Characterization and Diversity Analysis of Bacterial Isolates from Rhizosphere of Sewan (*Lasiurus scindicus*) and Dhaman (*Cenchrus setigerus*) Grasses

सेवण (लासियुरस सिंडिकस) और धामण (सेनक्रस सेटिगेरस) घासों के जड़ क्षेत्र से जीवाणु विलगों का चरित्र चित्रण और विविधता विश्लेषण

ASHA KUMARI

THESIS

Master of Science in Agriculture

(Biotechnology)

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DEPARTMENT OF BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
SWAMI KESHWANAND RAJASTHAN
AGRICULTURAL UNIVERSITY (SKRAU), BIKANER

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In partial fulfilment of the requirement for

The degree of

Master of Science in Agriculture

(Biotechnology)

By

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2022

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Place: Bikaner ***(Asha Kumari)***

Date:

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ABBREVIATIONS

TERMS	ABBREVIATIONS
%	Percent
+ve	Positive
◦c	Degree centigrade
µ	Micro
µg	Microgram
µl	Microlitre
A	Absorbance
bp	Base pair
CAT1	Catalase 1
CD	Critical difference
cfu	Colony forming unit
cm	centimeter
ddH_2O	Double distilled water
DHA	Dehydrogenage activity
DMSO	Dimethyl sulphoxide
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediamine tetraacetate di sodium salt
FDA	Fluorescence diacetate assay
g	Gram
ha	Hectare
HCN	Hydrogen cyanide
IAA	Indole acetic acid
iMEC	Marker efficiency calculater
ITS	Internal transcribed spacer
kg	Kilo gram
M	Molar
MBC	Microbial biomas carban
ml	Millilitre
mM	Millimolar
mm	Millimolar

TERMS	ABBREVIATIONS
N	Nitrogen
NA	Nutrient agar
NB	Nutrient broth
nm	nanometer
OC	Organic carban
PCR	Polymerase chain reaction
PGPR	Plant Growth Promoting Rhizobacteria
rpm	Rotations per minute
SCoT	Start codon targeted sequence
SEm	Standard error of the mean
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TE	Tris (10mM) EDTA (1mM)
UV	Ultra Voilet
V	Volts
-ve	Negative
W/V	Weight/Volume
Wt	Weight

1. INTRODUCTION

Soil is an outstanding medium for support of diverse microorganisms by providing nutrition and habitat. There are two types of organisms living in the soil namely soil flora and soil fauna. Hence soil is an effervescent medium for various life forms rather than merely being an inert static material. Soil is now considered to be active or dynamic system (Balasubramanian, 2017). Nutrient requirement in agriculture has been rising and is likely to increase further to enhance the agriculture productivity across the globe in order to keep pace with growing food demand. Soil microorganisms play an important role in nutrient acquisition for plants and with the increasing emphasis on environment friendly, low input agricultural practices there is increasing interest in the management of soil microbial communities to boost plant growth (Jacoby *et al.,* 2017).

Microbial diversity in the soil is a crucial environmental issue that concerns people from all fields of the society. Interest in microbial diversity has developed promptly in the scientific community (Wilson 1988; Franklin 1993 and Benizri *et al.,* 2002). Rising attention is being drawn to microorganisms due to the attribute that the fertility of soil depends on its chemical composition and on the qualitative and quantitative nature of microorganisms inhabiting it. Maintenance of live, population diversity and functional microbial communities in the soil is need for sustainable agriculture (Beare *et al*.,1995 and Benizri *et al*., 2002). Soil contains a wide range of microorganisms described as a ‘black box’ (Paul and Clark 1989).

Almost 30% of their fixed carbon by terrestrial plants is come out in the form of root exudates, consequently changing the content of surrounding soil (Beauregard, 2015). The root exudates composition reflects the contradictory-concomitantly attractive and repulsive behavior of the plant relation with microorganisms. Plants produce anti-

microbial, insecticidal and nematicidal compounds to resist pathogens and pests. They also produce edging cells that detach from roots and play an important role as biological and physical barrier against aggressors. Plants also produce metabolites used as source of carbon resulting in the attraction of plant beneficial soil microorganism that help plants in controlling diseases directly via the production of antimicrobial compounds or indirectly via the induction of plant systemic resistance (El Zahar Haichar *et al.,* 2014).

Microbial communities are groups of microorganisms that distributed and wide spread nearly in all habitats. The microbial populations that form the community can interact in different ways; play a crucial role in the productivity of plants by influencing their physiology and development. The determination of the activities of microbial communities in soil is important for an efficient acquisition of nutrient and mineral transformations. The determination of microbial communities C, N, P and S contents by fumigation techniques has permitted in a better quantification of nutrient activity in soil (Nannipieri *et al.,* 2003).

While many members of the rhizospheric soil microbiome are beneficial to plant growth but plant pathogenic microorganisms like bacteria, fungi, actinomycetes etc. colonize the rhizosphere bearing to break through the protective microbial shield and to mitigate the natural plant defense mechanisms to cause disease. A third group of microorganisms that can be found in the rhizosphere are named as neutral (Mendes *et al*., 2013). The relationships between microorganisms and plant roots can be classified into three categories: symbiotism, parasitism, and commensalism. These relationships are variable, depending upon environmental and physiological conditions. Soil microorganisms have been considered capable of affecting the growth of higher plank in numerous ways. (Clark *et al*., 1949).

The term ‘‘rhizosphere’’ (Greek rhiza = root, and sphere = field of influence) was first defined by Hiltner, 1904 as ‘‘the zone of soil immediately adjacent to plant roots that supports high levels of bacterial activity’’ in terms of higher microbial activity. Rhizosphere supports large and active microbial population capable of exerting beneficial, neutral and detrimental effects on the plants. Although the microbiome of the phyllosphere impact plant health and, often, food production, only a limited number of studies have been aimed at discovering these particular communities (Glick, *et al.,* 2021). The importance of the rhizosphere microbiome for plant growth has been widely recognized, for the vast majority of rhizosphere microorganisms the knowledge is scanty. Most members of the rhizosphere microbiome are part of a complex food web that utilizes the large amount of nutrients released by the plant. Given that these rhizodeposits (e.g. exudates, border cells, mucilage) are a major driving force in the regulation of microbial diversity and activity on plant roots, (Cook *et al*., 1995) postulated that plants may modulate the rhizosphere microbiome to their benefit by selectively stimulating microorganisms with traits that are beneficial to plant growth and health. Rhizosphere is a vital region of plant ecosystem; 2 mm from the root surface is termed as rhizosphere zone. It governs the chemistry of plant nutrient and affects growth of the plants. The rhizosphere microbiome usually refers to bacterial, archaeal and fungal community as well as their metagenomes of this microbiome has been referred to as the second genome of the plant (Berendsen *et al*., 2012). Culture-dependent approaches have shown that microbial diversity of soil and rhizosphere microbiomes is highly underestimated. Recent advances in next-generation sequencing and bioinformatics allow the unraveling of taxonomic composition and functions of complex communities in a wide range of habitats and environmental conditions (Hiraoka *et al.,* 2016). Rhizodeposits, root exudates, and root border cells are vital components of the rhizosphere that significantly affect root colonization

capacity and multiplication of rhizosphere microbes, as well as secretion of organic bioactive compounds (Hassan *et al*., 2019).

Plant growth promoting rhizobacteria (Kloepper and Schrot, 1978) have emerged as the best alternative of hazardous chemical fertilizers for sustainable and eco-friendly agriculture, because they are diazotrophs converting nitrogen into ammonia to be used by plants and also trigger plant growth via production of phytohormones, viz., IAA, gibberellic acid, cytokinins and ethylene. Around 80% of diazotrophic, indole producing rhizobacteria promotes plant growth directly via phosphate solubilization, production of plant enzymes, HCN, antibiotics, siderophores for sequestering of iron and by lowering ethylene concentration via ACC deaminase activity. IAA acts as an important signal molecule in the regulation of plant development by initiation, cell division and cell enlargement. The amino acid L-tryptophan, serves as a physiological precursor for biosynthesis of auxins in microbes and plants. Bacteria synthesize auxins to perturb host physiological processes for their own benefit by altering the auxin pool, depending upon the amount of IAA produced. Therefore, it becomes necessary to identify and incorporate those efficient bacterial strains, which reside in the rhizosphere of plants, utilize the rich source of substrates, released from roots and are expected to produce auxins as secondary metabolites. Several soil bacteria, particularly those belonging to the species of *Bacillus* and *Pseudomonas* have remarkable abilities to synthesize various beneficial substances, along with potent PGP activities. Diazotrophic rhizobacteria, trigger and enhance plant growth as well as yield through various mechanisms, so their use can reduce the application frequency of chemical fertilizers. Indole-3-acetic acid (IAA), a most common natural auxin influences several physiological processes of the plant's health (Kumari *et al*., 2018).

Sewan *(Lasiurus scindicus*) and dhaman (*Cenchrus setigerus*) grasses **-**Cenchrus- Lasiurus type grasslands are associated with sub-tropical arid and semi-arid regions comprising the northern part of Gujarat and Rajasthan (Sanadya *et al*., 2018). *Lasiurus scindicus* and *Cenchrus setigerus* is the primary grass of extremely arid parts of Jaisalmer, Jodhpur, Barmer and Bikaner districts of western Rajasthan in the Indian *Thar* Desert. It thrives well under moisture stress on sandy plains, low dunes and hummocks of this region (Sanadya *et al*., 2021).

The perennial indigenous sewan grass **(***Lasiurus scindicus***)** popularly known as the "king of desert grasses"**.** The only known species of sewan grass (*Lasiurus sindicus* Henr.) belongs to family: *Poaceae*, native to dry areas of north Africa, Sudanese and Sahelian regions, east Africa, and Asia (Assaeed, 1997). Sewan grass is extremely drought resistant and thrives even in areas receiving very low rainfall (100 to 150 mm) annually and extreme temperatures ranging from -3 to 50ºC. Regeneration and growth of sewan grass in relation to soil moisture levels in the root zone indicate that, for good growth and maximum yield, it is essential that the deeper root zone profile is charged with sufficient water to support continuous growth of plants (Mertia *et al*., 2006). This grass has developed a number of morphological, anatomical and biochemical strategies to withstand the extreme climatic conditions. The leaves show characteristic C4 NADP-ME type of anatomy and have developed sclerenchyma to impart mechanical strength during drought and high wind (Kalia *et al*., 2020). It is a diploid species with somatic chromosome number, 2n= 20. Sewan grass is a perennial grass that can live up to 20 years. Propagation is done by sowing or root slips. It grows best on alluvial soils or light brown sandy soils with a pH of 8.5. This grazing pasture is of utmost importance in areas where annual rainfall is below 250 mm. The crude protein in the sewan herbage is high (8.14 per cent) in the early vegetative stage of growth. A cutting interval of 30 days at a

height of 15 cm gives the best dry matter yields. Sewan grass yields 2.7 to 10.5 tones fresh forage/ha/year and up to 3.4 tones dry matter/ha in well-established swards. Species like sewan grass are very important in arid environments because they provide forage, which maintains both wild mammals and livestock, and soil cover (Sanadya *et al*., 2018). In deteriorated rangelands of Saudi Arabia, sewan grass helps to control the low value invasive species *Rhazya stricta* by smothering its seedlings. It is a useful tool to improve rangeland management (Assaeed and Al-Doss, 2001). In India, sewan grass covers 0.1 million hectares of area when little water is available (Bhagmal *et al.,* 2011). The crown (rhizome), in which food (starch and sucrose) is stored and protected by scaly layers, is highly compressed, which allows the plant to remain dormant for years without moisture (Kumawat *et al*., 2019).

Dhaman grass (*Cenchrus setigerus*) Poaceae; is one of the most suitable and highly nutritive grass for desert environmental conditions. This grass, fed green, turned into silage, or made into hay is said to increase flow of milk in cattle and impart a sleek and glossy appearance. Seeds of this grass are used as famine food by the tribal during severe conditions. However, estimation of antimicrobial activities and identification of any bio-active compound still not have been done in this grass. The chemical analysis of isopropyl alcohol extract of *Cenchrus setigerus* showed a mixture of long chain hydrocarbons, carboxyl esters, alcohols, acids, alkaloids, steroids, amino and nitro compound etc. Phytochemical screening using the pharmacognostic methods revealed the presence of flavonoids, steroids and alkaloids (Singariya *et al*., 2014). The heterotrophic microbial populations were found to be higher in the mycorhizosphere soil of co-cropping system of *Cenchrus setigerus* and *Pennisetum pedicellatum* as compared to individual mycorhizospheres of *Cenchrus setigerus* and *Pennisetum pedicellatum* (Dubhey *et al.,* 2011). GC–MS analyses showed that

majority of these identified compounds in various crude extracts contain normal hydrocarbons, fatty acid, fatty acid esters, terpeniods, phytosterols, alkaloids and glycosides (Arora and Kumar, 2017).

Microbial diversity totally depend upon the soil characteristics such as moisture content, temperature, nutrient fertility status because of the direct or indirect relationship with the microbes. Microbial ecology has undergone a profound change in the last two decades with regard to methods employed for the analysis of natural communities. Emphasis has shifted from culturing to the analysis of signature molecules including molecular DNA-based approaches that rely either on direct cloning and sequencing of DNA fragments (shotgun cloning) or often rely on prior amplification of target sequences by use of the polymerase chain reaction (PCR). The pool of PCR products can again be either cloned and sequenced or can be subjected to an increasing variety of genetic profiling methods, including amplified ribosomal DNA restriction analysis, automated ribosomal intergenic spacer analysis, terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, single strand conformation polymorphism, and denaturing high-performance liquid chromatography (Nocker *et al.,* 2007). Advanced molecular technology is a promising manner for studying microbial communities (O'Brien *et al.,* 2019).

It is very essential to investigate and characterize the useful rhizobacteria from the rhizosphere region of sewan and dhaman grasses. Nevertheless, rhizospherie of these grasses is not well explored for the study of microbial populations for beneficial uses. Therefore, the present study was designed to isolate the bacteria and its delineation for PGPR activities and molecular phylogeny from rhizosphere region of these grasses with the following objectives:

Objectives

1. Isolation and characterization of bacteria from rhizosphere and adjacent region of sewan **(***Lasiurus scindicus***)** and dhaman (*Cenchrus setigerus*) grasses
2. Estimation of plant growth promoting potential of the isolates
3. To determine the molecular diversity in bacterial population (ITS region amplification, SCoT/CBDP analysis)
4. To validate sequence of ITS region of the isolate

Diverse microbiota reside in the soil and influenced by plant roots known as 'rhizosphere' which represents a unique biological niche with a diverse micro flora comprising of bacteria, fungi, protozoa and algae. This community is supported nutritionally by a high amount of organic materials derived from the plant roots and root exudates that are necessary for microbial growth (Lynch, 1990).

Plants have evolved with a plenty of microorganisms having important roles for plant growth and health. A considerable amount of information is now available on the structure and dynamics of plant microbiota as well as on the functional capacities of isolated community members. Due to the interesting functional potential of plant microbiota as well as due to current challenges in crop production there is an urgent need to bring microbial innovations into practice. Different approaches for microbiome enhancement exist. On the one hand microbial strains or strain combinations can be used, however, field success is often variable and improvement is urgently required. Smart, knowledge-driven selection of microorganisms is needed as well as the use of apposite delivery approaches and formulations are essential. On the other hand, farming practices or the plant genotype can influence plant microbiota and thus functioning. Therefore, selection of appropriate farming practices and plant biotechnology leading to improved plant microbiome interactions are avenued to enhance the benefit of plant microbiota (Compant *et al*., 2019).

2.1 Importance of sewan and dhaman grasses in arid ecosystem

Indian hot arid zone covers an area of 32 million ha called '*Thar* Desert'. 85 percent of the hot desert lies in India and the rest of the 15 percent in Pakistan. It represents the most inhospitable arid zone of the world spreading mostly in the states of Rajasthan, Gujarat, Punjab,

Haryana, Karnataka, and Andhra Pradesh in India. About 91 percent of the Indian desert falls in Rajasthan covering about 61 percent geographical area of the state. High wind velocity, huge dune, semi-stabilized and stabilized dunes of different nature, high diurnal variation in temperature, scanty and poor rainfall, intense solar radiation, and high rate of evaporation are the peculiar characteristics of the Thar desert. The natural grasslands lie in desert areas are highly deteriorated stage with the productivity of only 300–400 kg/ha/year. Western Rajasthan is the major part of the *Thar* desert where low and erratic rainfall, high temperature and dust storms in summer impose severe restrictions for arable cropping. Natural grasslands in the arid and semi-arid zones of Rajasthan are rich in grasses that supply bulk of forage to grazing stock. The perennial grasses with better rooting ability especially in the top 15 cm soil profile are able to bind the soil particles, check soil erosion, add to soil fertility through decayed roots and foliage parts and thus help in the conservation of soil. The microbial communities associated with the plant rhizosphere play a vital role in maintaining the plant-soil ecological cycle in desert ecosystem (Rao, 2000).

Sewan *(Lasiurus sindicus Henr*.), an important perennial grass cover of this region, flourishes well on sandy plains, low dunes and hummocks. Sewan grasslands have a vital role in forage production, in situ grazing and soil stabilization in the arid region. It is found mainly in wastelands, dunes, hammocks, and sandy plains but less popular for cultivation in farmer's fields. Sewan grass has many features like good nutritional value, soil binder, tolerance to high temperature, high digestibility and palatability, and prolonged drought conditions contributed greatly towards its success as a potential forage species in arid environments. It contains significant quantities of crude fibres, lignin, minerals and crude protein, and varies in the proportion of their tissue that can be digested by ruminants (Sanadya *et al*., 2021).

Dhaman grass is indigenous to India and is adapted to arid and semi arid tropical climates with long dry season,Tufted, perennial (sometimes behaving as an annual and flowering in the first year), non-rhizomatous or shortly rhizomatous with deep root system (Bogdan *et al*.,1997). It is very tolerant towards drought and grows in areas of annual rainfall less than 200 mm, making it excellent for improvement of low rainfall grazing land. It is more tolerant than *C. ciliaris*. It is a leafy fodder having a tender stem which provides abundant foliage. It is adapted to arid and semi arid tropical climates with long dry season. About 8-10 tonnes of forage may be obtained in 3-4 cuttings per hectare. Sowing just before the usual rainy periods in summer at the rate of 1.5-3.0 kg per hectare depends up on seed supplies, cost and rapidity of cover desired. Nutritive value of dhaman grass includes 4.5% crude protein and 38% crude fibre. Neutral detergent fibre and acid detergent fibre content is 72.0 and 33.0%, respectively (Narain *et al*., 2008).

2.2 Isolation and characterization of bacteria from Rhizosphere and adjascent region of sewan and dhaman grass

2.2.1 Soil as habitat for flora and fauna

Soil is a complex and dynamic biological system. We are limited in the determination of microbial mediated reactions because present investigations for determining the overall rate of entire metabolic processes (such as respiration) or specific enzyme activities (such as urease, protease and phospho mono esterase activity) do not allow any identification of the microbial species directly involved in the measured processes.

Soil represents a highly heterogeneous environment for the microflora and fauna inhabiting it; the diverse components in the solid fractions in the soil (sand, silt, clay and organic matter) provide multitude of microhabitats. Soil habitat is defined as the totality of living

organisms inhabiting soil including plants, animals, and microorganisms and their abiotic environment. Soil is a complex habitat where interaction of large number of different microorganisms including bacteria, fungi, algae and protozoa takes place. They can be found free living or attached to the exterior of soil particles in bulk soil, but a mass of bacteria also interact with plant roots, named as rhizosphere.

The central problem posed by the link between microbial diversity and soil function is to understand the relations between genetic diversity and community structure and between community structure and function. Soil seems to be characterized by a redundancy of functions; for example, no relationship has been shown to exist between microbial diversity and decomposition of organic matter. Generally, a reduction in any group of species has less effect on overall processes in soil because other microorganisms can take on its function.

Soil is fundamental and irreplaceable part of life and it governs productivity of terrestrial ecosystems and maintains biogeochemical cycles utilizing microorganisms to degrade, ultimately, all organic compounds including persistent xenobiotics and naturally occurring polyphenolic compounds. The soil provides support to variety of life forms including macrofauna, mesofauna, microfauna, macroflora and microflora. Around 80–90% of the processes in soil are reactions mediated by microorganisms (Coleman & Crossley, 1996, Nannipieri & Badalucco, 2003). Soil is highly heterogeneous and complex microhabitat, which is reflected in the spatial distribution and enormous diversity of microorganisms and their metabolic versatility. The major contributors of soil microbial diversity that is bacteria and fungi are extremely flexible and can carry out almost all known biological reactions. The presence of microorganisms alters the habitat and

makes it possible for other life forms to survive and function (Maestre *et al.,* 2015).

2.2.2. Soil microbial diversity

The term biodiversity has been defined in diverse ways. In terms of microorganisms, it describes the number of different types (species), and their relative abundance in a given community in a given habitat. In molecular phylogenetic terms, it may be defined as the number and distribution of different sequence types present in the DNA extracted from the individuals in the habitat. There are three major factors considered as determinants for microbial life (structure of microbial communities) in the soil which can be listed as (i) plant type, responsible for providing specific carbon and energy source for the microorganisms (ii) soil type, as the combination of soil texture and structure, organic matter, micro aggregate stability, pH and the presence of the key nutrients e.g. N, P, Fe etc. determines the suitability of niche for microorganisms (iii) agricultural management practices, such as crop rotation, tillage, irrigation, fertilizer application etc. are the key determinants of microbial community structure. Microorganisms are omnipresent, inhabit in diverse extreme sites across various ecosystems. Due to the extreme variations in geoclimatic patterns, the global arid biome consists of highly diversified and heterogeneous system. Microbial diversity in desert soils is mostly dependent on characteristics such as temperature, moisture and availability of the organic carbon (Buyanovsky *et al*.,1982; Parker *et al*., 1983). Gram-positive spore formers are dominant and the populations do not decline significantly even during summers (Rao *et al*., 1983). Actinomycetes may constitute 50% of the total microbial bacterial population in desert soils (Hethener *et al*., 1967). Cyanobacteria also contribute significantly to the biota of hot arid regions in terms of primary productivity and nitrogen fixation (Bhatnagar *et al*., 2005).

Soil bacteria and fungi play important roles in the functioning of terrestrial ecosystems, yet our understanding of their responses to climate change lags significantly behind that of other organisms. This gap in our understanding is particularly true for dry lands, which occupy~41% of Earth´s surface, because no global, systematic assessments of the joint diversity of soil bacteria and fungi have been conducted in these environments till date (Upton *et al.,* 2018).

Maestre *et al.* (2015) reported that in a survey of drylands worldwide and DNA-sequencing approaches revealed that increases in aridity reduce the diversity and abundance of soil bacteria and fungi and also represents an important advancement in our understanding of soil microbial communities and their likely responses to ongoing climate change.

Bach *et al.* (2018) reported soil microbial community composition and structure of both bacteria and fungi at a microbially-relevant scale; results showed micro aggregates support highly diverse microbial communities, including several unidentified genera. Isolating aggregates with a microbially sensitive approach provides new opportunities to explore soil microbial communities and the factors shaping them at relevant spatial scales.

2.2.3 Activity of microorganisms

Microorganisms are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in key processes like soil bacteria and fungi play crucial roles in various biogeochemical cycles (Molin and Molin, 1997, Wall and Virginia, 1999) and are responsible for the cycling of organic compounds. Soil microorganisms also influence aboveground ecosystems contributing to plant nutrition (George *et al*. 1995), plant health (Filion *et al*. 1999, Smith and Goodman, 1999**)**, soil structure (Dodd *et al*. 2000) and soil fertility (Yao *et al*. 2000). Soil microorganisms are important for the

continuing cycling of nutrients and for driving above ground ecosystems (Klironomos *et al*., 2000). In natural ecosystems, plants experience complex interactions with microorganisms on physical, metabolic and hardly a single plant family has been recognized, which is not living in symbiosis with microorganisms (Frey-Klett *et al*., 2007).

Lange *et al.* (2015) studied that the higher plant diversity increases rhizosphere carbon inputs into the microbial community resulting in both increased microbial activity and carbon storage. Increases in soil carbon were related to the enhanced accumulation of recently fixed carbon in high-diversity plots, while plant diversity had expressed less effect on the decomposition rate of existing carbon.

Isobe *et al.* (2018) studied the relationship between microbial community dynamics and bio-available N dynamics throughout a year in a cool-temperate deciduous forest, with a focus on the dormant season and analyzed temporal changes in abundances of N-cycling microbial populations and N concentrations and transformations in soils.

2.2.4 The rhizosphere and non rhizoshere region in plant

German agronomist ‘Hiltner’ was first to define the ‘rhizosphere’, in 1904, as the effect of the roots of legumes on the surrounding soil in terms of higher microbial activity. The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. This zone is about 1 mm wide, but has no distinct boundary. Rather, it is an area of intense biological and chemical activity influenced by compounds exudates by the root, and by microorganisms feeding on the compounds. The roots exude water and compounds broadly known as exudates. Root exudates include amino acids, organic acids, carbohydrates, sugars, vitamins, mucilage and proteins. The exudates act as messengers, which stimulate biological and physical interactions between roots and soil organisms.

They modify the biochemical and physical properties of the rhizosphere and also contribute to root growth and plant survival. High levels of moisture and nutrients in the rhizosphere attract more number of microorganisms than elsewhere in the soil. The composition and pattern of root exudates affect microbial activity and population numbers which, in turn, affect other soil organisms that share this environment. It is an estimate that a plant releases 20 and 50% of their photosynthates through root. A large number of macroscopic organisms and microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates (Lynch, 1990), creating a very selective environment where diversity is low (Lucas *et al*, 2001). The rhizosphere is a centre of intense biological activity due to the food supply provided by the root exudates. In soils that are well colonized by plants, the plant roots may affect all the soil present in a particular area, and little non-rhizosphere soil (bulk soil) may be present (Vanpeer and Schippers, 1989).

2.2.5 Evaluation of Plant growth promoting rhizobacteria

The term PGPR (Kloepper and Schrot, 1978) refers to those plants root (‘rhizosphere’)-associated bacteria that are capable of stimulating plant growth, *e.g*. by improving plant nutrition, by the production of plant growth regulators or by preventing the attack of pathogenic microorganisms. PGPR vary in their degree of intimacy with the plant, from intracellular, *i.e.* existing inside root cells, to extracellular, *i.e.* free living in the rhizosphere. Some PGPR are commercially available as inoculants and have applicability in agriculture, forest regeneration, and phytoremediation of soils. A putative plant growth promoting rhizobacteria (PGPR) qualifies as PGPR when it is able to produce a positive effect on the plant upon inoculation, hence demonstrating good competitive skills over the

existing rhizosphere communities. Generally 2-5% of rhizospheric bacteria are PGPR (Antoon and Prevost, 2005).

Gholve *et al*. (2006) isolated sixty fluorescent *Pseudomonas* bacteria from rhizosphere soil and rhizoplane surface of chilli, cotton, soybean, safflower, moong, groundnut and sorghum crops on King's B medium under aseptic condition by following dilution plate technique.

Fischer *et al*. (2007) studied isolation and characterization of bacteria from the rhizosphere of wheat in which morphology and gram staining were determined by using a light microscope (1,000×) (Zeiss, Argentina S.A).

Suresh *et al.* (2010) isolated 10 strains of fluorescent pseudomonads from the rhizosphere soils of bajra (*Pennisetum glaucum*), jowar (*Sorghum vulgare*), rice (*Oryza sativa*) and maize (*Zea mays*) and screened for their plant growth promoting activity based on their ability to produce hydrogen cyanide (HCN), siderophores, proteases, indole acetic acid (IAA), broad spectrum antifungal activity against pathogenic fungi and phosphate solubilization and finally their results indicated that most of the isolates tested to possess plant growth Bergey's manual of determinative bacteriology promoting traits..

Kumar *et al.* (2012) isolated large number of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium and Serratia* and reported that to enhance plant growth from six soil samples from French bean rhizosphere were collected from different location of Shimla and Solan of H.P (India).

Zhao *et al.* (2012) isolated actinobacteria from seven medicinal plants rhizosphere by enrichment culture technique where the isolates were identified by cultural and morphological characteristics, including morphology and colour of aerial mycelium, characteristics of colonies

on the plate, spore mass colour, colour of diffusible pigments, and sporophore and spore chain morphology.

Malleswari and Bagyanarayana (2013) isolated total 219 bacterial strains from the rhizosphere soil samples of some medicinal plants viz., Coleus forskohlii, Andrographis paniculata, Withania somnifera, Ocimum sanctum, Aloe vera, Tagetes erecta, Mimosa pudica, Artemisia vulgaris, Acorus calamus and Mentha spicata by serial dilution plate technique using nutrient and King's B agar media. Four isolates were found to be promising PGP activities and they were identified as Pantova sp., Bacillus sp. and Pseudomonas sp. on the basis of colony morphology and Gram's staining.

Mohite (2013) isolated 10 bacterial isolates as IAA producer from rhizosphere soil among which 5 were selected based on IAA production ability. The isolates were coded as br1, br2, br3 (from banana rhizosphere), wr2 (from wheat rhizosphere) and mr2 (from maize rhizosphere). The isolates were identified based on morphological observation and biochemical characterization.

Ashokvardhan *et al*. (2014) isolated rhizospheric actinomycetes by using serial dilution method on the SCA (starch casein agar) medium and chitin agar medium from the rhizosphere. The organism was identified by various biochemical tests *viz*. casein and starch hydrolysis.

Sharma *et al.* (2014) studied 32 bacteria which were isolated from pearl millet, *Pennisetum glaucum* (L.) R. Br. rhizosphere and associated non-rhizospheric areas. Pearl millet rhizosphere and non-rhizosphere areas were dominated by gram negative bacterial population with rod shaped creamy colonies. Salinity and temperature tolerance study of bacterial isolates showed that most of the bacterial isolates (rhizospheric as well as non-rhizospheric) were less halophilic. The overall findings indicated that the most effective bacterial isolate

with maximum PGPR activity is PM-14 that was confirmed by 16S rDNA gene sequencing method and BLAST analysis of the sequence revealed that the predominant microorganism is the *Acetinobacter sp*.

Chatterjee *et al.* (2015) studied isolates morphologically and biochemically characterized through phenotypic tests (Gram staining, catalase, oxidase, urease test, microscopic observation, spore production, and motility).

Tiwari *et al.* (2016) isolated, screened and characterized the PGPR from the rhizosphere soil of pigeonpea growing in different areas of Bijarkala and Kumarganj, U.P., India. All the nine bacteria were isolated and characterized for various plant growth promoting activities. The results suggested that higher percentage of phosphate solubilizers, IAA, and HCN producers were also found in rhizospheric soils of pigeonpea as comparison to bulk soils and shown potential as biofertilizers or microbial inoculants pigeon pea crop.

Bibi *et al.* (2020) isolated total 48 bacteria *viz*., *Erwinia stewartii, Klebsiella terrigena, Klebsiella pneumonia spp.* Ozaene*, Serratia plymuthica, Yersinia, Escherichia blattae, Edwardsiella ictaluri,* and *Obesumbacterium proteus* by serial dilution plate technique using nutrient agar medium strains from the rhizosphere soil samples. Morphological studies including colony colour, bacterial shape and gram staining were performed.

Kones *et al.* (2020) studied the presence of both bacteria and fungi benefiting plants health and promotes growth. Biochemical and morphological characterization of the organisms revealed *Trichoderma*, *Aspergillus*, *Fusarium*, and *Phytophthora* spp. as the most abundant fungi in the rhizoplane of bacterial wilt tolerant tomatoes just like *Bacillus*, *Burkholderia*, *Micrococcus* and *Pseudomonas* spp.

2.2.6 Estimation of plant growth promoting potential of the isolates

Kloepper *et al.* (1989) reported that plant growth promoting rhizobacteria (PGPR) as heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and or indirectly. In last few decades a large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance plant growth.

Juanda *et al.* (2005) reported that plant growth promoting rhizobacteria (PGPR) could be used to induce specific changes to root system architecture (RSA) which could impart growth benefits in specific environmental conditions.

Kumar *et al.* (2012) examined a total of thirty bacteria which were isolated screened *in vitro* for different plant growth promotion activities such as phosphate solublization, IAA production, ammonia production, ACC deaminase activity, HCN production and catalase.

Bhattacharyya *et al.* (2012) reported the plant growth-promoting rhizobacteria (PGPR) as the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), quorum sensing (QS) signal interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance, promoting beneficial plant-microbe symbioses, interference with pathogen toxin production etc.

Sharma *et al*. (2013) examined several soil microbial species exhibiting P-solubilization capacity, playing an important role in the whole soil P cycle. The main P-solubilization mechanisms employed by soil microorganisms include release of complexing or mineral-dissolving compounds, e.g., organic acid anions, siderophores, protons, and hydroxyl ions; liberation of extracellular enzymes (biochemical P-mineralization); and the release of P during substrate degradation (biological P-mineralization).

Salcedo *et al*. (2014) isolated actinobacteria from rhizosphere of wild plants such as *Vallea, Weinmannia, Vaccinium, Drimys, Rosmarinus,* as well as from legumes grasses (Rye Grass or Bromus) and clovers (*Trifolium repens* and *Trifolium pratense*). They reported that all isolates were able to solubilize tricalcium phosphate or aluminium phosphate.

Singh *et al.* (2014) isolated bacteria with phytase activity from rhizosphere and proposed as PGPB to be used in soils with high content of organic P. Bacterial isolates identified as *Advenella* were positive for phytase production, and increased the P content and growth of Indian mustard (*Brassica juncea*)).

El-Said *et al*. (2016) isolated ninety one bacterial strains from the rhizosphere of maize, wheat, clover and rice plants were screened for their plant growth promoting activities. The results of *in vitro* assays showed that all rhizobacterial isolates had the ability to produce indole acetic acid (IAA) in presence or absence of tryptophan (L-TRP) with a wide variation observed among them. The results also showed that 11 rhizobacterial isolates were able to solubilize calcium super phostphate CaR 3 R(POR 4R)R 2 Ron Bunt and Rovira medium. These isolates were tested for nitrogenase activity and cyanide production.

Noumavo *et al.* (2016) studied the use of bio-resources such as PGPB (plant-growth-promoting bacteria) to enhance plant growth and bio-control produce wheat.

Dida *et al.* (2018) isolated strains were picked up from each plate containing pure culture and streaked in straight lines in starch agar plates as carbon source. The plates were incubated at 32ºC for 48 h.

Kashyap *et al.* (2020) isolated twenty three rhizospheric soil samples of chilli plants from southern plateau and hills region agro-climatic zones of Karnataka and Andhra Pradesh (states of India) were collected where rhizobacterial isolates showing plant growth promoting activities along with having biocontrol potentials were characterized using morphological, biochemical and physiological attributes.

2.3 Analysis of soil samples for nutrient content and microbiological parameters

Fterich *et al.* (2014) investigated that enzymatic activity (dehydrogenase, phosphatase and β-glucosidase) expressing soil microbial activities were significantly higher under *Acacia* canopies. Soil organic carbon, microbial biomass and microbial coefficient were found to be significantly greater in canopied soil, compared with uncanopied soil whereas the highest microbial density and activity were redistricted in sandy-loam soil microbial biomass and activity increased gradually and significantly to a maximum at 20–30°cm soil depth and subsequently decreased at 30–50°cm.

Gehlot *et al.* (2013) reported *Acacia* as a good soil binder, prevents soil erosion, good colonizer of poor soil and is able to nodulate and fix nitrogen and thus improve soil fertility and although the nodulation of native legumes growing in alkaline soil in the *Thar* Desert of India.

Choudhary *et al.* (2017) isolated 57 bacterial strains and purified from root nodules of *Vachellia (Acacia) leucophloea* growing in soils of different districts of Western Rajasthan. Their carbon utilization and

intrinsic antibiotic resistance patterns were different from the closely related type strains, host range analysis suggests that some of the strains were promiscuous and nodulate other members of the genus *Acacia spp.* and *Prosopis species.*

Kumar *et al*. (2018) investigated the cultivation of leguminous crops and trees which offers multiple advantages including augmentation of crop and soil productivity and adapting to climate change by increasing resilience of agro ecosystems. Legumes have the ability to reduce the CO_2, emitted during the manufacturing of chemical nitrogenous fertilizers through their biological nitrogen fixation (BNF) capacity, improve soil health and soil carbon content as per the nature of the specific crops.

Ye *et al. (*2020) investigated that legume plantation had greater enrichment of middle- and high-mass and condensed aromatic-like DOM components in soils and higher abundance of microbial-derived molecules (e.g., protein-like and carboxyl-rich alicyclic molecules) was found at the legume plantation relative to the non-legume plantation, which suggests a faster microbial turnover of DOM (dissolve organic matter).

2.4 Molecular characterization of sewan and dhaman grass rhizospheric bacterial population

DNA markers have various applications in plant molecular genetics research (Semagn *et al.*, 2006). Start codon targeted (SCoT) polymorphism is one of the novel, simple and reliable gene-targeted marker systems. This molecular marker offers a simple DNA-based marker alternative and reproducible technique which is based on the short conserved region in the plant genes surrounding the ATG (Sawant *et al*., 1999) translation start codon (Collard and Mackill et al., 2009). This technique involves a polymerase chain reaction (PCR)

based DNA marker with many advantages such as low-cost, high polymorphism and extensive genetic information.

Borneman *et al.* (1996) analysed 16S rRNA gene clone libraries from East Amazonian soil. Of 124 clones sequenced, 98.4% fell within the Bacteria the remainder being mitochondrial DNA. There was considerable diversity, with only 40 of sequences being duplicated, and approximately 25% of sequences fell within five groups that could not be assigned to previously characterised groups within the Bacteria. For example, only 19% and 0.8% of sequences were representative of Bacilli and Actinomycetes, respectively, and no sequences were found that were representative of pseudomonads, Agrobacteria, Alcaligenes or Flavobacterium.

Felske *et al.* (1998) sequencing of 16S rRNA covering a stretch of approximately 1500 nucleotides. About half of the sequences found in the clone library showed only slight relationships to other known sequences, while the other half were highly similar (approximately 95% sequence identity) to other database entries (mainly Bacillus species).

Dunbar *et al.* (1999) compared the levels of bacterial community diversity in two pinyon rhizosphere soil samples and two between-tree (interspace) soil samples by analyzing 179 cultivated bacterial isolates and 801 16S rRNA genes amplified from extracted soil DNA. Phylotypes were defined by performing a restriction fragment length polymorphism analysis of 16S rRNA gene sequences with the enzymes RsaI and BstUI.

Felske *et al*. (2000) studied a total of 240 drill cores were taken at 12 sampling sites from the Drentse A grasslands. TGGE analysis of 16S rRNA amplicons demonstrated previously that five drills within a 2 meter circle showed a highly similar bacterial community composition and pooling of such samples did not cause a considerable loss of

information (Felske and Akkermans 1998). However, the efficiency of ribosome extraction from soil was expected to vary.

Tejera *et al.* (2005) amplified 16S rDNA from six *A. chroococcum* isolates from soil samples (AS1, AS2 and BS4 isolates) and rhizosphere isolates (AR2, BR3 and BR4 isolates), and from four *Azospirillum spp*. isolates from rhizosphere soils (AR8) and roots (BR1, CR3, DR1). In all cases isolates produced ARDRA patterns typical of their *A. chroococcum* and *A. brasilense* when they were compared with the patterns of reference strains. According to these results we confirm the presence of *A. chroococcum* and *A. brasilense* species in isolates.

Chowdhury *et al.* (2007) performed PCR fingerprinting and amplified ribosomal DNA restriction analysis of isolates from Sewan grass. PCR amplification of 16S rRNA gene in the 60 bacterial isolates resulted in amplification of 1.5-Kb amplicon, which was subjected restriction digestion with AluI and Sau3A I. Both of the tetra-cutting restriction to endonucleases yielded distinct banding patterns, which were used as molecular markers to construct a composite dendrogram to find phylogenetic similarities among the isolates.

Sharma *et al.* (2008) carried out RAPD analysis of DNA isolated from soils under Ker and associated open areas of Bikaner, Kodamdesar and Nagaur regions of western Rajasthan was carried out using six primers to find out the effect of Ker (*Capparis deciduas*) plants on microbial diversity. The average Jacard's coefficient similarity within Ker samples was less (0.250) than that within adjoining open area (0.337), indicating that the soil under Ker supports more diverse microbes compared to the open areas. The soils from the three locations were quite uniform both under Ker (94.53%) and in open area (94.76%).

Collard and Mackill et al. (2009) studied RAPD markers have been used for numerous applications in plant molecular genetics research despite having disadvantages of poor reproducibility and not generally

being associated with gene regions. A novel method for generating plant DNA markers was developed based on the short conserved region flanking the ATG start codon in plant genes. This method uses single 18-mer primers in single primer polymerase chain reaction (PCR) and an annealing temperature of 50℃. PCR amp licons are resolved using standard agarose gel electrophoresis. This method was validated in rice using a genetically diverse set of genotypes and a backcross population. Start codon targeted (SCoT) markers were generally reproducible but exceptions indicated that primer length and annealing temperature are not the sole factors.

Ambrosini *et al*. (2012) isolated 299 strains from the roots and rhizosphere of sunflower cultivated in five different areas using N-free media. 16S rDNA PCR-RFLP and 16S rRNA partial sequencing were used for identification and the Shannon index was used to evaluate bacterial diversity.

Bell *et al.* (2014) sequenced bacterial 16S rRNA genes and the fungal internal transcribed spacer (ITS) region to compare the community composition of 66 soil samples from the rhizosphere of planted willows (*Salix* spp.) and six unplanted control samples at the site of a former petrochemical plant.

Franke-Whittle *et al.* (2015) studied differences in bacterial and fungal communities between replant and closely situated control non-replant (fallow) soils, the V1-V3 region of the bacterial 16S rRNA gene and the ITS1 region of fungi from the different soils were sequenced using 454 pyrosequencing and data were analysed using the MOTHUR pipeline.

Gislin *et al.* (2018) studied amplified 16s rRNA PCR products of two antagonistic bacteria were purified and sequenced. The sequence of bacterial isolates S1A1 and S7A3 were subjected to NCBI blast. The isolates S1A1 and S7A3 BLAST results showed 99% and 95% respectively, similarity with the available database sequence of *Bacillus*

amyloliquefaciens. The sequences were deposited in Gene Bank and the accession numbers KY864390 (S1A1) and KY880975 (S7A3) were obtained.

Yeoh *et al.* (2016) and Li *et al.* (2016) studied the diversity of the bacterial community sampled in the rhizosphere and rhizomes of plants of *Micanthus giganteus* through the 16S rRNA and *nif*H genes verified the formation of distinct structural groups in these different habitats of plant/bacteria association.

Rathi *et al.* (2017) investigated genetically diverse root nodule bacteria associated with *Alysicarpus vaginalis* from alkaline soil on the basis of PCR-RFLP (ARDRA) pattern ten different genetic groups were formed showing significant genetic diversity among the strains.

Shankla *et al*. (2018) studied root nodules out of 72 bacterial (RNB) strains isolated from *Crotalaria burhia*, 51 rhizobia-like strains were examined for genetic diversity based on ARDRA and RAPD patterns and used BLASTn sequence similarity results based on 16S rRNA gene of selective thirteen strains representing four ARDRA types revealed that they were related to genera Ensifer, Rhizobium and Bradyrhizobium.

Golkar and Mokhtari (2018) studied the genetic diversity of safflower was assessed using 12 polymorphic sequence-related amplified polymorphism (SRAPs) and 11 polymorphic start codon targeted (SCoT) markers in 100 genotypes of safflower gathered from different geographical regions of the world. The 23 primers generated a total of 227 polymorphism fragments with a mean of 68.2% within the range of 3 (SCoT 31 and SCoT 35) to 13 (SCoT 35) bands per primer. The results of the analysis of molecular variance showed a significant difference across cultivated safflower genotypes possessing a high intra-population variation.

Berlanas *et al.* (2019) examined rhizosphere bacterial and fungal microbiota across five grapevine rootstock genotypes cultivated in the

same soil at two vineyards and sampling dates over two years by 16S rRNA gene and ITS high-throughput amplicon sequencing and used quantitative PCR (qPCR) approach to measure the relative abundance and dynamic changes of fungal pathogens associated with black-foot disease.

Guo *et al.* (2019) studied rhizosphere microbial (bacteria, fungi and arbuscular mycorrhizal) diversity and composition using Illumina sequencing of the 16S rRNA and ITS genes for comparison to plant and soil properties and results showed that bacterial and fungal alpha diversity was significantly higher at mid-elevation, while AMF alpha diversity decreased monotonically.

Gagnon *et al.* (2020) analysed the main edaphic parameters that structured microbial populations associated with the indigenous woody plants that had naturally colonized the site and study also revealed that many of the most abundant fungal genera (e.g., *Claussenomyces, Eupenicillium* and *Trichoderma)* were more abundant in the rhizosphere than in the root endosphere.

Taye *et al*. (2020) analysed rhizosphere soils of 16 diverse genotypes of *Brassica napus*; samples were analyzed using 16S rRNA gene amplicon sequencing over a 10-week period at single location as well as at three time points at two additional locations.

Wen *et al.* (2020) studied the rhizosphere bacterial communities of soyabean were analyzed by using high-throughput sequencing of V4 hypervariable regions of 16S rRNA gene amplicons via Illumina MiSeq. The results of alpha diversity analysis showed that the BRH and SRH of BX10 were significantly lower in community richnesss than that of BD2, while the WRH exhibited no significant difference between BX10 and BD2.

Ghobadi *et al.* (2021) studied fifteen SCoT and fifteen CBDP primers produced 262 and 298 fragments which all of them were polymorphic in wheat, respectively. The number of polymorphic bands (NPB),

polymorphic information content (PIC), resolving power (Rp), and marker index (MI) for SCoT primers ranged from 14 to 23, 0.31 to 0.39, 2.55 to 7.49, and 7.56 to 14.46with an average of 17.47, 0.34, 10.44, and 5.69, respectively, whereas these values for CBDP primers were 15 to 26, 0.28 to 0.36,3.82 to 6.94, and 4.74 to 7.96 with a mean of 19.87, 0.31, 5.35, and 6.24, respectively. Based on both marker systems, analysis of molecular variance (AMOVA) indicated that the portion of genetic diversity within species was more than among them.

dos santos *et al.* (2021) examined the diversity and genetic structure in *Dalbergia nigra* mini-garden. For this, eleven primers were selected, which generated 180 fragments (70.76% polymorphism). The polymorphic information content (PIC) for the markers used averaged 0.38, considering them as moderately informative. The number of ISSR fragments to obtain desired precision in genetic diversity analyses was shown to be 122 polymorphic fragments, which was attained by examining 11 ISSR markers with clear amplification patterns. Molecular variance analysis (AMOVA) for *D. nigra* populations indicated moderate genetic differentiation ($\Phi_{ST} = 0.118$). The ΔK statistic corroborates the analysis of the structure, indicating the convergence of two Bayesian groups (K = 2). The ISSR markers were efficient in detecting genetic diversity in *D. nigra*.

2.5 To validate sequence of ITS region of the isolates

Prosser *et al.* (2002) analysed 16S rRNA genes is now widely used for analysis of bacterial populations, and analysis of 18S rRNA genes and internal transcribed spacer (ITS) regions is increasingly being used to analyse fungal populations. Ribosomal rRNA genes are ideal for this purpose in that they possess regions with sequences conserved between all bacteria or fungi, facilitating alignment of sequences when making comparisons, while other regions exhibit different degrees of variation, enabling distinction between different groups.

Brown *et al.* (2005) showed that the ITS sequence obtained for all 512 clones examined, along with 248 associated 16S rRNA gene sequences (either full length or >1000 base pairs), resulting in the compilation of an extensive ITS sequence database coupled to 16S rRNA gene phylogenetic information.

Franke-Whittle *et al.* (2015) studied differences in bacterial and fungal communities between replant and closely situated control non-replant fallow soils, the V1-V3 region of the bacterial 16S rRNA gene and the ITS 1 region of fungi from the different soils were sequenced using 454 pyro-sequencing (Titanium chemistry), and data were analysed using the MOTHUR pipeline.

Berlanas *et al.* (2019) examined rhizosphere bacterial and fungal microbiota across five grapevine rootstock genotypes cultivated in the same soil at two vineyards and sampling dates over two years by 16S rRNA gene and ITS high-throughput amplicon sequencing and used quantitative PCR (qPCR) approach to measure the relative abundance and dynamic changes of fungal pathogens associated with black-foot disease.

Guo *et al.* (2020) studied rhizosphere microbial (bacteria, fungi and arbuscular mycorrhizal fungi) diversity and composition using Illumina sequencing of the 16S rRNA and ITS genes for comparison to plant and soil properties and results showed that bacterial and fungal alpha diversity was significantly higher at mid-elevation, while AMF alpha diversity decreased monotonically.

Karim *et al*. (2022) studied molecular analysis based on 16S rRNA markers and showed three isolates belonging to the *Bacillus*. The LC648364 isolates are closely related to species *Bacillus* sp. strain LLB-17, LC648365 is closely related to *B. subtilis* strain S11 and LC648366 are closely related to *B. cereus* strain EM6.

3. MATERIALS AND METHODS

The present investigation Research entitled **"Characterization and Diversity Analysis of Bacterial Isolates from Rhizosphere of Sewan (*Lasiurus scindicus*) and Dhaman (*Cenchrus setigerus*) Grasses"** was carried out under laboratory conditions at Department of Biotechnology, Plant Biotechnology Centre, College of Agriculture, *Swami Keshwanand Rajasthan Agricultural University*, Bikaner and ICAR-Central Institute for Arid Horticulture (ICAR-CIAH), Bikaner. In order to characterize bacterial population under the sewan (*Lasiurus scindicus*) and dhaman (*Cenchrus setigerus*) grasses morphological, microscopic, biochemical, soil analysis, PGPR activity and SCoT markers analysis were carried out. The materials and protocol adopted in the investigation are described in this chapter.

Materials and Methods

3.1. Materials

3.1.1. Glass-wares and chemicals

Glass-wares and plastic items used during study were from Borosil, Durasil, Polylab and Eppendorf etc. All the chemicals and reagents used in the present investigation were of analytical and molecular biology grade manufactured by standard and internationally reputed companies like Hi-Media, CDH etc. **Appendix-I.**

3.1.2. Equipments

The instruments like autoclave, deep freezer, ice maker, microscope, weighing balance, laminar airflow, BOD incubator, centrifuge, vortex, shaker, microwave oven, micropipettes (10µl, 100µl, 1000µl), hot air oven, agarose gel electrophoresis system, gel documentation system, etc. were used in this study. The details of instrument used throughout the study were listed in **Appendix- II.**

3.1.3 Oligos

The primers used in this study were selected from Collard and Mackill (2009) study and customly synthesized from Erufins, Indian/Europe (International Group of Life Science). The details about the oligos are given in **Table 3.1.**

Table 3.1: SCoT primer used for profiling in among 13 isolates in the present study

S. No.	Primer ID*	Sequence (5' - 3')	Tm (℃)
1.	ScoT02	CAACA**ATG**GCTACCACCC	45
2.	ScoT13	ACGAC**ATG**GCGACCATCG	45
3.	ScoT14	ACGAC**ATG**GCGACCACGC	45
4.	ScoT15	ACGAC**ATG**GCGACCGCGA	45
5.	ScoT16	ACC**ATG**GCTACCACCGAC	45
6.	ScoT18	ACC**ATG**GCTACCACCGCC	45
7.	ScoT19	ACC**ATG**GCTACCACCGGC	45
8.	ScoT20	ACC**ATG**GCTACCACCGCG	45
9.	ScoT21	ACGAC**ATG**GCGACCCACA	45
10.	ScoT22	AACC**ATG**GCTACCACCAC	45
11.	ScoT23	CACC**ATG**GCTACCACCAG	45
12.	ScoT25	ACC**ATG**GCTACCACCGGG	45
13.	ScoT26	ACC**ATG**GCTACCACCGTC	45

*Collard and Mackill, 2009

3.2. Methodology

3.2.1 Isolation of morphologically different colonies and further biochemical identification of bacterial isolates

3.2.1.1 Source of isolation

The experimental material in the present study consisting of soil samples which were collected from the rhizosphere of sewan and dhaman grasses growing from different sites rhizosphere and non Rhizosphere soil from Bikaner ARS (28.10° N latitud e and 73.18° E longitude), Jodhpur CAZRI (26.24° N latitude and 73 .00° E longitude) and Jaisalmer (27.52° N latitude and 70.18° E longi tude) District of Rajasthan in India. Intact root system was dug out and the rhizospheric soil samples were carefully taken in aseptic bags and immediately transported laboratory under cold condition at 4℃ for further processing. Soil samples were collected from 10-25 cm depth and allowed to pass through 2.0 mm sieve separately and stored at -80 ℃ in deep freezer for further analysis.

3.2.1.2 Cultural characterization

Bacteria were isolated from the rhizospheric and non-rhizospheric soil. Soil samples were obtained from a depth of 10 to 25 cm and placed in sterile poly bags. Samples were kept at ambient temperature during the expedition and 4℃ upon re turn to the laboratory. Sample (about 10 g) of air-dried soil was mixed with sterilized distilled water (90 ml) and considered as 10^{-1} dilution. The soil samples suspensions were subjected to vortex at 200 rpm for 30 minutes. One ml of soil mixtures were transferred to 9 ml of sterile distilled water to get 10^{-2} dilution and similarly stepwise final dilution of 10^{-4} to 10^{-6} was attained. About 100 µl of each suspension was spread on basic nutrient agar plates and incubated at 28℃ temperature for 24 hrs. These colonies were plated separately in fresh

Petri plates containing nutrient agar medium and purified by streak plate method, code number were allocated accordingly and maintained (Table 3.2). Bacterial isolates having different morphological characteristics on agar plates were selected and preserved on nutrient agar slants. NA plates were observed for morphological characters and number of bacterial colonies (cfu/g).

$$\text{Final count} = \frac{\text{Number of well-isolated colonies X Dilution factor}}{\text{Aliquot taken}}$$

Table 3.2: List of cultural characters observed

S. No.	Colonial characters	S. No.	Colonial characters
1.	Size	5.	Texture
2.	Shape	6.	Opacity
3.	Margin	7.	Pigment
4.	Elevation		

3.2.1.3. Morphological characterization of bacterial isolates

3.2.1.3.1. Gram's staining

Gram staining or differential staining is used for categorizing the bacteria into two distinct groups either gram positive or gram negative. Hi Media gram's staining kit was used for Gram staining. Firstly, thin Smears of isolates were prepared ith sterilized water on clean glass slides and the smears were dried and fixed (slightly heated), afterward fixed smears were flooded with crystal violet stain for 1 min and washed with distilled water now smear were flooded with iodine solution for 1 min and washed with distilled water and decolorized with alcohol for 5-10 seconds and again washed with distilled water. Finally, smear were subjected to the counter stain safranin for 10 seconds, washed, stained slides and left for air dry for observing under research microscope at 40X,100 X.

3.2.1.4. Biochemical characterization:

For biochemical tests, kits available from Hi-Media (Hi-Pure Bacterial Identification Kit) were used. Individual bacterial suspension (100 µl having 10^6 CFU/ml) was inoculated in each well of test strips and allowed to incubate for 24 h at 28 ± 2˚C. After incubation, observations were recorded by applying appropriate reagents provided with the kit. Many biochemical tests were carried out for characterization of rhizospheric isolates were discussed below.

3.2.1.4.1. Catalase activity:

Bacterial cultures were grown in nutrient broth medium for 48 h at 28℃. 48 hr old bacterial colonies were added with 2-3 drops of hydrogen peroxide (3%) on a clean glass slide and mixed using a sterile tooth pick. The evolution of oxygen as effervescence indicated positive catalase activity.

3.2.1.4.2. Oxidase test:

Bacterial cultures were grown in nutrient broth medium for 48 h at 28℃, than by a loop pick a well isolated colony and rub on oxidase disc and observed the color changes Microorganism are oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

3.2.1.4.3. Starch hydrolysis:

On the starch agar plates, the bacterial cultures were streaked and allowed the microbes to grow at 37℃ for 48 hrs. Pour iodine solution in the plate. The blue-black color appears due to formation starch iodine complex. If the area around streaked culture remains clear it indicates the degradation of starch has occurred due to production of amylase.

3.2.1.4.4. Urease activity:

Bacterial cultures were grown in Christensen's Urea Agar medium for 24- 48 hour at 28℃. A heavy inoculum used from an 18- to 24-hour pure culture to streak the entire slant surface. The butt (without stab) served as a color control. Tubes with loosened caps incubated at 35℃. The slants observed for a color change at 6 hours, 24 hours, and every day for up to 6 days. Urease production was indicated by a bright pink (fuchsia) color on the slant. Any degree of pink considered as positive reaction. Prolonged incubation was avoided as it may result in a false-positive test due to hydrolysis of proteins in the medium. To eliminate protein hydrolysis as the cause of a positive test, a control medium lacking urea was also used.

3.3. Characterization of bacterial isolate for plant growth promoting properties

Bacterial isolates were analyzed for the following PGP traits:-

3.3.1. HCN production:

Bacterial isolates were screened for the HCN production by adapting the method of Lorck, (1948) Color change of the Whatman filter paper from deep yellow to reddish-brown color indicated production of HCN.

3.3.2. IAA production:

IAA production was detected by the modified method as described by Brick *et. al.* (1991). Soil microorganisms including bacteria, fungi, and algae are also capable of producing physiologically active quantities of auxin. The entire cultures were incubated in the peptone broth enriched with tryptophan broth to check for production of indole acetic acid a precursor of auxin which is an important plant hormone. The quantitative estimation of IAA is performed by using Salkowaski method by using the reagent (50 ml, 35% of perchloric

acid, 1 ml of 0.5 M $FeCl_3$ solution and mixture were incubated at room temperature for 30 min, and 90 min and observe for pink color production and read calorimetrically at OD 530 nm.

3.3.3. Phosphate solubilization:

A loop full of fresh bacterial cultures was streaked on the centre of agar plates containing Pikovskaya agar with insoluble tricalcium phosphate (TCP) and incubated for 120 h at 28±2℃. The presence of halo zone around the bacterial colonies indicated positive phosphate solubilization ability. (Pikovskaya, 1948).

3.4. Analysis of soil samples for microbiological and nutrient parameters

Soil was analysis on the following properties such as:

3.4.1. Dehydrogenase activity (DHA)

Dehydrogenase activity was assayed by the method of Casida *et. al.* (1964). One gram of fresh processed (<2 mm) soil sample, determined moisture content gravimetrically was taken in a screw cap test tube (15 ml capacity). To it, 0.2 ml of 3% 2,3,5-Triphenyl tetrazolium chloride and 0.5 ml of 1% glucose were added. After mixing the content, the tubes were incubated for 24 hours at 30℃. Once the process of incubation was over, it was followed by the addition of 10 ml of methanol to it. The whole material was mixed thoroughly for 1 minute. After mixing, the tubes were placed in a refrigerator for 3 hours. The production of triphenyl formazon was determined by measuring absorbance at 485 nm.

3.4.2. Fluorescein diacetate (FDA) hydrolysis

The hydrolysis of FDA in soil is linear with time and soil up to 3 hours and 2 g dry weight, respectively (Schnurer and Rooswall 1982. Field-moist soil seived (2 mm mesh) and stored at 4℃ before analysis. Soil (2g dry weight) placed in an Erlenmeyer flask (250 ml) and treated with 25-100ml phosphate butter, then fluorescein diacetate (final

concentration of 10 micro gram/ml) is added, and the mixture incubated on a rotary shaker at 24℃ for up to 3 hours. After the incubation the reaction was stop by adding acetone to reach a final concentration of 50% v/v. The soil suspension is the centrifuged at 4000 rev. min for 10-15 min and the optical density of the clear supernatant is measured at 490 nm.

3.4.3. Microbial Biomass Carbon (MBC)

Soil microbial biomass carbon (SMB-C) was determined by chloroform fumigation method, (Nunan *et al.,* 1998), moist soil (7 g dry weight) split into two samples (each 3.5 g dry weight). The fumigated control placed in a 250 ml bottle and then immediately extracted with 100 ml 0.5 M K_2SO_4, (ratio extractant: soil (dry weight is 4:1 v/w) for 30 min in an oscillating shaker at 200 rev min (or 45 min for an overhead shaker at 40 rev min and then filtered through a paper filter (Whatman no. 42). The fumigation is carried out using a 50 ml glass vial which contains the moist soil; the vial is placed in a desiccators lined with wet tissue paper and a vial with soda lime. A beaker containing 25 ml ethanol-free $CHCl_3$, and a few boiling chips is added, and the desiccator evacuated until the $CHCl_3$ has boiled vigorously tor 2 min. The desiccator is then incubated in the dark at 25°C for 24 h. After fumigation, $CHCl_3$ removed by repeated six-fold) evacuations and the soil is transferred to 250ml bottles for extraction with 0.5 M K_2SO_4 as mentioned for the non-fumigated sample. All extracts are stored at -15℃ prior to analysis.

3.4.4. Organic Carbon

Rapid titration method (wet digestion method) was used for organic carbon determination (Walkley and Black 1934). In this determination 2 gm of dried soil was treated with 10 ml of 1N $K_2Cr_2O_7$ solution in a 250 ml conical flask. A 20 ml of concentrated H_2SO_4 was slowly added to the flask. After 30 minutes, about 0.5 gm of NaF, 100 ml of distilled water and 10 drops of diphenylamine indicator were

added to the flask. These contents were titrated against 0.5N ferrous ammonium sulphate solution. The change from violet to bright green through blue colour was the end point. The value of ferrous ammonium sulphate used for titration was adopted for calculating organic carbon and was expressed as percentage. In another flask, 10 ml of 1N $K_2Cr_2O_7$ solution was titrated without soil against 0.5N ferrous ammonium sulphate solution to determine blank reading.

3.4.5. Available Nitrogen

Nitrogen in soil sample was determined by distillating soil in alkaline permanganate method given by Subbiah and Asija (1956). Weighing 5.0 g of soil and transfer it to macro-digestion tube (250ml dip the receiver tube end in boric acid solution. Preset the time for 6 minutes and switch-on for adding 35 ml of 2.5% NaOH solution. On the timer switch and run the distillation for 6 minutes. Remove the conical Mask after the timer switch goes off automatically i.e. after 6 min. Titrate the content of the flask against 0.02 N H_2SO_4 till color changes from bluish green to brick red. Run a blank set simultaneously.

$$\text{Available N} = \frac{\text{reading ml (Sample blank) X strength of } H_2SO_4 \text{X } 0.014 \text{ X } 2.24 \text{ X } 10^6 \text{ (kg/ha)}}{\text{Weight of the oven dry soil in gram}}$$

3.5. Molecular characterization of bacterial isolates

3.5.1. Bacterial DNA isolation and Quantification

1) DNA was isolated by freeze and thaw method with some modifications. (Shanker and Kaur, 2018)

2) Single colony from each purified bacterial culture was inoculated into 10 ml nutrient broth (**Appendix-II**) at 28 ± 2˚C for 24 h on environmental shaker at 160 rpm.

3) 2.0 ml bacterial culture was taken into 2.0 ml centrifuge tube and then centrifuged at 10,000 rpm for 10 min at 4˚C.

4) The supernatant was discarded and the pellet was used for extraction of DNA.

5) 200µl nuclease free water was added into the pellet and mixed it well by gentle tapping.

6) The solution was kept at a -40℃ for 20 minutes. Thereafter, boiled at 90℃ in water bath for 10 min.

7) The solution was kept again at -40℃ for 20 min.

8) Then, kept at room temperature for 5-10 min and centrifuged at full speed (12000 rpm) for 2 min.

9) Finally, supernatant was transferred to fresh micro centrifuge tube 1.5ml and store DNA at -20℃.

The integrity of DNA was assessed by agarose gel electrophoresis analysis by casting of 0.8% agarose gel in 150 ml 0.5X TBE (Tris Borate EDTA) buffer containing 0.5 pg/ml of ethidium bromide. Electrophoresis was carried out at 120V for 28 min. The quality of DNA in the gel was visualized under UV light using UV-transilluminator. The gel images were documented using Bio-imaging System (Syngene, UK).

DNA was also quantified using Spectrophotometer (LabMate Asia Pvt Ltd, India). The quality of DNA was assessed at A_{260} nm absorbance and the 1.8 ratio of A_{260}/A_{280} was considered of quality parameter of the DNA.

3.5.2. PCR profiling of ScoT markers

For assessing molecular diversity among the bacterial strains, functional molecular marker such as ScoT was profiled using PCR amplification on genomic DNA of bacteria. The standard PCR amplification process was adopted in the present study. The PCR reaction mixture was prepared in 10 µl reaction volume by adding the

following reaction components **(Table: 3.3)** and added to the solution of priming step:

Table 3.3: PCR reaction mixture

Component	Quantity in µl
Go Taq reaction mixture (2X)	5
$MgCl_2$ (25mM)	0.4
Primers (10mM)	1.5
DNA (50ng/ml)	2
Nuclease free water	1.1
TOTAL	**10µl**

The tubes were placed in the Thermal Cycler (Eppendorf, Germany) for amplification. The thermal cycling conditions for amplification were programmed in Thermal cycler (GeneMate) as mentioned in **Table 3.4.**

Table 3.4: PCR conditions for amplification:

S. No.	Step	Temp.	Time	Cycle(s)
1.	Initial Denaturation	95℃	5 min	1
2.	Denaturation	95℃	0.30 sec	40
	Primer annealing	45℃	0.30 sec	
	Primer extension	72℃	2 min	
3.	Final Extension	72℃	10 min	1

3.5.3. Gel electrophoresis of PCR products

The PCR product were subjected to gel electrophoresis on 1.5% agarose gel by casting 100-150 ml agarose gel in 0.5X TBE (Tris

Borate EDTA) buffer and boiled to dissolve the agarose. When the agarose reached at room temperature then 0.5 pg/ml of ethidium bromide was added and kept it for solidify. After that the samples were loaded in the wells of agarose gel. The gel was run at 120 V for 1-2 h. To determine the fragment size, standard 1 kb DNA ladder markers (Fermentas, USA, **Appendix-II**) was loaded apart from the PCR samples. Gels were visualized under UV gel reader and photographed using Gel Documentation system (SynGene, UK).

3.5.4. Band Scoring

The bands were score as binary matrix (0, 1). The '0' represented as absence of the band, whereas '1' represented as presence of the band. Faintly visible bands were not scored but the major bands corresponding faint bands were considered for scoring. In order to confirm the presence of bands and determine reproducibility all the primers were replicated two times.

3.5.5. Polymorphism analysis

The polymorphism information of profiled ScoT markers was assessed by using iMEC (Amiryousefi *et. al.,* 2018) online software by feeding of binary matrix data. The value inferred by iMEC software was recorded and interpretation was infered.

3.5.6. Phylogenetic analysis

The phylogenetic analysis assessed by using binary matrix in NTSys 2.0 software (Rohlf, 1998). The pair-wise association coefficients were calculated from qualitative data matrix using jaccard's similarity coefficient. Cluster analysis for the genetic distance was carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships among the clones.

3.5.7. Population structure analysis

The scored binary matrix data from molecular analysis was used to determine the number of populations present in bacterial strains of the Sewan and Dhaman rhizosphere using STRUCTURE 2.0 software (www.stanford.edu).

3.6 Statistical Analysis

The data collected on different experiments and parameters from field were subjected to statistical analysis by using Completely Randomized Block Design (CRD) at *p*-value <0.05 and within the isolates, ANOVA was done.

4. RESULT

The present research entitled **"Characterization and Diversity Analysis of Bacterial Isolates from Rhizosphere of Sewan (*Lasiurus scindicus*) and Dhaman (*Cenchrus setigerus*) Grasses"** was conducted under well managed laboratory conditions at Department of Biotechnology, Plant Biotechnology Centre, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University (SKRAU), Bikaner and ICAR-Central Institute of Arid Horticulture, Bikaner. The soil samples from rhizosphere and adjoining region of sewan (*Lasiurus scindicus*) and dhaman (*Cenchrus setigerus*) grasses were collected from three places were used in this study. In present era organic and sustainable agricultural production, the interaction with in microbes and rhizosphere root of crops result such as mineralization, solubilization and mobilization etc. The main aim was to isolates and to evaluate plant growth promoting potential of these bacteria from rhizosphere and non rhizosphere soil of sewan and dhaman grasses and further may be used as inoculants for growth and yield.

The various observations were taken from bacterial isolates such as morphological, biochemical, microscopic, PGP potential and molecular characterization along with soil attributes such as nutrient status and soil microbiological activities are discussed in this chapter.

4.1 Collection of soil sample from rhizosphere and non rhizosphere soil of sewan and dhaman

Soil sample were collected randomly from the rhizosphere and non rhizosphere region of selected plants of sewan and dhaman grasses growing at different sites such as (i) Agricultural Research Station (ARS), Bikaner, (ii) CAZRI and adjoining places, Jodhpur and (iii) Jaisalmer. Total 12 categories of samples collected from rhizosphere and non rhizosphere zone of sewan and dhaman grasses growing at ARS, Bikaner; CAZRI and adjoining places, Jodhpur;

Jaisalmer of Rajasthan and designated as BKSNR, BKDR, BKDNR, JUSR, JUSNR, JUDR, JUDNR JSMSR, JSMSNR, JSMDR, JSMDNR (Table 4.1) (Fig. 4.1).

Table 4.1: Soil sample collected from Rhizosphere and non-Rhizosphere soil

One letter Code	Sample Code	Plant Name	Location	Soil type
A	BKSR	Sewan	BIKANER	Rhizosphere
B	BKSNR			non- Rhizosphere
C	BKDR	Dhaman		Rhizosphere
D	BKDNR			non- Rhizosphere
E	JUSR	Sewan	JODHPUR	Rhizosphere
F	JUSNR			non- Rhizosphere
G	JUDR	Dhaman		Rhizosphere
H	JUDNR			non- Rhizosphere
I	JSMSR	Sewan	JAISALMER	Rhizosphere
J	JSMSNR			non- Rhizosphere
K	JSMDR	Dhaman		Rhizosphere
L	JSMDNR			non- Rhizosphere

4.1.1 Isolation of bacteria from rhizosphere and adjacent soil of sewan and dhaman

A total of 30 bacterial isolates were identified based on the different morphological characteristics and designated from AS-1 to AS-30. Isolations were made by taking one gram air dried, sieved soil

A

B

Fig. 4.1 A. Sewan and B. Dhaman grasses

and plating on nutrient agar (NA) media after making appropriate serial dilution.

4.1.2 Morphological characterization (colony character) of bacterial isolates

A total 30 isolates were observed for morphological characterization by growing on nutrient agar plates and these isolates were examined for colony characters such as shape, margin, elevation, texture, opacity, size and pigmentation (Table 4.2). After incubating for up to 72 hours bacterial isolates shown variable growth patterns. Out of 30 isolates 23 isolates detected with circular shaped colony (AS-1, AS-2, AS-3, AS-5, AS-6, AS-8, AS-9, AS-10, AS-11, AS-12, AS-14, AS-15, AS-16, AS-17, AS-19, AS-20, AS-21, AS-22, AS-25, AS-26, AS-28, AS-29 and AS-30) and six isolates detected with irregular shaped colony (AS-7, AS-13, AS-18, AS-23, AS-24 and AS-27) and one isolate detected as filamentous shaped colony (AS-4).

Five distinct types of colony margin were found. Among these 18 bacterial isolates detected with entire colony margin (AS-1, AS-2, AS-3, AS-5, AS-6, AS-10, AS-11, AS-12, AS-14, AS-15, AS-16, AS-20, AS-21, AS-22, AS-25, AS-26, AS-28 and AS-29), 7 isolates detected filiform colony margin (AS-4, AS-8, AS-13, AS-17, AS-23, AS-24 and AS-30), two isolates detected curled colony margin (AS-19 and AS-27), two isolates detected with lobate colony margin (AS-7 and AS-9), and one as undulate colony margin (AS-18).

Colony elevation of bacterial isolates were found as convex, flat and raised. Out of total 30 isolates, 21 were observed as convex colony elevation (AS-1, AS-2, AS-4, AS-5, AS-6, AS-10, AS-11, AS-12, AS-13, AS-15, AS-16, AS-19, AS-20, AS-21, AS-22, AS-25, AS-26, AS-27, AS-28, AS-29 and AS-30), six isolates were observed having flat colony elevation (AS-7, AS-8, AS-9, AS-14, AS-18 and AS-23) and

three isolates were detected with raised colony elevation (AS-3, AS-17 and AS-24).

Colony textures were identified as smooth and rough in the isolates. Out of total 30 bacterial isolates, 20 isolates were identified as smooth (AS-1, AS-2, AS-3, AS-4, AS-5, AS-6, AS-10, AS-11, AS-12, AS-13, AS-16, AS-19, AS-20, AS-21, AS-22, AS-25, AS-26, AS-27, AS-28 and AS-29) and rough texture was found in 10 isolates (AS-7, AS-8, AS-9, AS-14, AS-15, AS-17, AS-18, AS-23, AS-24 and AS-30).

Out of these 30 bacterial isolates, three types of colony opacity opaque, transparent and translucent were observed. Among which 17 bacterial isolates were observed opaque colony opacity (AS-1, AS-3, AS-7, AS-8, AS-9, AS-10, AS-14, AS-15, AS-17, AS-18, AS-22, AS-23, AS-26, AS-27, AS-28, AS-29 and AS-30) and 12 isolates were observed having translucent colony opacity (AS-2, AS-4, AS-5, AS-11, AS-12, AS-13, AS-16, AS-19, AS-20, AS-21, AS-24 and AS-25). One isolated depicted transparent opacity.

When analyzed for colony size out of the total 30 isolates made, 23 showed medium size (AS-1, AS-2, AS-3, AS-4, AS-5, AS-6, AS-7, AS-8, AS-9, AS-12, AS-13, AS-14, AS-16, AS-17, AS-19, AS-20, AS-21, AS-22, AS-24, AS-27, AS-28, AS-29 and AS-30), four isolates were observed large size (AS-15, AS-18, AS-23 and AS-26) and three isolates were observed with small size (AS-10, AS-11 and AS-25).

Table 4.2: Morphological features in terms of isolates from Rhizosphere and non Rhizosphere

S. NO.	Isolates	Colony Shape	Colony Margin	Colony Elevation	Colony Texture	Colony opacity	Colony Size	Pigment Production
1.	AS-1	Circular	Entire	Convex	Smooth	Opaque	Medium	Off white
2.	AS-2	Circular	Entire	Convex	Smooth	Translucent	Medium	Orange
3.	AS-3	Circular	Entire	Raised	Smooth	Opaque	Medium	White
4.	AS-4	Filamentous	Filiform	Convex	Smooth	Translucent	Medium	Light yellow
5.	AS-5	Circular	Entire	Convex	Smooth	Translucent	Medium	Light yellow
6.	AS-6	Circular	Entire	Convex	Smooth	Transparent	Medium	Transparent
7.	AS-7	Irregular	Lobate	Flat	Rough	Opaque	Medium	White
8.	AS-8	Circular	Filiform	Flat	Rough	Opaque	Medium	Light orange
9.	AS-9	Circular	Lobate	Flat	Rough	Opaque	Medium	Light yellow
10.	AS-10	Circular	Entire	Convex	Smooth	Opaque	Small	Orange
11.	AS-11	Circular	Entire	Convex	Smooth	Translucent	Small	Light yellow
12.	AS-12	Circular	Entire	Convex	Smooth	Translucent	Medium	White
13.	AS-13	Irregular	Filiform	Convex	Smooth	Translucent	Medium	Light yellow
14.	AS-14	Circular	Entire	Flat	Rough	Opaque	Medium	Off white
15.	AS-15	Circular	Entire	Convex	Rough	Opaque	Large	White
16.	AS-16	Circular	Entire	Convex	Smooth	Translucent	Medium	Light yellow
17.	AS-17	Circular	Filiform	Raised	Rough	Opaque	Medium	Light yellow
18.	AS-18	Irregular	Undulate	Flat	Rough	Opaque	Large	White
19.	AS-19	Circular	Curled	Convex	Smooth	Translucent	Medium	White

S. NO.	Isolates	Colony Shape	Colony Margin	Colony Elevation	Colony Texture	Colony opacity	Colony Size	Pigment Production
20.	AS-20	Circular	Entire	Convex	Smooth	Translucent	Medium	White
21.	AS-21	Circular	Entire	Convex	Smooth	Translucent	Medium	White
22.	AS-22	Circular	Entire	Convex	Smooth	Opaque	Medium	Off white
23.	AS-23	Irregular	Filiform	Flat	Rough	Opaque	Large	Light orange
24.	AS-24	Irregular	Filiform	Raised	Rough	Translucent	Medium	White
25.	AS-25	Circular	Entire	Convex	Smooth	Translucent	Small	Orange
26.	AS-26	Circular	Entire	Convex	Smooth	Opaque	Large	Light yellow
27.	AS-27	Irregular	Curled	Convex	Smooth	Opaque	Medium	Pink
28.	AS-28	Circular	Entire	Convex	Smooth	Opaque	Medium	Orange
29.	AS-29	Circular	Entire	Convex	Smooth	Opaque	Medium	Light yellow
30.	AS-30	Circular	Filiform	Convex	Rough	Opaque	Medium	Light yellow

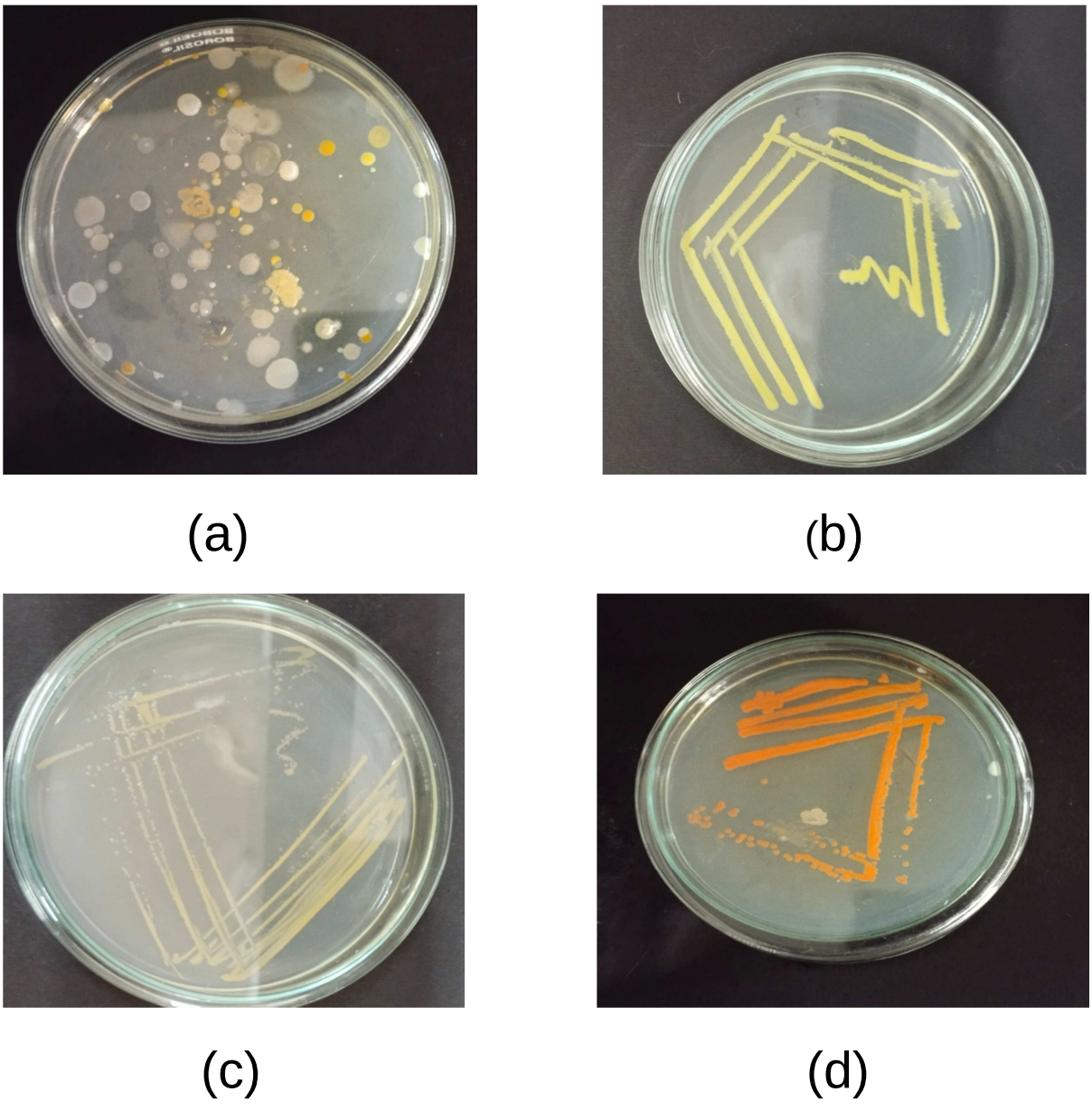


Fig. 4.2: (a) Culturable diversity of bacteria isolatesd from sewan and dhaman grasses and bulk soil samples in terms of colony morphology as observed on nutrient agar plates, (b) AS-11, (c) AS-23 and (d) AS-28

Production of different pigments were found among these bacterial isolates, nine isolates shown white color (AS-3, AS-7, AS-12, AS-15, AS-18, AS-19, AS-20, AS-21 and AS-24), ten isolates produced light yellow pigmentation (AS-4, AS-5, AS-9, AS-11, AS-13, AS-16, AS-17, AS-26, AS-29 and AS-30), four isolates produced orange coloration (AS-2, AS-10, AS-25 and AS-28), two produced light orange (AS-8 and AS-23), three isolates shown the off white coloration (AS-1, AS-14 and AS-22), only one isolate produced pink coloration (AS-27) and one was observed as transparent colony (AS-6) (Fig. 4.2).

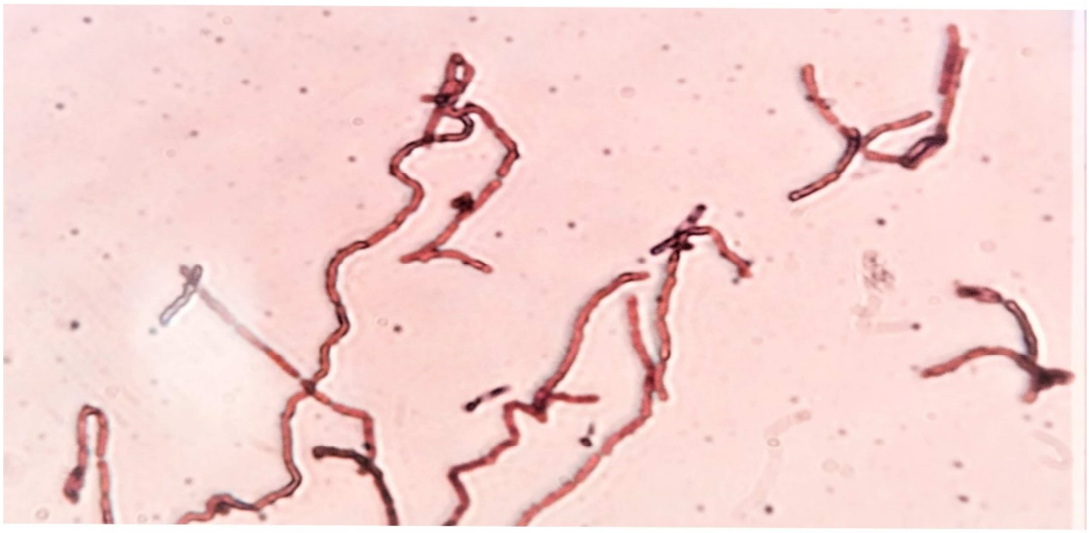
4.1.3 Enumeration of bacteria from Rhizosphere and non Rhizosphere soil samples

Total 12 type of soil sample collected from three districts of Rajasthan (Table 4.1) designated as A to L sample, in which A and B sample were from rhizosphere and non rhizosphere soil from sewan grass, C and D sample from rhizosphere and non rhizosphere soil from dhaman grass, growing at ARS Bikaner. E and F sample were rhizosphere soil and non rhizosphere soil from sewan grass, G and H sample from rhizosphere soil and non rhizosphere soil from dhaman grass collected from CAZRI and adjacent area at Jodhpur. I and J sample from rhizosphere region and non rhizosphere region of sewan grass, K and L sample from rhizosphere and non rhizosphere region from dhaman grass growing at Jaisalmer of Rajasthan. Bacterial population ranged from 1.17×10^6 cfu/g in non rhizosphere soil to 5.71×10^6 cfu/g in rhizosphere soil (Table 4.3). A compile study of these three districts of Rajasthan higher to low bacterial population, 8.8×10^6 cfu/g rhizosphere soil from dhaman grass in Jodhpur followed by, 7.63×10^6 cfu/g rhizosphere soil from sewan and 6.0×10^6 cfu/g rhizosphere soil from dhaman grass in ARS Bikaner and 4.2×10^6 cfu/g rhizosphere soil and 5.33×10^5 cfu/g non rhizosphere soil from sewan grass, 6.33×10^5 cfu/g non rhizosphere soil from dhaman grass in jaisalmer (Table 4.3). In Conclusion relative study of bacterial

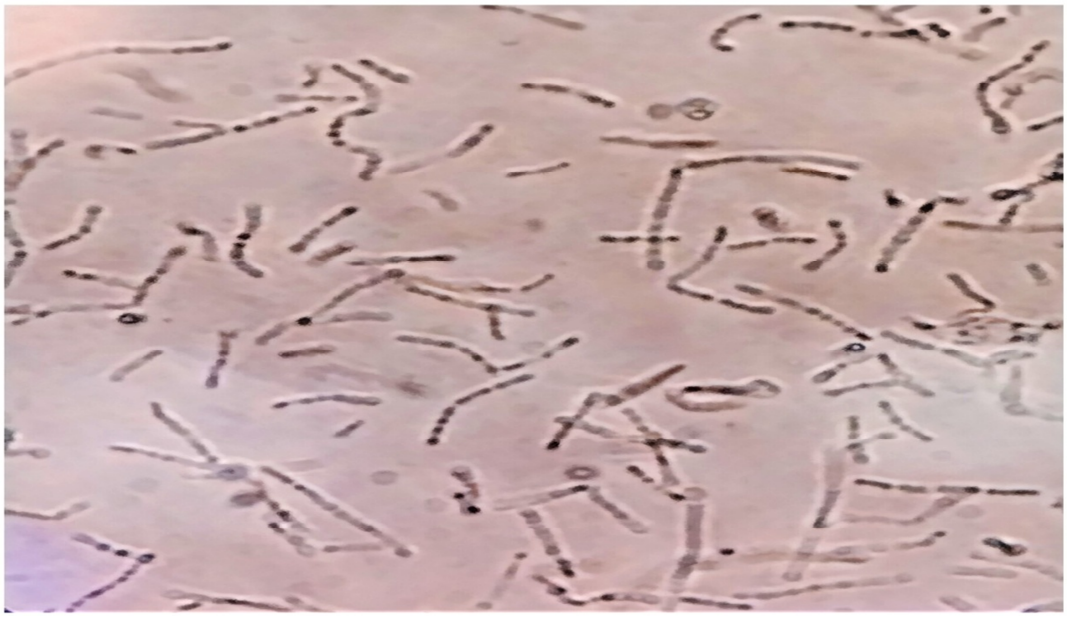
population of rhizosphere soil found higher than non rhizosphere soil of these three districts of Rajasthan. The highest bacterial population were recorded in Jodhpur dhaman rhizosphere soil and when analyzed for sewan grass, ARS Bikaner rhizosphere soil evaluated the highest bacterial population density (76.33 cfu/g soil) (Table 4.4).

Table 4.3: Effect of sampling site and host plant in accord with proximity to plant roots on cultivable bacterial population of soil

	Population count
A. Location	**cfu/g($x10^5$)**
A1	40.58
A2	40.42
A3	22.33
S Em±	0.99
CDat5%	2.91
B. Host plant	
B1	31.06
B2	37.83
S Em±	0.82
CDat5%	2.38
C. Distance from root zone	
C1	57.11
C2	11.78
S Em±	0.82
CDat5%	2.38



(a)



(b)

Fig. 4.3: Microscopic image of isolates upon Gram staining (400X): (a) Rod shaped bacteria (AS-18) and (b) Coccus shaped bacteria (AS-29).

Table 4.4: Combined effect of sampling site and host plant in accord with proximity to plant roots on cultivable bacterial population of soil

	cfu/g(x10^5)					
A x B x C	**A_1**		**A_2**		**A_3**	
	B_1	**B_2**	**B_1**	**B_2**	**B_1**	**B_2**
C_1	76.33	60.00	40.67	88.00	42.33	35.33
C_2	9.33	16.67	12.33	20.67	5.33	6.33
S Em±				1.99		
CDat5%				5.83		

*****Conoly forming unit (cfu/g)

*A1= Bikaner, A2= Jodhpur, A3= Jaisalmer, B1= Sewan (*Lasiurus scindicus*),

B2= Dhaman (*Cenchrus setigerus*), C1= rhizosphere soil, C2 = non-rhizosphere soil

4.1.4 Microscopic observations of isolates

Total 30 bacterial isolates were observed through Gram`s staining for cell morphological characterization (Table 4.5). Among these, 28 isolates were found gram negative (AS-1, AS-2, AS-3, AS-5, AS-7, AS-8, AS-9, AS-10, AS-11, AS-12, AS-13, AS-14, AS-15, AS-16, AS-17, AS-18, AS-19, AS-20, AS-21, AS-22, AS-23, AS-24, AS-25, AS-26, AS-27, AS-28, AS-29 and AS-30) and two isolates were gram positive (AS-4 and AS-6). When observed under compound microscope, 21 bacterial isolates showed as coccus (AS-2, AS-3, AS-4, AS-5, AS-6, AS-8, AS-10, AS-11, AS-12, AS-13, AS-16, AS-17, AS-19, AS-20, AS-22, AS-23, AS-25, AS-26, AS-28, AS-29 and AS-30) and nine isolates were found as rod shaped (AS-1, AS-7, AS-9, AS-14, AS-15, AS-18, AS-21, AS-24 and AS-27) (Fig. 4.3).

4.2: Biochemical characterization of bacterial isolates

A total 30 bacterial isolates were studied for various biochemical studies and results are presented in Table 4.6. Out of these, 29 isolates were found positive for catalase activity, of which 15 isolates showed high activity (AS-2, AS-3, AS-4, AS-6, AS-7, AS-12, AS-14, AS-15, AS-17, AS-20, AS-23, AS-24, AS-25, AS-26 and AS-28), four bacterial isolates showed medium activity (AS-9, AS-10, AS-18 and AS-22), While ten showed relatively less catalase activity (AS-1, AS-5, AS-11, AS-13, AS-16, AS-19, AS-21, AS-27, AS-29 and AS-30) and one isolate found negative for this activity (AS-8). All the 30 bacterial isolates were detected positive for oxidase enzyme activity. Among these 13 were found high in oxidase activity (AS-2, AS-4, AS-8, AS-13, AS-14, AS-15, AS-17, AS-21, AS-22, AS-24, AS-25, AS-29 and AS-30), ten isolates showed medium (AS-3, AS-5, AS-6, AS-9, AS-12, , AS-16, AS-20, AS-23, AS-26 and AS-28) and high starch hydrolysis (AS-13 and AS-30) and 16 isolates were showed (AS-1, AS-3, AS-4, AS-6, AS-9, AS-11, AS-14, AS-15, AS-16, AS-17, AS-18, AS-20, AS-23, AS-24, AS-28 and AS-29) less starch hydrolysis and 12 bacterial isolates were not shown starch hydrolysis activity (AS-2, AS-5, AS-7, AS-8, AS-10, AS-12, AS-19, AS-21, AS-25, AS-26 and AS-27). All the 30 bacterial isolates were studied for urease activity; among these 17 bacterial isolates showed positive for urease activity (AS-1, AS-4, AS-6, AS-7, AS-11, AS-14, AS-15, AS-17, AS-18, AS-20, AS-21, AS-22, AS-23, AS-24, AS-25, AS-29 and AS-30) and 13 bacterial isolates showed negative for urease activity (AS-2, AS-3, AS-5, AS-8, AS-9, AS-10, AS-12, AS-13, AS-16, AS-19, AS-26, AS-27 and AS-28) (Fig. 4.4).

Table 4.5: Microscopic characteristics of microscope

S. NO.	Isolates	Shape	Gram's Reaction
1.	AS-1	Rod	Gm –ve
2.	AS-2	Coccus	Gm –ve
3.	AS-3	Coccus	Gm –ve
4.	AS-4	Coccus	Gm +ve
5.	AS-5	Coccus	Gm –ve
6.	AS-6	Coccus	Gm +ve
7.	AS-7	Rod	Gm –ve
8.	AS-8	Coccus	Gm –ve
9.	AS-9	Rod	Gm –ve
10.	AS-10	Coccus	Gm –ve
11.	AS-11	Coccus	Gm –ve
12.	AS-12	Coccus	Gm –ve
13.	AS-13	Coccus	Gm –ve
14.	AS-14	Rod	Gm –ve
15.	AS-15	Rod	Gm –ve
16.	AS-16	Coccus	Gm –ve
17.	AS-17	Coccus	Gm –ve
18.	AS-18	Rod	Gm –ve
19.	AS-19	Coccus	Gm –ve
20.	AS-20	Coccus	Gm –ve
21.	AS-21	Rod	Gm –ve
22.	AS-22	Coccus	Gm –ve
23.	AS-23	Coccus	Gm –ve
24.	AS-24	Rod	Gm –ve
25.	AS-25	Coccus	Gm –ve
26.	AS-26	Coccus	Gm –ve
27.	AS-27	Rod	Gm –ve
28.	AS-28	Coccus	Gm –ve
29.	AS-29	Coccus	Gm –ve
30.	AS-30	Coccus	Gm –ve

Table 4.6: Biochemical characterization of isolates

S. No.	Isolates Name	Catalase Activity	Oxidase Activity	Starch Hydrolysis	Urease Activity
1.	AS-1	+	+	+	+
2.	AS-2	+++	+++	-	-
3.	AS-3	+++	++	+	-
4.	AS-4	+++	+++	+	+
5.	AS-5	+	++	-	-
6.	AS-6	+++	++	+	+
7.	AS-7	+++	+	-	+
8.	AS-8	-	+++	-	-
9.	AS-9	++	++	+	-
10.	AS-10	++	+	-	-
11.	AS-11	+	+	+	+
12.	AS-12	+++	++	-	-
13.	AS-13	+	+++	+++	-
14.	AS-14	+++	+++	+	+
15.	AS-15	+++	+++	+	+
16	AS-16	+	++	+	-
17.	AS-17	+++	+++	+	+
18.	AS-18	++	+	+	+
19.	AS-19	+	+	-	-
20	AS-20	+++	++	+	+
21.	AS-21	+	+++	-	+
22.	AS-22	++	+++	-	+
23.	AS-23	+++	++	+	+
24.	AS-24	+++	+++	+	+
25.	AS-25	+++	+++	-	+
26.	AS-26	+++	++	-	-
27.	AS-27	+	+	-	-
28.	AS-28	+++	++	+	-
29.	AS-29	+	+++	+	+
30.	AS-30	+	+++	+++	+

-=no production, + = less production, ++ = moderate production and +++ = High production

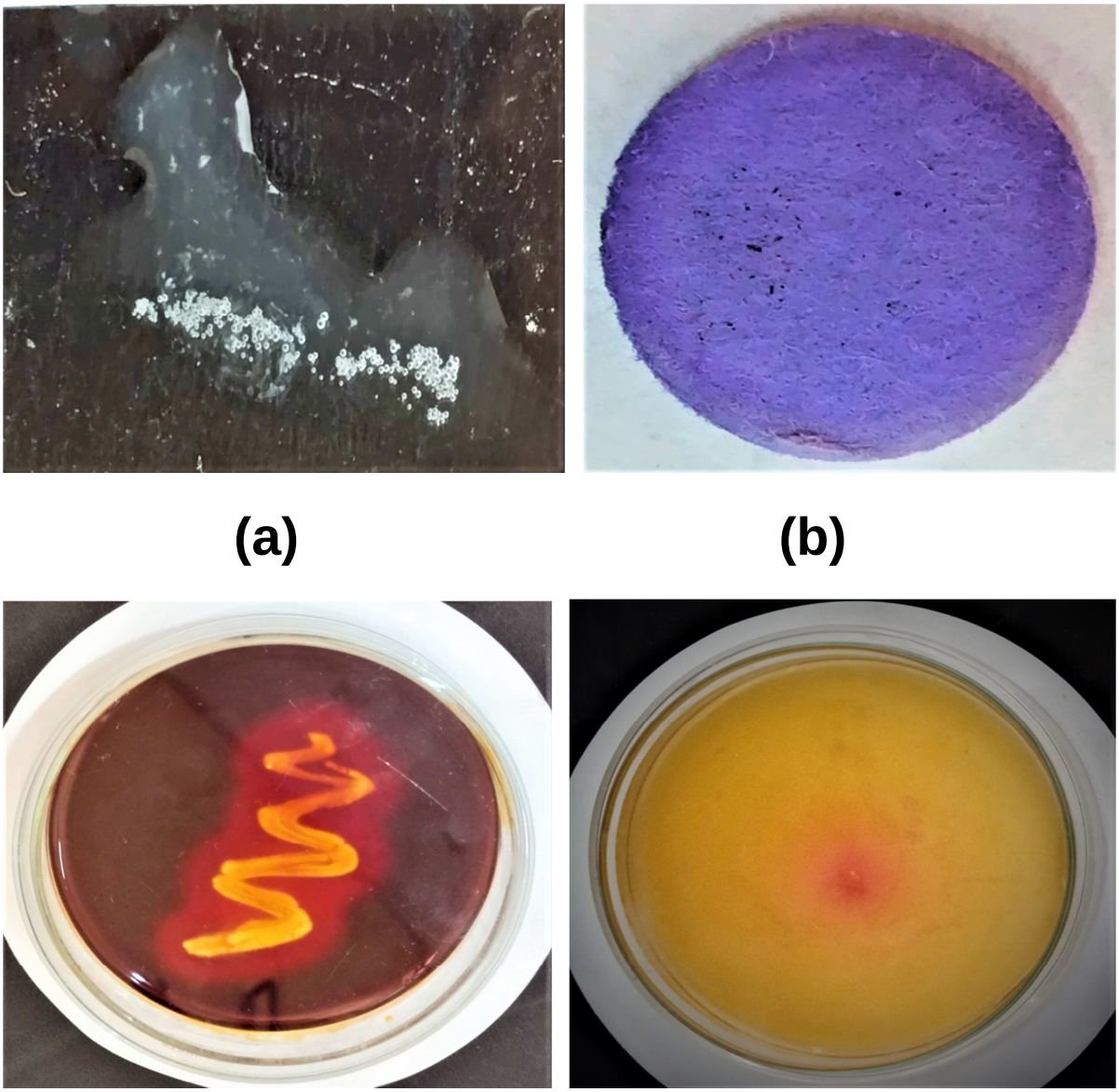


Fig. 4.4: Biochemical characterization of isolates:

- **(a) Catalase activity (AS-24),**
- **(b) Oxidase activity (AS-23),**
- **(c) Starch hydrolysis (AS-13) and**
- **(d) Urease activity (AS-18)**

4.3 Characterization for plant growth promoting properties of the isolates

All the isolates were characterized for their plant growth promoting potential activities such as production of HCN, IAA and phosphate solubilization of tricalcium phosphate (Fig. 4.5).

4.3.1 HCN production

The isolates were tested for plant growth promoting potential in terms of HCN production on 200 ml Luria broth in 25 ml tubes with 0.88 g glycine. Among these six isolates were detected with high HCN production activity (AS-1, AS-14, AS-15, AS-16, AS-17 and AS-26), seven isolates were detected moderate HCN production activity (AS-3, AS-4, AS-6, AS-13, AS-20, AS-25 and AS-28), eight isolates were detected less HCN production activity (AS-9, AS-12, AS-18, AS-19, AS-21, AS-22, AS-27 and AS-29) and nine bacterial isolates did not show HCN production (AS-2, AS-5, AS-7, AS-8, AS-10, AS-11, AS-23, AS-24 and AS-30). All the 30 bacterial isolates HCN production activity were summarized in Table 4.7.

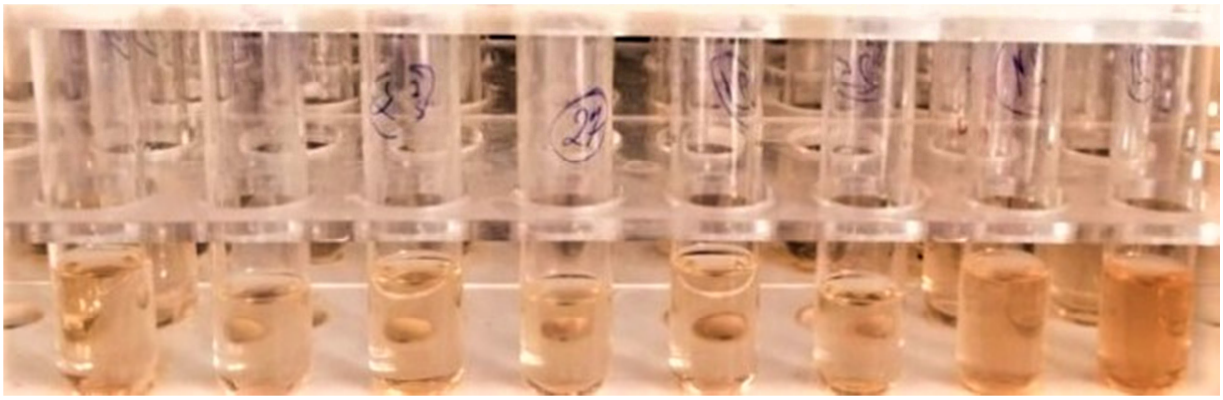
4.3.2 IAA production activity

For estimation of the IAA (indole acetic acid) production potential (Table 4.7) all of these isolates were grown on 300 ml peptone broth containing 0.75 mg tryptophan (precursor of IAA). Contrasting results were detected for IAA production. All the 30 isolates found capable of IAA production. The activity ranged from 0.636µg/ml (AS-16 and AS-26) to 0.848 µg/ml (AS-21). After 48 to 72 hrs. of incubation; it was recorded that the highest concentration was in the isolate AS-21 (0.848 µg/ml) followed by AS-6 (0.821 µg/ml) and bacterial isolates having least concentration were AS- 16 (0.636 µg/ml), AS- 26 (0.636 µg/ml) and AS- 29 (0.642 µg/ml).

4.3.3 Phosphate solubilization activity

All the 30 bacterial isolates were detected for their plant growth promoting potential in terms of phosphate solubilizing prospective on Pikovskaya agar medium (Table 4.7). Among these, total of 14 bacterial isolates showed phosphate solubilization activity (AS-1, AS-4, AS-6, AS-8, AS-11, AS-13, AS-15, AS-17, AS-18, AS-20, AS-21, AS-23, AS-24 and AS-27) and 16 isolates did not show solubilization (AS-2, AS-3, AS-5, AS-7, AS-9, AS-10, AS-12, AS-14, AS-16, AS-19, AS-22, AS-25, AS-26, AS-28, AS-29 and AS-30).

AS-13 was detected to produce the largest size hallow zone of 38mm with 30mm colony diameter respectively. The remaining isolates found to depict lesser size hallow zone with smaller size colony diameter showing moderate phosphate solubilization activity. After AS-4, AS-6 and AS-15 each were detected to produce 18mm hallow zone with 14mm, 16mm and 15 mm colony diameter respectively. The remaining isolates having lesser hallow zone with small colony diameter are categorized as moderate phosphate solubilizing activity as these isolates found able to solubilizing insoluble form of phosphate.



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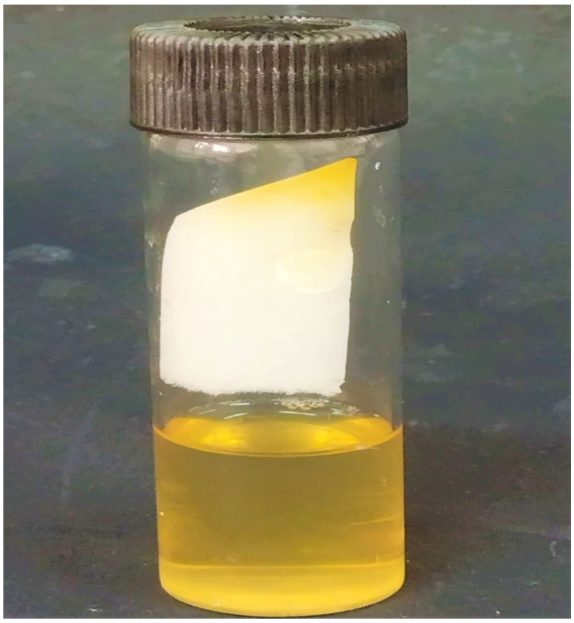
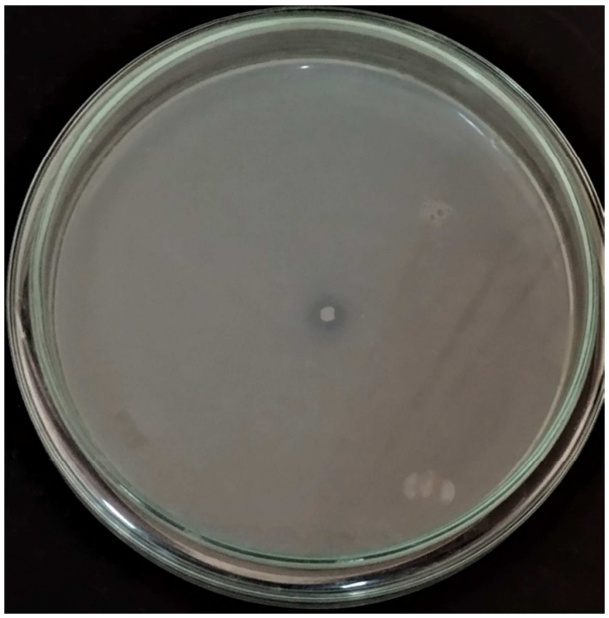




(b) (c)

Fig. 4.5: Characterization of the isolates for plant growth promoting (PGP) traits (a) IAA production as generation of pink colour in tryptophane enriched peptone broth, (b) HCN production as indicated by yellow color of filter paper strips on glycine enriched nutrient media (AS-15) and (c) Phosphate solubilization as clear halo-zone surrounding the bacterium inoculuim on pikovskaya agar plate (AS-17).

Table 4.7: Plant growth promoting properties of the isolates

S. NO.	Isolates	HCN Production	IAA Concentration		Tri -calcium Phosphate Solubilization		
			(µg/ml)	Pink coloration	Qualitative Response	Colony Diameter in (mm)	Hallow Zone Diameter in (mm)
1.	AS-1	+ + +	0.654	+	++	15	17
2.	AS-2	-	0.727	++	-	3	-
3.	AS-3	+ +	0.655	+	-	5	-
4.	AS-4	+ +	0.656	+	++	14	18
5.	AS-5	-	0.695	++	-	4	-
6.	AS-6	+ +	0.821	+++	++	16	18
7.	AS-7	-	0.701	+	-	2	-
8.	AS-8	-	0.664	+	+	11	14
9.	AS-9	+	0.661	+	-	4	-
10.	AS-10	-	0.663	+	-	6	-
11.	AS-11	-	0.703	+	+	8	11
12.	AS-12	+	0.683	+	-	5	-
13.	AS-13	+ +	0.725	++	+++	30	38
14.	AS-14	+ + +	0.722	++	-	4	-
15.	AS-15	+ + +	0.654	+	++	15	18
16.	AS-16	+ + +	0.636	+	-	3	-
17.	AS-17	+ + +	0.717	++	+	4	6
18.	AS-18	+	0.648	+	+	11	14
19.	AS-19	+	0.648	+	-	2	-
20.	AS-20	+ +	0.735	++	+	10	14
21.	AS-21	+	0.848	+++	+	11	13
22.	AS-22	+	0.744	++	-	5	-
23.	AS-23	-	0.699	+	+	10	12
24.	AS-24	-	0.806	+++	+	11	13
25.	AS-25	+ +	0.658	+	-	6	-
26.	AS-26	+ + +	0.636	+	-	7	-
27.	AS-27	+	0.679	+	+	9	11
28.	AS-28	+ +	0.686	+	-	3	-
29.	AS-29	+	0.642	+	-	7	-
30.	AS-30	-	0.700	+	-	6	-
	S Em±		0.004				
	CDat5%		0.012				

- = no production + = less production, ++ = moderate production and +++ = High production

4.4 Fertility status of the soils

All the soil samples collected from three different sites of the ARS, Bikaner; CAZRI and nearby area, Jodhpur and Jaisalmer; analyzed for fertility status by estimating organic carbon and available nitrogen in Table 4.8 and 4.9.

4.4.1: Soil organic carbon

Organic carbon (O.C.) values ranged from 0.161% to 0.201% in bulk (non rhizosphere) soil and rhizosphere soil respectively. When analyzed the samples location wise, the utmost value of organic carbon found in Jodhpur soils (0.227%) followed by Bikaner soils (0.191%) and least in samples collected from Jaisalmer district (0.125%) (Table 4.10). With respect to host plant the organic carbon values in the soils found to be at par. In combined studies it was observed that rhizosphere soil of sewan recorded the highest values of O.C. (0.251%), followed by rhizosphere soil of dhaman (0.245%) collected from Jodhpur. Soil samples from ARS Bikaner also shown higher O. C. values in rhizosphere soil of sewan (0.225%) and dhaman (0.210%) in contrast to non rhizosphere soil samples of these two grass species (0.169% and 0.160% respectively) (Table 4.9). Overall these soils recorded with low organic carbon content, so fall under category of low organic carbon and low fertility status as well.

4.4.2: Available nitrogen

Available nitrogen content ranged from 113.26 to 134.59 kg/ha in bulk soil (non- rhizosphere) and rhizosphere respectively. Overall the rhizosphere soil recorded higher amount of available N (134.59) in contrast to bulk soil (113.26) (Table 4.10). The combined study of available nitrogen reveals that the highest amount of available N in the sewan rhizosphere soil (172.10 kg/ha) followed by dhaman rhizosphere soil (161.64 kg/ha) collected from Jodhpur.

Table 4.8: Effect of sampling site and host plant in accord with proximity to plant roots on status of soil available nitrogen and soil organic carbon

	Nutrition status of soil	
	Available N (kgha^{-1})	**O.C. (%)**
A. Location		
A1	122.76	0.191
A2	155.84	0.227
A3	93.18	0.125
S Em±	0.56	0.001
CDat5%	1.62	0.002
B. Host plant		
B1	126.27	0.181
B2	121.58	0.180
S Em±	0.45	0.001
CDat5%	1.32	N/A
C. Distance from root zone		
C1	134.59	0.201
C2	113.26	0.161
S Em±	0.45	0.001
CDat5%	1.32	0.002

*****Available Nitrogen (kgha^{-1})

*****Organic Carbon (%)

*A1= Bikaner, A2= Jodhpur, A3= Jaisalmer, B1= Sewan (*Lasiurus scindicus*),

B2= Dhaman (*Cenchrus setigerus*), C1= rhizosphere soil, C2 = non-rhizosphere soil

Table 4.9: Combined effect of sampling site and host plant in accord with proximity to plant roots on status of soil available nitrogen and soil organic carbon

	Available N (kgha^{-1})						O.C. (%)					
AxB xC	**A_1**		**A_2**		**A_3**		**A_1**		**A_2**		**A_3**	
	B_1	**B_2**	**B_1**	**B_2**	**B_1**	**B_2**	**B_2**	**B_1**	**B_1**	**B_2**	**B_1**	**B_2**
C_1	127.43	130.66	172.10	161.64	114.00	101.71	0.225	0.210	0.251	0.245	0.139	0.132
C_2	116.53	116.42	146.75	142.86	80.82	76.21	0.169	0.160	0.195	0.215	0.107	0.120
S Em±			1.11						0.002			
CDat5%			3.24						0.005			

*****Available Nitrogen (kgha^{-1})

*****Organic Carbon (%)

*A1= Bikaner, A2= Jodhpur, A3= Jaisalmer, B1= Sewan (*Lasiurus scindicus*),

B2= Dhaman (*Cenchrus setigerus*), C1= rhizosphere soil, C2 = non-rhizosphere soil

Among soil samples of bulk soils of sewan grass from Jodhpur showed the higher N content (146.75 kg/ha) followed by non rhizosphere soil from dhaman grass (142.86 kg/ha) of Jodhpur (Table 4.9). Samples collected from ARS, Bikaner and Jaisalmer consisting of rhizosphere soil and non rhizosphere bulk soil also revealed identical tendency of available soil N content. Overall low amount of available nitrogen content were found in these soils. So, we can say low category in available nitrogen and poor fertility status in these soils of three districts of Rajasthan (Fig. 4.6).

Higher amount of carbon content and available nitrogen is considered valuable for presence and survival of microorganisms and bacterial population in soil.

4.5 Soil biological analyses

4.5.1 Dehydrogenase activity

Dehydrogenase enzyme activity (DHA) (Fig. 4.7c) in these soil ranged 7.50 to 8.02 µg/g TPF released/g/day in non rhizosphere to rhizosphere soil respectively (Table 4.10). The highest was found in Jodhpur soil which was significantly higher than Bikaner and Jaisalmer soils. The effect of host plant (7.36% higher activity in sewan soil than dhaman soil) and proximity to plant roots (6.93% higher activity in rhizosphere soil than non rhizosphere soils) was comparatively less when comparison the effect of sampling site (27% higher in Jodhpur soil than Bikaner and Jaisalmer soils). In combined effect studies the highest amount of dehydrogenase enzyme activity was detected in Jodhpur sewan rhizosphere soil (10.24) followed by Jodhpur sewan non rhizosphere soil (9.32), Jodhpur dhaman rhizosphere soil (8.9) and Jodhpur dhaman non rhizosphere soil (7.75) µg/g TPF released/g/day) (Table 4.11). In conclusion the ratio of dehydrogenase activity of rhizosphere to non rhizosphere soil from sewan and dhaman grasses

is 1.069 which shows trivial effect of plant roots on microbial activity (Table 4.12).

4.5.2 Fluorescein diacetate hydrolysis activity

The fluorescein diacetate (FDA) hydrolysis activity (Fig. 4.7e) ranged from 5.14 µg/g soil in Jaisalmer soil to 8.17 µg/g soil in Jodhpur soil (Table 4.10). FDA hydrolysis activity recorded higher in rhizosphere soil (6.69 µg/g soil) as comparison to non rhizosphere soil (5.59µg/g soil) i.e. 17.57 % higher values of rhizosphere over non rhizosphere buk soil which shows evident impact of plant roots on this parameter. In combined effect studies the highest value of FDA activity was found in Jodhpur dhaman rhizosphere soil (9.24 µg/g soil) followed by Jodhpur dhaman non rhizosphere soil (8.59 µg/g soil),Jodhpur sewan rhizosphere soil (7.78 µg/g soil) and Bikaner dhaman rhizosphere soil (7.73 µg/g soil). The least activity was found in Jaisalmer dhaman non rhizosphere soil (Table 4.11).

4.5.3 Microbial biomass carbon

Soil microbial biomass carbon (MBC) is an important attribute for analysis of soil health. The values of MBC ranged from 6.71µg/g soil in bulk soil to 8.43µg/g soil in rhizosphere soil (Fig. 4.7d). The MBC value of rhizosphere soil was measured as 1.26 of the non rhizosphere bulk soil (Table 4.10) which reveals momentous increase in microbial biomass due to rhizospheric effect. When analysis as done according to site out of the three locations, Jodhpur soil depicted the highest microbial biomass carbon content (8.5µg/g soil) which was 13.9% more than Bikaner and 26.97 % more than Jaisalmer soil. In pooled effect studies the highest value of MBC was found in Jodhpur dhaman rhizosphere soil (11.36 µg/g soil) followed by Jaisalmer sewan rhizosphere soil (9.20 µg/g soil). The least activity was found in Jaisalmer dhaman non rhizosphere soil (4.13 µg/g soil) (Table 4.11).

Table 4.10: Effect of sampling site and host plant in accord with proximity to plant roots on status of soil biological properties

	Microbiological properties in soil		
	MBC	**FDA**	**DHA**
A. Location			
A1	7.48	5.26	7.11
A2	8.52	8.17	9.05
A3	6.71	5.14	7.12
S Em±	0.70	0.04	0.15
CDat5%	N/A	0.11	0.43
B. Host plant			
B1	7.91	5.90	8.05
B2	7.22	6.47	7.47
S Em±	0.57	0.03	0.12
CDat5%	N/A	0.08	0.35
C. Distance from root zone			
C1	8.43	6.69	8.02
C2	6.71	5.69	7.50
S Em±	0.57	0.03	0.12
CDat5%	1.67	0.08	0.35

*Microbial Biomass Carbon (µg/g soil)
*Fluorescein diacetate hydrolysis (Fluorescein produced µg/g soil)
*Dehydrogenase (µg TPF release/g/day)
*A1= Bikaner, A2= Jodhpur, A3= Jaisalmer, B1= Sewan (*Lasiurus scindicus*), B2= Dhaman (*Cenchrus setigerus*), C1= rhizosphere soil, C2 = non-rhizosphere soil

Table 4.11: Combined effect of sampling site and host plant in accord with proximity to plant roots on status of soil biological properties

A x B x C	MBC						FDA						DHA					
	A_1		A_2		A_3		A_1		A_2		A_3		A_1		A_2		A_3	
	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
C_1	8.26	8.26	8.26	11.36	9.20	5.16	4.91	7.73	7.78	9.24	5.64	4.86	7.38	7.15	10.24	8.90	7.41	7.04
C_2	6.19	7.22	7.23	7.23	8.26	4.13	4.60	3.79	7.11	8.59	5.42	4.62	6.96	6.96	9.32	7.75	7.01	7.01
S Em±	1.398						0.071						0.294					
CDat5%	N/A						0.208						N/A					

*Microbial Biomass Carbon (µg/g soil)

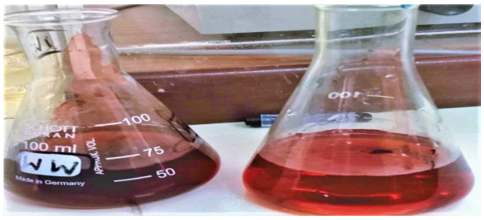
*Fluorescein diacetate hydrolysis (Fluorescein produced µg/g soil)

*Dehydrogenase (µg TPF release/g/day)

*A1= Bikaner, A2= Jodhpur, A3= Jaisalmer, B1= Sewan (*Lasiurus scindicus*),

B2= Dhaman (*Cenchrus setigerus*), C1= rhizosphere soil, C2 = non-rhizosphere soil

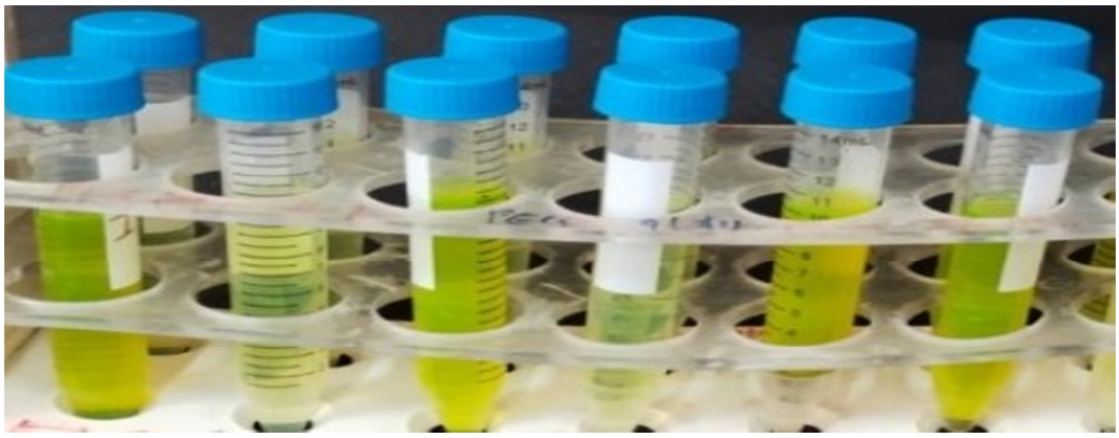
(a)



(b)

(d)

(e)



(f)

Fig. 4.6: Fertility status of the soils: (a) Organic carban and (b) Available nitrogen,

Fig. 4.7: Soil biological analyses: (c) DHA activity, (d) MBC activity and (e) FDA activity.

4.6 Molecular characterization of bacterial Isolates from rhizosphere and adjacent region of sewan and dhaman grasses

4.6.1 Isolation and purity check of bacterial genomic DNA

Genomic DNA isolated from 13 bacterial isolates (AS-4, AS-6, AS-9, AS-11, AS-12, AS-13, AS-14, AS-17, AS-18, AS-21, AS-23, AS-25 and AS-27) using modified freeze and thaw method and quantified by UV-VIS spectrophotometer. The quality of DNA was measured at A_{260} nm absorbance and A_{260}/A_{280} was recorded in between 1.75 to 1.80. The observed quality parameters of genomic DNA were considerably very well.

4.6.2 Amplification of SCoT markers

For molecular characterization of bacterial isolates, 13SCoT markers were selected from public literature (Collard and Mackill, 2009) (Table 3.1). For profiling in 13 bacterial isolates, the selected primers were custom synthesized. All the 13SCoT markers were produced significant number of polymorphic bands (Table 4.12, Figure 4.8). These 13SCoT markers were analysed for further study.

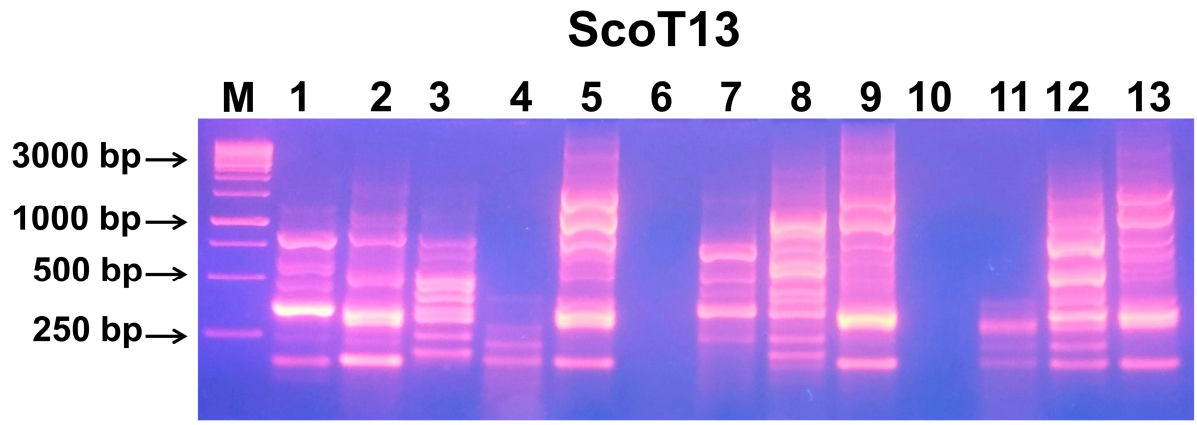
4.6.3 Assessment of polymorphism and marker efficiency

Polymorphism of ScoT markers and their efficiency was calculated using iMEC marker efficiency calculator. The binary matrix (0 and 1) were scored from the electrophoretic gel image of all the markers. Where 1 was indicating as presence and 0 was indicating as absence of the alleles. the binary matrix (0 and 1) values were used for detecting polymorphism and marker efficiency calculating with the help of marker efficiency calculating with the help of marker efficiency calculator (iMEC) [Amiryousefi *et al.,* 2018]. The results detected by analysis of 13SCoT markers were ranged from 50bp to 3000bp. All the 13SCoT primers were amplified a total of 133 scorable bands of which all were polymorphic (Table 4.14). The number of polymorphic bands ranged from 7 (ScoT26) to 17 (SCoT13) with an average of 10.23

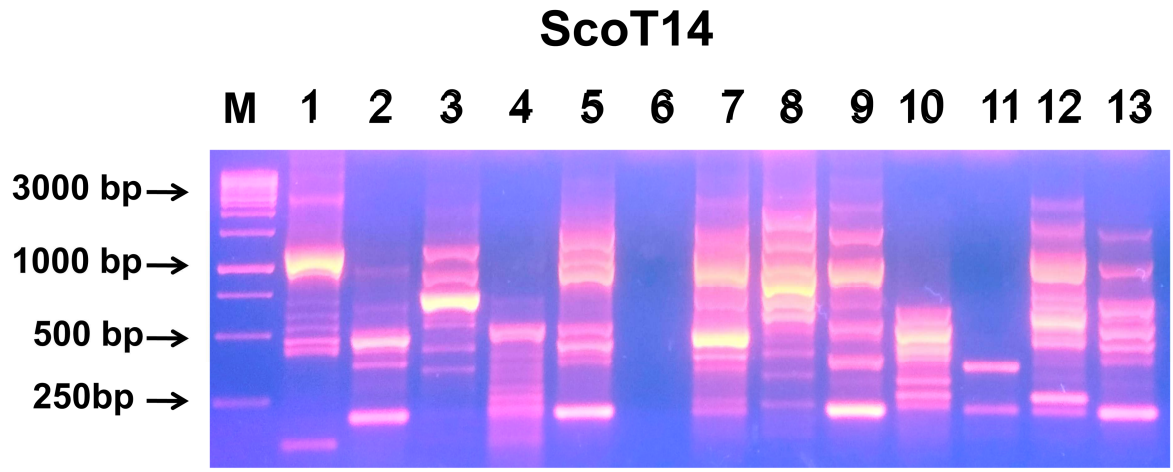
polymorphic bands per primer. All the SCoT markers showed 100% polymorphism. The polymorphism Information Content (PIC) value detected from the markers were ranged from 0.42 (SCoT15) to 0.75 (SCoT02). The average percentage of PIC value was 55%. The Marker Index (MI) values detected from the markers were ranged from 0.49 (SCoT15) and 0.75 (SCoT02). The average Marker Index (MI) value was 0.62. The average Heterozygosity index (H) was 0.62. The H values obtained from 13SCoT markers were ranged from 0.49 (SCoT15) to 0.75 (SCoT02). The discriminating power (D) ranged from 0.26 (SCoT19) to 0.69 (SCoT25) with an average of 0.56. The effective multiplex ratio (E) was obtained as one indicating by 100% efficiency of all 13 markers used in this study. The further was study of all the 13ScoT markers in the Table 4.12.

4.7 Genetic diversity analysis of bacterial isolates

To detect the genetic variability among the 13 bacterial isolates, the marker data were analysed in NTSys software using binary matrix (0 and 1). Based on SCoT markers analysis, the high genetic variability within rhizospheric bacterial populations was revealed in the present study. The neighbour-joining (NJ) dendrogram generated based on SCoT markers showed a clear clustering pattern of genetically closer individual (Figure 4.9). The 13 isolates were clustered in to three major Groups. Among them, Group-I was consisted with eight isolates (AS-4, AS-9, AS-12, AS-14, AS-17, AS-18, AS-25 and AS-27) and Group-II was consisted with four isolates (AS-6, AS-11, AS-21 and AS-23). The Group-III was consisted with only one isolate (AS-13) as outlier of the dendrogram (Figure 4.9). Group-I was differentiated at coefficient 0.48 similarly coefficient. The Group-II was bifurcated at 0.31 similarly coefficient, whereas, the last Group-III was divided at 0.17 similarly coefficient in the UPGMA dendrogram.



(a)



(b)

Fig. 4.8: Representing photograph of SCoT markers profiling in 13 bacterial isolates (a) SCoT 13 and (b) SCoT 14; M= 1kb ladder and the no. of 1 to 13 depict PCR products from bacterial isolates AS-1 to AS-13 respectively.

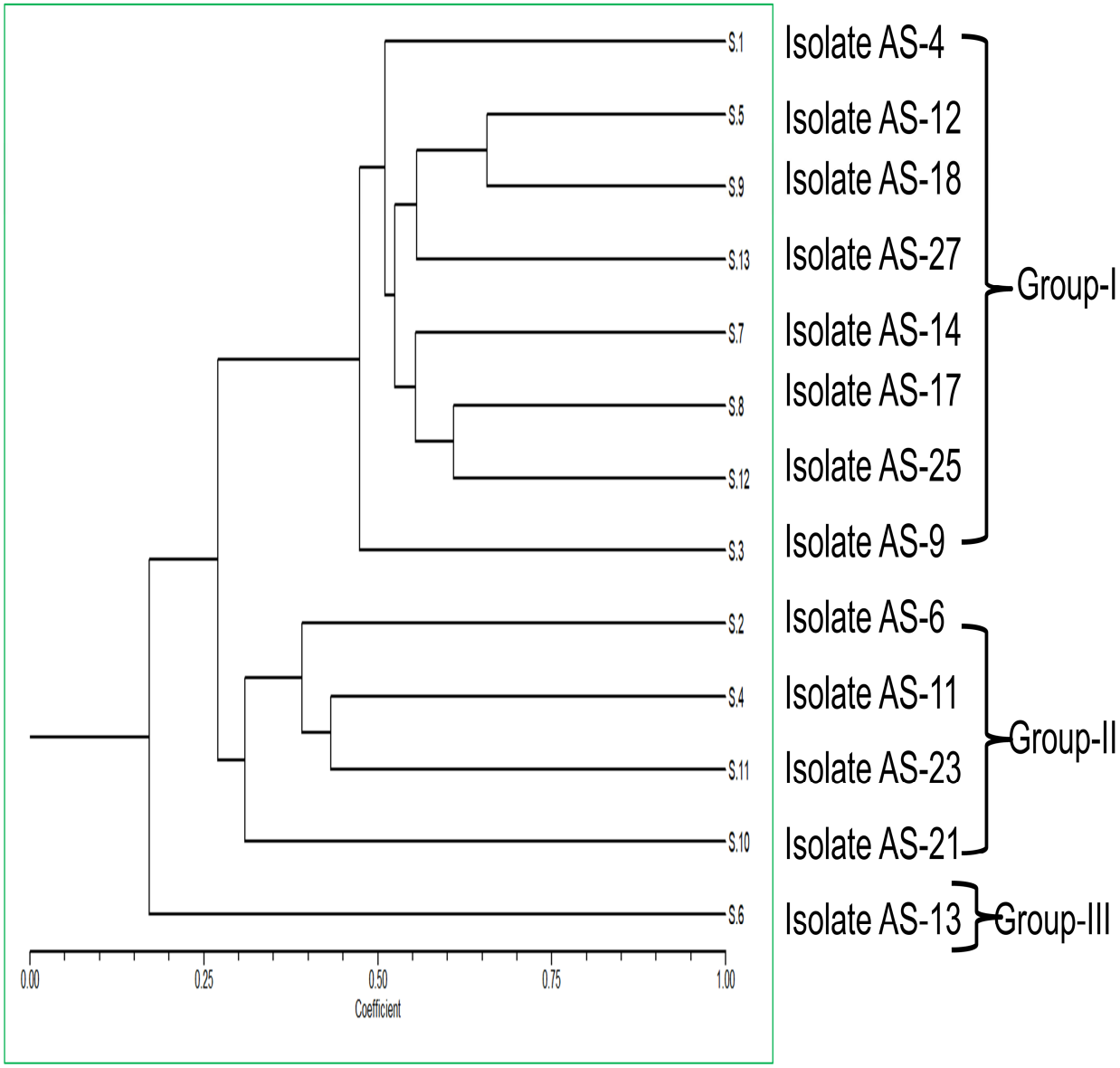


Fig. 4.9: Dendrogram based on SCoT data using the neighbour- joining (NJ) Method

Table 4.12: Characteristics of SCoT markers analyzed in this study

Markers	Primers (5'-3')	Allele Size (bp)	NTP	NPB	%P	PIC	H	E	MI	D
SCoT2	CAACA**ATG**GCTACCACCC	50-2500	10	10	100	0.69919	0.7464	1	0.7464	0.47929
SCoT13	ACGAC**ATG**GCGACCATCG	50-3000	17	17	100	0.51384	0.58508	1	0.58508	0.57768
SCoT14	ACGAC**ATG**GCGACCACGC	50-2700	14	14	100	0.51997	0.6018	1	0.6018	0.57875
SCoT15	ACGAC**ATG**GCGACCGCGA	50-2500	9	9	100	0.42399	0.49076	1	0.49076	0.46296
SCoT16	ACC**ATG**GCTACCACCGAC	50-2500	9	9	100	0.58473	0.65907	1	0.65907	0.66524
SCoT18	ACC**ATG**GCTACCACCGCC	250-3000	10	10	100	0.50929	0.59065	1	0.59065	0.51795
SCoT19	ACC**ATG**GCTACCACCGGC	50-3000	9	9	100	0.53125	0.5771	1	0.5771	0.25484
SCoT20	ACC**ATG**GCTACCACCGCG	50-3000	9	9	100	0.54636	0.62415	1	0.62415	0.5812
SCoT21	ACGAC**ATG**GCGACCCACA	100-2500	10	10	100	0.57475	0.64982	1	0.64982	0.63974
SCoT22	AACC**ATG**GCTACCACCAC	100-2500	9	9	100	0.53162	0.61012	1	0.61012	0.5812
SCoT23	CACC**ATG**GCTACCACCAG	100-2500	9	9	100	0.58151	0.65512	1	0.65512	0.60399
SCoT25	ACC**ATG**GCTACCACCGGG	50-2000	10	10	100	0.5876	0.66166	1	0.66166	0.69103
SCoT26	ACC**ATG**GCTACCACCGTC	250-1500	7	7	100	0.57859	0.6521	1	0.6521	0.68864
Mean			10.23	10.23	100	0.54472	0.616039	1	0.616039	0.562872

NTB = No. of total band, NPB = No. of polymorphic band, %P = percent of polymorphism, PIC = Polymorphism information content,

H = Heterozygosity index, D = Discriminating power, E = Effective multiplex ratio, MI = marker index, bp = base pair

Among the 13 isolates, six isolates were found to be more similar at molecular level, making three distinct pairs. Among which four isolates belong to Group-I; first two isolates (AS-12 and AS-18) showed more similarity at 0.68 coefficient and another 2 isolates (AS-17 and AS-25) showed more similarity at 0.63 coefficients. Remaining 2 isolates belong to Group-II (AS-11 and AS-23) showed more similarity at 0.43 coefficient. Low similarity among the bacterial isolates indicated low relationship, whereas, high similarity indicates showed high relationship.

4.8 Bacterial population structure analysis

The population structure analysis of the 13 bacterial isolates was carried out using STRUCTURE 2.0 software by putting K value from 1 to 10 with 9 iterative replicates. The K value from 0 to 10 indicated the possible number of population where as the highest value of delta K indicating the possible structural population.

The delta K value was identified by interpreting structure software result using STRUCTURE HARVESTOR TOOL (http://taylor0. biology.ucla.edu/structureHarvester/). The peak at K6 value was seen very high followed by K2, which indicating there are 6 populations among the 13 isolates (Figure 4.10).

The population structure analyses data represented in Evanno Table output file (Figure 4.11). The Evanno Table indicates delta K value ranged from 0.230465 to 2.123260 it indicates the 3 and 6 numbers of populations, respectively. It suggests that the K6 has the highest delta K value. Thus, the population structure analysis of the 13 bacterial isolates using 13SCoT markers was indicating the 6 major populations with admixture in each population (Figure 4.12). The population 1 was consisted with the two isolates (AS-4 and AS-6). The population 2 was consisted with the two isolates (AS-9 and AS-11), whereas, population 3 was consisted with the two isolates (AS-12 and

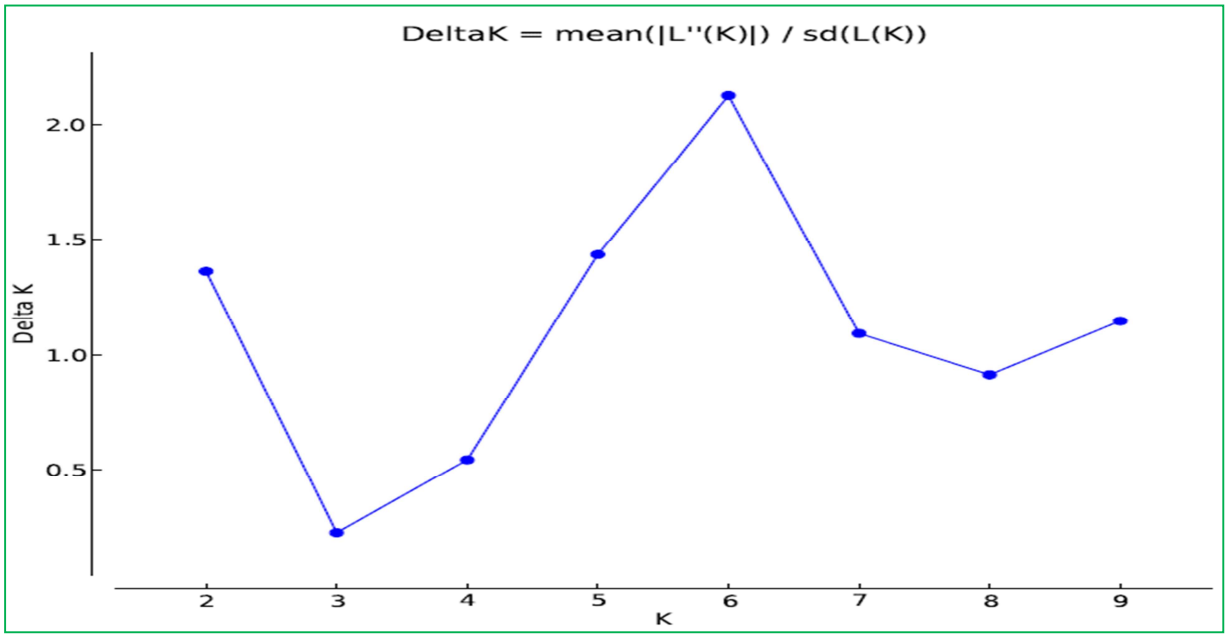


Fig. 4.10: Structural analysis by profiling 13 ScoT markers in 13 bacterial Delta K Value

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	9	-1825.544444	2.976202	—	—	—
2	9	-10580.377778	1967.654327	-8754.833333	2678.533333	1.361282
3	9	-16656.677778	6123.745556	-6076.300000	1411.311111	0.230465
4	9	-21321.666667	7536.143621	-4664.988889	4103.233333	0.544474
5	9	-30089.888889	9423.198784	-8768.222222	13518.877778	1.434638
6	9	-25339.233333	11638.908430	4750.655556	24712.433333	2.123260
7	9	-45301.011111	23772.115741	-19961.777778	25947.144444	1.091495
8	9	-39315.644444	17842.896759	5985.366667	16315.244444	0.914383
9	9	-49645.522222	25179.486998	-10329.877778	28833.700000	1.145127
10	9	-31141.700000	24727.247183	18503.822222	—	—

Fig. 4.11: Structural analysis by profiling 13 ScoT markers in 13 bacterial Evanno Data

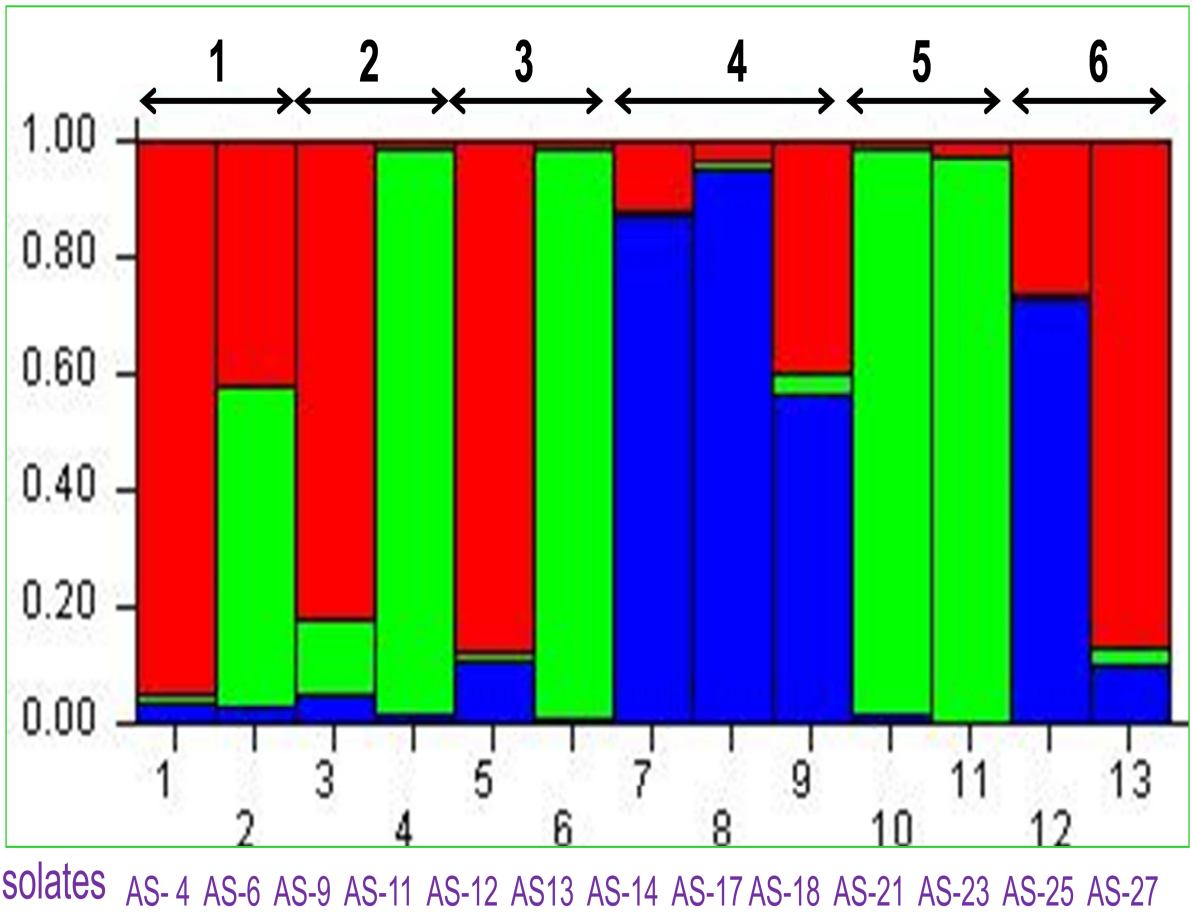


Fig. 4.12: Structural Analysis By Profiling 13 ScoT Markers In 13 Bacterial Isolates Population

AS-13). Similarly, the population 4 consisted with the three isolate (AS-14, AS-17 and AS-18). Population 5 and 6 consisted with the two isolates (AS-21 and AS-23) and (AS-25 and AS-27), respectively. Among the 6 population observed three populations indicates pure form of bacterial isolates, which means isolate AS-11 in population 2, isolate AS-13 in population 3 and isolates AS-21 and AS-23 in population 5 seems to be in pure form. Further, the population 5 is consisted with highly pure form of isolates.

4.9 Sequences validation of pure form of isolates

For sequence validation of the bacterial isolates, the *Streptococcus* specific ITS1 and ITS2 primer set as forward and reverse primers respectively was profiled on the genomic DNA of 13 bacterial isolates. As a result, around 200 bp size fragment was amplified in AS-11, AS-13, AS-21 and AS-23 each. Further, the sequence of PCR products were validated through Sanger sequencing by providing ITS1-ITS2 primer set. All the four bacterial isolates were shown significant level of sequence similarity with sequence available of *Streptococcus* strains in public genomic database (Figure 4.13).

Fig. 4.13: Sequences validation of pure form of isolates

5. DISCUSSION

All the while bringing up the results of the experiment baptized **"Characterization and Diversity Analysis of Bacterial Isolates from Rhizosphere of Sewan (*Lasiurus scindicus*) and Dhaman (*Cenchrus setigerus*) Grasses".** This study was designed to isolate the potent plant growth promoting bacteria from of rhizosphere and bulk soil from to native grasses of the arid region viz. sewan (*Lasiurus scindicus*) and dhaman (*Cenchrus setigerus*). Further these isolates were distinguished the best PGP isolates so that elite isolates may be developed as bio-inoculants which may serve as bio-fertilizers for Bikaner and Jodhpur and Jaisalmer regions to support crop growth and yield under organic cultivation after standard studies of plant microbe interactions. The discussions on results obtained from various aspects of the investigations are presented in this chapter.

5.1. Isolation of bacteria from rhizosphere and adjacent bulk soil and their characterization

Soil fertility, vigor and vital functions are supported and maintained by diverse microbial populations since these actively participate in key biogeochemical processes including nutrients cycling, decomposition or else mineralization of organic residue, supporting soil structure and removal of pollutants. Today, In the course of emergent emphasis on sustainable and low input agricultural practices; there is also a growing concern to investigate soil microbial populations to facilitate enhanced growth and fitness of crop plants in the era of climate change. However, with the purpose of making the most of the valuable properties of microorganisms; at all times it is essential to relate to the favorable factors for the microbial diversity and activity. There are different edaphic (soil type, nutrient status, pH, moisture), plant (age, species, cultivars) and management (cultivation, inputs etc.) factors influencing the structural and functional diversity of soil

microorganisms. In the present investigation the effect of various sites, plant types and management practices is clearly pragmatic on bacterial population structure, functions and qualitative traits.

In our study we isolated 30 bacterial isolates from rhizosphere and bulk soils of sewan (*Lasiurus scindicus*) and dhaman (*Cenchrus setigerus*) grasses collected from Bikaner, Jodhpur and Jaisalmer districts of Rajasthan. Further, we found that colonies were in color appearance such as orange, light yellow, white or off-white etc. All the isolates, except two shown positive grams staining reaction. Bacterial enumeration revealed multifold increase in population count as well as morphological diversity from sewan and dhaman rhizosphere than the bulk soils which showing clear rhizospheric effect as a result of volatile compounds (root exudates) such as organic acids, sugars, proteins and amino acids etc. secreted by root cells and root associated microflora (Tkacz *et. al.,* 2020). These results are supported by the work of Choi *et al.* (2017) that reported greater microbial population in rhizosphere soils. In broad-spectrum, the rhizosphere effect greatly decline when distance is increased from the root Edwards et.al. (2015). Since more than 70% isolates of our study, isolated from rhizosphere soil indict suitability of rhizosphere of these two grasses for bacterial growth and development. Furthermore, physicochemical properties of the rhizosphere region are greatly distinct in contrast to the adjacent bulk soil. Both the property of soil as well as host plant species have tremendous effect on number and diversity of associated microorganisms (Oliver et al., 2008; Donn et al, 2015; Liu et al.2020).

All the 30 isolates and 29 isolate resulted positive for oxidase and catalase activity respectively., Additionally, 18 isolates were able to hydrolyse starch and 17 isolates were found positive for urease enzyme activity. Kumar *et al*. (2012) shown that root associated bacteria depicting catalase activity must be strongy resistant against chemical, mechanical and environmental stress. Our data are

supporting results of Majeed *et al.* (2015) who studied wheat rhizosphere,made bacterial isolation found all nine isolates positive for catalase activity. Also, Chatterjee *et al.* (2015) isolated rhizospheric bacteria and stated 30 percent isolates positive for starch hydrolysis, 90 percent isolates positive for urease activity and all the isolates positive for catalase activity and only one isolates reported positive for oxidase activity.

5.2. Analyses of soil samples for C and N contents

Carbon and nitrogen contents are the two chief nutrients and key elements for growth and survival of microorganisms in the soil. We found organic carbon varied from 0.161% to 0.201% with the greater value was recorded in rhizospheric soil and the lesser value in bulk soil, consistently soil available nitrogen was also measured comparatively more in rhizospheric soil (134.59 kg/ha) and the lower value was calculated in non rhizospheric soil (113.26 kg/ha). The available N content of the collected soil samples was found to be low. Organic C content of all soils were in the very low range. The lower level of N may be attributed due to numerous climatic and edaphic factors, such as lower amount of organic carbon, emanating from minimal vegetation cover along with raised pH, favoring higher volatilization losses (Kumar *et al*., 2013, Gupta *et al.* 2014). Carbon content plays an imperative role in the regulation of the diversity and structure of soil microbiome (Zhou *et al.* 2002). Studies of Chatterjee *et al.* (2015) accounted higher bacterial population of in Bakkhali and least in Purba Amarabati owing to more content of organic carbon and water holding capacity in comparison to the other areas having lower organic carbon. Further, Zhang *et al.* (2019) also reported enhanced N absorption by crop as well as increased crop yield resulting due to additional soil microbial biomass.

5.3. Microbiological analyses of soil samples

Soil microbial biomass is both the source as well as sink of nutrients and also mineralization of organic substrates. In this investigation, we concluded significant variation among the samples in terms of all three soil biological attributes when analyzed with respect to place, proximity from plant roots and the species of host plant. We found samples from rhizosphere and Jodhpur sites favored higher activities of both the soil enzymes DHA, FDA as well as amount of MBC in contrast to counterpart soils. Interestingly FDA activities were lower in soil samples from sewan while the activities of DHA and MBC recorded higher in contrast to soil samples from dhaman. Overall low microbial activities rhizosphere and bulk soils may be ascribed to higher pH and very low OC status of these soils. Chao (2011) also described more dehydrogenase and β-glucosidase enzymes activities in rhizosphere soils than in the non rhizosphere soil. Xu *et al* (2020) reported the high influence of soil salinity on amount of organic matter, nitrogen, dissolved organic carbon, and microbial biomass carbon (MBC) of soil. Soil moisture is also one of the key determinants of the organisms living in the soil in several ways. The soil water content influences the pH, the diffusion rates of solvents and gases and the nutrients availability as well (Wolińska A., 2010).

5.4. Characterization of isolates for PGP traits

In the present investigation, five isolates were found to be efficient phosphate solubilizers while nine other were also able to grow on Pikovskaya agar plates and hence able utilize insoluble tricalcium phosphate. Out of 14 PSB 12 isolates belong to rhizosphere and only two isolate from bulk soil. Reyes *et* al. (2006) reported high population of phosphate-solubilizing bacteria (PSB) in the rhizosphere in contrary to bulk soil. Phosphate solubilizing microbial isolates from rhizosphere and bulk soils release phosphatases to hydrolyze organic phosphorous in the soil (Bi *et al*., 2018). The role of root associated bacteria in

sustaining sufficient nutrients including phosphorus levels in crop production earlier reported by Saravanan *et al.* (2007). Desale *et al*. (2014) also reported PGPR capable of solubilizing phosphate in salinity conditions and exerting beneficial traits for plant growth under these conditions. Zheng et al. (2019) reported better competence to solubilize inorganic phosphorus, exude organic anions and phosphate uptake when consortium of PSB applied to *Brassica napus* rather than individual strains.

In this study, 21 bacterial isolates found positive for HCN production among which six were high and nine were medium producer two third belong to dhaman and remaining to sewan grass. Among the HCN producers (cyanogenic) isolates Various other studies have also reported function of HCN in disease suppression in different crops (Defago et al., 1990; Ramette et al., 2006). Kremer and souiss (2001) suggested a potential and environmentally friendly method for bio-control of weeds through HCN produced in the rhizospheres of seedlings by scrutinized PGPR. Further same mechanism was supported by Kamei *et al*. (2014). PGP bacteria enhance plant growth directly or indirectly by phytohormones production, nitrogen fixation, mineral solubilization (P, Zn and K) and production of siderophores and HCN Mukhtar *et al*. (2019).

Indole acetic acid (IAA) is one of the elementary plant hormones, considered to be the primary native auxin in general. In our findings it is revealed that all the bacterial isolates were able to produce IAA in the varying from 0.636 to 0.848 µg/ml; indicating a substantial inevitability among these isolates for IAA production. It was suggested that rhizobacteria synthesize auxins consecutively to agitate physiological processes of the host plant for their personal advantage (Shih-Yung, 2010). The IAA production by rhizospheric bacterial isolates from diverse oil seed and cereal crops, had already been reported in various studies including Cakmakci *et al*. (2007) and Mehnaz *et al.* (2010).

Although all the isolates were able to produce IAA, results shown less amount in contrast to the earlier investigations where the rhizobacteria from *Brassica campestris* reported production of the IAA in the tune of 6.02–29.75 µg.ml^{-1} by Poonguzhali *et al.* (2008). The production of IAA is influenced by various factors like pH, utilization of carbon and nitrogen and availability of ample amount of the precursor L- tryptophan in the media (Mohite, 2013; Chandra et al., 2018). These isolates may need to be further optimized for IAA production potential quantitatively, however qualitatively all of these have fitness for this particular attribute.

5.5. Molecular characterization of bacterial isolates

5.5.1. Genetic Diversity Analyses

In the present investigation, 13SCoT markers were used for molecular diversity analyses among 13 selected isolates and the dendrogram constructed which confirmed three major groups. Here these three main groups owing to three different sites, two grass species and also the samples belong to rhizosphere and bulk soil. Cavaglieri *et al.* (2009) reported that the diversity in exudates from plant roots sustain a collection of diverse microorganisms, adding to a host's compliance and recruit rhizospheric in addition to endophytic microbial populations according to its requirements. Ladygina *et al.* (2010) also observed that rhizospheric microbial diversity and carbon allocation was subjective by plant species. Our results are also coherent with Liu *et al.* (2015) who proposed that tree species influenced microbial diversity and nitrogen availability in rhizosphere soil. Wang *et al.* (2018) reported the top ten bacterial phyla in the rhizosphere soil and found the greatest relative abundance of Proteobacteria and Actinobacteria among the top ten phyla. Edwards *et. al.* (2018) after screening of the rice metagenome reported that the sampling segment (bulk soil or rhizosphere) and soil are the principal factors in determining the structure of plant bacterial diversity.

Pouralibaba *et al.* (2019) analyzed the diversity of *F. oxysporum* f. sp. *lentis* using eight SSR markers. Their findings showed more molecular variability of this fungal species within regions. Tkacz *et. al.* (2020) conducted global microbial kingdom analysis simultaneously on multiple plants and found related prokaryotic and comparable eukaryotic communities inside and on the root surface of cereals, legumes, and crucifers. However the profile of bacterial micro biota is more influenced by the root fraction than by soil or plant species.

5.5.2. Polymorphism and efficiency detection of SCoT markers

The various environmental factors may have an effect on variability in the performance of PGPR. A large number of studies applied morpho-physiological and biochemical methods aimed on the isolation and identification of microbes. A comprehensive assessment of genetic and functional diversities can be helpful for predicting the suitability of new beneficial strains of microorganisms into the new location (Joseph *et al*., 2007). Currently, polyphasic approaches being exploited involving techniques to determine both phenotypic and genotypic characteristics Getahun *et al.* (2020). The phenotypic profiling in this study demonstrated notable metabolic adaptability with three bacterial species viz. *Ochrobactrum* spp, *Pseudomonas* spp and *Klebsiella* spp, further results of BOX-PCR and partial sequencing of 16S rRNA genes showed better discriminatory influence in favor of fingerprinting of root associated bacterial isolates through prominent scale of polymorphism. Dos santos *et al.* (2021) characterized the diversity and genetic structure in *Dalbergia nigra* mini-garden where eleven primers were selected, which generated 180 fragments (70.76% polymorphism) and the polymorphic information content (PIC) for the markers used averaged 0.38, considering them as moderately informative. Similarly, the high number of polymorphic bands and huge polymorphic fragment achieved in our investigation show prospective of SCoT marker analyses. We obtained polymorphic bands from 7 to

17 in these markers showing 100 % polymorphism. The polymorphism Information content (PIC) was detected as 0.42 to 0.75 and also the average value of this index 55%.

5.5.3. Population Structure Analysis

Huzar-Novakowiski and Dorrance (2018) suggested several factors such as host range, environmental conditions, and application of agrochemicals etc. influence the population structure and genetic diversity of microorganisms including pathogens. In the present study, the population structural analyses of 13 selected bacterial isolates using 13SCoT markers signifying the 3 main populations amid admixture in each population. Additionally, we also found four isolates are there as pure culture of population, may serve as prominent resources for further detailed studies in terms of functional and structural characterization. Proteobacteria and Actinobacteria have been reported as the utmost abundant groups in various soil microbial communities in cucumber fields (Tian *et al.,* 2014). The findings of diversity analyses of our study show superiority in contrast to results of Kodisang *et. al.* (2013) who analyzed cultivable diversity of 17 isolates from field grown crops using RAPD markers and reported 12 isolates represents similar banding pattern. Gagnon *et al.* (2020) also studied microbial populations in the rhizosphere, bulk soil and root endophytes using Illumina sequencing. Wu *et al.* (2021) demonstrated that changes in soil nutrition level causes the consecutive transformation in microbial communities in the *Populus alba* plantations. Our investigation also agrees with these findings.

6. SUMMARY AND CONCLUSION

The present study entitled experimentation **" Characterization and Diversity Analysis of Bacterial Isolates from Rhizosphere of Sewan (*Lasiurus scindicus*) and Dhaman (*Cenchrus setigerus*) Grasses"** was accomplished under laboratory environment at Department of Biotechnology, Plant Biotechnology Centre, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University (SKRAU), Bikaner and Indian Council of Agricultural Research - Central Institute of Arid Horticulture, Bikaner (Raj.). With the aim of achieving the objectives of present investigation, the data pertaining to various traits viz. microbial morphological, biochemical, microscopic, microbiological, soil nutrient status, PGPR activities and molecular level characterization are summarized as below:

- Total 30 bacteria were isolated on nutrient agar media from rhizosphere and adjacent soil of sewan and dhaman grasses collected from Jodhpur, Jaisalmer and Bikaner district of Rajasthan. Those isolated bacteria are primarily characterized as single/filament small to big coccus or rod shaped showing white, light yellow, to light orange pigmentation.
- Bacterial population in the samples ranged from 5.33×10^5 to 8.8×10^6 cfu/g on the nutrient agar plates.
- Gram staining was used for categorizing the bacteria into two groups: Gram +ve or Gram –ve, we found all the isolates are gram –ve either rods or cocci except two (AS-4, AS-6 which identified as Gram +ve cocci).
- Out of 30 isolates, 18 isolates were able to hydrolyse starch and 17 isolates were found positive for urease enzyme activity. All the isolates were found positive for oxidase activity and positive for catalase except (AS-8).

- Additional analyses of these bacterial isolates were carried out for their PGP traits. Total fourteen isolates confirmed solubilization of tri-calcium phosphate (TCP) on Pikovskaya agar however isolate AS-13 showed the biggest 38mm halo zone with 30mm diameter followed by isolates AS-21 showed 18mm halo zone with 15mm diameter.
- Further, these cultures when inoculated in the peptone broth (with tryptophan), all the isolates were found positive for IAA production and the highest concentration of IAA found in the isolate AS-21(0.848 µg/ml) and the least concentration in isolate AS-16 and AS-26 (0.636 µg/ml). HCN production screened by adapting the Lorck method which detected the highest HCN production in the six isolates.
- The fertility status of soils revealed that the soils are low in organic carbon (0.161% to 0.201%) and available nitrogen (113.26 kg/ha to 134.59 kg/ha).
- Soil biological analyses revealed that the soil enzyme activities such as dehydrogenase enzyme activity ranged from 7.5 to 8.02 µg TPF release / g / day. Furthermore FDA hydrolysis ranged from 5.14 to 8.17 µg fluorescein / g and microbial biomass carbon ranged from 6.71 to 8.43 µg / g soil.
- Fine characterization of rhizosphere and bulk soil bacterial isolates at molecular level revealed the crucial findings in this present study. Three major groups of bacterial communities were observed by phylogenetic analyses.
- In the current study, 100% polymorphism and genetic relationship obtained which indicate the authority of the SCoT marker analyses and it has been confirmed valuable in the genetic diversity studies and detection of polymorphism in bacterial communities.

- Thirteen SCoT primers were amplified 133 scorable bands of size ranged from 50bp to 3000bp among these all were detected polymorphic.
- In the present study, the number of polymorphic bands observed ranged from 7 (SCoT26) to 17 (SCoT13) with an average of 10.23 polymorphic bands per primer.
- The polymorphism information content (PIC) value detected from the marker were ranged from 0.42 (SCoT15) to 0.75 (SCoT02) with an average percentage of PIC value of 55%.
- The average Heterozygosity index (H) among the bacterial isolates was detected as 0.62.
- The discriminating power (D) ranged from 0.26 (SCoT19) to 0.69 (SCoT25) with an average of 0.56 and effective multiplex ratio (E) was obtained as unit indicating 100% efficiency of markers used in this study.
- In the Population structure analysis of the study, six major populations were identified. Among the six populations observed three populations indicate pure form of bacterial from four pure isolates. This means isolate AS-11 in population 2, isolates AS-13 in population 3 and isolate AS-21 and AS-3 in population 5 seems to be in pure form. Further, the population 5 is consisted with highly pure form of isolate.

Conclusion

From the present study it can be concluded that rhizosphere and adjacent region of sewan and dhaman grasses are habitat for several bacteria possessing plant growth promoting activities. Pure bacterial isolates (AS-11, AS-13, AS-21 and AS-23) obtained from rhizosphere of dhaman grass showing plant growth promoting (PGP) activities such as production of HCN, IAA and phosphate solubilizatioin. These

isolates may be used as a bio-fertilizer and bio-decomposers which help in nutrient transformation and stimulate plant growth by production of various substances. Our finding shows the existence of more genetic variability in the rhizospheric bacterial populations. Notably, the results revealed that the SCoT technique could be used in evaluation of genetic relationships, which ultimately would be supportive in delineation of various other isolates. The study can also be used in bioinformatics studies for further analysis of microbial population by means of diverse high-throughput techniques.

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Characterization and Diversity Analysis of Bacterial Isolates from Rhizosphere of Sewan (*Lasiurus scindicus*) and Dhaman (*Cenchrus setigerus*) Grasses

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ABSTRACT

The present investigation aimed at isolation and characterization of potent bacterial isolates from rhizosphere and adjacent area of two native grasses (*Lasiurus scindicus* and *Cenchrus setigerus*) of arid region covering three districts of Rajasthan (India). These soils characterized with low organic carbon (0.161% to 0.201%) and low available nitrogen (113.26 kg/ha to 134.59 kg/ha). Total 30 bacterial isolates obtained shown plant growth promoting (PGP) activities among which all the isolates were positive for IAA production and 70% isolates were cyanogenic of which six isolates found to produce high HCN. Total fourteen isolates confirmed solubilization of tri-calcium phosphate (TCP) including AS-13 producing 38 mm halo zone on Pikovskaya agar. Soil biological analyses revealed that the soil enzyme activities such as dehydrogenase enzyme, FDA hydrolysis and microbial biomass carbon found 1. 07 to 1.26 fold more in rhizosphere than bulk soil revealing positive impact on plant roots on soil microflora. Further, SCoT markers found to be best effective technique to estimate the genetic diversity and detection of polymorphism in bacterial communities. Thirteen SCoT primers were amplified 133 scorable bands of size ranged from 50bp to 3000bp among these all were detected 100% polymorphism, the number of polymorphic bands observed ranged from 7 (SCoT 26) to 17 (SCoT 13) with an average of 10.23 polymorphic bands per primer. The polymorphism information content (PIC) value detected from the marker were ranged from 0.42 (SCoT15) to 0.75 (SCoT02) with an average percentage of PIC value of 55% and effective multiplex ratio (E) was obtained as unit indicating 100% efficiency of markers used in this study. In the Population structure analysis of the study, six major populations were identified. Among the six populations observed three populations indicate pure form of bacterial from four pure isolates. Our finding shows the existence of more genetic variability in the rhizospheric bacterial populations. Isolates AS-11, AS-13, AS-21 and AS-23 are in pure form and showing good plant growth promoting traits may be used in development of bio-inoculants for arid region.

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सेवण (लासियुरस सिंडिकस) और धामण (सेनक्रस सेटिगेरस) घासों के जड़ क्षेत्र से जीवाणु विलगों का चरित्र चित्रण और विविधता विश्लेषण

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विकास शर्मा** प्रमुख शोध सलाहकार

सार

प्रस्तुत अन्वेषण का उद्देश्य राजस्थान (भारत) के तीन जिलों को सम्मिलित करते हुए शुष्क क्षेत्र की दो देशी घासों (लासियुरस सिंडिकस और सेनक्रस सेटिगेरस) के जड़ क्षेत्र और आस-पास के क्षेत्र से शक्तिशाली जीवाणुओं के विलगन और चरित्र चित्रण करना है। निम्न जैविक कार्बन **(0.161%** से **0.201%)** और कम उपलब्ध नाइट्रोजन **(113.26** किग्रा/हेक्टेयर से **134.59** किग्रा/हेक्टेयर) की विशेषता वाली इन मिट्टी से प्राप्त कुल **30** जीवाणु विलगों जो कि पौधों की वृद्धि को बढ़ावा देने वाली (पीजीपी) गतिविधियों को दर्शाते हैं, जिनमें से सभी विलग आई.ए.ए. उत्पादन के लिए सकारात्मक थे और **70%** विलग सायनोजेनिक थे, जिनमें से छह विलग उच्च एच.सी.एन. का उत्पादन करने योग्य पाए गए। पिकोवस्काया अगार पर **38** मि.मी. पारदर्शी क्षेत्र का उत्पादन करने वाले **AS-13** सहित ट्राई-कैल्शियम फॉस्फेट **(TCP)** घुलनशीलता में सक्षम कुल चौदह विलगों की पुष्टि की गई। मृदा जैविक विश्लेषणों से पता चला है कि मृदा एंजाइम गतिविधियां जैसे डिहाइड्रोजनेज एंजाइम, एफडीए हाइड्रोलिसिस और सूक्ष्मजीवो की कार्बन जैव मात्रा मिट्टी के सूक्ष्मजीवों पर पौधों की जड़ों का सकारात्मक प्रभाव को प्रकट करते हुए थोक मिट्टी की तुलना में जड़ क्षेत्र में **1.07** से **1.26** गुना अधिक पाया गया। तेरह एस.सी.ओ.टी. प्राइमरों को **50** क्षार-युग्म से **3000** क्षार-युग्म तक के आकार के **133** प्रशंसनीय पट्टीओं में प्रवर्धित किया गया, इन सभी में **100%** बहुरूपता पायी गयी, औसत **10.23** बहुरूपी पट्टी के साथ **7** (एससीओटी **26**) से **17** (एससीओटी **13**) तक प्रति प्राइमर बहुरूपी पट्टी की संख्या देखे गए थी। मार्कर से पता चला कि बहुरूपी सूचना सामग्री **(PIC)** का मान **0.42** (एससीओटी**15**) से **0.75** (एससीओटी**02**) तक था, जिसका औसत **55** प्रतिशत था। और प्रभावी बहुभागी अनुपात **(E)** इकाई के रूप में प्राप्त किया गया था जो कि इस अध्ययन में प्रयुक्त मार्करों की **100%** दक्षता को इंगित करता है । अध्ययन के जनसंख्या संरचना विश्लेषण में छह प्रमुख आबादी की पहचान की गई। अवेक्षित छह आबादी में से तीन आबादी चार शुद्ध अलगाव से जीवाणु के शुद्ध रूप को दर्शाती है। हमारी खोज जड़क्षेत्र के जीवाणु आबादी में अधिक आनुवंशिक परिवर्तनशीलता के अस्तित्व को दर्शाती है। अलगाव **AS-11, AS-13, AS-21** और **AS-23** जो कि शुद्ध रूप में हैं और शुष्क क्षेत्र के लिए जैव- टीकों के विकास में पौधों की अच्छी वृद्धि को बढ़ावा देने वाले लक्षणों का उपयोग किया जा सकता है।

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APPENDIX-I

Chemicals and instruments used

A. Chemicals

S.No.	Chemical	Source	Role
1.	Bromophenol blue	GCC Biotech Pvt Ltd., India	Used as a gel loading dye
2.	Ethidium bromide	Hi-media, India	Used as a tracking dye
3.	Ethanol	Hi-media, India	Used for surface sterilization
4.	$MgCl_2$	Hi-media, India	Used in PCR
5.	Agarose	Hi-media, India	For gel electrophoresis
6.	SCoT	Eurofins	Used in PCR
7.	Go *Taq* reaction mixture (2X)	Promega	Used in PCR

B. Instruments and plasticware used

1. Agarose gel electrophoresis system (Hoefer Inc. US)

2. Autoclave (Sandeep Instruments Pvt. Ltd. India)

3. Deep freezer (Vestfrost solutions, Esbjerg)

4. Gel documentation system (Syngene, UK)

5. Ice maker (Chino, Japan)

6. Weighing balance (Sartorius, Germany)

7. Microwave oven (LG, India)

8. Microcentrifuge (Eppendorf, Germany)

9. Refrigerated centrifugation machine (Plasto Crafts, India)

10. U.V absorbance spectrophotometer (LabMate Asia Pvt Ltd., India)

11. Vortex mixer (Sartorius, Germany)

14. PCR (Thermo Scientific, USA)

15. Micropipettes (Nichiryo, Japan)

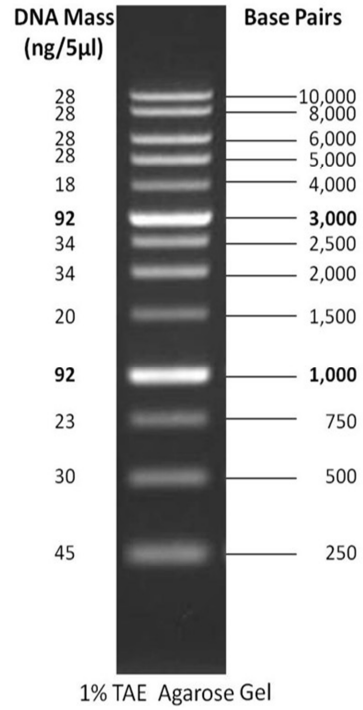
16. PCR tips, tubes, PCR racks, etc. (Tarson, India)

C. Preparation of 10XTBE buffer – 1litre

1. 108 g Tris base
2. 55 g Boric acid
3. 7.5 g EDTA (disodium salt)

D. Molecular markers (Ladders)

1 kb DNALadder



APPENDIX-II

A. Media:

B. Nutrient Broth

Beef Extract	3g
Peptone	5g
Sodium chloride	5g
Distilled Water make to volume	1000 ml
Final pH	7.0

a) Nutrient Agar

Beef Extract	3g
Peptone	5g
Agar	15g
Sodium chloride	5g
Distilled Water make to volume	1000ml
Final pH	7.0

b) Starch agar medium

Starch	20g
Beef extract	3g
Peptone	5g
Agar	15g
Distilled water make to volume	1 000 ml

Iodine solution	(as used in Gram staining)
pH	7.0

d) Pikovaskya medium for isolation of phosphate solubilizing microorganisms

Glucose	10g
Tricalcium phosphate	5g
$(NH_4)_2$, SO_4	0.5g
KCl	0.2g
$MgSO_4$. $7H_2O$	0.1g
$MnSO_4$	Trace
$FeSO_4$	Trace
Yeast Extract	0.5g
Distilled Water make to volume	1000ml
pH	7.0

B. Chemicals

1. $(NH_4)_2HPO_4$ (0.5%)
2. Acetonitrile (0.4%)
3. Ammonium Acetate (7.5M)
4. Chilled absolute alcohol
5. Chloroform
6. Concentrated H_2SO_4
7. CTAB (10%) in 0.7M NaCl
8. Ethanol (70%)
9. Ethidium bromide
10. H_3PO_4

11. $HgCl_2$ (0.2%)
12. Potassium dichromate (1N)
13. Sodium chloride (5M)
14. Tris-saturated phenol (pH 8.0)

C. Stains

(i) Gram Stain

(ii) Crystal violet

Solution A

Crystal violet (90% dye content)	2g
Ethanol, 95%	20ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

The solutions A and B were mixed and stored for 24h and filtered through coarse filter paper.

(iii) Gram's iodine

Iodine	1.0g
Potassium iodide	2.0 g
Distilled water	300ml

(iv) Ethyl alcohol (decolorizer)

(v) Counter stain (stock solution)

Safranin O (certified)	2.5g
Ethanol, 95%	100 ml

Working solution: 10ml stock solution added to 90ml distilled water.

D. Reagents

a) Salkowski's reagent:

Ingredients	ml
Perchloric acid (35%)	50
$FeCl_3$ (0.5M)	1

Add 1ml of 0.5M $FeCl_3$ to 50ml of 35% perchloric acid.

a) IAA Standard solution (30ppm):

Add 30mg of IAA standard into1000ml of distilled water. Prepare just before use.