterial populations (Brady 1984). The high fungal counts might be due to the base nature of the soils pH above 7.15–8.06 (Moore 1990). Actinomycete has been on the increase due to the acidic nature of the soils that favor for the proliferation. Soil management influences soil microorganisms and soil microbial processes through changes in the quantity and quality of plant residues entering the soil, and its spatial distribution. In general, it has been accepted that there is a decrease in microbial activity with the increase in cultivation. This is similar to that was observed by Gupta and Germida (1988), who found lower enzyme activities in all aggregates size fractions under cultivated soils than native soils.

Microorganisms are important for the soil fertility, and it has the function to produce organic matter, mineral soil particles and natural nutrients from atmosphere and supply to the plants for their growth. The organic matter and content were on the increase significantly in the study report; similar to this, Danso et al. (1992) reported that the continuous application of farm yard manure (FYM) increased the organic carbon content as well as nitrogen contents. The mineral nutrients such as phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and all trace elements were broken down by the process of weathering from water, temperature changes and weak acids by the decomposition of organic matter from microorganism. Nonmineral nutrient of nitrogen (N) converts the atmospheric gas and supply to the plants. However, certain soil bacteria can change the atmospheric N for cell structure; this N was utilized by plant growth after the death of the microorganisms.

Soil nutrients interlinked with each other in our study with significant level at $p < 0.05$, and it was coincide with previous studies showed that a relation between the soil microbial biomass C, soil organic C and total N. The soil zinc has been increased in the treated soil than in the infertile soil with 0.84 ppm. The same as report was reported by continuous application of FYM at 15 t ha-I for 3 years increased zinc level from 0.48 to 0.87 % (Sankaranarayanan 2004). Calcium and magnesium have been analyzed for the soil nutrient fertility, and they are positively correlated with the chlorophyll content of the crop; this showed that soil has produced sufficient levels of Z and Mg nutrients for the plant growth. The retention of nutrients in soil is highly dependent on the cation exchange capacity of the soil, which thus is a key function for soil fertility.

In our study, the amylase and cellulase are increased in all treatments than in infertile soil, as well as the enzymes and organic carbon was directly proportional to each other. The previous studies also suggested equivalent to our results that amylase and cellulase did not decrease with soil depth and decrease in organic C as observed (Deng and Tabatabai 1996). These enzymes have an important role in residue decomposition. For example, cellulose is the most abundant compound in the biosphere, comprising almost half of the biomass synthesized by photosynthetic fixation of CO_2 (Eriksson et al. 1990). So, it is important to understand the factors that affect the degradation of cellulose in soils because the reactions involved provide readily available C for the growth of microorganisms. In acid and alkaline phosphatase, all the treatments showed reduced amount of enzyme activity, when compared with infertile soil, because the phosphatase enzyme depends upon the phosphorous when it decreases the enzyme activity also be decrease.

The similar report has been reported that there is no correlation between acid and alkaline phosphatase activities with available P, which is consistent with other studies (Eriksson et al. 1990; Baligar et al. 1999). This lack of correlation between phosphatases and available P may be due to the suppression of soil phosphatase activity from long-term application of phosphate fertilizer (about 125 kg P_2O_5 per ha per year) as suggested by Haynes and Williams et al. (1992). Thus, it seems phosphatases are stimulated when phosphate levels are low in soils. Both acid and alkaline phosphatase activity varies widely due to soil management (Dick 1994), fertilizer (Dick et al. 1988) and tillage. In

conclusion, the present study exhibits that the use to agro inputs has improved the soil fertility in various levels, even though combined chemical and biological inputs have significantly increased the *C. juncea* growth.

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Isolation and Structural Characterization of N-(naphthalene-1-yl) Propanamide, a Herbicidal Compound from *Streptomyces* sp. KA_1-3

P. Priyadharsini, D. Dhanasekaran, and B. Kanimozhi

Abstract

Totally 15 actinobacterial isolates were screened for herbicidal activity against the weed. Among the 15 isolates, only five isolates KA_1-3, KA_1-4, KA_1-7, KA_2-6 and KA_{23}A showed significant activity, particularly KA_1-3 had shown excellent herbicidal activity against *Cyperus rotundus*. The herbicidal effect of *Streptomyces* sp. KA_1-3 culture filtrates on germination and seedling growth of *C. rotundus* was severely affected when compared to control. *Streptomyces* sp. was extracted and purified. Chemical structure of the compound N-(naphthalene-1-yl) propanamide was established on the basis of spectroscopic studies such as UV, FT-IR, H^1NMR and MS. The present study concludes that *Streptomyces* sp. isolate will be used as bioherbicide against *C. rotundus.* Further studies are required to confirm the activity of N-(naphthalene-1-yl) propanamide against *C. rotundus* under field conditions.

Keywords

Streptomyces sp. KA_1-3 · Weeds · Herbicidal compound · Purification · Chemical structure

Introduction

Microorganisms being the pioneer colonizer of this earth planet, as come to stay, have cosmopolitan conglomerates of highly compatible organisms. Microorganisms with its 3.8 billion year biosynthetic experience remain nature's best chemists and treasure house for a variety of novel biologically active metabolites. The term microbial bioprospecting refers to the search of microorganisms for biologic products or the utilization of microbial cell as a whole for human benefit and environmental applications. In general, the microbial bioprospecting starts from the collection of environmental samples to the identification and application of specific bioproducts.

Of the scores of soilborne microorganisms, actinobacteria have been reported to be prolific producers of a variety of biochemicals. Herbicidal activity of the compounds excreted by the

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streptomyces group came into the limelight with the discovery of bialaphos from *S. hygroscopicus* and *S. viridochromogenes* (Ogawa et al. 1973). Anisomycin from *Streptomyces* sp. is the first microbial product used for the development of the synthetic herbicide methoxyphenone which exhibits excellent activity against barnyard grass and crabgrass (Yamada et al. 1974; Ito et al. 1974). Herboxidiene from *Streptomyces* sp. is a potent soil-based herbicide for many weed species (Issac et al. 1992). Other promising phytotoxins from *Streptomyces* sp. are isoxazole-4-carboxylic acid, nigericin, vulgamycin, ansamitocin, herbimycin and pyridazocidin (Babczinski et al. 1991; Kobinata et al. 1991; Gerwick et al. 1997).

Since weeds are opportunistic species, rapidly modifying their behavior with a high phenological plasticity to suit better any newly prevailing conditions, concern is growing among farmers and weed scientists regarding recent developments in weed control technology. There is a great fear about the future situation and what solutions will be required, if some species of weeds become well adapted to this technology. Omura et al. (1979, 1984) have reported the herbimycin, antibiotic *Streptomyces* isolates. We have already reported that the bioherbicidal activity of *Streptomyces* isolates against *Ehinochilora crus-galli* L, *Gynandropsis pentaphylla* L, *Amaranthus spinosus* L, *Cyperus rotundus* L, *Amaranthus viridis* L, *Cassia occidentalis* L and *Echinochloa oryzicola* L (Dhanasekaran et al. 2012). The present study describes the extraction, purification and the structural elucidation of the bioactive compound from a liquid culture of *Streptomyces* sp. KA_1-3. The herbicidal activity of the compound is also addressed.

Materials and Methods

Collection of Samples

The whole plant seeds of *C. occidentalis* L and rhizomes of *C. rotundus* L were collected from paddy fields in and around Tiruchirappalli. Seeds of *Sesamum indicum* L, *Eleusine coracana* L and *Zea mays* L were collected from Tamilnadu Agricultural College and Research Institute, Tiruchirappalli, Tamilnadu. The collected weeds and crops were identified based on morphological and taxonomical characteristics of stem, leaf, flower and seeds of the plant (Tadulingam et al. 1932).

Actinobacterial Collection

About 15 isolates of *Streptomyces* sp. KA_1-3 were collected from Germplasm, Department of Microbiology, Bharathidasan University; collected isolates were subcultured on starch casein agar slants and incubated at 28 ± 2 °C for 10 days and is used for further analysis.

Production of Herbicidal Compounds by Submerged Fermentation

All the *Streptomyces* isolates were inoculated on starch casein agar plates and incubated at 28 °C for 7 days for mass preparation of mycelium. After the growth, *Streptomyces* sp. mycelium was scraped and aseptically transferred into 50 ml of starch casein broth (starch 1 %; casein 0.03 %; calcium carbonate 0.002 %; potassium nitrate 0.2 %; sodium chloride 0.2 %; magnesium sulfate 0.005 %; pH 7.0 ± 0.2) prepared in 250-ml Erlenmeyer flasks and incubated in a rotary shaker at 120 rev/min for 48 h at 28 °C. The broth cultures were transferred to sterile centrifuge tubes and centrifuged at 10,000 rpm for 10 min. The supernatants were collected and used as a test sample to screen the herbicidal activity.

Extraction of Herbicidal Compound from *Streptomyces* sp.

After fermentation, the production medium was collected and centrifuged at 10,000 rev/min for 30 min at 4 °C to separate the supernatant and

Table 1 Screening for herbicidal activity of *Streptomyces* sp. KA_1-3 by moist chamber technique

Sl. No	Isolates	% of seed germination	
		Cassia occidentalis L	*Cyperus rotundus* L
1	KA_1-3	30	25
2	KA_1-7	60	33
3	KA_2-6	60	33
4	KA_2-3A	45	66
5	Control	92	87

mycelium. Extracellular metabolites present in fermentation broth were extracted by liquid–liquid extraction using equal volume of ethyl acetate in a separating funnel for 24 h. Then, the ethyl acetate portion was kept under reduced pressure for evaporation to obtain the extracellular metabolite (Radhakrishnan et al. 2007).

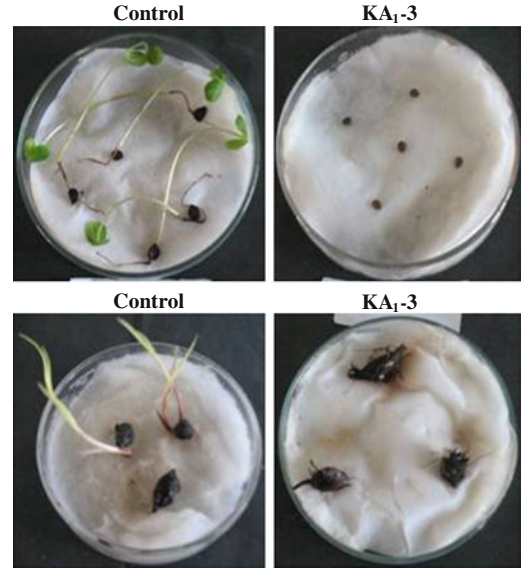

Fig. 1 Screening for herbicidal activity of *Streptomyces* sp. KA_1-3 by moist chamber technique

Screening for Herbicidal Activity of *Streptomyces* sp. KA_1-3 by Moist Chamber Technique

It was performed by placing a sterile filter paper moistened with 3 ml crude extract in a Petri dish. Fine seeds of *Cassia occidentalis* L and *C. rotundus* were placed on the each plate. The moist chamber was incubated at 28 °C for 4 days in dark and observed for the germination. Similar procedure was followed for control plate without culture filtrate. The experiment was repeated 3 times for the isolates that expressed the herbicidal activity to confirm the observation (Mallik 1997).

Screening of Herbicidal Activity of *Streptomyces* sp. by Rolled Towel Paper Assay

The rolled paper towel assay was used to evaluate the efficacy of coating crop and weed seeds with spores of *Streptomyces* sp. strain to test the growth inhibition of seeds. *Streptomyces* sp. spore suspensions were obtained by flooding 10-day-old starch casein agar plate culture with sterile distilled water. Surface sterilized seeds of crops and weeds were soaked for 30 min in *Streptomyces* sp. spore suspensions and air-dried in a laminar flow hood. The seeds were spread on a moistened sterile 26- to 30-cm paper towel. The towel was rolled, placed in a plastic bag and incubated for 5 days at room temperature. Three towels were used for each treatment, and the experiment was carried out three times.

Growth and Vigor Index of Seedlings

From each replication, seedlings were selected for at random and shoot height and root length were measured. The shoot height was measured from collar to the tip of the long primary leaf and root length from the collar to tip of the primary root, and respective mean valves are calculated. The vigor index of the seedlings was calculated by using the formula suggested by Abdualbaki and Anderson (1973).

$$\text{VI} = (\text{Root length} + \text{Shoot height}) \times \% \text{ of seed germination}$$

Table 2 Screening of herbicidal activity of *Streptomyces* sp. KA1-3 by rolled paper towel assay

Sl. No	Plants	Growth parameters	Isolate	
			KA_1-3	Control
1	*Cassia occidentalis* L	% of seed germination	25	100
		Root length (cm)	5.7 ± 1.06	9.4 ± 1.20
		Shoot length (cm)	7.4 ± 0.84	11.7 ± 0.8
		Dry weight (gm)	0.04 ± 0.1	0.05 ± 0.1
		Vigor index	327.5	2110
2	*Cyperus rotundus* L	% of seed germination	0	67
		Root length(cm)	0 ± 0	5.5 ± 4.9
		Shoot length (cm)	0 ± 0	7.7 ± 5.3
		Dry weight (gm)	0 ± 0	0.74 ± 4.5
		Vigor index	0	884.4
3	*Sesamum indicum* L	% of seed germination	100	75
		Root length (cm)	6.5 ± 1.0	5.3 ± 3.7
		Shoot length (cm)	4.7 ± 1.4	3.6 ± 2.8
		Dry weight (gm)	0.06 ± 0.2	0.03 ± 2.3
		Vigor index	1120	667.5
4	*Zea mays* L	% of seed germination	100	100
		Root length (cm)	16.3 ± 1.0	14.7 ± 1.3
		Shoot length (cm)	15.5 ± 1.2	14.3 ± 1.6
		Dry weight gm	0.09 ± 0.6	0.04 ± 0.4
		Vigor index	3180	2870
5	*Eleusine coracana* L	% of seed germination	100	100
		Root length (cm)	4.5 ± 0.4	3.6 ± 0.3
		Shoot length (cm)	4.1 ± 0.5	4.4 ± 0.5
		Dry weight (gm)	0.09 ± 0.1	0.05 ± 0.2
		Vigor index	800.6	800

Purification of the Compound by Thin-Layer Chromatography

The herbicidal compound was purified by using thin-layer chromatography. The compound was dissolved in 200 µl of ethyl acetate. With the help of capillary tube, the sample was spotted at the bottom of the silica gel, and then, it was placed in the developing beaker containing mobile phase. The solvent was allowed to run till it reaches about half a centimeter below the top of the plate. Then, the sheet was kept in a closed chamber to visualize the separated compounds as clear spots. *Rf* value of the spot separated on the TLC plate was determined. *Rf* value is equal to the movement of solute from the origin/movement of the solvent from the origin.

Herbicidal Effect of N-(naphthalene-1-yl) Propanamide

The compound was reconstituted 0.1 gm in 10 ml water, and the sample was loaded on Whatman's No. 4 filter papers inside 9-cm glass Petri plates. Filter paper was air-dried. Seeds of *C. occidentalis* and *C. rotundus* were placed over the moistened

filter paper. Similar procedure was followed for control plate with distilled water. The moist chamber was incubated at 28 °C for 7 days in dark and observed for the seed germination.

Structure Elucidation of the Compound

The melting point of the compound was determined on a Buchi-540 melting point apparatus. The UV spectra of the compound were measured at 200–400 nm using Ultraviolet–visible spectrophotometer (Shimadzu UV 1601). Infrared spectra of the compound were obtained using a Fourier transform infrared spectrometer (Bruker FT-IR instrument equipped with AT-XT Golden gate accessories). The spectra were collected within a scanning range of 400–4,000 cm^{-1}. The spectra were analyzed for various functional groups.

The proton NMR (JEOL GSX-400 MHZ-GSX 500) spectra of the compound were obtained by using a dimethyl sulfoxide as a solvent and further confirmed by mass spectroscopy (Finnigan MAT 8230). The structure of the compound was established with the help of spectral data obtained from various spectroscopic techniques. The 3D structure of the compound was obtained using ChemDraw software (Ultra 8.0)

Results

All weed and crop seeds were collected from paddy fields in Tiruchirappalli district, and fifteen *Streptomyces* isolates among that five isolates showed significant activity and one isolate *Streptomyces* sp. showed excellent activity which were obtained from Germplasm, Department of Microbiology, Bharathidasan University. The herbicidal effect of extracellular metabolites of *Streptomyces* sp. was screened for crop seeds and weeds (Table 1 and Fig. 1). One isolate was chosen for further herbicidal activity. The *Streptomyces* sp. biomass–treated crop seeds such as *S. indicum*, *E. coracana*, *Z. mays* and weed seeds of *C. occidentalis* L and rhizomes of

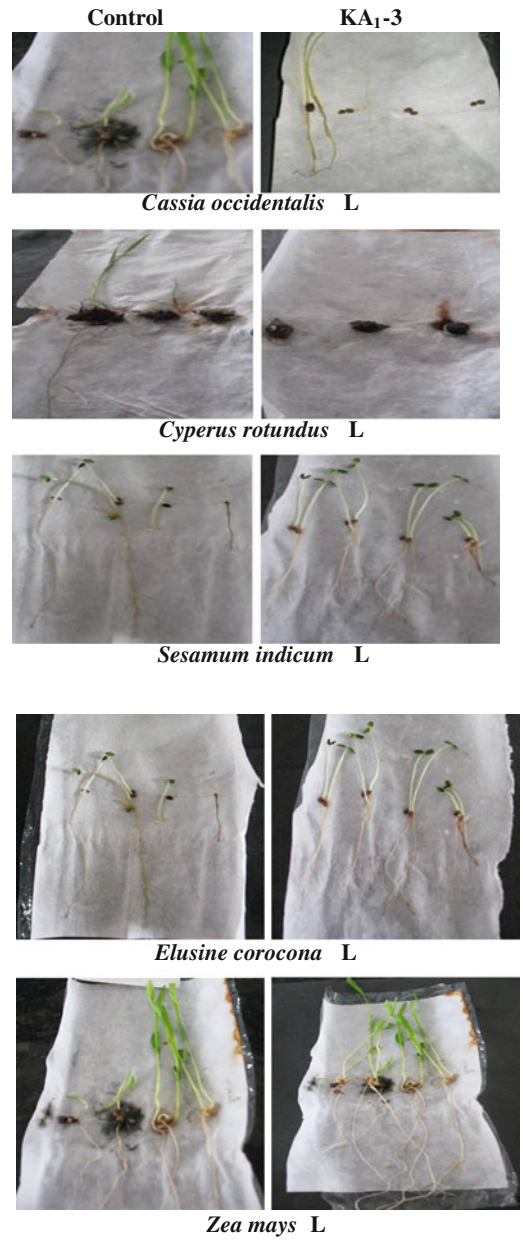

Fig. 2 Screening of herbicidal activity for *Streptomyces* sp. KA_1-3 by rolled paper towel assay

C. rotundus L were tested for plant growth germination and herbicidal assay. The roll towel method was implemented to discover the growth parameter variation in the above-mentioned crop plants and weeds (Table 2 and Fig. 2). The result of the growth-promoting assay has shown that there is no growth variation in normal plants

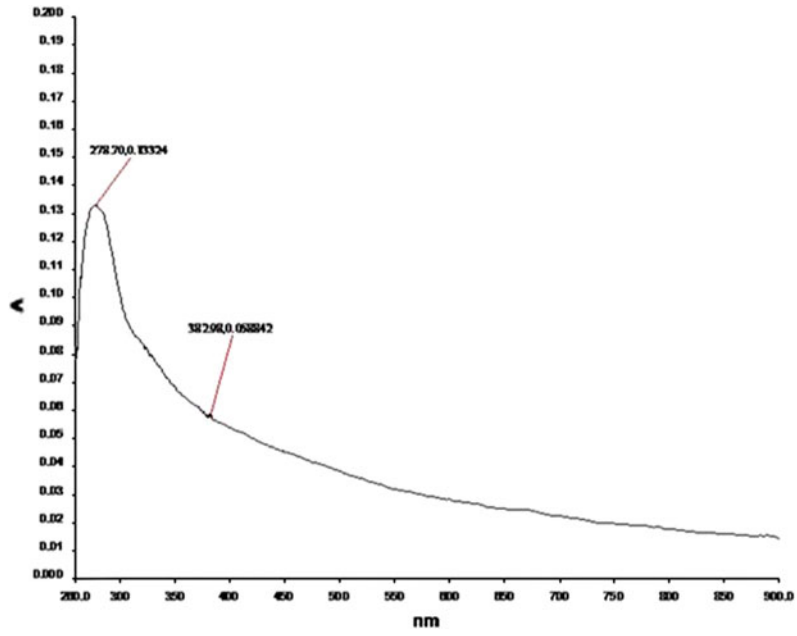

Fig. 3 UV spectrum of N-(naphthalene-1-yl) propanamide

when compared to the control. But distinct growth variation was observed in weeds. The shoot and root growth of *C. rotundus* was severely affected when compared to control. The present result has clearly indicated that the extracts of *Streptomyces* sp. culture significantly inhibit the root and shoot growth in *C. rotundus* under in vitro condition.

The solvent system chloroform:methanol (30:70) was found to have good separation with single spot. The *Rf* value of the spot was found to be 0.678. The compound was readily soluble

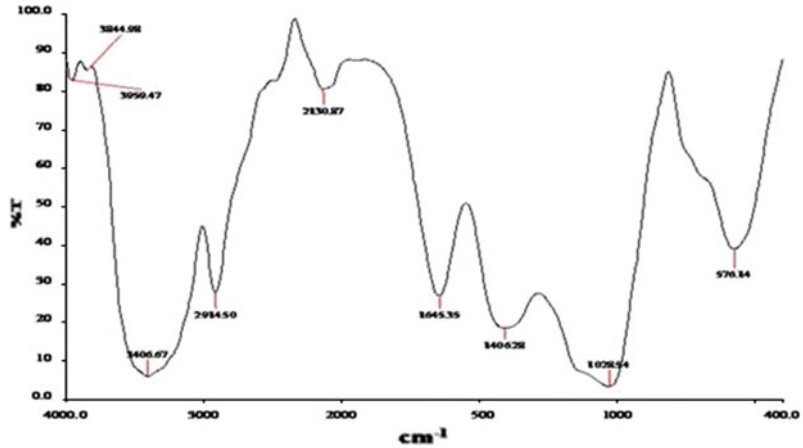

Fig. 4 FT-IR spectra of N-(naphthalene-1-yl) propanamide

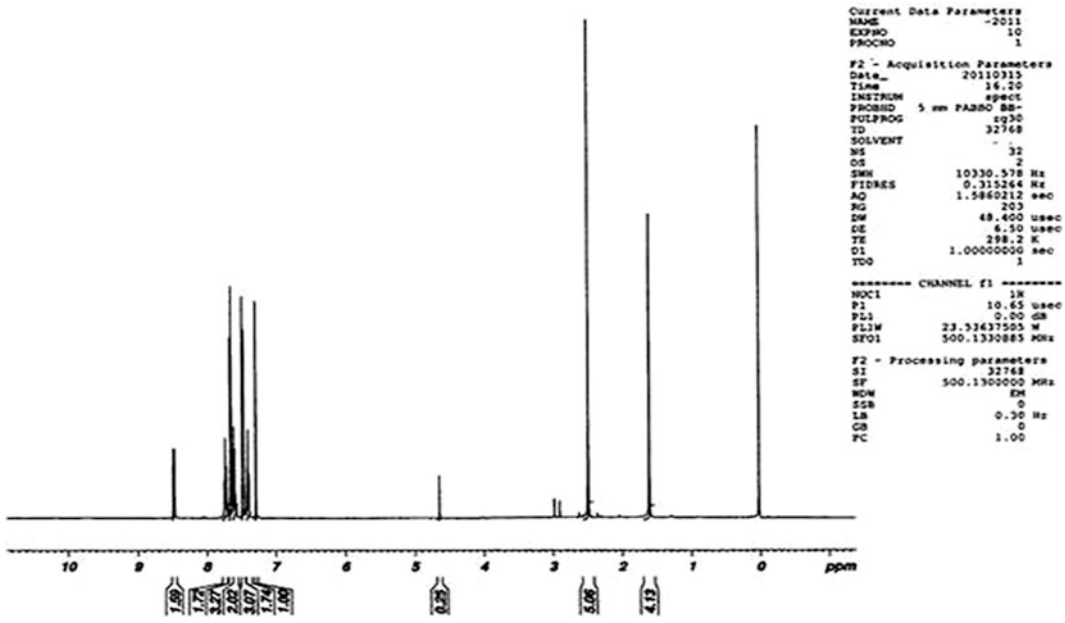

Fig. 5 H^1NMR (DMSO, 500 MHZ) of N-(naphthalene-1-yl) propanamide

in water, methyl sulfoxide but insoluble in methanol, ethanol, chloroform and acetone. The spectral data UV, FT-IR, H^1NMR and MS obtained for the compound were used to establish the structure of the compound UV-Vis Figs. 3 and 4 (λmax 382; FT-IR cm^{-1} 3406.67 (NH), 2914, 1645, 1406 (C = O)); H^1NMR Fig. 5 (DMSO 500 MHz); 8.5 (1H, S, NH), 7.6–7.7 (3H, m, Arh), 7.6–7.5 (1H, Arh), 7.5–7.4 (2H, Arh), 7.4–7.2 (1H, Arh) and 2.6–2.7 (2H, q, CH2), 1.3–1.4 (3H, t, CH3). Based on the spectral data, the structure of the compound extracted from *Streptomyces* sp. was identified as N (naphthalene-1-yl) propanamide, and the molecular formula was determined as $C_{13}H_{13}NO$ and molecular weight obtained by MS as 199 (Figs. 6, 7 and Table 3).

Discussion

The biochemical analysis and allelopathic activity of the *Streptomyces* sp. revealed that the compound was potential to reduce 80 % inhibition of *C. rotundus*. These findings are similar to the findings of Bae et al. (1993) and parallel to that of Dhanasekaran et al. (2012). It has been analyzed and reported that the potential actinobacterial isolates inhibit the germination, and root and shoot growth of *C. rotundus*. Bataineh and Hameed (2008) had reported that the culture filtrate of *Streptomyces* caused significant phytotoxic effect against *Amaranthus retroflexus* seeds indicated by complete inhibition for seed germination, when applied in 1:1 dilution with sterilized distilled water. Kadir and Charudattan (2000) determined that *Dactylaria higginsii* had potential as a bioherbicide agent for purple nutsedge. *D. higginsii* resulted in significant reductions in shoot numbers (72 %), shoot dry weight (73 %) and tuber dry weight (67 %).

Finally, the present study identifies that the potential herbicidal compound extracted from *Streptomyces* sp. can pave way for further analysis of the compounds to discover the mechanism and mode of actions. The discovery of novel bioherbicides with new chemical structures and new site of actions suggests the possibility of a genetically manipulating of microorganism to yield more utilizing. It is clear

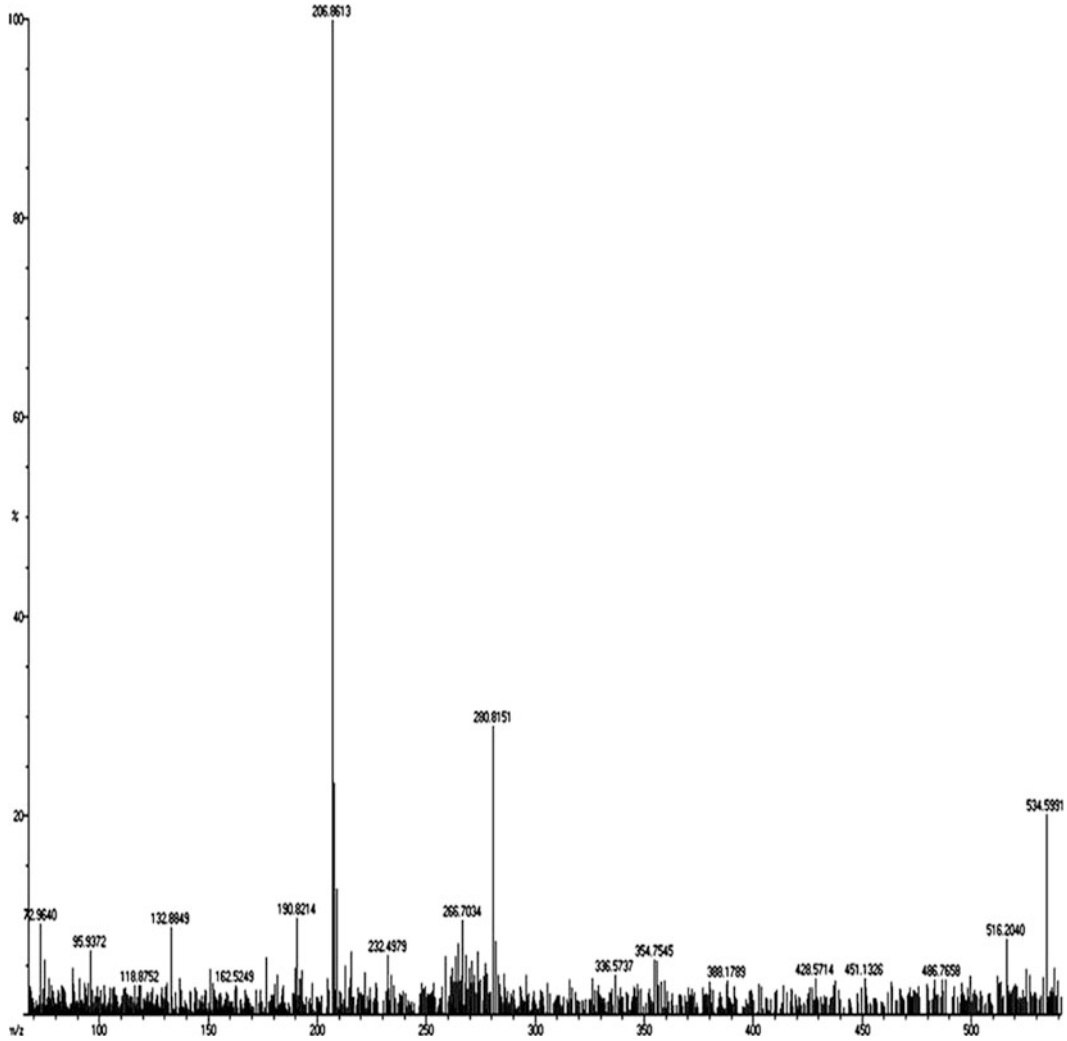

Fig. 6 Mass spectra for N-(naphthalene-1-yl) propanamide

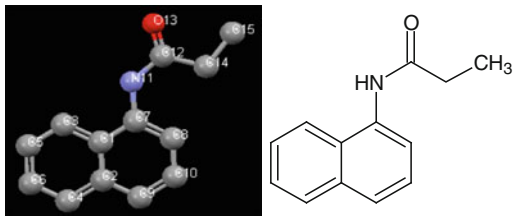

Fig. 7 3D and 2D structure of N-(naphthalene-1-yl) propanamide

Table 3 Herbicidal effect of the N-(naphthalene-1-yl) propanamide

Sl. No	Isolate	% of seed germination inhibition	
		Cassia occidentalis L (%)	*Cyperus rotundus* L (%)
1	KA_1-3	80	80
2	Control	100	100

that the mechanisms of plant growth promotion and biocontrol are not mutually exclusive but interdependent processes. Many of the properties exhibited by actinobacteria are also those exhibited by effective biocontrol agents, which make their use in agriculture as an attractive alternative to agrochemicals.

Conclusion

From this work, it is finally concluded with the inhibitory effect of *Streptomyces* sp. KA_1-3 against the weeds by its compound. This study will lead and support to produce bioherbicides for weed inhibition, growth and improvement, yield of economically important crops.

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Evaluation of *Streptomyces* spp. for the Biological Control of *Phomopsis* Canker Disease of Tea Plants

P. Ponmurugan and D. Saravanan

Abstract

An experiment was conducted to study the efficacy of a bioformulation containing *Streptomyces* species to control *Phomopsis* canker disease of tea under field condition. Among the various treatments, soil application and wound dressing of *Streptomyces* species was found to be superior to other biocontrol agents and systemic fungicides in controlling *Phomopsis* canker. Various treatments healed the wound to varying degrees and improved the plant health. The bud break, tipping weight and green leaf yield also increased in plants to some extent after treatments. In the case of untreated control, the canker size increased and the plants were found unhealthy exhibiting yellowing of leaves and stunted growth with heavy flowering.

Keywords

Streptomyces sp · Biological control · *Phomopsis* · Canker disease · Tea plant

Introduction

Tea is a perennial woody plant that is characterized by a single main stem from which numerous branches are developed to a crown of leaves to get a bushy appearance. Being a monocultural crop, it provides a stable microclimate for a number of pests and diseases. Perennial habit of the tea plant, peculiar cultural conditions and warm humid climate of the tea growing areas are highly conducive for disease development (Muraleedharan and Chen 1997). A large number of pathogenic organisms which are specialized in attacking different parts of the plant are available in this ecological niche. As in any other crop, many diseases affect the roots, stems and leaves of the tea plant. Crop loss in tea due to pests, diseases and weeds recorded as high as 43 % (Baby 2001). The majority of diseases in tea are of fungal origin, and a few are caused by bacteria and one each of virus and algae. In a recent monograph on tea diseases, nearly 400 pathogens were described.

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Irrespective of the pathogen and the parts affected, the disease symptoms manifest as debilitation, defoliation and sometimes death of the bushes.

Among the stem diseases, *Phomopsis* canker (Collar canker) is very important which is caused by the fungus *Phomopsis theae* Petch, which belongs to the family Sphaeropsidaceae of order Sphaeropsidales, from the class Deuteromycetes. It is a facultative parasite occurring in almost all tea growing areas of the world. The disease is more common in south India, especially in young tea (2–8 years old) plantations (Ponmurugan and Baby 2007) than in mature tea (Bore 1996). Infection occurs both on the main stem and on the lateral branches of the plant, the former being more serious than the latter (Baby et al. 2002).

The disease starts as a small lesion on the bark and enlarges in size killing the bark which dries up to from a slight depression. A thick callus develops all along the border of the fungus-invaded area by the growth of the meristematic tissue of healthy bark, thus forming a typical canker. In severe attacks on the main stem, the collar is girdled completely and the affected plant dies. Yellowing of leaves (Chlorosis), stunted growth, early flowering and presence of more 'banji' shoots are the other common symptoms of the disease (Arunachalam 1995).

Phomopsis infection occurs when the bark moisture of the stem falls below a critical level (Venkataram 1992). Infection can also occur through wounds on the collar during various agronomic practices like surface watering during dry weather, fertilizer application and mulching close to collar, deep planting and weeding implements. Wounds can also be caused due to heavy wind, hail, sunshine, falling shade trees, etc. The disease is associated with dry weather and poor soil conditions and is particularly bad in areas subjected to long drought. Infection can occur on stems of any size by means of spores, which are produced on cankered bark. The spores of *P. theae* are dispersed by wind or rain. The fungus cannot penetrate into undamaged bark, and infection takes place through wounds (Shanmuganathan 1985).

Although *Phomopsis* canker is known for over 40 years in southern India, the disease has become a major problem only in recent years as the estates now resort to large-scale replanting infilling and inter-row planting to increase production and productivity. Unfortunately, majority of the clones and seedlings used for this are susceptible to the disease. Further, the change in climatic conditions aggravates the situation, despite its importance; no effective measures, especially using *Streptomyces* spp. belongs to actinomycetes group, have been worked out to control the disease under field condition.

The biology of the pathogen and the aetiology of the disease are known. Moreover, clonal susceptibility, nature of the pathogen, its life cycle and host parasite interactions were studied. Based on these, attempts were also made to develop effective management strategies for the disease. In order to control *Phomopsis* canker, soil applications of biocontrol agents such as *Trichoderma harzianum* and *Gliocladium virens* at 2 kg per bush and/or soil drenching of Bavistin at 5 l per bush along with wound dressing with COC as an effective disease management strategy (Ponmurugan 2002).

Actinomycetes have been identified as one of the major groups of soil microbes. They have been widely used as biocontrol agents. They are capable of secreting antibiotics, enzymes, vitamins, growth promoting and other biologically active substances (Krishnakumari et al. 2006). It has been reported that plant growth is enhanced to great extent due to the production of growth-promoting substances and suppress the pathogens by producing antibiotics. Actinomycete groups of biocontrol agents are able to parasitize and degrade spores of fungal and bacterial plant pathogens (Keiser et al. 2000).

Actinomycetes, particularly *Streptomyces* spp. by virtue of their wide distribution and antibiotic production, may participate actively in establishing the microbiological equilibrium in soil (Moreno et al. 2003). It has been proved by Windham et al. (1986) that biocontrol agents not only control the disease but also increase the plant growth. Enhanced plant growth due to amendment of tea soil with *T. harzianum*,

G. virens, Bacillus subtilis and *Pseudomonas fluorescence* has been reported (Agnihothrudu 1999; Ponmurugan and Baby 2005a, b, 2007). The increased plant growth has been ascribed to the control of minor pathogens in rhizosphere soil and/or due to increased soil fertility. Production of growth-promoting substances by biocontrol agents is known (Saadoun and Gharaibeh 2002; Bonjar et al. 2005).

The present work envisaged in exploiting actinomycetes, especially *Streptomyces* spp. in controlling the stem disease like *Phomopsis* canker in tea plants. Efforts were already made to isolate actinomycetes from rhizosphere soil samples collected from various agroclimatic zones of the tea plantations of southern India. The isolated actinomycete cultures were already identified based on morphological, biochemical and molecular characterization traits from which an efficient isolate was selected based on various bioassays. Efficiency of the secondary metabolites of the actinomycete isolates in suppressing the pathogens was tested. The mass production of secondary metabolites was carried out using biofermenter and antifungal compounds present in it where further characterized, and its efficacy was subsequently evaluated against *Phomopsis* canker disease in tea plantations.

Materials and Methods

Streptomyces strains were screened based on agar disc method (Acar and Goldstein 1996), well-diffusion method (Dhingra and Sinclair 1995), dual culture bioassay (Dennis and Webster 1971a) and antibiosis method (Dennis and Webster 1971b) for checking the efficacy of culture filtrate of *Streptomyces* strains against *P. theae* and *P. hypolateritia*. The efficacy of *Streptomyces* spp. culture filtrate was bioassayed in vitro at 10 % level. The culture filtrate was mixed with molten, cooled PDA medium, so as to obtain the required concentration, and dispersed uniformly into petri plates. The plates were inoculated with 5 mm mycelial disc of the pathogen. Pathogens were inoculated in unmodified medium served as control. The radial growth of the pathogens was measured till the pathogens in control plates completely covered the plates. The percentage inhibition of the pathogens growth was calculated.

Sterilized filter paper discs impregnated with *Streptomyces* broth culture were used as an antifungal metabolite substance. It was placed onto the PDA plates on diametrically opposite points after the pathogen colony grew considerably (20–30 mm). The plates were incubated under room temperature up to 5 days and were observed for zone of inhibition, which indicates a positive reaction for antifungal activity.

Mass Production of *Streptomyces* spp. Mode of Application and Survivability

Based on the in vitro activity, an indigenous *Streptomyces* strain was selected for greenhouse and field evaluation. VA13 strain was grown in nutrient broth using a biofermentor (Bioconsole, London) setting up the optimum pH (5.0), temperature (25 °C) with agitation speed (120 rpm) for about 48 h. Finally, cultures were withdrawn from the outlet valve after discarding the effluents and mixed with suitable carriers such as vermicompost, lignite, decomposed coconut coir pith and commercial talc powder. Around 2 kg of the biopreparations per bush was applied after mixing with dried cow dung (1:1 ratio). The spore load in the preparation was adjusted to a maximum extent, and shelf life period of bioformulations containing *Streptomyces* spp. was studied periodically. Similarly, soil samples and wood scrapings were collected periodically for the enumeration of *Streptomyces* spp. to know the survival (Malarvizhi 2006).

Evaluation of *Streptomyces* spp. Against *Phomopsis* Canker Disease

Two field trials were conducted in a naturally infected field containing *Phomopsis* canker disease to evaluate *Streptomyces* spp. bioformulation at UPASI Tea Research Institute Experimental Garden (Tamil Nadu) and Munnar

Table 1 Antagonistic effect of *Streptomyces* on tea pathogens such as *P. theae* and *P. hypolateritia*

Days after inoculation	Antibiosis method (radial growth in mm)		Paper disc method (linear growth in mm)	
	P. theae	*P. hypolateritia*[a]	*P. theae*	*P. hypolateritia*[a]
1	0.0 (100)	0.0 (100)	28.3	13.7
3	0.0 (100)	0.0 (100)	35.7	21.4
5	1.8 (93)	0.0 (100)	–	27.3
7	5.8 (81)	1.5 (95)	–	33.3
8	10.1 (71)	2.7 (90)	–	36.7
9	13.3 (65)	3.9 (86)	–	–
10	10.0 (53)	5.5 (80)	–	–
CD@$P = 0.05$	2.38	1.30	3.25	2.51

[a] Values in parentheses denote percentage growth inhibition on radial growth

Table 2 Effect of chemical and biological control on the development of *Phomopsis* canker under greenhouse condition

Treatments	Canker size (cm)					
	Pre-treatment		Post-treatment			
	0 months		12 months		24 months	
	Length	Width	Length	Width	Length	Width
Carbendazim[a]	2.18	0.56	2.02	0.46	2.00	0.34
Carbendazim[b]	1.80	0.56	1.36	0.48	1.06	0.38
Streptomyces[a]	1.68	0.58	1.40	0.46	1.20	0.40
Streptomyces[b]	1.98	0.54	1.60	0.26	1.24	0.18
Trichoderma[a]	2.10	0.58	1.64	0.34	1.34	0.28
Trichoderma[b]	2.78	0.56	2.00	0.26	1.64	0.14
Control (untreated)	2.48	0.66	3.24	1.04	4.58	1.46
SE±	0.76	0.15	0.60	0.14	0.40	0.07
CD at $P = 0.05$	1.55	0.30	1.24	0.29	0.85	0.15

[a] Wound dressing
[b] Soil applications

(Kerala). Field trail was conducted in a tea estate in Munnar, Kerala State, India, lying at an elevation of 2,134 m above mean sea level, with a highly susceptible clone, YK-7 planted in 1988 at a spacing of $4' \times 2' \times 2'$. In order to control the stem/root pathogens, the above biopreparations containing *Streptomyces* spp. along with different types of contact and systemic fungicides and biocides (vermicompost) for comparison were used. Treatments were imposed soon after pruning in which two types of pruning were adopted, that is, cut across (24 inches from ground level) and rejuvenation pruning (to the healthy wood).

The experiments were randomized block design with 12 treatments, each replicated with three plots, and each plot consisted of 10 bushes. Recovery of the pruned bushes is monitored by counting the number of bud break and recoded three successive tippings weight and measuring green leaf yield and thereby calculated productivity index (Sharma and Sathyanarayana 1990). Canker size was calculated based on the area of lesion. Visual assessment was performed with respect to bush health characteristics such as leaf colour, number of plucking points, plucking surface area, flowering and growth of shoots (Ponmurugan 2002).

Table 3 Evaluation of indigenous *Streptomyces* spp. on the control of *Phomopsis* canker in tea plants (after 2 years of assessment)

S. no.	Treatment details	Pre-treatment canker size (cm)		Post-treatment canker size (cm)	
		Length	Width	Length	Width
1.	100 % *Streptomyces*[a]	7.15	2.70	6.05	2.18
2.	100 % *Pseudomonas*[a]	7.53	3.05	6.31	2.88
3.	100 % *Bacillus*[a]	7.05	3.03	6.25	2.18
4.	100 % *Trichoderma*[a]	7.88	2.15	6.18	2.15
5.	50 % *Streptomyces* + 50 % vermicompost[a]	7.85	2.77	6.25	2.17
6.	50 % *Streptomyces* + 50 % *Pseudomonas*[a]	7.03	3.26	6.43	2.86
7.	50 % *Streptomyces* + 50 % *Bacillus*[a]	7.85	2.99	6.45	2.19
8.	50 % *Streptomyces* + 50 % *Trichoderma*[a]	8.88	3.14	6.45	2.04
9.	100 % *Streptomyces*[a] + COC[b]	7.84	2.18	6.08	2.00
10.	Bavistin soil drenching + COC[b]	7.11	2.24	6.11	2.04
11.	Bavistin soil drenching + *Streptomyces*[a]	7.28	2.24	6.18	2.00
12.	Untreated control	7.44	2.44	8.87	3.41
CD at $P = 0.05$		0.23	0.13	0.25	0.12

[a] Soil application
[b] Wound dressing

Results

Evaluation of *Streptomyces* spp. Against *Phomopsis* Canker Under Field Condition

The results showed that there was a significant decrease in the area of canker size in cut across pruned bushes (Table 2). The reduction in canker size was maximum (6.45 × 2.04 cm) with treatments where biological control agents were given both as soil application and as wound dressing, especially 50 % *Streptomyces* and 50 % *Trichoderma* combination. This clearly showed the inhibitory effect of antagonists on the growth and reproduction of *P. theae* (Table 1).

The maximum green leaf yield, banji per cent and productivity index were observed in 50 % *Streptomyces* and 50 % *Trichoderma* combination in soil application followed by 50 % *Streptomyces* and 50 % *Bacillus* treatments in both cut cross and rejuvenation pruning fields (Tables 7 and 8). The yield potential and productivity index were increased in all the treated plots after imposing treatments except control plots (Table 2). The improvement of 5035.34 made tea kg ha^{-1} was recorded in a treatment where 50 % *Streptomyces* and 50 % *Trichoderma* combination as soil application was applied. The productivity index was 1.7 ± 0.2 in the same treatment followed by 1.6 ± 0.1 in 50 % *Streptomyces* and 50 % *Bacillus* combination (Table 3).

Both the biological and the chemical treatments have reduced the size of canker to substantial size than that of untreated control. It renovated the lesion, caused by *P. theae* efficiently and reduced the size of canker which registered 6.45 × 2.04 cm in length and width, respectively. Individual treatments were found to be inferior to combination treatments followed by integrated schedule. In the case of untreated control, the canker size was enlarged from 7.44 × 2.44 cm to 8.87 × 3.41 cm where bushes were found to be unhealthy with chlorosis.

The banji content along with bud break and tipping weight was found to be higher in untreated control bushes with low productivity index in yield potential in both cut cross and rejuvenation pruning fields (Tables 4 and 5). Application of biocontrol agents along with chemical fungicides gave deprived results and unhealthy conditions remained for control plant with increased canker size and deduced chlorophyll content with

Table 4 Evaluation of indigenous *Streptomyces* spp. in cut across pruned field containing *Phomopsis* canker on growth performance of tea (after 2 years of assessment)

S. no.	Treatment details	Bud break	Tipping weight[a]	Yield (made tea kg ha^{-1})	Banji content (%)	Productivity index
1.	100 % *Streptomyces*[b]	20.05	162.70	3,456.32	30.52	1.4 ± 0.1
2.	100 % *Pseudomonas*[b]	18.31	156.57	3,389.87	32.41	1.4 ± 0.1
3.	100 % *Bacillus*[b]	18.05	152.87	3,258.74	32.28	1.4 ± 0.1
4.	100 % *Trichoderma*[b]	16.11	157.45	3,256.58	33.32	1.4 ± 0.1
5.	50 % *Streptomyces* + 50 % Vermicompost[b]	22.20	162.95	4,489.54	27.20	1.5 ± 0.2
6.	50 % *Streptomyces* + 50 % *Pseudomonas*[b]	28.43	182.27	4,658.54	27.89	1.5 ± 0.2
7.	50 % *Streptomyces* + 50 % *Bacillus*[b]	26.45	172.00	4,889.23	25.47	1.6 ± 0.1
8.	50 % *Streptomyces* + 50 % *Trichoderma*[b]	36.73	196.23	5,035.34	20.56	1.7 ± 0.2
9.	100 % *Streptomyces*[b] + COC[c]	23.08	168.55	3,241.14	38.62	1.3 ± 0.1
10.	Bavistin soil drenching + COC[c]	25.00	162.65	3,247.98	37.17	1.3 ± 0.2
11.	Bavistin soil drenching + *Streptomyces*[b]	26.10	162.87	3,058.54	40.35	1.2 ± 0.1
12.	Untreated control	8.23	104.13	2,014.14	50.07	1.0 ± 0.1
CD at $P = 0.05$		10.41	112.54	154.58	15.89	00.02

[a] Average of three tippings
[b] Soil application
[c] Wound dressing

profuse flowering. Application of Bavistin as soil drenching and COC for wound dressing showed least progress in the percentage of canker reduction. However, it was unable to improve the yield and other physiological parameters.

Tea Canopy Architectural Analysis

Recovery of the plant metabolism was highlighted from the results of morphological and physiological attributes were observed in the tea bushes (Table 6). The morphological attributes were studied by means of analysing tea bush canopy architecture such as plucking surface and number of plucking points. Similarly, physiological attributes such as total root carbohydrate, dry matter and bark moisture contents were estimated. Almost all the data of morphological and physiological attributes were found to be maximum in 50 % *Streptomyces* and 50 % *Trichoderma* combination in soil application wherein the plucking surface and number of plucking points were 6,255.5 cm^2 and 67.23 Sq. ft, respectively. The total root carbohydrate was 16.31 %, and bark moisture content was 83.50 % in the same treatment.

Enumeration of *Streptomyces* spp. in Tea Soil Samples and Prune Cuts

Enumeration of *Streptomyces* spp. showed that they could survive well in tea soil and prune cuts (Table 7). The population level was 8.0×10^4 g^{-1} soil, while on prune cuts, it was 5.3×10^3 g^{-1} wood scraping after 24 months of their application in rejuvenated plots. In the case of cut across fields, the population density of *Streptomyces* spp. was 5.8×10^4 g^{-1} soil, while on prune cuts, it was 4.8×10^3 g^{-1} wood scraping after 24 months.

Table 5 Evaluation of indigenous *Streptomyces* spp. in rejuvenation pruning field containing *Phomopsis* canker on growth performance of tea (after 2 years of assessment)

S. no.	Treatment details	Bud break/ bush	Tipping weight[a]	Yield (Made tea kg ha^{-1})	Banji content (%)	Productivity index
1.	100 % *Streptomyces*[b]	9.88	65.23	1,466.30	20.12	1.4 ± 0.2
2.	100 % *Pseudomonas*[b]	8.31	60.47	1,254.81	22.44	1.5 ± 0.1
3.	100 % *Bacillus*[b]	7.23	62.24	1,405.56	25.32	1.5 ± 0.2
4.	100 % *Trichoderma*[b]	9.33	58.17	1,350.23	22.30	1.4 ± 0.1
5.	50 % *Streptomyces* + 50 % vermicompost[b]	9.56	65.85	1,589.14	23.27	1.5 ± 0.2
6.	50 % *Streptomyces* + 50 % *Pseudomonas*[b]	10.43	73.65	2,114.55	18.78	1.7 ± 0.2
7.	50 % *Streptomyces* + 50 % *Bacillus*[b]	10.40	70.52	2,087.23	17.65	1.6 ± 0.1
8.	50 % *Streptomyces* + 50 % *Trichoderma*[b]	13.70	82.23	2,356.33	15.62	1.8 ± 0.2
9.	100 % *Streptomyces*[b] + COC[c]	9.71	70.50	1,987.00	17.98	1.4 ± 0.1
10.	Bavistin soil drenching + COC[c]	9.74	68.67	1,887.07	19.90	1.3 ± 0.2
11.	Bavistin soil drenching + *Streptomyces*[b]	8.41	68.68	2,014.50	18.34	1.3 ± 0.1
12.	Untreated control	3.89	15.32	914.27	42.00	1.0 ± 0.2
CD at $P = 0.05$		2.13	10.72	114.47	14.23	00.02

[a] Average of three tippings
[b] Soil application
[c] Wound dressing

Discussion

The effective decrease in canker size by the application of the bioformulation portends. *Trichoderma* bioformulations were proved to be effective in controlling some of the primary and secondary root diseases (Onsando 1990), thorny stem blight disease of tea (Chandramouli and Baby 2002) and *Phomopsis* canker disease (Ponmurugan and Baby 2007). *Trichoderma* spp. is known to produce antibiotic substances (Papavizas 1985) which would have helped to suppress the development of the pathogen in situ.

The increase in productivity on application of bioformulations containing *Streptomyces* buttresses our hypothesis. Bioformulations containing *Streptomyces* were effective in controlling bird's eye spot disease of tea (Gnanamangai and Ponmurugan 2011). Actinomycetes particularly *S. sannanensis* tend to grow in acidic soils, and they are more suitable for tea plantation and production. They perform well as biocontrol agent when supplied with adequate source of carbon and nitrogen. It enhances the rate of multiplication (Ponmurugan et al. 2011).

Bavistin did not significantly reduce the cankers and did not contribute to increase in yield. These results promote the findings of

Table 6 Evaluation of indigenous *Streptomyces* spp. against *Phomopsis* canker on tea bush architecture and biometric parameters (after 2 years of assessment)

S. no.	Treatment details	Plucking surface (cm^2)	Plucking points (Sq. ft)	Dry matter (%)	Total root carbohydrate (%)	Bark moisture (%)
1.	100 % *Streptomyces*[a]	4,995.5	55.37	15.98	09.87	73.36
2.	100 % *Pseudomonas*[a]	4,895.0	50.67	15.03	08.56	70.00
3.	100 % *Bacillus*[a]	4,732.5	52.67	14.00	09.05	71.56
4.	100 % *Trichoderma*[a]	4,887.7	53.23	15.87	09.05	72.36
5.	50 % *Streptomyces* + 50 % vermicompost[a]	4,889.7	58.87	15.98	10.47	74.34
6.	50 % *Streptomyces* + 50 % *Pseudomonas*[a]	6,012.3	62.00	17.45	13.07	78.90
7.	50 % *Streptomyces* + 50 % *Bacillus*[a]	6,008.3	60.23	17.00	13.56	78.00
8.	50 % *Streptomyces* + 50 % *Trichoderma*[a]	6,255.5	67.23	19.56	16.31	83.50
9.	100 % *Streptomyces*[a] + COC[b]	4,214.3	52.74	14.56	08.41	74.74
10.	Bavistin soil drenching + COC[b]	4,365.7	52.67	15.23	08.67	74.00
11.	Bavistin soil drenching + *Streptomyces*[a]	4,565.5	54.17	14.95	09.32	72.32
12.	Untreated control	1,234.0	21.91	07.27	05.37	58.58
CD at $P = 0.05$		5,46.23	24.24	5.23	3.47	10.48

[a] Soil application
[b] Wound dressing

Table 7 Survival of *Streptomyces* spp. in tea soil and on wood scrapings

Month after treatment	*Streptomyces* spp. population			
	Rejuvenated field		Cut across field	
	Soil[a]	Wood[b]	Soil[a]	Wood[b]
Initial	23.0	17.5	22.0	18.5
6	15.5	12.4	15.6	13.8
12	12.7	10.1	10.7	08.2
18	10.8	07.2	07.5	07.5
24	08.0	05.3	05.8	04.8

[a] cfu $\times 10^4$ g^{-1} soil dry wt
[b] cfu $\times 10^3$ g^{-1} wood dry wt

Karthikeyan et al. (2008). Fungicide treatments were able to suppress the severity of the disease on par with antagonist treatment; conversely, it was not able to enhance plant growth.

Mixture of bavistin for soil drenching and COC for wound dressing treated plots gave increased green leaf yield moderately similar to that of individual treatments as 100 %

antagonist (*Streptomyces*, *Pseudomonas*, *Bacillus* and *Trichoderma*). The trend remained the same where different microbial consortia were used. Increasing growth parameters may be due to the recovery of plants from *Phomopsis* infection. Enhanced plant growth due to amendments of soil with biocontrol agents has been investigated (Sanjay et al. 2008). Suppression of charcoal stump rot and wood rot disease of tea as well as enhanced plant growth was reported due to the application of biocontrol agents. Amendments of soil with biocontrol agents resulted in increase in soil fertility, control of minor pathogens and increase in general health of plants, ultimately leading to increase plant metabolism.

Among the biocontrol agents tested, *Streptomyces* was found to be better than *Trichoderma* bioformulation in terms of curing cankers as well as increasing growth parameters in trail plots. It was due to rapid growth acidic soils, production of phytohormones, extracellular enzymes and immediate action upon the cankered region in tea bushes which in turn improve general metabolism of the plants. According to Lo and Lin (2002), several possible factors for increased plant growth by application of antagonists may be due to the control of deleterious root microorganisms, production of growth stimulating factors and increased uptake of nutrients.

Competitive saprophytic ability of *Streptomyces* spp. has been reported already which confirms this view (Ahmad and Baker 1987). Due to their saprophytic nature, they could survive well in tea soil and on wood and thus act as effective barrier to fresh entry of the pathogen. Moreover, biocontrol agents are producing cell wall degrading enzymes upon infection with host plants (Siven and Chet 1989). Survival of different biocontrol agents in tea soils and bushes after using bioformulations was well reported earlier by Sanjay et al. (2008) in grey blight, Ajay et al. (2004) in blister blight and Chandramouli and Baby (2002) in thorny stem blight diseases. Thus, this work shows that *Streptomyces* spp. can be effectively harnessed for treating *Phomopsis* canker in tea plantations.

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Influence of Bioinoculants on Growth and Nutrient Uptake in *Dalbergia latifolia* Roxb. under Tropical Nursery Condition

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Abstract

Studies on bioinoculants interaction with *D. latifolia* seedlings rhizosphere experiments were carried out in tropical nursery condition on CRBD up to 180 DAT. Initially, host plant native microbial profiles were analyzed and the associative bioinoculants were identified. Beneficial bioinoculants such as N_2-fixing (*Rhizobium* and *Azospirillum*), phosphate-solubilizing bacteria, *Trichoderma* and AM fungi are inoculated individually or in various combinations and uninoculated, totally 25 treatments (T_1–T_{25}). The bioinoculants efficiency for growth, biomass, nodulation, soil and tissues macro and micronutrients, NUE, chlorophyll and Leghemoglobin contents, rhizosphere microbial populations, SQI of *D. latifolia* seedlings was significantly increased at 60, 120 and 180 DAT. In general, combined treatments of T_{25}, followed by T_{24} seedlings, are significantly better in performance compared to others including uninoculated control.

Keywords

Bioinoculants · *Dalbergia latifolia* · Combined inoculation · Tropical nursery conditions

Introduction

Dalbergia latifolia (Rose wood) is one of the prime timber trees are commonly found in tropical and subtropical countries. It is commercially exploited for furniture making and panel work. Whereas the demand for supply of this wood is on the increase with increasing population, the area of forestland is consistently decreases. The demand–supply gap and rising prices of industrial wood are making farm-forestry and agro-forestry plantations

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a viable and attractive alternative land use option. Farm forestry in expanding the area will play a very important role of the rose wood growth in meeting the country's future wood requirement.

The green revolution has been achieved through increased chemical inputs, particularly fertilizers and pesticides. The excessive use of inorganic fertilizers and plant protection chemicals in agriculture and agro forestry has resulted in a gradual loss of soil organic matter with a subsequent reduction in water-holding capacity and aggravates the causing residual problem to the agriculture produces the environmental pollution. However, the alternative method has been developed to discourage the chemical inputs by using bioinoculants and biocontrol agents.

Over the past 40 years, our awareness of the symbiotic relationship between plant species and the group of ubiquitous bioinoculants and biocontrol agents has increased significantly. The potential economic importance of these microorganisms based on the productivity of agricultural crops, particularly when species requiring high levels of nutrients are grown on nutrient-deficient or nutrient-fixing soils.

In *D. latifolia*, the influence of AM fungi on nodulation (Crush 1974), seedling quality in farm nurseries and growth and nutrient uptake has also been investigated (Sumana and Bagyaraj 1998). However, studies on the influence of bioinoculants and biocontrol agent on the growth, nutrient uptake and seedling quality of *D. latifolia* and the compatibility of these organisms in its rhizosphere are lacking. The paucity of information on the interactions of bioinoculants and biocontrol agent in the rhizosphere of *D. latifolia* has prompted in the present investigation with the following objectives: (1) to assess the plant growth response to specific microbes either individually or in combinations; (2) to identify the best combination of specific microbes for the planting situation; verify their compatibility and efficacy under nursery conditions; and (3) to assess the role of bioinoculants and biocontrol agent individually and in combination in improving the seedling quality.

Materials and Methods

Maintenance of the Experimental Plants

The experiments were carried out in the nursery of the Botany Department, Bharathiar University, Coimbatore, Tamilnadu, India. Plants inoculated with biocontrol agent (*Trichoderma*), AM fungi, N_2-fixing and phosphate-solubilizing bacteria individually or in various combinations and the control (uninoculated) plants were raised in polybags and arranged in a completely randomized block design (Fig. 1). Plants were watered as and when necessary, throughout the duration of the experiment. The positions of the polybags were altered once in every 15 days to expose seedlings to uniform conditions.

Substrate and Transplanting

Dalbergia latifolia Roxb. seeds were obtained from the Institute of Forest Genetic and Tree Breeding (IFGTB), Coimbatore. The seeds were sown in trays containing heat-sterilized (121 °C for 3 h) sand:soil (1:1 W/W) mixture. One thirty-day-old, uniform, healthy, disease- and pest-free seedlings were transplanted one to each 23 × 13 cm-sized polythene bags, filled with ca 3 kg of sand: red soil: cow dung (1:2:1) mix. The soil had a pH of 7.8 and electric conductivity of 42.35 $mScm^{-1}$, 0.14 mg Kg^{-1} total nitrogen (N), 0.016 mg Kg^{-1} of total phosphorus (P), 0.12 mg Kg^{-1} exchangeable potassium (K) and 3.68 % organic carbon prior to cow dung amendment. The indigenous AM fungi, *Rhizobium*, *Azospirillum*, phosphate-solubilizing bacteria and *Trichoderma* populations were 8.84, 0.23, 0.27, 0.25 and 2.37 propagules, respectively, g^{-1} soil.

Inoculum

AM fungi

To inoculate the *C. equisetifolia* seedlings, AM fungal inoculum at the rate of 10,000

22	10	12	14	15
23	9	20	8	18
5	16	11	1	2
7	25	13	24	17
6	19	3	21	4

Harvest – 1

25	19	15	14	3
13	7	20	4	8
23	1	24	6	17
21	2	22	16	5
18	11	10	12	9

Harvest – 2

17	1	24	21	11
20	22	2	12	10
7	9	23	19	6
25	4	16	5	8
14	13	3	15	18

Harvest - 3

Place of study: Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India. (11°01'N, 76°93'E, altitude 410 m.a.s.l)
Design: CRBD
Treatment: 25
Replicate: 3

Treatment:
T1 – control, T2 – *Glomus intraradices* + *Trichoderma harzianum*, T3 – *Glomus fasciculatum* + *Trichoderma harzianum*, T4 – *Rhizobium* + *Trichoderma harzianum*, T5 – *Azospirillum* + *Trichoderma harzianum*, T6 – Phosphate solubilizing bacteria + *Trichoderma harzianum*, T7 - *Glomus intraradices* + *Glomus fasciculatum* + *Trichoderma harzianum*, T8 - *Glomus intraradices* + *Rhizobium* + *Trichoderma harzianum*, T9 - *Glomus intraradices* + *Azospirillum* + *Trichoderma harzianum*, T10 - *Glomus intraradices* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T11 - *Glomus fasciculatum* + *Rhizobium* + *Trichoderma harzianum*, T12 - *Glomus fasciculatum* + *Azospirillum* + *Trichoderma harzianum*, T13 - *Glomus fasciculatum* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T14 – *Frankia* + *Azospirillum* + *Trichoderma harzianum*, T15 – *Rhizobium* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T16 – *Azospirillum* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T17 - *Glomus intraradices* + *Glomus fasciculatum* + *Rhizobium* + *Trichoderma harzianum*, T18 - *Glomus intraradices* + *Glomus fasciculatum* + *Azospirillum* + *Trichoderma harzianum*, T19 - *Glomus intraradices* + *Glomus fasciculatum* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T20 - *Glomus intraradices* + *Rhizobium* + *Azospirillum* + *Trichoderma harzianum*, T21 - *Glomus intraradices* + *Rhizobium* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T22 - *Glomus fasciculatum* + *Rhizobium* + *Azospirillum* + *Trichoderma harzianum*, T23 - *Glomus fasciculatum* + *Rhizobium* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T24 - *Rhizobium* + *Azospirillum* + Phosphate solubilizing bacteria + *Trichoderma harzianum*, T25 - *Glomus intraradices* + *Glomus fasciculatum* + *Rhizobium* + *Azospirillum* + Phosphate solubilizing bacteria + *Trichoderma harzianum*

Fig. 1 Nursery trail on *Dalbergia latifolia* seedlings inoculated with bioinoculants and biocontrol agent

propagules of a single or equal proportion (5,000 each) of two species was placed in the planting hole before planting the seedlings. *C. equisetifolia* seedlings not inoculated with AM received the same amount of sterile inoculum, which had been autoclaved at 121 °C for 90 min. three times at regular intervals. Soil microbes in AM fungal inoculum were equalized across treatments by applying 25 ml of "microbial wash" to each bag.

This "microbial wash" was prepared by blending 100 mg AM fungal inocula in 1,000 ml deionized water and filtering it three times through a 25-µm sieve. Arbuscular mycorrhizal fungal inoculum consisted of soil and root containing spore from a pot culture of cowpea which was colonized with *Glomus intraradices* Schenck and Smith or *Glomus fasciculatum* (Gerd. and Trappe) Walker and grown for 9 months. Both AM fungal species originated from a pure stand of *C. equisetifolia* located adjacent to the area where the soil used in the experiment was collected.

Bacteria

Inocula of *Rhizobium*, *Azospirillum* and phosphate-solubilizing bacteria were obtained from the Department of Agricultural Microbiology, Tamilnadu Agricultural University, Coimbatore. Five g of charcoal-based bacterial inocula (10^9 CFU g^{-1}) was placed as a thin layer about 2 cm below the soil surface in each bag of specific treatment before seedlings transplantation.

Trichoderma

Trichoderma harzianum was isolated from the rhizosphere of the respective hosts and maintained in a special medium (Elad and Chet 1983) containing the following: g l^{-1}, $MgSO_4$—0.20, K_2HPO_4—0.90, NH_4NO_3—1.00, KCl-0.15, Glucose—3.00, Metalaxyl—0.30, PCNB—0.20, Rose Bengal—0.15, Chloromphenicol—0.25, Agar—15.00, Dis. H_2O—1,000 ml. Five g of

peat-based *T. harzianum* spore inoculum containing 65 × 10^3 spores g^{-1} was placed in the planting hole at the time of seedling transplantation.

Harvest and Measurements

Dalbergia latifolia seedlings were harvested every 60 days up to 180 days after transplantation (DAT) with their entire root systems. Growth parameters such as shoot length, root length, shoot dry weight, root dry weight, nodule number, nodule dry weight and leaf number were measured. The harvested root subsamples were processed for microscopic observation following the procedure of Phillips and Hayman (1970), and the AM colonization was quantified according to the magnified interaction method (McGonigle et al. 1990). Arbuscular mycorrhizal fungal spores in the rhizosphere soil samples were estimated by a wet-sieving and decanting technique of Gerdemann and Nicolson (1963). Populations of Azospirillum, *Rhizobium*, phosphate-solubilizing bacteria and biocontrol agent (*Trichoderma*) were estimated by dilution plate method (Wollum 1982).

Quantitative Estimation of the Microorganisms

Rhizosphere soil samples collected were placed in polythene bags sealed, brought to the laboratory, shade dried and stored at 4 °C until further analysis. Dilution plate method was employed for the enumeration of microbial population in the soil samples. Appropriate dilution, viz., 10^{-3} for fungus, 10^{-5} for *Rhizobium*, *Azospirillum* and phosphobacteria, was chosen for respective organism [fungus—potato agar medium; *Rhizobium*—Yeast extract agar (Subba Rao 1986); *Azospirillum*—N-free semi solid malate medium (Dobereiner et al., 1976); Phosphobacteria—Pikovskayas's medium (Sundara Rao and Sinha 1963)].

Preparation of Roots and AM Fungal Assessment

Fixed roots were washed free of FAA and observed under a dissection microscope (×20) for AM fungal spores attached to them. After examination, the roots were cut into 1-cm bits, cleared in 2.5 % KOH (Koske and Gemma 1989), acidified with 5 N HCl and stained with trypan blue (0.5 % in lactoglycerol). The roots were kept overnight immersed in stain for staining. The stained roots were examined with a compound microscope (×200–400) for AM fungal structures, and the percentage of root length colonization was estimated according to magnified intersection method (McGonigle et al. 1990).

Enumeration and Isolation of AM Fungal Spores

A total of one hundred gram soil was dispersed in 1L water and the suspension was decanted through 710 to 38 μm sieves. The residues in the sieves were washed into beakers.

The sievates were dispersed in water and filtered through gridded filter papers. Each filter paper was spread on a petri dish and scanned under a dissection microscope ×40 magnification and all intact spores were counted. Sporocarps and spore clusters were considered as one unit. Intact AM fungal spores were transferred using a wet needle to polyvinyl alcohol-lactoglycerol with or without Melzers reagent on a glass slide for identification. Spores were identified based on spore morphology and subcellular characters and compared with original descriptions (Schenck and Perez 1990). Spore morphology was also compared with the culture database established by INVAM (http://invam.cag.wvu.edu).

Leaf Area

The leaf areas were recorded using the LI—3,000 portable leaf area meter (Li—Cov, USA).

Estimation of Chlorophyll

Chlorophylls (*a*, *b* and total) were extracted in 80 % acetone and estimated according to Yoshida et al. (1971). The homogenate was filtered through a cheesecloth. The residue was re extracted with 80 % acetone and filtered. The filtrates were pooled and centrifuged at 7,000 g for 10 min. The clear supernatant was made up to 20 ml with 80 % acetone and its optical density (OD) was measured at 645 and 663 nm. Total chlorophyll was calculated by using the following formula and the results were expressed in mg g^{-1} fresh weight.

$$\text{Total chlorophyll}\ \left(\text{mg g}^{-1}\right) = \frac{20.2A_{645} + 8.02A_{663}}{a \times 1,000 \times W} \times V$$

$$\text{Chlorophyll}\ a\left(\text{mg g}^{-1}\right) = \frac{12.7A_{663} - 2.69A_{645}}{a \times 1,000 \times W} \times V$$

$$\text{Chlorophyll}\ b\left(\text{mg g}^{-1}\right) = \frac{22.9A_{645}\ \textit{minus};\ 4.68A_{663}}{a \times 1,000 \times W} \times V$$

where

- a Distance travelled by light in the cell (1 cm)
- V Volume of the extract in ml
- W Fresh weight of the sample in g

Leghemoglobin

Fresh nodules were macerated with double volumes of phosphate buffer and filtered through two layers of cheesecloth. The nodule debris was discarded. The turbid reddish-brown filtrate was centrifuged at 10,000 g for 15 min. and diluted suitably. Three ml of the extract was added to an equal volume of alkaline pyridine reagent and mixed thoroughly. The solution turns greenish yellow due to the formation of ferric hemochrome. The hemochrome was divided equally into two tubes. To one portion, few crystals of sodium dithionite were added to reduce the hemochrome, stirred without aeration and read at 556 nm. To the second portion, a few crystals of potassium hexacyanoferrate were added to oxidize the hemochrome and read at 539 nm. Leghemoglobin was calculated by using the formula mentioned below and the results were expressed in mM (Appleby and Bergersen 1980).

$$\text{Lb concentration}\ (\text{mM}) = \frac{A_{556} - A_{539} \times 2\text{D}}{23.4},$$

where D the initial dilution.

Analysis of Soil Nutrient Content

The total nitrogen (N) and available P were determined, respectively, by micro-Kjeldahl and molybdenum blue methods of Jackson (1973). Exchangeable K was extracted from the soil in ammonium acetate solution (pH 7) and measured with a digital flame photometer (Jackson 1973). Soil organic carbon was determined according to Piper (1966).

Analysis of Plant Nutrient Content

The plant samples were oven-dried and ground to a fine powder in Willy ball mill. These samples were used for the estimation of N, P and K. One hundred milligrams of tissue samples were wet-digested in 2 ml of conc. H_2SO_4 containing catalyst ($CuSeO_3$). The digested samples were made up to 50 ml, and N content in the extract was estimated by micro-Kjeldahl method. Two hundred milligram of plant tissue was wet-digested in 10 ml of a triple-acid mixture (nitric acid, sulfuric acid and perchloric acid in the ratio of 9:2:1, respectively). The digested samples were made up to 100 ml for P estimation. Phosphorus was estimated colorimetrically following the vanadomolybdate method (Jackson 1973). Potassium content in the aliquot of the triple-acid extract was estimated by the emission spectrophotometry using EEL flame photometer (Jackson 1973).

Nutrient Use Efficiency

Nutrient use efficiencies (NUE) were calculated as unit of biomass produced per unit of nutrient content (Koide 1991) using the formula:

$$\text{NUE} = \frac{\text{Plant dry weight g (shoot + root)}}{\text{Tissue nutrient content g (shoot + root)}}$$

Seedling Quality Index

Seedling quality index (SQI) was calculated according to Dickson et al. (1960) using the formula:

$$= \frac{\text{Total dry weight g } \left(\text{plant}^{-1}\right)}{\frac{\text{Height (cm)}}{\text{Stem girth diameter (cm)}} + \frac{\text{Shoot dry weight g } \left(\text{plant}^{-1}\right)}{\text{Root dry weight g } \left(\text{plant}^{-1}\right)}}$$

Statistical Analysis

The data were statistically analyzed by analysis of variance (ANOVA), and treatment means were separated using Duncan's multiple range test (DMRT). Pearson's correlation analysis was used to assess the relationships between seedling biomass, microbial population and plant tissue and soil nutrients (Zar 1984).

Results

Growth and Biomass

Shoot Height

Dalbergia latifolia seedling growth was significantly higher in T_{25} soil at all the stages (Table 1), followed by T_{20}, T_4 and T_{12} treatments at 60,120 and 180 DAT, respectively. Although seedlings growth in T_6 soil was least among microbial inoculations at 60 and 120 DAT and T_2 at 180 DAT, these were still 1.61-, 1.94- and 2.05-fold higher than control seedlings at the respective harvests (Plates 1, 2 and 3).

Tap Root Length

The tap root length of *D. latifolia* seedlings was higher in T_{23} soil; however, seedlings in T_3 and T_9 soils had the least tap root length among the inoculated seedlings though these were 1.18-, 2.24- and 1.35-fold higher than uninoculated control seedlings at 60, 120 and 180 DAT (Table 1).

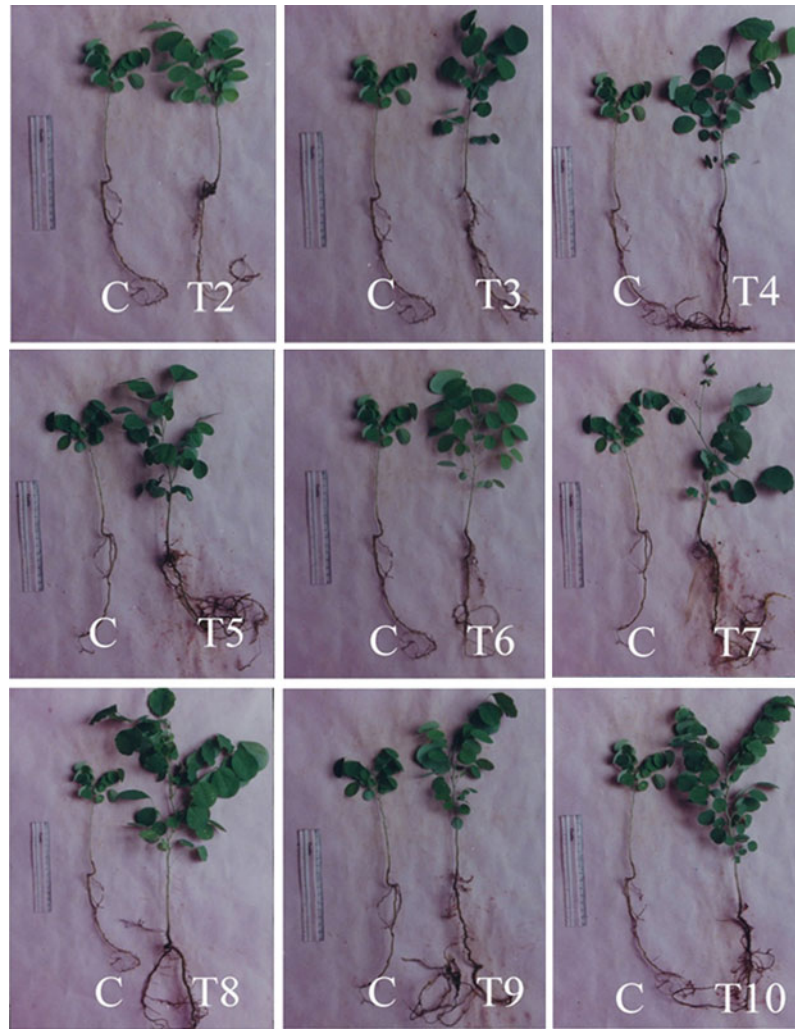
Leaf Number

Higher leaf number occurred in T_{24} seedlings at all the stages of seedling growth, which were 2.12-, 1.85- and 2.38-fold more than control (Table 2). These were followed by T_6 and T_{22} seedlings at 60 DAT and T_{15} at 180 DAT. In contrast, T_4, T_{25} and T_3 seedlings had the least leaf numbers among inoculated treatments in spite of being 1.12-, 1.05- and 1.3-fold higher than control seedlings.

Leaf Area

Higher leaf area was observed in T_{24} seedlings followed by T_{16} (60 DAT), T_{12} (120 DAT) and T_{16} (180 DAT) at different stages of seedling growth (Table 2). In contrast, the least leaf area was recorded in T_{15}, T_2 and T_{22} seedlings among inoculated treatments even though these were 2.12-, 1.36- and 1.18-fold higher than uninoculated control seedlings at 60, 120 and 180 DAT.

Plate 1. Treatments 2 to 10 with control plants



Nodule Number

Nodulation was higher in T_{25} seedlings at 60 DAT and T_{24} seedlings at 120 and 180 DAT (Table 3). Overall, seedlings in T_{22}, T_{25} and T_{20} were found to follow this at 60, 120 and 180 DAT. Nodule numbers of T_3, T_6, T_{10}, T_{12} and T_{23} seedlings were almost similar to that of control seedlings at 120 DAT. Although T_6 seedlings had the least nodule number among inoculated treatments at 180 DAT, it was still 1.91-fold more than control seedlings.

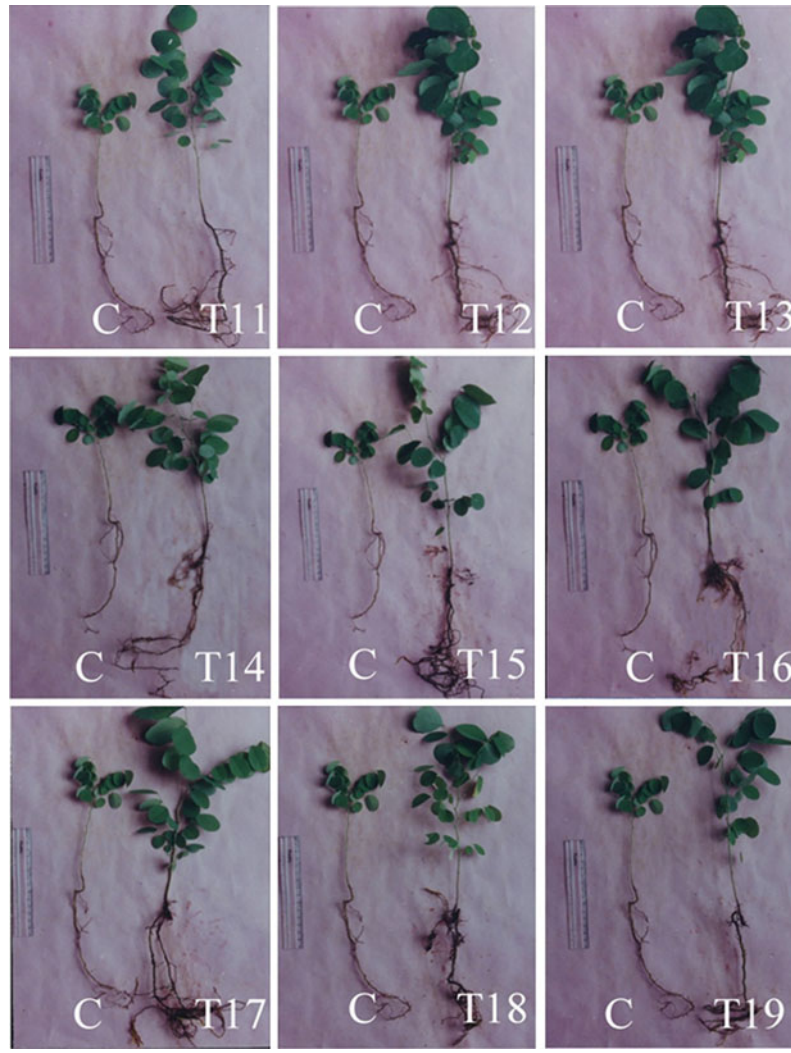
Nodule Dry Weight

Nodule dry mass was significantly higher in T_{25} seedlings at 60 DAT and T_{22} seedlings at 120 and 180 DAT (Table 3). Low nodule mass occurred in T_{10}, T_{17} and T_6 seedling at 60 DAT and T_3 seedlings at 120 and 180 DAT, respectively. However, these low nodular masses recorded among treatments were 1.32-, 1.13- and 1.57-fold greater compared to uninoculated control seedlings at the respective harvests.

Shoot Dry Weight

Seedlings in T_{25} soil had significantly higher shoot dry weight (SDW) than control at 60, 120 and 180 DAT (Table 4). Similarly, seedlings in T_{16} and T_5 recorded the second higher overall SDW among inoculated seedlings at 120 DAT.

Plate 2. Treatments 11 to 19 with control plants



Least SDW among inoculated seedlings was observed in seedlings in T_{17}, T_2 and T_{22} soils. However, the SDW of these treatments was 1.08-, 1.89- and 2.28-fold higher than control seedlings.

Root Dry Weight

Microbial inoculations either individually or in combinations significantly increased root dry weight (RDW) (Table 4). Seedlings in T_{23} soil recorded highest RDW at 60 and 120 DAT, however, at 180 DAT seedlings in T_{25} soil had the maximum RDW, which was 8.94-fold greater than control seedlings. The high RDW'S of T_{25} seedlings were followed by seedling in T_{25} at 60 and 120 DAT, T_{24} at 180 DAT. Even as seedlings in T_{21} at 60 DAT and T_{21} and T_2 at 120 and 180 DAT had the least RDW among inoculated seedlings, these were, respectively, 1.56-, 6.5- and 1.12-fold higher than control seedlings.

Root/Shoot Ratio

Root/shoot ratio (R/S ratio) of seedlings in T_{23}, T_{12} and T_9 was higher at 60, 120 and 180 DAT (Table 4), respectively. These were followed by seedlings in T_{14}, T_{23} and T_{21} soils at 60, 120 and 180 DAT, respectively. The lowest R/S ratio was recorded, respectively, in T_9, T_1 and T_5 at 60, 120 and 180 DAT.

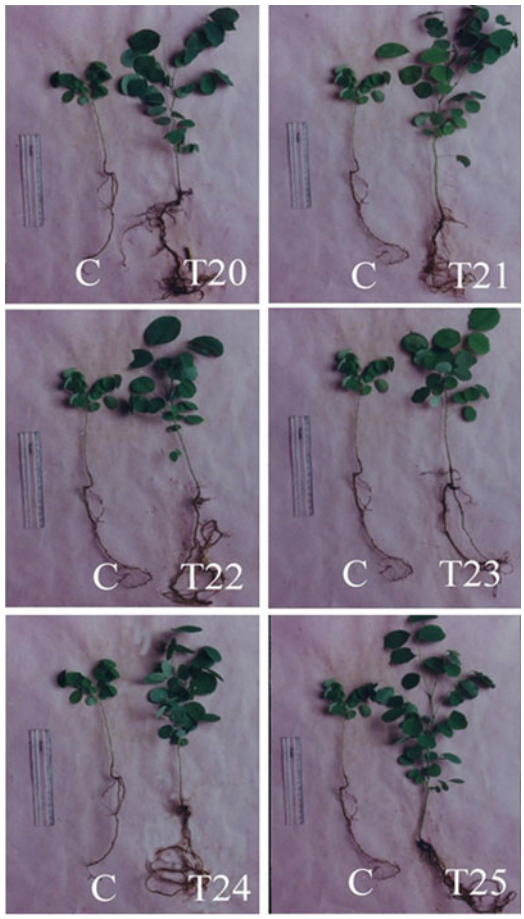


Plate 3. Treatments 20 to 25 with control plants

Seedlings Chlorophyll Content

Chlorophyll *a*

Seedlings in T_{25} soil recorded higher chlorophyll *a* at 60 and 180 DAT (Table 5). However, the concentration of chlorophyll *a* was the highest in T_{24} seedlings at 120 DAT. These were followed by seedlings in T_{17}, T_{25} and T_{24} soils during the respective harvests. The lowest chlorophyll *a* was recorded in T_6, T_4 and T_7 seedlings, but these concentrations were still 1.04-, 1.02- and 1.06-fold high than control seedlings.

Chlorophyll *b*

The chlorophyll *b* was higher in seedlings raised in T_{24} soil closely followed by seedlings in T_{25} soil at all the stages of seedling growth (Table 5). The low chlorophyll *b* content occurred in seedlings in T_{11} soil at 60 and 120 days and in T_7 seedlings at 180 DAT. These least concentrations observed, however, were significantly different and were 1.02-, 1.07- and 1.08-fold higher when compared to control seedlings.

Total Chlorophyll

D. latifolia seedlings in T_{25} followed by T_{20} soils had the highest total chlorophyll content at 60, 120 and 180 DAT (Table 5). In contrast seedlings in T_4 soil had low total chlorophyll at 60 and 120 DAT. Likewise, seedlings in T_3 soil had the low total chlorophyll content at 180 DAT. However, these low levels of total chlorophyll in these treatments were significantly higher and were 1.04-, 1.06- and 1.14-fold higher compared to control seedlings at their respective harvests.

AM Fungal Colonization

Arbuscule (%RLA)

Arbuscule production was found to be higher in seedling roots grown in T_{17} soil at 60 and 180 DAT (Table 6). A similar increase in arbuscule production occurred in seedlings in T_{23} and T_{25} soils at 120 DAT. These maximum increases in treatments were followed by seedlings in T_{25}, T_{24} and T_7 at 60, 120 and 180 DAT, respectively. The %RLA was low in seedlings raised in T_5 and T_{15} soils at 60 DAT. A similar reduction in arbuscules production occurred in T_6 and T_4 seedlings at 120 and 180 DAT. However, these reductions in %RLA were significantly higher

Table 1 Effect of bioinoculants and biocontrol agent interactions on shoot height (cm) and tap root length (cm) of *Dalbergia latifolia* at 60,120 and 180 days after transplantation

Treatment	Shoot height (cm)			Tap root length (cm)		
	Days after transplantation			Days after transplantation		
	60	120	180	60	120	180
T1	10.27 a	14.40 a	19.57 a	12.37 a	15.50 a	27.97 a
T2	16.30 e	19.33 c	27.63 b	29.27 j	37.27 d	39.80 c
T3	14.63 c	26.73 hi	28.73 bc	22.53 g	34.77 c	41.70 d
T4	14.30 c	34.73 m	36.87 k	19.43 de	49.13 h	70.67 l
T5	19.20 hi	30.83 l	34.23 ghi	24.43 h	57.40 j	63.33 i
T6	13.33 b	18.37 b	33.80 gh	35.13 n	37.77 d	76.07 m
T7	17.37 f	27.73 j	28.67 bc	19.50 de	60.47 kl	68.27 k
T8	19.43 i	23.87 e	38.80 l	36.40 o	59.73 k	61.50 i
T9	15.63 d	26.60 hi	30.73 de	33.50 lm	56.60 j	37.73 b
T10	13.53 b	25.53 fg	37.27 k	20.77 f	61.53 l	78.80 n
T11	16.53 e	26.80 hi	35.50 ij	18.60 d	52.60 i	54.97 h
T12	14.60 c	29.10 k	46.13 n	22.30 g	44.20 g	52.10 g
T13	18.70 gh	31.53 l	37.53 kl	34.33 mn	52.70 i	85.67 o
T14	13.53 b	27.70 j	29.40 cd	19.57 e	42.30 f	47.57 ef
T15	14.53 c	27.20 ij	31.77 ef	28.33 i	32.00 b	53.23 gh
T16	19.37 i	27.73 j	32.97 fg	33.17 kl	57.50 j	68.60 k
T17	17.60 f	29.47 k	35.23 hi	32.40 k	40.43 e	76.33 m
T18	16.47 e	21.47 d	36.70 jk	14.57 b	40.57 e	46.87 e
T19	13.57 b	26.53 hi	29.57 cd	20.60 f	34.43 c	68.47 k
T20	20.53 j	26.33 gh	34.53 hi	36.47 o	44.73 g	65.60 j
T21	17.47 f	25.57 fg	42.37 m	29.10 ij	49.53 h	85.77 o
T22	18.60 g	23.73 e	31.50 e	24.50 h	52.50 i	62.13 i
T23	16.53 e	25.43 f	31.33 e	40.17 p	65.47 m	87.40 o
T24	18.30 g	21.50 d	33.90 gh	15.93 c	41.60 ef	49.13 f
T25	21.47 k	35.77 n	56.70 o	35.17 n	37.57 d	48.40 ef

Means followed by a common letter are not significantly different at the 5 % level by DMRT

and were 3.57- and 1.74-fold higher than control seedlings at their respective harvests.

Vesicle (%RLV)

The %RLV was significantly higher in seedlings raised in T_8 soil at 60 DAT, and T_{19} soil at 120 and 180 DAT (Table 6). These were followed by seedlings in T_{25} soil at 60 and 180 DAT. At 60 DAT, %RLV was minimum in T_2 seedlings and no vesicle formation was observed in T_1, T_4, T_5, T_6, T_{19}, T_{20} and T_{22} seedlings at this period. Likewise, least %RLV was recorded in T_{14} and T_{15} seedlings among treatments, but this reduced %RLV levels were significant and 1.35- and 1.43-fold higher than control at 120 and 180 DAT.

Total Colonization

The percent root length colonization (%RLC) was higher in seedlings raised in T_{17} soil followed by T_{19} soil at 60 to 180 DAT (Table 6). In contrast, a minimum %RLC occurred in T_4 seedlings during 60 DAT and T_2 seedlings at 120 and 180 DAT. However, these reduced

Table 2 Effect of bioinoculants and biocontrol agent interactions on Leaf number and Leaf area (cm^2) of *Dalbergia latifolia* at 60,120 and 180 days after transplantation

Treatment	Leaf number			Leaf area (cm^2)		
	Days after transplantation			Days after transplantation		
	60	120	180	60	120	180
T1	13.67 a	24.67 a	26.00 a	9.05 a	20.19 a	56.95 a
T2	18.33 bc	37.67 e–h	46.00 ef	28.30 efg	27.38 b	117.26 m
T3	24.00 efg	27.00 ab	33.67 b	28.43 efg	57.32 e	87.07 ef
T4	26.67 f–i	44.67 jk	54.00 h	23.78 b–f	85.39 l	95.41 ij
T5	25.67 f–i	31.00 bc	42.67 de	37.40 hi	97.06 p	124.54 n
T6	28.67 hi	32.67 cd	43.00 de	33.91 gh	59.07 f	75.86 cd
T7	25.67 f–i	36.67 d–g	37.00 bc	33.40 gh	80.58 j	92.97 hi
T8	25.00 e–h	27.33 ab	48.33 fg	41.49 ij	51.88 c	84.49 e
T9	25.00 e–h	27.33 ab	51.00 gh	27.06 def	80.48 j	132.54 o
T10	23.00 def	38.33 ghi	50.33fgh	22.36 b–e	70.16 i	98.42 j
T11	21.33 cde	33.33 cde	35.33 b	26.29 c-f	89.10 m	95.67 ij
T12	24.67 efg	42.00 ijk	46.33 ef	29.65 fg	98.35 q	108.20 kl
T13	25.33 f–i	41.33 hij	46.00 ef	20.66 bc	95.44 o	111.32 l
T14	16.67 ab	36.67 d–g	35.67 b	19.24 b	83.29 k	87.85 efg
T15	26.00 f–i	45.00 jk	54.67 h	19.20 b	69.49 i	77.86 d
T16	27.00 ghi	34.00 c–g	34.33 b	51.23 k	91.71 n	134.19 o
T17	26.00 f–i	38.00 f–i	38.00 bc	36.65 hi	81.67 j	106.72 k
T18	19.67 bcd	36.33 d–g	54.00 h	42.19 ij	80.41 j	120.29 m
T19	27.33 ghi	31.00 bc	35.67 b	44.58 j	64.76 g	90.81 fgh
T20	26.67 f–i	32.67 cd	45.67 ef	41.50 ij	64.64 g	87.86 efg
T21	26.00 f–i	33.67 c–f	34.67 b	38.78 hij	66.67 h	75.29 cd
T22	28.67 hi	34.33 c–g	36.00 b	36.03 hi	53.61 d	67.39 b
T23	18.00 bc	42.00 ijk	51.33 gh	21.79 bcd	67.54 h	92.01 ghi
T24	29.00 i	45.67 k	62.00 i	51.28 k	126.16 r	143.42 p
T25	26.33 f–i	26.00 a	41.00 cd	37.59 hi	56.59 e	72.50 c

Means followed by a common letter are not significantly different at the 5 % level by DMRT

%RLC levels recorded among inoculated treatments were 1.05-, 1.10- and 1.06-fold higher compared to control seedlings at their respective harvests.

Population of Microorganisms

Rhizobium

Increased *Rhizobium* population was found in the rhizosphere of *D. latifolia* seedlings in T_{24} soil, followed by T_{22} soil at 180 DAT (Table 7).

Low rhizobial populations existed in T_2 and T_{10} soils among treatments and were statistically insignificant compared to control seedlings.

Azospirillum

Unlike *Rhizobium,* population density of *Azospirillum* (Table 7) was higher in the rhizosphere of T_{22} seedlings at 180 DAT. In contrast, low population levels, which were insignificant to control, occurred in the rhizosphere of T_2, T_4 and T_7 seedlings.

Table 3 Effect of bioinoculants and biocontrol agent interactions on nodule number and nodule dry weight (mg) of *Dalbergia latifolia* at 60,120 and 180 days after transplantation

Treatment	Nodule number			Nodule dry weight (mg)		
	Days after transplantation			Days after transplantation		
	60	120	180	60	120	180
T1	0.33 a	0.33 a	0.67 a	1.9 a	2.3 a	6.5 a
T2	0.67 ab	0.67 ab	2.00 abc	6.2 a-d	5.4 abc	10.6 abc
T3	0.33 a	0.33 a	2.33 abc	3.1 abc	2.6 ab	11.2 a–d
T4	3.00 bcd	6.33 fgh	12.00 i	11.2 b–e	10.2 a–f	31.4 j
T5	1.67 abc	4.33 def	5.33 de	6.6 a–e	19.0 fgh	19.8 fg
T6	0.33 a	0.67 ab	1.33 ab	3.0 abc	6.0 a–d	10.2 ab
T7	1.33 abc	1.33 abc	3.67 bcd	7.1 a–e	14.2 c–g	17.1 ef
T8	2.00 a–d	3.00 a–e	23.33 k	6.1 a–d	10.4 a–f	13.3 b–e
T9	3.33 cd	4.67 efg	3.33 bcd	12.7 de	16.7 fg	12.9 b–e
T10	0.33 a	2.33 a–e	3.67 bcd	2.5 ab	10.9 a–f	13.8 b–e
T11	2.33 a-d	2.67 a–e	18.67 j	11.8 cde	12.8 c–g	49.2 l
T12	0.33 a	2.33 a–e	7.67 fg	12.7 de	12.1 b–g	23.9 ghi
T13	3.00 bcd	4.67 efg	17.67 j	11.3 b–e	15.3 d–g	42.6 k
T14	4.00 de	3.67 cde	9.67 gh	15.5 ef	19.9 fgh	27.5 hij
T15	1.00 abc	2.33 a–e	3.33 bcd	5.1 a–d	10.7 a–f	15.9 def
T16	1.33 abc	3.67 cde	3.67 bcd	9.0 a–e	15.4 d–g	15.6 c–f
T17	2.00 a–d	3.00 a-e	7.67 fg	2.5 ab	14.9 c–g	22.6 g
T18	0.67 ab	1.67 a–d	2.67 abc	6.2 a–d	11.8 a–g	28.0 ij
T19	1.33 abc	1.33 abc	6.67 ef	6.7 a-e	16.4 fg	23.1 gh
T20	2.67 a–d	3.67 cde	18.33 j	11.2 b–e	16.6 fg	49.1 l
T21	3.00 bcd	3.33 b–e	2.33 abc	13.5 de	16.2 efg	12.2 b–e
T22	5.67 ef	9.00 i	24.67 k	22.0 f	26.6 h	54.1 m
T23	0.33 a	2.00 a–e	4.33 cd	2.7 abc	10.0 a–f	14.6 b–e
T24	1.33 abc	7.00 ghi	3.00 a–d	6.7 a–e	6.3 a–e	13.7 b–e
T25	6.33 f	8.33 hi	10.33 hi	22.5 f	21.1 gh	54.0 m

Means followed by a common letter are not significantly different at the 5 % level by DMRT

Phosphate-Solubilizing Bacteria

Maximum and minimum population of phosphate-solubilizing bacteria (PSB) occurred in the rhizosphere of T_{24} and T_{23} seedlings, respectively (Table 7). The low PSB population recorded in the T_{23} treatment was 2.3-fold higher compared to control seedlings.

AM Fungal Spore Number

AM fungal spore number was high in T_{17} soil, which was 4.25-fold higher than control at 180 DAT (Table 7). Subsequent higher AM fungal spore density was observed in T_{18} soil. Though the least AM fungal spore number occurred in T_5 soil among inoculated soils, it was still 1.5-fold higher than control soil.

Trichoderma

In *D. latifolia,* higher *Trichoderma* population was recorded in T_{24} soil followed by T_{23} and T_{25} soils at 180 DAT (Table 7). However, no significant increase in *Trichoderma* population occurred when co-inoculated with *G. fasciculatum* (T_2).

Table 4 Effect of bioinoculants and biocontrol agent interactions on shoot dry weight (g), root dry weight (g) and root/shoot ratio (g) of *Dalbergia latifolia* at 60,120 and 180 days after transplantation

Treatment	Shoot dry weight (g)			Root dry weight (g)			Root/shoot ratio (g)		
	Days after transplantation			Days after transplantation			Days after transplantation		
	60	120	180	60	120	180	60	120	180
T1	0.0485 a	0.16 a	0.18 a	0.0095 a	0.02 a	0.2092 a	0.20 ab	0.13 a	1.16 fg
T2	0.0736 c	0.30 b	0.43 c	0.0158 b	0.13 b	0.2346 b	0.21 ab	0.43 bc	0.55 ab
T3	0.0902 d	0.42 f	0.46 d	0.0396 f	0.20 c	0.4410 d	0.44 d	0.48 c	0.96 e
T4	0.1453 g	0.66 n	0.82 k	0.0561 hi	0.29 i	1.3034 o	0.39 cd	0.44 bc	1.59 i
T5	0.1749 h	0.75 t	0.77 j	0.0761 m	0.25 f	0.3616 c	0.44 d	0.33 b	0.47 a
T6	0.1248 f	0.41 f	1.08 q	0.0472 g	0.21 d	1.1755 m	0.38 cd	0.51 c	1.09 f
T7	0.1071 e	0.59 i	0.72 g	0.0201 c	0.40 o	0.7922 g	0.19 ab	0.68 d	1.10 f
T8	0.1726 h	1.10 v	1.27 r	0.0411 f	0.26 g	1.0956 l	0.24 b	0.24 ab	0.89 d
T9	0.0952 d	0.68 p	0.61 e	0.0103 a	0.32 j	1.4526 p	0.11 a	0.47 c	2.38 kl
T10	0.0965 d	0.62 k	1.75 u	0.0856 n	0.33 k	1.1746 m	0.89 g	0.53 c	0.67 bc
T11	0.2074 ij	0.64 m	0.76 i	0.0403 f	0.43 p	1.0914 l	0.19ab	0.67 d	1.44 h
T12	0.0620 b	0.38 d	1.69 t	0.0318 e	0.44 q	1.2744 n	0.51 de	1.16 g	0.75 c
T13	0.1194 f	0.73 s	1.54 s	0.0732 l	0.43 p	0.8186 h	0.61 e	0.59 cd	0.53 a
T14	0.0631 b	0.71 q	1.08 q	0.0733 l	0.44 r	1.0335 k	1.16 hi	0.62 cd	0.96 e
T15	0.1490 g	0.61 j	0.85 l	0.0856 n	0.24 f	1.0848 l	0.57 e	0.39 bc	1.28 g
T16	0.0528 a	0.75 t	0.98 m	0.0253 d	0.33 k	0.8912 i	0.48 de	0.44 bc	0.91 de
T17	0.0524 a	0.73 r	1.06 p	0.0582 i	0.43 p	0.7293 f	1.11 h	0.59 cd	0.69 bc
T18	0.2123 jk	0.67 o	0.75 h	0.0583 i	0.38 n	1.0988 l	0.27 bc	0.57 cd	1.47 hi
T19	0.2011 i	0.60 i	1.01 n	0.0676 k	0.45 s	0.6636 e	0.34 c	0.75 e	0.66 b
T20	0.2205 k	0.50 g	1.06 p	0.0862 n	0.23 e	1.0117 j	0.39 cd	0.46 c	0.95 e
T21	0.1255 f	0.64 l	0.67 f	0.0148 b	0.35 l	1.5380 q	0.12 a	0.55 cd	2.30 k
T22	0.1446 g	0.33 c	0.41 b	0.0634 j	0.27 h	0.4433 d	0.44 d	0.82 ef	1.08 f
T23	0.0941 d	0.54 h	1.02 o	0.1384 p	0.62 u	1.0410 k	1.47 j	1.15 g	1.02 ef
T24	0.0759 c	0.40 e	1.02 o	0.0547 h	0.37 m	1.7423 r	0.72 f	0.93 f	1.71 i
T25	0.2696 l	0.89 u	3.05 v	0.1011 o	0.46 t	1.8711 s	0.38 c	0.52 cd	0.61 b

Means followed by a common letter are not significantly different at the 5 % level by DMRT

Total Fungal Species

Nine non-mycorrhizal fungal species were isolated from the rhizosphere of *D. latifolia* seedlings in various treatments (Table 8). Across all treatments *A. niger* was the most frequent species followed by *Fusarium* sp. and *Mucor* sp. *Botrytis* sp. was the infrequent species which occurred in T_7, T_9, T_{13}, T_{16}, and T_{22} treatments. Maximum colony-forming units (CFU) were found in T_4 soil, which were 1.5-fold higher compared to control soil.

Soil Nutrient Level

Nitrogen

Inoculation of bioinoculants and/or biocontrol agent significantly increased the N concentration of the soils at 180 DAT. Higher N accumulation was found in T_7 soil followed by T_6 soil. T_5 soil had the least N content among treatments, but was significantly higher compared to control soil (Fig. 2).

Table 5 Effect of bioinoculants and biocontrol agent interactions on Chlorophyll a (mg), chlorophyll b (mg) and chlorophyll t (mg) of *Dalbergia latifolia* at 60,120 and 180 days after transplanting

Treatment	Chlorophyll *a* (mg)			Chlorophyll *b* (mg)			Chlorophyll *t* (mg)		
	Days after transplantation			Days after transplantation			Days after transplantation		
	60	120	180	60	120	180	60	120	180
T1	35.70 a	34.80 a	36.58 a	46.38 a	49.19 a	48.79 a	43.41 a	46.17 a	45.00 a
T2	49.04 j–m	45.58 g–j	50.30 g	67.31 hi	72.95 ijk	74.33 f–i	65.98 gh	69.71 g–j	71.90 fgh
T3	38.79 abc	35.98 abc	39.61 b	47.66 ab	52.95 ab	53.29 ab	45.29 ab	51.11 abc	51.48 b
T4	38.36 abc	35.33 ab	39.35 b	70.07 i	69.26 f–j	53.75 ab	45.20 ab	48.95 ab	51.96 b
T5	48.59 i–m	47.71 hij	49.69 g	64.61 ghi	72.40 h–k	75.07 ghi	60.44 e–h	68.97 f–j	70.22 e–h
T6	37.21 ab	35.45 ab	47.31 ef	55.48 cde	71.22 g–k	65.72 c–f	51.98 bc	68.30 f–j	65.64 d–g
T7	41.37 c–f	39.49 b–e	38.84 b	66.66 hi	59.11 bcd	52.90 ab	63.81 fgh	57.24 b-e	55.88 bc
T8	47.56 h–m	45.84 –g–j	48.47 fg	69.48 hi	68.91 e–i	70.69 e–i	66.24 gh	65.77 e–j	66.57 d–h
T9	44.87 f–i	47.57 hij	46.90 def	63.38 e–i	74.56 ijk	72.18 e–i	59.92 d–g	71.11 hij	70.57 e–h
T10	45.26 ghi	43.69 e–i	46.52 def	63.77 f–i	65.42 d–h	65.63 cde	61.17 e–h	62.77 e–j	61.89 cd
T11	46.13 g–l	43.46 e–i	46.99 def	47.37 ab	52.43 ab	67.90 e–h	46.68 ab	65.04 e–j	64.49 def
T12	38.84 abc	36.63 a–d	39.26 b	48.26 abc	54.65 ab	53.85 ab	46.77 ab	51.55 a–d	52.94 b
T13	44.65 e–h	41.45 efg	45.15 de	58.13 d–g	65.64 d–h	67.52 e–h	55.53 cde	62.34 e–j	61.66 cd
T14	45.40 g–j	43.03 e–h	46.28 def	62.71 d–i	68.66 e–i	68.56 e–i	60.64 e–h	65.56 e–j	64.29 de
T15	45.58 g–k	44.74 f–j	45.93 de	65.90 ghi	69.35 f–j	67.94 e–h	62.40 e–h	66.96 e–j	64.81 d–g
T16	46.68 g–m	45.08 g–j	47.03def	64.61 ghi	71.37 g–k	71.80 e–i	64.15 fgh	63.17 e–j	67.20 d-h
T17	49.49 lm	42.34 efg	45.59 de	58.41 d–g	64.47 c–g	66.86 d–g	56.92 c–f	62.00 e–i	64.96 d–g
T18	49.17 klm	42.61 efg	49.83 g	61.38 d–h	64.59 c–g	67.45 e–h	65.88 gh	49.35 ab	52.30 b
T19	43.19 d–g	41.08 d–g	44.78 d	56.05 def	62.09 c–f	65.28 cde	52.57 bcd	59.72 c–g	61.34 cd
T20	46.27 g–l	45.11 g–j	42.07 c	47.47 ab	52.65 ab	58.54 bc	66.30 gh	72.31 ij	72.23 gh
T21	41.21 cde	40.08 c–f	42.73c	55.49 cde	61.95 cde	58.91 bcd	59.92 d–g	58.61 b–f	54.672 bc
T22	40.47 bcd	39.38 b–e	42.10 c	54.68 bcd	58.24 bc	57.88 bc	52.18 bc	57.24 b–e	55.61 bc
T23	45.78 g–l	43.55 e–i	46.37 def	63.51 e–i	67.58 e–i	68.08 e–h	58.68 c–g	65.12 e–j	67.26 d–h
T24	44.66 e–h	48.53 j	50.63 g	70.81 i	77.64 k	76.65 i	52.26 bc	61.03 d–h	64.06 de
T25	50.17 m	47.97 ij	50.65 g	70.62 i	76.28 jk	75.96 hi	67.75 h	72.65 j	73.57 h

Means followed by a common letter are not significantly different at the 5 % level by DMRT

Table 6 Effect of bioinoculants and biocontrol agent interactions on arbuscule (%), vesicle (%) and hyphae (%) of *Dalbergia latifolia* at 60,120 and 180 days after transplanting

Treatment	Arbuscule (%)			Vesicle (%)			Hyphae (%)		
	Days after transplantation			Days after transplantation			Days after transplantation		
	60	120	180	60	120	180	60	120	180
T1	–	0.21 a	0.62 a	–	3.13 a	2.46 a	5.51 a	28.79 a	46.17 a
T2	–	2.81 f	8.42 g	0.040 a	6.19 e	10.40 d	16.27 j	31.79 b	49.35 b
T3	–	3.87 g	9.18 g	0.190 b	15.49 l	25.23 l	7.18 c	59.49 o	83.78 o
T4	–	0.85 bc	1.08 b	–	11.51 i	16.73 h	5.79 b	47.33 j	70.71 k
T5	0.00 a	1.56 d	1.71 c	–	10.46 h	20.80 j	7.51 d	35.50 c	52.56 c
T6	–	0.75 b	1.69 c	–	28.64 t	30.64 o	11.89 f	66.63 s	84.59 p
T7	7.47 g	11.66 l	32.44 p	4.613 f	20.30 q	19.29 i	38.30 t	69.62 u	79.67 m
T8	–	2.57 ef	5.90 f	0.183 b	17.70 n	29.72 n	13.38 g	52.64 m	68.56 j
T9	–	8.74 k	22.56 l	0.243 b	13.76 k	16.53 h	16.54 j	44.53 h	66.55 i
T10	–	5.55 i	15.57 i	0.427 c	25.57 s	35.61 p	13.66 g	59.58 o	84.28 p
T11	1.34 e	7.70 j	11.15 h	1.297 e	8.60 f	10.73 d	33.63 r	42.85 g	54.80 e
T12	–	4.86 h	11.66 h	10.333 h	9.11 g	45.15 q	15.74 i	65.62 r	89.46 r
T13	0.71 c	2.37 e	10.66 h	0.877 d	5.75 d	6.87 c	29.23 p	37.56 d	53.69 d
T14	–	0.95 c	4.32 e	0.437 c	4.23 b	6.62 c	22.84 l	50.94 k	76.74 l
T15	0.00 a	2.73 f	3.38 d	10.603 h	8.66 f	3.52 b	20.33 k	39.61 e	59.63 f
T16	0.34 b	2.80 f	5.53 f	0.797 d	4.74 c	6.62 c	36.18 s	46.22 i	52.36 c
T17	16.20 i	20.93 p	38.66 q	10.640 h	32.59 u	20.60 j	69.61 v	72.46 w	94.64 s
T18	6.63 f	17.63 m	23.20 lm	21.853 i	11.38 i	23.55 k	31.53 q	66.77 s	84.24 p
T19	0.95 d	8.69 k	24.38 m	–	36.41 v	50.40 s	58.65 u	71.84 v	89.67 r
T20	0.94 d	17.53 m	20.39 k	–	11.67 i	14.51 f	27.73 o	63.70 q	85.62 q
T21	–	8.92 k	14.45 i	10.217 h	16.19 m	19.36 i	9.55 e	40.56 f	61.66 g
T22	–	19.39 o	18.52 j	–	19.81 p	12.66 e	13.70 g	51.72 l	62.34 h
T23	0.34 b	24.63 q	23.63 lm	9.587 g	18.60 o	27.51 m	23.51 m	61.59 p	85.42 q
T24	6.39 f	18.58 n	26.33 n	10.137 h	12.85 j	15.31 g	14.46 h	55.71 n	66.67 i
T25	8.43 h	24.63 q	28.66 o	10.653 h	24.22 r	49.57 r	26.69 n	67.32 t	82.55 n

Means followed by a common letter are not significantly different at the 5 % level by DMRT

Table 7 Bioinoculants and biocontrol agent interaction spore density of *Dalbergia latifolia* seedlings in nursery condition

Treatment	180 days after transplantation				
	Rhizobium (10^{-5})	*Azospirillum* (10^{-5})	Phosphate-solubilizing bacteria (10^{-5})	VAM spore (10 g)	*Trichoderma* (10^{-3})
T1	0.3 a	0.3 a	0.3 a	12.0 a	2.3 a
T2	0.3 a	0.3 a	1.0 a–e	22.0 cde	2.7 a
T3	0.7 ab	0.7 abc	0.7 ab	24.3 e	4.7 b
T4	2.0 abc	0.3 a	1.0 a–e	20.7 bcd	7.0 b–f
T5	1.7 abc	1.3 a–e	1.3 a–d	18.0 b	7.7 c–f
T6	1.3 abc	0.7 abc	2.0 a–e	19.7 bc	7.7 c–f
T7	0.7 ab	0.3 a	1.0 abc	35.7 hij	5.0 bc
T8	1.3 abc	0.7 ab	1.3 a–d	32.0 fgh	9.3 f
T9	1.0 abc	1.3 a–e	1.3 a–e	32.0 fgh	4.7 b
T10	0.3 a	0.7 abc	2.0 a–e	30.7 fg	4.7 b
T11	2.3 bc	0.7 ab	1.7 a–e	29.7 fg	5.0 bc
T12	0.7 ab	1.7 a–e	2.0 a–e	28.3 f	5.7 bcd
T13	1.0 abc	1.0 a–d	3.0 a–e	36.0 h–k	5.7 b–e
T14	1.7 abc	2.0 b-e	2.0 a–e	22.7 cde	6.7 f
T15	2.0 abc	1.7 a–e	4.0 cde	21.3 cde	6.7 f
T16	0.7 ab	2.7 de	3.3 b–e	24.0 de	5.7 b–e
T17	2.3 bc	0.7 abc	1.3 a–e	51.0 m	5.7 b–f
T18	1.0 abc	1.7 a–e	0.7 abc	44.7 l	7.7 c–f
T19	1.3 abc	1.3 a–e	3.0 b–e	41.3 kl	8.3 def
T20	1.7 abc	2.3 cde	1.3 a–e	38.0 ijk	9.7 f
T21	1.7 abc	2.0 a–e	3.3 b–e	33.3 ghi	8.7 ef
T22	3.0 c	3.7 e	1.0 a–e	33.7 ghi	9.0 f
T23	2.7 abc	1.0 a–d	4.0 de	33.3 ghi	10.0 f
T24	3.3 c	2.3 a–e	4.3 e	33.0 ghi	10.3 f
T25	2.3 bc	3.3 e	4.0 de	40.3 jkl	10.0 f

Means followed by a common letter are not significantly different at the 5 % level by DMRT

Phosphorus

High soil P content among treatments was found in T_6 soils followed by T_4 soils at 180 DAT (Fig. 3). Among the treatments, T_{21} soil had the least soil P content which was insignificant compared to control in spite of it being 1.75-fold higher.

Potassium

Higher K content was found in T_{25} soil followed by T_9 soil at 180 DAT (Fig. 4). Among the treatments, T_4 and T_{16} soils had the least K content and was 1.01-fold more compared to uninoculated control.

Organic Carbon

The percent organic carbon was the highest in the rhizosphere of T_{23} followed by T_{14} seedlings at 180 DAT (Fig. 5). Although the percent organic carbon in the rhizosphere of T_2 seedlings was the least among treatments, it was still 1.08-fold significantly higher compared to uninoculated control rhizosphere soil.

Table 8 Effect of bioinoculants and biocontrol agent on occurrence of non-mycorrhizal soil fungi in *Dalbergia latifolia* seedlings under nursery conditions

Treatment	Fungal species							
	Aspergillus flavus	*A. niger*	*A. terreus*	*Botrytis* sp.	*Fusarium* sp.	*Mucor* sp.	*Penicillium* sp.	*Rhizopus* sp.
T_1	+	+	–	–	+	+	–	–
T_2	+	+	+	–	+	–	–	–
T_3	–	+	–	–	+	–	+	+
T_4	–	+	–	–	–	–	–	–
T_5	–	–	–	–	–	–	+	+
T_6	–	+	+	–	–	–	–	–
T_7	–	–	+	+	+	–	+	–
T_8	–	+	+	–	–	+	–	–
T_9	+	–	–	+	+	+	+	–
T_{10}	+	+	+	–	+	–	–	+
T_{11}	–	+	–	–	+	–	+	–
T_{12}	–	–	–	–	+	+	–	+
T_{13}	+	+	+	+	+	–	–	–
T_{14}	–	+	+	–	–	–	+	+
T_{15}	–	+	+	–	–	+	–	+
T_{16}	+	–	–	+	+	+	+	–
T_{17}	+	–	+	–	+	+	–	+
T_{18}	–	+	–	–	+	+	–	–
T_{19}	+	+	+	–	+	+	–	–
T_{20}	–	+	–	–	–	+	+	+
T_{21}	+	+	–	–	–	+	+	–
T_{22}	–	+	–	+	–	+	–	–
T_{23}	+	+	+	–	–	–	+	–
T_{24}	+	+	–	–	–	–	+	–
T_{25}	+	+	+	–	–	+	+	+
Frequency (%)	48	76	48	20	52	52	48	36

+ Present, – Absent

Tissue Nutrients

Nitrogen

Dalbergia latifolia seedlings in T_{14} soils had maximum tissue N among treatments, followed by seedling in T_{22} soil at 180 DAT and least tissue N content occurred in T_2 seedlings and the increase over control seedlings was significant (Fig. 3).

Phosphorus

Maximum tissue P was observed (Fig. 3) in seedlings raised in T_1 and T_{25} soils followed by

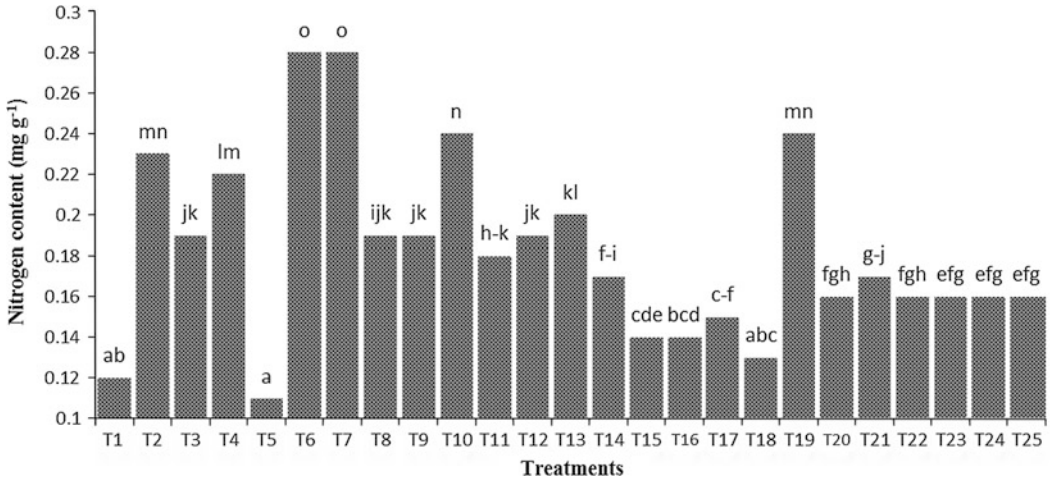


Fig. 2 Soil nitrogen content in *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

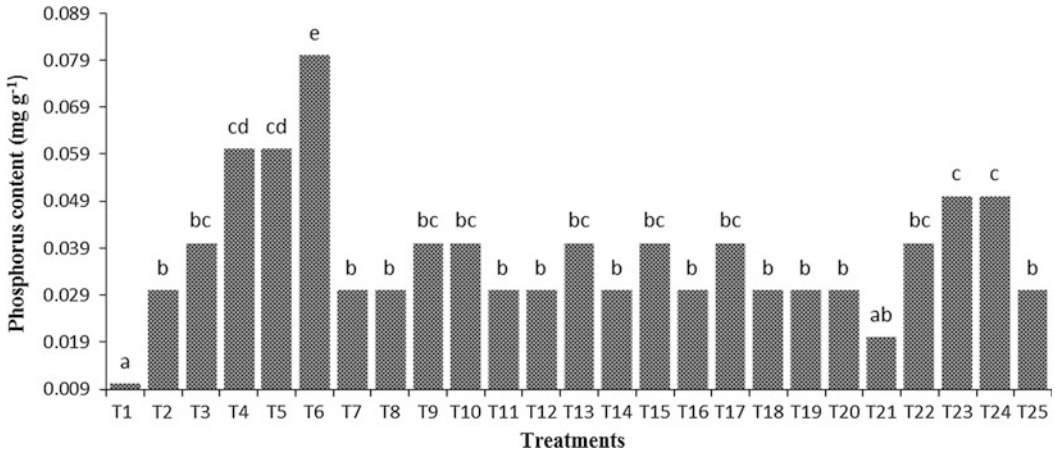


Fig. 3 Soil phosphorus content in *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

seedlings in T_{18}, T_{19}, T_{23} and T_{24} soils at 180 DAT. The least tissue P content was observed in seedlings raised in T_4 soils and the increase over control seedlings was insignificant.

Potassium

Dalbergia latifolia seedlings in T_7 soil had the maximum tissue K contents followed by T_{22} at 180 DAT (Fig. 3). The least K tissue concentrations among treatments occurred in seedlings raised in T_4 soil and the increase over control seedlings was insignificant.

Leghemoglobin Concentration

Nodule leghemoglobin content of *D. latifolia* seedlings was high in the seedlings raised in T_{24} soil at 60 and 120 DAT (Table 9). However, seedlings in T_{25} soil had maximum leghemoglobin content at 180 DAT. Though nodule leghemoglobin contents of seedlings in T_{11} soil

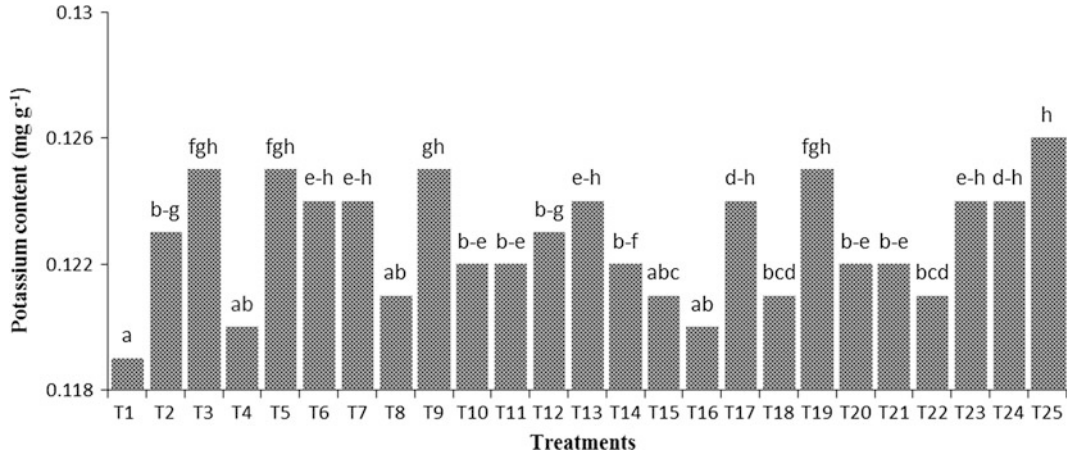


Fig. 4 Soil potassium content in *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

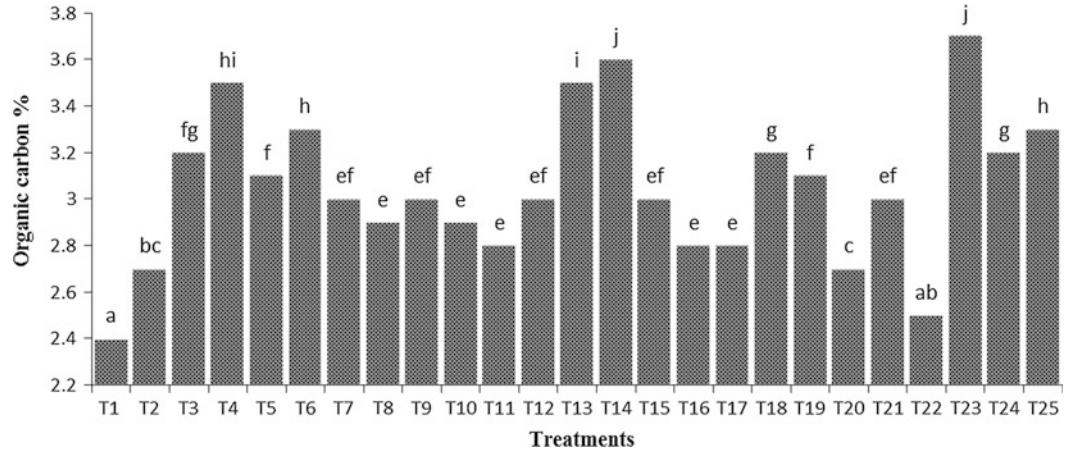


Fig. 5 Soil organic carbon content in *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

was low among inoculated seedlings at 60 DAT, it was still 1.06-fold higher than control seedlings.

Nutrient Use Efficiency

Nitrogen

Maximum nitrogen use efficiency (NUE) occurred in T_{19} seedlings at 60 DAT and in T_{25} seedlings at 120 and 180 DAT (Table 10). Generally, seedlings in T_{25}, T_{19} and T_{10} soils had the subsequent maximum NUE at 60, 120 and 180 DAT. In contrast, T_{22} seedlings had the lowest NUE among inoculated treatments even though it was 1.62- and 1.05-fold more over control at 120 and 180 DAT. At 60 DAT, least NUE was recorded in T_{16} seedlings, among the treatments, which were 1.16-fold less than uninoculated control.

Phosphorus

Phosphorus use efficiency (PUE) was significantly higher in T_{25} seedlings at all harvests

Table 9 Effect of bioinoculants and biocontrol agent interactions on Leghemoglobin (mM) of *Dalbergia latifolia* at 60,120 and 180 days after transplanting

Treatment	Leghemoglobin (mM)		
	Days after transplantation		
	60	120	180
T1	0.0048 a	0.00 a	0.0133 a
T2	0.0117 ab	0.01 a	0.0223 b
T3	0.0057 a	0.00 a	0.0158 a
T4	0.0709 abc	0.09 abc	0.0928 h
T5	0.0188 ab	0.03 a	0.0359 c
T6	0.0054 a	0.02 a	0.0739 g
T7	0.0310 ab	0.05 ab	0.0545 e
T8	0.1626 cd	0.17 cde	0.2679 n
T9	0.0447 ab	0.05 ab	0.0493 d
T10	0.0133 ab	0.05 ab	0.0550 e
T11	0.0051 a	0.19 de	0.1974 l
T12	0.2058 d	0.22 def	0.2204 m
T13	0.0816 abc	0.02 a	0.1651 k
T14	0.0872 abc	0.09 abc	0.1045 j
T15	0.0445 ab	0.05 ab	0.0737 g
T16	0.0612 ab	0.06 ab	0.0634 f
T17	0.0744 abc	0.07 ab	0.0745 g
T18	0.0627 ab	0.09 abc	0.0977 i
T19	0.0493 ab	0.08 ab	0.0767 g
T20	0.2426 d	0.05 ab	0.2937 o
T21	0.0386 ab	0.14 bcd	0.0174 a
T22	0.2220 d	0.25 ef	0.2668 n
T23	0.0940 abc	0.21 def	0.0666 f
T24	0.3839 e	0.41 g	0.3279 p
T25	0.1071 bc	0.28 f	0.4284 q

Means followed by a common letter are not significantly different at the 5 % level by DMRT

(Table 10), followed by T_{20}, T_{16} and T_{12} treatments at 60, 120 and 180 DAT, respectively. Seedlings in T_{16} at 60 DAT and T_2 seedlings at 120 and 180 DAT had the least PUE among treatments; however, these were still 1.23-, 2.35- and 1.68-fold higher than control seedlings at the respective harvests.

Potassium

Potassium use efficiency (KUE) was significantly higher in T_{25} at all the harvests (Table 10), followed by T_{20}, T_{23} and T10 seedlings at 60, 120 and 180 DAT at the respective harvests. In contrast, T_{12} seedlings at 60 DAT and T_2 seedlings has the least KUE among inoculated treatments at 60, 120 and 180 DAT, which were 1.04-, 1.88- and 1.26-fold more than uninoculated control seedlings.

Seedling Quality Index

Seedling quality was significantly better in T_{25} seedlings, followed by T_{24}. In contrast, the quality of seedlings in T_2 soil was low as indicated by the seedling quality index (SQI), which was higher compared to control seedlings (Fig. 5). Single and combined inoculation had excellent seedling quality than uninoculated control seedlings (Fig. 6).

Discussion

This study shows the importance of bioinoculants and biocontrol agents for the seedling growth, biomass, nutrient uptake and quality index of *D. latifolia*. It demonstrates the synergistic effect of AMF, N_2-fixing and PSB in *D. latifolia* for the first time and confirms these organisms in different crop species (Fallik et al. 1988; Negi et al. 1990; Kim et al. 1998; Muthukumar et al. 2001). *D. latifolia* seedling growth and biomass significantly increased when bioinoculants were added to unsterilized soil. Generally, the effect of AM association on plant growth in native soils is well documented (Muthukumar et al. 2001). In the present study, inoculation of AM combination (*G. intraradices* + *G. fasciculatum*) had significantly increased the growth and biomass of seedlings than individual inoculation. As additional evidence, the plant dry weight was significantly positively correlated with %RLV ($r = 0.542$; $P < 0.01$; $n = 25$) (Table 11). This is further supported by the existence of significant positive correlation with plant P ($r = 0.575$; $P < 0.01$; $n = 25$) and plant dry weight ($r = 0.587$; $P < 0.01$; $n = 25$). Relatively individual

Table 10 Nutrient use efficiency of *Dalbergia latifolia* seedlings inoculated with bioinoculants and biocontrol agent at 60, 120 and 180 days after transplanting (DAT)

Treatment	Nitrogen (mg g^{-1})			Phosphorous (mg g^{-1})			Potassium (mg g^{-1})		
	60 DAT	120 DAT	180 DAT	60 DAT	120 DAT	180 DAT	60 DAT	120 DAT	180 DAT
T_1	230.2 a	651.3 a	1,394.6 a	597.9 a	1,672.1 a	3,499.1 a	513.3 a	1,499.1 a	3,207.5 a
T_2	339.7 ab	1,515.2 bc	2,214.8 ab	8,64.1 a	3,933.9 ab	5,892.9 a	601.4 a	2,821.1 ab	4,049.1 a
T_3	347.7 b	1,615.8 c	2,363.4 ab	1,228.6 b	5,581.8 bc	8,082.1 ab	772.5 ab	3,528.7 b	4,919.6 a
T_4	512.8 cd	2,323.7 e	5,086.1 cd	1,932.7 d	8,640.0 c	19,107.2 cd	1,703.4 d	7,854.5 e	16,967.2 d
T_5	571.8 de	2,191.9 de	2,460.1 ab	2,367.9 e	9,046.4 de	10,125.9 b	1,901.5 de	7,210.9 de	7,930.8 d
T_6	535.8 cd	1,895.4 cd	6,783.2 e	1,607.5 c	5,533.9 bc	19,641.7 d	1,201.5 b	4,187.8 bc	14,572.9 c
T_7	371.9 bc	2,827.4 f	4,253.1 c	1,167.0 ab	8,915.3 d	13,281.6 bc	617.5 a	4,690.0 bc	7,075.2 ab
T_8	483.4 cd	2,248.6 de	5,128.1 cd	1,942.7 d	9,136.0 de	20,966.4 d	1,548.6 cd	7,192.2 de	16,339.3 d
T_9	240.3 a	2,212.2 de	4,486.5 c	976.9 ab	9,090.0 de	18,064.0 cd	703.3 a	6,451.0 d	12,556.7 c
T_{10}	469.3 c	2,372.2 e	7,150.6 f	1,640.5 c	8,535.7 d	25,431.3 e	1,433.9 c	7,188.0 de	21,192.8 e
T_{11}	574.7 de	2,467.6 e	4,180.5 c	2,359.0 e	9,670.3 de	1,6208.8 c	1,965.9 de	8,256.9 e	13,586.7 c
T_{12}	219.2 a	1,868.5 cd	6,679.5 e	876.6 a	7,389.1 cd	26,014.9 e	533.0 a	4,393.5 bc	15,287.1 cd
T_{13}	466.3 c	2,785.6 f	5,545.4 d	1,800.0 cd	10,346.4 e	20,673.7 d	1,203.8 b	7,109.2 de	13,702.3 c
T_{14}	239.3 a	1,999.5 d	3,550.5 b	1,274.8 b	10,566.1 e	18,830.4 cd	915.4 b	7,527.5 de	13,018.4 c
T_{15}	563.9 d	2,030.6 d	4,466.9 c	2,172.2 de	7,650.9 cd	16,896.5 c	1,675.7 cd	5,950.7 cd	13,218.4 c
T_{16}	197.7 a	3,031.4 fg	4,529.6 c	736.8 a	1,1160.9 ef	16,515.4 c	565.9 a	8,645.8 e	12,358.9 bc
T_{17}	264.0 a	2,746.2 f	4,102.5 bc	1,033.6 ab	10,581.7 e	15,792.9 c	732.5 a	7,208.8 de	10,881.7 bc
T_{18}	512.5 cd	1,966.8 d	3,384.2 b	2,482.6 e	9,277.0 de	15,900.0 c	1,804.0 d	6,677.1 d	11,315.3 bc
T_{19}	802.1 fg	3,066.4 fg	4,796.6 c	2,465.1 e	9,363.4 de	14,389.7 bc	1,562.2 cd	5,638.2 c	8,516.3 b
T_{20}	544.8 e	1,282.7 b	3,557.2 b	2,921.0 fg	6,481.4 c	17,694.9 cd	2,306.0 f	5,231.4 c	14,277.9 c
T_{21}	263.7 b	1,833.3 cd	4,044.4 bc	1,336.2 bc	8,839.3 d	18,839.3 cd	797.2 ab	5,469.6 c	11,850.5 bc
T_{22}	368.1 bc	1,055.0 ab	1,458.2 a	2,000.0 d	5,464.9 bc	7405.2 a	1124.3 b	3,159.4 ab	4,322.8 a
T_{23}	458.6 c	2,233.3 de	3,894.3 bc	2,152.8 de	10,257.5 e	17,725.9 c	1,905.7 de	8,985.3 ef	15,119.1 cd
T_{24}	235.7 a	1,364.3 b	4,796.2 c	1,176.6 ab	6,773.7 c	23,774.1 de	1,044.8 b	5,940.0 cd	20,217.9 e
T_{25}	797.2 fg	3,271.2 g	10,168.4 g	3,309.8 g	13,540.0 f	42,064.1 f	2,745.9 f	11,202.2 g	34,416.1 f

Means followed by a common letter(s) are not significantly different at 5 % level according to DMRT

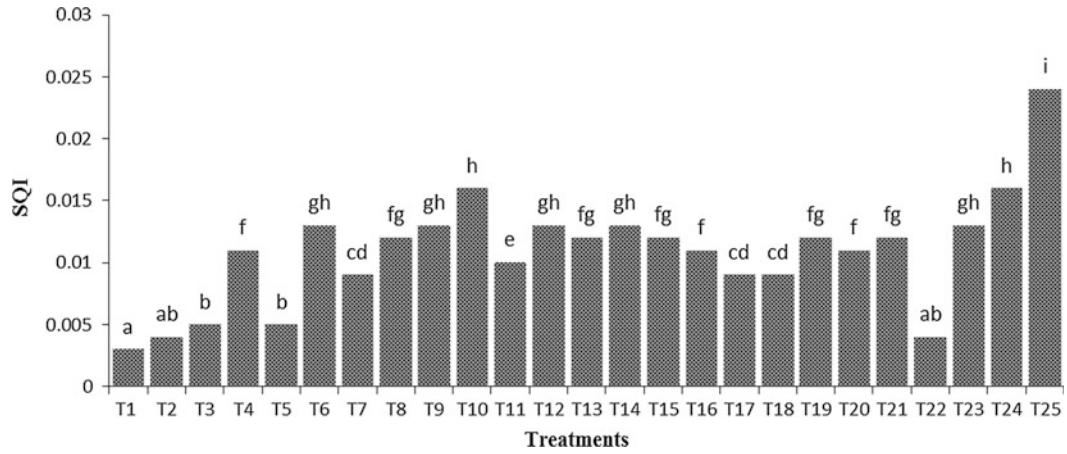


Fig. 6 Seedling quality index of *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

inoculation of *G. fasciculatum* significantly increased stem girth than *G. intraradices*. However, Sumana and Bagyaraj (1998) reported that the best AM fungi for inoculation *D. Latifolia* are *G. leptotichum* and *G. fasciculatum* in forest nurseries (Fig. 7).

Combined inoculation of N_2-fixing bacteria and mycorrhizal fungi increased seedlings growth, biomass and nodule formation when compared to control. Rhizobial inoculation enhanced nodulation and N fixation in *D. latifolia* which is in accordance with Gupta and Rahangdale (1999) who also reported increased nodulation and N_2 fixation in *Dalbergia sissoo* inoculated with AMF and *Rhizobium*. The results of the present study also supports the observations made elsewhere where AM fungi has been reported to influence nodulation and N_2 fixation in leguminous and actinorhizal plants (Russo 1989; Isopi et al. 1994; Osundina 1998). Other microbes like *Azospirillum* and PSB also improved AM formation as shown for other rhizosphere microorganisms (Azcon-Aguilar and Barea 1992; Barea et al. 1998). The precise mechanisms that account for such microbial stimulation of AM formation have not been clearly identified yet (Fig. 8).

However, rhizobial inoculation increased the AMF colonization. This increase in AM colonization levels can be attributed to the increase in P demand of the host brought about by the bacterial symbiont, since nodulation and nitrogen fixation require a high level of P in the host tissue (Barea and Azcon-Aguilar 1983). Nodule biomass was significantly positively correlated with *Rhizobium* ($r = 0.439$; $P < 0.05$; $n = 25$) and *Azospirillum* ($r = 0.510$; $P < 0.01$; $n = 25$) populations in the soil. Dual inoculation of *Rhizobium* sp. and *G. mosseae* has been reported to enhance growth and nodulation in *Prosopis cineraria* (Rani et al. 1998). Combined inoculation of AM fungi and *Rhizobium* increased the leaf area. An increase in cytokinin activity in the shoots due to mycorrhizal colonization promotes leaf growth by increasing cell division and cell expansion (Baas and Kupier 1989).

Inoculation of *Rhizobium* increased *Dalbergia*-seedling N content when co-inoculated with *Azospirillum*. The increase in N content of *D. latifolia* seedlings in *Rhizobium* and *Azospirillum* combination indicates that the increased N in the soil due to *Azospirillum* activity is taken up by the roots. This is further supported by the existence of a strong correlation between *Rhizobium* ($r = 0.649$; $P < 0.01$; $n = 25$) and *Azospirillum* ($r = 0.675$; $P < 0.01$; $n = 25$) population with plant N. In contradiction, Reverkar and Konde (1988) reported superior N_2 uptake in single inoculation compared to mixed inoculation of *Rhizobium* and *Azospirillum*. One possible reason is an increased nodule number and dry weight, which

Table 11 Correlation between soil and tissue nutrient, plant biomass, arbuscular mycorrhizal root colonization and microbial populations in *Dalbergia latifolia* inoculated with bioinoculants and biocontrol agent

	Soil N	Soil P	Soil K	Plant N	Plant P	Plant K	Nodule Number	Nodule DW	SDW
Soil N									
Soil P	–								
Soil K	–	–							
Plant N	−0.441[a]	–	–						
Plant P	–	–	–	0.502[a]					
Plant K	0.398[a]	–	–	–	–				
Nodule Number	–	–	–	0.411[a]	–	–			
Nodule DW	–	–	–	0.443[a]	–	–	0.464[a]		
SDW	–	–	–	–	0.415[a]	–	–	–	
RDW	–	–	–	0.459[a]	0.573[b]	–	–	–	0.585[b]
Plant DW	–	–	–	–	0.542[b]	–	–	–	0.919[b]
%RLA	–	–	–	–	0.552[b]	–	–	–	–
%RLV	–	–	0.477[a]	–	0.414[a]	–	–	–	0.608[b]
%RLC	–	–	0.516[b]	–	0.406[a]	–	–	–	0.398[a]
Rhizobium	–	–	–	0.649[b]	–	–	0.428[a]	0.439[a]	–
Azospirillum	-0.442*	–	–	0.675[b]	0.528[b]	–	–	0.510[b]	–
PSB	–	–	–	–	0.575[b]	–	–	–	0.495[a]
Trichoderma	–	–	–	0.698[b]	0.674[b]	–	0.414[a]	–	–

	RDW	Plant DW	%RLA	%RLV	%RLC	*Rhizobium*	*Azospirillum*	PSB	*Trichoderma*
Soil N									
Soil P									
Soil K									
Plant N									
Plant P									
Plant K									
Nodule Number									
Nodule DW									
SDW									
RDW									
Plant DW	0.857[b]								
%RLA	–	–							
%RLV	–	0.542[b]	–						
%RLC	–	–	0.546[a]	0.689[b]					
Rhizobium	–	–	–	–	–				
Azospirillum	–	–	–	–	–	0.471[a]			
PSB	0.560[b]	0.587[b]	–	–	–	0.437[a]	0.427[a]		
Trichoderma	0.503[a]	0.474[a]	–	–	–	0.713[b]	0.576[b]	0.504[a]	

[a] Correlation is significant at the 0.05 level (2-tailed)
[b] Correlation is significant at the 0.01 level (2-tailed)
–Not significant

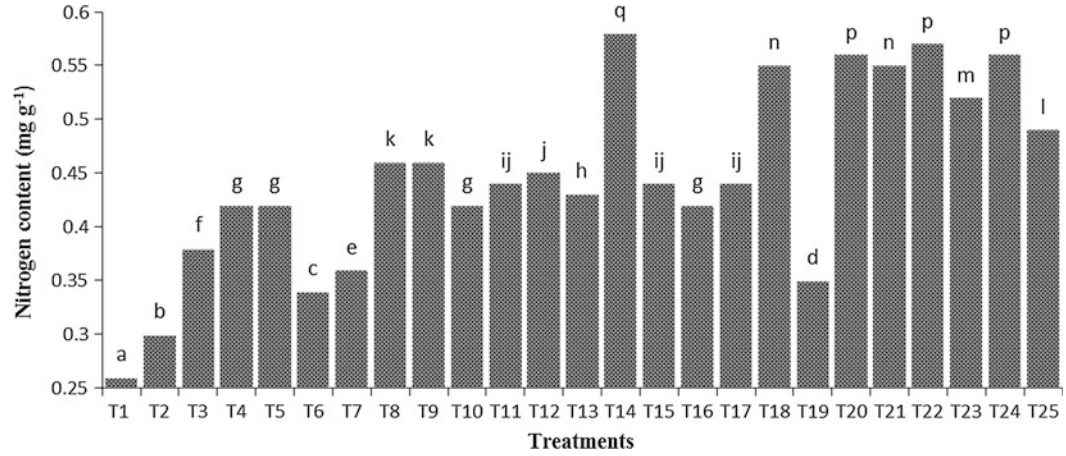


Fig. 7 Tissue nitrogen content in *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

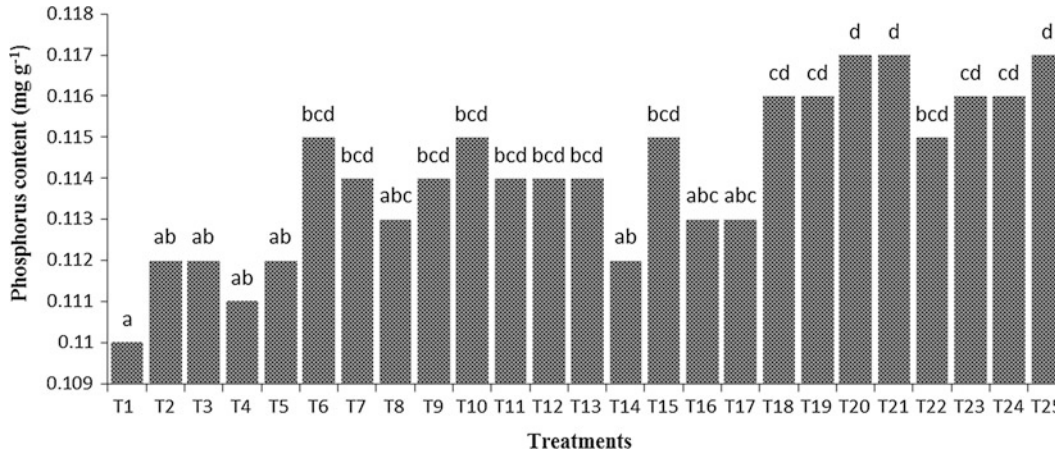


Fig. 8 Tissue phosphorus content in *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

might have, enhanced the amount of nitrogen in the plants (Nambiar et al. 1983) which is supported by the existence of a positive correlation of Plant N to nodule number ($r = 0.411$; $P < 0.05$; $n = 25$) and nodule biomass ($r = 0.443$; $P < 0.05$; $n = 25$).

These studies do indicate that dual inoculation of different AM fungi enhance P content (Bolan et al. 1987), significant differences in P content of *D. latifolia* seedlings were evident in this study when AM fungal species were inoculated individually. The strong positive correlation of %RLA ($r = 0.552$; $P < 0.01$; $n = 25$), and %RLC ($r = 0.406$; $P < 0.01$; $n = 25$) with plant P, clearly suggests that AM fungi could aid *D. latifolia* seedlings in the uptake of P from the soil. As AMF is able to access sparingly soluble P in the substrate more readily, uptake and transport of soil P by AM fungal hyphae is a well-known response (Mosse et al. 1976; Elgala et al. 1995).

In the present study %RLC, populations of *Rhizobium*, *Azospirillum*, PSB and *Trichoderma* were significantly correlated to plant N and/or P. The population of specific group of microbes in the soil inoculated with different bioinoculants varied with treatments or their combinations. Soil

microbial growth and activity is usually limited by the availability of carbon (Wardle 1992; Lynch 1990). In contrast, when AM colonization had no or negative effect on plant growth, it could result in less carbon being exuded. Further, the nutrient status of plants is known to influence root exudates composition (Kraffczyk et al. 1984; Lipton et al. 1987). The composition and the amount of microorganisms present in the rhizosphere of *D. latifolia* seedlings in different treatments could be due to the changes in the quality and quantity of compounds exuded by seedlings in different treatments.

Inoculation with PSB increased plant biomass of *Dalbergia* seedlings. The PSBs are known to release several organic acids, which solubilize bound phosphates (Kim et al. 1998). Organic acid released by the PSB results in a fall in pH, and as a consequence, the P concentration in the substrate increases (Sperber 1957), which is reflected by a drop in soil pH in PSB-inoculated soils. Further, PSB can also influence plant growth through their ability to synthesize plant hormones (Sumner 1990; Rodriguez and Fraga 1999).

Several mechanisms have been proposed by which *Trichoderma* is known to influence plant growth. These include production of growth hormones (Windham et al. 1986), solubilization of insoluble major and minor nutrients in the soil (Altomare et al. 1999; Yedidia et al. 2001) and increased uptake and translocation of low-available minerals (Baker 1989; Kleifeld and Chet 1992; Inbar et al. 1994).This is evidenced by the existence of a strong positive correlation between populations of *Trichoderma* and seedlings dry weight of *D. latifolia* ($r = 0.698$; $P < 0.01$; $n = 25$). *Trichoderma* population was significantly higher in AM fungi–inoculated soils than in uninoculated soils. This contradicts the observations of Green et al. (1999) where the AM fungus *G. intraradices* has been shown to suppress the population density of *T. harzianum*. Further, *Trichoderma* had positive effect on mycorrhization (%RLA, $r = 0.297$; %RLV, $r = 0.348$; %RLC, $r = 0.330$) in the present study which contrasts the results of Green et al. (1999) who reported a negative effect of *T. harzianum* on *G. intraradices* colonization in cucumber (*Cucumis sativus* L.).

In the present study, population of *Rhizobium* was strongly correlated with other populations of *Azospirillum*, PSB and *Trichoderma*. Most current evidence indicates that many microorganisms develop function in the rhizosphere, which may influence not only plants but also the co-occurring microbial members of the soil community (Lynch 1990; Kloepper 1991). Specialized activities such as production of vitamins, amino acids, hormones may be operating

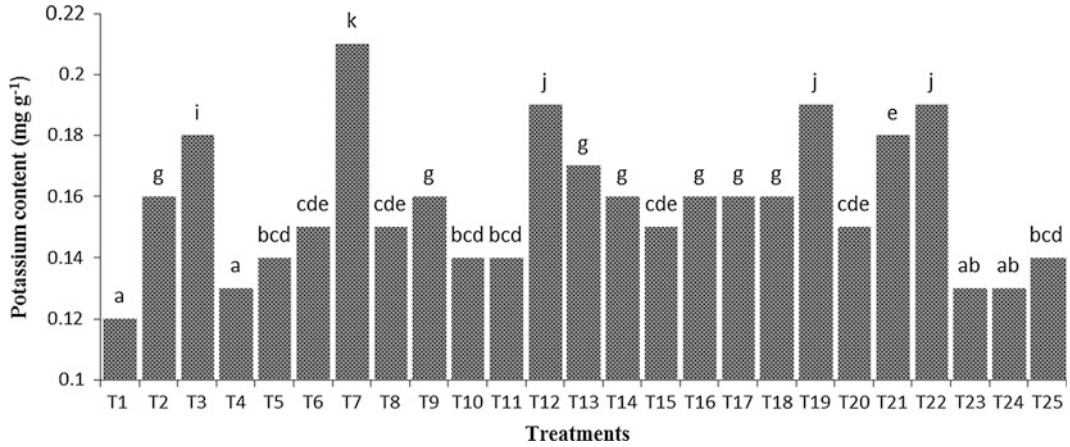


Fig. 9 Tissue potassium content in *D. latifolia* seedlings inoculated with bioinoculants and biocontrol agents at 180 DAT

in microbe–microbe interactions involving AM fungi and other soil microorganisms.

The results show the root/shoot (R/S) ratio of *D. latifolia* seedlings during later stages of growth was similar or higher to that of uninoculated control. The decreased R/S ratios indicate the host's ability to channel energy to shoot production, due to an increased efficiency of the roots by AM fungi (Bethlenfalvay et al. 1982). An increased allocation to roots to increase absorptive surface area could result in an increased rate of nutrient absorption, increasing the R/S ratio. However, shoot N, P and K contents in combined inoculated plants differed significantly from individual inoculation of the endophytes. The increased N, P and K status can be attributed to the role of extramatrical hyphae of AM fungi in making available the inaccessible nutrients available to the plant roots (Muthukumar and Udaiyan 1995).

Generally, bioinoculations significantly increased the efficiency of nutrient usage in *Dalbergia* seedlings. These results indicate that *Dalbergia* seedlings inoculated with different microbes used nutrients more efficiently which is in accordance with studies where microbial inoculations are known to increase nutrient use efficiencies (Menge et al. 1978; Graham and Syvertsen 1985). Though increased nutrient use efficiencies are assumed to be associated with nutrient deficiency, *Dalbergia* seedlings inoculated with different microbes always had more nutrients than the uninoculated control. So, one explanation could be that nutrients other than N, P or K can be limiting these nutrient usages (Koide et al. 2000).

At the end of the nursery experiment, the seedling quality (as assessed by SQI) of *D. latifolia* seedlings in multiple inoculated treatments (*G. intraradices*/*G. fasciculatum*/*Rhizobium*/*Azospirillum*/PSB/*Trichoderma*) was significantly higher. A similar increase in nursery seedling quality inoculated with different bioinoculants has been reported for neem seedlings (Muthukumar et al. 2001).

In conclusion, *D. latifolia* seedlings inoculated with a combination of AM fungi, *Rhizobium*, *Azospirillum* and PSB show substantial increase in seedling growth nutrient content and seedling quality. These responses of inoculated seedlings are several folds higher when compared with uninoculated seedlings. Inoculation of *D. latifolia* seedlings with combined bioinoculants confirms higher seedling vigor in the nursery, which raises the possibility of better the out plant survival of these seedlings in the field (Fig. 9).

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Isolation and Characterization of Microcystin-Producing *Microcystis aeruginosa* MBDU 626 from a Freshwater Bloom Sample in Tamil Nadu, South India

A. M. P. Anahas, M. Gayathri, and G. Muralitharan

Abstract

Toxin-producing cyanobacteria are a worldwide threat to both human and animal health. Microcystins (MCs) are the most commonly occurring toxins produced by bloom-forming cyanobacteria, especially *Microcystis* sp. This study describes the occurrence of bloom-forming toxigenic *Microcystis aeruginosa* MBDU 626 from Manjalar Dam, Theni District, Tamil Nadu, South India. Two microcystin (MC) variants, MC-LR and [D-Asp^3] MC-LR were identified from the isolated strain using high-performance liquid chromatography and gas chromatography coupled mass spectrometry (GC/MS). Four peptides such as aeruginosin, microginin, kasumigamide and anabaenopeptin were also co-produced along with these MC variants. Our results show that the presence of cyanobacterial toxins in essential water resources requires rapid remedial action and needs to develop a national program for regular monitoring of toxigenic blooms in freshwater bodies of South India, in general, Tamil Nadu, in particular.

Keywords

Cyanobacteria · *Microcystis aeruginosa* · Microcystin-LR · Manjalar dam · Theni · Tamil Nadu · South India

Introduction

Cyanobacteria (blue-green algae) are the prominent cause of water blooms in eutrophic lakes and drinking water reservoirs worldwide (Carmichael 1994; Sivonen 1996). Toxic bloom-forming cyanobacteria have been reported causing animal death and also adversely affecting human health (Carmichael 1994, 2001; Codd et al. 1997). Microcystins (MCs) are the most commonly encountered cyanotoxins (Sivonen

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1996). *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Hapalosiphon*, *Anabaenopsis*, etc., are common MC-producing cyanobacterial genera (Carmichael 1992; Sivonen and Jones 1999). However, majority of the MCs-producing blooms are dominated by *Microcystis* (Codd 1999; Kabernick et al. 2000; Lehman et al. 2005; Li et al. 2007; Dai et al. 2008; Xu et al. 2008).

The general structure of MC is cyclo-(D-alanine-*X*-D-MeAsp-*Z*-Adda-D-glutamate-Mdha), where D-MeAsp is D-erythro-*β*-methyl-aspartic acid, and Mdha is *N*-methyldehydroalanine (Mdha). *X* and *Z* are variable L-amino acids (Sivonen and Jones 1999). To date, more than 90 structural variants of MCs have been reported (Zurawell et al. 2005; Wood et al. 2008). The most common MC congener (MC-LR) is characterized by the presence of leucin (L) and arginin (R) as L-amino acids in positions 2 and 4 (Xaa^2 = L: Ala, Yaa^4 = R: Arg) (Gulledge et al. 2002). Since the first elucidation of MC structure by Botes et al. (1984), extensive structural characterizations of other MC variants have been the subject for many studies (Sivonen et al. 1990; Namikoshi et al. 1992a, b; Luukkainen et al. 1994; Namikoshi et al. 1995; Sano and Kaya 1995, 1998; Beattie et al. 1998) and resulted in the identification of different structural variants of MCs to date. MC-LR is the most toxic and widely encountered MC variant for which World Health Organization (WHO) set a guideline value of 1 μg L^{-1} for drinking water (WHO 1998). Based on the review of all the toxicity data, the International Agency for Research on Cancer (IARC) classified MC-LR as a potential carcinogen (Group 2B) (Grosse et al. 2006).

Beside MCs, various other linear and cyclic oligopeptides such as aeruginosins, anabaenopeptilides, cyanopeptolins, anabaenopeptins and microginins are found within the genus *Microcystis* (Namikoshi and Rinehart 1996). As like MCs, the structures of these peptides generally include unusual amino acids residues, such as 3-(4-hydroxyphenyl)-lactic acid (Hpla) and 2-carboxy-6-hydroxy-octahydroindole (Choi) in aeruginosins, or 3-amino-6-hydroxy-2-piperidone (Ahp) in cyanopeptolins, *β*-amino-*α*-hydroxy-decanoic acid in the linear microginins (Neumann et al. 1997; Fukuta et al. 2004; Harada 2004; Welker et al. 2004a, b).

In fact, no consistent hypothesis has been developed so far to explain the high structural variability and patchy distribution of cyanopeptides (Welker et al. 2006). This is partly due to the still very limited knowledge on the occurrence of individual peptides and peptide classes in environmental samples (Fastner et al. 2001). These peptides have been found to exhibit a wide range of biochemical and pharmacological activities (Fastner et al. 2001; Bister et al. 2004; Welker et al. 2004a).

While there have been lengthy investigations regarding the occurrence of toxic cyanobacteria in many countries, there are only a few reports on their occurrence in India (Prakash et al. 2009). This might be due to the prevalence of less toxic variants like MC-RR, or in some cases, a lack of awareness and knowledge to correlate properly the toxicity with the prevailing cyanobacterial blooms (Sangolkar et al. 2009). Cyanobacterial blooms that produce MC-LR, MC-RR and its demethylated variant have been reported in India (Agrawal et al. 2006; Prakash et al. 2009), and adjacent tropical countries including Korea (Kim et al. 1999; Oh et al. 2001) and Thailand (Wang et al. 2002). In this study, we have reported the investigations into the occurrence of MC-producing *Microcystis* sp. in Theni District, Tamil Nadu, South India.

Materials and Methods

Bloom Sampling and Strain Culture Conditions

Cyanobacterial bloom sample was collected from Manjalar Dam (10°11′37.15″N 77°37′55.86″E), Theni District, Tamil Nadu, India (Fig. 1). The sample was identified as containing primarily of *Microcystis aeruginosa*. Generic assignment of the isolate was based on morphological criteria (Rippka et al. 1979). The bloom sample was initially grown in BG-11 medium with nitrate source. The culture was

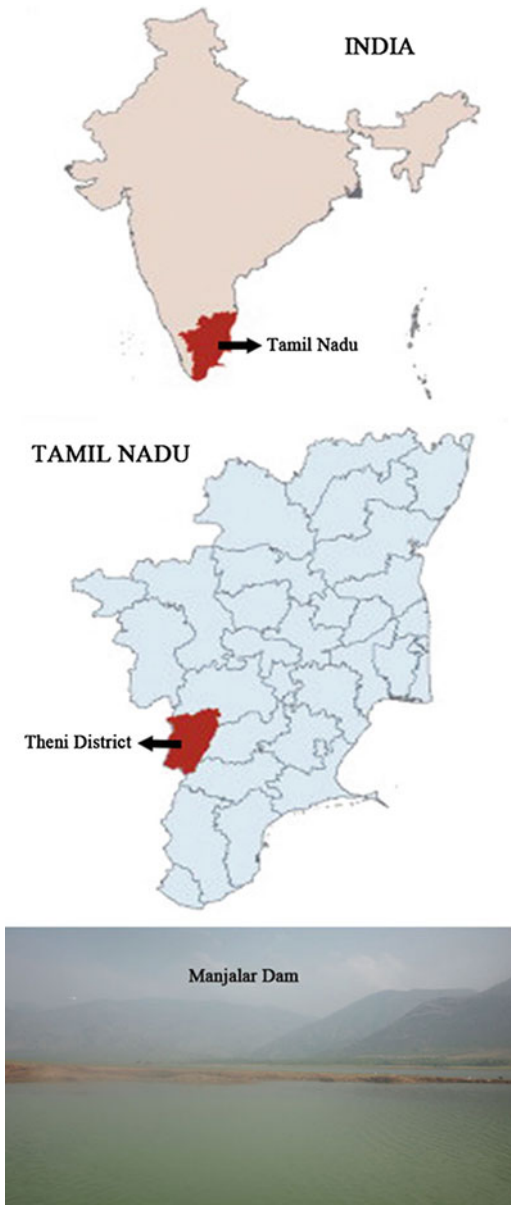

Fig. 1 Map of Tamil Nadu, India, showing Theni District (in *red*) and the Manjalar Dam was shown in the picture (10°11′37.15″N 77°37′55.86″E), where the bloom sample was collected

incubated under constant light intensity (50 μE m^{-2} S^{-1}) for up to 10 days at 25 °C. No bacterial contamination was detected during microscopic observation of the culture.

Extraction and Analysis of Microcystins

Toxin was extracted as described previously (Frias et al. 2006). Briefly, late log phase culture (15 days old) of *Microcystis aeruginosa* MBDU 626 was centrifuged at 5,000 × g for 15 min at 4 °C (Remi, India), and the pellets were freeze dried and stored at −20 °C until further analysis. MC was extracted with MeOH/H_2O (3:1, v/v) from frozen samples (∼1 g) submitted to sonic disruption for 25 min. Extract was centrifuged (10,000 × g for 15 min) and the supernatant collected. The pellet obtained was re-extracted. The supernatant was combined and evaporated to dryness in a rotary evaporator (40 °C). The dried material was resuspended in MeOH and partitioned with $CHCl_3$:MeOH:H_2O (7:6:3, v/v/v) to remove hydrophobic compounds and pigments. The hydro-alcoholic phase was evaporated and dissolved in 1 ml of MeOH/H_2O (7:3 v/v). The extract was filtered through 0.45 μm millipore membranes and injected into the HPLC system.

HPLC Analysis

A high-performance liquid chromatography equipped with a constant flow pump (Shimadzu LC 8A, Japan) was used. Separation was accomplished under reversed phase isocratic conditions with (Shim-Pack CLC-Octa decyl silane) ODS-C_{18} column (4.6 mm ID × 25 cm) and guard column (Shim-Pack G-ODS) (4 mm ID × 1 cm) and mobile phase of 100 % methanol. The flow rate was 1 ml/min for analysis, and UV absorbance at 254 nm was used as detector.

Acid Hydrolysis and Derivatization of the Toxin

The isolated compound was mixed with 6 N HCl (100/900 μl) and heated at 110 °C for 22 h. The reaction mixture was cooled to room temperature and evaporated in a stream of N_2, then

equal volume (200 µl) of dichloromethane (CH_2Cl_2) and trifluoroacetic anhydride (TFAA) was added, and again the mixture was heated at 110 °C for 5 min, then evaporated by N_2 (Namikoshi et al. 1992b). The residue was dissolved in MeOH for GC/MS analysis.

GC/MS Analysis

GC/MS was performed with an Agilent gas chromatograph coupled to a JEOL GC/MS II MATE ion trap mass spectrometer. HP5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm) was operated in a split less mode, and the injector temperature was 220 °C. The carrier gas (He) flow was adjusted to 1 ml min^{-1}. Samples of 1 µl in MeOH were injected into the GC/MS. The program rate for the analysis of amino acid derivatives was 80–250 °C at 20 °C/min.

Molecular Analysis

Total genomic DNA was isolated from the tested cyanobacterial strain following the previously described method (Neilan 1995) and was used as a template in PCR; 16S rDNA was amplified from the genomic DNA using the cyanobacterial specific primers (Wilmotte et al. 1993; Nelissen et al. 1994). Purified PCR product was sequenced using the BigDye Terminator Cycle Sequencing v2.0 kit on an ABI 310 automatic DNA sequencer (Applied Biosystems, CA, USA). The 16S rDNA gene sequence determined in this study was deposited in the GenBank database under the accession number JN542384.

Results

Strain Characterization

The results of the present study revealed the occurrence of *M. aeruginosa* in Manjalar dam bloom samples. The isolated strain was characterized by both morphological and 16S rDNA sequence analysis. Figure 2 shows the typical morphology of *M. aeruginosa* strain isolated from the sampling site. 16S rDNA sequence analysis revealed that the isolated strain was having 95 % similarity to *M. aeruginosa* LMECYA 106 (EU078498) and *M. aeruginosa* UWOCC 019 (AF139295), confirming its identity.

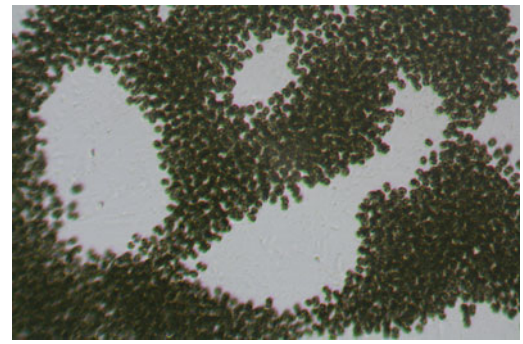

Fig. 2 Photomicrograph illustrating the morphological features of *Microcystis aeruginosa* MBDU 626 isolated from water bloom sample

GC/MS Analysis of Microcystin

MCs were generally detected as singly protonated molecular ions. GC/MS analysis revealed the presence of two different MC isoforms. Both isoforms showed the characteristic fragment ion peak 135 [M + H^+] (Tables 1 and 2), the Adda side-chain [$PhCH_2CH(OMe)^+$], which is a key indicator for the presence of MCs. Further investigation into the fragment ion peaks enabled the identification of the isoforms as MC-LR and [D-Asp^3] MC-LR.

Product assignation of the fragment at *m/z* 994.5[M + H^+] in GC/MS spectrum revealed the presence of MC-LR. The detected fragment ions at *m/z* 86 and 112 show the presence of immonium ions. These *m/z* values indicate the presence of Leu and Arg residues, and this result is also corroborated by the molecular mass of 994 Da. The ion fragment at *m/z* 553.4 corresponds to [Mdha-Ala-Leu-MeAsp-Arg + H]$^+$ evidencing the presence of other amino acid residue characteristic of MCs, Mdha and also

Table 1 Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 994 corresponding to MC-LR obtained from *M. aeruginosa* MBDU 626 strain

Fragment ions	MC-LR (*m/z*)
[Immonium of Arg]$^+$	70.2
[Leu]$^+$	86.2
[Immonium of Arg]$^+$	112.2
[$PhCH_2CH(OMe)$]$^+$	135.1
[Glu-Mdha + H]$^+$	213.3
[Mdha-Ala-Leu + H]$^+$	268.1
[Arg-MeAsp + H]$^+$	286.2
[$C_{11}H_{14}O$-Glu-Mdha + H]$^+$	375.2
[Arg-MAsp-Leu-Ala + H]$^+$	470.1
[$C_{11}H_{14}O$-Glu-Mdha-Ala + H]$^+$	466.2
[Adda-Arg-Masp + H]$^+$	599.8
[Arg-Masp-Leu-Ala-Mdha + H]$^+$	553.4
Loss of PhCH = CH(OMe)	861.5
$[M + H]^+$	994.5

Table 2 Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 981 corresponding to [D-Asp^3] MC-LR were obtained from *M. aeruginosa* MBDU 626 strain

Fragment ions	[D-Asp^3]MC-LR (*m/z*)
[$PhCH_2CH(OMe)$]$^+$	135.2
[Mdh-Ala + H]$^+$	155.1
[$C_{11}H_{14}O$ + H]$^+$	163.3
[(Arg + NH_2) + 2H]+	174.4
[Glu-Mdha + H]$^+$	213.2
[MeAsp-Arg + H]$^+$	272.1
[(Arg + NH_2)-MeAsp + 2H]+	289.1
[$C_{11}H_{14}O$-Glu-Mdha + H]$^+$	375.3
[$C_{11}H_{14}O$-Glu-Mdha-Ala + H]$^+$	466.3
[Mdha-Ala-Leu-MeAsp-Arg + H]$^+$	539.2
[MeAsp-Arg-(Adda-MeOH) + H]$^+$	553.3
[MeAsp-Arg-(Adda-MeOH)-Glu + H]$^+$	682.4
[(M-$PhCH_2CH(OMe)$) + H]$^+$	847.4
$[M + H]^+$	981.5

indicating the presence of the residues Leu and Arg at positions 2 and 4, respectively. Additionally, the following molecular ion species have provided full confirmation of MC-LR identity: [Glu-Mdha + H]$^+$ at *m/z* 213.1, [M + H-Adda]$^+$ at *m/z* 861.5, [Arg-Adda-Glu + H]$^+$ at *m/z* 599.8, [M + H-Glu]$^+$ at *m/z* 866.6 and [$C_{11}H_{14}O$-Glu-Mdha]$^+$ at *m/z* 375.2. A complete list of the detected fragment ion peaks for MC-LR is shown in Table 1.

The second MC isoform identified in the strain tested was [D-Asp^3] MC-LR at *m/z* 981.5[M + H^+]. This MC isoform has a molecular weight of 981 Da. The detected fragment ion peak at *m/z* 539[Arg-Asp-Leu-Ala-Mdha + H]$^+$ is characteristic for this demethylated MC-LR isoform (Table 2). Indeed, the fragmentation pattern of the *m/z* ion completely matched with that expected from [D-Asp^3] MC-LR. Table 2 shows the product assignation of the fragment produced in GC/MS. The *m/z* ions at 213.2[Glu-Mdha + H]$^+$, 155.2[Mdha-Ala + H]$^+$ and 446.3[$C_{11}H_{14}O$-Glu-Mdha-Ala + H]$^+$ indicated the presence of Mdha and

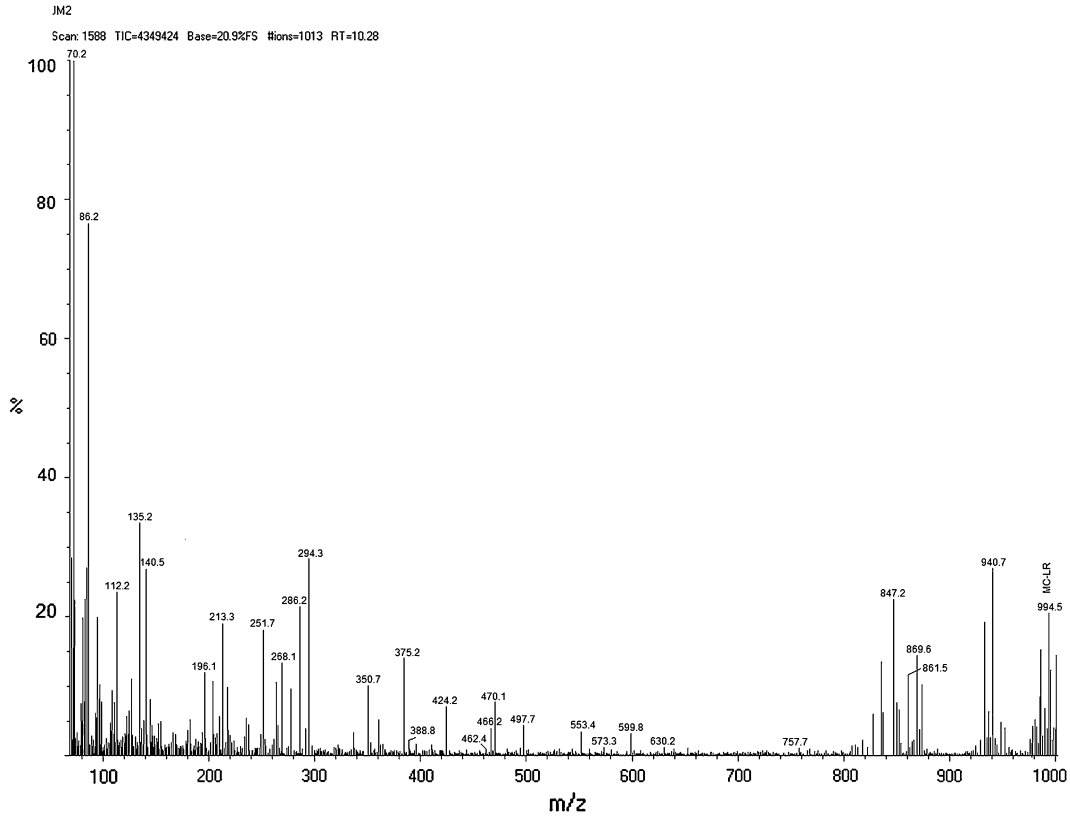

Fig. 3 GC/MS spectrum of MC-LR detected from *Microcystis aeruginosa* MBDU 626. (See Table 1 for fragment ion identifications)

Ala in position 7 and 1, respectively. On the other hand, the *m/z* ions at 289.1[(Arg + NH_2)-Asp + 2H]$^+$, 272.1[Asp-Arg + H]$^+$, 553.3[Asp-Arg-(Adda-MeOH) + H]$^+$ and 682.4[Asp-Arg-(Adda-MeOH)-Glu + H]$^+$ strongly indicated the presence of Asp instead of MeAsp in position 4, proving the demethylated MC to be [D-Asp^3] MC-LR. The mass spectrum of both MC-LR and [D-Asp^3] MC-LR MC isoforms detected in this study shown in Figs. 3 and 4, respectively.

It is interesting to note that the tested *M. aeruginosa* MDBU 626 had shown five peptides identical to microginin, aeruginosin 602, aeruginosin 101, anabaenopeptin and kasumigamide at 698.3 *m/z*, 603.3 *m/z*, 645.6 *m/z*, 851.5 *m/z* and 788.6 *m/z*, respectively. Microginin are linear peptides with a characteristic N-terminal 3-amino-2 hydroxydecanoic acid (Ahda). The fragment ions at *m/z* 698.3 (Ahda-Thr-Pro-Tyr-Trp) from the side chain of Ahda were observed with the same ions in the mass spectrum (Fig. 6).

The other peptide aeruginosin is linear tetrapeptides with the unique moiety 2-carboxy-6-hydroxy-octahydroindole (Choi) and a C-terminal Arg derivative. Fragment spectra of two peptides characteristically show an intense mass signal detected at *m/z* 140 Da, the Choi-immonium ion which is indicative of aeruginosins. A peptide with [M + H]$^+$ at *m/z* 645.36 could also be identified as an aeruginosin (aeruginosin 644) with a number of fragments identical to fragments of aeruginosin 602: *m/z* 86, 140, 250, 266 and 350 Da. The fragment ions at *m/z* 86.2 (Leu- or Ile-immonium ions), *m/z* 140.1 (Choi-immonium ions), *m/z* 250.1 (Hpla-Leu-Choi-Argal), *m/z* 266.2 (Choi-Arginal-CH_3N_2–H_2O + H) and *m/z* 350.4 (Choi-Ac Argininal −NH_2 +H) were observed together with the same ions in the mass spectrum (Fig. 5). The predominant fragment

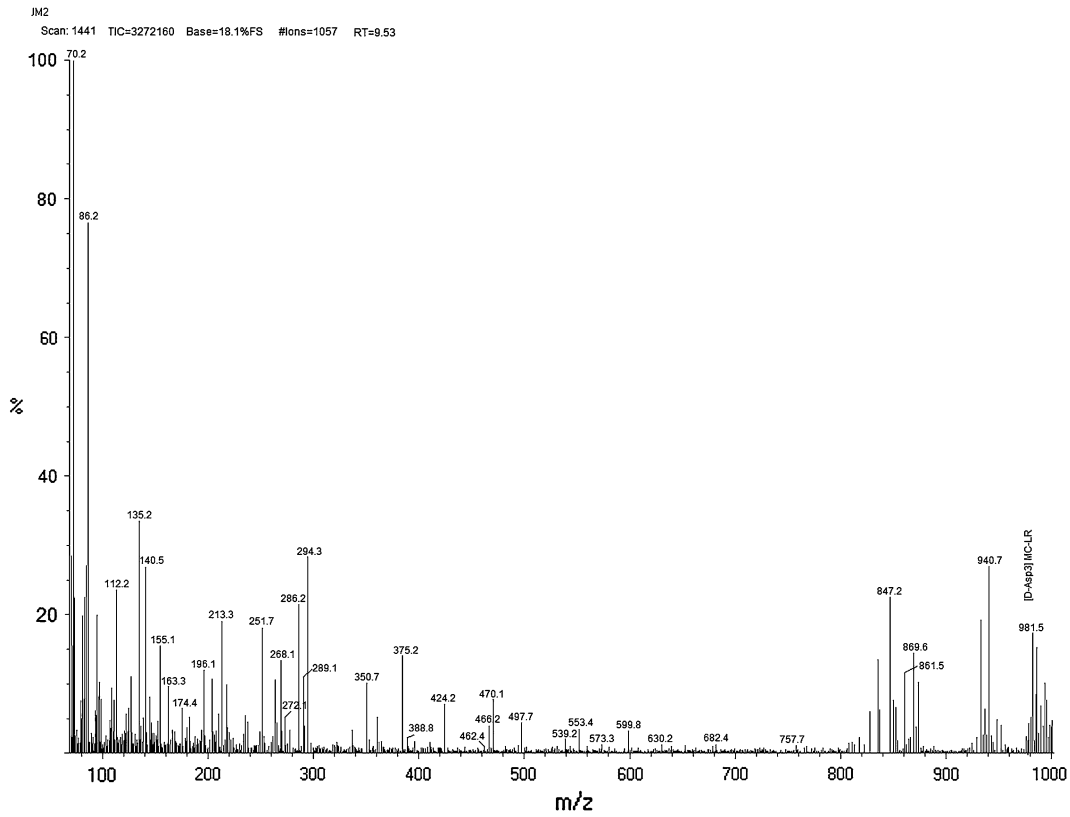

Fig. 4 GC/MS spectrum of [D-Asp^3] MC-LR detected from *Microcystis aeruginosa* MBDU 626. (See Table 2 for fragment ion identifications)

ions were observed in the MS spectra of the related aeruginosin, which are summarized in the Table 3.

Kasumigamide, a linear tetrapeptide containing an N-terminal α-hydroxyl acid with *m/z* 787.38 (Pla-*β*Ala-Ahipa-Arg-phSer) having the C-terminal moiety, that is, hydroxy-group of phenyl-serine was observed in the mass spectrum (Fig. 5).

Anabaenopeptins, a group of cyclic hexapeptides are characterized by a 19-membered peptide ring that is formed by cyclization between the C-terminal amino acid and the ε-amine of a lysine residue. The α-amine of the lysine is further linked through an ureido group to a side-chain amino acid. Two anabaenopeptin variants with similar mass have been identified in this study. Fragmentation spectrum by GC/MS was indicated that the peak at m/z 851[M + H$^+$] corresponded to two isobaric anabaenopeptin variants, that is, anabaenopeptin B1 and F. The fragment ions at *m/z* 57 (MAla-Immonium ion) *m/z* 70 (Arg-/Lys-related ions), *m/z* 84 (Lys-Immonium ion), *m/z* 112 and 129 (Arg-Immonium ion), *m/z* 201 (CO + Arg) (side chain) *m/z* 233 (MAla + Phe + H), *m/z* 286 (Lys + CO + Arg-CN_2H_2), *m/z* 291(HTry + Ile + H), *m/z* 376 (MAla + HTyr + Ile + H), *m/z* 417 (HTyr + Ile + H), *m/z* 538 (Lys + Phe + MAla + HTyr + 2H) and *m/z* 651 (Lys + Phe + MAla + HTyr + Ile + 2H) were observed together with the same ions in the mass spectrum (Fig. 6 and Table 4).

Discussion

Occurrence of cyanobacterial blooms and associated animal and human poisoning has been documented from over sixty-five countries

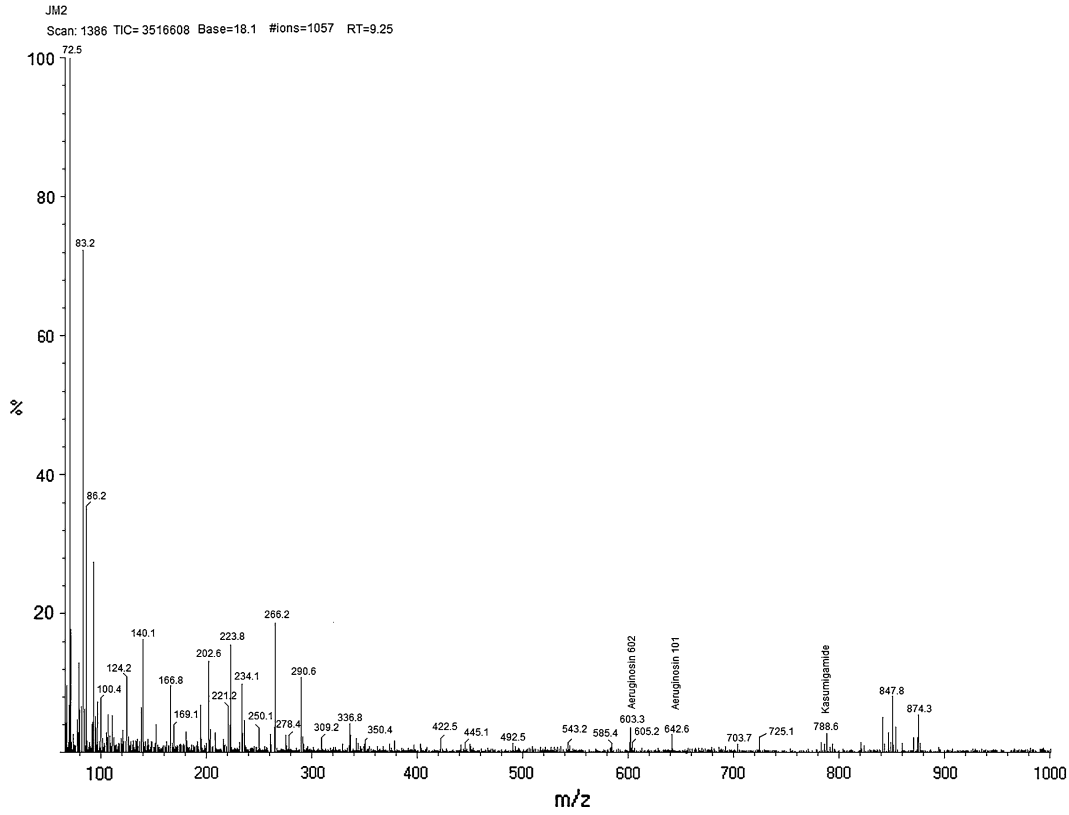

Fig. 5 GC/MS spectrum of aeruginosin peptide detected from *Microcystis aeruginosa* MBDU 626; peak at *m/z* 603 and *m/z* 642 corresponding to aeruginosin 602 and aeruginosin 101; peak at *m/z* 788 identified as kasumigamide. (See Table 3 for fragment ion identifications)

Table 3 Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 603 corresponding to aeruginosin peptide obtained from *M. aeruginosa* MBDU 626 strain

Fragment ions	Aeruginosin (*m/z*)
Leu-immonium ion	86.2
Argal-fragment	100.4
Choi-immonium fragment	140.1
Choi + H	169.1
(Leu-Choi)fragment	221.2
$R_{1,2}$-Hpla-Leu-CO + H[b]	250.1
Hpla-Leu-H	266.2
Choi-Argininal-CH_3N_2-H_2O + H[a]	278.4
Choi-Argininal-NH_2-H_2O + H	290.6
Choi-Argininal-NH_2 + H	309.2
Choi-AcArgininal-NH_2 + H	350.4
M-Argal	445.1
M-CH_3N_2-H_2O + Ha	543.2
M-H_2O + H	585.4
M + H	603.3

[a] CH_3N_2 is the ureido group of Argininal; [b] R_1 and R_2 are either a hydrogen or a chlorine in the non-, mono- or di-chlorinated variant, respectively

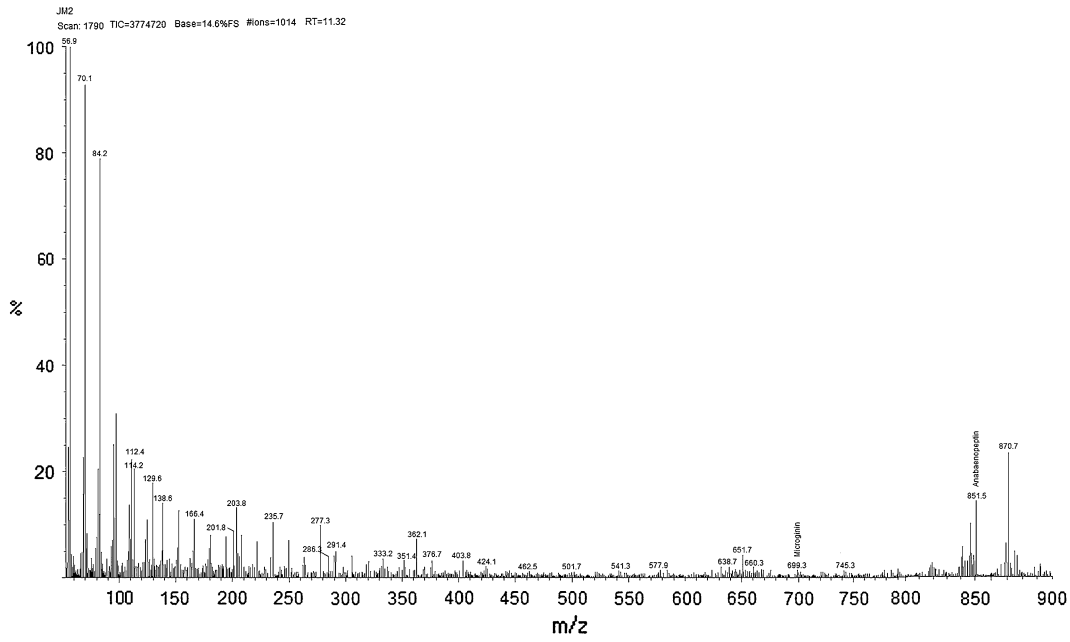

Fig. 6 GC/MS spectrum of anabaenopeptin peptide detected from *Microcystis aeruginosa* MBDU 626; peak at *m/z* 851 corresponding to a mixture of anabaenopeptin F and [HArg^6]-anabaenopeptin B; peak at *m/z* 699 identified as microginin. (See Table 4 for fragment ion identifications)

Table 4 Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 851 corresponding to [HArg^6]-anabaenopeptin B and anabaenopeptin F peptides obtained from *M. aeruginosa* MBDU 626 strain

Fragment ions	[HArg^6]-Anabaenopeptin B *m/z*	Anabaenopeptin F *m/z*
MAla-Immonium ion	57	57
Arg/Lys-related ions	70	70
Lys-Immonium ions	84	84
Arg-Immonium ions	112	112
[MAla + CO + H]	114	114
Arg-Immonium ions	129	129
[CO + Arg](side chain)	201	201
[HTyr + Val + H]	277	–
Lys + CO + Arg-CN_2H_2	286	286
[HTyr + Ile + H]	–	291
[MAla + HTyr + Val + H]	362	–
[MAla + HTyr + Ile + H]	–	376
[HTyr + Val + Lys]	403	–
[HTyr + Ile + Lys]	–	417
[Lys + Phe + MAla + HTyr + 2H]	–	538
[Lys + Phe + MAla + HTyr + Val + 2H]	637	–
[Lys + Phe + MAla + HTyr + Ile + 2H]	–	651
[M + H]+	851	851

(Codd et al. 2005), including India (Agrawal et al. 2006), Sri Lanka (Jayatissa et al. 2006) and Bangladesh (Welker et al. 2005). The warm water temperature in India promotes dense *Microcystis* growth almost throughout the year (Parker et al. 1997; Agarwal et al. 2001). There have been few reports of MC occurrence in India (Sangolkar et al. 2009), and information about the evidence of bloom formation and toxicity in South Indian water bodies is particularly scarce. During the biodiversity survey of different freshwater ponds of Thanjavur District, Tamil Nadu, it is reported that potentially toxic cyanobacterial blooms are common in the freshwater ponds of Tamil Nadu region (Muthukumar et al. 2007). Out of the five ponds investigated, Dabeerkulam pond showed low diversity of cyanobacteria which was attributed to a massive bloom of *Microcystis aeruginosa* (Muthukumar et al. 2007). Similarly, this study indicates the presence of toxigenic *M. aeruginosa* MBDU 626 in the fresh water of Manjalar Dam in Periyakulam, Theni District. The freshwater bodies of South India in general, Tamil Nadu in particular, have so far been given less attention. This work was an extension of our earlier report on the presence of MC-LA-producing *Microcystis aeruginosa* MBDU 013 in Kuttappar Lake at Tiruchirappalli District, Tamil Nadu (communicated data).

GC/MS method has been developed for screening MCs, in complex samples such as sediments. It is based on the detection of 2-methyl-3-methoxy-4-phenylbutyricacid (MMPB), which is formed when the Adda residue is split following oxidation of MCs (Harada et al. 1996; Kaya and Sano 1999; Tsuji et al. 2001). Mass spectrometry (MS) has proved to be a valuable technique for providing structural information on MCs (Harada 1995; Kondo and Harada 1996; Meriluoto et al. 2000), without need for toxin standards or specific retention times that are required for HPLC analyses (Jungblut et al. 2006).

The [D-Asp^3] MC-LR and MC-LR have been shown to form [M + H] $^+$ ion of *m/z* 981 and 995 (Diehnelt et al. 2005; Jungblut et al. 2006; Del Campo and Ouahid 2010). Similar fragment ions for [D-Asp^3] MC-LR and MC-LR were reported from an Antarctic cyanobacterial mat community by Q-Star quadrupole-TOF hybrid mass spectrometer (Jungblut et al. 2006). The characteristic fragment ion for MC-LR has also been reported by Diehnelt et al. (2005). In Uttar Pradesh, India, five eutrophic temple ponds in the vicinity of Varanasi city were reported for MC-LR-producing *Microcystis* blooms (Prakash et al. 2009). In addition to *Microcystis,* MC-LR forming *Nostoc* sp. BHU001 was reported from the agricultural pond of Banaras Hindu University, Varanasi, India (Bajpai et al. 2009). Frias et al. (2006) have reported that the occurrence of MC-LR in a bloom in the eutrophic reservoir Billings, Sao Paulo City, Brazil, by ESI–MS/MS analysis. In a similar study, ten out of 12 MCs, including [D-Asp^3] MC-LR and MC-LR, were detected from International Culture Collections strains of *Microcystis* (Del Campo and Ouahid 2010) and reported the fragment ions for [D-Asp^3] MC-LR at *m/z* 155.2, 213.2, 289.1, 553.3 and 682.4. Similar fragment ions were obtained from our experiment (Figs. 4 and 5). [D-Asp^3] MC-LR also has been detected in bloom samples from Morocco (Oudra et al. 2001) and the Philippines (Baldia et al. 2003). The characteristic Adda fragment for MCs was seen at 135.2 *m/z* (Figs. 3 and 4), possibly generated by the α-cleavage at the methoxy group of the Adda β-amino acid moiety (Ortea et al. 2004).

Five peptides were identified as to aeruginosin, microginin, anabaenopeptin and kasumigamide (Figs. 5 and 6), and these were also identified from the *m/z* of GC/MS analysis. The MS approach was successful in detecting a multitude of known and new peptides from very small samples of cyanobacterial cells. Detectability of individual peptides depends partly on the efficiency with which they can be protonated (Karas et al. 2000). Further information on the identity of oligopeptides was gained from the comparison with published fragmentation data from pure substances and from a fragment database (Haande et al. 2007).

The co-occurrence of MCs and cyanopeptolins in *Microcystis* spp. dominated field samples was reported previously (Jacobi et al. 1996; Neumann et al. 2000). Many of the substances detected

belong to well-known groups of cyanobacterial peptides like MCs, anabaenopeptins, microginins, cyanopeptolins and aeruginosins, of which many have been discovered in *Microcystis* spp. (Namikoshi et al. 1996). With respect to known peptides, combinations of anabaenopeptins, microginins and aeruginosins were observed, while MCs were found along with aeruginosins. This correlates to the detection of aeruginosins as well as cyanopeptolins in both toxic and nontoxic *Microcystis* culture strains (Namikoshi et al. 1996; Dittmann et al. 1997).

A fragment ion *m/z* at 698.3$[M + H]^+$, characteristics of microginin, was reported from bloom material of lake Tegamura, Japan (Kodani et al. 1999). Our experiments identified similar fragment ions from the tested organism. A peptide with a molecular mass of *m/z* 603$[M + H]^+$ is probably a new variant of an aeruginosin-type peptide, as suggested by the fragment ion of *m/z* 140, indicating the presence of the unusual amino acid Choi, which is unique to aeruginosin-type molecules (Murakami et al. 1995; Matsuda et al. 1997; Erhard 1999). Ishida et al. (1999) have reported that aeruginosin 101 was originally isolated from *Microcystis aeruginosa* (NIES 101). Agarwal et al. (2006) have reported the presence of aeruginosin by MALDI-TOF/MS analysis, in the *Microcystis* blooms from Gosalpur Lake of Jabalpur in Central India. Kasumigamide, a novel antialgal peptide which shows a characteristic fragment *m/z* at 787.3$[M + H]^+$ was originally isolated from freshwater cyanobacterium, *Microcystis aeruginosa* (NIES-87) (Ishida and Murakami 2000). *Microcystis* colonies isolated from lakes Müggelsee, Pehlitzsee and Parsteiner See in and around Berlin, Germany, were shown to possess mainly of kasumigamide linear peptide (Welker et al. 2004a).

The co-occurrence of both MCs and anabaenopeptins in natural populations has been well documented (Kodani et al. 1999; Fastner et al. 2001; Grach-Pogrebinsky et al. 2003). In the samples dominated by *Microcystis* spp., anabaenopeptins were found only when MCs also were present (Gkelis et al. 2005) and similar results have been reported from natural population samples studied (Kodani et al. 1999; Fastner et al. 2001; Grach-Pogrebinsky et al. 2003). However, it is still unclear whether cyanobacterial strains produce both types of peptides simultaneously or produce only MCs. Our results support the hypothesis of the coexistence of toxic MC with nontoxic peptides.

This study reinforces the earlier investigations into cyanobacterial blooms in Central India on the occurrence of toxigenic species in freshwater bodies of Indian ecosystem and states that major concern should be given for the screening program at least for those freshwater bodies used for animal or human consumption.

For a variety of reasons, the harmful impact of cyanobacteria on human health was always been a topic of interest (Falconer 1996, 1997). Concern about the MCs health risk to humans through drinking water, led the WHO to develop and suggest a provisional guideline level of MC-LR at 1 $\mu g/L^{-1}$. Up to now, this value has been considered as a safe level in drinking water (Falconer et al. 1999). Further research and data analysis are needed to generate the information on MC occurrence, diversity and distribution with reference to climatic zones, namely temperate, tropical and subtropical regions (Sangolkar et al. 2009). This study clearly revealed that toxigenic *Microcystis* strains are present in the freshwater bodies of Southern Indian region and major attention should be given for the effective screening and mitigation strategies.

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Microbial Bioinoculants Potential on the Growth Improvement of *Curcuma longa* L. under Tropical Nursery Conditions

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Abstract

The aim of the present study is to assess the impact of microbial bioinoculants application on turmeric (*Curcuma longa* L.) plant productivity under nursery conditions. The understanding of the growth performances of turmeric plants inoculated with microbial bioinoculants under nursery conditions is a key to improve crop production further in field. Turmeric plants with 13 different combinations of microbial bioinoculants in the liquid formulations were applied as singly, dual and multiple combinations. The experimental plants were maintained based on complete randomized block design (CRBD). Since both biotic and abiotic factors influenced the plant growth including soil nutrient status, plant hypotrophy, phytochemical composition, microbial population density and mycorrhizal proteins, these were taken into consideration and those were analyzed. Among the bioinoculant combinations applied, multiple combinations worked out better than the single inoculation. There occurred significant positive as well as negative correlation among all these biotic and abiotic factors at 5 and 1 % levels. The treatments such as T_5, T_{10} and T_{13} were assessed as best combinations of bioinoculants and those were also found as compatible.

Keywords

Microbial bioinoculants · Turmeric · Glomalin · Soil fertility · Biotic factors · Abiotic factors · PGPR

Introduction

Industrialization and population explosion are the two major drawbacks confined to reduction in agricultural productivity. To increase the agricultural productivity to meet the needs of global population, there should be a change in the current agricultural practices. Because, the

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high usage of chemical fertilizers has made the soil infertile, accumulation of toxic chemicals in the soil and food products and imbalance in the nutrient cycling and ecosystem. In order to maximize the agricultural productivity with minimum soil loss, a cheap, better and safest way is necessary. All these criteria can be achieved through the application of microbial bioinoculants. Indeed, these microorganisms are known to possess vast range of capabilities by producing growth-promoting substances and enhancing the plant nutrients, biologic N_2 fixation and protection against stress and diseased conditions. These PGPR have been shown to cause very real and positive effects when matched correctly to the right plant and the right environmental situation.

Turmeric (*Curcuma longa* L.) which is a perennial crop belongs to Zingiberaceae family and is widely cultivated in India and other parts of the world. Curcumin, a bioactive component of turmeric, is responsible for the wide spectrum of medicinal properties and has commercial applications. The microbial bioinoculants such as *Azospirillum lipoferum*, *Trichoderma viride*, *Bacillus megaterium*, *Pseudomonas fluorescens* and arbuscular mycorrhizal fungi (AM fungi) were chosen for the study due to the metabolic activities they carry out. An important aim of nursery experiments is to produce consistent results of target morphological, biochemical and microbiological characteristics with bioinoculant treatment on turmeric growth. Successful field cultivation can be achieved based on the results of nursery trials (Jacobs et al. 2003). If a positive effect of microbial bioinoculants is seen on a specific crop in nursery studies, there is a strong likelihood that those benefits will carry through to field conditions (Lucy et al. 2004). The present study is focused at identifying the suitable combination of microbial bioinoculants that successfully increase the yield and quality of turmeric.

In addition to this, the influences of microbial bioinoculants on the soil microbial population as well as colonization of AM fungi in the roots of turmeric were studied. It is found that the ecologically important role played by AM fungi is due to the production of mycorrhizal proteins, namely total glomalin and easily extractable glomalin. These proteins possess the capability to enhance the soil quality by making the soil particle clump together, involve in carbon cycling and sequester the heavy metals in the soil. This study is the maiden attempt to find out the variations in the concentrations of glomalin in the soil and root. The present study has given special context to analyze whether there is any significant correlation with glomalin and other biotic and abiotic factors.

Materials and Methods

Experimental Setup

The experiments were carried out at Elathur village, Gobichettipalayam, Erode District in Tamil Nadu, India. The polybag media were prepared by mixing farmyard manure, soil and sand in the ratio of 1:2:1. The nursery plants were maintained based on CRBD. Seeds were inoculated with bioinoculants and biocontrol agents (*A. lipoferum*, *B. megaterium*, AM fungi, *T. viride* and *P. fluorescens*) individually or in various combinations, and the control plants were raised in polythene bags. Plants were watered as and whenever necessary throughout the experimental duration. Various combinations of bioinoculants were introduced viz: T_1—control, T_2—*A. lipoferum*, T_3—*T. viride*, T_4—*P. fluorescens*, T_5—*A. lipoferum* + *T. viride*, T_6—*A. lipoferum* + *B. megaterium*, T_7—*P. fluorescens* + AM fungi, T_8—*T. viride* + *B. megaterium*, T_9—*A. lipoferum* + *T. viride* + *B. megaterium*, T_{10}—*T. viride* + *B. megaterium* + AM fungi, T_{11}—*A. lipoferum* + *B. megaterium* + AM fungi, T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*, T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AM fungi.

Sample Collection

Root, rhizome and rhizosphere soil samples were collected from nursery experiment turmeric polybag at the harvesting period. The rhizosphere soils from the individuals were mixed to form a composite soil sample, air dried in room temperature, packed in polythene bags and kept at 4 °C for further analysis.

Methodologies Adopted

The soil physicochemical properties such as pH, EC and nitrogen (N), phosphorus (P) and potassium (K) were analyzed (Jackson 1973). Organic carbon matter was estimated according to Walkley and Black method (Walkley and Black 1934). The micronutrients such as Cu, Zn, Fe and Mn were estimated as described by Lindsay and Norvell (1978). The plant shoot height, root length, shoot and root biomass and number of leaves present in each plant were recorded. The shoot and root dry weights were obtained from each sample by oven drying at 80 °C to get a constant weight.

The estimation of chlorophyll *a*, chlorophyll *b* and total chlorophyll in leaves was made (Witham et al. 1971). The total carbohydrate and protein concentration was determined by the methods of Hedge and Hofreiter (1962) and Lowry's (1951), respectively. The concentration of phenol content present in the leaves was analyzed according to the method of Malick and Singh (1980). Easily extractable glomalin (EEG) and total glomalin (TG) extractions from turmeric root and rhizosphere soil were performed based on the method of Wright and Upadhyaya (1996). The quality of turmeric product was assessed by estimating the curcumin (Cur) based on spectrophotometric method (Sadasivam and Manickam 1991).

The microorganisms such as bacteria, actinomycetes and fungi were isolated and enumerated to assess the population density by using standard microbiological techniques. The percentage of mycorrhizal colonization in turmeric roots was determined using trypan blue method (Phillips and Hayman 1970).

Statistical Analysis

All data were subjected to analysis of variance (ANOVA) and the means separated using Duncan's multiple range test (DMRT). Pearson's bivariate correlation analysis (SPSS version 11.5) was used to assess the relationships between biotic and abiotic variables.

Results

Influence of Microbial Bioinoculants on the Soil Edaphic Factors

The rhizosphere soil pH of the various microbial bioinoculants existed from 8.5 to 9.2.

The combination of *A. lipoferum* and *T. viride* (T_5) was involved in the increment of rhizosphere soil pH to the maximum level when compared to all other treatments including control. However, the trials of T_4, T_9, T_{10} and T_{13} maintained pH level equivalent to control. The electrical conductivity of the rhizosphere soils varied from 0.1 to 0.16 among all the treatments assessed. In turn, the combination of *A. lipoferum* and *B. megaterium* (T_6) inoculation had raised the EC value to its highest extent of 0.16. Rather than the single inoculation, multiple inoculations expressed some effect on increasing the EC values. The maximum amount of OC % was accumulated when inoculated with combined inoculation of *P. fluorescens* and AM fungi (T_7). The second maximum OC content was observed in the treatments T_8 (*T. viride* and *B. megaterium*). Almost all the treatments had shown remarkable and significant variation when compared to control (Table 1).

The influence of microorganisms in increasing N content at the second level was observed in the treatment of T_9 (*A. lipoferum*, *B. megaterium* and AM fungi), and T_{13} had showed maximum total N content in their rhizosphere soils. Of all the

Table 1 Influence of microbial bioinoculants on rhizosphere soil edaphic factors

Treatments	pH	EC	OC	N	P	K	Fe	Mn	Zn	Cu
T_1	8.6 ± 0.3^{b}	0.1 ± 0.004^{a}	0.16 ± 0.006^{a}	60 ± 2^{a}	4 ± 0.12^{a}	$62 \pm 2^{e\text{-}f}$	4.4 ± 0.1^{a}	3 ± 0.1^{a}	0.66 ± 0.01^{a}	0.82 ± 0.02^{a}
T_2	$8.9 \pm 0.35^{e\text{–}h}$	0.1 ± 0.003^{a}	0.84 ± 0.04^{f}	70 ± 3^{fg}	4.4 ± 0.2^{b}	45 ± 2.1^{a}	5.4 ± 0.22^{e}	3.28 ± 0.12^{a}	0.94 ± 0.04^{c}	1.04 ± 0.02^{e}
T_3	8.5 ± 0.4^{a}	0.1 ± 0.002^{a}	$0.75 \pm 0.03^{b\text{-}e}$	70 ± 2^{fg}	5.2 ± 0.2^{cde}	63 ± 3^{g}	5.92 ± 0.15^{h}	5.14 ± 0.22^{ghi}	0.72 ± 0.02^{a}	0.92 ± 0.02^{c}
T_4	8.6 ± 0.31^{b}	0.1 ± 0.002^{a}	1 ± 0.04^{g}	64 ± 2^{c}	5.4 ± 0.2^{f}	75 ± 3.2^{k}	5.86 ± 0.18^{g}	5.12 ± 0.2^{ghi}	0.68 ± 0.01^{a}	0.86 ± 0.03^{a}
T_5	$9.2 \pm 0.4^{k\text{–}m}$	0.1 ± 0.003^{a}	0.9 ± 0.02^{a}	62 ± 3^{b}	6.2 ± 0.1^{hi}	70 ± 2^{i}	5.24 ± 0.2^{d}	4.24 ± 0.1^{e}	0.94 ± 0.03^{c}	0.91 ± 0.04^{b}
T_6	8.5 ± 0.2^{a}	$0.16 \pm 0.004^{j\text{–}m}$	$0.2 \pm 0.08^{l\text{–}m}$	72 ± 3^{hi}	5.6 ± 0.2^{g}	65 ± 3^{h}	5.39 ± 0.1^{e}	4.4 ± 0.12^{f}	1.8 ± 0.02^{klm}	1.1 ± 0.05^{fgh}
T_7	8.5 ± 0.3^{a}	0.1 ± 0.003^{a}	1.72 ± 0.06^{k}	67 ± 2^{de}	5.2 ± 0.1^{cde}	68 ± 3.2^{hi}	5.86 ± 0.22^{g}	4.12 ± 0.2^{d}	0.77 ± 0.01^{a}	0.84 ± 0.02^{a}
T_8	8.7 ± 0.2^{cd}	0.1 ± 0.004^{a}	1.42 ± 0.05^{k}	70 ± 3^{fg}	4.2 ± 0.2^{a}	70 ± 3^{j}	5.24 ± 0.2^{d}	3.4 ± 0.1^{b}	1.11 ± 0.04^{de}	0.94 ± 0.01^{d}
T_9	8.6 ± 0.4^{b}	$0.12 \pm 0.003^{b\text{-}e}$	1.2 ± 0.04^{i}	78 ± 3^{m}	7 ± 0.3^{l}	72 ± 2^{h}	4.7 ± 0.2^{b}	6.2 ± 0.2^{lm}	0.81 ± 0.02^{b}	1.15 ± 0.02^{i}
T_{10}	8.6 ± 0.3^{b}	0.13 ± 0.002^{fg}	1.32 ± 0.06^{j}	74 ± 2^{i}	7.1 ± 0.3^{m}	66 ± 1.5^{b}	6.1 ± 0.1^{i}	4 ± 0.1^{cd}	0.88 ± 0.04^{bc}	$1.3 \pm 0.03^{l\ m}$
T_{11}	9 ± 0.4^{ij}	0.1 ± 0.004^{a}	1.1 ± 0.05^{h}	63 ± 1^{bc}	6.8 ± 0.2^{kl}	50 ± 2^{a}	5.11 ± 0.1^{c}	3.14 ± 0.12^{a}	0.8 ± 0.02^{b}	0.94 ± 0.02^{d}
T_{12}	8.7 ± 0.3^{cd}	0.1 ± 0.003^{a}	1.22 ± 0.04^{i}	70 ± 2^{fg}	4.2 ± 0.3^{a}	70 ± 3^{l}	5.24 ± 0.24^{d}	3.4 ± 0.14^{b}	1.11 ± 0.02^{de}	0.94 ± 0.02^{d}
T_{13}	8.6 ± 0.3^{b}	0.14 ± 0.002^{hi}	1.01 ± 0.04^{g}	76 ± 2^{kl}	6.4 ± 0.1^{l}	80 ± 1^{lm}	7 ± 0.17^{lm}	5.5 ± 0.2^{jk}	1.29 ± 0.03^{fg}	1.2 ± 0.03^{jk}

Note T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The values bearing the same letters are not significantly different at 5 % level according to DMRT

treatments tested, four treatments viz. T_2, T_3, T_8 and T_{12} had shown their performance similarly in increasing the soil N content. The available phosphorus content of all the treatments occurs between the ranges of 4.0 and 7.0 kg/acre. The combined effort of *T. viride*, *Pseudomonas* and AM fungi had elevated the available phosphorus level to maximum compared with the control. The treatment of T_9 was followed by T_{10}, which stayed in the second place with a slight variation in the available phosphorus content. The net effect of all the five bioinoculants (T_{13}) exerted its potency with the highest K content. Even the single inoculation of *P. fluorescens* (T_4) present the results comparable with the multiple inoculations, and this is an additional point for further experiment.

On considering the Fe content, the input of microbial bioinoculants had put their work toward the inflation of Fe based on their capability and the values exist from 4.40 to 7.0 ppm (Table 1). It was demonstrated that T_{13} exhibited the maximum values, respectively. The treatments namely T_3, T_4, T_7 and T_{10} had shown corresponding values. The treatment of T_9 leads to all other treatments with the maximum Mn content of 6.2 ppm, which was followed by T_{13} with 5.5 ppm. The maximum and minimum range of Zn existed between 0.65 and 1.81 ppm. T_6 had greater value of Zn content among all other treatments (1.8 ppm). Indeed, the second maximum results were obtained in the case of T_{13} (1.29 ppm). Both treatments of T_8 and T_{12} retained the same and higher Zn content values substantially. The maximum value of Cu was present in the rhizosphere soil of $T_{10.}$ Next to that, T_{13} found to be efficient in increasing the Cu content. With the special note that all the treatments showed Cu content higher than the control. The root length of the treatments varied from 15 to 25.3 cm. T_{12} had expressed drastic elevation in its root length. The treatments of T_6 and T_7 were well known for producing lengthier roots. The triple combinations of microbial bioinoculants were perceptible in performance compared with single and multiple combinations.

Turmeric Growth and Yield Improvement Through Microbial Bioinoculant Application

The impact of microbial bioinoculants application on the growth and yield of turmeric was assessed by measuring morphological parameters they exhibit. The primary factor which was taken into consideration was number of leaves present in the turmeric plant (Table 2). Maximum number of leaves were present in the treatment of T_{12}, whereas the minimum number of leaves were shown by treatment T_2. Several other treatments such as $T_{6,}$ T_9 and T_{13} had the number of leaves next to that of T_{12}. Generally, intercalary shoots appear from the primary or mother rhizomes. The measurement of intercalary shoots denotes the growth intensity of plants. The treatments appeared to have 0–3 intercalary shoots. The multiple combinations of biofertilizers had helped in attaining maximum number of intercalary shoots than the single or multiple inoculations. Based upon the type of inoculation made, the shoot heights were found to differ from each other. Both T_{10} and T_{12} were acting superior by growing to a greater extent than any other treatments and also showed only least variation among them. Likewise, the treatment of T11 and T13 captured second maximum position, and statistically, they were equal to each other in their performance level.

Impact of Microbial Bioinoculants on Biochemicals of Turmeric

It is indeed necessary to understand the biochemistry of plants in addition to its morphological parameters. The single inoculation of *T. viride* (T_4) was involved largely in accumulation of carbohydrates in the turmeric leaves to its higher extent (Fig. 1). The second maximum carbohydrate level was shown by T_{11} (33.5 μg/0.1 g). The total phenol concentration among the bioinoculant treatments lies between 4.31 and 120.8 μg/0.5 g leaf. The combined

Table 2 Impact of microbial bioinoculants on growth and yield of turmeric under nursery trials

Treatments	No. of leaves	Intercalary shts	Sht Height (cm)	Root length (cm)	1° rhizomes	2° rhizomes	3° rhizomes	Sht biomass (g)	Root biomass (g)	Rhi biomass (g)
T_1	5 ± 1^{a}	0 ± 0^{a}	73 ± 3.0^{a}	15.1 ± 0.3^{a}	7 ± 2^{hij}	25 ± 3^{k}	2 ± 0^{a}	14.3 ± 0.3^{a}	8.0 ± 0.1^{a}	98.51 ± 4^{a}
T_2	5 ± 1^{a}	0 ± 0^{a}	80 ± 2.0^{bc}	16 ± 0.4^{a}	1 ± 0^{a}	6 ± 1^{a}	8 ± 1^{d}	19.1 ± 0.4^{a}	9.50 ± 0.22^{a}	112.7 ± 3^{a}
T_3	$7 \pm 0^{d\text{-}g}$	$1 \pm 0^{b\text{-}d}$	88 ± 2.2^{fg}	16.8 ± 0.2^{b}	5 ± 1^{fg}	23 ± 2^{j}	17 ± 2^{i}	27.4 ± 0.5^{b}	11.0 ± 0.4^{b}	175.3 ± 5^{bc}
T_4	$6 \pm 1^{b\ c}$	$2 \pm 1^{e\text{-}i}$	84 ± 4.0^{de}	18.5 ± 0.5^{cd}	4 ± 1^{de}	20 ± 1^{h}	15 ± 1^{h}	49.2 ± 0.9^{de}	14.7 ± 0.5^{e}	185.0 ± 3^{d}
T_5	6 ± 1^{bc}	$1 \pm 0^{b\text{-}d}$	94 ± 2.0^{hi}	19.5 ± 0.4^{f}	2 ± 0^{b}	25 ± 2^{k}	9 ± 1^{e}	35.6 ± 1.1^{c}	13 ± 0.2^{d}	182.2 ± 4^{d}
T_6	$8 \pm 2^{h\text{-}j}$	$1 \pm 1^{b\text{-}d}$	98 ± 1.0^{k}	21.6 ± 0.3^{h}	3 ± 0^{c}	7 ± 1^{a}	10 ± 2^{ef}	50.2 ± 1.3^{f}	12.2 ± 0.3^{c}	214.2 ± 7^{de}
T_7	$6 \pm 1^{b\ c}$	$2 \pm 1^{e\text{-}i}$	97 ± 3.0^{l}	20 ± 0.25^{g}	2 ± 0^{b}	13 ± 1^{bcd}	12 ± 2^{fg}	61.7 ± 1.8^{g}	16.9 ± 0.3^{f}	335.7 ± 12^{fj}
T_8	$7 \pm 2^{d\text{-}g}$	$1 \pm 0^{b\text{-}d}$	95 ± 2.5^{j}	19 ± 0.15^{e}	9 ± 2^{klm}	13 ± 2^{bcd}	0 ± 0^{a}	75.4 ± 2.2^{j}	18.3 ± 0.5^{g}	307.5 ± 9^{i}
T_9	$8 \pm 1^{h\text{-}j}$	$2 \pm 1^{e\text{-}i}$	93 ± 3.0^{hi}	19.2 ± 0.21^{e}	2 ± 1^{b}	18 ± 3^{efg}	8 ± 2^{d}	82.3 ± 1.8^{k}	21.0 ± 0.4^{hi}	327.7 ± 10^{j}
T_{10}	$7 \pm 0^{d\text{-}g}$	$1 \pm 1^{b\text{-}d}$	101 ± 1.2^{m}	18.5 ± 0.3^{cd}	9 ± 1^{klm}	18 ± 2^{efg}	5 ± 1^{c}	95.5 ± 3.1^{lm}	20.6 ± 0.5^{hi}	307.0 ± 12^{i}
T_{11}	$7 \pm 1^{d\text{-}g}$	$3 \pm 1^{j\text{-}m}$	99 ± 3.3^{l}	18.6 ± 0.2^{cd}	7 ± 2^{hij}	28 ± 3^{lm}	24 ± 3^{klm}	70.8 ± 1.4^{i}	26.3 ± 0.7^{m}	337.7 ± 11^{j}
T_{12}	9 ± 2^{klm}	$2 \pm 0^{e\text{-}i}$	102 ± 2.6^{m}	$25.3 \pm 0.5^{i\text{-}m}$	5 ± 1^{fg}	24 ± 2^{k}	18 ± 2^{j}	62.7 ± 2.0^{h}	24.1 ± 0.8^{jkl}	364.0 ± 15^{k}
T_{13}	$8 \pm 1^{h\text{-}j}$	$3 \pm 1^{j\text{-}m}$	100 ± 2.0^{l}	18.5 ± 0.2^{cd}	7 ± 1^{hij}	21 ± 3^{i}	3 ± 0^{b}	50.9 ± 0.9^{f}	16.9 ± 0.3^{f}	$403.7 \pm 18^{l\ m}$

Note T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The values bearing the same letters are not significantly different at 5 % level according to DMRT

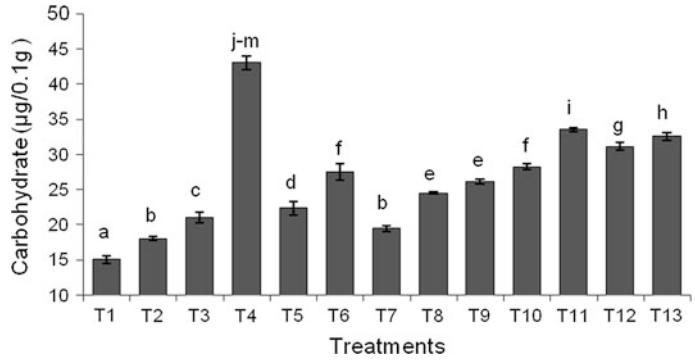

Fig. 1 Carbohydrate concentration variations in response to microbial bioinoculants. *Note* T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The *bars* bearing the same letters are not significantly different at 5 % level according to DMRT. The *error bars* represent the standard deviation values at 5 % level of significance

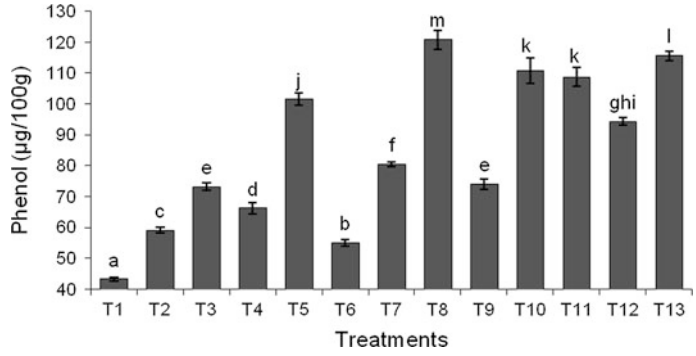

Fig. 2 Treatments of microbial bioinoculants showing phenol content variations. *Note* T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The *bars* bearing the same letters are not significantly different at 5 % level according to DMRT. The *error bars* represent the standard deviation values at 5 % level of significance

effect of all five different microbial bioinoculants had raised the total phenols to its second maximum compared with all other treatments (Fig. 2). But the treatment of T_8 took up its first place with the concentration of 120.7 µg/0.5 g leaf. In the case of protein, variations in the concentration occurred were depending on the efficacy of biofertilizers applied, thus maintaining the protein concentrations from 0.45 to 0.66 mg/0.1 g (Fig. 3). The maximum protein concentration was achieved when rhizomes were treated with *A. lipoferum*, *T. viride*, *B. megaterium* and *P. fluorescens* together. The treatment of T_{13} was the one which showed second maximum value not only in the case of phenol but also in the case of protein concentration. The multiple combinations were found to be more effective than the single or dual inoculation. Curcumin is the major bioactive component, and its level determines the quality of turmeric. The high-quality turmeric rhizomes were produced by two treatments (T_{12} and T_{13}),

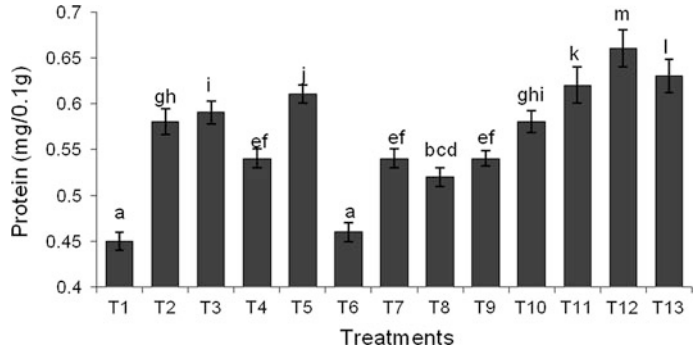

Fig. 3 Variations in the protein concentrations according to microbial bioinoculant treatments. *Note* T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The *bars* bearing the same letters are not significantly different at 5 % level according to DMRT. The *error bars* represent the standard deviation values at 5 % level of significance

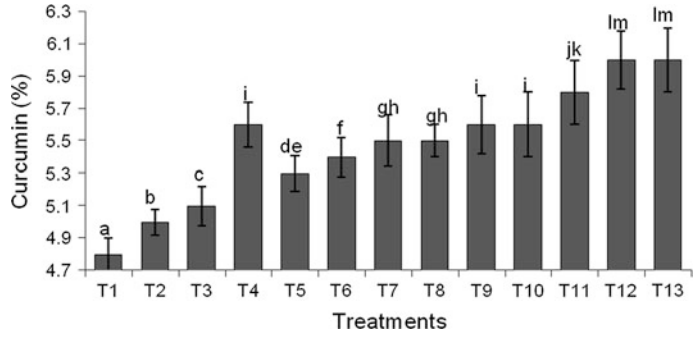

Fig. 4 Microbial bioinoculants showing increasing concentrations of curcumin. *Note* T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The *bars* bearing the same letters are not significantly different at 5 % level according to DMRT. The *error bars* represent the standard deviation values at 5 % level of significance

involving multiple combinations of bioinoculants (Fig. 4). Then, the treatment of T_{11} is also capable of providing quality turmeric rhizomes with 5.8 % curcumin.

Status of Microbial Population in the Turmeric Rhizosphere Soil

The rhizosphere soil that contained bacterial load to its maximum was present in the samples of T_{12} (Table 3). The soil samples of treatment T_9 were also potentially colonized by bacteria (20×10^{-5}/g soil). The soil samples of T_{13} contained less bacterial number finally when compared to T_{12} even when it was inoculated with an additional microorganism. The fungal colonization was higher in the rhizosphere soils of T_{10} and T_{13}. The dual inoculations maintained the fungal population level moderately when relating to control and individual inoculation. But the case was in contrast to actinomycetes

Table 3 Impact of microbial bioinoculants on microbial density of turmeric rhizosphere soil under nursery trials

Treatments	Bacteria ($\times 10^{-5}$/g soil)	Actinomycetes ($\times 10^{-4}$/g soil)	Fungi ($\times 10^{-3}$/g soil)	Azospirillum ($\times 10^{-4}$/g soil)	P. fluorescens ($\times 10^{-4}$/g soil)	PSB ($\times 10^{-3}$/g soil)	T. viride ($\times 10^{-3}$/g soil)	AMF (%)
T_1	8 ± 1^{a}	7 ± 2^{ij}	4 ± 1^{a}	5 ± 1^{bc}	0 ± 0^{a}	$1 \pm 0^{b-d}$	0 ± 0^{a}	68 ± 2^{a}
T_2	9 ± 1^{a}	4 ± 1^{de}	4 ± 0^{a}	7 ± 2^{fgh}	0 ± 0^{a}	$2 \pm 1^{e-g}$	0 ± 0^{a}	78.4 ± 3^{d}
T_3	10 ± 0^{b}	9 ± 2^{m}	6 ± 1^{ab}	4 ± 0^{a}	$1 \pm 0^{b-g}$	$1 \pm 0^{b-d}$	$1 \pm 0^{b-d}$	67.8 ± 2^{a}
T_4	12 ± 1^{d}	9 ± 1^{m}	6 ± 1^{ab}	6 ± 1^{de}	$2 \pm 1^{h-m}$	$2 \pm 1^{e-g}$	0 ± 0^{a}	83.8 ± 4^{ef}
T_5	9 ± 1^{a}	3 ± 1^{c}	9 ± 2^{c}	6 ± 1^{de}	0 ± 0^{a}	4 ± 2^{klm}	$2 \pm 1^{e-g}$	100 ± 4^{m}
T_6	14 ± 2^{e}	2 ± 1^{b}	8 ± 2^{bc}	8 ± 2^{ijk}	0 ± 0^{a}	$3 \pm 1^{h-j}$	0 ± 0^{a}	73.6 ± 2^{b}
T_7	10 ± 1^{b}	6 ± 2^{h}	12 ± 3^{de}	6 ± 1^{de}	$1 \pm 0^{b-g}$	$3 \pm 1^{h-j}$	$3 \pm 1^{h-j}$	75 ± 1^{c}
T_8	8 ± 1^{a}	9 ± 3^{m}	15 ± 3^{fg}	5 ± 1^{bc}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	$92.8 \pm 2^{i\ j}$
T_9	20 ± 4^{jk}	9 ± 2^{m}	18 ± 2^{i}	9 ± 2^{lm}	0 ± 0^{a}	$3 \pm 1^{h-j}$	4 ± 1^{klm}	87.9 ± 3^{gh}
T_{10}	11 ± 2^{c}	1 ± 0^{a}	24 ± 3^{kl}	5 ± 1^{bc}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	98 ± 3^{l}
T_{11}	16 ± 2^{fg}	8 ± 2^{kl}	16 ± 2^{h}	7 ± 1^{fgh}	$1 \pm 1^{b-g}$	$1 \pm 0^{b-d}$	4 ± 1^{klm}	94.3 ± 2^{k}
T_{12}	23 ± 3^{lm}	5 ± 1^{fg}	20 ± 2^{j}	8 ± 2^{ijk}	$1 \pm 1^{b-g}$	4 ± 2^{klm}	4 ± 2^{klm}	94.8 ± 1^{k}
T_{13}	18 ± 2^{hi}	9 ± 2^{m}	25 ± 4^{m}	6 ± 1^{de}	0 ± 0^{a}	2 ± 1^{-g}	$2 \pm 0^{e-g}$	100 ± 4^{m}

Note T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The values bearing the same letters are not significantly different at 5 % level according to DMRT

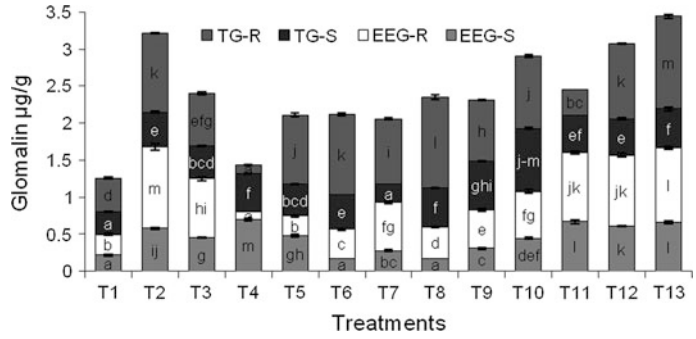

Fig. 5 Variations in the glomalin concentrations in turmeric root and rhizosphere soil. *Note* T_1—control; T_2—*A. lipoferum*; T_3—*T. viride*; T_4—*P. fluorescens*; T_5—*A. lipoferum* + *T. viride*; T_6—*A. lipoferum* + *B. megaterium*; T_7—*P. fluorescens* + AM; T_8—*T. viride* + *B. megaterium*; T_9—*A. lipoferum* + *T. viride* + *B. megaterium*; T_{10}—*T. viride* + *B. megaterium* + AMF; T_{11}—*A. lipoferum* + *B. megaterium* + AMF; T_{12}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens*; T_{13}—*A. lipoferum* + *T. viride* + *B. megaterium* + *P. fluorescens* + AMF. The *bars* bearing the same letters are not significantly different at 5 % level according to DMRT. The *error bars* represent the standard deviation values at 5 % level of significance

population, that is, even the control had appreciable number of organisms. However, it was counted that T_{10} have very less actinomycetes in number although it showed more fungal population. Firstly, *Azospirillum* sp. found T_9 rhizosphere as suitable shelter for its survival. In such a way, T_6 and T_{12} accommodated more *Azospirillum* sp. populations. *T. viride* was found to be highly colonized in the rhizosphere of T_9, T_{11} and T_{12} treatments. Some of the treatments responded with nil or few colony numbers. On correlation with populations of *P. fluorescens* and *T. viride*, the treatments enable comfortable stay for phosphate solubilizers. Of all the 13 treatments analyzed, it was observed that the roots of all treatments were colonized by AM fungi. The treatments of T_3 had least AM fungi colonization levels.

Variations in the Concentrations of Glomalin in Turmeric Rhizosphere Soil and Root

The present study has proposed to analyze the influence of inoculated microorganisms on biochemistry of AM fungi, because such a novel approach could be useful to understand the ecology of AMF. The AM fungal-released glomalin protein was quantified. It was observed that the treatment of T_4 released EEG-S at its highest level, and also the other two multiple bioinoculant combinations (T_{11} and T_{13}) were also effective. But the lining of EEG inside the hyphal walls of AMF that thrive inside the roots of turmeric plants took place mostly in the treatment of T_1. The combinatorial effect of *A. lipoferum*, *B. megaterium* and AMF, that is, T_{12} treatment had produced significant raise in the concentration of EEG-R, whereas replacing the AMF with both *T. viride* and *P. fluorescens* also maintained the concentration of EEG-R as almost the same. The wholesome work of bioinoculants in the treatment of T_{13} had helped to retain EEG-R 1.01 µg/g root. The concentration of TG-S was observed to vary from 0.25 to 0.85 µg/g soil. The minimum and maximum protein concentrations were observed in the soil samples of T_7 and T_{10}, respectively. The treatments of T_2 and T_6 had shown their protein concentrations exactly alike. Two of the treatments, namely T_4 and T_{11}, were unable to secrete TG-R combinations higher like control (T_1). The potency of all microorganisms applied (T_{13}) had elevated the TG-R concentration in roots to its peak extent (Fig. 5). The correlation coefficient of biotic and abiotic variations in turmeric under nursery conditions is given in the Table 4. It was found that those parameters had

Table 4 Correlation coefficient of biotic and abiotic variations in turmeric under nursery conditions

Variables	pH	EC	OC	N	P	K	Mn	Zn	Cu	Leaf No.	Inter shts	Sht Hgt	Rt lgth	Sht Bio	Rt Bio	Rhi Bio
pH	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EC	– 0.388	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
OC	0.039	– 0.305	–	–	–	–	–	-	-	-	-	-	-	-	-	-
N	– 0.440	0.640[a]	0.274	–	–	–	–	–	–	–	–	–	–	–	–	–
P	0.134	0.442	0.215	0.360	–	–	–	–	–	–	–	–	–	–	–	–
K	– 0.573[a]	0.282	0.209	0.350	0.115	–	–	–	–	–	–	–	–	–	–	–
Mn	– 0.376	0.390	0.081	0.433	0.525	0.664[a]	–	–	–	–	–	–	–	–	–	–
Zn	– 0.119	0.772[b]	– 0.279	0.435	0.011	0.125	0.040	–	–	–	–	–	–	–	–	–
Cu	– 0.158	0.742[b]	0.078	0.876[b]	0.614[a]	0.136	0.369	0.426	–	–	–	–	–	–	–	–
Leaf No.	– 0.262	0.493	0.187	0.585[a]	0.298	0.495	0.362	0.538	0.457	–	–	–	–	–	–	–
Inter shts	– 0.022	0.133	0.475	0.148	0.520	0.388	0.383	0.048	0.132	0.562[a]	–	–	–	–	–	–
Sht Hgt	0.011	0.413	0.529	0.516	0.500	0.293	0.150	0.472	0.457	0.766[b]	0.633[a]	–	–	–	–	–
Rt Lgth	– 0.036	0.213	0.305	0.217	0.015	0.306	0.020	0.495	0.068	0.745[b]	0.416	0.724[a]	–	–	–	–
Sht Bio	– 0.140	0.261	0.670[a]	0.519	0.547	0.282	0.170	0.10	0.495	0.564[a]	0.507	0.741[a]	0.457	–	–	–
Rt Bio	0.124	– 0.004	0.648[a]	0.260	0.446	0.111	– 0.005	– 0.006	0.257	0.627[a]	0.728[b]	0.745[b]	0.566[a]	0.833[b]	–	–

(continued)

Table 4 (continued)

Variables	pH	EC	OC	N	P	K	Mn	Zn	Cu	Leaf No.	Inter shts	Sht Hgt	Rt lgth	Sht Bio	Rt Bio	Rhi Bio
Rhi Bio	−0.142	0.254	0.676[a]	0.499	0.423	0.430	0.224	0.226	0.389	0.727[b]	0.804[b]	0.850[b]	0.572[a]	0.775[b]	0.840[b]	–

Variables	OC	N	P	Zn	Cu	Leaf No.	Inter shts	Sht hgt	Rt lgth	1° Rhi	3° Rhi	Sht Bio	Rt bio	Rhi Bio
Car	0.206	0.139	0.387	0.133	0.187	0.455	0.690[a]	0.403	0.388	0.260	0.391	0.444	0.533	0.429
Phe	0.647[a]	0.316	0.387	0.096	0.316	0.406	0.517	0.726[b]	0.285	0.554[a]	−0.041	0.620[a]	0.666[a]	0.722[b]
Prtn	0.472	0.193	0.276	−0.112	0.174	0.356	0.509	0.489	0.321	−0.002	0.445	0.181	0.522	0.473
Cur	0.555[a]	0.402	0.429	0.278	0.353	0.752[b]	0.868[b]	0.815[b]	0.675[a]	0.328	0.282	0.695[a]	0.836[b]	0.900[b]
EEG-S	0.174	−0.030	0.244	−0.224	0.065	0.071	0.488	0.111	0.049	−0.019	0.521	−0.054	0.297	0.159
EEG-R	0.246	0.406	0.034	0.097	0.316	0.277	0.275	0.301	0.084	−0.022	0.272	0.026	0.313	0.384
TG-S	0.237	0.679[a]	0.588[a]	0.092	0.805[b]	0.407	0.119	0.387	0.084	0.392	−0.147	0.672[a]	0.459	0.327
TG-R	0.192	0.652[a]	−0.054	0.643[a]	0.519	0.381	−0.121	0.465	0.307	0.048	−0.509	0.178	0.016	0.348
Bac	0.163	0.367	0.310	0.277	0.345	0.823[b]	0.675[a]	0.538	0.653[a]	0.010	0.372	0.413	0.661[a]	0.659[a]
Fun	0.556[a]	0.644[a]	0.521	0.209	0.647[a]	0.685[a]	0.618[a]	0.785[b]	0.432	0.539	−0.102	0.775[b]	0.771[b]	0.899[b]
Pseudo	0.247	−0.357	−0.101	−0.409	−0.526	−0.008	0.422	−0.024	0.193	−0.162	0.733[b]	0.001	0.216	0.045
Tricho	0.447	−0.031	0.336	−0.13	−0.052	0.484	0.692[b]	0.496	0.493	−0.174	0.526	0.324	0.693[b]	0.638[a]
PSB	−0.018	−0.134	0.000	0.259	−0.153	0.253	0.191	0.194	0.602[a]	−0.664[a]	0.273	−0.159	0.013	0.079
AMF	0.495	0.272	0.488	0.140	0.429	0.406	0.499	0.635[a]	0.375	0.435	−0.058	0.578[a]	659[a]	620[a]

Variables	Car	Phe	Prtn	Cur	EEG-S	EEG-R	TG-S	TG-R	Bac	Fun	Act	Azo	Pseudo	Tricho	PSB	AMF
Car	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phe	0.328	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Prtn	0.325	0.616[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cur	0.743[b]	0.666[b]	0.576[a]	–	–	–	–	–	–	–	–	–	–	–	–	–
EEG-S	0.602[a]	0.268	0.776[b]	0.448	–	–	–	–	–	–	–	–	–	–	–	–
EEG-R	−0.068	0.276	0.652[b]	0.304	0.452	–	–	–	–	–	–	–	–	–	–	–
TG-S	0.413	0.424	0.235	0.385	0.183	0.079	–	–	–	–	–	–	–	–	–	–
TG-R	−0.310	0.384	0.165	0.175	−0.285	0.378	0.180	–	–	–	–	–	–	–	–	–
Bac	0.518	0.197	0.463	0.761	0.365	0.394	0.290	0.096	–	–	–	–	–	–	–	–
Fun	0.387	0.762[a]	0.507	0.818[b]	0.207	0.383	0.607[a]	0.422	0.609[a]	–	–	–	–	–	–	–

(continued)

Table 4 (continued)

Variables	Car	Phe	Prtn	Cur	EEG-S	EEG-R	TG-S	TG-R	Bac	Fun	Act	Azo	Pseudo	Tricho	PSB	AMF
Act	0.221	0.072	0.032	0.146	0.143	0.013	−0.201	−0.341	0.151	0.002	-	-	-	-	-	-
Azo	0.245	−0.164	0.072	0.384	0.064	0.163	0.127	0.110	0.713[b]	0.186	−0.136	-	-	-	-	-
Pseudo	0.552[a]	−0.098	0.224	0.258	0.493	−0.067	−0.230	−0.673[b]	0.140	−0.189	0.369	−0.074	-	-	-	-
Tricho	0.162	0.307	0.548	0.574[a]	0.265	0.387	−0.067	−0.036	0.709[b]	0.455	0.237	0.0536	0.204	-	-	-
PSB	0.040	−0.210	0.215	0.205	0.109	−0.015	−0.332	0.134	0.452	−0.067	−0.230	0.643[a]	0.093	0.473	-	-
AMF	0.487	0.847[b]	0.639[a]	0.715[b]	0.437	0.168	0.557[a]	0.298	0.396	0.753[b]	−0.094	0.174	−0.156	0.450	0.074	-

[a] Correlation is significant at the 0.05 level (2-tailed). [b] Correlation is significant at the 0.01 level (2-tailed)

pH—potential hydrogen; *EC*—electrical conductivity; *OC*—organic carbon; OM—organic matter; *N*—nitrogen; *P*—phosphorus; *K*—potassium; *Mn*—manganese; *Zn*—zinc; *Leaf No.*—leaf number; *Sht Hgt*—plant height; *Rt lgth*—root length; *Sht Bio*—shoot biomass; *Rt Bio*—root biomass; *Rhi Bio*—rhizome biomass; 1° *Rhi*—primary rhizomes; 3° *Rhi*—tertiary rhizomes; *Car*—carbohydrate; *Prtn*—protein; *Phe*—phenol; *EEG-S*—easily extractable soil glomalin; *EEG-R*—easily extractable root glomalin; *TG-S*—soil total glomalin; *TG-R*—root total glomalin; *Bac*—bacteria, *Fun*—fungi; *Act*—actinomycetes; *Azo*—*Azospirillum* sp.; *Pseudo*—*P. fluorescens*; *Tricho*—*T. viride*; *PSB*—phosphate-solubilizing bacteria; *AM*—arbuscular mycorrhizal fungi. *Note*—Non-significant results were omitted

significantly positive as well as negative correlations at both 5 and 1 % confidence levels.

Discussion

The initial assessment of microbial bioinoculants application in order to increase the turmeric yield and quality under nursery conditions is essential since the outcome of nursery trials would almost express the same even when tested under field conditions (Lucey et al. 2004). Taking this into consideration, the nursery trials were made using microbial inoculants based on their ability to mobilize soil nutrients and conferring biocontrol activity to plants. The multiple strain products are generally recommended for particular hosts, and single strains are preferable to avoid the strategies of antagonistic effects (Thompson 1980; Keyser et al. 1993). Additionally, the research on crop cultivation under the local climate and edaphic factors is necessary to attain high yield with high quality (Ishimine et al. 2003).

The soil pH is the main factor for colonization of these agriculturally important microorganisms. Some of the treatments had shown that the pH found to get reduced when compared to this indirectly enhanced microbial activity that happens in the rhizosphere region, finally reducing the soil pH due to production of organic acids (Rengel and Marschner 2005). Several treatments effectively raised the soil pH were T_5 and T_{11}. The microorganisms involved in these treatments played a classical phenomenon in raising soil pH. Increasing the soil pH is related to the availability of soil nutrients (Luizao et al. 2007). The treatment involving *P. fluorescens* and AMF had resulted in accumulation of OC and OM at greater extent. This combination represents its compatibility toward each other (Edwards et al. 1998), and the role of AMF in carbon cycling has been reported previously. The combination of *A. lipoferum* along with *T. viride* and *P. fluorescens* had increased total nitrogen in soil.

The biologic nitrogen fixation carried out by *A. lipoferum* was responsible for the increment of the total content. The performance of *A. lipoferum* was better even when inoculated individually. There occurs a possibility of increasing the nitrogen fixation by *A. lipoferum* with the aid of other agriculturally important microorganisms when inoculated combined. Some PGPR secrete some molecules, acting as inducers/signals to help the process of nitrogen fixation (Parmar and Dadarwal 1999).

Like the combination involving *T. viride*, *P. fluorescens* and AMF can strongly influence the phosphorus content of soil. *P. fluorescens* was known to be an efficient solubilizer of complex phosphates, releasing inorganic phosphates. In turn, the only slightest variation was observed between T_9 and T_{10}. In the case of T_9, which involves the work of *B. megaterium* had raised the available phosphorus content. Thus, the strategies followed by microorganisms were previously explained by Rodriguez et al. (1999). Simultaneously, the phenomenon of phosphate solubilization and precipitation is largely depending on the soil type or pH. The mycorrhizae inoculated plants have shown significantly higher levels of P, and various micronutrients most notably Zn and Cu (Kothari et al. 1991) support our results.

P. fluorescens exhibited its significant effect on tertiary rhizomes of turmeric (Table 2). *Pseudomonas* sp. as the aggressive colonizers of root and its effect in plant growth had been discussed previously (Antoun and Prevost 2005; Gravel et al. 2007).

The treatment of T_{13} has shown that macro and micronutrient contents such as K, Fe and Mn raised the crop yield, quality and number of intercalary shoots to its maximum.

The concentrations of these nutrients in improving the crop yield are in accordance with the results of Rengel et al. (1999). Although the nutrients are present in soil, the mobility of those nutrients into the vegetative tissues is a big concern (Pearson and Rengel 1994). Hence, the mobility of nutrients can be achieved by microbial fertilizers especially AMF rather than the application of chemical fertilizers.

The single inoculation of *T. viride* has increased the carbohydrate content of the plant than any other combinations. Under greenhouse conditions, vegetative growth parameters like height, fresh and dry weight, leaf area and yield were significantly enhanced over the control when inoculated with *Trichoderma* sp. (Raj et al. 2005). *T. viride* jointly with *P. fluorescens* increased the total phenol content of plants. The suitable explanation of this observation was given by Mathivanan et al. (2005). Rich in phenolic composition of plants provides defense against various diseases (van Loon et al. 1998). The colonization of *Trichoderma* sp. in the rhizosphere was significantly correlated with intercalary shoots, root and rhizome biomass, curcumin and native soil bacteria. *T. viride* inoculum has been positive, and this significantly influenced the turmeric quality (curcumin) than any other microorganisms involved.

Irrespectively, *T. viride* and AM fungal had increased the accumulation of EEG in soil to its higher extent. Such appearance of AM fungi in individual inoculation of *T. viride* treatment may be due to the occurrence of viable AM fungal spores in the soil used for filling the pot. But this irrelevant appearance of AMF characters is unavoidable since the usage of sterilized soil does not encourage the sustainability of inoculated and seed germination due to lack of inducing activity of beneficial microorganisms. The maximum concentration of TG-S and TG-R was observed in the treatments of T_{10} and T_{13}. Even these treatments include the input of AMF. The evidence of high levels of glomalin is related to higher microbial activity as stated by Wright and Upadhyaya (1998). EEG was strongly significantly correlated with the protein concentration of plant, and this may be due to translocation of phosphate and C by AMF necessary for the synthesis of proteins (Johansen et al. 1993; Miller and Jastrow 2000). Lutgen et al. (2003) had correlated glomalin concentrations (TG & EEG) with mycorrhizal variables and obtained significant results, whereas treatments with high EEG-S and EEG-R were known to have significantly influenced by the AMF colonization, since T_4 and T_{13} showing both higher EEG

concentrations and total root colonization by AMF. The same was also applicable to the TG concentrations (Table 4). Controls on the production of glomalin are unknown. Soil and climate factors, AMF species type, host plant and their productivity could be important contributions. In turn, it is indeed necessary to have detailed study on the interactions of AMF with beneficial microbes and their interference in the biochemical aspects of glomalin production. Of all the biotic and abiotic factors analyzed, the treatments such as T_5, T_{10} and T_{13} were concluded as best combinations of bioinoculants and those were also found as compatible. So, these combinations of bioinoculants can be recommended for the application in turmeric fields.

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Microbial-Treated Sago Mill Effluent: A Potential Water Resource for Agroecosystem Management

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Abstract

All over the world, sago mill is one of the important agro-based industries which release large amount of wastewater containing organic and inorganic solid wastes. This wastewater commonly is referred as effluent which has obnoxious odor, irritating color, lower pH, higher BOD and COD. It affects the health of soil, natural ecosystem, animals, plants and human beings. Hence, proper remediation is necessary before the release of the wastewater. In the present study, we have attempted two investigational systems: one is treatment of sago industry effluent by aerobic bacterial consortium, and the other is effect of treated and untreated effluents on soil microflora and growth of legume plants. For the treatment system, the starch-degrading bacteria were isolated from sago industry wastewater and waste-contaminated soil. The genera, *Flavobacterium* (A), *Enterobacteriaecea* (B), *Alcaligenes* (C), *Acenitobacter* (D), *Corynebacterium* (E), were found efficient in starch degradation, and they were applied in the treatment of sago mill effluent under aerobic condition. The physico-chemical parameters such as pH, TS, TSS, TDS, BOD, COD, DO, magnesium, calcium and potassium were found decreased in effluent after 7 days. In the second phase of investigation, pot culture experiment was carried out to find out effect of treated and untreated wastewater on the survival of soil microbes and the growth of legume plants. Shoot height, root length, fresh weight, dry weight and chlorophyll content of the legume plants and soil micro flora showed an increase when treated wastewater was tested. The results revealed that wastewater treated by aerobic microorganisms can be effectively used for irrigation.

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Effluent · Degradation · Green gram · Maize · Starch

Introduction

Industrialization causes a serious problem of pollution of water, soil and air based on the type of industry, nature of raw materials, processes involved and type of equipment used. One of the most important pollutants is the effluent released from various industries. Each and every process in all kinds of industries is in need of water, as a result, a large amount of water after processing was released as effluent. This effluent with number of hazardous components causes deleterious effect to the agroecosystems.

Sago mill is one of the food processing industries and releasing large amount of effluent after the processing with tapioca. Tapioca is an important tuber crops cultivated in more than 80 countries of the world. Tapioca which is also known as cassava and biologically named as *Manihot esculenta* coming under the family Euphorbiaceae. It is one of the most important starch root crops in the tropics. The tuber (root) of tapioca plants are edible and serve as staple food in many tropical countries and are also important source of starch. A survey conducted by The Central Tuber Crops Research Institute (CTCRI) has indicated that more than three-fourth of the cassava area is in Salem, South Arcot and Dharmapuri districts of Tamil Nadu. There are nearly about 1,000 sago and starch processing units in Salem district alone (Ayyasamy et al. 2008).

The manufacturing process consists of washing, peeling, crushing, slurring, settling, sizing, roasting, drying and polishing. The wastewater arising out from the washing of the roots and of the peeled tapioca roots and the supernatant from the settling tanks, where the starch is settled, constitutes the trade effluent from the sago industry (Nandy et al. 1991a, b). These units discharge about 40,000–50,000 liters of sago per ton of sago processed (Saroja and Sastry 1972). The wastes aroused from tapioca processing are of different forms, bark, hampers and wastewater. The bark is either thrown or burnt while hampers with 66 % of fermentable carbohydrates may be used as animal feed or utilized for mushroom cultivation. In modern industries, decanters are added to the processing line to separate more water from the starch. The wastewater discharged directly from the decanters is high in organic substances and contains not only starch but also fat and protein as well (Tanticharoen and Bhumiratana 1995).

Nearly 30–50 m^3 of water is required to extract about one ton of sago starch (Chew and Shim 1993). The wastewater contains 11,000–13,500 mg/l in terms of COD, 4,200–7,600 mg suspended solids/l and pH 4.5–5.0. Carbon and nitrogen and also has high concentration of suspended solids and insoluble fibers. The effluent also has obnoxious odor, irritating color, lower pH and higher BOD (Ayyasamy et al. 2008). The effluents, while released to the land without treatment, can reduce groundwater quality, and when discharged to river, it affects aquatic ecosystem and causes severe devastation of fresh water lives (Vikineswary et al. 1994).

In common, the effluents are used by farmers for irrigation purpose. But, applications of domestic and industrial wastewater on soil resulted in accumulation of various anions and cations. In plants, these cations and anions in higher concentration induce morphological, physiological disorders, reduced growth and inhibition of germination (Vijayakumari 2003). Also, a study conducted by Rajannan et al. (1998) reports that among various strains *Bacillus* sp. was found to be more tolerant followed by *Pseudomonas* sp. at high concentration of the effluent. This suggests the toxic nature of the industry effluent on soil microbial population. Thus, the disposal of effluent without treatment diminishes the soil fertility and the

rate of germination, growth and production of the crop are found to be decreased. Number of studies has given supportive results, a study by Sahai and Neelam (1987) shows that both the seed of germination and seedling growth of rice seeds were retarded while higher concentration of spent wash was used for irrigation. Dixit et al. (1986) reported that the continuous irrigation of cardboard factory effluent to rice seeds inhibits the germination with increasing the concentration of the effluent. However, there was a limited literature concerning plant growth to develop agroecosystem irrigated with sago mill effluent.

So, suitable treatments are necessary to bioconvert or recycle the wastewater before discharged it to the environment. But considering the socioeconomic profile of each small-scale industrial farming operations, it was necessary to develop an appropriate low-cost technology for the treatment of starch extraction wastewaters. Hence, the study is focused to design a treatment method of high efficiency with low cost.

Numbers of microorganisms are capable of degrading the starch present in the sago effluent (Balagopalan et al. 1991). Some organisms under anaerobic conditions will yield methane (Tanticharoen and Bhumiratana 1995). Number of study was reported for anaerobic treatment of organic effluent (Saravanan et al. 2001). But, the use of sago effluent was limited. Hence, the study was carried out for both aerobic and anaerobic treatment.

In the absence of effective measures to treat sago mill effluent, it was felt that it would be more appropriate to perform aerobic treatment with potent microorganisms and anaerobic treatment through biogas technology to tackle the problems of contaminants in sago mill effluent. Hence, this present study is made with various objectives, namely (1) to assess the presence of starch-degrading bacteria in sago mill effluent and effluent-contaminated soil, (2) to find out the efficiency of selected starch-degrading bacteria in the reduction in starch from synthetic media and sago mill effluent, (3) to find out the effect of aeration on the reduction in starch and treatment of sago effluent, (4) to perform anaerobic treatment through biogas technology for the reduction in starch and other physico-chemical characteristics of sago effluent and (5) to find out the effect of microbial-treated and microbial-untreated sago mill effluent on the growth of leguminous plants and survival of soil microbes.

Materials and Methods

Sample Collection

The effluent and effluent-contaminated soil/sediments were collected from a sago industry located in Salem district of Tamil Nadu. The sago factory effluent was collected in a sterile glass bottles from the point of release and from pond where the effluent was stored for a longer time. The sediment was collected in sterile polyethylene bags from the bottom of the ponds. The soil was collected from nearby garden and from maize field. All the samples were transported to the laboratory within 2 h for the analysis of microorganisms.

Physico-Chemical Characteristics of Sago Effluent

The physico-chemical parameters of the effluent such as color, odor, pH, electrical conductivity (EC), temperature, total solids (TS), total suspended solids (TSS), total dissolved solids (TDS), dissolved oxygen (DO), BOD, COD, hardness, chloride, phosphate and magnesium were analyzed by standard methods (Table 1).

Effluent Treatment

Aerobic and anaerobic methods were followed to treat sago mill effluent efficiently.

Aerobic Treatment

The consortium was made with the bacterial culture enriched in sago mill effluent. For

Table 1 Analytical methods followed for the estimation of physico-chemical parameters of the effluent

Sl. no	Parameters	Method followed	References
1	pH	pH meter (Elico)	APHA (2005)
2	EC	Conductivity meter	APHA (2005)
3	Total solids (TS)	Evaporation and drying (105 °C)	APHA (2005)
4	Total suspended solids (TSS)	Filtration and drying (105 °C)	APHA (2005)
5	Total dissolved solid (TDS)	Evaporation and drying (105 °C)	APHA (2005)
6	Dissolved oxygen	Dissolved oxygen meter	APHA (2005)
7	BOD	Winkler's method (titration)	APHA (2005)
8	COD	Reflex method (titration)	APHA (2005)
9	Alkalinity	Titration	APHA (2005)
10	Total hardness	Versenate method (titration)	APHA (2005)
11	Calcium	Versenate method (titration)	APHA (2005)
12	Magnesium	Versenate method (titration)	APHA (2005)
13	Chloride	Argentometric method (titration)	APHA (2005)
14	Sulfate	Turbidity method	APHA (2005)
15	Phosphate	Stannous chloride method	APHA (2005)
16	Sodium	Flame emission photometric method	APHA (2005)
17	Potassium	Flame emission photometric method	APHA (2005)
18	Starch	Standard method Sadasivam and Manickam (1996)	
19	Amylase	Standard method Sadasivam and Manickam (1996)	
20	Glucose	Standard method Sadasivam and Manickam (1996)	

consortium, a protocol (A + B + C + D + E) was made. Where A— *Flavobacterium* sp.; B— Enterobacteriaceae; C— *Acinetobacter* sp.; D— *Alcaligenes* sp. and E— *Corynebacterium* sp.

A loopful of culture (presumptively selected from starch hydrolysis test) was inoculated individually in presterilized 100 ml nutrient broth. The flask was kept in a shaker at 120 rpm for 16–18 h at 30 °C. The culture broth was centrifuged at 10,000 rpm for 20 min. Cell suspension was prepared using sterile distilled water and adjusted to 0.5 OD using UV Visible spectrophotometer (Model : Hitachi). One percent (10^5 CFU/ml) of the above suspension was used as inoculums for the degradation of starch.

Five liter effluents were taken under aseptic condition in 10-l bottle having a side-arm outlet and sterilized. To this, 1 % inoculum containing 10^5 CFU/ml of bacterial consortium (A + B + C + D + E), the highly efficient organisms among tested, was inoculated, and sterile dry air was passed continuously using an aerator. Every 24 h for 7 days, the sample was taken and analyzed the concentration of starch and production of amylase and glucose (Sadasivam and Manickam 1996). On the final day, the whole sample was filtered under aseptic condition through a sand filter column. For sand filtration, a glass column having 75 cm in length and 10 cm in diameter was taken. The bottom of the column was closed with a glass stopper. To this, large stones (2.0–2.3 cm) were packed in bottom regions followed by small stones (0.7–1.5 cm), gravel (0.4–0.6 cm), coarse sand (0.05–0.1 cm) and fine sand (0.15–0.3 mm). The packed column was washed three times with distilled water and sterilized for 15 min at 15 lb pressure. Then, bacterially treated effluent was transferred to the column. The filtrate was collected in a sterile conical flask and used for germination study. Physico-chemical parameters such as, color, odor, pH, EC, temperature, TS, TSS, TDS, DO, BOD, COD, hardness, phosphate, chloride and magnesium were estimated before and after sand filtration.

Anaerobic Treatment

The effluent was treated under anaerobic condition by a biogas technology. Bottles of 2.5 l capacity were taken and about one-third of the bottles was filled with substrates, leaving a head space for the accumulation of gas produced. Five setups with different combination and composition was made for the production of biogas as shown in the following protocol: (1) raw effluent alone = 1,500 ml (without amendments); (2) raw effluent + Tapioca peelings (10 g/100 ml) = 750 ml + 750 ml (1:1 ratio); (3) raw effluent + Tapioca peelings (10 g/100 ml) = 1,000 ml + 500 ml (2:1 ratio); (4) raw effluent + cow dung (10 g/100 ml) = 750 ml + 750 ml (1:1 ratio); and (5) raw effluent + cow dung (10 g/100 ml) = 1,000 ml + 500 ml (2:1 ratio).

All the five setups were tightly closed with a holed cork. A silicon tube was taken and one end was fitted to cork and another was closed with pinch clips, to maintain anaerobic condition. The setup was thoroughly shaken and allowed to react at room temperature. For every 3 days, the gas was released from all setups and was measured by downward displacement of water. This experiment was continued, till the gas production getting down. The gas released was expressed in ml according to the report by Saravanan et al. (2001). The potential set was chosen and analyzed for methane concentration through gas chromatography. The characteristic analysis, germination study and impact on beneficial organisms were again repeated with both the aerobically and anaerobically treated effluent (Manivasakam 1987).

Impact of Sago Mill Effluent on Agroecosystem Management Through Pot Culture Study

A pot culture study was conducted to study the effect of different concentrations (20, 40, 60, 80 and 100 %) of sago industry effluent on seed germination and soil microorganisms. The beneficial organisms and the seed for study were chosen as *Rhizobium*, *Azotobacter* and *Phosphobacterium* and black gram, green gram and red gram, respectively.

Impacts on Seed Germination and Plant Growth of Legume Plants

Tarson cups filled with 250 gm of fine red soil was taken for germination studies, for each seed variety about six cups were maintained, representing a control, 20, 40, 60, 80 and 100 % effluent. Equal numbers of seeds were placed in all the cups. The control was supplied with tap water and the remaining cups were supplied with respective concentration of effluent regularly. The seed germination was noted on the 2nd day. The day from seed germination, the growth of the plant was measured every day for a week, and finally, the plants were uprooted and the height of the root and number of nodules were recorded (Ayyasamy et al. 2008). The experiment was conducted in triplicates, unless otherwise stated. Data points in the tables and figures represent the means, with all the error bars shown (1 standard error of mean). Both the mean and standard deviation were performed with appropriate usage of statistical package on Microsoft Excel Version 2007.

Impact on Soil Beneficial Organisms

The fertile soil was collected in a healthy agricultural land and filled in cups. Control (tap water), 20, 40, 60, 80 and 100 % was labeled and maintained for each organism, respective concentration of the effluents was supplied to respective cups and tap water was supplied for control. Every day, soil sample from each cup was serially diluted and plated in selective medium such as YEMA, Ashbeys and Pikovasky for *Rhizobium, Azotobacter* and *Phosphobacterium,* respectively. After incubation, all the plates were counted and the numbers of colonies were observed and recorded.

Statistical Analysis

For comparison, all the experiments were carried out in triplicate. Both the mean and standard deviation were determined where appropriate, using the statistical package in Microsoft® Excel (Version 2007). In batch-mode study performed in mineral salts medium amended with 1, 2 and 3 % starch, the parameters such as bacterial growth, degradation of starch, production of amylase and glucose are closely related to each other. Therefore, the correlation between the bacterial growth, degradation of starch, production of amylase and glucose was analyzed using the statistical package of SPSS version 12.

Results

Physico-Chemical Characteristics of Sago Mill Effluent

Significant physiochemical characteristics were analyzed in sago mill effluent and the results are given in Table 2. The color of the raw effluent when collected was dirty white and has obnoxious odor. It has acidic pH around 4.22, oxidation reduction potential around 144 mv, and its EC was 5.1 ms. The value of TS was 6,200 mg/l, a total suspended solid was 4,000 mg/l and TDS was 2,200 mg/l, the BOD of the sample was found to be 1,486 mg/l, and the COD was 5,760 mg/l. The other parameters, such as magnesium, chloride, phosphate and calcium, were also analyzed with the respective methods, and it was 2,623, 5,698, 2.12, 205 mg/l, respectively. As the study was determined to treat the sago effluent which is rich in starch. The concentration of starch, reducing sugar, amylase activity was also estimated and found as 2,200, 700, 1,000 mg/l, respectively.

Table 2 Physio-chemical parameters of sago mill effluent

Sl. No	Parameter	Untreated effluent
1	Color	Dirty white
2	Temp	37.5 °C
3	Odor	Obnoxious
4	pH	4.22
5	TS (mg/l)	6,200
6	TSS (mg/l)	4,000
7	TDS (mg/l)	2,200
8	BOD (mg/l)	1,486.5
9	COD (mg/l)	5,760
10	Alkalinity (mg/l)	2,500
11	Conductivity	5.11 ms
12	OR potential	144 mv
13	Salinity (mg/l)	5,000
14	Total Hardness (g/l)	10.85
15	Calcium (mg/l)	204.75
16	Magnesium (mg/l)	2,623.2
17	Phosphate (mg/l)	2.12
18	Chloride (mg/l)	5,698.23
19	Starch (mg/l)	2,200
20	Amylase (mg/l)	1,000
21	Glucose (mg/l)	700

Microbial Quality of the Sago Mill Effluent

The number of bacterial colonies on nutrient agar plate was estimated in sago mill effluent as 7.8×10^6, 3.4×10^7, and 0.7×10^8 CFU/ml for the dilutions 10^{-3}, 10^{-4} and 10^{-5}, respectively. Among the fungi, 5, 4 and 2 CFU/ml of fungal colonies were observed on rose bengal agar for 10^{-2}, 10^{-3} and 10^{-4} dilutions. The actinomycetes were 4.2×10^5, 1.8×10^6 and 0.2×10^7 CFU/ml on glucose asparagine agar for the dilutions of 10^{-2}, 10^{-3} and 10^{-4}.

Heterotrophic Bacteria in Sago Mill Samples

Among the bacterial population, the Enterobacteriaceae holds a maximum percentage of 21.87 %, then the *Alcaligenes* spp that has around 19 % (Fig. 1a), and the other genera such as *Lactobacillus, Bacillus, Pseudomonas, Serratia, Flavobacterium* and *Acinetobacter* hold a percentage of 16, 13, 12, 9, 9 and 6 %, respectively.

Plate 1 Effect of untreated and microbial-treated sago mill effluent on the growth of legume plants. **a** Effects of untreated effluent. **b** Effects of aerobically treated effluent. **c** Effects of anaerobically treated effluent

(a)

(b)

(c)

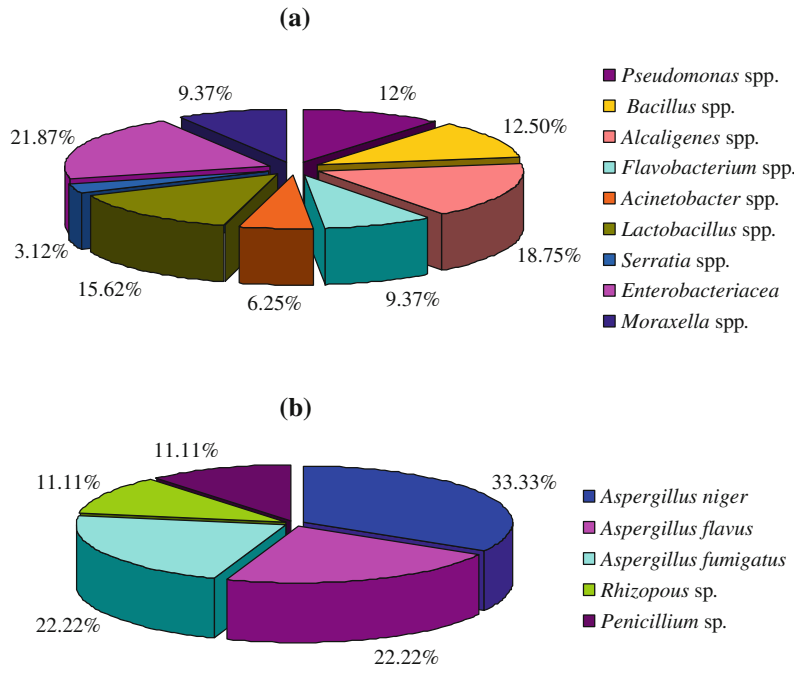

Fig. 1 Microbial population in sago mill effluent. **a** Bacterial population. **b** Fungal population

Fungal Species in Sago Mill Samples

Considering the fungal population, the majority of them are *Aspergillus* spp. Most predominant genus was *Aspergillus niger* (33 %), represented in Fig. 1b, and secondly, the *Aspergillus flavus* and *Aspergillus fumigatus* which holds a percentage of 22 and 22 %, respectively. The other organisms, namely *Rhizopous* spp. and *Penicillium* spp., hold a level of 11 %.

Starch Degraders

All the isolated and identified bacterial colonies were subjected to starch hydrolysis to identify the starch degraders. All the organisms were spotted on starch hydrolysis medium.

The organisms such as *Moraxella, Bacillus, Serratia, Lactobacillus, Flavobacterium, Acinetobacter,* Enterobacterieacea*, Alcaligenes* and *Corynebacterium* were found to degrade starch by the production of clear zone around their colonies. Among the starch degraders, five organisms come under the family Enterobacteriaceae and they produce the zone with diameter 25, 17, 24, 26, 20 and 27 mm. Among the total starch degraders, only five were identified as more efficient and they alone were used for further studies. They are A-*Flavobacterium* sp. (EF-1), B-Enterobacterieacea (SL-7), C-*Acinetobacter* sp. (EF-3), D-*Alcaligenes* sp. (EF-4), E-*Corynebacterium* sp. (EF-17).

Aerobic Treatment of Sago Mill Effluent with Microbial Consortium

The microbial consortium was made and inoculated in 5 l of effluent and the study was done for about a week, and the amylase activity, starch concentration and reducing sugars were measured regularly for a week and the results are given in Fig. 2. On the initial day, the amylase activity was 200 mg/ml and it gradually increased and attained a drastic activity 1,700 mg/ml on 6^{th} day followed by 2,200 mg/l for as the amylase activity increased with the time of study, there was the production of reducing sugar by the degradation of starch; thus, the starch concentration was found as 1,552 for 0^{th} day and gradually decreases, and

finally on the last day of study, the concentration of starch was 300 mg/l. The reducing sugar concentration was determined as 600 mg/l on the initial day and was 2,000 mg/l on the 7th day.

Physico-Chemical Characteristics of Aerobically Treated Effluent

In the physico-chemical characteristics of the aerobically treated effluent, most of the parameters was found to be decreased when compared with the raw effluent (Table 3). The pH seemed to be 6.70 which was neutral, and color of the treated effluent was found to be pale white and its odor was not irritable. The treated effluent has a temperature of 35.9 °C, oxidation reduction potential of 12 mv and EC of 5.46 ms, and its other characteristics like TS, TDS, TSS were 3,000, 2,310, 690 mg/l, respectively. The biological characteristics like BOD, COD were also found to be decreased when compared with the untreated effluent 849, 3,210 mg/l. Its magnesium, chloride, phosphate, calcium and total hardness were 931, 6,298, 1, 168 and 4 mg/l, respectively. The concentration of starch, reducing sugar, amylase activity was found to be 300, 2,000 and 2,200 mg/l, respectively.

Anaerobic Treatment by Production of Biogas

The sago mill effluent was an aerobically treated for about 3 days, gas was released from the set up and measured for an interval of each week with different combination, and the quantity of gas was expressed in ml. The biogas production from sago mill effluent under anaerobic condition was represented in Fig. 3. The raw effluent acting as control produces no gas on 3rd, 6th, 9th and 12th day; but on 15th and 18th day, there was a small quantity of gas production on 15th day, which was 20 ml, and on 18th day, gas production was 30 ml. The 1,000 ml of effluent was mixed with 500 ml of crushed tapioca peeling, and this shows better results for gas production

Table 3 Physico-chemical parameters of aerobically treated sago mill effluent

Sl. No	Parameter	Untreated effluent	Aerobically treated effluent
1	Color	Dirty white	Pale white
2	Temp	37.5 °C	35.9 °C
3	Odor	Obnoxious	Obnoxious
4	pH	4.22	6.70
5	TS (mg/l)	6,200	3,000
6	TSS (mg/l)	4,000	690
7	TDS (mg/l)	2,200	2,310
8	BOD (mg/l)	1,486.5	849.8
9	COD (mg/l)	5,760	3,210
10	Alkalinity (mg/l)	2,500	350
11	Conductivity	5.11 ms	5.46 ms
12	OR potential	144 mv	12 mv
13	Salinity (mg/l)	5,000	5,400
14	Total Hardness (g/l)	10.85	4.02
15	Calcium (mg/l)	204.75	168
16	Magnesium (mg/l)	2,623.2	931.18
17	Phosphate (mg/l)	2.12	1.09
18	Chloride (mg/l)	5,698.23	6,298.04
19	Starch (mg/l)	2,200	300
20	Amylase (mg/l)	1,000	2,200
21	Glucose (mg/l)	700	2,000

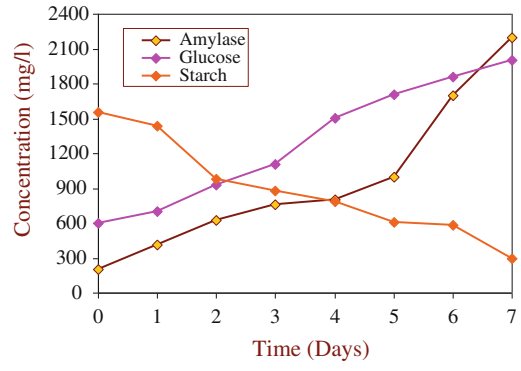

Fig. 2 Biodegradation of starch and synthesis of amylase and glucose during aerobic treatment of sago mill effluent

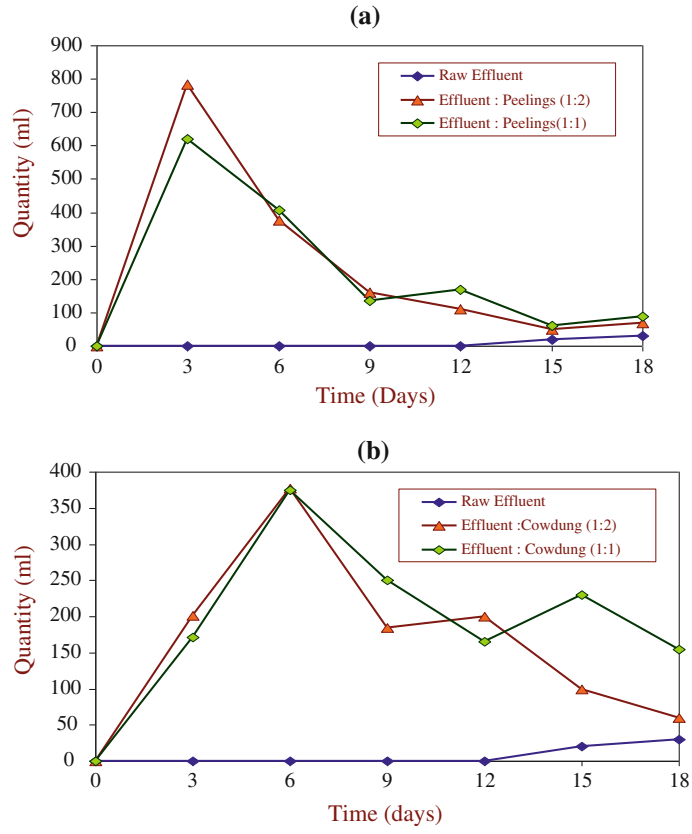

Fig. 3 Production of biogas during anaerobic treatment of sago mill effluent. **a** Effluent with tapioca peelings. **b** Effluent with cow dung

on 3rd day by 785 ml, and on 6th day it was 378, then the gas production decreased gradually and reached 70 ml on 18th day. The equal quantity of effluent and peelings also gave better result but lesser than the previous, its 3rd day production was about 620 and on 8th day, it was 406 ml, and for 9th, 12th, 15th and 18th day, the production was 135, 170, 60 and 90 ml. respectively, when compared with the effluent with peelings. The biogas production in the set was low at 3rd, 6th and 9th day: the production rate on those days were, 201, 377 and 185 ml, and again there was an increase in the production on 12th day, it was 200 ml, and again the production rate gradually decreased at 15th and 18th day as 100 and 60 ml, respectively. The effluent with cow dung, in the ratio 1:1 gave a same result as the previous: its production rate was 171, 375, 250, 165, 230 and 185 ml on 3rd, 6th, 9th, 12th, 15th and 18th day, respectively.

Physico-Chemical Characteristics of Anaerobically Treated Effluent

The physico-chemical characteristics of anaerobically treated effluent also was analyzed and stated in Table 4. It is concluded that it is more or less similar when compared with the untreated effluent. Its pH is acidic. It has a dirty white color and has no irritable odor. It has a temperature of 35.9 °C, oxidation reduction potential of 133 mv and EC of 5.26 ms and its other characteristics like TS, TDS, TSS are 4,210, 2,210, 2,000 mg/l, respectively. The parameters such as BOD, COD are found to be 478, 1,600 mg/l, respectively. The magnesium, chloride, phosphate, calcium levels of an aerobically treated effluent are 2,141, 6,698, 1 and 189 mg/l, respectively. The concentration of starch, reducing sugar, amylase activity is found as 1,295, 600 and 900 mg/l, respectively.

Table 4 Physico-chemical parameters of anaerobically treated sago mill effluent

Sl. No	Parameter	Untreated effluent	Anaerobically treated effluent
1	Color	Dirty white	Dirty white
2	Temp	37.5 °C	37.5 °C
3	Odor	Obnoxious	Obnoxious
4	pH	4.22	4.22
5	TS (mg/l)	6,200	4,210
6	TSS (mg/l)	4,000	2,000
7	TDS (mg/l)	2,200	2,210
8	BOD (mg/l)	1,486.5	478.5
9	COD (mg/l)	5,760	1,600
10	Alkalinity (mg/l)	2,500	1,600
11	Conductivity	5.11 ms	5.26 ms
12	OR potential	144 mv	133 mv
13	Salinity (mg/l)	5,000	5,700
14	Total Hardness (g/l)	10.85	9.04
15	Calcium (mg/l)	204.75	189
16	Magnesium (mg/l)	2,623.2	2,141.1
17	Phosphate (mg/l)	2.12	1.36
18	Chloride (mg/l)	5,698.23	6,697.92
19	Starch (mg/l)	2,200	1,295
20	Amylase (mg/l)	1,000	900
21	Glucose (mg/l)	700	600

Impact of Sago Mill Effluent on Seed Germination

The sago mill effluent has impact on seed germination. There is a notable difference in using the treated and untreated effluents on seed germination of green gram, black gram and red gram. The different concentration of both treated and untreated effluents is used for seed germination, and a pot culture study is made the growth of the plant is measured regularly for 10 days, and at the final day, the plant is uprooted and the root length and also the nodule are counted (Plate 1).

Impact of Untreated and Treated Effluents on Black Gram

The use of untreated effluent for irrigation decreases the plant growth of black gram at higher concentrations like 100, 80 and 60 % (Fig. 4a). The growth of the plant increases day by day but at a very low rate and reaches a maximum shoot length of 3.96 cm on the 10^{th} day and on irrigating 80 % of sago mill effluent the germination. The shoot length varies from 0.5 cm on the 2^{nd} day and 4.17 cm on the 10^{th} day when 60 % effluent is used. The germination is observed on 3^{rd} day and has a shoot length of 1.02 cm, and the shoot length 7.37 cm is observed on the final day of study. The growth of black gram irrigated with 40 and 20 % of the effluents was 0.22 cm on the 3^{rd} day, where germination initiated, and on the 7^{th} day, the shoot length was 7.45 cm; 1.12 cm on 3^{rd} day 7.82 cm on the 7^{th}, respectively. While that of control was 1.37 cm, and on the final day, the shoot length was measured as 10.4 cm. Thus, the shoot length in the control were found as increased in centimeters. The root length and the nodule count were 9.57, 8.6, 8.2, 7.35, 7.07 cm and 16, 13, 9, 10, 8 for 20, 40, 60, 80, 100 %, respectively, while that of control was 10 and 19 cm (Tables 5 and 6).

The use of aerobically treated effluent for irrigation does not affect the growth of black gram (Fig. 4b). In some cases, the growth increases with that of the control shoot length of 100 % aerobically treated effluent and is found as 2.75 cm, on 3^{rd} day and 10.62 cm on 10^{th} day; and for 80 % treated effluent, it was 2.17 cm on 3^{rd} day and on 10^{th} day the shoot length was observed as 10.82 cm while irrigating 60 % treated effluent the shoot length was 1.62 cm on 3^{rd} day and the shoot length was 11 cm on the final day; the shoot length on 3^{rd} day was 2.7 cm and that of 10^{th} day was 11.17

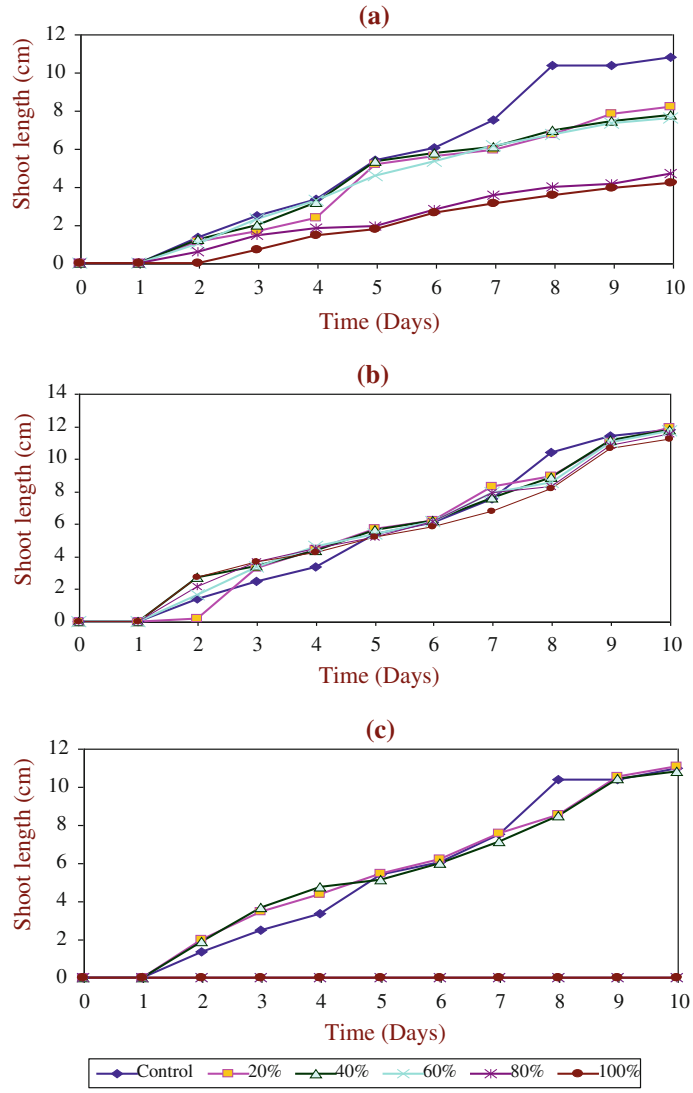

Fig. 4 Growth of black gram in soil irrigated with treated and untreated sago mill effluents at various concentrations. **a** Raw effluent. **b** Aerobically treated effluent. **c** Anaerobically treated effluent

when 40 % of treated effluent was irrigated; 20 % treated effluent on irrigation shows a shoot length of 3 cm on the 3rd day and on 10th day 11.05 cm. The nodule count of the black gram for 20, 40, 60, 80, and 100 % was 22, 19, 15, 10 and 9, respectively. The root length was measured as 11.82, 10.75, 10.95, 11.12, 10.97 cm (Tables 5 and 6). Thus, for the aerobically treated effluent, the germination was found on 3rd day for all the concentrations. And almost all the diluted effluent shows a similar growth.

In the soil pots irrigated with anaerobically treated effluent, the shoot length was denoted in Fig. 4c. The anaerobically treated effluent was found to decrease the plant growth at higher concentration as its pH was acidic. There was no growth observed while irrigating higher concentration such as 100, 80 and 60 %, but shows a decreased shoot length; while irrigating 40 and 20 % effluent, the germination was observed on 3rd day and the shoot length was 1.9 cm on 3rd day and 10.45 cm on 10th day. The nodule count for those concentrations was 11, 14.66 and the root length was found as 10.87 and 10.6 cm (Tables 5 and 6). Thus, it is concluded that the anaerobically treated effluent has influence on seed germination and growth of black gram.

Table 5 Effect of untreated and microbial-treated sago mill effluent on the nodulation of legume roots

Concentrations	Black gram			Green gram			Red gram		
	Untreated effluent	Aerobically treated effluent	Anaerobically treated effluent	Untreated effluent	Aerobically treated effluent	Anaerobically treated effluent	Untreated effluent	Aerobically treated effluent	Anaerobically treated effluent
Control	19 ± 2.2	19 ± 2.2	19 ± 2.2	21.7 ± 2.5	21.7 ± 2.49	21.7 ± 2.5	14 ± 2.7	14 ± 2.7	14 ± 2.7
20 %	16.6 ± 2.2	22 ± 1.6	14.7 ± 2.6	14.5 ± 3.4	19.5 ± 1.1	14.66 ± 2.6	9.75 ± 1.5	13.75 ± 2.3	10.66 ± 1.2
40 %	13.3 ± 1.7	19 ± 0.8	11 ± 2.44	12 ± 1.9	16 ± 2.5	11 ± 2.4	7.25 ± 1.5	12 ± 0.7	9 ± 0.81
60 %	9.5 ± 1.8	15 ± 0.8	0	8.75 ± 2.4	13.3 ± 2.8	0	5.3 ± 0.5	11.75 ± 1.8	0
80 %	10 ± 1.2	10 ± 1.2	0	5.33 ± 2.1	12 ± 1.9	0	4	11 ± 0.8	0
100 %	8 ± 1.6	9.33 ± 1.7	0	4.5 ± 0.5	13 ± 2.8	0	2.5 ± 0.5	11.6 ± 1.24	0

Table 6 Effect of untreated and treated sago mill effluent on the root length of legume plates

Concentration	Untreated effluent			Aerobically treated effluent			Anaerobically treated effluent		
	Black gram	Green gram	Red gram	Black gram	Green gram	Red gram	Black gram	Green gram	Red gram
Control	10.8 ± 0.2	11.8 ± 0.6	6.5 ± 0.3	10.8 ± 0.2	11.8 ± 0.6	6.5 ± 0.3	10.8 ± 0.2	11.8 ± 0.6	6.5 ± 0.3
20 %	9.5 ± 0.2	10.8 ± 0.3	5.1 ± 0.6	11.8 ± 0.4	12.2 ± 0.3	6.5 ± 0.2	10.8 ± 0.1	4.5 ± 0.4	5.4 ± 0.2
40 %	8.6 ± 0.3	10.7 ± 0.3	4.6 ± 0.7	10.8 ± 0.2	11.9 ± 0.3	6.5 ± 0.2	10.6 ± 0.3	4.1 ± 0.3	5.1 ± 0.3
60 %	8.2 ± 0.1	9.8 ± 0.5	3.7 ± 0.4	10.9 ± 0.4	11.2 ± 0.1	6.2 ± 0.4	0	0	0
80 %	7.3 ± 0.3	9.0 ± 0.7	3.6 ± 0.3	11.1 ± 0.6	11.4 ± 0.05	5.9 ± 0.3	0	0	0

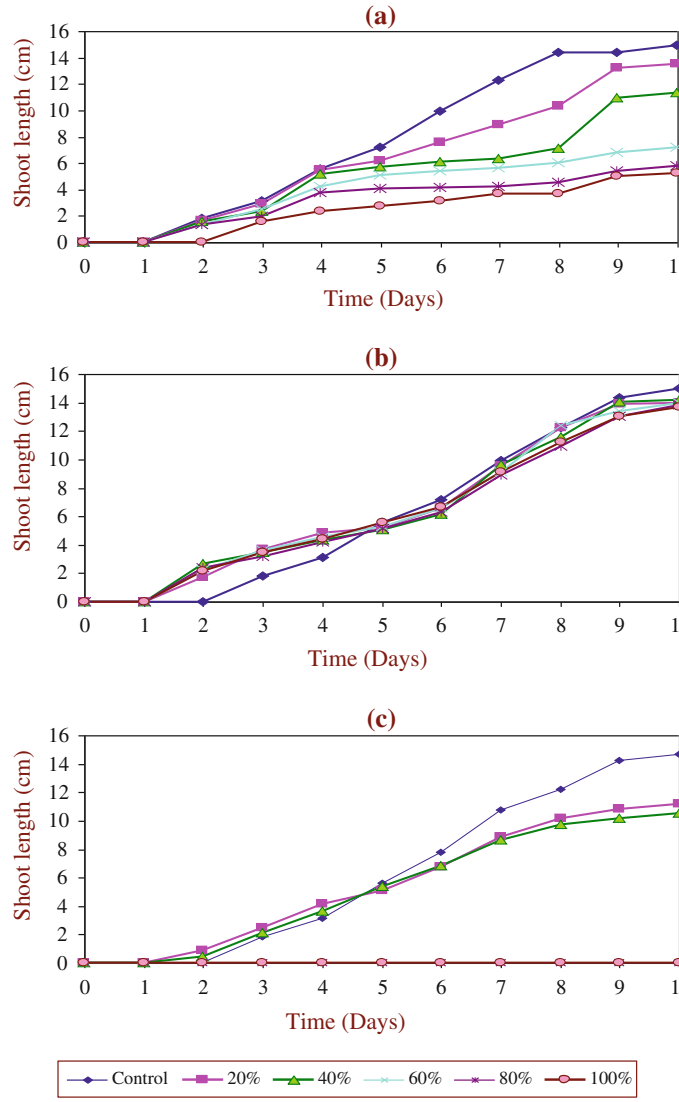

Fig. 5 Growth of green gram in soil irrigated with treated and untreated sago mill effluents at various concentrations. **a** Untreated effluent. **b** Aerobically treated effluent. **c** Anaerobically treated effluent

Impact of Untreated and Treated Effluents on Green Gram

The untreated effluent is found to decrease also the plant growth of green gram. The shoot length while irrigating higher concentration of effluent (100 %) there is no seed of germination till the 4th day, after the growth was observed and measured as 1.6 cm on the 5th day and the shoot length was 3.7 cm on 10th day (Fig. 5a). But on irrigating 80 and 60 % raw effluent, the germination was commented on the 3rd day and the shoot length was 1.33 cm, and on final day, it was 4.53 cm; 1.3 and 6 cm, respectively, and the shoot length of lower concentration such as 40 % was 1.57 cm on the 4th day and 7.1 cm on 10th day and that of less concentration such as 20 % was commented as 1.65 cm and a maximum of 10.31 cm on 4th and 10th day, respectively. While the control shows germination on 4th day and the shoot length was measured as 1.8 cm, on the final day, the shoot length was at maximum of 14.35 cm. The root length and the nodule count for 100, 80, 60, 40, 20 % was found as 10.82, 10.72, 9.75, 9.04, 9.05 cm and 4, 5, 9, 12, 14, respectively. Where as in control the

nodule count was 21, the root length was 11.77 cm. The above-stated results show that the sago mill effluent affects the plant growth at higher concentration.

The aerobically treated effluent does not reduce the growth of green gram (Fig. 5b).

The effluent of concentration of 100 % shows a shoot length 2.15 cm on 3rd and on the 10th day the shoot length was measured as 13.02 cm; and on irrigating 80 % effluent, the seed germinated on 3rd day and its shoot length was 2.37 cm and on the 10th day, it was 13 cm. While irrigating 60 %, the seed germination was on 3rd day which gives a shoot length of 2.22 cm and on 10th day it was observed as 13.37 ± 0.28 cm. The shoot length on 3rd day was 2.65 cm and attains a maximum of 14.05 cm while irrigating 40 % treated effluent. On irrigating 20 % treated effluent, the shoot length on 3rd day was 1.75 cm and on the final day, the shoot length was 13.92 cm. The nodule count of green gram irrigated with aerobically treated effluent with different concentration such as 100, 80, 60, 40, 20 % were 13, 12, 13, 16,

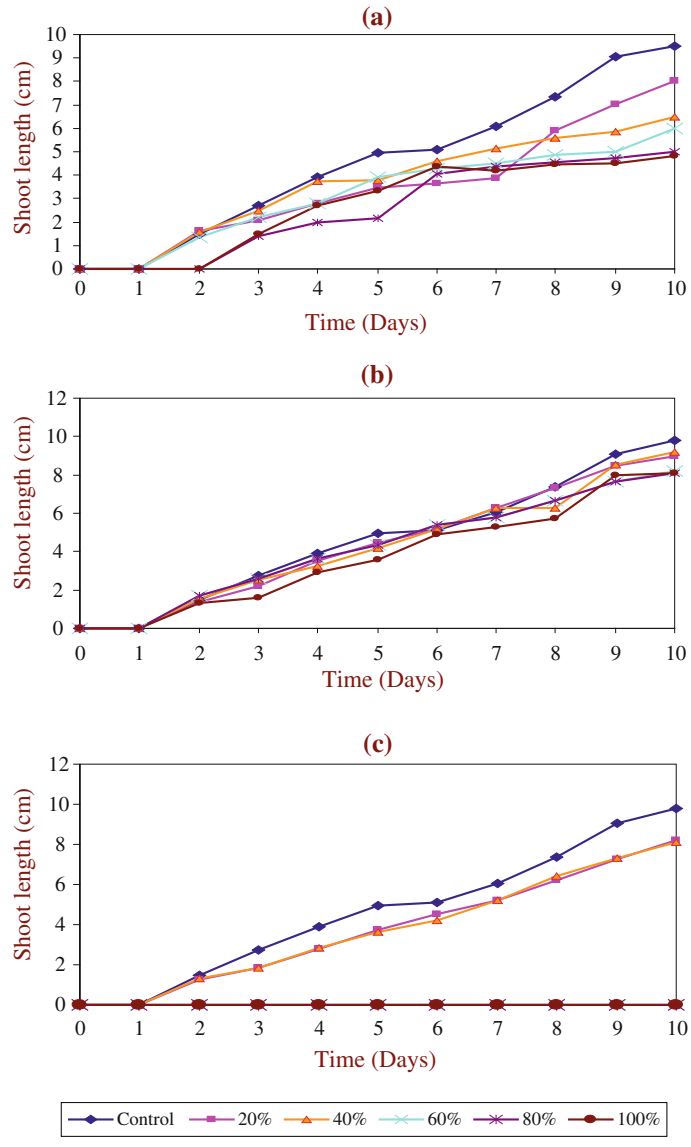

Fig. 6 Growth of red gram in soil irrigated with treated and untreated sago mill effluents at various concentrations. **a** Raw effluent. **b** Aerobically treated effluent. **c** Anaerobically treated effluent

19 cm, respectively, and the root length was 12.2, 11.95, 11.22, 11.4, 10.8 cm (Tables 5 and 6). When irrigating the aerobically treated effluent, there is no inverse effect on seed germination.

The anaerobically treated effluent retarded the growth of the plant and arrests germination. High concentration of the effluent such as 100, 80 and 60 % was used. The use of 40 and 20 % of effluent shows a shoot length of 0.95 and 13.77 cm; 1.9 and 13.2 cm on 3rd and 10th day, respectively (Fig. 5c). Its nodule count and root length were found as 11, 15 and 0.4, 0.35 cm for 40 and 20 %, respectively (Tables 5 and 6).

Impact of Untreated and Treated Effluents on Red Gram

The untreated effluent has a greater influence on red gram and the observations are given in Fig. 6a. The growth of red gram was highly affected on irrigating raw effluent at higher concentration of 100 % shows a shoot length of 1.5 cm on 3rd day, and finally on 10th day, the shoot length was 4.5 cm. When irrigating the shoot length of red gram was 1.4 cm, and its maximum length was 4.75 cm. For both the concentrations of 100 and 80%, the seed germination was observed on 4th day. For 60 %, the germination was observed on 3rd day and its length was 1.36 cm and on 10th day 5 cm. While the lower concentrations such as 40 and 20 % show germination on 3rd day and its shoot length was 1.56, 5.86 cm; 1.6, 7.03 cm on 3rd and 10th day, respectively. The nodule count of the control was 14 and that of 20, 40, 60, 80 and 100 % was 10, 7, 5, 4 and 2, respectively (Tables 5 and 6). Root length was 5.12, 4.55, 3.7, 3.65, 3.05 cm on irrigating the raw effluent of concentrations 100, 80, 60, 40, 20 % while that of the control was 6.5 cm.

The aerobically treated effluent shows a similar growth as control, the shoot length while using the different concentration of effluent such as 100, 80, 60, 40, 20 % was more or less similar with only a minor difference (Fig. 6b). On irrigating the 100 % treated effluent, seed germinated on the 3rd day and shows a shoot length of 1.3 cm and on final day, the shoot shows a maximum of 8 cm; the 80 % treated effluent also let the seed to germinate on the 3rd day which has a shoot length of 1.72 cm and its length on the 10th day was 7.65 cm; 60 % of the treated effluent shows a length of 1.72 cm and 7.65 cm on 3rd and 10th day, respectively. Both the 40 and 20 % of treated effluent shows germination on 3rd day; its shoot lengths were observed as 1.55 cm and 8.55 cm; 1.4 cm and 8.47 cm on 3rd and 10th day, respectively. The nodule count and the root length by irrigating the different concentrations such as 20, 40, 60, 80 and 100 % was 13, 12, 11, 11, 11 and 6.52, 6.52, 6.22, 5.95, 5.8 cm, respectively (Tables 5 and 6).

The anaerobically treated effluent has an inverse effect on red gram; it suppresses the germination at higher concentrations such as 100, 80 and 60 %. The germination was observed only on 3rd day in lesser concentrations such as 40 and 20 %. The shoot length measured while irrigating 40 % was 1.26 cm on 3rd day and 7.26 cm on the final day; when 20 % treated effluent was irrigated, the shoot length was 1.3 cm on 3rd day and 7.3 cm on the final day (Fig. 6c). The nodule was counted as 10.7 and 9 cm for 20 and 40 %, and the root length was measured as 5.4, 5.14 cm for 20 and 40 %, respectively (Tables 5 and 6).

Impact of Sago Mill Effluent on Soil Microbes

The raw sago mill effluent has number of subversive impacts on the environment. One of the major impacts was the change in the microbial load of the soil to exhibit such impacts; the soil was irrigated with different dilutions of raw effluent, and a pot culture study was made. The same study was done for both aerobically and anaerobically treated effluent. At an interval of every 24 h, the soil sample was taken, serially diluted, spread plated, and after incubation, the colonies were counted. The beneficial soil microorganisms for the study were selected as *Rhizobium, Azotobacter* and phosphobacteria.

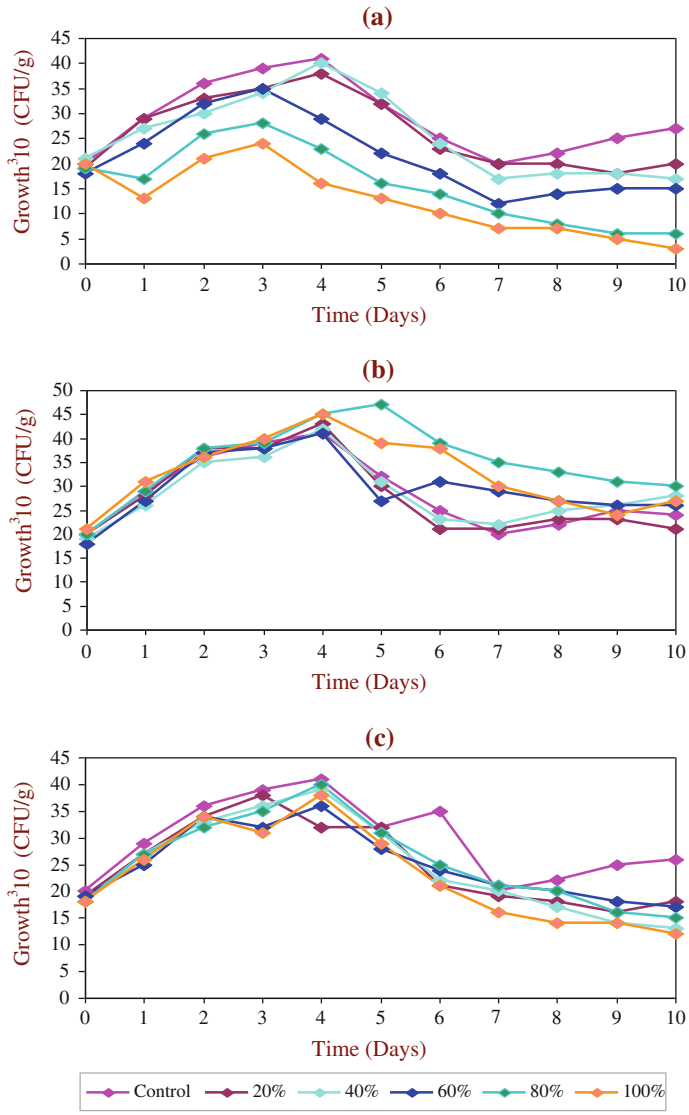

Fig. 7 Survival of *Rhizobium* sp. in soil pots irrigated with treated and untreated sago mill effluents at various concentration. **a** Raw effluent. **b** Aerobically treated effluent. **c** Anaerobically treated effluent

Those bacteria were counted on YEMA, Ashbeys agar and Pikovaskayas medium, respectively.

Impact of Untreated and Treated Sago Effluents on the Survival of *Rhizobium* spp. in soil

The raw effluent at higher concentration reduces the microbial count of *Rhizobium* spp (Fig. 7a). In the control, the *Rhizobium* count was 20×10^3 CFU/g on the initial day, and on the final day of study, the colony count was found as 27×10^3 CFU/g which shows that on irrigation, tap water shows an increased count on final day. While its count in treating the untreated effluent as such without dilution decreases as the time of incubation increases, this is shown by the colony count. It gives a count of 20×10^3 CFU/g on the initial day and on the final day the count was 5×10^3 CFU/g. Its count was also found to decrease when irrigating the dilutions such as 80 and 60 %; the CFU/g of soil was 19 on initial day and decreases to 6 on the 10^{th} day, 18 and 15

on the initial and final day, respectively. The colony count when irrigating the 40 % of sago effluent shows a result of 21×10^3 and 17×10^3 CFU/g on initial day and 10^{th} day, respectively. The lower concentration such as 20 % shows a decreased but not a drastic difference, when compared with the control. It gives a colony count of 19×10^3 CFU/g on the initial day, and on the final day, it was increased by 20×10^3 CFU/g.

The aerobically treated effluent has no deleterious effects on the survival of *Rhizobium* spp. (Fig. 7b). The colony count on the initial and final day was similar as that of the control and the colony count on the 0^{th} and 10^{th} day for different concentration of treated effluent as follows: The 100 % of aerobically treated effluent shows the enhancement of colonies from 21 to 27×10^3 CFU/g and 80 % effluent gives from 20 to 30×10^3 CFU/g and 60 % of effluent had 18–26×10^3 CFU/g and that of 40 % was 19–28×10^3 CFU/g and for 20 % also shows an enhancement from 20 to 21×10^3 CFU/g.

The anaerobically treated effluent also has more or less an equal number of colonies as that of the aerobically treated effluent (Fig. 7c). However, a slight variation showed when the pot irrigated with anaerobically treated effluent from 60 to 100 % concentrations. The 100 % effluent has the colony count of 18 and 12×10^3 CFU/g on 0^{th} and 10^{th} day, respectively. The 80 % effluent has the colony count of 18×10^3 CFU/g initially, and it was suddenly increases to 27×10^3 CFU/g, and finally, the colony count was found as 15×10^3 CFU/g. The colony count was more or less equal when the pot was irrigated with lower concentration of effluent.

Impact of Untreated and Treated Sago Effluents on the Survival of *Azotobacter* spp. In soil Pots

The untreated effluent at higher concentration such as, 100, 80 and 60 % shows a greater influence on the microbial growth. On irrigating 100 % effluent, the colony count was decreases at a greater extent on the 0^{th} day and on the 7^{th} day, there is a severe decrease in the count of 8×10^3 CFU/g (Fig. 8a). On the 8^{th}, 9^{th} and 10^{th} day, the count was 6, 3 and 0×10^3 CFU/g, respectively. On irrigation with 80 % sago effluent, the count was found as 22×10^3 CFU/g. From the 8^{th} day, the colony count was diminished. The count was found as 8, 8 and 6 on 8^{th}, 9^{th} and 10^{th} day. While in control, the colony count of *Azotobacter* was 24 and 5×10^3 CFU/g on 0^{th} and 10^{th} day, respectively. There is a colony count of 21 and 4×10^3 CFU/g on 0^{th} and 10^{th} day, respectively, while using 40 % effluent for irrigation, and the irrigation with 20 % effluent shows a colony count of 23 and 11×10^3 CFU/g on 0^{th} and 10^{th} day, respectively.

By the irrigation of aerobically treated effluent, it was concluded that there is no greater influence on microbial population even the higher concentration (100 and 80 %) shows a similar microbial load as control (Fig. 8b). On irrigation, the 100 % effluent shows a colony count of 21×10^3 CFU/g on the initial day and has a colony count of 16×10^3 CFU/g on the final day. In lower concentrations such as 20 %, it was found as 22 and 10×10^3 CFU/g on 0^{th} and 10^{th} day, respectively.

The study conducted with anaerobically treated effluent proved that there was no inverse impact on microbial population of *Azotobacter* while irrigating the effluent treated under anaerobic condition. The colony count when irrigating the undiluted treated effluent was 24 and 2×10^3 CFU/g on 0^{th} and 10^{th} day, respectively (Fig. 8c). While irrigating 80 % of anaerobically treated effluent, the colony count was 24×10^3 CFU/g on the 0^{th} day and 4×10^3 CFU/g on 10^{th} day. On irrigating the 60 %, the soil sample gives a colony count of 23 and 5×10^3 CFU/g on 0^{th} and 10^{th} day. On irrigating 40 % of an anaerobically treated effluent, the colony count was 21 and 7×10^3 CFU/g on 0^{th} and 10^{th} day, respectively. In 20 % concentration, the influence was less when compared with control.

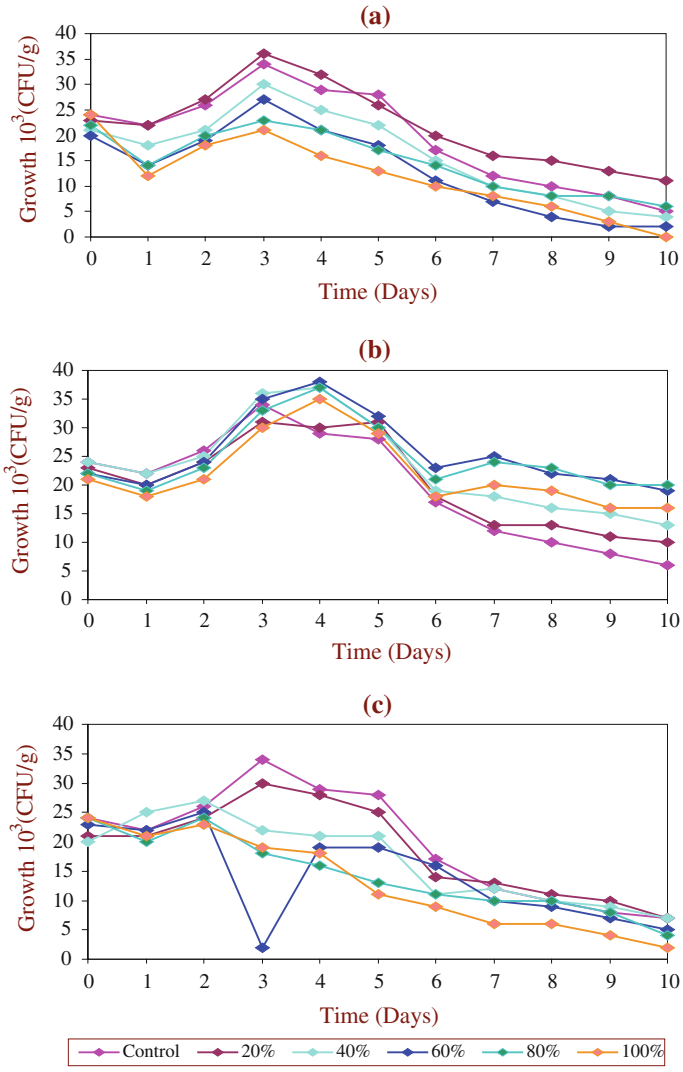

Fig. 8 Survival of *Azotobacter* sp. in soil pots irrigated with treated and untreated sago mill effluents at various concentration. **a** Raw effluent. **b** Aerobically treated effluent. **c** Anaerobically treated effluent

Impact of Untreated and Treated Sago Effluents on the Survival of Phosphobacteria in Soil Pots

The effect of raw effluent on phosphobacteria was found to be of greater, which eliminates the phosphobacteria at the 10th day. The colony count observed on irrigating the undiluted effluent was 12 × 10^3 CFU/g on the initial day, and the count was sweeped off on the 4th day itself (Fig. 9a). The count of phosphobacteria was 10 × 10^3 CFU/g on the initial day, but the organisms were totally eliminated on the 6th day itself while irrigating 80 % effluent. The count was 13 × 10^3 CFU/g on the initial day and the organisms eliminated on 5th day on irrigating 60 % sago mill effluent. When irrigating 40 % effluent, the colony count was 10 × 10^3 CFU/g. On the initial day, the organisms were eliminated; on the 9th day and at the lower concentration 20 %, the count was 11; on initial day and at the final day, the count was 1 × 10^3 CFU/g, while that of control was 12 and 3 × 10^3 CFU/g on initial and final day.

Aerobically treated effluent shows a colony count as the control (Fig. 9b). At higher concentration such as 100 %, the colony count was 10, 1 × 10^3 CFU/g on the initial and final day,

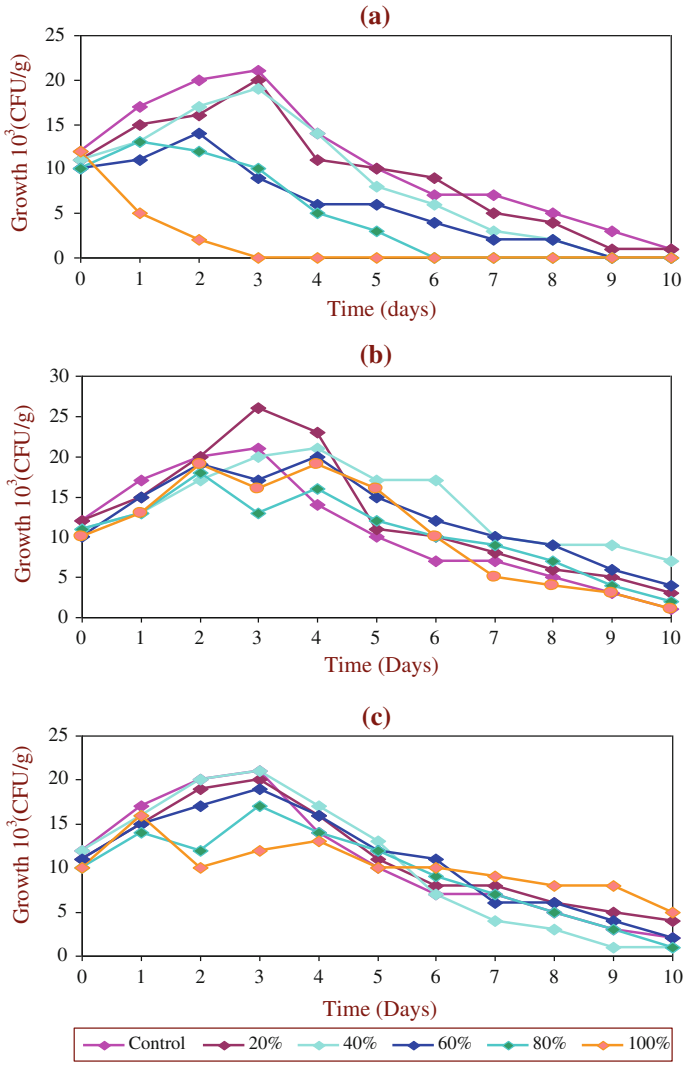

Fig. 9 Survival of phosphobacteria in soil pots irrigated with treated and untreated sago mill effluents at various concentration. **a** Raw effluent. **b** Aerobically treated effluent. **c** Anaerobically treated effluent

respectively. The colony count was observed as 11 and 2 × 10^3 CFU/g on initial and final day, respectively, while using 80 % treated effluent. The colonies of 10 and 2 × 10^3 CFU/g was commented on 0^{th} and 10^{th} day, respectively, while using 60 % treated effluent. When irrigating lower concentrations such as 40 and 20 % treated effluent, the colony count was same with only minor fluctuations and they give the colony count of 11 and 7 × 10^3 CFU/g and 12, 3 × 10^3 CFU/g on 0^{th} and 10^{th} day, respectively.

The anaerobically treated effluent shows a result as the aerobically treated effluent and has a minor change in the colony count (Fig. 9c). On irrigating the higher concentration of the effluent such as 100, 80, 60 %, the colony count was 10 and 5 × 10^3 CFU/g; 10 and 1 × 10^3 CFU/g; 11 and 2 × 10^3 CFU/g on initial and final day, respectively. At lower concentration such as 40 and 20 % concentration of effluent, the colony count was 12 and 1 × 10^3 CFU/g and 11 and 4 × 10^3 CFU/g on 0^{th} and 10^{th} day, respectively.

Discussion

Pollution is one of the major problems in the world. Various industries play an important role in causing pollution by releasing effluent. There is also minor contribution of organic waste for

the pollution of water and land. Numbers of microbes are capable of degrading starch and convert them into simple sugars. Some microorganisms under anaerobic condition can produce methane. Hence, the study is focused on the aerobic and anaerobic treatment of sago industry waste, which is simple, economical and ecofriendly when compared to the physical and chemical treatment. Another advantage is the easy disposable of digested sludge.

The aerobic treatment effectively reduces all the characteristics such as solids, calcium, magnesium, chloride, and because of treatment, its pH increases and attains neutral pH. The above-mentioned characteristics also were found to decrease in the treatment of sago wastewater (Ayyasamy et al. 2008). The microbial consortium degrades the starch by amylase enzyme and produces large number of glucose molecule. Hence, after treatment, the quantity of glucose is high and the starch content is low in the treated effluent.

The previous study in the aerobic treatment of sago industrial effluent was made with consortium of *Alcaligenes, Bacillus* and *Corynebacterium* by Ayyasamy et al. (2008). This combination gives a result of maximum degradation of starch (89 %), but the consortium of *Flavobacterium*, *Enterobacteriacae*, *Alacaligenes*, *Acinetobacter*, *Corynebacterium* in this study shows a maximum degradation.

The anaerobic treatment is a model for treating the wastewater through a biogas technology. The use of raw effluent alone shows a decreased, moreover no gas production till the 9^{th} day, but after it shows a meager amount of gas production, on 12^{th} and 15^{th} day. But the combination of tapioca peelings and the raw effluent is in the ratio 1:2, very effective for gas production. From the 3^{rd} day, there is an increase in the production on 6^{th} and 9^{th} day and the production gradually decreased on the 12^{th} and 15^{th} day. But the same combination in the ratio 1:1 shows a relatively decreased biogas production, the quantity of the tapioca peeling is equal to the quantity of the effluent, hence, there are no sufficient microbes to degrade the tapioca peelings; so, the partial digestion of the tapioca peelings occur which results in the decreased biogas production. But in the case of tapioca peelings in the ratio (1:2), the substrate was low and the organism load was high. Thus, there is complete degradation of starch in tapioca peeling which leads to the higher rate of biogas production.

Another combination is cow dung with raw effluent in the ratio 1:2, at early stages. There is no significant gas production but at later stages, from 12^{th} to 15^{th} day, the gas production attains a maximum level exceeds that of the production in the combination of tapioca peeling and raw effluent in the ratio 1:2. The cow dung with raw effluent in the ratio 1:1 also shows relatively a similar production with that of the former. The cow dung was diluted with raw effluent; the low and high production at early and later stages, respectively, because of the anaerobic condition might be started only later in those two sets. The study concluded that the maximum rate of production was 786 and 620 ml on 3^{rd} day in tapioca peelings with effluent in ratio 1:2 ratio and 1:1 ratio, respectively. The quantity of the gas was measured in liters as in the study done by Saravanan et al. (2001). Thus, the effluent treated in anaerobic condition gets digested completely, and it has the following characteristics: acidic pH, reduced solids, reduced BOD, COD, phosphate, alkalinity, hardness, calcium and magnesium level. A supportive anaerobic study was done by Saravanan et al. (2001). The COD gets reduced and the pH reduces from neutral to acidic, and the suspended solids increases in the treated effluent.

The treated wastewater was analyzed for recycling and reuse to ensure an alternative for sustainable waste resource management. When the aerobically treated effluent was irrigated to the seed, the seed germination and the growth of the plant as well as the root length and nodules count was found to be similar, moreover increased in some cases. This reveals that the wastewater was effectively treated by aerobic

method and thus, it has dual advantages: one is the efficient, low-cost, ecofriendly treatment technology and the other is the water may be recycled and used for irrigation. The treated effluent not only acts as a source of water, it also induces the growth of the plant and thereby done a role of bio fertilizer. The anaerobic treatment was aimed to treat concomitant utilization of wastewater by the production of biogas, so the efficient set alone was irrigated. The efficient set containing the high-starch solid substances retarded the plant growth and delayed the seed germination at lower concentration (20 and 40 %). But at higher concentration (60, 80 and 100 %), no growth was observed.

Saravanan et al. (2001) recommended the use of nitrogen enriched sludge for agricultural use. But, the anaerobically treated effluent reduces the growth in this study. In this study, the effluent was not enriched with nitrogen and the acidic nature of the treated effluent may also be the reason for controversy. The germination study with raw effluent and treated effluent suggests the use of aerobically treated effluent for irrigation, as it shows a better result. The effect of those effluents on microbial population of the soil is also analyzed by pot culture study. The untreated and treated effluents were irrigated to the soil, and the number of *Rhizobium*, *Azotobacter* and phosphobacteria population was estimated regularly for 10 days. While irrigating the untreated effluent, the microbial population gets gradually decreased as the time of treatment increases. When compared to the population count on initial day, the count decreases nearly 2-fold at the 9^{th} day. The untreated effluent has greater influence on phosphobacteria, which was eliminated at the final day of study. But the aerobically and anaerobically treated effluents show an equal microbial population in all the dilution. The growth in both treated effluents was also similar.

Thus, this study shows that the treated effluent does not affect the microbial population. This study reveals the impact of treated effluent on the plant growth and soil microorganisms. By the entire study, it is concluded that the use of untreated effluent decreases the plant growth, seed germination and microbial population. The aerobically treated effluent has positive influence on plant growth and microbial population, but the effluent treated under aerobic condition shows an inverse effect on plant growth and germination, but it does not alter the microbial load, thus it is suggested not treating the anaerobically treated effluent for irrigation. But the advantages in this treatment are the low-cost technology and production of bio gas. Through aerobic treatment, the production of value-added product such as glucose and amylase can also be possible, which have a wide application in various industries such as paper, brewing, textile, bakery, food and distillery. Thus, the low cost, ecofriendly nature, water recycling, production of valuable products are the merits of the study, and moreover, it is a combined remediation and bioconversion.

India is one of the major countries for the production of starch from tapioca. The sago starch processing units discharge a large quantity of effluent, with the following characteristics: acidic pH, irritating color and obnoxious odor, higher solid, DO, BOD, COD, magnesium, chloride and potassium which is nuisance to the society. If this effluent is used for irrigation, the plant growth and the seed germination is affected thus causing land pollution. To overcome this problem, the raw effluent is treated in two aspects: one is to recycle the wastewater and the other is to produce biogas from the effluent.

Aerobic method was adopted for the former, and anaerobic method was attempted for the later. For the aerobic treatment, the microbial consortium was made with organisms, such as *Flavobacterium*, Enterobacteriaecea, *Alcaligenes*, *Acenitobacter*, *corynebacterium*, which were isolated from sago effluent and effluent-contaminated sludge. More than 50 strains were isolated and the maximum of the isolates fell under the family Enterobacteriaceae, and secondly, the *Alcaligenes* sp. All the isolates were subjected to starch hydrolysis, to screen the

potential organisms. The potential strain was selected on the basis of the diameter of the zone formed due to the utilization of the starch on the medium. The diameter of *Flavobacterium* was found as maximum and then the Enterobacteriaceae. The microbial consortium shows a degradation rate better than the degradation of starch by individual strains.

So, the microbial consortium was used for the aerobic treatment of the sago industry effluent. The treatment procedure was simple and efficient, and the production of valuable products such as amylase and glucose can also be obtained. In the aerobically treated effluent, the effluent characteristics like BOD, COD, DO, magnesium, calcium and potassium reduces effectively in aerobic treatment with microbial consortium. Thus, these effluents act as a source of irrigation and also enhance the growth in some cases, which was revealed by pot culture study. The entire three seeds, black gram, green gram and red gram, show a better growth which is similar to the control where tap water was irrigated, while the sago effluent reduces the growth of all the plants at higher concentrations such as 100, 80 and 60 %. But, the anaerobically treated wastewater was unfit for irrigation due to its acidic nature. The acidic pH creates high osmotic pressure which causes wilting of seeds and no germination occurs. On irrigation, the effluent arrests the seed germination and the plant growth at higher concentration such as 100, 80 and 60 %. But, at lower concentration, the germination and plant growth occurs.

The raw effluent also has influence on microbial load, and it decreases the microbial population at higher concentration. But, both the treated effluents have no adverse effect on microbial population. The effluent has enough potential to produce bio gas under an aerobic condition as it is rich in starch. The use of tapioca peelings with effluent in the ratio 1:2 produces a maximum rate of bio gas; the use of cow dung with effluent in the ratio 1:2 also produces a maximum rate of bio gas but on the later stage. Thus, by the aerobic treatment, the effluent can be recycled and used for irrigation and also the products such as glucose and amylase can be obtained. But, the anaerobic treatment suits only for the production of biogas and not for irrigation. From the study, it is concluded that the microbial-treated sago mill effluent is a potential resource for agroecosystem management.

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Nodulation and Nitrogen Fixation in Rooted Stem Cuttings of *Casuarina junghuhniana* Miq. by *Frankia* Inoculation

A. Karthikeyan

Abstract

Casuarina junghuhniana Miq. fixes atmospheric nitrogen (N) through the symbiotic relationship with *Frankia*, a soil actinomycete group. The roots of *C. junghuhniana* produce root nodules where the bacteria fix atmospheric N, which is an essential nutrient for all plant metabolic activities. High-yielding and genetically superior trees of *C. junghuhniana* are selected and propagated vegetatively for commercial use. Yet, as the vegetative propagation uses inert material (vermiculite) for rooting, there is no chance for *Frankia* association that results absence of root nodules in rooted stem cuttings. Therefore, after planting of these stocks, there is a necessity to apply chemical fertilizers for N supply that increase the planting cost. To reduce the chemical fertilizers costs and to establish the N fixation in vegetatively propagated rooted stem cuttings of *C. junghuhniana*, the isolated actinomycete *Frankia* from root nodules of *C. junghuhniana* was cultured in artificial liquid P media and applied in this study. Application of the *Frankia* inoculum at the rate of 5 ml during the root initiation stage resulted in the development of an average of 12 nodules, weighing 43-mg/rooted stem cuttings of *C. junghuhniana* after 25 days. The rooted stem cuttings of *C. junghuhniana* were also on increase in shoot and root growth, number of lateral roots, shoot biomass, root biomass and tissue N content due to inoculation of *Frankia*. In this study, the acetylene reduction assay on *Frankia* liquid culture was also made and found the release of 150.69 nmol of C_2H_4/mg of protein/h in gas chromatography. This study supports the inoculation of *Frankia* in rooted stem cuttings of *C. junghuhniana* for biological N fixation so as to reduce the chemical fertilizers.

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Frankia · Nitrogen · *Casuarina junghuhniana* · Rooted stem cuttings · Root nodules

Introduction

Actinorhizal plants usually form root nodules in association with the nitrogen (N)-fixing actinomycete *Frankia* that helps them to survive even in nutrient-poor soils by N fixation. *Frankia* is an actinomycetes organism, which interacts with the roots of appropriate host plants to form N-fixing nodules also called actinorhizae (Benson and Silvester 1993). Actinorhizal plants include Casuarinaceae which is a major family of trees that have been disseminated throughout the tropics owing to their ability to grow in adverse conditions (Echhab et al. 2007). *Casuarina junghuhniana* Miq. belongs to Casuarinaceae, and on account of its high economic value, farmers are interested in planting this tree as an agroforestry crop in the states of Tamil Nadu and Pondicherry (India). It is useful to wind break, soil improvement, an ornamental live fencing and building construction material (Jayaraj 2010). *Frankia* is associated with *C. junghuhniana* for N fixation, and it has been estimated that *Frankia* fixes atmospheric N up to 362 kg N/ha/yr, which is an essential nutrient for all plant metabolic activities and growth (Shantharam and Mattoo 1997).

In *Frankia* inoculum, generally farmers used to collect the root nodules from mature Casuarina trees and then crush and add at the time of planting in new sites along with seedlings/cuttings of *Casuarinas.* This practice is often unsuccessful if the crushed root nodules contain dead or inactive *Frankia*. Further, for pulp and paper production, high-yielding and genetically superior trees of *C. junghuhniana* are selected and multiplied by rooted stem cuttings through farmers of Tamil Nadu and Pondicherry. But the rooted stem cuttings are being propagated in an inert material (vermiculite) so that there is no chance for *Frankia* association. Though inoculation of *Frankia* is essential in rooted stem cuttings of *C. junghuhniana*, there is no report found on nodulation of rooted stem cuttings in *C. junghuhniana.* Hence, there is an urgent need to find an alternate solution for the use of these chemical fertilizers for the rooted stem cuttings of *C. junghuhniana* during plantation. We attempted to study the effect of inoculation of cultured *Frankia* strain in rooted stem cuttings of *C. junghuhniana* on growth, biomass and nodulation, which could reduce the use of chemical fertilizers. Further, it is intended to decide the effect of *Frankia* on the efficiency of N uptake of *C. junghuhniana* rooted stem cuttings.

Materials and Methods

Location of the Study

The study was conducted at the model nursery of Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore (11°01′N and 96°93′E; altitude 410 m.a.s.l), Tamil Nadu, India. The climate is monsoonal with an annual precipitation of 640 mm and a dry season between January and April. The maximum and minimum monthly temperatures are 31 and 21 °C, respectively.

Isolation and Multiplication of *Frankia*

The *Frankia* used in this study was isolated from *C. junghuhniana* root nodules collected from the coastal area, and the location and characteristics of collected nodules are described in the Table 1.

The nodules were collected in ice box and stored in frozen condition at −4 °C. Afterwards, the nodules were surface sterilized with 30 % H_2O_2 and kept in shaker for 40 min. Under

Table 1 Source of *Frankia*

Place	Soil type	Source of nodules	Nodules colour	Nodules diameter
Cuddalore (TN) coastal zone	Sandy clay loam	Coastal plantations of *Casuarina junghuhniana*	Brown	1–1.5 cm

aseptic conditions, the nodules were rinsed with sterile water and 0.2 g of nodule was ground manually in sterile mortar and pestle. Then, the nodule solutions were centrifuged at 1,000 rpm for 20 min, and the supernatant was filtered through Whatman's No.1 filter paper. The suspension was then plated in P media and incubated at 25 °C for 4 weeks. One litre of P medium was prepared as follows (Shipton and Burgraff 1983): 10 g $CaCl_2.2H_2O$, 20 g $MgSO_4$, 0.46 g propionic acid, 0.15 g H_3BO_3, 0.15 g $ZnSO_4.7H2O$, 0.45 g $MnSO_4.H_2O$, 0.004 g $CuSO_4.5H_2O$, 0.028 g $Na_2MoO_4.2H_2O$, 0.009 g $CaCl_2.6H_2O$, 0.04 g Biotin, 100 g K_2HPO_4, 67 g $NaH_2PO.2H_2O$, 0.1 g FeNa EDTA and 8 g agar. The pH of the medium was adjusted to 6.8. After 30 days of incubation, the *Frankia* growth was observed as fluffy white colonies on P media plates. These colonies were transferred to P media broth for mass multiplication.

Analysis of Nitrogenase Activity

The nitrogenase activity of *Frankia* was determined in 21-day-old culture in N-free P media broth by using the acetylene reduction technique (Hardy et al. 1968) to confirm the presence of nitrogenase in the *Frankia* strain which is essential to break down the triple bond of N. 30 ml of culture is placed in 130 ml capacity of sterilized vials and sealed with rubber stoppers. About 10 % of the airspace in each vial was replaced by pure acetylene and allowed to stand for 1-h incubation at room temperature. About 0.5 ml of the gas was removed from each vial and injected into a gas chromatography (GC: Model: Nucon 910980) equipped with a flame ionization detector and a 2 m × 2.1 mm stainless steel column packed with Porapak Q on 80–100 mesh. The oven temperature was adjusted to 70 °C; injector temperature 50 °C; detector temperature 120 °C. The N carrier gas flow rate was adjusted to 30 ml/min to measure ethylene production. Blanks comprised air from bottles to which no acetylene was added. Peaks of ethylene were compared with ethylene standard (purity 99.9 %) injected into the GC to calculate concentrations. The nanomoles of ethylene produced per time unit was standardized to total cell protein. The protein concentration of the cells was determined as described by Lowry et al. (1951) with bovine serum albumin as the standard. The specific activity of nitrogenase was expressed as nanomoles of ethylene produced per mg of protein per hour. The rate of N fixation was calculated using the formula:

$$\text{Nitrogenase activity} = \frac{\text{Peak area count} \times 0.0006 \times \text{volume of gas injected into vial}}{\text{Incubation time} \times \text{volume of gas injected into GC} \times \text{total mg of protein in the sample}}$$

Collection and Propagation of *C. junghuhniana* Stem Cuttings

The stem cuttings were collected from the clones Cj 18 at model nursery, IFGTB, and treated with 0.1 % carbendazim fungicide and 2,000 ppm of indole butyric acid (IBA). After the treatment with IBA, the cuttings were placed in 100-cc root trainers that contain the inert media vermiculite. The rooted cuttings were thereafter placed in polytunnels made of polythene sheets (32–35 °C and 60–65 % RH) for 30 days. After 25 days, the cuttings showed initiation of 2–3 lateral roots with 1–1.5 cm length. In this stage, the rooted stem cuttings were transferred to shade house and watered regularly.

Inoculation of *Frankia* in *C. junghuhniana* Rooted Stem Cuttings

The cultured *Frankia*-strained P media broth was inoculated in the root zone of rooted stem cuttings of *C. junghuhniana* clone Cj 18 at the rate of 5-ml^{-1} cutting and maintained 15 replicates. Root trainers containing inoculated cuttings and uninoculated controls were placed in the shade house and watered regularly. The initiation of nodules and nodule numbers in each rooted stem cuttings was assessed. These planting stocks were maintained for 3 months in the model nursery of IFGTB and harvested for analysis of growth and biomass. The dry weights of *Frankia* inoculated these planting stocks were determined after oven drying at 50 °C to a constant weight.

Analyses of Growth, Biomass and Tissue N Content

The growth of *Frankia*-inoculated rooted stem cuttings and uninoculated cuttings was analysed in terms of shoot height, root length, number of lateral roots, collar diameter, dry weights of shoot, root, number of nodules and nodule biomass. The dry weights were determined after oven drying at 50 °C to a constant weight. The total N content was estimated in root and shoot sample using KELPLUS auto-analyser to determine the N fixation by inoculation of *Frankia* in the rooted stem cuttings of *C. junghuhniana.* The dried plant sample (0.25 gm) was digested with 3 gm of catalyst mixture: (potassium sulphate and cupric sulphate in the ratio of 5:1) and 10 ml of H_2SO_4 in Kjeldahl digestion system (KELFLOW) at 420 °C for 1 h. Then, the digested sample was diluted with 10 ml of distilled water before distillation. After distillation, the collected distillate was titrated against 0.1 N hydrochloric acid.

Statistical Analysis

Each measured variable in the nursery experiment was subjected to analysis of variance, and means were separated using Duncan's multiple range test (SPSS version 10).

Results

Morphological Characteristics of *Frankia* Isolate

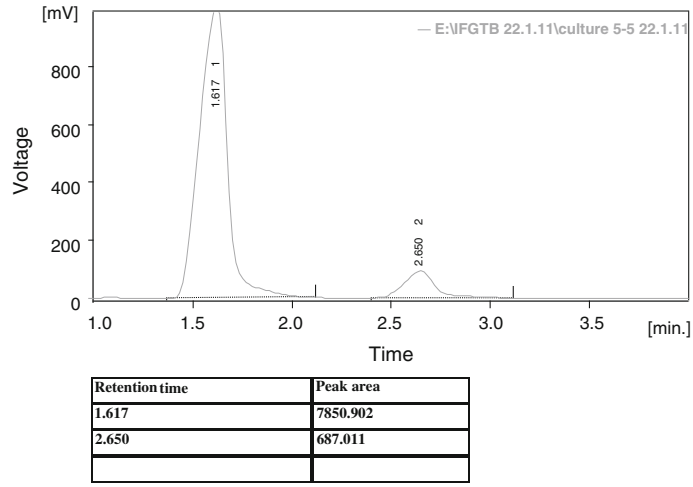
Under optimum conditions (28–32 °C), the growth of the isolate that formed white fluffy colonies on the P media plates was examined under light microscope. It showed branched and septate hyphae and round vesicles. The morphometrics of *Frankia* is shown in Table 2.

Nitrogenase Activity

The nitrogenase activity of *Frankia* was measured at various intervals. The experiment was repeated three times, and the mean value of the isolate was calculated. The observation of ability to reduce acetylene in vitro supports the fact that actinomycete was isolated in this experiment which is able to fix N in the *Frankia*-inoculated

Table 2 Morphometric of *Frankia*

Hyphal width (in µm @ 40x)	Vesicle dimension (in µm @ 40x)	Sporangia shape	No. of days grown in media
1–1.5	2–3	Circular	25 days



Retention time	Peak area
1.617	7850.902
2.650	687.011

ARA: 150.69 n mol.

Fig. 1 GC analysis for nitrogenase activity of *Frankia*

rooted stem cuttings. The *Frankia* showed the nitrogenase activity at 25-day-old liquid culture that results an amount of 150.69 nmol of ethylene produced per mg of protein per hour (Fig. 1).

Growth and Biomass of *C. junghuhniana* Rooted Stem Cuttings

Nodulation of *Frankia* was observed in 25 days after inoculation in the rooted stem cuttings of *C. junghuhniana*. The initial infections at 20 days showed clubbed roots in the rooted stem cuttings, and the nodule development occurred at 25 days. The rooted stem cuttings of the inoculated with *Frankia* strain showed significantly increased growth in shoot height, root length, collar diameter and biomass than the uninoculated control seedlings. The rooted stem cuttings showed higher nodule biomass than the uninoculated control. *Frankia*-inoculated cuttings showed dense root nodules in the root region, whereas the uninoculated cuttings showed absence of nodules. The root nodules developed in the rooted stem cutting weighed up to 43 mg, and 12 root nodules per cutting were obtained. The R/S ratio was significantly lower in *Frankia*-inoculated rooted stem cuttings than in the uninoculated control (Table 3). The new finding in the present study is the successful nodulation establishment in the *C. junghuhniana* rooted stem cuttings in inert media without using soil.

Tissue N Content

Significant differences in total N content in comparison with uninoculated controls were observed. The total N content was found 5.64 mg/g dry weight in the rooted stem cuttings, whereas the uninoculated control rooted stem cuttings showed a mean value of 0.41 mg/g dry weight (Fig. 2).

Discussion

The results of this study have clearly shown that *Frankia* can improve the plant growth through increased uptake of N. *Frankia* results in positive effect on the rooted stem cuttings of

Table 3 Growth and biomass of *C. junghuhniana* rooted stem cuttings to *Frankia* inoculation at 90 days under nursery conditions

Clone No.	Treatments	Collar diameter (cm plant^{-1})	Shoot length (cm plant^{-1})	Root length (cm plant^{-1})	No. of lateral roots/ plant	Shoot dry weight (mg / plant^{-1})	Root dry weight (mg plant^{-1})	R/S ratio	Nodulation time	No. of nodules	Nodule biomass (mg^{-1}nodule)
Cj 18	*Frankia*	1.871 b	18.89 b	14.3 *b*	15.1 b	0.905 b	0.557 b	0.615 b	30 days	12.12	43
	Control	0.542 a	5.9 a	4.8 *a*	1.8 a	0.288 a	0.199 a	0.690 a	–	–	–

Data were mean of 15 replicates; means followed by same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test

C. junghuhniana growth through improvement in growth and biomass. Earlier studies also reported that the increase in growth and biomass of casuarinas due to inoculation of *Frankia* might be strongly correlated with improved accumulation of N due to *Frankia* (Reddell et al. 1988). This study further supports the positive response of *C. junghuhniana* rooted stem cuttings in the nursery to *Frankia* application and strengthens the *Frankia* dependency of *C. junghuhniana* in low fertility. Similar results were reported for *Frankia* (nodule suspension) inoculation employed in *C. equisetifolia* seedlings (Muthukumar and Udaiyan 2010). In several studies (Lesueur and Duponnois 2005; Yamanka et al. 2003), the *Frankia* effects on the plant growth promotion have been demonstrated in sterile soil substrates. However, the growth-promoting effect of *Frankia* on *C. junghuhniana* rooted stem cuttings in inert media has not been reported. It has been repeatedly reported that spontaneous nodulation of the genera Casuarina is unlikely outside their natural habitat. This may be attributed to the fact that *Frankia* is not possible to transmit with the seed either within or on its surface (Torrey 1983).

Inoculation experiments of this kind in nursery conditions are essential for *C. junghuhniana* rooted stem cuttings which bring together the root system and nodulation as they propagated in inert media. In this study, nodulation occurs in 30 days in the rooted stem cuttings of *C. junghuhniana*. However, Vergnaud et al. (1985) have obtained axenic nodulation in *Alnus glutinosa* within 10 days. This also has shown that there is a difference in nodulation behaviour between *Alnus* and *C. junghuhniana.* Nodulation biomass and nodule number increased the rooted stem cuttings of *C. junghuhniana* raised in inert media. This reflects that the symbiotic N fixation depends on host photosynthesis (Arnone and Gordon 1990), where the ATP is supplemented to *Frankia*. The increased biomass in the rooted stem cuttings of both the clones could be the result of increased nutrient inflow rates through *Frankia*. The nitrogenase activity of *Frankia* in this study has shown that the *Frankia* culture contains more vesicles. Because vesicles of

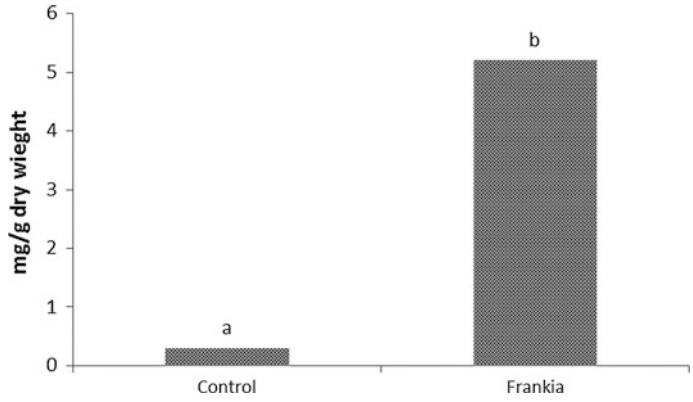


Fig. 2 Total N content in the rooted stem cuttings of *C. junghuhniana* inoculated with *Frankia*. Means followed by same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test

Frankia have been considered as the sites of nitrogenase for many years (Gauthier et al. 1981; Fontaine et al. 1984). The nitrogenase activity of *Frankia* also corroborates supply of Mg-ATP from the *Frankia*-inoculated *C. junghuhniana* cuttings that give energy for N fixation (Huss-Dannel and Hablin 1988). Increased tissue N content of *Frankia*-inoculated rooted stem cuttings of *C. junghuhniana* raised in inert media than the uninoculated control plants showed more influence of *Frankia* in N fixation.

Conclusion

The results from this study support the inoculation of cultured *Frankia* to the rooted stem cuttings of *C. junghuhniana* for enhancement of growth, biomass and nutrient uptake. It is essential to introduce potential *Frankia* in the rooted stem cuttings of *C. junghuhniana* as they propagated in inert media. This method of inoculation of *Frankia* in the rooted stem cuttings of C. *junghuhniana* will be beneficial for early establishment in the field without additional chemical fertilizers even in low-fertile lands.

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Studies on the Saprophytic Survival and Suppression of *Fusarium moniliforme* J. Sheld, *Helminthosporium oryzae* Breda De Haan and *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksw., Causing Diseases in Paddy.

A. Panneerselvam and R. Saravanamuthu

Abstract

Saprophytic behaviour and the conditions that limit the activity of the pathogens, *Fusarium moniliforme*, *Helminthosporium oryzae* and *Sarocladium oryzae*, causing diseases at different stages of growth of paddy, are lacking. The present article deals with (a) the population dynamics of soil microfungi in the paddy field;(b) the antibiotic interactions between the pathogens and soil fungi;(c) the saprophytic survival and competitive saprophytic colonization (CSC) of the paddy straw as influenced by physical factors of the soil;(d) cellulolysis rate of the pathogens; and (e) the amendments of the soil with oil cakes on the saprophytic behaviour and suppression of the pathogens.

Keywords

Saprophytic behaviour · *Fusarium moniliforme* · *Helminthosporium oryzae* · *Sarocladium oryzae* · Antibiotic interactions · Cellulolysis rate · Competitive saprophytic colonization · Saprophytic survival

Introduction

Understanding the modes of pathogen survival and the ways by which they could be suppressed is important, especially in evolving strategies for the control of plant diseases. The pathogens, in the absence of their hosts, survive either as dormant propagules or actively as saprophytes on dead organic substrates of the host in the soil Papavizas and Klag (1975). The survival structures of the pathogen in the soil are suppressed either due to natural suppressiveness of the soil or due to manipulation of the soil environment. The 'pathogen suppression' in the soil is considered as an important step in the control of diseases as it involves the direct disinfection of the soil (Lockwood 1977, 1986).

The soil fungi include both 'soil inhabitants' and 'soil invaders'; the interaction between these two groups of organisms is the determinant factors of the competitive survival of the

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pathogens in soil which include antagonism and symbiosis (Wicklow 1981). Fungal antagonism is usually characterized as the mechanism that protects the prior colonists of the substrates from colonization by other organisms (Brian 1960). Garrett (1970) has suggested that the production of antibiotics and the tolerance to the antibiotics produced by the other organisms are the factors that influence the saprophytic survival and competitive saprophytic colonization (CSC). Antibiotics are produced by most of the soil micro-organisms in in vitro conditions. Contrarily, most of the attempts to isolate and characterize such substances from natural soil, other than organic substrates, proved futile Nash and snyder (1962). Nevertheless, the in vitro production of antibiotics by fungi could provide valuable information as to how the pathogens survive in soil as saprophyte. It is also accepted, in general, that the antibiotics are produced in soil and furnish protection in competitive situation by eliminating other microorganism in soil (Gottlieb 1976; Alexander 1977).

Garrett's (1970) concept of saprophytic behaviour includes (a) saprophytic survival in the dead host tissues and (b) CSC of the substrate. The saprophytic growth and activity of the pathogens vary depending upon the environmental conditions and soil. The differences in the saprophytic behaviour of the pathogens in the soil should be due to variations in the 'cellulolysis rate' of the organisms as suggested by Garrett (1985). Though Garrett (1980) has provided valuable information regarding various aspects of the saprophytic behaviour of pathogens in soil, Lockwood (1988) has emphasized that there are much works needed to be done on these aspects.

The knowledge accumulated regarding the conditions that inhibit the saprophytism of the pathogens could be exploited for the biological control in several ways. Control of plant diseases through amendment of the soil with organic substrates like plant material, crop residues and food bases has been achieved (Papavizas and Lumsden 1980). The impact of the amendments on the control of plant diseases has also been emphasized (Cook and Baker 1983; Khan et al. 1974).

The agricultural pesticides and by-products of their decomposition enter into the soil could have either direct or indirect effect on the pathogens. The direct effect of pesticides can result in either impairment or enhancement of the growth of the pathogen; inoculums density, competitive saprophytic ability, saprophytic survival and virulence of the pathogens and the indirect effects are less clearly defined because they are mediated through various ecological processes and population of microorganisms Mukerji (1966). Hence, the saprophytic survival, suppression and population dynamics, cellulolysis rate and antibiotic interactions between the pathogens were studied in *F. moniliforme*, *H. oryzae* and *S. oryzae* disease causing microbes in paddy Park (1967).

Materials and Methods

Description of the Study Site

The study site of paddy field at Mannampandal village, Mayiladuthurai taluk of Thanjavur district, Tamil Nadu, is located in the Cauvery Basin at 79.6 N and 11.2 E latitude; the MSL is 43°. The soil is of alluvial type. The climate of the area is tropical and monsoonic. It received fairly good rainfall during the period of north-east monsoon (October–November, extends up to January) and south-west monsoon (June–July, extends up to August). The minimum temperature was 20 °C during December–January, and the maximum temperature was 41 °C during May–June. The field was under cultivation of paddy during the months August to January. Black and green grams were cultivated as alternate rainfed crop during the months of January–April.

Test Organisms

Fusarium moniliforme J. sheld (IMI 333615), *Helminthosporium oryzae* Breda de Haan, *Sarocladium oryzae* W. Gams and D. Hawksw (IMI 336355) were isolated from the infected host tissues of paddy; the identity was confirmed

with International Mycological Institute, London, and used as test organisms in the present study.

Collection of Soil Samples

Soil samples were collected at a depth of 15 cm from the paddy field in the first week of every month and brought to the laboratory. Samples were hand-picked, air-dried, stored and used for further study.

Physico-Chemical Properties of the Soil

Soil moisture, temperature and pH were determined as described by Mishra (1968). Moisture content of the soil was determined by drying the soil sample in an electric oven at 105 °C for 24 h. Soil temperature was measured using soil thermometer. Total organic carbon was estimated by rapid titration methods of Walkley and Black (1934) as described by Piper (1944), and the total organic matter was calculated by multiplying the organic carbon with constant factor 1.7241 as it is presumed that the organic matter of soil contains 58 % carbon (Robinson 1952). The total organic nitrogen was estimated by the micro-Kjeldahl distillation method (Jackson 1958).

Population Dynamics of Soil Fungi in the Paddy Field

Isolation of Fungi

Ten grams of hand-picked and air-dried soil sample was taken into 250-ml conical flask containing 100 ml sterile distilled water. The flask was shaken on an electric shaker to get a homogeneous suspension, and thereafter, 1:100 and 1:1000 dilutions were prepared by transferring serially in sterile distilled water. One millilitre of each dilution was plated in Petri dishes containing Martin's agar (dextrose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4.7H_20$ 0.5 g, rose bengal 0.066 g, agar–agar 18 g and distilled water 1,000 ml; the pH was adjusted to 5.6 before adding rose bengal and agar–agar) and potato dextrose agar (PDA) medium (potato 250 g, dextrose 15 g, agar–agar 18 g and distilled water 1,000 ml; pH 5.6). Streptomycin sulphate (100 mg 1^{-1}) was added to the media to prevent the bacterial growth. The plates were incubated at 25 ± 2 °C for five days, and the fungi appearing on the said agar media were recorded.

$$\text{Population of fungi dry g}^{-1} = \frac{\text{Mean no. of propagles in dilution plate}}{\text{Weight of the dry soil}} \times \text{Dilution factor}$$

$$\text{Percentage frequency} = \frac{\text{No. of soil samples from which fungi were recorded}}{\text{No. of soil samples}} \times 100$$

Identification of fungi was done by using the standard manuals (Gillman 1957; Ellis 1971, 1976; Subramanian 1971).

Studies on the Antibiotic Interactions

Colony Interaction Between Soil Fungi and the Test Pathogens

Colony interaction between the test pathogens and soil fungi was studied in in vitro by dual-culture experiments. The individual test organisms were grown on agar medium individually. Then, the agar blocks (~5 mm diameter) cut from the actively growing margin of the individual species of soil fungi and test organisms were inoculated juxtaposed to each other, approximately 3 cm apart, on PDA medium in Petri plates. Three replicates for each set were maintained. Control was set in single- and dual-inoculated cultures of the fungus. The position of the colony margin on the back of the disc was recorded daily. The percentage inhibition of growth was calculated as follows:

$$\text{Percentage inhibition} = \frac{r - r^2}{r} \times 100$$

r growth of the fungus measured from the centre of the colony towards the centre of the plates in the absence of antagonistic fungus

r^2 growth of the fungus measured from the centre of the colony towards the antagonistic fungus.

Colony interactions between the test pathogens and soil fungi were assessed following the model proposed by Porter (1924) and Dickinson and Boardman (1971). Five types of interaction grades as proposed by Skidmore and Dickinson (1976) have been used as follows:

Grade 1. Mutual intermingling growth without any macroscopic sights of interaction.
Grade 2. Mutual intermingling growth where the growth of the fungus is ceased and is being over grown by the opposed fungus.
Grade 3. Intermingling growth where the fungus under observation is growing into the opposed fungus either above or below.
Grade 4. Slight inhibition of both the interacting fungi with a narrow demarcation line (1–2 mm).
Grade 5. Mutual inhibition of growth at a distance of >2 mm.

Effect of Cell-free Culture Filtrates: Food Poisoning Technique

Cell-free culture filtrates of the individual species of soil fungi were obtained by growing them individually in broths, and the stalling growth products were used after passing through G5 Seitz filter.

The culture filtrates were added separately to the cooled PDA medium to give the concentrations of 5, 10, 15 and 20 %. The amended nutrient agar medium was dispersed in Petri plates and allowed to solidify. After solidification, 5-mm agar blocks cut from the actively growing margin of the test fungi were inoculated at the centre of the plates. The plates were incubated at 25 ± 2 °C for five days. The radial growth was measured periodically, and the mean growth rate was calculated. Control was maintained. The percentage inhibition of growth was calculated as follows:

$$\text{Percentage inhibition of growth} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in Control}} \times 100$$

Saprophytic Survival of Test Organisms

Preparation of Pre-Colonized Substrate Units

Saprophytic survival of the test organisms was studied using pre-colonized substrate units of the paddy straw with individual species of the pathogen. The pre-colonized substrate units were prepared following the method described by Garrett (1956). Effect of moisture, pH and temperature was also studied.

Competitive Saprophytic Colonization

CSC of the test organisms of *F. moniliforme* and *H. oryzae* was studied. Pure sand inoculum of the test pathogens, *F. moniliforme* and *H. oryzae*, was prepared as described by Garrett (1963), and the CSC was studied by Garrett (1975). Effect of moisture, pH and temperature was studied.

Cellulolysis Rate and Weight Losses of the Filter Paper Caused by Test Organisms

Determination of Cellulolysis Rate

The cellulolysis rate of the pathogenic fungi was assessed by determining percentage loss in dry weight of filter paper discs as described by Garrett (1983).

Amendments of Soil with Oil Cakes

Effects of Oil Cakes of Groundnut and Neem on the Saprophytic Survival of the Test Organisms

Hand-picked and air-dried field soil containing organic carbon (0.03 %), organic nitrogen (0.058) and pH (7.1) was used for the amendment. The containers with soil were amended with oil cakes of groundnut and neem separately. Control was maintained without amendment. The percentage survival, suppression and S_{50} values were calculated:

Effect of Oil Cake of Groundnut and Neem on the Competitive Saprophytic Colonization of the Pathogenic Fungi

The inoculum of *F. moniliforme*, *H. oryzae* and *S. oryzae* was prepared as described in Cambridge method; number of polythene bags (3) for each ratio was prepared and amended with oil cakes of groundnut and neem at 0.5, 1.0 and 2.0 % each separately. The moisture was adjusted to 45 % of moisture-holding capacity. Control was maintained without amendment. The percentage colonization of the substrate units by the pathogen and the C_{50} values for each pathogen were calculated.

$$\text{Percentage survival} = \frac{\text{Total no. of substrate units in which the pathogen recorded}}{\text{Total no. of units recorded}} \times 100$$

$$\text{Percentage suppression} = \frac{\text{Total no. of bits recovered} - \text{Total no. of bits in which the pathogen recorded}}{\text{Total no. of units recovered}} \times 100$$

Population Dynamics of Soil Bacteria, Actinomycetes and Fungi

The populations of bacteria, actinomycete and fungi were studied by conventional dilution plate technique in soil under different physico-chemical conditions and amendments. Bacteria were isolated from soil extract agar medium containing agar—15.0 g, KH_2PO_4—0.2 g and soil extract—1,000 ml (James 1958) by plating one millilitre of the aliquot from 10^{-5} dilution. Actinomycetes were isolated from soya bean meal—glucose agar medium containing agar—17.0 g, soya bean meal—5.0 g, glucose—5.0 g, $CaCo_3$—0.4 g and distilled water 1,000 ml (Tsao 1960) by plating one millilitre of the aliquot from 10^{-4} dilution. Fungi were isolated from the soil by the conventional dilution plate technique on PDA agar and Martin's rose bengal agar medium.

Results

Physico-Chemical Properties of the Soil

Soil temperature ranged between 27 and 39 °C. The maximum range of temperature (35–45 °C) was recorded in the months of April to July and the minimum temperature (25–30 °C) in November to January. The minimum moisture content of the soil (14 %) was recorded in the months of May and June. The level of soil pH of soil was narrow which ranged between 7.2 and 7.9. The total organic carbon and organic matter of the soil varied from 0.16 to 0.51 % and 0.28 to 0.88 %, respectively. The maximum organic carbon content (0.51 %) was recorded in the month of February and minimum (0.16 %) in November. The total organic nitrogen content of the soil varied from 0.02 to 0.097 %. Relatively maximum percentage of organic nitrogen content was recorded in the month of December (0.097 %) and minimum in the month of May (0.02 %).

Population Dynamics of Soil Fungi in the Paddy Field

Monthly Variation in Soil Fungi Population

Totally, thirty-nine species of fungi including black and white sterile mycelia were isolated from the paddy field soil. The total number of colonies isolated varied from 25.2 to 55.6 ($\times 10^3$ g^{-1} dry wt. of the soil). Maximum number of colonies were found in the months of July, August, September and November. There was a decline in the number of colonies during the months of January to June (Table 1). The stepwise multiple regression analysis of the physicochemical parameters of soil fungi was showed that the variation in the total organic nitrogen and moisture content of the soil was statistically significant ($P > 0.0005$). Though there were variations in pH organic carbon and soil temperature, they are not statistically significant in the present investigation.

Antibiotic Interactions Studies

Colony Interactions Between Soil Fungi and Test Pathogens

The maximum percentage inhibition of *F. moniliforme* growth was with *A. niger* (Table 2), but in case of *H. oryzae* and *S. oryzae*, it was with *T. viride* (60.7) (Table 3)

Culture Filtrates Effect of Soil Fungi on the Growth of Test Pathogens

The maximum percentage inhibition of *F. moniliforme* growth was on the PDA amended with 20 % of culture filtrate of *Gliocladium* sp. It was comparatively more sensitive to the culture filtrates than to *F. moniliforme*. The percentage inhibition of growth of the pathogen was maximum on the nutrient agar medium added with 20 % of the culture filtrate of *A. candidus* (Table 4).

Physical Factors Effect on the Soil Saprophytic Behaviour of Test Pathogens

Soil Moisture Content Effect on the Saprophytic Survival

The percentage survival of *F. moniliforme* was maximum (80) in the soil with moisture content of 10 % MHC (Table 5) and decreased with the increase in the moisture content. The percentage survival of *H. oryzae* was comparatively more in the soil with moisture content of 10 % MHC (Table 6). The percentage survival of *S. oryzae* was relatively high in the soil with moisture content of 10 % MHC (Table 7).

Moisture Effect on the Competitive Saprophytic Colonization of the Substrate by the Pathogens

The CSC of the substrate by *F. moniliforme*, *H. oryzae* and *S. oryzae* was relatively maximum in the inoculum: soil mixture with moisture content 50 % of MHC (Tables 8, 9 and 10).

Soil Moisture Effect on the Microbial Population

The total number of bacteria colonies increased with the increase in the moisture content and the period of incubation. The total number of actinomycete colonies isolated from the soil with the moisture content of 10, 25, 50 and 75 % MHC was 40.42, 45 and 53 ($\times 10^4$ g^{-1} dry wt. of the soil), respectively, after 4 weeks of incubation. Quantitatively, 19, 22.3, 29.6 and 33.3 ($\times 10^3$ g^{-1} dry wt. of the soil) colonies of fungi were isolated from the soil with moisture content of 10, 25, 50 and 75 % MHC, respectively, after 4 weeks of incubation (Table 11).

Table 1 Monthly variation in the population of soil fungi (number of colonies × 10^3 g^{-1} dry wt. of the soil) in the paddy field (1988–1989)

Name of the fungi	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	% Frequency
1. *Absidia glauca*	–	–	–	–	–	–	–	–	–	2.3	4.7	1.0	25.0
2. *Acrophialophora fusispora*	0.3	2.3	3.0	–	–	–	–	–	2.0	1.7	1.7	–	50.0
3. *Alternaria alternata*	0.7	–	–	–	–	1.7	–	2.0	–	3.0	1.7	–	41.7
4. *Aspergillus candidus*	–	–	–	–	3.3	–	–	–	–	3.0	4.0	–	25.0
5. *A. flavus*	3.0	3.3	4.0	–	0.6	0.3	0.3	0.3	0.7	3.3	4.3	5.0	91.7
6. *A. fumigatus*	1.7	3.0	1.7	–	–	–	–	–	–	–	3.3	4.7	41.7
7. *A. luchuensis*	–	–	–	–	–	2.3	–	4.0	–	1.0	–	2.0	33.3
8. *A. niger*	1.0	4.3	1.0	1.0	1.0	3.0	0.7	0.3	1.0	4.0	2.3	–	91.7
9. *A. nidulans*	–	–	1.7	–	2.0	–	3.0	1.7	2.7	3.0	1.3	2.7	66.7
10. *A. oryzae*	–	–	–	1.3	–	–	1.0	–	–	–	–	–	16.7
11. *A. sulphureus*	–	–	–	–	1.3	–	3.0	–	–	–	2.0	–	25.0
12. *A. sydowii*	2.0	4.3	–	–	–	2.0	–	5.7	–	–	–	2.0	41.7
13. *A. terreus*	9.3	10.3	11.7	9.0	8.3	5.3	4.3	6.3	5.0	6.3	8.0	8.7	100
14. *A. variecolor*	–	–	2.0	–	–	–	–	–	1.7	–	–	–	16.7
15. *A. versicolor*	–	–	–	3.0	–	–	–	–	–	–	–	–	8.3
16. *Cephalosporium* sp.	–	–	–	2.7	2.3	1.0	–	–	–	–	–	–	25.0
17. *Chaetomium* sp.	–	–	–	–	–	–	–	–	–	4.0	–	–	8.3
18. *Cladosporium* sp.	–	–	1.7	2.0	1.7	–	–	–	–	–	–	–	25.0
19. *Cunninghamella* sp.	2.0	5.0	2.0	–	–	3.3	–	–	4.7	3.0	–	–	58.0
20. *Curvularia lunata*	–	2.0	–	–	0.7	–	1.3	1.7	–	1.3	1.7	–	50.0
21. *C. senegalensis*	–	–	–	–	–	–		–	–	2.0	–	1.0	16.7
22. *Drechslera australiensis*	–	2.0	1.3	1.7	–	–	–	–	–	–	–	–	25.0
23. *Fusarium* sp.	3.3	4.3	–	3.7	0.3	0.7	0.3	1.0	2.3	2.0	1.7	3.0	91.7
24. *F. semitectum*	2.3	2.0	0.7	–	–	–	–	–	–	1.7	0.7	1.3	50.0
25. *Gliocladium* sp.	–	–	0.3	0.7	–	–	–	–	–	–	0.7	–	25.0
26. *Humicola* sp.	–	–	–	–	–	–	–	–	–	3.3	–	3.0	16.7
27. *Helminthosporium oryzae*	–	1.3	–	–	–	–	–	–	–	–	–	–	8.3
28. *Memnoniella* sp.	–	–	–	–	2.3	1.0	1.7	–	–	–	–	–	25.0

(continued)

Table 1 (continued)

Name of the fungi	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	% Frequency
29. *Mucor hiemalis*	–	–	–	–	–	2.3	–	–	–	2.0	–	2.3	25.0
30. *Paecilomyces* sp.	–	–	–	–	2.0	–	–	1.3	–	1.7	–	–	25.0
31. *Penicillium* sp.	2.0	3.0	1.7	1.3	–	–	–	–	0.3	1.0	1.7	1.0	66.7
32. *Penicillium citrinum*	2.3	2.0	1.0	0.7	1.0	0.3	–	–	–	–	–	1.7	58.3
33. *P. funiculosum*	0.3	0.7	1.3	–	–	–	–	2.7	–	–	1.7	1.0	50.0
34. *P. purpurogenum*	2.0	–	1.0	–	–	–	1.7	–	–	–	2.0	–	33.3
35. *Rhizopus stolonifer*	1.3	–	2.7	1.0	–	–	2.3	–	1.3	–	2.0	3.0	41.7
36. *Trichoderma viride*	2.7	2.0	1.7	–	–	–	1.3	–	1.3	–	2.0	3.0	58.3
37. *Verticillium* sp.	–	1.3	–	–	–	–	–	1.3	1.0	2.7	3.0	–	41.7
38. Black sterile mycelium	–	–	–	–	–	–	–	–	–	–	4.7	–	8.3
39. White sterile mycelium	2.3	–	–	–	5.0	3.3	4.3	–	–	3.3	–	–	41.7
Total number of colonies	38.5	54.8	40.8	27.8	30.1	26.5	25.2	28.3	27.4	55.6	53.2	47.7	
Total number of species	17	16	18	12	13	13	13	12	12	21	20	17	

Table 2 Colony interaction between *F. moniliforme* and some soil fungi in dual-culture experiments

Growth response of the antagonistic and test fungus	Antagonistic fungi tested											
	AC	Afl	Afu	And	Ang	As	At	Av	Gl	Pc	Pf	T
1. Colony growth of the pathogen towards antagonist (mm)	25	12	9	8	7	16	15	22	10	10	25	8
2. Colony growth of the pathogen away from the antagonist (mm)	30	22	22	26	18	25	23	27	25	25	30	20
3. % growth inhibition of the pathogen in the zone of interaction	16.7	60	70	73.3	76.7	46.7	50.0	26.7	66.7	66.7	16.7	73.3
4. Colony growth of the antagonist in control, that is, growth towards the centre of the plate in the absence of the pathogen (mm)	16.0	28.0	32.0	31.0	33.0	14.0	28.0	24.0	30.0	20.0	15.0	32.0
5. Colony growth of the antagonist towards the pathogen (mm)	10.0	19.0	22.0	24.0	19.0	9.0	11.0	13.0	15.0	9.0	8.0	26.0
6. Colony growth of the antagonist away from the pathogen(mm)	15.0	25.0	30.0	30.0	31.0	12.0	26.0	25.0	28.0	19.0	13.0	26.0
7. % growth inhibition in the zone of interaction	37.5	32.1	31.3	22.6	42.4	35.7	60.7	45.8	55.0	55.0	46.7	18.8

Growth of *F. moniliforme* towards the centre of the plate in the absence of any antagonistic fungus (control) was 28 mm. Measurement was taken on the sixth day
Ac *Aspergillus candidus*, Afl *A. flavus*, Afu *A. fumigates*, And *A. nidulans*, Ang *A. niger*, As *A. sulphureus*, At *A. terreus*, Av *A. variecolor*, Gl *Gliocladium* sp, Pc Penicillium citrinum, Pf *P. funiculosum*, T *Trichoderma viride*

Table 3 Colony interaction between *H. oryzae* and some soil fungi in dual-culture experiments

Growth response of the antagonistic and test fungus	Antagonistic fungi tested											
	AC	Afl	Afu	And	Ang	As	At	Av	Gl	Pc	Pf	T
1. Colony growth of the pathogen towards antagonist (mm)	21	23	25	16	21	20	22	24	12	16	19	11
2. Colony growth of the pathogen away from the antagonist (mm)	25	28	29	25	27	30	26	25	28	26	27	30
3. % growth inhibition of the pathogen in the zone of interaction	25	17.9	10.7	42.9	25	28.6	21.4	14.3	57.1	42.9	32.1	60.7
4. Colony growth of the antagonist in control, that is, growth towards the centre of the plate in the absence of the pathogen (mm)	10	28	32	33	35	14	25	23	30	25	22	43
5. Colony growth of the antagonist towards the pathogen (mm)	7	19	12	16	18	7	13	11	21	15	14	36
6. Colony growth of the antagonist away from the pathogen (mm)	9	26	31	31	33	12	23	21	25	24	20	42
7. % growth inhibition in the zone of interaction	30	32.1	62.5	51.5	48.6	50	48	52.2	30	40	36.4	16.3

Growth of *F. moniliforme* towards the centre of the plate in the absence of any antagonistic fungus (control) was 28 mm. Measurement was taken on the sixth day
Ac *Aspergillus candidus*, Afl *A. flavus*, Afu *A. fumigates*, And *A. nidulans*, Ang *A. niger*, As *A. sulphureus*, At *A. terreus*, Av *A. variecolor*, Gl *Gliocladium* sp, Pc *Penicillium citrinum*, Pf *P. funiculosum*, T *Trichoderma viride*

Table 4 Effect of the culture filtrate of some soil fungi on the growth of *F. moniliforme*, *H. oryzae* and *S. oryzae*

Name of the Culture filtrate	pH	Concentration (%)	*F. moniliforme*		*H. oryzae*		*S. oryzae*	
			Growth rate (mm/day)	% Inhibition	Growth rate (mm/day)	% Inhibition	Growth rate (mm/day)	% Inhibition
Control			10.6 ± 0.163	0	15.6 ± 0.163	0	4.06 ± 0.094	0
1. *Aspergillus candidus*	9.0	5	9.07 ± 0.09	14.46	13.6 ± 0.16	13.92	3.07 ± 0.09	21.73
		10	8.3 ± 0.25	21.38	12.87 ± 0.09	18.98	2.73 ± 0.25	31.01
		15	7.93 ± 0.09	25.16	12.33 ± 0.09	22.15	1.93 ± 0.19	52.46
		20	4. 27 ± 0.09	59.74	13.6 ± 0.28	31.65	1.2 ± 0.16	83.74
2. *A. flavus*	4.0	5	8.67 ± 0.09	18.24	13.07 ± 0.09	13.92	3.07 ± 0.09	26.10
		10	8.07 ± 0.09	23.89	12.02 ± 0.16	22.78	2.73 ± 0.09	32.75
		15	7.67 ± 0.09	27.67	11.66 ± 0.09	25.95	2.13 ± 0.09	47.53
		20	4.93 ± 0.09	53.46	14.87 ± 0.25	69.62	1.8 ± 0.15	54.18
3. *A. fumigates*	8.0	5	9.33 ± 0.19	11.95	15.6 ± 0.16	1.23	2.33 ± 0.09	42.61
		10	7.27 ± 0.09	31.45	14.47 ± 041	8.23	2.73 ± 0.09	32.76
		15	6.8 ± 0.16	35.85	13.4 ± 0.16	15.19	1.9 ± 0.19	77.09
		20	5.93 ± 0.25	44.03	12.0 ± 0.33	24.05	1.7 ± 0.09	82.01
4. *A. nidulans*	4.5	5	9.13 ± 0.09	13.84	12.13 ± 0.09	23.42	3.67 ± 0.09	9.85
		10	8.73 ± 0.09	17.61	11.06 ± 0.09	26.58	3.2 ± 0.16	21.18
		15	8.27 ± 0.25	22.01	10.47 ± 0.09	33.54	2.73 ± 0.09	32.75
		20	8.0 ± 0.16	24.52	10.0 ± 0.16	36.71	2.53 ± 0.09	37.68
5. *A. niger*	4.0	5	7.13 ± 0.09	32.70	14.53 ± 0.41	8.23	3.93 ± 0.09	3.20
		10	6.67 ± 0.09	37.10	13.4 ± 0.16	15.19	3.73 ± 0.09	4.92
		15	6.33 ± 0.09	40.25	12.93 ± 0.09	18.35	3.47 ± 0.09	14.77
		20	6.07 ± 0.09	42.77	12.2 ± 0.28	22.78	3.1 ± 0.1	22.9
6. *A. terreus*	6.0	5	8.13 ± 0.09	23.27	14.27 ± 0.19	9.49	1.73 ± 0.09	57.38
		10	7.73 ± 0.09	23.89	13.7 ± 0.09	13.29	1.47 ± 0.03	64.04
		15	7.27 ± 0.09	31.45	12.27 ± 0.09	22.15	1.13 ± 0.09	72.16
		20	1.53 ± 0.09	85.53	10.2 ± 0.16	35.22	1.07 ± 0.09	73.89

(continued)

Table 4 (continued)

Name of the Culture filtrate	pH	Concentration (%)	*F. moniliforme*		*H. oryzae*		*S. oryzae*	
			Growth rate (mm/day)	% Inhibition	Growth rate (mm/day)	% Inhibition	Growth rate (mm/day)	% Inhibition
7. *A. variecolor*	6.5	5	9.89 ± 0.25	6.91	14.0 ± 0.16	11.39	3.07 ± 0.09	24.63
		10	9.4 ± .16	11.32	12.87 ± 0.09	18.99	3.0 ± 0.28	26.10
		15	9.07 ± 0.09	14.47	11.67 ± 0.25	25.95	2.67 ± 0.09	34.48
		20	8.07 ± 0.09	23.89	09.6 ± 0.16	39.24	2.47 ± 0.09	39.40
8. *Gliocladium* sp.	7.0	5	1.6 ± 0	84.90	2.27 ± 0.09	82. 91	2.0 ± 0	50.73
		10	1.4 ± 0	95.89	2.13 ± 0.09	86.70	1.6 ± 0	60. 59
		15	1.2 ± 0	96.23	1.6 ± 0	89.87	1.2 ± 0	70.44
		20	1.07 ± 0.09	96.86	1.07 ± 0.09	93.67	1.2 ± 0	70 .44
9. *Penicillium citrinum*	7.5	5	6.73 ± 0.25	36.48	11.0 ± 0.16	25.13	4.73 ± 0.09	0
		10	5.13 ± 0.09	51.57	8.13 ± 0.19	48.73	4.23 ± 0.09	10.83
		15	2.27 ± 0.19	77.99	2.27 ± 0.09	85.44	3.67 ± 0.09	24.63
		20	2.07 ± 0.09	80.50	2.07 ± 0.09	87.34	2.93 ± 0.09	43.59
10. *P. funiculosum*	6.5	5	8.8 ± 0.16	16.98	13.67 ± 0.09	13.29	4.6 ± 0.09	17.24
		10	7.33 ± 0.09	31.13	10.0 ± 0.16	36.70	3.8 ± 0.16	19.70
		15	5.27 ± 0.09	50.0	6.73 ± 0.09	57.59	3.0 ± 0.16	26.10
		20	4.2 ± 0.16	60.37	6.07 ± 0.09	61.65	2.07 ± 0.09	49.26
11. *Trichoderma viride*	5.0	5	8.13 ± 0.19	23.58	15.87 ± 0.09	2.08	2.47 ± 0.09	39.40
		10	7.27 ± 0.09	31.13	15.27 ± 0.09	3.16	2.33 ± 0.09	42.61
		15	6.8 ± 0.16	38.67	14.8 ± 0.16	6.32	2.13 ± 0.09	47.53
		20	5.07 ± 0.09	52.83	4.2 ± 0.16	73.42	2.07 ± 0.09	49.26

Table 5 Effect of soil moisture, pH and temperature on the survival of *F. moniliforme* pre-colonized in substrate unit's paddy straw and buried in the soil

Treatments	Samplings			S_{50} value (Weeks)
	I	II	III	
Moisture (% MHC)				
10	80	78	75	>12
25	71	68	62	>12
50	68	63	51	>12
75	66	59	49	11–12
pH				
5	51	38	32	5
6	55	48	35	6–7
7	65	57	49	11–12
8	79	65	63	>12
9	80	69	61	>12
Temperature (°C)				
15 ± 2	59	55	47	9–10
30 ± 2	67	59	51	>12
42 ± 2	63	47	35	7–8

Table 6 Effect of soil moisture, pH and temperature on the survival of *H. oryzae* pre-colonized in substrate unit's paddy straw and buried in the soil

Treatments	Samplings			S_{50} value (Weeks)
	I	II	III	
Moisture (% MHC)				
10	73	69	63	>12
25	70	66	59	>12
50	68	61	57	>12
75	47	44	39	<4
pH				
5	69	64	54	<12
6	68	60	51	<12
7	70	61	48	11–12
8	62	43	38	6–7
9	54	41	32	5–6
Temperature (°C)				
15 ± 2	59	52	39	8–9
30 ± 2	71	63	51	>12
42 ± 2	58	44	31	6

Soil pH Effect on the Saprophytic Survival

The saprophytic survival of *F. moniliforme* was more favoured by alkaline range than by the acidic range of pH of the soil (Table 5). On the contrary, the saprophytic survival of *H. oryzae* was more favoured by acidic to neutral range of pH than by alkaline range (Table 6). The saprophytic survival of *S. oryzae* was more favoured by acidic range than by the alkaline range of pH of the soil (Table 7).

Table 7 Effect of soil moisture, pH and temperature on the survival of *S. oryzae* pre-colonized in substrate unit's paddy straw and buried in the soil

Treatments	Samplings			S_{50} value (Weeks)
	I	II	III	
Moisture (% MHC)				
10	70	66	54	>12
25	68	59	52	>12
50	63	57	51	12
75	35	31	29	<4
pH				
5	63	57	53	>12
6	67	61	57	>12
7	59	55	48	10–11
8	49	33	27	4
9	24	19	15	4
Temperature (°C)				
15 ± 2	68	62	59	>12
30 ± 2	64	61	56	>12
42 ± 2	40	34	28	4

Effect of pH on the CSC of the Substrate

The CSC substrate by the pathogens, *F. moniliforme* and *H. oryzae*, was maximum in the inoculum: soil mixture adjusted to pH 7. The CSC of the substrate by the pathogen was maximum in the inoculum: soil mixture adjusted to pH 5 (Tables 8, 9 and 10).

pH Effect on the Soil Microbial Population

The number of bacterial colonies isolated from the soil pH 5, 6, 7, 8 and 9, was 36, 38, 44, 53 and 54 ($\times 10^5$ g^{-1} dry wt. of the soil), respectively, after 4 weeks of incubation. The total number of actinomycete colonies isolated from the soil with the pH of 5, 6, 7, 8 and 9 was 35, 46, 53 and 59 ($\times 10^4$ g^{-1} dry wt. of the soil), respectively, after 4 weeks of incubation. Quantitatively, 44.6, 35.3, 25.3, 22.3 and 20.9 ($\times 10^3$ g^{-1} dry wt. of the soil) colonies of fungi were isolated from the soil with the pH of 5, 6, 7, 8 and 9, respectively, after 4 weeks of incubation (Table 12).

Soil Temperature

Soil Temperature Effect on the Saprophytic Survival

The saprophytic survival of *F. moniliforme* was maximum in the soil incubated at 30 ± 2 °C. The percentage survival of the pathogen was 59, 67 and 63 in the pre-colonized substrate recovered from the soil incubated at 15 ± 2, 30 ± 2 and 42 ± 2 °C, respectively, after 4 weeks of incubation (Table 5). The saprophytic survival of *H. oryzae* was also maximum in the soil incubated at 30 ± 2 °C (Table 6). But the saprophytic survival of *S. oryza*e was maximum in the soil incubated at 15 ± 2 °C (Table 7).

Temperature Effect on the CSC Substrate

The CSC substrate by *F. moniliforme*, *H. oryzae* and *S. oryzae* was relatively maximum in the inoculum: soil mixture incubated at 25 ± 2 °C (Tables 8, 9 and 10).

Table 8 Effect of soil moisture, pH and temperature on competitive saprophytic colonization of paddy straw substrate by *F. moniliforme* in the soil

Treatments	Inoculum : field soil							C_{50} value
	100:0	98:2	90:10	50:50	10:90	2:98	0:100	
	1.	2.	3.	4.	5.	6.	7.	8.
Moisture (% MHC)								
25	92	88	80	55	21	7	0	56
50	96	92	88	63	28	12	0	64
75	88	78	70	44	16	4	0	40
pH								
4	63	58	48	34	17	2	0	8
5	92	90	87	48	12	6	0	48
7	100	100	95	62	16	9	0	60
9	100	100	92	53	26	11	0	50
Temperature (°C)								
15 ± 2	100	96	92	43	18	5	0	43
25 ± 2	100	99	98	68	43	16	0	77
30 ± 2	100	95	88	56	32	12	0	60
42 ± 2	66	45	14	9	7	2	0	2

Temperature Effect on the Soil Microbial Population

The total number of bacterial colonies was decreased with the increase in the temperature and the period of incubation. The total number of actinomycete colonies isolated from the soil incubated at 25 ± 2, 30 ± 2 and 42 ± 2 °C was 37, 35 and 27 ($\times 10^4$ g^{-1} dry wt. of the soil), respectively, after 4 weeks of incubation. Quantitatively, 28.6, 21.7 and 15.5 ($\times 10^3$ g^{-1} dry wt. of the soil) fungal colonies were isolated from the soil incubated at 15 ± 2, 30 ± 2 and 42 ± 2 °C, respectively, after 4 weeks of incubation (Table 13).

Cellulolysis Rate

Colonization of Unsterilized and Sterilized Filter Paper and Weight Loss Caused by the Pathogens

The mean radial growth of *F. moniliforme*, *H. oryzae* and *S. oryzae* varied over unsterilized and sterilized filter paper discs. The percentage weight loss (WL) of the unsterilized filter paper discs was 2.16, 3.06 and 0.75 and of the sterilized filter paper disc was 2.3, 3.5 and 1.6, caused by the growth of *F. moniliforme*, *H. oryzae* and *S. oryzae*, respectively. The radial growth (diameter) of the three organisms over the sterilized filter paper disc was 44, 44 and 14 mm and 20, 22 and 10 over the unsterilized filter paper, respectively, after 22 days of incubation. The mean loss in dry weight of the filter paper due to the colonization by *F. moniliforme*, *H. oryzae* and *S. oryzae* was 9.2, 14.8, and 6.8 and 9.6, 15 and 6.8 mg over unsterilized and sterilized filter papers, respectively, at 2 ml moisture level (Tables 14, 15).

pH Effect on the Growth Rate of the Pathogens and the Weight Loss Caused by the Pathogens

The maximum growth rate and WL caused by *F. moniliforme*, *H. oryzae* and *S. oryzae* were at pH 8, 7 and 6–7 of the nutrient solution, respectively (Table 16).

Table 9 Effect of moisture, pH and temperature on competitive saprophytic colonization of paddy straw substrate by *H. oryzae* in the soil

Treatment	Inoculum : field soil							C_{50} value
	100:0	98:2	90:10	50:50	10:90	2:98	0:100	
	1.	2.	3.	4.	5.	6.	7.	8.
Moisture (% MHC)								
25	92	84	76	49	20	4	0	48
50	100	96	83	60	31	15	0	63
75	84	76	64	40	16	7	0	32
pH								
4	68	52	49	20	10	2	0	7
5	100	100	92	55	23	11	0	56
7	100	100	96	58	28	14	0	61
9	100	92	81	49	19	8	0	42
Temperature (°C)								
15 ± 2	100	99	87	49	19	9	0	48
25 ± 2	100	100	96	62	29	15	0	64
30 ± 2	100	100	82	60	26	14	0	62
42 ± 2	69	51	39	22	8	2	0	3

Table 10 Effect of moisture, pH and temperature on competitive saprophytic colonization of paddy straw substrate by *S. oryzae* in the soil

Treatment	Inoculum: field soil							C_{50} value
	100:0	98:2	90:10	50:50	10:90	2:98	0:100	
	1.	2.	3.	4.	5.	6.	7.	8.
Moisture (% MHC)								
25	93	84	76	49	15	4	0	47
50	100	92	84	56	25	7	0	57
75	84	77	68	44	13	0	0	38
pH								
4	100	96	84	40	6	1	0	40
5	100	98	96	45	9	2	0	45
7	100	92	76	44	5	2	0	42
9	97	84	72	40	4	0	0	38
Temperature (°C)								
15 ± 2	97	92	83	39	8	1	0	40
25 ± 2	100	98	93	51	15	4	0	52
30 ± 2	100	95	82	44	13	3	0	43
42 ± 2	86	74	64	18	4	0	0	22

Table 11 Effect of moisture on the population dynamics of bacteria

Treatments	Moisture (% MHC)	Samplings		
		I	II	III
10	Bacteria	31.0	33.0	36.0
	Actinomycetes	40.0	42.0	49.0
	Fungi	19.0	23.3	23.9
25	Bacteria	34.0	38.0	41.0
	Actinomycetes	42.0	47.0	53.0
	Fungi	22.3	24.1	29.0
50	Bacteria	37.0	42.0	49.0
	Actinomycetes	45.0	51.0	59.0
	Fungi	29.6	32.9	37.0
75	Bacteria	41.0	47.0	55.0
	Actinomycetes	53.0	57.0	63.0
	Fungi	33.3	37.6	45.7

(number of colonies $\times 10^5$ g^{-1} dry wt. of the soil), actinomycetes (number of colonies $\times 10^4$ g^{-1} dry wt. of the soil) and fungi (number of colonies $\times 10^3$ g^{-1} dry wt. of the soil) in the soil

Table 12 Effect of pH on the population dynamics of bacteria

Treatments		Samplings		
		I	II	III
pH 5	Bacteria	26	29	36
	Actinomycetes	35	44	36
	Fungi	44.6	42.4	61.2
pH 6	Bacteria	38	47	52
	Actinomycetes	46	52	55
	Fungi	35.3	39.6	61.9
pH 7	Bacteria	44	56	62
	Actinomycetes	53	55	63
	Fungi	25.3	27.3	43.6
pH 8	Bacteria	53	56	65
	Actinomycetes	51	59	66
	Fungi	22.3	22.7	27.9
pH 9	Bacteria	54	56	61
	Actinomycetes	59	63	66
	Fungi	20.9	21.6	26.3

(number of colonies $\times 10^5$ g^{-1} dry wt. of the soil), actinomycetes (number of colonies $\times 10^4$ g^{-1} dry wt. of the soil) and fungi (number of colonies $\times 10^3$ g^{-1} dry wt. of the soil) in the soil

Nitrogen Effect on the Colonization of Sterilized Filter Paper by the Test Pathogens

The mean radial growth and the WL caused due to colonization by *F. moniliforme*, *H. oryzae* and *S. oryzae* progressively increased with the increase in the nitrogen ($NaNO_3$) content of the nutrient solution (Table 17). C_{50} values of *F. moniliforme*, *H. oryzae* and *S. oryzae* were statistically analysed in relation to percentage loss in dry weight of the filter paper, growth rate

Table 13 Effect of temperature on the population dynamics of bacteria

Treatments		Samplings		
		I	II	III
15 ± 2 °C	Bacteria	57.0	69.0	73.0
	Actinomycetes	37.0	47.0	40.0
	Fungi	28.6	38.3	47.1
30 ± 2 °C	Bacteria	44.0	50.0	58.0
	Actinomycetes	35.0	39.0	44.0
	Fungi	21.7	34.0	40.2
42 ± 2 °C	Bacteria	30.0	38.0	42.0
	Actinomycetes	27.0	30.0	32.0
	Fungi	15.5	22.1	24.4

(number of colonies × 10^5 g^{-1} dry wt. of the soil), actinomycetes (number of colonies × 10^4 g^{-1} dry wt. of the soil) and fungi (number of colonies × 10^3 g^{-1} dry wt. of the soil) in the soil

Table 14 Colonization of unsterilized filter paper by the test pathogens and the weight losses caused by them

Name of the pathogens	Nutrient solution (ml)	Mean radial growth in diameter after			Mean loss in dry wt. by single paper (mg)	Radial growth rate over PD agar mm 24 h^{-1} (diameter)	% loss in dry wt. of the filter paper (mg)	CAI
		7 days	15 days	22 days				
F. moniliforme	2.0	12	16	20	9.2	11.0	2.16	0.20
H. oryzae	2.0	14	20	22	14.8	13.0	3.06	0.24
S. oryzae	2.0	6	8	10	6.8	3.2	0.75	0.23

CAI cellulolysis adequacy index

Table 15 Colonization of sterilized filter paper by the test pathogens and the weight losses caused by them

Name of the pathogens	Nutrient solution (ml)	Mean radial growth mm after (diameter in mm)			Mean loss in dry wt. by single paper (mg)	Radial growth rate over PDA agar mm 24 h^{-1} (diameter)	% loss in dry wt. of the filter paper (mg)	CAI
		7 days	15 days	22 days				
F. moniliforme	2.0	14	30	44	9.6	11.0	2.3	0.21
	3.0	14	32	48	10.0	–	–	–
H. oryzae	2.0	15	27	44	15.0	13.0	3.5	0.27
	3.0	14	26	46	15.1	–	–	–
S. oryzae	2.0	7	12	14	6.8	3.2	1.6	0.5
	3.0	7	12	15	7.0	–	–	–

CAI cellulolysis adequacy index

Table 16 Effect of pH of the nutrient solution on the colonization of sterilized filter paper by the test pathogens and the weight losses caused by them

Name of the pathogens	pH	Nutrient solution (ml)	Mean radial growth mm after (diameter in mm)			Mean loss in dry wt. by single paper (mg)	Radial growth rate over PDA agar (mm) 24 h^{-1} (diameter)	% loss in wt. of the filter paper (mg)
			7 days	15 days	22 days			
F. moniliforme	5	2	14	17	24	10.8	12.0	2.21
	6	2	16	20	25	12.8	12.3	2.45
		2	14	18	22	10.4	11.0	2.54
	8	2	11	15	19	9.4	12.0	3.01
H. oryzae	5	2	14	16	20	9.4	13.0	2.21
	6	2	17	19	21	10.4	13.3	2.45
	7	2	20	23	27	14.8	13.6	3.48
	8	2	16	19	24	10.8	13.0	2.51
S. oryzae	5	2	6	8	12	5.6	3.2	1.32
	6	2	7	8	14	6.8	3.5	1.6
	7	2	6	9	14	6.8	3.2	1.6
	8	2	5	7	9	5.2	3.0	1.22

Table 17 Effect of nitrogen content of the nutrient solution on the colonization of sterilized filter paper by the test pathogens and the weight losses caused by them

Name of the pathogens	Nitrogen $NaNo_3$	Nutrient solution (ml)	Mean radial growth mm after (diameter in mm)			Mean loss in dry wt. by sterilized filter paper (mg)	Radial growth rate over PDA agar mm 24 h	% loss in dry wt. of the filter paper
			7 days	15 days	22 days			
F. moniliforme	S/4	2	6	19	32	16.2	10.0	3.27
	S/2	2	12	23	37	18.8	10.2	3.79
	S/3/4	2	16	27	41	19.8	10.4	4.0
	S	2	17	29	43	21.2	10.5	4.28
	$S_{1.5}$	2	19	34	46	21.6	11.0	4.36
	S_2	2	22	37	48	23.2	11.2	4.48
H. oryzae	S/4	2	6	17	31	16.6	13.0	3.35
	S/2	2	9	21	37	18.8	13.4	3.79
	S/3/4	2	13	25	41	20.2	13.6	4.08
	S	2	15	27	45	20.8	14.0	4.20
	$S_{1.5}$	2	18	32	47	21.8	14.2	4.40
	S_2	2	19	35	50	22.8	14.6	4.60
S. oryzae	S/4	2	6	7	8	4.4	3.0	0.89
	S/2	2	5	8	9	5.4	3.2	1.09
	S/3/4	2	7	9	13	6.8	3.4	1.37
	S	2	7	11	15	7.2	3.6	1.45
	$S_{1.5}$	2	9	13	17	7.6	3.8	1.53
	S_2	2	13	17	20	8.2	3.8	1.65

Table 18 Comparison of saprophytic behaviour and cellulolysis rate of the test pathogens

Name of the organisms	Average S_{50} value (weeks)	S_{50} value	CAI	% loss in dry wt. of the filter paper
F. moniliforme	12	62	0.21	2.3
H. oryzae	12	64	0.27	3.5
S. oryzae	12	50	0.5	1.6

Table 19 Percentage survival of *F. moniliforme* in pre-colonized paddy straw substrate units buried in the soil amended with oil cakes of groundnut and neem

Treatments	Samplings			S_{50} value (weeks)
	I	II	III	
Control	85	80	76	>12
Groundnut (%)				
0.5	75	48	35	7–8
1.0	64	45	30	7
2.0	55	25	8	4–5
3.0	34	10	5	<4
Neem (%)				
0.5	40	35	28	<4
1.0	32	25	16	<4
2.0	25	18	12	<4
3.0	14	10	5	<4

over filter paper and loss in dry weight multiplied by growth rate over PDA agar. Positive correlation with all the five parameters was obtained. All are significant at 1 % level (Table 18).

Organic Amendments of the Soil with Oil Cakes

Groundnut and Neem Oil Cakes on the Saprophytic Survival of Test Pathogens

The survival of *F. moniliforme*, *H. oryzae* and *S. oryzae* was suppressed, and the S_{50} value was very much reduced by the amendment of the soil with oil cakes of groundnut and neem. The oil cake of neem was more effective than groundnut (Tables 19, 20 and 21).

Groundnut and Neem Oil Cakes Effect on the CSC Substrate by Test Pathogens

The CSC substrates by *F. moniliforme*, *H. oryzae* and *S. oryzae* decreased in the inoculums: soil mixture amended with the oil cakes of groundnut and neem. The oil cake of neem was more effective than groundnut in all cases (Tables 22, 23 and 24).

Groundnut and Neem Oil Cakes Effect of on the Soil Microbial Population

The population of bacteria, actinomycetes and fungi increased in the soil amended with oil cake of groundnut, but in the soil amended with oil cake of neem, the number of colonies of fungi isolated decreased, when compared with control.

Table 20 Percentage survival of *H. oryzae* in pre-colonized paddy straw substrate units buried in the soil amended with oil cakes of groundnut and neem

Treatments	Samplings			S_{50} value (weeks)
	I	II	III	
Control	75	68	61	>12
Groundnut (%)				
0.5	70	68	66	9–10
1.0	54	46	13	6–7
2.0	48	43	8	<4
3.0	45	32	5	<4
Neem (%)				
0.5	48	19	7	<4
1.0	44	15	0	<4
2.0	13	8	0	<4
3.0	5	0	0	<4

Table 21 Percentage survival of *S. oryzae* in pre-colonized paddy straw substrate units buried in the soil amended with oil cakes of groundnut and neem

Treatments	Samplings			S_{50} value (weeks)
	I	II	III	
Control	65	61	57	12
Groundnut (%)				
0.5	58	45	19	6
1.0	50	27	17	4
2.0	44	16	4	<4
3.0	24	8	0	<4
Neem (%)				
0.5	34	27	18	<4
1.0	26	14	6	<4
2.0	14	3	0	<4
3.0	7	0	0	<4

Table 22 Effect of the amendments of oil cakes on the competitive saprophytic colonization of the substrates by *F. moniliforme*

Treatment	Inoculum: field soil							C_{50} value
	100:0	98:2	90:10	50:50	10:90	2:98	0:100	
Control	100	95	88	56	36	12	0	62
Groundnut (%)								
0.5	55	46	41	25	13	8	0	2
1.0	48	44	32	21	9	5	0	0
2.0	29	22	13	17	8	0	0	0
Neem (%)								
0.5	46	39	35	21	3	2	0	0
1.0	27	20	20	19	9	0	0	0
2.0	15	9	6	5	0	0	0	0

Table 23 Effect of the amendments of oil cakes on the competitive saprophytic colonization of the substrates by *H. oryzae*

Treatment	Inoculum: field soil							C_{50} value
	100:0	98:2	90:10	50:50	10:90	2:98	0:100	
Control	100	100	84	61	29	14	0	64
Groundnut (%)								
0.5	61	54	43	26	9	4	0	5
1.0	49	42	32	21	5	4	0	0
2.0	26	22	21	19	0	0	0	0
Neem (%)								
0.5	49	46	33	14	6	4	0	0
1.0	32	25	18	9	5	0	0	0
2.0	17	6	4	0	0	0	0	0

Table 24 Effect of the amendments of oil cakes on the competitive saprophytic colonization of the substrates by *S. oryzae*

Treatment	Inoculum: field soil							C_{50} value
	100:0	98:2	90:10	50:50	10:90	2:98	0:100	
Control	100	95	87	51	15	3	0	50
Groundnut (%)								
0.5	55	47	39	27	11	0	0	4
1.0	44	39	27	19	8	0	0	0
2.0	31	25	17	9	4	0	0	0
Neem (%)								
0.5	43	39	31	19	7	0	0	0
1.0	27	23	11	7	0	0	0	0
2.0	11	7	3	0	0	0	0	0

Groundnut Oil Cake

The percentage colonization of paddy straw substrates by indigenous species of *Fusarium* increased in the soil amended with oil cake of groundnut after 12 weeks of incubation.

Neem Oil Cake

The number of colonies of *Fusarium* decreased with the increase in the percentage of oil cake amendment. On the other hand, the number of *Aspergillus* sp., *Cunninghamella* sp., *Gliocladium* sp. and *Trichoderma* sp. colonies increased.

Discussion

Soil is the most complex and heterogeneous environment, which is inhabited by diverse array of microorganisms including soil algae, fungi, bacteria, protozoa and also by micro- and macrofauna. Among them, fungi play a vital role in the soil as potential pathogens of plants, decomposer of organic matter, potential antagonists and allergens. They generally show variation, both quantitatively and qualitatively, in their pattern of distribution, which is often influenced by the physico-chemical parameters of the soil, by prevailing environmental

condition, by cropping pattern, by vegetation cover and also by certain unknown factors.

Agricultural lands are often exposed to intensive shifting cropping pattern, irrigation and application of chemical fertilizers and pesticides, which influence the soil microbial population either directly or indirectly and also beneficially or adversely (Katan and Lockwood 1970).

In the present study, the population dynamics of soil fungi in paddy field was studied both quantitatively and qualitatively. Thirty-nine species of fungi were isolated during the course of investigation, of which four belonged to phycomycetes, one ascomycete and the remaining belonged to deuteromycetes. Quantitatively, maximum number of colonies were isolated during the months of July, August, September and November. The species of *Aspergillus* were isolated most frequently. They include *A. candidus*, *A. flavus*, *A. fumigates*, *A. luchuensis*, *A. niger*, *A. nidulans*, *A. oryzae*, *A. sulphureus*, *A. sydowii*, *A. terreus*, *A. variecolor* and *A. versicolor*.

It has been reported that peaks in the density of fungal population occurred during the rainy season when the soil moisture were significantly high (Deka and Mishra 1984; Kamal and Bhargawa 1973; Saksena et al. 1967; Warcup 1957).The environmental factors such as pH, temperature and moisture content of the soil have been reported as the important factors affecting the microflora over 21 arctic and alpine tundra sites (Bissett and Parkinson 1979; Dowding and Widden 1974).

The range of variation in pH of the soil was also narrow, and in general, it does not allow wide fluctuation in the population of fungi. The stepwise multiple regression analysis of soil space, fungal population versus soil temperature, moisture, pH and organic nitrogen has been shown that the soil moisture and the total organic nitrogen were statistically significant. The effect of temperature and moisture content of the soil cannot be separated, since they are complementary with each other; so the important factors influencing the variation in the population of fungi in the present study could possibly be due to temperature, organic nitrogen and moisture content of the soil. As it was stated earlier, the paddy field soil was subjected to disturbances such as agricultural practices of irrigation and fertilization, which resulted in more homogeneity of soil which did not allow relatively wide fluctuation in the population of fungi.

The antibiotic interactions of some soil fungi namely *Aspergillus candidus*, *A. flavus*, *A. fumigates*, *A. nidulans*, *A. niger*, *A. terreus*, *A. variecolor*, *Gliocladium sp*. *Penicillium citrinum*, *P. funiculosum* and *Trichoderma viride* with *F. moniliforme*, *H. oryzae* and *S. oryzae* were studied individually. The test organism varied in their sensitivity to the stalling growth product of antagonistic fungi. The order of sequence of the inhibitory effect of the culture filtrates on the growth of *F. moniliforme* was *Gliocladium sp*, *A. terreus*, *Penicillium citrinum*, *P. funiculosum*, *A. candidus*, *A. flavus*, *T. viride, A. fumigatus*, *A. niger*, *A. nidulans*, *A. variecolor*; while on the growth of *H. oryzae* was *Gliocladium sp*, *P. citrinum*, *T. viride*, *A. flavus*, *P. funiculosum*, *A. variecolor A. nidulans*, *A. terreus*, *A. candidus*, *A. fumigatus*, *A. niger*; while on the growth of *S. oryzae* was *A. candidus, A. fumigatus*, *A. terreus*, *Gliocladium sp*, *A. flavus*, *T. viride*, *P. funiculosum*, *P. citrinum*, *A. variecolor*, *A. nidulans*, *A. niger*.

The inhibition of stalling growth products has been attributed to change in the nutrient medium from acidity to alkalinity, and volatile and non-volatile substances secreted by the organism (Robinson 1969). The inhibition of pathogen growth on the stalled nutrient agar may be argued as it was due to the exhaustion of nutrients and the possible change in the pH of the medium by the activity of the antagonistic fungi. But the inhibition of growth of *F. moniliforme*, *H. oryzae* and *S. oryzae* on nutrient rich medium (PDA) amended with culture filtrates disowns the argument and provided a clear evidence that the inhibition of growth was not due to lack of nutrients. Moreover, pH of the most culture filtrates except *A. candidus*, *A. fumigatus* and *P. citrinum* tested was in acidic to neutral range. So, the inhibitory effect of stalling growth products is due to the presence of volatile and

non-volatile substances rather than change in pH of the media and lack of nutrients. In spite of the fact that there are mounting evidences for the in vitro production of antibiotics, most of the attempts on the isolation and characterization of such substances from natural soil proved useless. Nevertheless, general consensus of opinion agrees that antibiotics are produced in soil and furnish protection in competitive situations by eliminating other organisms in soil (Gottlieb 1976; Stallings 1954).

Many microbial ecologists also believe that antibiotics are active in nature (Fravel 1988). Some of the strongest evidences upon which this conclusion is based include the following as summarized by Brock (1966): (a) antibiotics can be extracted from non-sterile soil; (b) antibiotic production will occur in non-sterile soil inoculated with antibiotic-producing organism and supplemented with organic enrichments such as straw; (c) antibiotic-producing microbes can be isolated easily from natural soils; (d) antibiotic-producing organisms are able to be under conditions to antagonize other organisms sensitive to antibiotics; and (e) conditions unfavourable for the accumulation of antibiotics reduce the antagonistic activity of the organism producing the antibiotics.

The saprophytic survival, CSC and suppression of the test pathogens were studied in relation to soil moisture, pH and temperature. The survival of the pathogens in the pre-colonized substrates buried in the soil prolonged in the presence of very low moisture contents (10 % MHC) and decreased with the increase in the moisture content. It was comparatively very low in the soil with 75 % MHC. The S_{50} value of *F. moniliforme* was relatively greater (11–12 weeks) than *H. oryzae* (<4 weeks) and *S. oryzae* (<4 weeks) in the soil with 75 % MHC.

Papavizas and Davey (1961) found that the saprophytism of *Rhizoctonia* was significantly higher when moisture was maintained at 20–60 % MHC. There was appreciable reduction in the colonization at 70 and 80 % MHC. It has also been reported that the growth of pathogens was best in soils with relatively low moisture content in the range of 33 to 60 % MHC, and growth of the pathogens in wet soil was greatly restricted or even suppressed completely; the reduction in growth of high soil moisture was attributed by Blair (1943) to a decline in soil aeration with an increase in the moisture content.

The response of *F. moniliforme*, *H. oryzae* and *S. oryzae* varied depending on soil reaction (pH). Relatively, *F. moniliforme* survived for a long period in the alkaline range of pH (8–9 and S_{50} value = >12 weeks), whereas *H. oryzae* and *S. oryzae* survived better in the acidic to neutral pH than in alkaline range.

Soil does not permit wide ranges of fluctuation as it is better buffered than the subaerial habitat (Garrett 1970). However, the fungi have limits of tolerance to the various factors, and it varies depending upon the species. It was found that the maximum percentage survival of *F. moniliforme* in the pre-colonized substrates buried in the soil was at pH 8–9, whereas *H. oryzae* and *S. oryzae* survived better in the soil at the pH range of 5 to 7. Papavizas and Davey (1961) emphasized that the competitive saprophytic activity of the pathogen is influenced by soil reactions. In the present study, also it was found that C_{50} values of *F. moniliforme* and *H. oryzae* were maximum at pH 7 and that of *S. oryzae* was also maximum at pH 5. Papavizas and Davey (1961) found that the saprophytic growth of *R. solani* was good at the reaction pH range of 4.5 to 8.1. Colonization declined to 10 % at pH value approximately 4. Optimum saprophytism occurred at neutral to slightly alkaline reaction. Similar observation was also reported with *F. udum* by Upadhyay and Rai (1983a, b). It is difficult to distinguish between direct effects of pH on growth and saprophytic colonization of pathogen in soil and the indirect effects mediated by the changed physicochemical and biological environment as a result of pH changes in soil (Papavizas 1970). However, the stimulation of the population of bacteria and actinomycetes in the soils adjusted to alkaline range of pH was recorded in the present study.

The maximum percentage survival of *F. moniliforme* and *H. oryzae* in the pre-colonized straw bits buried in the soil was at 30 ± 2 °C (S_{50} value = 12 weeks), whereas S. *oryzae* at 15 ± 2 °C. The survival of the organisms decreased with the increase in the temperature. S_{50} value of *F. moniliforme*, *H. oryzae* and *S. oryzae* decreased to 7–8, 6 and 4 weeks, respectively, at 42 ± 2 °C.

The temperature as a factor influences the saprophytic survival and colonization substrate by the pathogens. Burges and Griffin (1967) reported that the percentage of straw saprophytically colonized by *Fusarium roseum*, *Gibberella zeae*, *Cochliobolus* and *C. spicifer* generally appeared to increase with the reduction in the incubation temperature from 30 to 20 down to 10 °C. A similar effect of temperature was also reported by Gerlagh (1968). In the present investigation, also it was found that the percentage survival of *S. oryzae* increased with a reduction in the incubation temperature from 42 to 15 °C, whereas the maximum percentage survival of *F. moniliforme* and *H. oryzae* was at 30 °C. However, percentage survival was greater at 15 °C than at 42 °C.

The population of bacteria, actinomycetes and fungi quantitatively increased with the increase in the moisture content of the soil. However, the population of bacteria and actinomycetes increased but fungi decreased in alkaline range of pH. The CSC of paddy straw substrates by *F. moniliforme*, *H. oryzae* and *S. oryzae* was favoured by 50 % MHC. The alkaline range of pH was favourable for the colonization of the substrates by at pH 5–7. The percentage colonization of the substrates for the pathogens was maximum at 25 ± 2 °C. The S_{50} values of *F. moniliforme*, *H. oryzae* and *S. oryzae* were 77, 64 and 52, respectively.

The CSC of the substrates by the pathogens was also found maximum at 25–30 °C. However, 25–2 °C was found optimum for the colonization. Papavizas and Davey (1961) reported that the optimum soil temperature for saprophytic activity varied from one soil to another. *Rhizoctonia solani* showed greater saprophytic activity in greenhouse loamy sand at 20 °C than at other temperature tested and decreased at 30 °C, whereas its saprophytic activity was more at 26–30 °C in fine sand. Deacon (1973) found that a reduction in the incubation period at 21 °C from 28 to 9 days produced increase in the apparent percentage of straws colonized by *Gaeumannomyces graminis* and *Cercosporella herpotrichoides*. This increase was an order comparable with that produced by keeping the incubation period at 28 days but lowering the incubation temperature to 10 °C.

The cellulolysis rate of *F. moniliforme*, *H. oryzae* and *S. oryzae* was assessed in terms of percentage loss in dry weight of the filter paper. The cellulolysis rate of *H. oryzae* was comparatively greater than *F. moniliforme* and *S. oryzae*. The percentage loss in dry weight of the filter paper and growth of the pathogens was also influenced by the pH and the nitrogen content in the nutrient solution. Relatively, maximum percentage loss in dry weight of the filter paper due to colonization by *F. moniliforme* and *H. oryzae* was obtained at pH 8, 5–6, 6–7 of *H. oryzae* and *S. oryzae* was obtained at pH 8, 5–6, 6–7 of the nutrient solution, respectively. The percentage loss in dry weight was also increased with the increase in the nitrogen content of the nutrient solution.

Garrett (1978) suggested that the intrinsic growth rate has no apparent effect of cellulolysis rate. He also pointed out that the radial growth of the fungal colony across the paper circle need not be directly correlated with WL of the paper because WL must vary with area of the fungal colony and not with its radius. So, if WL varies with r^2, then r must vary with $\sqrt{WL}$. Thus, in a correlation between $\sqrt{WL}$ and growth rate of *Cochliobolus sativus* over filter paper circle, the value of correlation coefficient was 0.9192 (Garrett 1984). Further, he suggested that the growth rate over filter paper is controlled by cellulolysis rate and intrinsic radial growth rate.

Soil amendments with oil cakes of groundnut and neem suppressed *F. moniliforme*, *H. oryzae* and *S. oryzae* in the pre-colonized straw substrates buried in the soil. The oil cake

of neem was more effective than groundnut. S_{50} value declined to <4 weeks even in the presence of relatively low concentration of neem cake (0.5 %). *S. oryzae* was more sensitive to the amendment than the other test pathogens. Though the oil cake of groundnut suppressed *F. moniliforme*, other species of *Fusarium* (Chlamydospore producing species) in the soil increased. The percentage colonization of sterilized straw substrates buried in the soil by indigenous fusaria rapidly increased. The populations of antagonistic fungi like *Gliocladium* sp and *Trichoderma* sp. were not only increased in the soil treated with oil cake, but also competitively colonized the straw bits buried in the soil.

The oil cakes obtained after extraction of oil from several oil seeds are used as organic manures in traditional agriculture of Tamil Nadu. Amendment of soil with oil cakes may influence the growth and survival of microorganisms in several ways. It may be due to suppression of pathogens either through the toxic principle of decomposition products or through antagonism. It was found that the percentage survival of *F. moniliforme*, *H. oryzae* and *S. oryzae* was very much declined in the soil amended with oil cakes of groundnut and neem. S_{50} values were also declined to less than 4 weeks. Among the oil cakes of groundnut and neem, the oil cake of neem was more effective than groundnut. The CSC activity of the pathogens very much declined in soil amended with oil cakes. The reduction in population of parasitic fungi in the rhizosphere of plants and in the soil amended with the oil cakes has been reported (Khan et al. 1973; Singh and Singh 1970; Sing and Pandey 1965).

Conclusion

The present investigation dealt with the population dynamics of soil fungi in paddy field; antibiotic ability of some soil fungi against test pathogens, *F. moniliforme*, *H. oryzae* and their tolerance to antibiotics; saprophytic survival and suppression of the pathogen in pre-colonized paddy straw substrates and CSC under the influence of moisture, pH and temperature of the soil; the cellulolysis rate of the test organisms; and the effect of oil cakes of groundnut and neem on the saprophytic survival of the pathogens and on the population dynamics of indigenous micro flora of the soil.

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